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THE EFFECT OF ALLERGEN DOSE ON ALLERGIC RESPONSES

THE IMPACT OF THE DOSE AND TIMING OF EPICUTANEOUS
ALLERGEN EXPOSURE ON THE MANIFESTATION OF ALLERGIC
RESPONSES *IN VIVO*

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ABSTRACT

Exposure to sufficiently high allergen levels of some allergens appears to protect against the development of allergic diseases, and has also been used to treat established allergic diseases. A reduced prevalence of sensitization and, in some cases, asthma was reported among individuals living in homes with high allergen levels. Furthermore, allergic patients treated with high allergen doses during immunotherapy experienced improved allergic symptoms. The mechanisms for how high allergen exposure may prevent disease are not known and thus, a model in which these mechanisms could be studied in detail is warranted. The objective of this thesis was to model and further characterize the phenomenon of high allergen dose-dependent protection against the development of allergic responses, using a mouse model of epicutaneous sensitization. The impact of exposure to a high dose of cat dander in established allergic disease was also evaluated. Epicutaneous exposure to a high dose of cat dander (150 µg) prevented airway inflammation and airway hyperresponsiveness in genetically different strains of mice, including Th2 prone BALB/c mice. The protective effects against airway inflammation appeared to wane between 10 and 120 days after exposure. When the high dose of cat dander was applied with a sensitizing dose of peanut, peanut-induced anaphylaxis was prevented. Exposure to a high dose of another allergen, ovalbumin, was also able to prevent the development of allergic airway disease. In contrast, the high dose of cat dander did not improve established states of allergic disease. Thus, in our mouse model, a high dose of cat dander prevented the development of allergic responses and reflected characteristics of the phenomenon reported in humans. As such, this model may be useful for investigating mechanisms and potential prophylactic options for allergic diseases. As the high dose of cat dander either had no effect or worsened established allergic responses in our model, it is possible that the dose was not high enough to

improve disease in sensitized mice. It is also possible that established disease cannot be modified by epicutaneous exposure.

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ABBREVIATIONS

AHR	airway hyperresponsiveness
CDE	cat dander extract
Fel d 1	<i>Felis domesticus</i> 1
FLG	filaggrin
GC	goblet cell
GM-CSF	granulocyte macrophage-colony stimulating factor
GWAS	genome wide association studies
Ig	immunoglobulin
IFN	interferon
IL	interleukin
KLH	keyhole limpet hemocyanin
LPS	lipopolysaccharide
OVA	ovalbumin
PAMPs	pathogen associated molecular patterns
PAR	protease activated receptor
Th	T helper 2
TLR	toll like receptors
TSLP	thymic stromal lymphoietin
SPT	skin prick tests

CHAPTER 1: BACKGROUND

INTRODUCTION

Asthma is a chronic respiratory disease largely distinguished by variable airflow obstruction, which can be reversed spontaneously or with treatment. Asthma is diagnosed by the presence of recurring symptoms of wheeze, dyspnea, shortness of breath, chest tightness, and productive cough especially during the night or early in the morning (Morosco and Kiley, 2007). Features that are commonly associated with asthma are airway inflammation, airway hyperresponsiveness (AHR), and remodelling. In individuals with hyperresponsive airways, asthmatic symptoms can be triggered by a variety of stimuli that are specific such as allergens or nonspecific such as cold air or exercise (Morosco and Kiley, 2007).

In 2010, asthma was reported to affect more than 1.8 million Canadians in whom asthmatic symptoms were only well controlled in a third (Public Health Agency, 2014). It was reported to be one of the most significant chronic diseases amongst children worldwide (World Health Organization, 2013). Furthermore, asthma is associated with a reduced quality of life. In the United States, adults with asthma were less likely to be employed or lead physically active lifestyles, and spent approximately \$1900 (USD) more on healthcare expenses compared to nonasthmatics (Sullivan et al., 2011). Despite substantial progress in our understanding of the pathophysiology of asthma, there are currently no curative treatments or known preventative measures for asthma. While asthmatic symptoms can be well controlled by the use of drugs such as bronchodilators and nonspecific corticosteroids in most patients, these protective effects do not last after the treatment period (Guilbert et al., 2006) and airway remodelling is not slowed or attenuated (Warner and Knight, 2008). These drugs are also not

effective in the proportion of asthmatics with uncontrolled asthma (Wenzel et al., 2007), further highlighting the complexity of the disease.

ASTHMA ENDOTYPES

Asthma varies by the age of onset, severity, factors that contribute to disease progression, and responses to treatment (Lötvall et al., 2011). Asthmatic subtypes were classically defined as allergic or non-allergic asthma, however they were then further refined using phenotypic characteristics. Different asthma phenotypes included early-onset mild allergic asthma, later-onset asthma associated with obesity and severe nonallergic asthma (Corren, 2013). However, more emphasis is now placed on defining endotypes instead of phenotypes due to considerable overlap between phenotypic characteristics (eg. eosinophilia in allergic and nonallergic forms of asthma). Endotypes are “defined by distinct functional or pathophysiological mechanisms” (Lötvall et al., 2011) that can contribute to the observed characteristics. Different asthmatic endotypes include aspirin sensitive asthma, allergic bronchopulmonary mycosis (ABPM), allergic asthma, children with asthma predictive indices, severe late-onset hypereosinophilic asthma, and asthma in cross-country skiers (Lötvall et al., 2011).

ALLERGIC ASTHMA

Most reported cases of asthma were found to begin during childhood, and were also commonly associated with atopy and allergies (Wenzel, 2012). Atopy is defined as the genetic propensity to generate allergen specific IgE, and is functionally determined by a positive skin prick test or elevated allergen specific IgE levels. T helper (Th) 2 cells are key orchestrators in driving the allergic responses underlying this form of asthma. Th2 cells generate cytokines such as IL-4, IL-5, and IL-13, which lead to key features of allergic asthma. IL-4 is

important in IgE production, IL-5 is important in the recruitment, maturation and survival of eosinophils, and IL-13 can contribute to increases in mucus-secreting goblet cells, airway narrowing and remodelling. Indeed, individuals with allergic asthma have elevated allergen specific IgE, positive skin prick tests, and local airway responses such as allergen-induced bronchoconstriction, increased mucus-secreting goblet cells and airway eosinophilia (Busse and Lemankse, 2001). Furthermore, asthmatic individuals with characteristic Th2 type responses were also responsive to therapies targeting IL-4 and IL-13, emphasizing the importance of Th2 cells in allergic asthma (Ray et al., 2015). Animal studies involving adoptive transfer and IL-4 deficiency have also demonstrated that CD4⁺ Th2 cells are key mediators of allergic inflammation (Herrick and Bottomly, 2003).

Eosinophils are believed to be important mediators of asthmatic symptoms as well. Upon activation, eosinophils release toxic granule proteins (eg. major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase), reactive oxygen species, cytokines, and lipid mediators that can collectively inflict considerable damage to the airways. These molecules can expose nerve endings contributing to AHR, enhance vascular leakage by damaging airway endothelial cells, and contribute to mucus secretion and airway remodelling (Galli et al., 2008). As eosinophils have not been found in the airways or lungs of healthy individuals, their presence in the airways (>5% of the cell differential) and in the tissues is indicative of pathology (Disayabutr et al., 2015). Indeed, eosinophils have been consistently associated with allergic asthma. Several groups have also shown that numbers of sputum and airway eosinophils were associated with the degree of AHR in asthmatic individuals (Kay 1991; Kay 2005). Furthermore, experimental models of asthma have also demonstrated a direct role for eosinophils in AHR, supporting that their presence is likely to be important in the manifestation of asthmatic symptoms.

PATHOPHYSIOLOGY OF ALLERGIC ASTHMA

There are two components that are involved in an allergic response: sensitization and the effector phase. Sensitization involves the initial production of allergen-specific IgE, whereas the effector phase is the inflammatory response triggered by allergen binding to IgE upon *subsequent* allergen exposure. During sensitization, allergen specific IgE are generated in an IL-4-dependent manner. The IgE bind to and stabilize surface expression of FcεRs (FcεRI or FcεRII) on effector cells, rendering these cells hyper-responsive or *sensitive* towards that allergen (MacGlashan, 2008). IgE binds to effector cells such as mast cells and basophils via its high affinity receptor FcεRI and to other hematopoietic cells including B cells, T cells, eosinophils, epithelial cells, and Langerhans cells via its low affinity receptor FcεRII (also known as CD23) (Stone et al., 2010). In allergic asthma, subsequent allergen inhalation in sensitized individuals triggers asthmatic signs and symptoms through IgE mediated responses. During the first 10-30 minutes of allergen inhalation, an immediate early phase response occurs that typically resolves within 1-2 hours (Galli et al., 2008). Multivalent binding of inhaled allergen to allergen-specific IgE results in crosslinking and aggregation of the bound Fc receptors, which triggers degranulation of effector cells such as mast cells and basophils. Degranulation of effector cells like mast cells results in the release of preformed mediators (eg. histamine, heparin), cytokines (eg. IL-4, IL-13), and chemokines (eg. CXCL8). Leukotrienes and prostaglandins are also rapidly synthesized and released. The collective effects of these mediators include acting on airway smooth muscle cells resulting in contraction, increasing the permeability of blood vessels, recruiting other inflammatory cells, and inducing mucus secretion. Allergen binding of IgE on dendritic cells or B cells also facilitates further antigen presentation (Stone et al., 2010). Some allergic individuals, typically those with less well-controlled asthma, also experience a late asthmatic response that can begin as early as 3 hours after the initial allergen

exposure and last for hours to days (Herxheimer, 1952). The late asthmatic response may be triggered to some extent by the cytokines and chemokines released during the immediate reaction, which facilitate recruitment of effector cells such as eosinophils, neutrophils, macrophages and T cells. These effector cells drive airway narrowing (Haselden et al., 1999), collectively enhance lung damage, oedema, mucus secretion and thus, airway obstruction (Galli et al., 2008).

DEVELOPMENT OF ALLERGIC ASTHMA

Despite substantial advances in understanding the pathophysiology of allergic responses, the processes leading to sensitization are not as clear. This is due to the fact that sensitization is largely asymptomatic, and therefore, difficult to recognize clinically. In addition, the complex interaction between genetic and environmental factors in allergic disease suggest that the pathogenesis of allergic asthma is likely different for each individual. Nonetheless, understanding how certain factors may skew immune responses towards exaggerated Th2 type inflammation will help determine risk factors, and guide treatment development.

Immune dysregulation of Th2 responses

Allergies are exaggerated immune responses to environmental substances to which most people are tolerant, and Th2 cells have been identified as key orchestrators of these responses. Although the origins of allergic responses are not understood, one of the more widely accepted theories is the hygiene hypothesis (Herrick and Bottomly, 2003). The increase in the prevalence of allergic diseases (Umetsu et al., 2002; Cookson, 1999) during the same time that many lifestyle changes were made (presumably associated with reduced exposure to microbial products) led to the hygiene hypothesis. According to the hygiene hypothesis, dysregulated Th2 responses, and thus allergic diseases, developed due to a lack of

immune stimulation from Th1 skewing or regulatory T (Treg) cell inducing microbial exposures in early life. The Th1/Th2 paradigm suggested that Th1 responses were necessary to establish immunological homeostasis during the maturation of immune systems. This was based on experimental studies demonstrating IFN- γ mediated suppression of IL-4 production, and further supported by epidemiological evidence of a reduced prevalence of atopy in areas with increased Th1-inducing infections. However, the Th1/Th2 paradigm was undermined with the 1) observations that the increased prevalence of Th1-mediated autoimmune diseases (e.g. Type I diabetes) coincided with the increase in allergic diseases and 2) evidence from many studies demonstrating exacerbated or only partial suppression of Th2 responses with the addition of Th1 cells and/or IFN- γ into naive or sensitized mice. Furthermore, populations with high rates of Th2 inducing parasite infections were protected from allergic diseases, also suggesting that the presence of increased Th2 responses may not necessarily induce allergic diseases (Herrick and Bottomly, 2003; Umetsu et al., 2002).

Treg cells have been shown to suppress both Th1 and Th2 immune responses. Treg cells are thought to serve two main functions. One of these functions is to suppress inflammatory responses during infection to maintain homeostasis; stimulation with certain pathogen-associated molecular patterns (PAMPs) has been implicated in this process (Herrick and Bottomly, 2003). Indeed, Treg cells have been observed in individuals exposed to high rates of Th2-inducing helminth infections, but did not have any allergic symptoms (Herrick and Bottomly, 2003). The second function is believed to be to prevent unwanted reactions to self and harmless foreign antigens. In this context, Treg cell differentiation is believed to be induced in the absence of PAMPs or inflammatory signals (Herrick and Bottomly, 2003). Indeed, many studies have demonstrated that high levels of PAMPs induce Th1- (Eisenbarth et al., 2002) or Treg- (Herrick and Bottomly, 2003) type responses. Low levels of PAMPs were shown to induce Th2-type

responses (Eisenbarth et al., 2002), and presentation of antigen in the absence of inflammatory signals (including PAMPs) was associated with tolerance (Herrick and Bottomly, 2003). Furthermore, tolerance in healthy nonallergic individuals also appeared to be mediated by Treg cells (von Hertzen et al., 2009). Thus, immune dysregulation of Th2 responses may also be influenced by interactions between microbial products and innate immune components (Herrick and Bottomly, 2003).

ROLE FOR ROUTE OF SENSITIZATION

The epithelium acts as a first line of defense against environmental antigens by providing a physical, chemical and anti-microbial barrier, and is an integral component of the innate immune response (Cookson, 2004). However, a compromised barrier, due to damage or inherent defects, can contribute to enhanced antigen permeability, and depending on the nature of the damage, also a Th2 polarizing environment. Elevated epithelial-derived cytokines such as IL-33, TSLP and IL-25 are Th2 polarizing cytokines that have been observed along with Th2 cytokines in areas of damaged epithelium (Paul and Zhu, 2010).

I. The airway epithelial barrier

While the prevalence of elevated total serum IgE levels was strongly correlated with asthmatic symptoms (Burrows et al., 1989), only a third of atopic children and young adults were found to later develop asthma (Simpson et al., 2010). This suggested that other factors also contributed to allergic asthma. Indeed, genome wide association studies (GWAS) have identified genes or loci linked to the airway epithelium (e.g. *ORMDL3*, *IL-33*) (Moffatt et al., 2010) that were consistently associated with an increased risk for asthma, compared to genes associated with atopy and serum IgE levels (e.g. HLA-DQ locus, *FCERIA*, *STAT6*, *IL-13*) (Granada et al., 2012). IL-33, among other epithelial-derived

cytokines (TSLP, IL-25 and GM-CSF), has been shown to mediate the development of Th2 immune responses (Chu et al., 2013; Wang and Liu, 2009; Stampfli et al., 1998). Expression of ORMDL3, involved in sphingolipid synthesis, was induced in airway epithelial cells by allergen and IL-4 or IL-13 exposure (Suzuki et al., 2011; Miller et al., 2012). Furthermore, analysis of bronchial biopsies of asthmatic individuals revealed inherent defects in the airway epithelium involving incomplete formation of tight junctions. Increased antigen permeability was demonstrated in asthmatic epithelial cell cultures compared to epithelial cells from nonasthmatics (Xiao et al., 2011). Other environmental factors such as respiratory viruses and air pollutants (ozone, diesel particles and tobacco) were also shown to disrupt tight junction formation, and have been suggested to facilitate allergen sensitization (Lambrecht and Hammad, 2014). Furthermore, stimulation of innate receptors (select toll-like receptors (TLRs) and protease-activated receptors (PARs)) on airway epithelial cells induced increased epithelial production of TSLP, IL-33 and IL-25 (Lambrecht and Hammad, 2014). As such, a compromised barrier, and the ability of epithelial cells to produce Th2 polarizing cytokines purport a plausible role for the airway epithelium in sensitization.

II. The skin epithelial barrier

The epithelial barrier of the skin has also been identified as an important route for sensitization, and may influence the pathogenesis of multiple allergic diseases. At the population level, signs of atopic dermatitis have typically appeared in the first years of life, followed by symptoms of asthma and allergic rhinitis. This pattern of disease onset led to the idea that there was a natural progression in the development of allergic diseases, and was termed the atopic march (Spergel and Paller, 2003). Furthermore, children with atopic dermatitis experienced more severe asthma compared to asthmatic children without a history of atopic dermatitis (Spergel and Paller, 2003; Beck and Leung, 2000). These findings

further suggested a role for epicutaneous sensitization in the development of allergic airway responses.

Filaggrin (FLG) is a protein that is involved in maintaining the integrity of the epidermis. In 2006, two loss-of-function *FLG* allelic variants (R501X and 2282del4) were identified, and were more frequent in patients with coexisting atopic dermatitis and asthma. In addition, children with the R501X *FLG* allele experienced more frequent asthmatic exacerbations, and were more likely to require oral steroids compared to children with the wildtype allele (Suzuki et al., 2011). FLG mutations were also strong risk factors for the development of multiple allergies and asthma (Henderson et al., 2008). As FLG was not detected in the human bronchial epithelium (Ying et al., 2006), it has been suggested that epicutaneous sensitization might contribute to asthma by enhancing systemic allergic responses (Zheng et al., 2011). Indeed, increased circulating IL-13⁺ Th2 and decreased IFN- γ ⁺ producing T cells have been detected in patients with severe atopic dermatitis compared to healthy subjects (Czarnowicki et al., 2015). Circulating eosinophils in atopic dermatitis were also found to have enhanced migratory abilities, compared to eosinophils from healthy controls (Beck and Leung, 2000).

Several animal studies have also demonstrated the influence of epicutaneous sensitization in allergic airway responses (Suzuki et al., 2011). In one particular study involving epicutaneous sensitization to ovalbumin (OVA), AHR and airway inflammation was observed after a *single* inhalation challenge (Spergel et al., 1998). This study also demonstrated that epicutaneous sensitization with OVA alone was not sufficient to increase airway eosinophilia or AHR, but the intranasal exposure was also necessary. Thus, these findings suggest that epicutaneous allergen exposure may contribute to allergic airway responses, and local exposure to these allergens to the lungs is also necessary.

Exposure to aeroallergens early in life has also been suggested to be a risk factor for the development of atopic dermatitis. In 2008, a prospective birth cohort study assessed whether exposure to pets and/or house dust mite in the first few months of life influenced the risk of developing eczema in children with FLG mutations. While FLG mutations were associated with an increased risk for developing eczema during the first year of life, this risk was accentuated with cat ownership (not dog ownership or house dust mite exposure) at birth. In this study, this enhanced risk by cat ownership was not attributed to sensitization, as no cat specific IgE were detected in these children during the first year of life (Bisgaard et al., 2008). A later study demonstrated that cat ownership at birth enhanced the risk for developing eczema and sensitization in children with the 2282del4 FLG mutation. There was also a significant association between the 2282del4 mutation and asthma in these children but this was not influenced by cat ownership at birth (Schuttelaar et al., 2009). Collectively, these studies support a role for the skin in sensitization, and a potential role in the development of allergic asthma.

ROLE FOR ALLERGEN DOSE IN THE DEVELOPMENT OF ALLERGIC DISEASES

The incidence of asthma increased dramatically in industrialized countries at a time that coincided with an increase in indoor allergens levels (Platts-Mills et al., 1997). This observation gave rise to the idea that increased exposure to allergens may have *caused* the increased incidence of allergic asthma, likely through increased sensitization. In support of this, many studies had demonstrated that asthma was more prevalent in atopic children compared to nonatopic children, and higher IgE titers were seen in children with asthma (Guibas et al., 2015). Furthermore, clinical models involving allergen inhalation challenges have also supported the role for allergens in asthma. Thus, many studies have investigated

the relationship between the level of allergen exposure, the prevalence of sensitization and the prevalence of asthma. As the onset of most allergic diseases was reported to occur during childhood, many studies assessed the effects of early allergen exposure in an effort to determine risk factors or develop strategies to prevent the development of allergic asthma.

The direct influence of antigen dose on T cell priming has been demonstrated using *in vitro* systems. Hosken and colleagues (1995) demonstrated a dose-dependent relationship between the dose of OVA peptide used to stimulate naive CD4⁺ T cells and the differentiation of these cells to Th1 or Th2 type cells. Low (<0.05 µM) and very high (10 µM-100 µM) peptide concentrations were associated with increased Th2 type responses (increased IL-4 production with poor proliferative capacity), whereas moderate (0.3-0.6 µM) peptide doses were associated with Th1 like responses (increased IFN-γ production and robust T cell proliferation). Using another antigen, Tao and colleagues (1997) demonstrated that low antigen doses (<0.02 µM) were associated with Th2 responses, and higher antigen doses induced Th1 responses (0.2-20 µM). It is suggested that the concentrations tested by Tao and colleagues were not high enough to observe Th2 like responses with higher concentrations (Constant and Bottomly, 1997). Furthermore, in another *in vitro* study, the impact of antigen dose on Th2 cell-dependent production of IgE was studied (Constant and Bottomly, 1997). Stimulation of naive T cells with low antigen concentrations was associated with increased IL-4 and IgE production. In contrast, high antigen concentrations were associated with increased levels of IFN-γ and decreased levels of IgE. Interestingly, anti-IFN-γ monoclonal antibody did not reverse the high antigen dose-dependent suppression of IgE levels. This suggested that the increased levels of IFN-γ associated with increased antigen did not suppress IgE synthesis. Overall, based on these studies, there appears to be a direct impact of antigen dose on the polarization of naive CD4⁺ T cells. Furthermore, each study demonstrated

Th2 polarization with lower doses of antigen, implicating a role for exposure to low levels of allergen in sensitization. It has been suggested that Th2 polarization with low doses of antigen may be due to transient signalling patterns (Constant and Bottomly, 1997).

Furthermore, Riedl and colleagues demonstrated that high doses of antigen may overcome conditions that were conducive to Th2 polarization. In a prospective study, they assessed whether antigen dose influenced the development of sensitization to the antigen keyhole limpet hemocyanin (KLH) administered intranasally to atopic individuals in the presence of diesel exhaust particles. New onset sensitization to KLH was inversely associated with antigen dose. In addition, while KLH specific IgG were detected in nasal lavages of all subjects, subjects receiving the highest dose produced the highest levels of KLH specific IgG (Riedl et al., 2005).

I. Epidemiological evidence of allergen dose dependent effects

A strong association was found between the prevalence of sensitization in individuals living with, what was referred to as, “high” allergen concentrations compared to those with “low” or no detectable levels (Munir et al., 1997; Murray et al., 2001). The concentration of allergen that was strongly associated with sensitization varied with the allergen, region and population studied. Nonetheless, these studies suggested that there existed a threshold, for the level of allergen exposure needed for the development of sensitization. This threshold was also typically lower for atopic individuals (Munir et al., 1997; Murray et al., 2001). However, to-date, the dose response relationship between allergen levels greater than the threshold and the subsequent development of sensitization is not as clear.

The influence of cat ownership on sensitization and asthma in some communities triggered several groups to further investigate the relationships between the level

of allergen exposure, sensitization and asthma. In regions where cat allergens were found to be the dominating allergen, sensitization to cats was suggested to be a major risk factor for the development of asthma (Woodfolk, 2005). While some epidemiological studies found no relationship between the levels of exposure to cats and the prevalence of sensitization, many others found that increased concentrations of cat allergen in homes was associated with increased rates of sensitization (Woodfolk, 2005). Sensitization to cats was strongly associated with asthma (Platts-Mills et al., 2001; Ingram et al., 1995) and wheeze (Polk et al., 2004). Furthermore, children with cat allergies were also reported to have more severe asthma (Sarpong and Karrison, 1998). However, beginning in the late 1990s, multiple groups found a reduced prevalence of cat-specific sensitization among school-aged children that were exposed to increased levels of cat allergens early in life (based on cat ownership or allergen concentrations in house dust) (Braback et al., 2001; Hesselmar et al., 1999; Sporik et al., 1999; Platts-Mills et al., 2001). In some studies, the degree of sensitization (amount of IgE) was also decreased in children living with cats (Erwin et al., 2005). Increased cat exposure was also negatively associated with the prevalence of asthma in some studies (Perzanowski et al., 2002; Hesselmar et al., 1999; Platts-Mills et al., 2001).

In light of these findings, it has been suggested that there is a bell-shaped relationship between the level of exposure to cats and the prevalence of sensitization. That is, the frequency of cat specific sensitization was highest with exposure to “intermediate” levels of cat allergen, and reduced at very “low” (below threshold) or very “high” levels of exposure (Platts-Mills et al, 2004). This bell-shaped relationship was very elegantly demonstrated in adults as well (Custovic et al., 2003). The highest prevalence of cat specific sensitization was seen in adults exposed to intermediate levels of Fel d 1, whereas prevalence was reduced at low and high concentrations. In another study, childhood exposure to

pets including cats was associated with reduced prevalence of sensitization during adulthood (Roost et al., 1999).

Among nonallergic children living with a cat, some of these children had increased circulating Fel d 1 specific IgG and IgG4 antibodies without IgE (Platts-Mills et al., 2001; Hesselmar et al., 2003; Platts-Mills et al., 2003). Due to the IL-4-dependent production of IgG4, and the lack of cat-induced symptoms in these individuals, this immunological response was termed the modified Th2 response (Platts-Mills et al., 2001). This modified Th2 response was also suggested to be an immunological form of tolerance that may be protecting these individuals from allergies to their cats. Furthermore, IgG4 is suggested to block IgE mediated responses (Aalberse, 2011). It should be noted that in some nonallergic individuals no immune responses to cat (no Fel d 1 specific serum Ig's) have been detected and in others, Fel d 1 specific IgG, IgG4 and IgE have been detected (Erwin et al., 2014; Roost et al., 1999). It has been suggested that nonallergic individuals with Fel d 1 specific IgE in addition to IgG and IgG4, may have been desensitized from exposure to increased cat allergen levels from their cat (Erwin et al., 2014). Increased allergen specific IgG and IgG4 have also been observed in patients receiving allergen immunotherapy and in beekeepers who were stung multiple times; these individuals also experienced improved allergic symptoms (Larché et al., 2006; Meiler et al., 2008). IL-10 producing T cells have also been found in these individuals (Larché et al., 2006; Meiler et al., 2008). Despite this association, it should be noted that a protective role for IgG4 has not been clearly demonstrated. As such, it is only *possible* that exposure to high levels of cat dander may have a *tolerogenic* effect on sensitization and this may or may not be mediated by IgG4.

i. Does increased allergen exposure only prevent sensitization in certain populations (role for genetics)?

Even though the threshold for sensitization to allergens has been reported to be lower for atopic or “high risk” individuals (Munir et al., 1997; Murray et al., 2001), some studies have reported a protective effect of high cat allergen exposure even in high risk individuals. Increased exposure to cats was associated with a decreased prevalence of cat specific sensitization later in life in cohorts with a positive history of atopy (Pohlabein et al., 2007; Sporik et al., 1999). One study conducted in Sweden found a protective effect of cat ownership in atopic children *only* (Braback et al., 2001). In other studies, while the effect of increased cat exposure was also observed in individuals with no familial history of atopy, the effect was more pronounced in atopic individuals (Cullinan et al., 2004; Perzanowski et al., 2002; Roost et al., 1999). Thus, increased cat allergen levels in homes may not necessarily be a risk factor for the general population *or* high-risk individuals based on these studies; increased exposure to cat allergens may even be protective for genetically susceptible individuals.

ii. Do the protective effects of increased allergen exposure wane with time?

Some studies have found that living in a home with a cat during childhood (before 18 years of age) was negatively associated with prevalence of sensitization to cats and respiratory symptoms to cats or current asthma in adults (Roost et al., 1999; de Meer et al., 2004). However, there is also anecdotal evidence of some college students that experienced new allergic symptoms to their cats, which were previously not a problem, when they returned to their home from college. As such, it is possible that the protective effects from cat ownership may diminish if exposure to cats is reduced for an extended period of time. Erwin and colleagues (2014) characterized changes in serum antibody profiles of *nonallergic* college students who came from homes with cats, but lived away during the academic year where relative cat exposure was low. Among nonallergic students with Fel d 1 specific IgG, IgG4 and no IgE, IgG and IgG4 titers decreased over the 8-month academic year. In nonallergic students who had Fel d 1 specific IgE in addition to

Fel d 1 specific IgG and IgG4, IgG and IgG4 levels also decreased during the 8-months. Although allergic symptoms in these individuals were not evaluated, in a subset of the students without Fel d 1 specific IgE followed up after two years, there were no new positive skin prick tests or evidence of serum Fel d 1 IgE. Furthermore, other studies have reported that the presence of IgG and IgG4 was associated with reduced allergic symptoms (Jeal et al., 2006; Jakobsen et al., 2005). Thus, it is possible that with reduced exposure to increased levels of allergen, the protective effects may diminish and this may involve reduced levels of allergen specific IgG and IgG4. In support of this, living with a cat up until adulthood, and thus continuous exposure to high levels of cat, was suggested to enhance the protective effect of cat ownership. Asthmatic adults with continuous exposure (childhood and current) to high levels of cat allergen (based on cat ownership) were more likely to have lower Fel d 1 specific IgE levels, a greater IgG4⁺/IgE⁻ ratio, and smaller wheal size to cat, compared to asthmatic individuals with only childhood or current exposure to cats (Oryszczyn et al., 2009).

iii. Are the protective effects of increased allergen exposure specific to cat allergens?

Studies have reported an increased prevalence of house dust mite and cockroach specific sensitization among individuals living in homes with relatively higher house dust mite and cockroach concentrations (Arshad, 2010; Sporik et al., 1990). This association for house dust mites was reported in several cities with similar climates, and in cities with different climates (Platts-Mills et al., 2000). In New Zealand, domestic concentrations of house dust mite and cat allergens were much higher compared to other regions in the world (Sawyer et al., 1998). Yet, children living in homes with cats were less likely to be sensitized to cats, whereas the prevalence of house dust mite specific sensitization was increased among children living in homes with increased concentrations of house dust mite (Erwin et al., 2005). As most of these studies did not find a similar relationship with exposure

to house dust mite, it was suggested that this was a cat allergen specific phenomenon. In support of this, individuals who lived with cats and did not have cat specific IgE still had elevated serum IgE to other allergens (Erwin et al., 2014).

Felis domesticus 1 (Fel d 1) is considered the major cat allergen (Grönlund et al., 2010), and was quantified in household dust to determine cat allergen levels in homes. Fel d 1 is a 35-kDa tetrameric glycoprotein that is easily airborne on small particles (<5µm) and has been found to remain airborne for long periods of time after disturbances in settled dust (Woodfolk, 2005). House dust mite-derived allergens, *Dermatophagoides pteronyssinus* (Der p) 1 and *Dermatophagoides farinae* (Der f) 1 however, were typically found on larger particles in the air and settled down to the ground sooner. As such, it has been suggested that inhalation of Fel d 1 may be much higher than that of house dust mite accounting for the differences in the dose response relationship (Platts-Mills et al., 2001; Platts-Mills et al., 2004; Woodfolk, 2005). Furthermore, Fel d 1 is commonly regarded as a very sticky protein. In communities where cats were prevalent, Fel d 1 was detected even in buildings where cats were never present; despite the fact that cats had never been in those buildings, Fel d 1 levels were also found to be greater than those of other allergens in these locations (Platts-Mills et al., 2000).

Despite the majority of findings, one study reported that the prevalence of house dust mite sensitization was highest in homes with intermediate concentrations (1.4 to 10.4 µg/g dust in mattress samples), and lower at higher and lower house dust mite concentrations (Schram-Bijkerk et al., 2006). The bell-shaped relationship was observed in both farm and nonfarm living children; in homes with increased microbial exposures (i.e. on a farm) the curve was shifted downwards. This was observed in Switzerland, Germany, Netherlands and Austria, but not in Sweden where house dust mite levels were generally very low. Thus, it appears that in

some communities, increased exposure to house dust mites was associated with a reduced prevalence of sensitization. Another study also reported trends of a bell shaped relationship between concentrations of house dust mite allergen in house dust and sensitization in children in the UK (Cullinan et al., 2004). Although the association was not statistically significant, the prevalence of sensitization was increased in homes with lower concentrations of house dust mite, and reduced in homes with higher allergen concentrations. As such, it has been suggested that a *sufficiently high* level of exposure to house dust mites, or allergens in general, might prevent sensitization (Linneberg, 2008).

A bell-shaped relationship has also been reported for other allergens. Exposure to dogs in the first year of life was associated with a decreased prevalence of sensitization in children (Holscher et al., 2002). One study also found that living with a dog during the first year of life was inversely associated with sensitization and asthma at 6-7 years (more pronounced effect was seen with 2 or more dogs or cats) (Ownby et al., 2002). Decreased rates of new onset sensitization were also reported in animal handlers exposed to relatively high levels of rodent urinary allergens (Jeal et al., 2006).

iv. Is increased allergen exposure to one allergen able to influence sensitization to other allergens?

Based on findings from some studies, it appears that exposure to increased allergen levels may prevent sensitization to other allergens as well. Children who lived with 2 or more cats or dogs during the first year of life were less likely to develop sensitizations to both indoor and outdoor allergens (Ownby et al., 2002). In a prospective study, adults who lived with a cat between 8-18 years of age had reduced prevalence of sensitization to outdoor allergens as well; the prevalence of AHR and current asthma was also reduced. Cat ownership after the age of 18 was associated with a greater prevalence of asthma and AHR in this study (de Meer et

al., 2004). Patients receiving allergen immunotherapy were less likely to develop new sensitizations compared to allergic individuals who did not receive immunotherapy in some studies (des Roches et al., 1997; Pajno et al., 2001; Purello-D'Ambrosio et al., 2001; Inal et al., 2007; Bozek et al., 2014), but not others (Asero, 2004; Gulen et al., 2007). However, in another study, while adults currently owning a cat were less likely to be sensitized to cats and dogs, there was no effect on sensitization to house dust mite or pollen (Custovic et al., 2003). In addition, in New Zealand, where house dust levels of cat and house dust mite allergens were higher than found in other parts of the world, children living with a cat were protected from cat specific sensitization, but there was no effect on house dust mite sensitization (Erwin et al., 2005). Furthermore, the effect of cat allergen sensitization on asthma has been suggested to only be significant in areas where cat allergens were the dominant allergen (eg. Sweden) (Woodfolk, 2005); in regions where other allergens such as house dust mite, cockroach, and pollen were also significant, the effects of cat allergen on asthma were relatively minor (Gelber et al., 1993; Sears et al., 1989). Thus, while high allergen exposure was associated with a reduced incidence of new sensitizations, this was not a consistent finding.

v. Can increased exposure to microbial products influence the development of allergic disease?

While allergens can influence immune responses, it is not clear whether levels of other co-existing factors such as lipopolysaccharides (LPS or endotoxin) are mediating these effects. In a series of *in vitro* experiments, Eisenbarth and colleagues (2002) assessed the effect of different LPS doses with a fixed concentration of ovalbumin (OVA) on the polarization of naive T cells. Low LPS concentrations were associated with Th2-like responses, while increased LPS levels resulted in Th1-like responses. These findings were consistent with epidemiological reports of decreased prevalence of sensitization in children raised

on farms where exposures to endotoxin levels were high (Braun-Farhländer et al., 2002). In addition, in a mouse model of allergic asthma, IL-4 and IL-13-dependent responses were differentially affected by the dose of LPS. While the asthmatic phenotypes induced by the low dose of LPS were affected in both IL-4 and IL-13 deficient mice, the phenotypes induced by the high dose of LPS were not (Kim et al., 2007; Bang et al, 2013). Furthermore, increased exposure to other bacterial components such as muramic acid and the fungal allergen (1-3)-beta-D-glucan was inversely associated with wheeze in children (Van Strien et al., 2004) and recurrent wheeze in infants, respectively (Iossifova et al., 2007). High concentrations of endotoxin were also associated with reduced rates of sensitization to other allergens such as house dust mite and cat dander (Gehring et al., 2002). In a study conducted by Lynch and colleagues (2014), it was demonstrated that although exposure to high levels of cockroach, mice and cat allergens was strongly associated with allergic sensitization at 3 years of age, exposure to these allergens in the first year of life was negatively associated with recurrent wheeze. Children who were neither atopic nor developed wheeze at the age of 3 years, were also exposed to the greatest diversity of microbes and highest levels of allergens during the first year of life. Increased exposure to two particular phyla, Firmicutes and Bacteroidetes, was associated with the greatest protection against atopy and wheeze (Lynch et al., 2014). Thus, it appears that the timing of exposure to allergens likely influences the outcome of allergic sensitization and associated diseases.

Collectively, all of these studies suggest that immune responses can also be determined by antigen dose, consisting of allergen or microbial components. Although the relationship between allergen exposure and the development of allergic asthma in humans may be complicated, several studies have implied a protective effect of high allergen exposure in various respects (in genetically susceptible populations, effects may not be specific to a single allergen, impact on

sensitization to other allergens, etc.). Thus, detailed characterization of how immune responses differ with antigen dose is warranted.

II. Evidence of dose dependent effects from experimental models

As the occurrence of asthma can be a result of a multitude of factors, it is difficult to delineate which factors, including allergen levels, are likely influencing the prevalence of this disease through population studies. As such, experimental models provide the opportunity to study the direct influences of allergen dose on the manifestation of allergic disease while controlling for multiple factors. In an experimental model of allergic asthma, Llop-Guevara and colleagues (2008) investigated the relationships between antigen dose, sensitization, and airway inflammation using a 10,000-fold dose range of house dust mite. This study demonstrated that a threshold level of exposure was necessary before dose dependent increases in the total airway inflammation (total cells in bronchoalveolar lavage), airway eosinophils and total IgG1 were observed. Furthermore, at the higher doses of house dust mite exposure, total cells plateaued whereas the number of eosinophils and total IgG1 appeared to be reduced. In a study by Sakai and colleagues (1999), the effect of different sensitizing doses of OVA (0, 10, 100, 1000 μg) on the development of allergic airway responses was assessed in BALB/c mice. A sensitizing dose of 10 μg OVA was associated with increased airway reactivity to methacholine, while airway responsiveness at the dose of 1000 μg was comparable to that observed in naive mice. Lung tissue and BALF eosinophils, OVA specific IgE, T cell proliferation, IL-4 and IL-5 production by OVA stimulated splenocytes were also inversely related to the sensitizing antigen dose. Interestingly, overall cytokine production was suppressed at the 1000 μg dose, including that of IFN- γ . Finally, in an experimental model of anaphylaxis, it was demonstrated that epicutaneous application of higher doses of house dust mite prevented house dust mite-induced anaphylaxis (Hirai et al., 2016). This was shown to occur in an IgG dependent

manner. As such, although the pathogenesis of allergic diseases is likely influenced by many factors, the dose of allergen is likely a contributing factor. Exposure to *sufficiently high* quantities may be protective against disease whereas exposure to intermediate levels may confer an increased risk for sensitization.

ROLE FOR ALLERGEN DOSE IN MODULATING ESTABLISHED ALLERGIC ASTHMA

Sensitized individuals experience exacerbations or worsening of symptoms upon natural exposure to allergen, or upon allergen inhalation challenge, a clinical model commonly used to study allergic asthma (Salo et al., 2008; Durham, 1991). However, responses characteristic of immunological tolerance and/or desensitization have been observed in patients receiving allergen immunotherapy, beekeepers and animal handlers as a result of exposure to high levels of allergen (Larché et al., 2006; Meiler et al., 2008; Jeal et al., 2006). As such it appears that the level of allergen exposure can also influence the manifestation of allergic responses in sensitized individuals. In support of this, the dose of pollen allergen administered during immunotherapy, associated with clinical benefit, was approximately 100-fold greater than the estimated natural exposure to pollen in a year (Løwenstein, 1991). Furthermore, *in vitro* stimulation of peripheral blood cells from allergic donors with dust mite allergen, rye grass pollen and bee venom phospholipase A₂, produced high levels of IL-4 with low antigen concentrations, and reduced IL-4 levels with high antigen concentrations (Constant and Bottomly, 1997).

MOUSE MODELS

Although mice are a common species used to study allergic asthma, they do not naturally develop asthma. Thus to study this disease, features typically associated

with allergic asthma (AHR, airway inflammation and remodeling, circulating IgE) are induced to model the disease as closely as possible. As such, sensitization and associated inflammatory responses have been initiated through intranasal administrations of allergen consisting of acute or chronic dosing schedules, depending on the objectives of the study (Nials et al., 2008). It is also common to induce systemic sensitization by administration of allergen in conjunction with Th2 skewing adjuvants such as aluminum hydroxide (alum), followed by intranasal administrations of allergen to observe enhanced responses in the lungs (Zhang et al., 1997). While intranasal administrations are able to result in airway inflammation, hyperresponsiveness and remodeling, the induction of robust serum IgE levels along with these local characteristics were typically achieved with systemic sensitization with adjuvants such as alum (Zhang et al., 1997). Robust allergic airway responses have also been generated after priming with allergen via epicutaneous application (Spergel et al., 1998). Using these procedures, features of allergic airway responses are induced in mice, providing an opportunity to perform detailed characterization and mechanistic studies within an *in vivo* system.

RATIONALE

Epidemiological and experimental evidence has supported a role for allergen dose, and potentially microbial components, in influencing the development of allergic diseases. Exposure to different levels of allergen has also impacted established allergic responses. Multiple studies reported a reduced prevalence of sensitization, and in some cases, asthma or allergic disease, among individuals living in homes with relatively higher levels of allergen. In individuals undergoing immunotherapy, administration of high allergen doses was associated with reduced allergic symptoms that persisted even after treatment was discontinued. Thus, exposure to sufficiently high levels of allergen *prior* to and

after the development of allergic diseases may be protective or therapeutic, respectively.

Intervention studies that evaluated the effects of reducing allergen exposure through the use of interventions such as pillow and mattress covers have reported conflicting results with respect to sensitization. For example, while these studies were able to effectively reduce house dust mite levels in the house, house dust mite specific sensitization was reduced in some studies, while others found no effect (Guibas et al., 2015). Interestingly, in one study the prevalence of house dust mite sensitization was greater. The frequency of sensitization to house dust mite was almost 2-fold greater in children raised in homes with reduced house dust mite levels (from 30.3 $\mu\text{g/g}$ household dust to 18.9 $\mu\text{g/g}$ with interventions), compared to children raised in homes in which house dust mite levels were not controlled (Woodcock et al., 2004). As such, exposure to high levels of allergens may not necessarily be a risk factor, and understanding what allergen levels pose a risk within each environment should be pertinent prior to implementing allergen reduction strategies.

Arguably, understanding how allergen dose mitigates the risk of sensitization and thus prevalence of allergic diseases mechanistically may be used prophylactically in high-risk individuals (i.e. exposed to intermediate allergen levels). This may also be beneficial as some epidemiological findings reported that individuals raised with pets in the first year of life were less likely to become sensitized to other allergens as well (Ownby et al., 2002). As it is difficult to conduct mechanistic studies in humans, mouse models provide the opportunity to perform detailed characterization and mechanism studies and are commonly used to study allergic diseases. However, as allergic asthma is artificially induced in mice, it is important to determine whether the model reflects the features of the human disease as closely as possible. Thus, the main objective of this thesis was to

characterize the phenomenon of high allergen dose-dependent protection from allergic responses using a mouse model in which mechanisms may be studied in the future.

We used a mouse model (Figure 1) in which the manifestation of allergic airway responses was dependent on the dose of cat dander extract used during sensitization. This observation was made in BALB/c mice. It should be noted that the cat dander extract contained Fel d 1, but also contained other proteins and microbial products, including LPS (or endotoxin); thus, the effects observed in this thesis cannot be solely attributed to Fel d 1. However, the use of this unpurified extract is more relevant to human exposures to allergen, as allergens are encountered among other components such as endotoxin. In our model, epicutaneous exposure to 15 µg of cat dander, a dose that we referred to as “low”, for 10 days was associated with increased inflammation in the airways and lungs, and worsening lung function compared to naive mice (Figures 2b, 3a, and 5a respectively). However, in mice that were epicutaneously exposed to 150 µg of cat dander for 10 days, a dose we classified as “high”, allergic airway responses (airway and lung eosinophils, and AHR) were attenuated. Thus, in this thesis, epicutaneous application of 150 µg of cat dander for 10 days was used as the form of high allergen exposure.

OVERARCHING OBJECTIVE

To characterize the effects of high allergen dose and the timing of this exposure on the manifestation of allergic responses, using a mouse model of epicutaneous sensitization. The impact of exposure before or after the development of allergic responses was evaluated.

Specific Aim 1: To determine if attenuation of allergic airway responses with epicutaneous exposure to the high dose of cat dander is strain-independent.

RATIONALE AND STUDY DESIGN: While inbred strains of mice are genetically syngeneic, humans are genetically divergent species. Findings from twin studies and the identification of asthma susceptibility genes support a role for genetic influences on the development of asthma (Ober and Yao, 2011). Furthermore, the effects of high allergen exposure appeared to be more pronounced in genetically susceptible populations. BALB/c mice, in which epicutaneous application of 150 µg cat dander was associated with attenuated allergic airway responses, are prone to mount robust Th2-like responses (Gorham et al., 1996; Reiner and Locksley, 1995). However it is not known whether other strains of mice that are genetically less susceptible to Th2-like responses would be similarly affected by allergen dose. Thus, to strengthen the generalizability of the dose dependent effects of cat dander, allergic airway responses were also assessed in other, genetically different, strains of mice: C57Bl/6, and HLA DRB1*0401 (DR4) mice. DR4 mice are transgenic for the human MHC class II HLA DR4 molecule, and lack endogenous murine MHC class II molecules (Ito et al., 1996). C57Bl/6 are commonly described as being prone to mount Th1-like responses (Gorham et al., 1996; Reiner and Locksley, 1995). It is likely that DR4 mice are also more prone to mount stronger Th1-like responses, as they were generated on a C57Bl/6 background, although this has not been demonstrated. Both strains of mice were epicutaneously exposed to the low (15 µg) or high dose (150 µg) of cat dander extract (CDE) for 10 days followed by intranasal challenges of 10 µg CDE (Figure 1) as done with the BALB/c mice. Allergic airway responses (local inflammation and lung function) were assessed 48 hours after the last intranasal challenge.

Specific Aim 2: To determine the long-term impact of epicutaneous exposure to the high dose of cat dander on allergic airway responses.

RATIONALE AND STUDY DESIGN: Based on anecdotal evidence, it appears that the protective effects of high allergen exposure may diminish if exposure is

discontinued over an extended period of time. In our mouse model, allergen recall 10 days after epicutaneous application of the high dose of cat dander was associated with attenuated allergic airway responses (Figures 2-5). However, whether this response changed with time was not known. Thus, we assessed whether the development of allergic airway responses would still be prevented when assessed upon allergen recall long after epicutaneous exposure to the high dose of cat dander (Figure 6). This Aim was addressed in two ways. In the first manner, it was determined whether the protective effects of the high dose of cat dander changed over time. Mice that were initially exposed to the high dose of cat dander were simply re-challenged intranasally with cat dander after 120 days, instead of after 10 days (Figure 6a). In the second manner, it was determined whether prior exposure to the high dose of cat dander had any influence on subsequent sensitization to cat dander and allergic airway disease. Mice were initially exposed to the high dose of cat dander epicutaneously from days 0-11, and then were exposed to a sensitizing dose of cat dander (15 µg) on days 120-131. (Figure 6a). Allergic airway responses were then assessed 48 hours after the two intranasal allergen re-exposures in both cases (Figures 6b-e).

Specific Aim 3: To establish whether dose-dependent attenuation of allergic airway responses is allergen-specific.

RATIONALE AND STUDY DESIGN: A reduced prevalence of sensitization with increased allergen exposure has been observed with multiple allergens (e.g. cat and dog dander, house dust mite, and rodent urinary allergens). Furthermore, while other experimental models demonstrated attenuation of Th2-like inflammation with different allergens, each model differed by routes of sensitization. Thus, to confirm the generalizability of the protective effects due to high allergen exposure, we assessed whether high doses of another allergen, ovalbumin (OVA), could also attenuate allergic airway responses using the same model of epicutaneous sensitization. Mice were sensitized in the same manner as

done with cat dander. Mice were shaved and tape-stripped on an area of their backs and epicutaneously exposed to two different doses of OVA (500 and 1000 µg) for 10 days. A series of 5 intranasal challenges with 100 µg OVA were administered (Figure 7a). Allergic airway responses were then assessed 48 hours after the last intranasal challenge (Figure 7b-e).

Specific Aim 4: To assess the impact of epicutaneous exposure to the high dose of cat dander on the development of allergic responses to other allergens such as peanut.

RATIONALE AND STUDY DESIGN: It has been reported that individuals living with pets were less likely to be sensitized to other allergens (Ownby et al., 2002; de Meer et al., 2004). Thus, we examined whether epicutaneous exposure to the high dose of cat dander could also influence the *development* of allergic responses to other allergens such as peanut. Mice were epicutaneously exposed to the high dose of cat dander before (days 0-11) and/or concurrently with a sensitizing dose of peanut (days 20-31), and anaphylactic responses were assessed after an intraperitoneal peanut challenge (Figures 8 and 9). It was also determined whether the high dose of cat dander *alone* had any impact on peanut-induced anaphylaxis (Figure 10). Mice were exposed to the high dose of cat dander from days 0-11 followed by peanut alone from days 20-31; anaphylactic responses were then assessed after peanut challenge.

Specific Aim 5: To determine whether epicutaneous exposure to the high dose of cat dander affects allergic responses in established disease.

RATIONALE AND STUDY DESIGN: Patients undergoing allergen immunotherapy, which involves the administration of high allergen doses, had reduced allergic symptoms. As epicutaneous exposure to the high dose of cat dander was able to attenuate allergic airway responses during pathogenesis and amidst Th2 polarizing conditions, we determined whether it had any influence on

allergic airway responses in established disease (Figure 11). Furthermore, since we had observed that concurrent exposure to the high dose of cat dander with a sensitizing dose of peanut prevented peanut-induced anaphylaxis (Figures 8-10), we assessed whether the high dose of cat dander could influence peanut-induced anaphylaxis in mice that were already sensitized to peanut. Mice were epicutaneously sensitized with peanut from days 0-11, followed by epicutaneous application of the high dose of cat dander with and without peanut on days 20-31 (Figure 12 and 13 respectively). Anaphylactic responses were then evaluated after an intraperitoneal challenge with peanut.

CHAPTER 2: METHODS

Mice

4-6 week old female BALB/c purchased from Charles River and C57Bl/6 purchased from Jackson laboratory were housed in an ultraclean facility and allowed to acclimatize for one week prior to beginning experiments. HLA DR4 breeding pairs were purchased from Taconic, and bred at the animal facility at McMaster University. Mice were weaned and transported to St. Joseph's animal facility, where they were aged to 4-6 weeks prior to use. All experimental procedures were conducted in accordance with the Guide for the Human Use and Care of Laboratory Animals and approved by the Animal Research Ethics Board at McMaster University.

Allergens

The cat dander extract (CDE; Greer, Lenoir, NC) was formulated using dander collected from a mixed breed of cats, extract at 1:20 w/v in 0.01 ammonium bicarbonate, dialyzed against distilled water and dried into a de-fatted, and powdered lyophilized cake. The CDE was dissolved to a concentration of 15 μ g/ μ L total protein in phosphate buffered saline (PBS). The low dose (15 μ g CDE) was prepared by performing a 10-fold dilution of the high dose (150 μ g CDE) preparation. The CDE was aliquoted for use at the appropriate concentration and stored at -20°C until use. The intranasal administrations of CDE contained 10 μ g CDE per 25 μ L PBS. These intranasal challenges were prepared using a purified extract of cat dander supplied generously by Prof. Marianne Van Hage (Karolinska Institute, Sweden). This CDE consisted of reduced albumin and endotoxin. All exposures without CDE were done with PBS, referred to as saline throughout my thesis.

Crude peanut extract (Greer) was also a defatted lyophilized powder that was dissolved in PBS at 20 µg/µL for epicutaneous applications and stored at -20°C until use. The peanut used to challenge the mice was dissolved at a 10mg/mL in PBS.

The cat and peanut mixture consisted of the high dose of cat dander (150 µg) combined with the sensitizing dose of peanut (200 µg) and was stored at -20°C until use. These allergens were mixed together at the appropriate concentrations such that each 10 µL epicutaneous application consisted of 150 µg CDE and 200 µg peanut.

Ovalbumin (OVA; Sigma), the albumin obtained from chicken egg whites, was also a lyophilized powder that was dissolved in PBS to a concentration 100µg/µL from which the other doses were prepared. The intranasals for OVA were also prepared from this stock at a concentration of 100 µg per 25 µL PBS. The allergens were stored at -20°C until use.

Sensitization protocol

At 7 weeks of age, mice were shaved in an area (approximately 1 cm²) on their backs near the base of their tails using an electric razor. The stubble was removed using a mechanical razor, and the area was then tape stripped using cellophane tape to disrupt the stratum corneum prior to application of allergen. A volume of 10 µL of dissolved allergen was applied to the area using a pipette tip and allowed to dry before the mice were returned to their cages for a total of 10 days (or as specified in the aim). The area was tape-stripped prior to application of allergen each day.

For allergic airway models, the mice were re-exposed to the allergen via intranasal administrations of the allergen in a volume of 25 μ L while the mice were under light anaesthetic (2% isoflurane); these mice received CDE intranasally once per day for a total of 5 days. The days are specified within each Aim. Features of allergic airway disease (lung function and eosinophilia) were assessed 48 hours after the last intranasal administration. Airway resistance measurements were determined using a mechanical ventilator and nebulized methacholine challenges. Bronchoalveolar lavage was collected to quantify the inflammation in the airways. Eosinophils within the lung tissue were determined after fixing the left lung lobe and staining lung sections with hematoxylin and eosin.

For anaphylaxis, mice were given an intraperitoneal injection consisting of 5 mg of crude peanut extract dissolved in PBS. Mice were monitored for clinical signs of anaphylaxis for 40 minutes (ear canal digging, reduced motion, puffy eyes and ears, motionless, no response to prodding and convulsion/tremors (endpoint)) following injection. Rectal temperatures were obtained every 10 minutes. Clinical scores were assigned based on severity of clinical signs (0 – no signs, 1-ear canal digging, 2- reduced motion and puffy eyes and ears, 3-motionless, 4-no response to prodding and 5- convulsion/tremors). As an indicator of vascular leakage, the percentage of packed red blood cells (RBC) was determined by collecting blood by a retroorbital bleed into heparinized microcapillary tubes while the mice were anaesthetized. The blood samples were centrifuged for 1 minute using a microhematocrit centrifuge and the percentage of packed RBCs was calculated based on the fraction of packed RBCs and plasma in the tube.

Lung function measurements

Airway responsiveness of the mice was assessed using airway resistance measurements to nebulized methacholine (MCh) challenges at 3.1, 6.25, 12.5, 25, and 50 mg/mL. Mice were weighed and anaesthetized with an intraperitoneal (IP) injection of xylazine hydrochloride (Bayer, Toronto, ON; 10 mg/kg) and sodium pentobarbital (Ceva Sante Animale, Lenexa, KA; 30mg/kg) diluted in PBS. Prior to performing surgeries, surgical plane of anaesthesia was assessed via a toe pinch test. Mice were tracheostomized with a blunted 18-gauge needle for BALB/c mice and a blunted 19-gauge needle for C57Bl/6 and DR4 mice, to assess airway resistance using the Flexivent (SCIREQ, Montreal, QC) apparatus. Flexivent is a mechanical ventilator used for small animals. MCh challenges were administered via the attached Aeroneb ultrasonic nebulizer (SCIREQ, Montreal, QC), which efficiently delivers the aerosolized MCh deep into the lungs. The blunted needles and Flexivent were calibrated prior to use. The cannulated mouse was connected to the Flexivent via a Y tubing and a script for data collection of lung mechanics was initiated; mice were ventilated at 150 breaths/min, at a tidal volume of 10 mL/kg with a positive end-expiratory pressure of 2 cm H₂O. To ablate endogenous respiratory efforts during measurements, mice were paralyzed with rocuronium (Zemuron; 20mg/kg). Prior to the nebulization of each MCh dose, lungs were inflated to total lung capacity by a 6 second inspiration to normalize the lung volume history. Baseline resistance measurements were established by adding saline to the nebulizer. A sequence of 8 measurements were recorded over a 3 minute period; specifically, 8 “Quicksnap-150” perturbations representing single inspiration/expiration of 0.4 seconds in duration with a volume amplitude of 10 ml/kg were recorded. MCh challenges were performed after baseline measurements. The nebulizer was dried carefully between each dose and washed between mice.

The airway resistance for each MCh dose was determined by taking the peak (maximal) resistance value from a defineable pattern found within the 8

perturbations recorded following each nebulization. The pattern consisted of increasing resistance until a peak, followed by a decrease in resistance; any airway resistance measurement that deviated considerably from this trend was excluded. Mice with baseline resistance values greater than 1 cm H₂O/mL/s due to airway obstruction or an artefact were excluded.

BALF processing

Bronchoalveolar lavage fluid (BALF) was collected to quantify the cellular inflammation in the airways. A volume of 250 µL of PBS was injected into the airways through the cannulated needle in the trachea using a 1 mL syringe. The chest area of the mouse was massaged gently and the BALF was aspirated back into the syringe. The process was then repeated with another 250 µL PBS. The BALF was centrifuged at 150g for 10 minutes and then the cells were resuspended to obtain a total cell count using 0.4% trypan blue. The BALF was then pelleted onto microscope slides using a cytopspin (Cytospin 3, Shandon). The pelleted samples were then stained with the Wright-Giemsa (W-G; Sigma) stain. Cells were fixed with 100% methanol (Caledon), air-dried, and stained with the W-G stain. 200 cells were counted per slide, and differentiated into either: neutrophil, lymphocyte, eosinophil or macrophage cells. Two slides per mouse were counted and counts were averaged between the two slides to obtain a percent differential. Absolute cell counts were obtained by multiplying the percent differential by the total cell count obtained using trypan blue.

Hematoxylin and Eosin stain

Eosinophil infiltration into the peribronchial area of the lung was quantified by histology. Left lungs were filled with 10% buffered formalin to a pressure of 20 cmH₂O, and allowed to fix for 48 hours. The lobes were then cut into inferior and

superior segments to obtain a cross section of the primary airway; the inferior left lobe was used. The section was processed and embedded in paraffin wax. 3-micron thick transverse cross sections were then collected on microscope slides. These sections were then stained with hematoxylin and eosin (H&E). Samples were rehydrated and stained in modified Mayer's hematoxylin (2g/L hematoxylin (Sigma-Aldrich), 60g/L AlK(SO₄)₂ (Sigma-Aldrich), 0.2g/L sodium iodate (Sigma-Aldrich), 1g/L citric acid, chloral hydrate 50g/L (Sigma-Aldrich)) for 10 minutes. The granules were stained with buffered 0.05% Eosin (pH 5.5; acetate buffer containing Eosin Y) for 5 minutes.

Images of all H&E stained sections were viewed under a light microscope (Olympus BX40; Carsen Group Inc., Markham Ontario) at 40x objective magnification. Images were collected and analyzed using a customized digital image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada). Images that satisfied the following criteria for analysis were included: i) airway was intact ii) there was no tethering (that is, areas of connective tissue) between the airway wall and vessels. Eosinophils were counted within a 50-micron wide band area extending from the smooth muscle layer-tissue interface towards the lung tissue. Eosinophils were counted based on the primary criteria of a clearly visible bilobal nucleus, with eosin (pink)-stained cytoplasm (granules). The band areas and eosinophil counts of all images of an airway were summed to express the eosinophil count as eosinophils/mm².

Statistical analyses

All values are expressed as averages with standard deviation. Outliers were determined based on the Graphpad Grubbs outlier test using a significance level where alpha = 0.05 and excluded. A statistically significant difference was detected using a one-way analysis of variance (ANOVA). Tukey post hoc analysis

was used when all means were compared against each other. When only selected means were compared, a Bonferroni correction was performed, as recommended by Graphpad Prism. A difference was considered statistically significant with a p value <0.05 .

CHAPTER 3: RESULTS

Specific Aim 1: To determine if attenuation of allergic airway responses with epicutaneous exposure to the high dose of cat dander is strain-dependent.

Studies analyzing family history or twins along with the identification of multiple asthma susceptibility genes have supported a significant role for genetics in asthma (Ober and Yao, 2011). Moreover, the degrees and nature of allergic airway responses have also been shown to vary in genetically different strains of mice despite identical sensitization protocols (Kodama et al., 2010; Zhang et al., 1997; Hirota et al., 2008). Thus, it was determined whether the attenuation of allergic airway responses with epicutaneous exposure to the high dose of cat dander was strain-specific. C57Bl/6 mice and mice that were transgenic for the human MHC class II molecule HLA DRB1*0401 (DR4) were primed and challenged in the same manner as the BALB/c mice. Briefly, at 4-6 weeks of age, these mice were shaved and tape stripped on a small area of their backs, followed by epicutaneous application of saline, 15 or 150 µg cat dander extract (CDE) for 10 days. These mice then received intranasal administrations of CDE, and allergic airway responses were assessed 48 hours after the last intranasal challenge (Figure 1). The dose of 15 µg of CDE on the skin was referred to as the low dose while 150 µg of CDE was referred to as the high dose. Overall, the trends observed in the BALB/c mice were also largely observed in C57Bl/6 and DR4 mice.

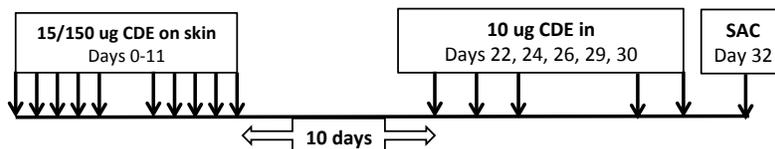


Figure 1. Sensitization timeline used in BALB/c, C57Bl/6 and DR4 mice. 4-6 week old mice were shaved and tape-stripped on an area of their backs. Either saline, 15 µg cat dander extract (CDE) or 150 µg CDE was then applied epicutaneously to the exposed area in a volume of 10 µL for 10 days as shown. Intranasal (in) administrations of 10 µg CDE or saline were given in 25 µL of volume as indicated. Allergic airway responses were assessed 48 hours after the last intranasal. SAC, sacrificed

Airway and lung eosinophilia was attenuated in all three strains with epicutaneous exposure to the high dose of cat dander

As observed in BALB/c mice (Figures 2b and 3a), BALF eosinophil counts and eosinophils in the peribronchial lung tissue were attenuated in C57Bl/6 mice receiving the high dose of CDE on the skin, compared to C57Bl/6 mice receiving the low dose of CDE (Figure 2d and 3b). However, the differences in C57Bl/6 were not statistically significant. BALF eosinophil counts reflected airway eosinophil counts. Mice receiving saline on the skin but CDE intranasally (referred to as saline skin) did not have significantly increased eosinophils in the BALF or in the peribronchial tissue compared to naive mice. This finding supported the notion that epicutaneous application of CDE was likely contributing to the responses observed instead of the intranasal challenges alone. In DR4 mice, total cell and eosinophil counts in the BALF were significantly lower in the high dose mice compared to the low dose mice (Figure 2e and f). Eosinophil counts in the peribronchial tissue of the DR4 mice were not increased in the high dose group, but were increased in the low dose group (Figure 3c). Thus, airway and lung eosinophilia appeared to be attenuated in both C57Bl/6 and DR4 mice, as observed in the BALB/c mice. When the data from all three strains were pooled (Figure 2h and 3d), eosinophilia was reduced in mice epicutaneously exposed to the high dose of CDE, despite different susceptibilities to Th2 type responses between the strains. This finding highlights the generalizability of the effect.

In contrast to BALF eosinophils, BALF neutrophils, lymphocytes and macrophages were comparable between low and high dose BALB/c mice, but increased compared to naive BALB/c mice (data not shown). In C57Bl/6 and DR4 mice BALF neutrophils, lymphocytes and macrophages did not differ between naive, low and high dose mice (only BALF neutrophils in DR4 mice were

significantly greater in the low dose mice compared to naive mice) (data not shown).

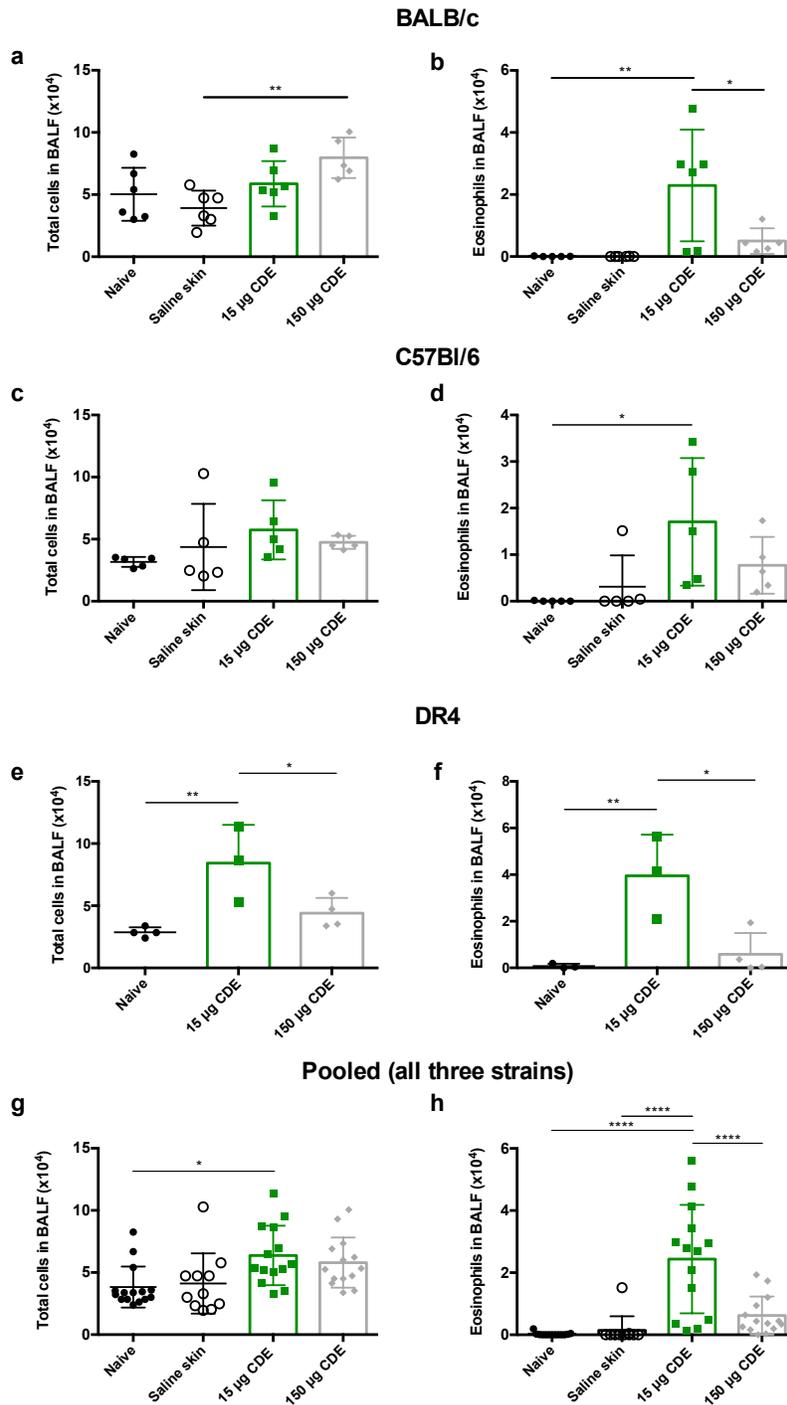


Figure 2. The effect of cat dander dose on airway inflammation in all three strains. Bronchoalveolar lavage cell fluid (BALF) was pelleted onto a microscope slide using the Cytospin. The cells were differentiated and quantified in BALF based on morphological characteristics after Wright-Giemsa staining. All values are expressed as means \pm standard deviations. *p<0.05; **p<0.01

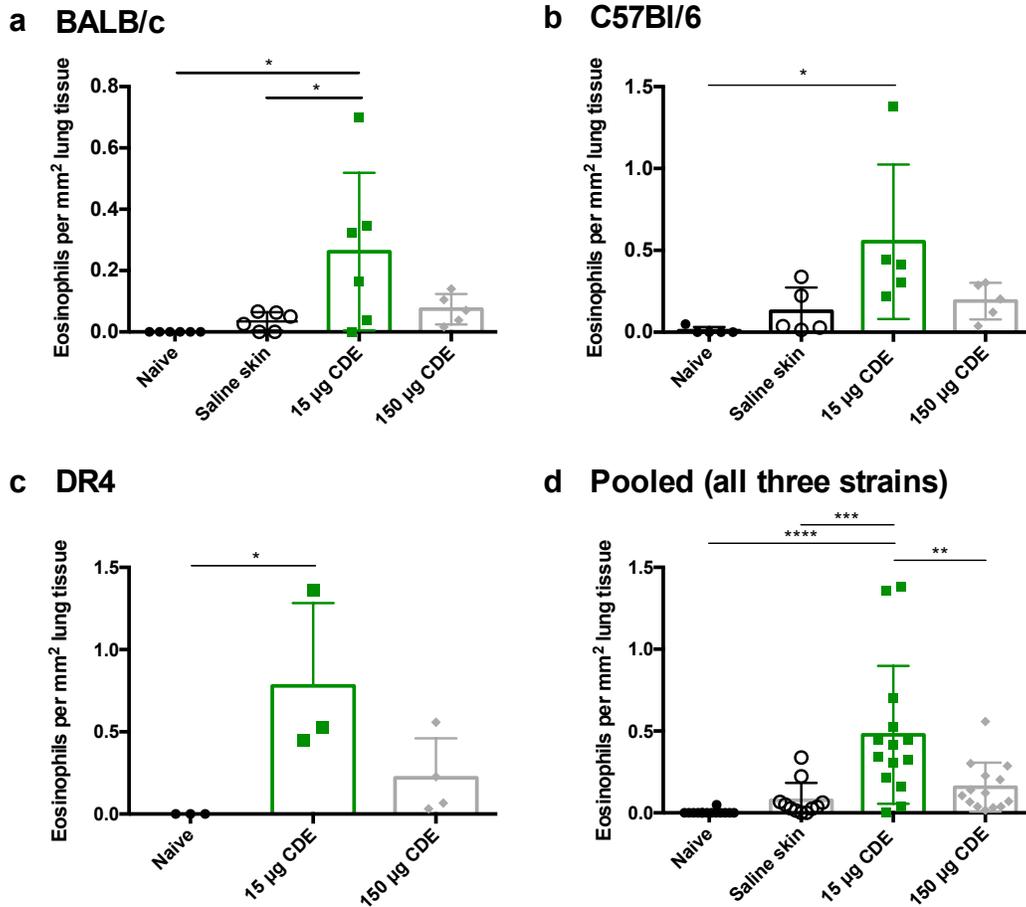


Figure 3. The effect of cat dander dose on lung eosinophilia in all three strains. Left lobes of the lung were fixed in 10% buffered formalin, embedded in paraffin wax and 3 micron sections were cut to obtain transverse sections of the lung. Sections were stained with hematoxylin and eosin, and eosinophils were quantified within 50 microns of the peribronchial lung tissue under 40x magnification. All values are expressed as means \pm standard deviations. * p <0.05

Mucus-producing goblet cell counts appeared to be attenuated in all three strains with epicutaneous exposure to the high dose of cat dander

Mucus-secreting goblet cell (GC) counts were attenuated in mice receiving the high dose of cat dander compared to mice receiving the low dose in all three strains, although the differences were not statistically significant. This difference was most apparent when the GC counts from all three strains were pooled (Figure 4d); when the strains were analyzed separately, the GC counts were not as reduced in mice receiving the high dose across all three strains likely due to a lack of statistical power (small number of mice and large variability). GC counts in BALB/c mice were only slightly lower in the high dose group compared to the low dose group (96 ± 40 GCs per mm airway and 78 ± 48 GCs per mm airway respectively) (Figure 4a). In C57Bl/6 mice, mucus-secreting GC counts were lower in the high dose group, but were significantly increased in the low dose group compared to naive mice (Figure 4b). Although the mucus-secreting GC counts were approximately 2-fold lower in the high dose group compared to the low dose group in C57Bl/6 mice (40 ± 37 GCs per mm airway and 81 ± 48 GCs per mm airway respectively), these differences were not statistically significant. Similar to the BALB/c mice, the average mucus-secreting GC counts in DR4 mice were slightly lower in the high dose group compared to the low dose group (23 ± 20 GC per mm of the airway and 33 ± 28 GC per mm of the airway respectively) (Figure 4c).

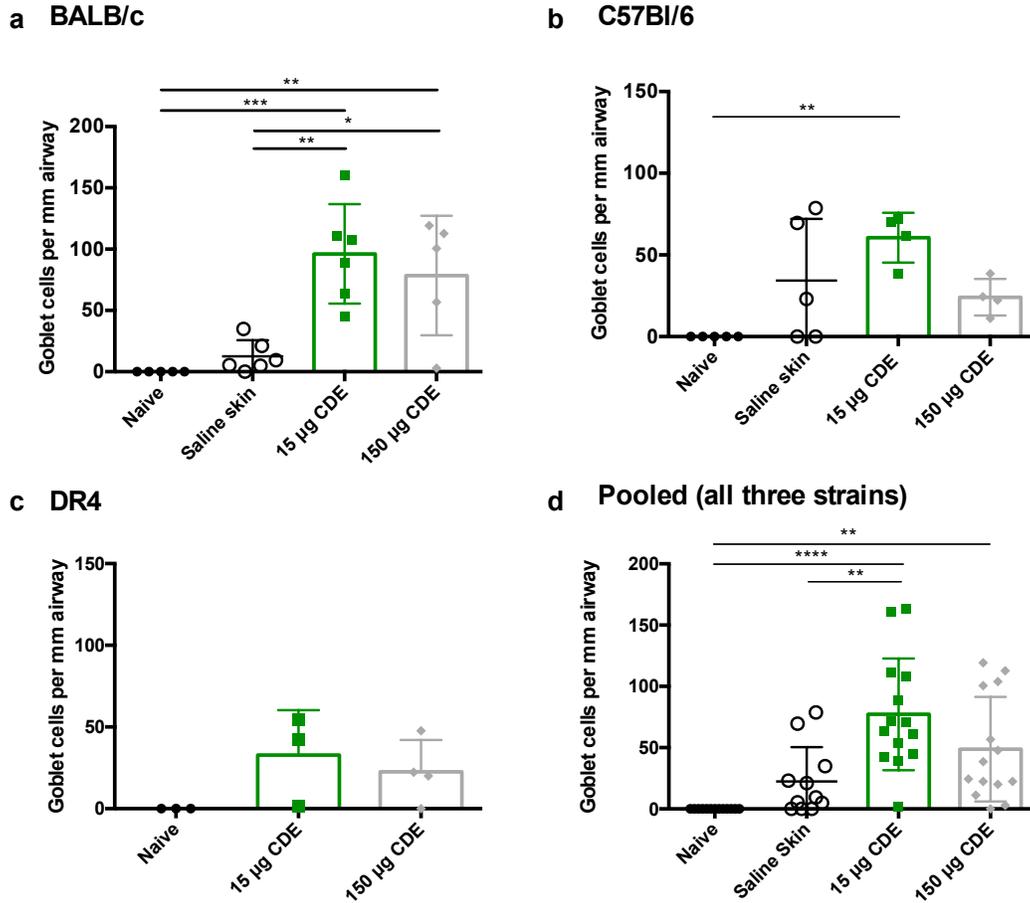


Figure 4. Mucus secreting goblet cell (GC) counts in primary airway of all three strains. Left lobes of the lung were fixed in 10% buffered formalin, embedded in paraffin wax and 3 micron sections were cut to obtain transverse sections of the lung. Sections were stained with periodic acid-Schiff, and GCs were quantified in the airway under 20x magnification. All values are expressed as means \pm standard deviations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Airway resistance was attenuated in all three strains with epicutaneous exposure to the high dose of cat dander

In C57Bl/c mice, airway resistance was not increased in the high dose mice compared to the naive mice, but was significantly increased in the low dose group at the indicated methacholine (MCh) doses (Figure 5b). In DR4 mice, airway resistance measurements also were not increased in the high dose mice compared to the naive mice, while they were significantly increased in the low dose group at the indicated MCh doses (Figure 5c). Thus, airway resistance was attenuated in mice receiving the high dose of cat dander across all three strains. Again, this was observed even when the measurements from all three strains were pooled (Figure 5d).

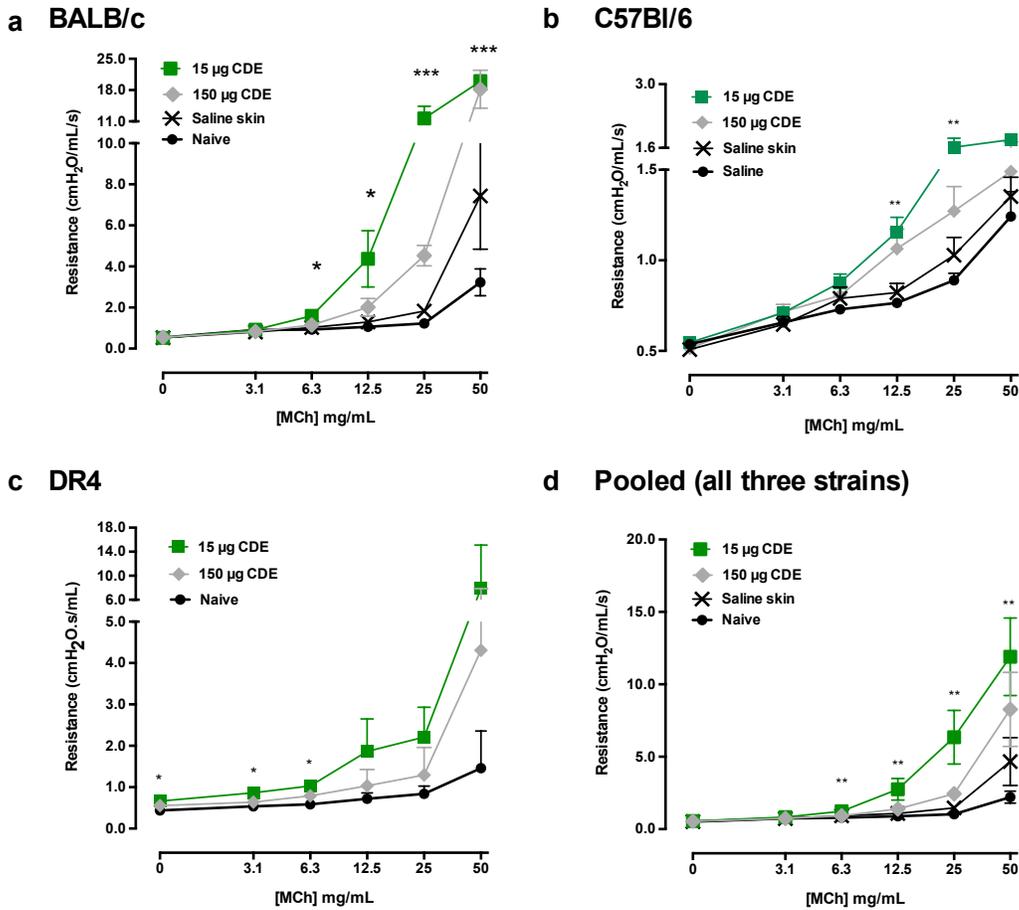


Figure 5. Airway resistance measurements in all three strains. Mice were anaesthetized and tracheostomized and ventilation was controlled using the Flexivent mechanical ventilator. To assess airway responsiveness, airway resistance was measured after intratracheal administrations of increasing nebulized methacholine (MCh) challenges. Baseline resistance was determined with nebulized saline (PBS). At each MCh concentration, a sequence of 8 measurements was recorded over a 3-minute period. Groups were compared against naive mice for statistical analyses of airway resistance measurements. All values are expressed as means \pm standard deviations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ reported for differences between mice exposed to the low dose of CDE and naive mice.

Specific Aim 2: To determine the long-term impact of epicutaneous exposure to the high dose of cat dander on allergic airway responses.

The protective effect of epicutaneous exposure to the high dose of cat dander against BALF eosinophilia diminished with time

Some college students who returned home for Thanksgiving reported developing allergic symptoms towards their cats to which they were not previously allergic. Although this evidence is anecdotal, it has been suggested that perhaps the protective effects from being raised with a cat may wane if no longer exposed to high levels of cat allergen for an extended period of time (Erwin et al., 2014). Thus, we sought to determine whether the protective effects of epicutaneous exposure to the high dose of cat dander also decreased with time in our murine model. DR4 mice were epicutaneously exposed to the high dose of CDE from days 0-11, followed by intranasal challenges with CDE on days 22, 24 and 26. Allergic airway responses were then assessed after intranasal recall challenges with CDE 120 days after the epicutaneous exposure (Figure 6a). DR4 mice that were recalled with allergen 10 days after the epicutaneous exposures (Figure 1) were also included in these analyses to determine whether the responses changed with time.

When mice were recalled with CDE 10 days after epicutaneous exposure to the high dose of CDE (150 µg (10d)), airway eosinophilia was attenuated compared to mice epicutaneously exposed to the low dose of CDE (15 µg (10d)) (Figure 6c). In addition, the airway eosinophilia in the 150 µg (10d) did not differ from the mice that only received CDE during the intranasal recall challenges (Recall (120d)). Interestingly, mice that were recalled with CDE 120 days after epicutaneous exposure to the high dose of CDE (150-Sal (120d)) appeared to have increased BALF eosinophil counts compared to mice that were recalled with

allergen 10 days later (150 μg (10d)); however, these differences were not statistically significant. The degree of BALF eosinophilia in the 150-Sal (120d) mice was also comparable to the levels observed in the 15 μg (10d) mice and in the age-matched Sal-15 (120d) mice. Similar trends were also observed with the percentage of BALF eosinophils (Figure 6d). Thus, our findings suggested that the protective effects against airway eosinophilia due to epicutaneous exposure to the high dose of cat dander decayed with time. It should be noted that although both the 150-Sal (120d) group and 150 μg (10d) groups received the same number of intranasal challenges with CDE in total, the 150 μg (10d) received these challenges consecutively, just prior to assessment of allergic airway responses. Interestingly, despite receiving 5 consecutive intranasal challenges of CDE, BALF eosinophil counts and percentages were still lower in the 150 μg (10d) mice, compared to the 150-Sal (120d) mice. Although this difference was statistically significant, the relatively increased numbers appear to support the notion that the protective effects of epicutaneous exposure to the high dose of cat dander diminished with time. Furthermore, the protective effects with high cat dander exposure were also observed when allergic airway responses were assessed 40 days after epicutaneous allergen exposure (data not shown). Thus, these data suggested that these protective effects began to decay between 40 and 120 days after epicutaneous exposure in our mouse model.

Airway resistance measurements did not differ between 150-Sal (120d) mice and Recall (120d) mice despite increased BALF eosinophilia in the former group (Figure 6e). Airway resistance measurements in these mice were also comparable to those of the 150 μg (10d) mice suggesting that all of the mice exposed to the high dose of cat dander did not demonstrate signs of AHR.

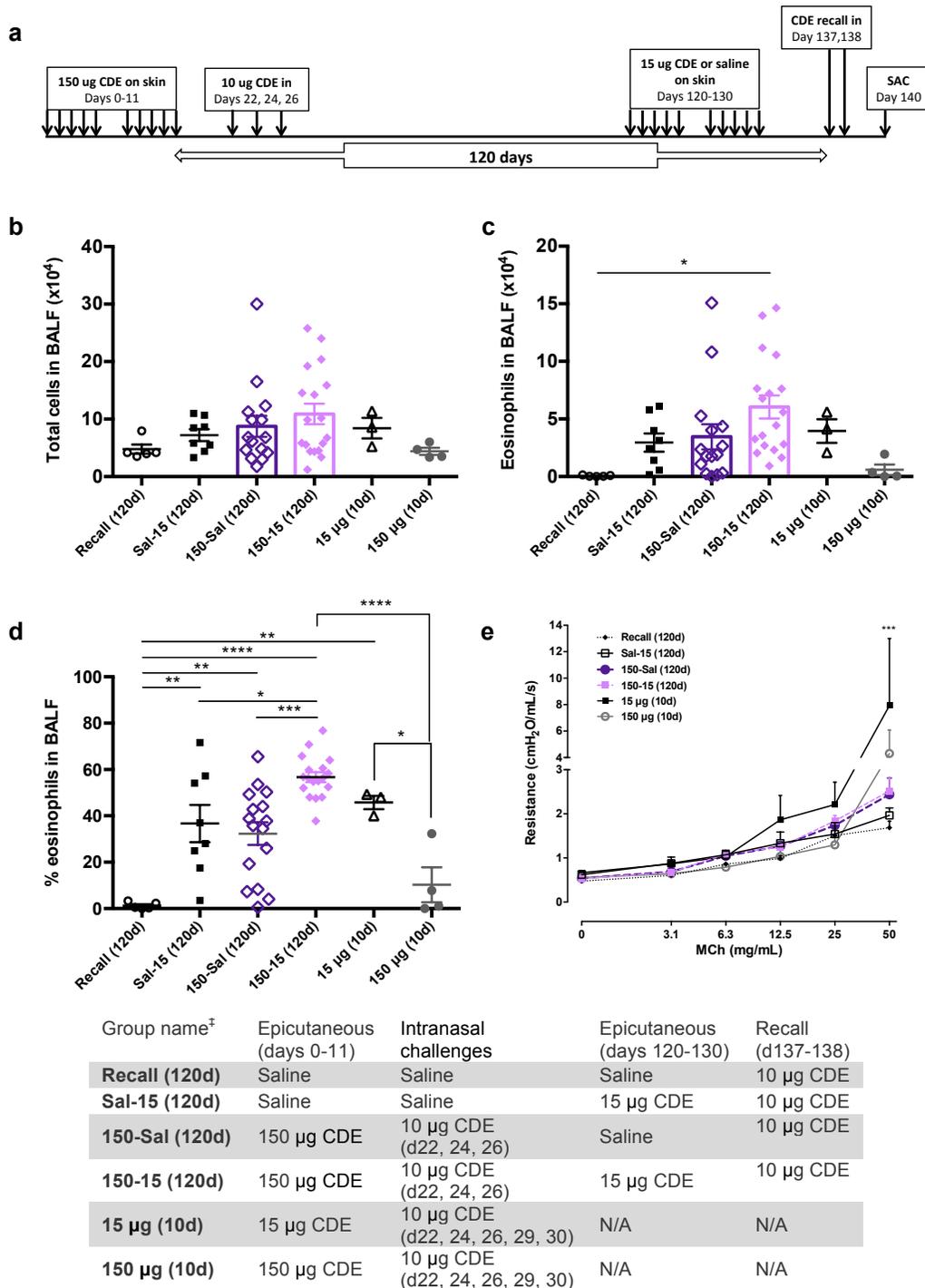


Figure 6. The change in allergic airway responses with time after short-term epicutaneous exposure to the high dose of cat dander. a) Sensitization schedule: 4-6 week old female DR4 mice were exposed to cat dander extract (CDE) as detailed in schedule. in, intranasal. SAC, sacrificed. Groups are outlined in the table. ‡d, days indicated refer to period of time between epicutaneous exposure and recall b) Total cell and c) eosinophil counts in BALF d) Percentage of eosinophils in BALF e) Airway

resistance measurements. Groups were compared against naive mice for statistical analyses of airway resistance measurements. All values are expressed as means \pm standard deviations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ reported for differences between mice exposed to the low dose of CDE and naive mice.

Prior acute epicutaneous exposure to the high dose of cat dander contributed to worsening airway eosinophilia with later exposure to a sensitizing dose of cat dander

We also hypothesized that, if the protective effect of epicutaneous exposure to the high dose of cat dander waned with time, later epicutaneous application of a sensitizing dose of cat dander should result in the development of allergic airway responses. To test this hypothesis, DR4 mice were shaved, tape-stripped, and received an epicutaneous application of saline or the high dose of CDE from days 0-11, followed by intranasal administrations of saline or CDE on days 22, 24 and 26. The low dose of CDE (also referred to as the sensitizing dose; 15 µg) or saline was then applied to the same area on the backs and in the same manner (i.e. after shaving and tape-stripping) from days 120-130. Allergic airway responses were assessed 48 hours after allergen re-exposure to the lungs (Figure 6a). The mice that were recalled with allergen 120 days later were much older at the time of challenge compared to the DR4 mice recalled 10 days later. Thus, to determine if the degree of eosinophilia was stable with increasing age, the 15 µg (10d) group was also included in these analyses.

As expected, greater numbers of BALF eosinophils were detected in the mice that received saline on the skin from days 0-11 and the sensitizing dose of 15 µg CDE from days 120-130 (Sal-15 (120d)) compared to the Recall (120d) mice (Figure 6c). BALF eosinophils were also comparable between the Sal-15 (120d) and 15 µg (10d) mice demonstrating the stability of the eosinophil response with increasing age. Although the Sal-15 (120d) mice only received 2 intranasal doses of CDE compared to the 5 intranasal doses received by the 15 µg (10d) mice, the number and percentage of BALF eosinophils were comparable between these two groups. This suggested that 2 intranasal challenges may have been sufficient for eosinophil influx into the airways. Interestingly, prior acute epicutaneous

exposure to the high dose of CDE on days 0-11, followed by epicutaneous application of the low dose of CDE from days 120-130 (150-15 (120d)) was associated with increased BALF eosinophil counts and percentages compared to Sal-15 (120d) mice (Figure 6c and d). Mean BALF eosinophil counts were approximately 2-fold greater in the 150-15 (120d) mice ($6.0 \times 10^4 \pm 4.2 \times 10^4$ BALF eosinophils) compared to the Sal-15 (120d) and 150-Sal (120d) mice ($3.0 \times 10^4 \pm 2.2 \times 10^4$ and $3.5 \times 10^4 \pm 4.2 \times 10^4$ respectively). The mean percentage of eosinophils was $56.7 \pm 9.1\%$ in the 150-15 mice compared to $36.7 \pm 22.7\%$ in the Sal-15 (120d) and $32.3 \pm 19.5\%$ in the 150-Sal mice. The mean percentage of BALF eosinophils in the 150 μg (10d) mice was only $10.2 \pm 15\%$. Thus, it appeared as if the diminished protective effects of the high dose cat dander exposure and the effects of the sensitizing dose of cat dander were additive, and collectively contributed to worsening airway eosinophilia.

Airway resistance measurements did not differ between the 150-15 (120d) and Recall (120d) mice despite worsening airway eosinophilia in the former group. Similarly, airway resistance measurements did not differ between Sal-15 (120d) and Recall (120d) mice, despite increased BALF eosinophilia in the Sal-15 (120d) group (Figure 6e).

Specific Aim 3: To establish whether dose-dependent attenuation of allergic airway responses occurs with other allergens.

Attenuation of airway eosinophilia was also observed with epicutaneous exposure to a high dose of ovalbumin

To determine whether the dose-dependent effects on the development of allergic airway responses were specific to cat dander, a different allergen, ovalbumin (OVA), was also tested. BALB/c mice were shaved and tape-stripped on their backs, and saline, 500 or 1000 µg of OVA was applied to the area from days 0-11; OVA was then administered intranasally as indicated, and allergic airway responses were evaluated 48 hours after the last intranasal challenge (Figure 7a). Naive mice received saline instead of allergen all throughout the sensitization protocol. While total BALF cells were comparable between all groups, BALF eosinophils were significantly increased in mice that received 500 µg of OVA epicutaneously compared to naive mice ($p = 0.0397$) (Figure 7b-c). As observed with CDE, BALF eosinophil counts were lower in the mice epicutaneously exposed to 1000 µg of OVA (higher dose) but did not significantly differ from naive mice due to lack of statistical power (small number of mice and large variability). Furthermore, although the average BALF eosinophil counts in the 1000 µg OVA mice was approximately 50% lower than that of the 500 µg OVA mice, this difference was also not statistically significant for the same reasons. The average eosinophil counts in the peribronchial tissue were comparable between the 500 µg OVA and the 1000 µg OVA mice (Figure 7d); this may have been due to the variability within the 1000 µg OVA group. Thus, eosinophilia was attenuated with high dose exposure to two sources of allergens, cat dander and OVA.

Airway resistance measurements did not differ between mice sensitized with OVA and naive mice (Figure 7e). Furthermore, the trends observed for the dose-dependent effects of OVA were similar to the trends seen for cat dander with respect to eosinophilia. However, the degrees of response to OVA (mean total cell and eosinophil counts in BALF and eosinophils counts in lung tissue) were somewhat greater than those observed for cat dander. Thus, our data suggest that the dose-dependent attenuation of eosinophilic inflammation may not be specific to a single allergen.

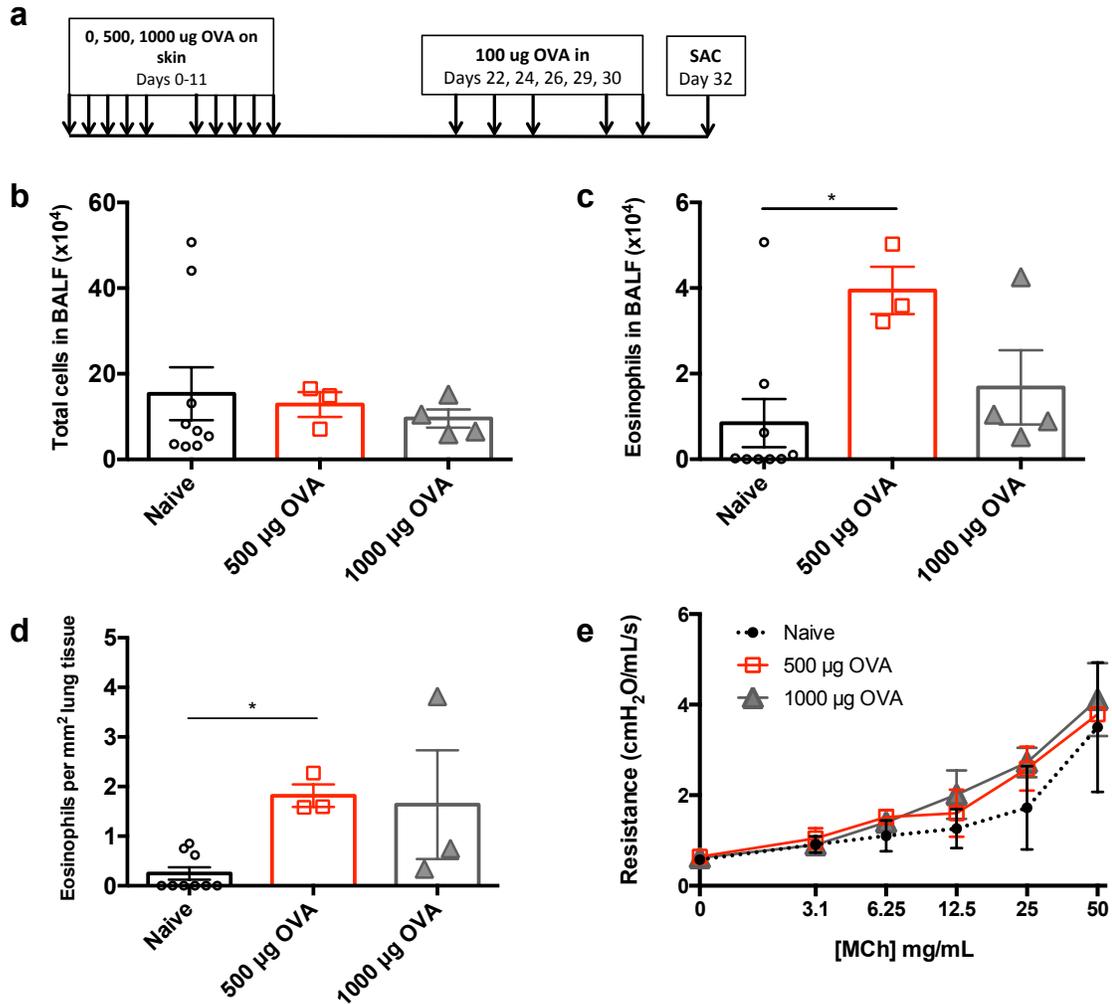


Figure 7. The dose-dependent effects of ovalbumin (OVA) on the development of allergic airway responses in BALB/c mice. a) 4-6 week old female BALB/c mice were shaved and tape-stripped, followed by epicutaneous application of saline, 500 µg OVA or 1000 µg OVA for 10 days as shown. These mice were administered 100 µg OVA intranasally (in) on days 22, 24, 26, 29, and 30. Allergic airway responses were assessed 48 hours after the last intranasal challenge. SAC, sacrificed b) Total cell and c) eosinophil counts in the BALF d) Eosinophils in the peribronchial lung tissue e) Airway resistance measurements with increasing doses of nebulized methacholine (n= 3-9 mice/group). Groups were compared against naive mice for statistical analyses of airway resistance measurements. All vals are expressed as means \pm standard deviations. *p<0.05 reported for differences between mice exposed to the low dose of OVA and naive mice.

Specific Aim 4: To assess the impact of epicutaneous exposure to the high dose of cat dander on the development of allergic responses to other allergens such as peanut.

Concurrent epicutaneous exposure to the high dose of cat dander and a sensitizing dose of peanut was sufficient and necessary to prevent peanut-induced anaphylaxis

Based on its protective effects against the development of allergic responses (Figures 2-5), we assessed whether epicutaneous exposure to the high dose of cat dander also influenced allergic responses to other allergens such as peanut. The high dose of cat dander (classified as “cat”) was applied to the skin from days 0-11, followed by epicutaneous application of a mixture consisting of the high dose of cat dander and a sensitizing dose of crude peanut extract (referred to as cat+peanut) on days 20-31 (Figure 8a). Mice sensitized with peanut only were used as a control. Anaphylactic responses to peanut after an intraperitoneal challenge with 5 mg of crude peanut extract were assessed, and the effect of the high dose of cat dander on these responses was evaluated. Compared to the peanut control group, mice receiving the high dose of cat dander followed by the cat and peanut mixture did not demonstrate any signs of anaphylaxis. Specifically, the rectal temperatures did not decrease, and no clinical signs such as ear canal digging or reduced motion were observed (Figure 8b-d). Hematocrit concentrations, indicative of vascular leakage, were also not increased (Figure 8e). Next, we assessed whether exposure to the high dose of cat dander was necessary prior to concurrent exposure to cat and peanut to prevent peanut-induced anaphylaxis. Mice received saline from day 0-11, followed by the cat+peanut mixture on days 20-31 (Figure 9a). Upon challenge with peanut, these mice also did not develop any signs of anaphylaxis compared to the peanut control group (Figure 9b-e). Finally, to determine whether this protective effect

was dependent on concurrent exposure to both allergens, the effects of the allergens applied separately were tested. Mice were epicutaneously exposed to the high dose of cat dander alone from days 0-11 followed by epicutaneous application of the sensitizing dose of peanut alone on days 20-31 (Figure 10a). Upon challenge, rectal temperatures decreased in these mice, and clinical scores and hematocrits were increased; these responses were comparable to the responses in the peanut control group (Figure 10b-e). Based on these findings, epicutaneous exposure to the high dose of cat dander in the presence of a sensitizing dose of peanut was sufficient (Figures 9a-e) and necessary (Figure 10a-e) to prevent peanut-induced anaphylaxis in our mouse model.

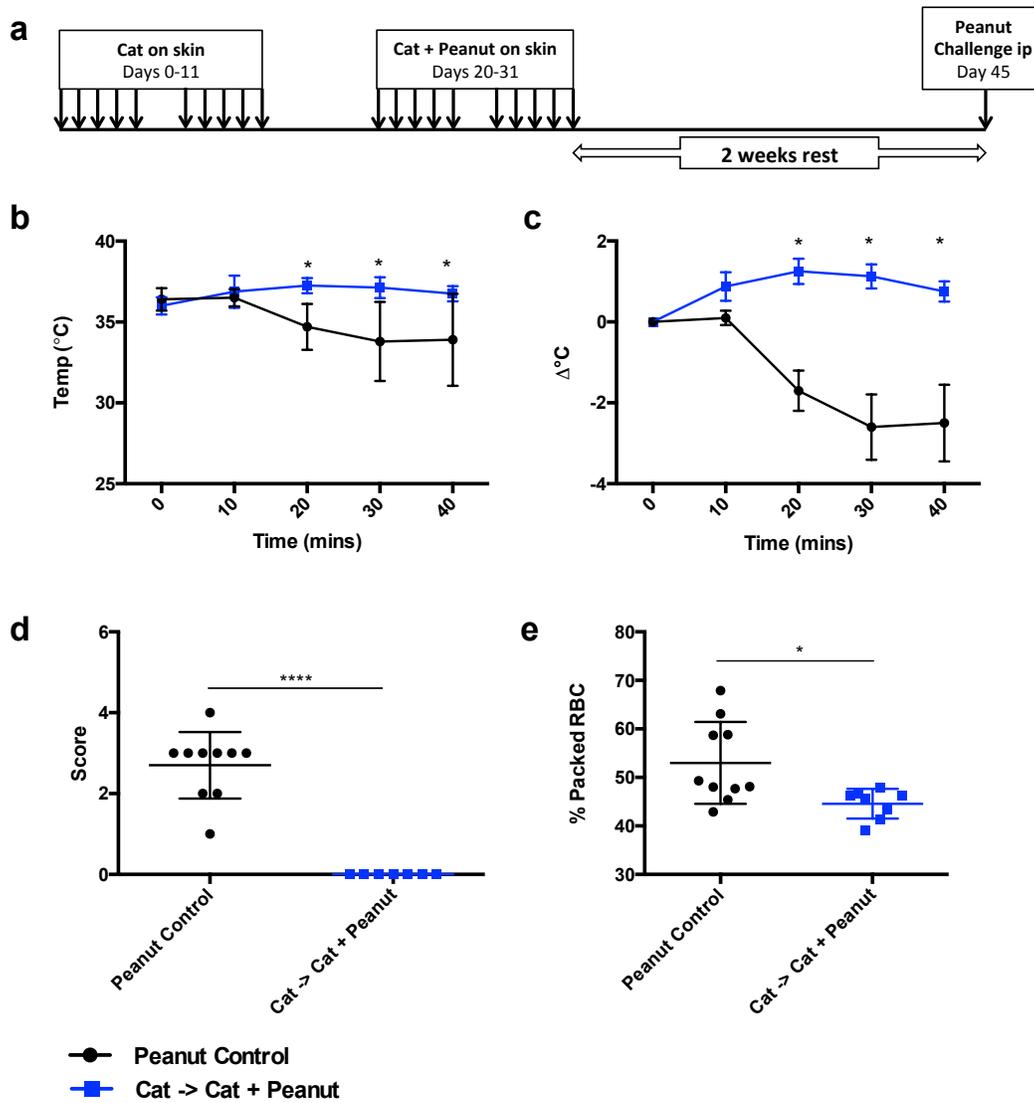


Figure 8. The effect of epicutaneous exposure to the high dose of cat dander prior to concurrent exposure with peanut on peanut-induced anaphylaxis. a) Sensitization schedule: female C57Bl/6 mice were shaved and tape-stripped followed by epicutaneous application of high dose cat dander (referred to as cat) from days 0-11. From days 20-31, the same area was shaved and tape-stripped, followed by epicutaneous application of a mixture consisting of high dose cat dander and a sensitizing dose of crude peanut extract (cat + peanut). Peanut control mice were shaved, tape-stripped on their backs, and saline was applied to the area from days 0-11; a sensitizing dose of peanut was applied in the same manner and area from days 20-31. Mice were intraperitoneally challenged with 5 mg crude peanut extract and signs of anaphylaxis were monitored: b) rectal temperatures over time, c) changes in rectal temperature over time, d) clinical scores, and e) hematocrit (%packed red blood cells (RBC)). All values are expressed as means \pm standard deviations. * $p < 0.05$; **** $p < 0.0001$

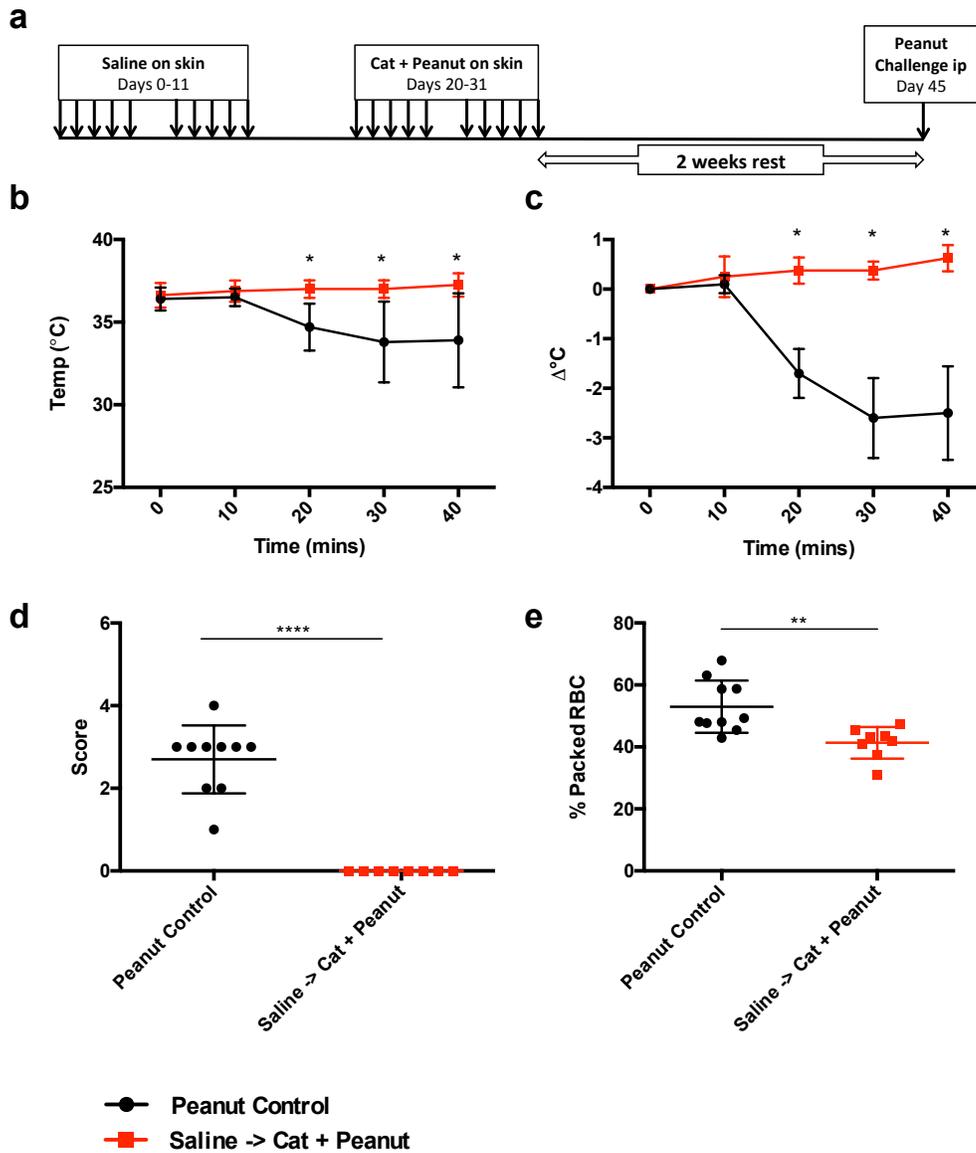


Figure 9. The effect of concurrent epicutaneous exposure to the high dose of cat dander and peanut on peanut-induced anaphylaxis. a) Sensitization schedule: female C57Bl/6 mice were shaved and tape-stripped followed by epicutaneous application of saline from days 0-11. From days 20-31, a mixture consisting of high dose cat dander and a sensitizing dose of crude peanut extract (cat + peanut) was applied epicutaneously in the same manner. Peanut control mice were shaved and tape-stripped followed by epicutaneous application of saline from days 0-11 and then received epicutaneous application of peanut on days 20-31. Mice were intraperitoneally challenged with 5 mg crude peanut extract and signs of anaphylaxis were monitored: b) rectal temperatures over time, c) changes in rectal temperatures over time, d) clinical scores, and e) hematocrit (%packed red blood cells (RBC)). * $p < 0.05$; **** $p < 0.0001$; ** $p < 0.01$

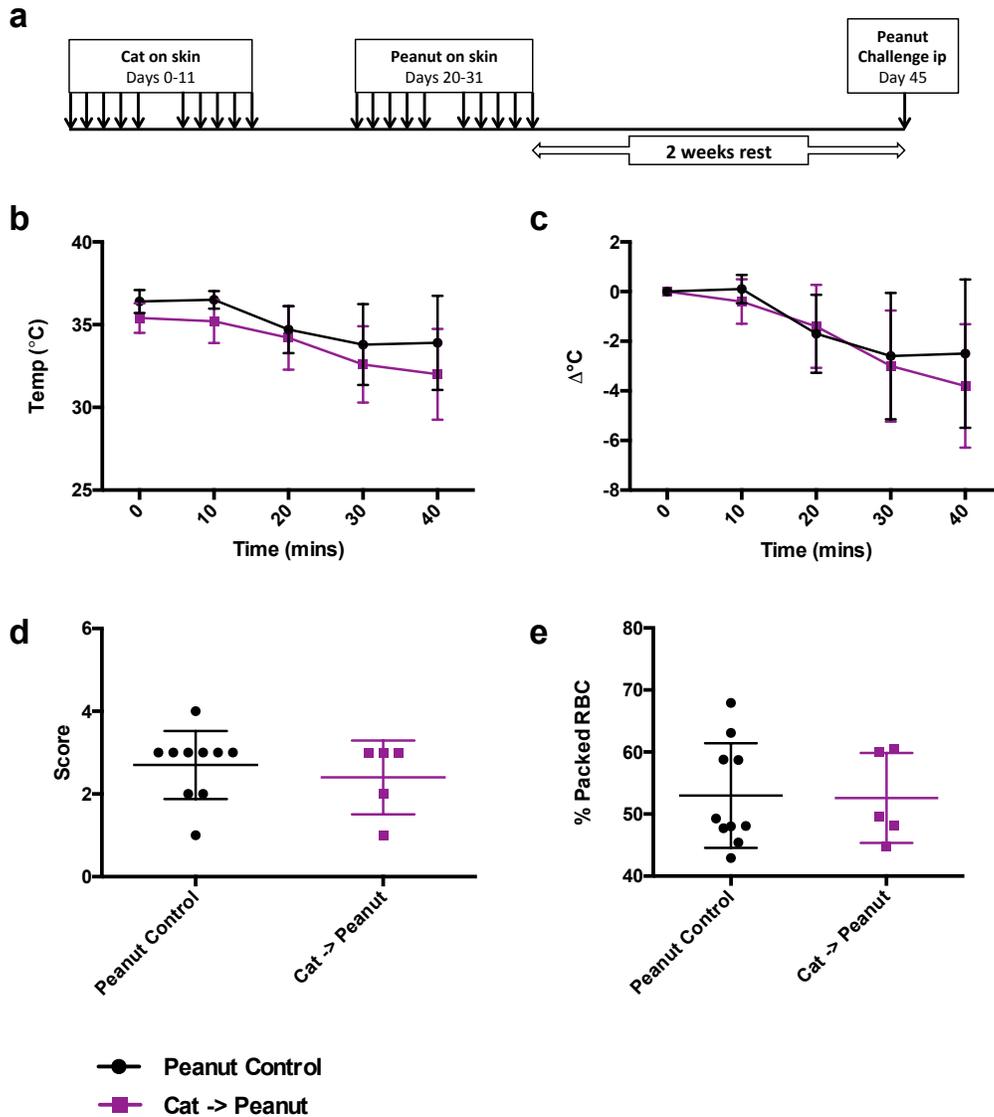


Figure 10. The effect of epicutaneous exposure to the high dose of cat dander alone on peanut-induced anaphylaxis. a) Sensitization schedule: female C57Bl/6 mice were shaved and tape-stripped followed by epicutaneous application of high dose cat dander (cat) from days 0-11, and then epicutaneous application of a sensitizing dose of crude peanut extract (cat + peanut) on days 20-31 in the same manner. Peanut control mice were shaved and tape-stripped followed by epicutaneous application of saline from days 0-11 and then received epicutaneous application of peanut in the same manner. Mice were intraperitoneally challenged with 5 mg crude peanut extract and signs of anaphylaxis were monitored: b) rectal temperatures over time, c) changes in rectal temperatures over time, d) clinical scores, and e) hematocrit (%packed red blood cells (RBC)). All values are expressed as means \pm standard deviations.

Specific Aim 5: To determine whether epicutaneous exposure to the high dose of cat dander affects allergic responses in established disease.

Epicutaneous exposure to the high dose of cat dander may have contributed to worsening airway eosinophilia in established allergic airway disease

Since epicutaneous exposure to high dose cat dander in naive mice lessened the degree to which allergic airway responses developed, we explored whether the high dose of cat dander also influenced responses in mice with established allergic airway disease. Mice were primed with epicutaneous application of the low dose of CDE (15 µg) from days 0-11, and intranasal administrations of CDE on days 22, 24 and 26 to induce allergic airway disease. The high dose of CDE or saline was then applied epicutaneously from days 33-44 (15-150 and 15-Sal respectively) and allergic airway responses were assessed after recalling the response to the lungs with CDE (Figure 11a). Naive mice received saline instead of CDE all throughout. While BALF eosinophil counts in the 15-150 mice were relatively greater than in the 15-Sal mice, these differences were not statistically significant (Figure 11c). This may have been due to the variability in the data points obtained in the 15-150 group. Although the percentage of BALF eosinophils was increased ($52.6 \pm 9.6\%$) in the 15-150 mice compared to the 15-Sal ($40.5 \pm 8.2\%$) mice, this difference was also not statistically significant (Figure 11d). Overall, while it appeared that epicutaneous exposure to high levels of cat dander may have contributed to worsening airway eosinophilia, these findings are not conclusive. Airway resistance measurements were not obtained in these mice.

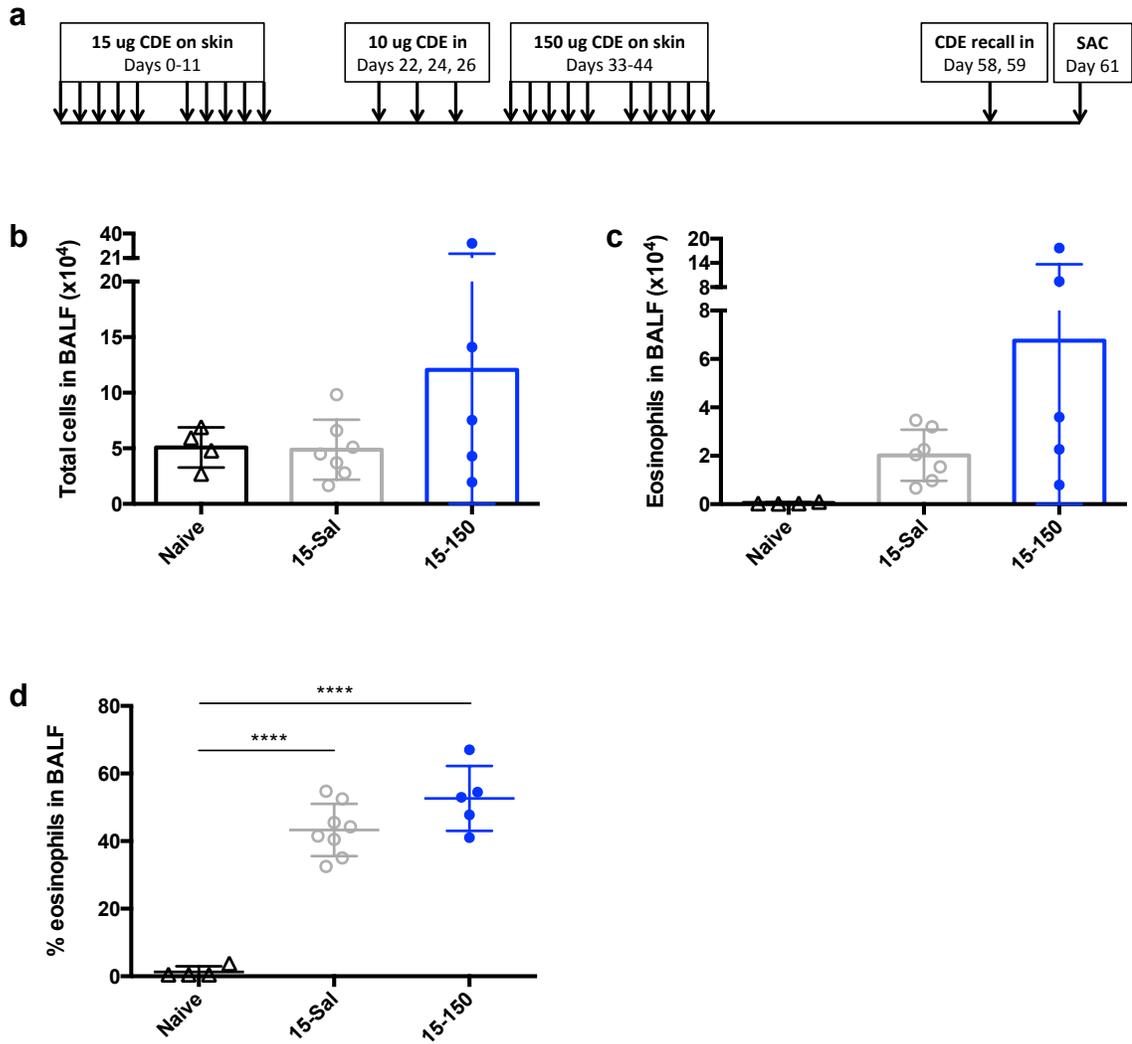


Figure 11. The effect of epicutaneous application of the high cat dander dose on established allergic airway responses. a) Sensitization schedule: 4-6 week old DR4 mice were shaved and tape-stripped followed by application of 15 μ g cat dander extract (CDE) from days 0-11 as shown. Intranasal (in) administrations of CDE were then given on days 22, 24 and 26 to establish allergic airway disease. High dose cat dander (150 μ g CDE) or saline was then applied to the same shaved and tape-stripped area of the back) from days 33-44. Allergic airway responses were assessed 48 hours after the last intranasal. Naive mice received saline throughout. SAC, sacrificed b) Total cell and c) eosinophil counts in BALF d) Percentage of eosinophils from cell differentials of BALF samples. All values are expressed as means \pm standard deviations. ****p<0.0001

Concurrent epicutaneous exposure to the high dose of cat dander with peanut did not prevent peanut-induced anaphylaxis in peanut sensitized mice

To determine whether the high dose of cat dander could influence anaphylactic responses in mice that were already sensitized to peanut, mice were epicutaneously exposed to peanut on days 0-11, followed by application of the cat+peanut mixture on days 20-31 (Figure 12a). Peanut challenge in these mice resulted in worsening of anaphylactic responses compared to the peanut control mice (Figures 12b-e). Rectal temperatures were significantly reduced compared to changes in temperature in peanut sensitized mice (Figure 12b and c). These mice also demonstrated more severe clinical signs such as no response to prodding than peanut control mice (Figure 12d); these signs of sickness were also observed sooner in these mice compared to peanut control mice. The hematocrit concentration was also significantly increased compared to the peanut control (Figure 12e). This worsening of responses was not observed when mice were sensitized with peanut alone from days 0-11, followed by epicutaneous application of cat alone on days 20-31 (Figure 13a-e). Although the rectal temperatures were lower than those of the peanut control mice (Figure 13b and c), these differences were not statistically significant. The clinical scores and hematocrit were also comparable (Figure 13d and e). These findings demonstrated that concurrent epicutaneous exposure to the high dose of cat dander with peanut in mice that were already sensitized to peanut contributed to worsening of anaphylactic responses. However, exposure to the high dose of cat dander alone had no effect on anaphylactic responses in peanut-sensitized mice.

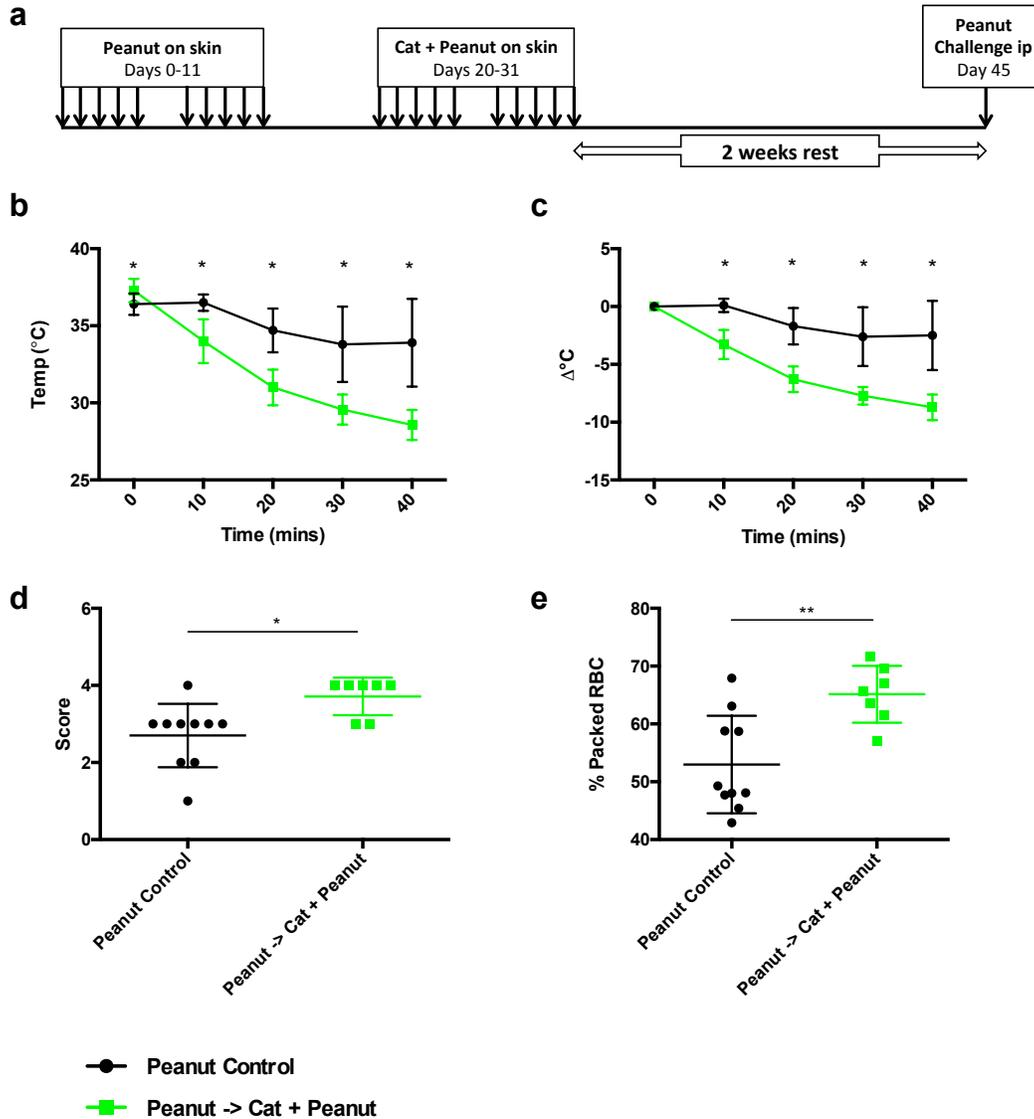


Figure 12. The effect of concurrent epicutaneous exposure to the high dose of cat dander and peanut in peanut sensitized mice. a) Sensitization schedule: female C57Bl/6 mice were shaved and tape-stripped, followed by epicutaneous application of a sensitizing dose of crude peanut extract (peanut) from days 0-11. On days 20-31, they received epicutaneous application of a mixture consisting of high dose cat dander and the sensitizing dose of crude peanut extract (cat + peanut) in the same area and manner. Peanut control mice were shaved and tape-stripped followed by epicutaneous application of saline on days 0-11, and then received epicutaneous application of peanut in the same area and manner. Mice were intraperitoneally challenged with 5 mg crude peanut extract and signs of anaphylaxis were monitored: b) rectal temperatures over time, c) changes in rectal temperatures over time, d) clinical scores, and e) hematocrit (%packed red blood cells (RBC)). All values are expressed as means ± standard deviations. *p< 0.05; **p<0.01

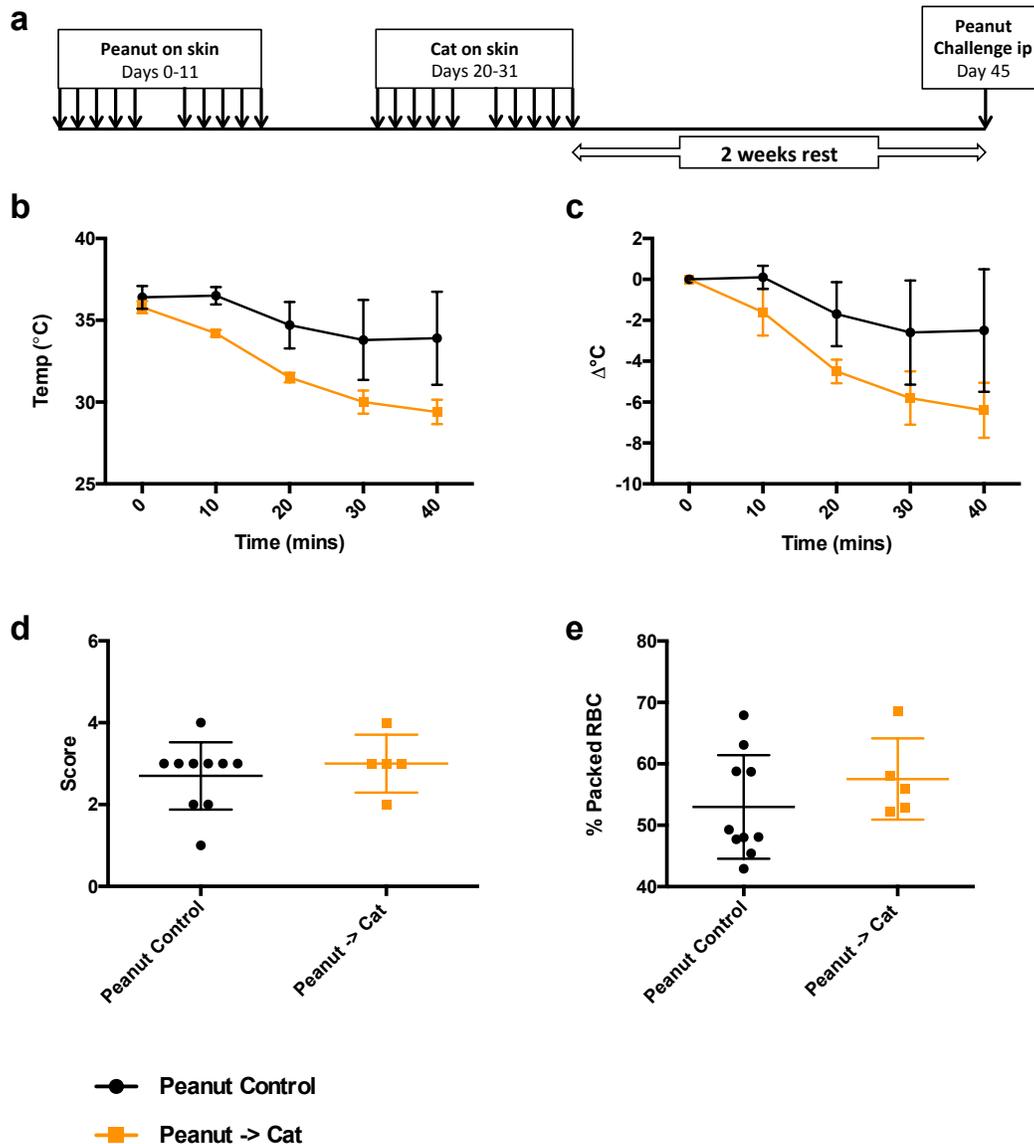


Figure 13. The effect of epicutaneous application of the high dose of cat dander alone in peanut sensitized mice. a) Sensitization schedule: female C57Bl/6 mice were shaved and tape-stripped followed by epicutaneous application of a sensitizing dose of crude peanut extract (peanut) from days 0-11. From days 20-31, they received epicutaneous application of high dose cat dander (cat) in the same manner and area. Peanut control mice were shaved and tape-stripped followed by epicutaneous application of saline from days 0-11 and then epicutaneous application of peanut on days 20-31 in the same area and manner. Mice were intraperitoneally challenged with 5 mg crude peanut extract and signs of anaphylaxis were monitored: b) rectal temperatures over time, c) changes in rectal temperatures over time, d) clinical scores, and e) hematocrit (%packed red blood cells (RBC)). All values are expressed as means ± standard deviations.

CHAPTER 4: DISCUSSION

A role for allergen dose and the timing of this exposure in influencing the manifestation of allergic responses has been suggested. Among other factors, allergen dose was implicated in influencing sensitization, and in some cases the development of allergic asthma. While epidemiological studies found that exposure to relatively high allergen levels was associated with a reduced prevalence of sensitization, experimental studies extended this observation to Th2-like inflammation as well. Some studies demonstrated that increased allergen exposure was associated with a reduced frequency of sensitization in genetically susceptible populations, to other allergens, and may not be allergen specific. This response was also suggested to diminish if allergen exposure was reduced for an extended period of time. Furthermore, exposure to sufficiently high allergen levels has also been shown to ameliorate allergic diseases, as is observed with immunotherapy or from beekeepers and animal handlers. Thus, in an effort to study the mechanisms by which high dose allergen exposures influence the manifestation of allergic diseases, we characterized the high allergen dose phenomenon in a mouse model of epicutaneous sensitization.

INFLUENCE OF ALLERGEN DOSE ON THE DEVELOPMENT OF ALLERGIC DISEASE

I. Genetics

Allergic airway responses have been shown to differ between different strains of mice, despite identical sensitization protocols (Yagi et al., 2006; Hirota et al., 2008). While BALB/c mice mounted stronger Th2 responses, C57Bl/6 mice have been shown to mount more robust Th1-type responses (Hayashi et al., 2001). DR4 mice were generated on a C57Bl/6 background and are commonly used to study Th1-mediated autoimmune diseases (Ito et al., 1996). The relative degree of Th1 or Th2 responses in DR4 mice has not yet been compared to other strains.

However, it is possible that DR4 mice may mount stronger Th1 responses, as they were generated on a C57Bl/6 background. In our study, the dose-dependent effects on the development of allergic airway responses were similar across BALB/c, C57Bl/6 and DR4 mice. The degrees of airway and lung eosinophilia, and airway resistance were enhanced with epicutaneous exposure to a low dose of cat dander in all three strains and attenuated with epicutaneous exposure to a high dose of cat dander. When the strains were analyzed individually, it appeared that the high cat dander dose-dependent attenuation of the number of mucus-secreting goblet cells was most evident in C57Bl/6 mice, but not in BALB/c and DR4 mice. In a model of OVA-induced allergic airway disease, more robust goblet cell hyperplasia was observed in BALB/c mice compared to C57Bl/6 mice (Hayashi et al., 2001). The differential effects of the high dose of cat dander on goblet cell hyperplasia in BALB/c and C57Bl/6 may also be due to inherent differences in IL-13 signaling between these strains, as IL-13 can influence goblet cell hyperplasia (Hirota et al., 2008). Thus, while it is possible that BALB/c mice might be susceptible to manifesting more robust goblet cell hyperplasia, when the counts from all three strains were pooled, the GC counts were generally greater in mice receiving the lower dose of cat dander compared to mice receiving the high dose. As such, it appears that the high cat dander dose may have attenuated goblet cell hyperplasia, however, a greater number of mice per group may be necessary to observe this effect more clearly. In our study, the effect of the high dose of cat dander on goblet cell hyperplasia in the DR4 mice did not differ from the low dose. However, this is also likely due to the low number of mice and variability in the counts within each group in this study.

Morokata and colleagues (2000) have demonstrated that with low sensitizing doses of OVA (100 ng and 8 µg) given intraperitoneally with alum, Th2 like responses (increased IgE and IgG1, decreased IgG2a, increased BALF eosinophils and IL-4, IL-5 production in the lungs, with decreased IFN-γ) were observed in

C57Bl/6 mice while Th1-like responses (decreased IgE and IgG1, increased IgG2a, reduced lung eosinophils and IL-4 and IL-5 production in the lungs, with increased IFN- γ) were observed in BALB/c mice. However at higher sensitizing doses (50 μ g and 1 mg), C57Bl/6 mice demonstrated Th1-like responses and BALB/c demonstrated Th2-like responses. While Morokata and colleagues observed differences in the dose-dependent responses between BALB/c and C57Bl/6 mice, the effects of low and high dose were similar across all three strains in our study; it is possible that the initial route of sensitization may have influenced this outcome. Morokata and colleagues sensitized mice with an intraperitoneal administration of allergen with alum, whereas we applied allergen epicutaneously.

Epidemiological studies (Cullinan et al., 2004; Perzanowski et al., 2002; Roost et al, 1999; Pohlabein et al., 2007) reported that increased exposure to cats was associated with a decreased prevalence in sensitization later in genetically susceptible and nonsusceptible individuals. In support of these epidemiological findings, we also observed that the high dose of cat dander was able to attenuate the development of allergic airway responses in Th2 prone BALB/c mice, and in two other genetically different strains (C57Bl/6 and DR4), one of which is more prone to mount Th1-like responses (C57Bl/6). The generalizability of the high cat dander dose dependent attenuation was also illustrated when the data from all three strains were pooled, and also appeared more pronounced for all of the outcomes measured.

II. Duration

Protection against allergic reactions conferred by high doses of allergen has been suggested to wane (Erwin et al., 2014). This was based on anecdotal evidence of college students developing new or increased allergic symptoms to their cats upon moving away from home during the academic year. In another natural model of

high allergen exposure, it was demonstrated that tolerogenic responses were only transiently observed after multiple beestings in nonallergic beekeepers. Cutaneous late phase responses to bee venom decreased after the first week of beekeeping season (when beekeepers were stung about 13 times on average), and antigen specific T cell proliferation also decreased drastically. However, antigen-specific T cell proliferation was increased again 2-3 months after beekeeping season (when antigen exposure was reduced), and before the next beekeeping season cutaneous late phase responses were also increased again (Meiler et al., 2008). Our data also suggest that the protective effects from exposure to a high dose of cat dander also decays with time. Mice recalled with allergen 120 days after receiving the high dose of cat dander demonstrated enhanced airway eosinophilia (150-Sal (120d); Figure 6c and d) this was not observed in mice that were intranasally re-exposed to allergen 10 days (150 µg (10d); Figure 6c and d) and 40 days (data not shown) after epicutaneous exposure. Although this increase was not statistically significant, this observation warrants further discussion. First, mice exposed to the high dose of cat dander that were recalled 120 or 10 days later both received a total of 5 intranasal administrations of CDE. However, airway eosinophilia was still lower in the mice recalled 10 days later despite having received all 5 intranasal exposures consecutively. Second, the mice recalled 120 days later demonstrated percentages of airway eosinophilia that were comparable to mice that were exposed to a sensitizing dose of cat dander. Although this is speculation, based on these two observations, it is possible that the high dose of cat dander may not be completely preventing sensitization from occurring. Rather, it may also be inducing another response that is capable of masking the sensitization. Then when high dose exposure is discontinued, the suppressive response weakens and the response elicited with sensitization dominates. In humans, it has been proposed by some (Aalberse and Platts-Mills, 2004; Davies et al., 2013) that low levels of cat allergen exposure could maintain IgE producing plasma cells while IgG production would be decreased. With

increased exposure, IgG and IgG4 production would increase (Erwin et al., 2014). Although the evidence to support a protective role for IgG antibodies is not clear, increases in IgG and IgG4 have been observed following allergen immunotherapy, which has been associated with clinical benefit (Akdis and Akdis, 2015). In addition, in mice, a high sensitizing dose of allergen was shown to prevent anaphylaxis in an IgG dependent manner (Hirai et al., 2016). As mice do not produce IgG4, it is possible that protective effects may be mediated through another IgG isotype in mice. Arguably, these dose dependent effects on immunoglobulin profiles may also explain why some individuals develop new allergic symptoms to their cats that were previously not a problem. Indeed, in some nonallergic students, it was observed that Fel d 1 specific IgG decreased over the academic year while Fel d 1 specific IgE was unchanged (Erwin et al., 2014); this study did not assess whether these students experienced new symptoms upon allergen re-exposure.

Epicutaneous exposure to a sensitizing dose of cat dander long after exposure to the high dose appeared to worsen airway eosinophilia, seemingly in an additive manner ((150-15 (120d); Figure 6c and d). This degree of airway eosinophilia was greater than that observed in sensitized mice (Sal-15 (120d) and 15 µg (10d)). Some students who were not allergic to their cats were found to have positive skin prick tests or Fel d 1 specific IgE. In these individuals who were likely exposed to relatively low levels of cats during the academic school year, Fel d 1 specific IgG were reduced although IgE levels did not change (Erwin et al., 2014). Thus, it is possible that the epicutaneous application of the high dose of cat dander may not have prevented sensitization in these mice, which was then enhanced with subsequent exposure to the sensitizing dose.

III. Type of allergen

Multiple epidemiological studies have found a decreased prevalence of allergen

specific sensitization or asthma with increased exposure to cats or dogs (Custovic et al., 2003; Sporik et al., 1999; Platts-Mills et al., 2001; Ownby et al., 2002; Hesselmar et al., 1999), fungal allergen (Iossifova et al., 2007), rodent urinary allergen (Jeal et al., 2006) and even house dust mite (Schram-Bijkerk et al., 2006; Cullinan et al., 2004). Experimental models have also demonstrated attenuated or prevention of allergic responses with increased allergen exposure with house dust mites (Llop-Guevara et al., 2008; Hirai et al., 2016) and OVA (Sakai et al., 1999). In support of these findings, we demonstrated that epicutaneous exposure to a higher dose of cat dander or OVA was associated with attenuated allergic responses in BALB/c mice. BALF eosinophils and eosinophils within the peribronchial tissue were increased with epicutaneous application of the “low” dose of OVA (500 µg) and attenuated with application of the “high” dose of OVA (1000 µg). However, no changes were observed in the airway resistance between OVA sensitized (at either dose) and naive mice. Thus, attenuation of allergic airway inflammation may occur irrespective of the type of allergen, however airway resistance does not appear to be influenced based on our findings. Our studies are the first to demonstrate the dose dependent effects of two different allergens on allergic airway responses using an identical sensitization protocol.

IV. Impact on other allergens

Allergen specific immunotherapy was associated with a reduced prevalence of new sensitizations in some studies (des Roches et al., 1997; Pajno et al., 2001; Purello-D’Ambrosio et al., 2001), but not others (Asero, 2004; Gulen et al., 2007). In our mouse model, epicutaneous exposure to the high dose of cat dander prevented peanut-induced anaphylaxis only when applied in the presence of peanut; however, pre-treatment with the high dose of cat dander was not required for this protective effect. Prevention of new sensitizations during immunotherapy is thought to be mediated by induced regulatory T cells through a mechanism known as bystander suppression. Bystander suppression involves the spread of

tolerance from one antigen to the next, if presented by the same antigen-presenting cell (APC) (Sakaguchi et al., 2009). In an experimental model, recall of immune responses with two antigens, where a tolerant response was induced to one of the antigens, was associated with reduced inflammatory responses to the other antigen as well (Navarro et al., 2015). However, in our model, bystander suppression is not a likely mechanism of action as pre-treatment with the high dose of cat dander was not necessary to prevent peanut-induced anaphylaxis.

Anaphylactic responses are mediated by allergen specific IgE and/or IgG triggered responses in mice (Arias et al., 2011). Since concurrent exposure to the high dose of cat dander and peanut prevented peanut-induced anaphylaxis, it is possible that initial sensitization to peanut was prevented, and thus, anaphylaxis did not occur. It is possible that sensitization to peanuts may have been prevented due to epitopes in cat dander that may be immunodominant. If cat allergens consisted of immunodominant epitopes, these may have been recognized by the immune system and prevented an immune response from developing against other epitopes (Akram and Inman, 2012) such as those derived from peanut. It is also possible that other constituents may have prevented sensitization to both cat allergens and peanut such as endotoxin in the extract. APCs may have also been activated differently with antigen dose. Thus, it would be interesting to determine how APCs were influenced by antigen.

INFLUENCE OF ALLERGEN DOSE *AFTER* THE DEVELOPMENT OF ALLERGIC DISEASE

I. Impact on established allergic airway disease

Allergen dose has also been shown to influence ongoing allergic disease. Immunotherapy, involving administration of high doses of allergen, has improved symptoms in patients with allergic rhinitis and asthma (Yukselen and Kendirli,

2014). A single dose in allergen immunotherapy can be 100-fold greater than the estimated natural exposure to that allergen in a year (Løwenstein, 1991; Larsen et al., 2016). Conversely, natural exposures to levels of allergens associated with sensitization are relatively lower, and continued exposure in sensitized individuals triggers exacerbations (Sporik et al., 1999). In our model, epicutaneous exposure to the high dose of cat dander did not ameliorate allergic airway responses in cat sensitized mice. Instead, airway eosinophilia appeared to be exacerbated with epicutaneous exposure to the high dose of cat dander in sensitized mice, compared to mice that were just sensitized. Although these differences were not statistically significant, it is apparent that the high dose of cat dander contributed to a relatively greater frequency in eosinophils, and did not attenuate this response. As such, in our model, application of the high dose of cat dander appears to exacerbate allergic airway responses. Airway resistance was not assessed in these mice, thus it is not known whether lung function was also worsened. It is possible that the “high” dose of cat dander used was not *sufficiently high* to attenuate allergic airway responses in our mouse model.

Epicutaneous application of allergen as another route for administering allergen in immunotherapy has also been explored. In a dose escalation trial for grass pollen-induced rhinoconjunctivitis, epicutaneous application of allergen to a tape-stripped area significantly reduced hay fever symptoms in the high dose group compared to the placebo group (Senti et al., 2012). It should be noted that treatment with higher doses was also associated with pruritis, erythema, wheal and eczema. However, in a model of oral peanut sensitization with cholera toxin, Mondoulet and colleagues (2012) demonstrated that intact skin was necessary to induce tolerance using an allergen patch known as Viaskin. Application of the patch on tape-stripped skin exacerbated Th2-like responses. Furthermore, while application of the Viaskin patch on intact skin only allowed passage of limited amounts of allergen (Dioszeghy et al., 2011), this was sufficient to prevent lesions

in the gastrointestinal tract that were associated with prolonged oral exposure to peanut (Mondoulet et al., 2012). In our model, the high dose of cat dander was applied to the same area where mice were initially sensitized and the area was also shaved and tape-stripped prior to application of the high dose of cat dander. This was done for two reasons. First, epicutaneous application of high dose of cat dander in this manner was observed to attenuate the development of allergic airway responses, despite application to a compromised skin barrier. Second, if indeed we observed that high doses of cat dander could attenuate features of established allergic disease, then perhaps high doses of cat dander could have therapeutic potential, especially in individuals with chronic underlying inflammation as seen in cases of atopic dermatitis. However, in our model, airway eosinophilia appeared to be exacerbated. It is possible that due to the method in which the high dose of cat dander was applied, allergic airway responses in these sensitized mice could not be attenuated.

II. Impact on peanut-induced anaphylaxis in sensitized mice

In light of the finding that concurrent exposure to the high dose of cat dander and the sensitizing dose of peanut was able to prevent peanut-induced anaphylaxis, we tested whether this mixture could also influence established peanut sensitization. However, administration of the high dose of cat dander, alone or with peanut, to mice that were peanut sensitized did not prevent peanut-induced anaphylaxis. Instead when the high dose of cat dander was given concurrently with peanut in peanut sensitized mice, anaphylactic responses were exacerbated. It is likely that the worsening of symptoms that was observed was because these mice essentially received a prolonged sensitization protocol to peanut. This may have occurred through activation of peanut specific memory T cells during the second epicutaneous application of peanut. It would be interesting to determine whether these mice were also sensitized to peanut to a greater degree.

Downregulation of immune responses to specific antigens has been shown to occur by inducing antigen specific regulatory cells (Akdis and Akdis, 2015). The ability of allergens to induce immune tolerance to other antigens has been suggested to occur through mechanisms of infectious tolerance such as bystander suppression. Studies have demonstrated that tolerance to one antigen was able to spread tolerance to another antigen (Moldaver et al., 2014; Navarro et al., 2015). In light of these mechanisms of antigen-specific immune suppression, it makes sense that peanut-induced anaphylaxis was not prevented when cat dander was given alone. However, the employed allergen application protocols were also not appropriately designed to determine whether tolerance to cat dander was induced and thus, whether bystander suppression could occur. One possible means to test this would be to sensitize mice to peanut, apply the high cat dander dose, and then apply the high cat dander dose with the sensitizing dose of peanut prior to peanut challenge. Furthermore, it would be also interesting to determine whether both peanut-->cat+peanut and peanut-->cat groups were still protected against cat (Figure 12a and Figure 13a respectively). Indeed, non-allergic individuals who were raised with cats were protected from cat specific sensitization while they were still sensitized to other allergens (Erwin et al., 2014).

LIMITATIONS OF STUDIES

A major limitation in this thesis was the lack of immunoglobulin data. While allergic reactions are mediated by various components, these responses are triggered by antigen binding to IgE and in mice, IgG1 as well. Furthermore, as eosinophilia can be induced in the absence of IgE and IgG1 in mice (Epstein, 2004), it is not possible to determine whether these mice were indeed sensitized without immunoglobulin measurements. One group also suggested that house dust mite-induced anaphylaxis was prevented in an IgG dependent manner. In their model, increased IgG was induced with epicutaneous exposure to high antigen

doses of house dust mite (Hirai et al., 2016). In humans, increases in antigen specific IgG and IgG4 have also been observed in patients after beginning allergen specific immunotherapy (Larché et al., 2006); the blocking of IgE mediated responses by IgG4 is suggested to be one of the mechanisms that may mediate protection against allergic reactions (Akdis and Akdis, 2015). Thus it is even difficult to merely speculate possible mechanisms without immunoglobulin data. As such, analysis of allergen specific immunoglobulin levels is pertinent when characterizing responses in models of allergic diseases. Many attempts at measuring cat dander and Fel d 1 specific immunoglobulin measurements have been made in the past by our lab, however, these efforts were not successful. At the time points that serum samples were collected (upon sacrifice on days 32 and 52), total Igs were low overall, and both total and cat dander specific Igs did not differ between naive, sensitized and high dose groups. In a more recent study, we were able to detect total IgE when serum was collected at day 27 (just after the intranasal challenges in the shorter model of allergic airway disease), however serum samples were not collected this early in the studies presented in this thesis. We have not been able to detect cat specific IgE at all. Finally, more recent attempts at optimizing the ELISA suggested that something in our samples was interfering with the measurements; OD readings from multiple dilutions of the sample were variable and did not reflect the dilution factor.

Another limitation in the allergic airway studies presented is the lack of a robust functional readout using our mouse models. While lung function can be determined from airway resistance measurements, increased airway resistance was not consistently reproduced in sensitized mice, despite increased airway eosinophilia. It was recently observed that airway resistance may correlate better with lung tissue eosinophilia rather than airway eosinophilia based on studies from our lab. However, this is not clear in all of the studies presented in this thesis, specifically in the OVA experiments (this may also be due to the low

number of mice per group in this study). Furthermore, it is possible that by increasing the number of intranasals administered or the dose of allergen in the intranasal challenge, we may consistently observe significantly increased airway resistance in sensitized mice and obtain a more reliable functional readout.

There are also inherent limitations in studying asthma in mice (Epstein, 2004). People with allergic asthma have a baseline response to methacholine while free of symptoms; however, methacholine-induced responses in mice are transient and only occur with allergen exposure. Furthermore, while IgE is necessary for allergen-induced inflammation in humans, it is not essential in mice. Finally, in humans, IL-4 induces IgE and IgG4, however in mice IL-4 induces IgE and IgG1 (Epstein, 2004); IgG4 isotype is also specific to humans, and is not generated in mice (Epstein, 2004). However, detailed characterization of disease is not possible in humans. Thus, animal models provide the best alternative for studying dynamic disease processes in an *in vivo* system. Furthermore, immune responses are integrated responses involving many components such as inflammatory cells and mediators and collectively impact physiological processes. Thus, studying phenomena in an *in vivo* system allows one to see how responses involved in maintaining homeostasis are altered. Thus, detailed characterization of changes in immunological and physiological processes with a high dose of allergen in a mouse model may provide helpful insight for developing therapeutics.

CONCLUSION

In summary, the high dose-dependent attenuation of allergic airway responses during development occurred irrespective of genetic differences in mice and was observed with two different sources of allergen (cat dander and ovalbumin). The protective effect of the high dose of cat dander also appeared to wane with time, and prevented peanut-induced anaphylaxis when applied with peanut. However,

in established disease, the high dose of cat dander appeared to exacerbate airway inflammation, and did not prevent peanut-induced anaphylaxis in peanut sensitized mice. Thus, it appears that the high dose of allergen used in these studies was sufficient to attenuate the development of allergic responses, but not responses in established disease states. Our findings are also the first to demonstrate this bell shaped relationship with cat dander in a model of epicutaneous sensitization. Finally, we were able to model many of the aspects of the high allergen dose phenomenon observed in humans within our mouse model. Thus, this model may be useful for studying the mechanisms by which high allergen doses may be able to mitigate allergenic immune responses.

Along with a myriad of studies conducted by other groups, our findings highlight that a bell shaped relationship between the manifestation of allergic airway responses and the dose of allergen is indeed observed over a wide range of exposures. This relationship has been demonstrated with different allergens, and has been seen independently of the route of sensitization (epicutaneous, intranasal exposures, systemic administration) in mice. Thus, even though in humans we do not know the most relevant route of exposure for different allergens, the site of exposure may be subject to a similar pattern of responses over a wide range of doses. Overall, our findings support that exposure to *sufficiently high* levels of allergens may not necessarily be a risk factor, and determining the *range of* allergen levels that pose a risk within each society should be pertinent prior to implementing allergen reduction strategies.

FUTURE DIRECTIONS

In addition to determining how immunoglobulin levels changed with allergen dose, further detailed characterization studies are also needed. For example, determining how the different allergen doses influenced key immunological

players (e.g. APCs and thus T cells, or immunoglobulin profiles). It would also be interesting to then determine whether this change is consistently seen with different antigens. It would also be interesting to determine whether high doses of house dust mite could prevent the development of allergic airway disease, as house dust mite-derived allergens have enzymatic activity. In addition, while mechanical damage of the skin barrier has been shown to induce a Th2 polarizing environment (Oyoshi et al., 2010), application of the high dose of cat dander on a damaged patch of skin still prevented allergic responses. Thus, it would be interesting to compare APC activation between low and high dose receiving mice, and also measure cytokine levels at the site of sensitization.

We demonstrated that the protective effects of the high dose of cat dander might decay with time. As such, it would be interesting to identify the protective components that decrease as a result of discontinued exposure, and to demonstrate that this decrease results in the appearance of allergic features or responses. Furthermore, it would be interesting to also determine whether exposure to the sensitizing dose of cat dander *shortly* after exposure to the high dose of cat dander could also influence the manifestation of allergic responses.

Finally, determining how the effects of high antigen dose can be harnessed may be of clinical significance (e.g. identifying the active component of CDE mediating these protective effects), potentially also as a prophylactic treatment. These studies would also provide greater insight into when, or if, exposure to high amounts of a potential allergen would be a risk factor for allergic disease.

CHAPTER 5: REFERENCES

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