IMMUNE MODULATION OF ALLERGIC RESPONSES

INVESTIGATING MECHANISMS OF PEPTIDE INDUCED IMMUNE MODULATION OF MURINE MODELS OF ALLERGIC AIRWAYS DISEASE

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TITLE Investigating mechanisms of immune modulation in murine models of allergic airways disease

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Lay Abstract

Asthma is a disease of the airways that can cause difficulties in breathing. In some people, asthma develops because their immune system reacts in an uncontrolled manner to common environmental proteins, called allergens. Whole allergen immunotherapy is a treatment for asthma, where asthmatic people are injected with doses of allergen until their immune system no longer responds to (or 'tolerates') the allergen. In some people, injection of allergen can lead to a life-threatening immune response known as 'anaphylaxis'. Peptide-immunotherapy is a form of whole allergen immunotherapy where people are given small fragments of the allergen (a 'peptide') rather than the whole allergen. The benefit of peptide immunotherapy is that the treatment peptides are too small to cause anaphylaxis, but remain large enough to teach the immune system. In this thesis, we examined how treatment with small peptides teaches the immune system to tolerate the larger and more complex whole allergen.

Abstract

Asthma is defined as reversible airflow obstruction and an estimated 1-in-3 Canadians will be diagnosed over their lifetime. Many clinical phenotypes of asthma exist, but allergic asthma is the most common presentation. Despite effective therapies, approximately 65% of Canadian asthmatics have poorly controlled disease. Thus, there remains pressing need to develop disease modifying therapies.

Allergen-specific immunotherapy (SIT) is a disease-modifying therapy for allergic disease that consists of repeatedly administering doses of allergen, to an allergic individual; over 100 years of clinical use, SIT has been demonstrated to reduce symptoms of disease both during and after cessation of therapy. Widespread clinical uptake of SIT has been limited by the risk of developing anaphylaxis as a response to therapy. Peptide immunotherapy is a derivation of SIT, that attempts to retain the disease-modifying benefits, while lessening the risk of anaphylaxis, by treating subjects with allergen-derived T-cell peptide epitopes. Peptide immunotherapy has been demonstrated to reduce symptoms of allergic disease in treated subjects; however, it remains unknown how administration of a single (or several) T-cell epitopes can modulate immune responses to entire complex allergens. Additionally, how genetic diversity in peptide epitope presentation effects the development of immune tolerance is unknown. In this thesis, we sought to characterize these mechanisms of peptide immunotherapy; the hypothesis was, "*The induction of immunosuppression by peptide immunotherapy involves the infectious spread of tolerance beyond the treatment epitope, and is dependent upon treatment peptide dose and affinity to MHC*".

Through the definition of these mechanistic traits we hoped to expedite and inform the design of future peptide based therapeutics. The studies presented within this thesis examine the

topic of immune modulation of allergic disease in mouse models, and have focused upon broadly pertinent characteristics of immune modulation, such as the number, dose and affinity of immunomodulatory epitopes.

Acknowledgements

This PhD thesis is a culmination of what seems like a life-time of events. I'm thrilled to be able to say that I was a part of Dr. Mark Larché's lab in McMaster University throughout many of my life's most momentous events, such as my marriage to Emily Husul and the birth of my son, Max. It speaks volumes that I was thrilled to spend these major events in Dr. Mark Larché's company. He's one of the most genuine and generous people I've had the privilege to know, and I consider myself lucky to have learned under his tutelage. As a supervisor, Mark always provided exactly what I needed – advice, and trust. It was Mark's trust in me to design and perform experiments as an independent researcher early in my career that has given me the confidence required to excel in this field, while Mark's advice is what shaped me into the scientist I am today. On top of all that, Mark has always practiced what he preached: family first, a skill that can often be lost in the professional world, and a skill I'm thankful to have learned.

Throughout my PhD thesis, Dr. Mark Inman was the voice of reason and well of knowledge that I could always rely on. From my earliest days in the lab, Mark ensured his door was open to chat and he was always willing to take time from his day to discuss experiments, lab and anything else.

I've always believed that you learn and grow the most when you're pulled out of your comfort zone. As such, it is natural that some of the clearest lessons that I learned during my PhD have come during interaction with Dr. Manel Jordana. I greatly appreciate his honesty and the effort he invested in driving myself and my work to the appropriate level. Likewise, Dr. Helen Neighbour could always be relied on to provide the valuable, grounding insights that drive a thesis forward.

Beyond my committee, I was lucky enough to learn beside many fantastic, inspirational people; more than I could list here. Dr. Chris Rudulier and Elisabeth Simms deserve special mention though, as I'll forever remember the great times and late nights at local (and distant) pubs spent chatting about life, the lab, and everything in between.

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List of Figures and Tables

Chapters 2, 3, and 4 & 6 have been prepared as manuscripts, and thus each have their figures that have been numbered for submission, and placed at the end of the text. As such, figures from these chapters are not presented in the table below. This list only presents figures from Chapter 5, which is representative of a classical thesis chapter, with figures interspersed throughout the text.

Abbreviations

Airway hyperresponsiveness (AHR)
Airway inflammation (AI)
Allergen-specific immunotherapy (SIT)
Aluminum hydroxide (Alum)
Antigen presenting cell (APC)
Bronchial-alveolar lavage fluid (BAL)
Bursa derived lymphocytes (B-cells)
Cat dander extract (CDE)
Cluster of differentiation (CD)
Complete Freund's adjuvant (CFA)
Cyclosporin A (CsA)
Cytotoxic T lymphocyte antigen-4 (CTLA-4)
Dendritic cell (DC)
Draining lymph nodes (DLN)
Early asthmatic reaction (EAR)
Endotracheal (ET)
Epithelial-derived cytokines (EDCs)
Enzyme-linked immunosorbent assays (ELISAs)
Eosinophil chemotactic factor-A (ECF-A)
Eosinophil derived neurotoxin (EDN)
Eosinophil peroxidise (EPO)
Eosinophillic cationic protein (ECP)
Experimental autoimmune encephalomyelitis (EAE)
Forced expiratory volume in one second (FEV1)
Granulocyte-macrophage colony-stimulating factor (GM-CSF)
Global Initiative for Asthma (GINA)
Haematoxylin and Eosin (H&E)
House dust mite (HDM),
House dust-mite[HDM]
Human leucocyte antigens (HLA)
IL-10 producing iTregs (Tr1)
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)
Immunoglobulin (Ig)
Immunoregulatory T-cells (Tregs)
Inducible Tregs (iTregs)
Inducible costimulatory [ICOS]
Inhaled corticosteroids (ICS)
Intercellular adhesion molecule (ICAM)
Interferon-gamma (IFN-γ)
Interleukin (IL)
Late-phase reaction (LAR)
Leukocyte function associated antigen (LFA)
Lympocytic choriomeningitis virus (LCMV)
Myelin basic protein (MBP)

Major histocompatibility complex (MHC)
Methacholine (MCh)
Natural tregs (ntregs)
Non-B-non-T cell (NBNT)
ORM1-like 3 (ORMDL3)
Ovalbumin (OVA)
Pathogen response receptor (PRR)
Peptide based-allergen specific immunotherapy (PIT)
Peptide-MHC (pMHC)
Periodic acid-schiff (PAS)
Phosphate-buffered saline (PBS)
Platelet-activating factor (PAF)
Provocative concentration of a stimulus needed to cause a decrease by 20% in the FEV1 (PC20).
Provocative concentration of methacholine causing a doubling in baseline respiratory system
Quality adjusted life year (QALY)
Radioallergosorbent test (RAST),
Resistance (PC200).
Regulatory T-cells (Tregs)
Rhinovirus (RV)
Respiratory syncytial virus (RSV)
Respiratory system resistance (RRS)
Signal transducer and activator of transcription (STAT)
Single-nucleotide polymorphisms (SNP)
Sphingosine 1-phosphate (S1P)
Standard error of the mean (SEM)
Stimulation index (SI)
T-box transcription factor (T-bet)
T-box transcription factor (T-bet)
T-cell helper (Th)
T-cell receptor (TCR)
Thymic stromal lymphopoietin (TSLP)
Thymus derived lymphocyte (T-cell)
Toll-like receptor (TLR)
Transforming growth factor (TGF)- β)
Transforming growth factor (TGF)-β- producing iTregs (TH3)
Tritiated(3[H])
Type 2 differentiated T-cells (Th2)
Tumour necrosis factor (TNF)
Venom immunotherapy (VIT)
Very late antigen (VLA)
World Health Organization (WHO)

Declaration of Academic Achievement

This thesis consists of 7 chapters and two appendices. Chapters 1 and 7 (introduction and conclusion) were conceived, written and edited by me, in their entirety. Dr. Mark Larché reviewed these chapters. Chapters 2-6 are original research that have been generated in conjunction with co-authors. Appendix 1 is a commentary piece, while Appendix 2 is a review articles. The contribution of each coauthor will be addressed at the start of each chapter.

Beyond the work presented in this thesis, I've also conceived, wrote (7 of 9 sections), developed and edited a second review article ("An Update on Lymphocyte Subtypes in Asthma and Airways Disease") that was published in the journal CHEST (2017) **151:** 1122-1130. M. Larché provided guidance to the development of this article while C. Rudulier contributed 2 (of 9) sections and edited. This article has not been included in the thesis as rights could not be retained.

Chapter 1: Introduction

1.1. Opening Statement

The research presented in this thesis examines two salient features of peptide immunotherapy; specifically, how peptide-major histocompatibility complex (MHC) binding characteristics influence the development of tolerance (a.k.a. tolerogenesis) and how treatment with a select few peptide epitopes can create immunological tolerance to a complex protein allergen. The generation of immunotherapeutics requires an understanding of the underlying disease, the immunological underpinnings of disease and the principles of tolerance induction. The introduction will summarize these, and related, fields of research to establish context and rationale for the work presented.

This thesis has been prepared as a 'Sandwich' document. This format entails the use of a global introduction and conclusory section that serve as the 'bread' that sandwiches published, or submitted, peer-reviewed articles that constitute the contents or 'meat' of the thesis.

The ensuing introduction will first review research describing the exaggerated, aberrant type 2 immune response that typifies allergic disease. The role of thymus derived lymphocytes (T-cells), and specifically type 2 differentiated T-cells (Th2) during exaggerated Th2 responses towards allergen will be examined, and linked to important landmarks associated with disease. The research in my thesis was performed to examine and establish novel mechanisms by which peptide immunotherapy subverts ongoing Th2 immune responses. The rationale for this procedure will be illustrated through a review of the history of allergen-specific immunotherapy, existing mechanisms of tolerogenesis and the merit of peptide-immunotherapy as a safer, efficacious alternative. Finally, the requirement to examine these phenomena in murine models will be discussed. The introduction will conclude by summarizing the findings of the presented research, and demonstrating the evolution of this work, and ultimately, myself.

1.2. The socioeconomic burden of asthma

The Global Initiative for Asthma (GINA) and Global Asthma Network has estimated that over 300-million people worldwide have asthma[1, 2], a disease that causes approximately 250,000 premature deaths yearly[3]. Asthma is predominantly a disease of industrialized countries, with clinically diagnosed asthma rates the highest in Australia (21.5%), Sweden (20.2%), the United Kingdom (18.2%), Netherlands (15.3%) and Brazil (13%)[4]. In comparison, less industrialized areas, like Africa had a combined asthma prevalence of 3.94%. The Public Health Agency of Canada indicate that, as of 2009-2010, 8.4% (2.4 million) of all Canadians over the age of 12 live with asthma[5]. These estimates demonstrate the commonality of asthma within the adult population. The prevalence of asthma spawned a study that sought to establish the lifetime risk of asthma. Study of over 9 million individuals in Ontario, Canada between 1996 and 2007 revealed that approximately 1 in 3 people will be diagnosed with asthma at one-point in their lives[6]. Taken together, these studies indicate that a large proportion of the global population, and more specifically, Canadians suffer from asthma as a chronic illness.

The Center for Disease Control (CDC) last estimated that each year asthma accounts for 500, 000 patient hospitalizations in the USA[7]. Canadian statistics are largely in-line with these numbers, as there were approximately 267,000 emergency room visits and 67,000 hospitalizations in 2011. These data suggest that current therapies are not effectively controlling disease. Indeed, in Canada, 65.6% of asthmatics report being "poorly controlled"[5], with 47% of Canadian asthmatics experience asthma symptoms 1 night per week. These figures illustrate how asthma impacts the quality of life of Canadians, despite a robust national medical care program. Treatments for asthma have existed for decades, but we lack the ability to rigorously control disease and thereby limit the associated financial burden. Novel, highly efficacious therapies,

like Omalizumab, may be able to better control disease, but they are expensive. Omalizumab therapy has an estimated cost of $63,895-71,309 (CDN) per quality adjusted life year (QALY)[8, 9], a value notably more than the unofficially 'acceptable' value of $50,000/QALY[10]. In contrast, the incremental cost-effectiveness ratio (ICER) of immunotherapy varies between 1,500 - 29,000 (CDN)/QALY[11, 12]. Furthermore, these analyses tend to ignore the long-term benefits associated with immunotherapy, a feature that would further enhance cost-effectiveness. Taken together, asthma-care remains problematic, making emerging, cost-effective strategies like immunotherapy attractive.

1.3. What is asthma

When discussing the burden of asthma, the issue of a universal, agreed upon definition arises. Asthma lacks a clear phenotype, as it presents as a heterogeneous, fluid disease, with many underlying immune mechanisms. The Global Initiative for Asthma (GINA) has attempted to unify the definition of asthma, citing the predominant clinical feature of asthma to be a history of episodic shortness of breath, commonly accompanied by a cough or wheeze[13]. This definition also refers to asthma as a chronic inflammatory disease, associated with airway hyperresponsiveness (AHR), atopy and other measures of sensitization. We are now entering an era where we seek to define asthma 'endotypes', wherein precise mechanisms responsible for disease pathology and symptoms are identified[14]. The recent success in phenotyping numerous forms of asthma has reinforced the notion that asthma is a heterogeneous disease, perhaps more aptly described as a 'syndrome', but importantly, allergic asthma remains the most common form of the disease[15].

AHR is a concept that will be visited throughout this introduction and the research presented, as such it deserves a brief explanation. AHR in allergic asthmatics can be induced by

both allergen and non-specific agents (acetylcholine, histamine etc.). AHR comprises airway narrowing and bronchospasm[16], features that indicate the airway to be more 'twitchy' and prone to exaggerated responses. Measurement of airway narrowing, in patients, is typically performed by assessment of a patients' forced expiratory volume in 1 second (FEV_1). AHR can be identified in patients through determining the "provocative concentration" of a stimulus (typically methacholine/histamine) producing a 20% reduction in baseline FEV_1 (PC_{20})[17]. As in humans, AHR is a readily measurable diagnostic in mice, although the method of quantification varies markedly. In mice, rodent ventilators and plethysmographs directly quantify airway resistance, from which AHR can be identified.

1.4. The immunology of allergic asthma, a historical perspective

The development of therapies for allergic asthma has benefited from a long history of exceptional research examining the mechanisms of allergic disease onset and exacerbation. Some of the earliest studies performed in the field of allergy effectively established key mechanisms of disease that continue to shape how we think of disease, and remain targets of therapeutics under development to this day. Here, the immunology underpinning allergic asthma will be introduced through a historical review; this strategy will introduce the conception of immunotherapy and highlight the long-acknowledged relevance of this treatment strategy.

The term "allergy" was used by von Pirquet, in 1906, to describe all biological effects that are the results of 'changed reactivity' to a molecule; however, this term has evolved (or to some, been corrupted) to describe IgE-mediated diseases[18, 19]. Asthma has long been associated with the term "allergy"[20], a term now used to describe an exaggerated immune responses to proteins that, in most people, are relatively harmless. Indeed, since Hippocrates' definition of asthma citing a seasonal relationship between disease and autumn, asthma and allergies have been

linked[21]. While von Pirquet is commonly perceived as the 'father' of allergy, Charles Blackley was the first to define hay fever as a hypersensitivity to pollen. In his 1873 *tour-de-force,* Blackley captured and identified pollen allergens in the air, replicated symptoms of hay-fever in patients, demonstrated skin reactivity to allergen and finally, observed that sneezing after allergen exposure typically lasted 6-8 hours post-exposure[22]. Therefore, it is largely Blackley's vision of allergy that has persisted, a view described 30 years prior to von Pirquet.

Dunbar was a researcher and clinician in the early 1900s who revisited the work of Blackley and pioneered many findings that remain relevant today. Dunbar purified allergens from wheat, rye, rye-grass and cat dander, then demonstrated that applying trace amounts of these allergens to the conjunctiva and mucus membrane of sensitive subjects induced the near instantaneous development of hay-fever symptoms[23]. This work critically reinforced the pollen hypothesis of hay-fever, which, at the time had fallen out of favour.

In 1921, Prausnitz and Küstner[24] demonstrated that a serum factor was sufficient to passively transfer allergic disease, in humans[25]. This experiment capped off a decade of work in animal models by Dunbar and others, wherein they demonstrated that the transfer of serum from pollen-sensitized Guinea Pigs or Humans into naïve Guinea Pigs passively sensitized these normal animals to pollen allergens[26]. Together, these observations formed the foundation for the hypothesis that asthma was more than a disease of the airway muscle. Shortly after the work of Prausnitz and Küstner, Coca and Cooke defined the term *atopy*[20], using it to describe the immediate wheal and flare reactions associated with allergen challenge[27] and described this process to be dependent upon heat-labile serum "bodies" or "reagins".

Knowledge pertaining to the Th2 response associated with asthma and allergies can largely be attributed to experiments attempting to define what "reagins" were. In 1966, reagins were

identified as immunoglobulin (Ig) E[28,29]. The term atopy evolved with our understanding of IgE. In 1971 and 1973, Dr. Pepys published a pair of reviews redefining the term atopy to describe patients that have detectable serum levels of IgE specific for common environmental allergens, with or without overt allergic disease[30, 31]. Today, atopy implies a hereditary predisposition to enhanced IgE production[32, 33] and the presence of atopic-spectrum clinical symptoms[34]. Much of our current understanding of asthma can be attributed to these initial observations made in the early 1900s. Furthermore, our more than a century-old focus on IgE and skin-prick testing illustrates why these factors remain at the forefront of clinical care.

In parallel to research identifying IgE/reagin, a distinct body of work began to formally link asthma to allergy. In 1922, Huber and Koessler[35] stated that: "in most cases of bronchial asthma the causative factor lies in the allergic reactibility of the individual". In addition to this concept, Huber and Koessler reviewed human lung samples, taken following death by asthma exacerbation. This seminal work established a series of observations that continue to shape our understanding of the pathology of asthma. Amongst their most important conclusions were: a) the primary cellular symptom of bronchial asthma is the influx of eosinophils into the blood, sputum and airway tissue and b) the asthmatic lung undergoes extensive remodeling, including: i) thickening of the airway wall, ii) increased size of airway mucus glands, iii) bronchospasm likely occurs due to irritation of lung nerves and iv) hypertrophy of airway smooth muscle[35].

Despite early acknowledgement of eosinophil accumulation in the lungs of asthmatics, attention was not paid to eosinophils until the advent of corticosteroids. In 1958, Dr. H. Morrow Brown performed a trial of prednisolone in 90 asthmatic individuals. He observed that those possessing sputum eosinophilia prior to the beginning of therapy were those most likely to receive clinical benefit from corticosteroid therapy; benefit that included cessation of

bronchospasm and reductions in blood eosinophils[36]. In the early 1970s, Drs. Kay and Frank Austen established a link between allergies and eosinophil recruitment, observing that antigenic challenge following passive sensitization induced eosinophil recruitment by a penta-peptide, eosinophil chemotactic factor-A (ECF-A)[37]. While ECF-A was subsequently demonstrated to be an inefficient eosinophil recruitment factor *in vivo*, this early study linked eosinophil recruitment to IgE and atopy. Once allergic disease was linked to eosinophilia, interest in eosinophils blossomed. In 1975, blood eosinophilia was found to inversely correlate with lung function in asthmatics, and resolve with corticosteroid therapy[38]. In 1979, Vieira and Prolla examined the sputum of 384 subjects with various lung diseases, finding that high sputum eosinophil counts was a predictive factor of allergic asthma[39]. Subsequent studies confirmed that recruitment of eosinophils to both the airway lumen[40, 41] and tissue[42] is a cardinal marker of allergic asthma. Thus, eosinophilic lung infiltration is a well-established mechanism of allergic asthma.

A link between eosinophilic inflammation and the development of AHR had been theorized in the 1920s. The boom in research examining eosinophils as biomarkers of disease prompted research into this putative link. In 1985, lung function decline 6-7 hours post allergen challenge was found to only occur in subjects with significant bronchoalveolar lavage (BAL) eosinophilia[41]. Moreover, examination of BAL from asthmatic and non-asthmatics at baseline, revealed that asthmatics with AHR had significantly more eosinophils and eosinophil granule products in their airways[43]. Similarly, severe asthma exacerbations were found to be accompanied by elevated levels of eosinophils and free eosinophil granules in the sputum, that did not wane until 48-hours post exacerbation[44]. Corroborating evidence, supporting the role of eosinophils as drivers of AHR came from a study of antigen avoidance. Following a 3-month period of allergen avoidance, house dust mite allergic children possessed increased airway

function and reduced sputum eosinophilia[45]. Thus, numerous studies have linked the presence of eosinophils to ongoing airway dysfunction.

The role of eosinophils in asthma has been debated. In mice, depletion of eosinophils has not yielded a consistent effect. Eosinophils have been deemed critical for airway tissue remodeling, but not airway dysfunction[46], critical for AHR induction, but not airway remodeling[47] and sometimes, entirely dispensable[48]. In humans, not all asthmatics have high levels of eosinophils in the blood[49] or bronchoalveolar lavage[50], nor can eosinophils always be found the lungs of asthmatics[35, 51, 52] . Consequently, eosinophils may not be solely responsible for driving airway dysfunction in asthma, but sufficient evidence exists to acknowledge eosinophils as a contributor to allergic asthma and a hallmark of Th2 lung inflammation.

In parallel with research linking IgE and eosinophils to allergic asthma, the role of T-cells as orchestrators of Th2 immune processes began to accumulate. IgE production was linked to T-cell activation in the 1980s through the discovery of IL-4 (originally named B-cell Growth Factor), as a molecule capable of inducing proliferation of activated B-cells[53], low-level secretion of IgG[54], and robust secretion of IgE, *in vitro*[55]. Subsequently, IL-4, in combination with IL-6, was confirmed to up-regulate IgE synthesis in human B-cells[56]. In 1985, Warren & Sanderson created a T-cell clone that induced eosinophil differentiation from bone marrow cultures, via a soluble mediator[57]. The responsible molecule was later cloned[58], and named IL-5[59]. IL-5 united cellular with humoral mechanisms in allergy, as IL-5 had first been recognized as T-cell Replacing Factor, as it induced Ig secretion in activated and proliferated murine B-cells[60]. Therefore, the discovery of both IL-4 and IL-5 emanated from their ability to induce immunoglobulin secretion and eosinophilic infiltration, critical features of allergy.

The biological properties of IL-4 and IL-5 suggested that T-cells secreting these cytokines (Th2 T-cells) were complicit in the elaboration of allergic lung inflammation. Indeed, observations of active Th2 T-cells in asthmatics quickly emerged to support this hypothesis. T-cells obtained from atopic individuals secreted large amounts of IL-4, when cultured with allergen[61]. *In vivo* evidence arose shortly thereafter, with observations that T-cells obtained from the BAL of atopic asthmatics were predominantly positive for IL-3, -4 and -5 mRNA[62] as were biopsies from late-phase reactions to allergen-skin prick tests. Similarly, bronchial biopsies taken from atopic asthmatics after allergen challenge revealed that eosinophil recruitment correlated with T-cell expression of IL-4 &-5 mRNA[63], and activated T-cells[64, 65]. These studies were corroborated by previous investigations that demonstrated the importance of T-cells during eosinophil accumulation. In mice, T-cell depleted animals were unable to clear helminth worm infection, nor mount an eosinophilic response, but adoptively transferring T-cells into deficient animals restored worm expulsion and eosinophil accumulation[66]. These studies indicated that T-cells were responsible for the eosinophilia and Ig responses that characterized allergic asthma.

The third archetypal Th2 cytokine IL-13 was cloned in 1993[67], and identified in the BAL of allergen challenged atopic asthmatics two years later[68] . In mice, administration of IL-13 was found to induce AHR, mucus production and goblet cell hyperplasia[69]. Mouse models have provided direct evidence for the role of IL-4, -5 and -13 in asthma, as selective cytokine upregulation gave rise to disease while ablation protected from allergic airways disease[70, 71].

The early success of targeted cytokine depletion in mice, combined with the identification of T-cells producing T_H2 cytokines in asthmatics, gave rise to the notion that antagonizing Th2 cytokines should protect asthmatics from symptoms and disease progression. This strategy led to the creation of numerous monoclonal antibodies and small-molecule

antagonists. In mice, blockade of IL-5 function via monoclonal antibody application (TRFK-5), lead to the complete abrogation of eosinophils. Early reports demonstrated that TRFK-5 abolished eosinophil accumulation in the lungs of *Nippostronglylus brasiliensis* infected mice[72], while subsequent studies demonstrated that IL-5 blockade (or deficiency) prevented allergen induced eosinophilia and AHR in mice[71, 73, 74] and guinea pigs[75, 76]. These preclinical results led to the testing of an IL-5 monoclonal antibody in mild asthmatics, with the expectation that antagonizing IL-5 would reduce eosinophilia and thereby ameliorate symptoms of asthma. Surprisingly, IL-5 blockade, despite significantly reducing eosinophilia, had no effect on symptoms or AHR[77]. Numerous other anti-Th2 cytokine therapies have demonstrated less than anticipated efficacy. We recently reviewed this topic (Appendix 1[78]) as the failure of these strategies highlights the complexity of disease progression, and potential pitfalls associated with anti-cytokine antibody therapy.

In the context of these failed strategies, interesting lessons can be taken from murine studies of IL-4, IL-5 and IL-13. For instance, IL-4 deficient mice can mount airway inflammation, remodeling and AHR after a series of allergen challenges[79], using a mechanism that is likely IL-5 dependent[74]. In contrast, others have observed that IL-4 and IL-13 are required for development of airway dysfunction in mice, but not IL-5[80]. Finally, while some models of helminth infection have demonstrated that IL-13 is required to facilitate robust T-cell differentiation, in the combined absence of IL-4 and -13, upregulated IL-5 can mediate worm expulsion[81, 82]. These studies reveal a high degree of redundancy amongst the Th2 cytokines. Such complexity and overlapping function supports the use of therapies that can induce immunosuppression, like immunotherapy, rather than a therapy targeting a single molecule.

Thus far, landmark studies that established the biological relevance of eosinophils, T-cells and IgE to asthma have been reviewed. The ensuing sections will discuss new mechanisms of asthma and the establishment and subversion of peripheral tolerance to allergens; through this discussion, the disruption of ongoing inflammation and restoration of immune tolerance by immunotherapy will be introduced.

1.5. ILC2s, a new cell implicated in Th2 disease

The discussion to this point has focused on historically relevant well established mediators of allergic disease. A potentially critical component of the allergic cascade that has not yet been introduced is the type 2 innate lymphocyte cell (ILC2). The role of ILC2s in the Th2 immune response and their relevance to emerging therapies will be discussed.

Together, thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 are considered epithelial-derived cytokines (EDCs), as they are robustly expressed at mucosal surfaces by epithelial cells (and perhaps macrophages[83] and keratinocytes[84]) and enable barriers to respond to danger[85]. EDCs contributed to the discovery of ILC2s. T1ST2 was identified in the 1980s as an orphan receptor[86], predominantly expressed on Th2 polarized T-cells[87]. In 2005, intranasal administration of IL-33, the T1ST2 endogenous ligand[88], was found to induce AHR, eosinophilia and goblet cell hyperplasia, in the presence or absence of T, B or NK cells[89] and function independently of IL-4[90]. In 2001, IL-25 was identified as a cytokine, and its administration to mice was found to induce IL-4, -5 and -13 production by a non-B-non-T cell (NBNT) cell, that in-turn drove IgE, IgG1secretion, eosinophil accumulation[91], and could mediate worm expulsion[92]. TSLP initially drew interest in the field of Th2 disease, due to its ability to potently activate human dendritic cells (DCs)[93] and facilitate Th2 differentiation[84, 94]. Upregulation of TSLP in the lungs of naïve mice potently induced airway inflammation, AHR and IgE

secretion[95], demonstrating a mechanism of TSLP-dependent inflammation not reliant on T-cells. Later, it was found that TSLP potently activates ILC2s[96].

Thus, the administration of these cytokines and study of mouse models with deficient or impaired T- cells, enabled the discovery of ILC2s[97-99]. Phenotypically, ILC2s are defined as lineage negative, sca-$1^{-/+}$, c-Kit^{+}, IL-$7R^{+}$, T1/$ST2^{+}$, $CD25^{+}$, Thy-1^{+}, MHC Class $II^{+/-}$and $ICOS^{+/-}$ [100] and have been identified in mouse and human lungs[101], nasal polyps, blood[102], lymph nodes, intestines[103] and skin[96]. These, and other studies, demonstrated that ILC2s can execute a complete Type 2 response, in the absence of T-cells. Indeed, ILC2s are now acknowledged as critical mediators of worm expulsion[99, 104] and inducers of lung inflammation following exposure to allergens[105], proteases[106] and viruses[107]. Moreover, in immune competent animals, sensitized and challenged with OVA, ILC2s produce similar amounts of IL-5 and IL-13 as T-cells[108], and more IL-13 than T-cells in response to direct lung challenge with IL-25 and IL-33[105]. Importantly, ILC2 lack antigen specific receptors, and cannot form a memory response[109]. Taken together, ILC2s are potent mediators of type 2 responses, with or without T- or B-cells.

ILC2s and T-cells share many responsibilities and may synergize during disease pathogenesis. Through the expression of MHC Class II on their surface[97], ILC2s *in vitro* and *in vivo* have been demonstrated to take up, process and present antigens to T-cells[110, 111]. MHC class II dependent dialogue between ILC2s and T-cells potentiates T-cell responses, while increasing ILC2 proliferation and cytokine release[111, 112]. This mechanism, and others[109], indicate that ILC2s aid T-cell differentiation and potentiate T-cell responses. Taken together, ILC2 appear to play a role in each phase of the type 2 immune response. The identification of ILC2s in the blood[113] and lungs of asthmatic patients[102, 114], supports their role in human disease as well.

ILC2s represent a new cell type that mimics the function of Th2 T-cells in their ability to induce tissue remodeling, mucus production and eosinophil accumulation. ILC2s can also perform roles classically attributed to the eosinophil (ie: worm clearance), synergize with T-cells and may be the dominant source of type 2 cytokines in the airways of severe asthmatics[115]. These findings indicate that new therapeutics developed to target Th2 pathologies will need to be cognizant of their impact upon ILC2s.

1.6. Early and late responses to allergen provocation

Understanding the kinetics of an immune response can reveal much about the mechanisms underlying the response. Study of the early and late response to allergen provocation has been critical to our understanding of allergic asthma. Intriguingly, evidence supporting the potency of peptide immunotherapy has been derived from an understanding of the disease kinetics.

McMaster University, through the work of Drs. Hargreave and Dolovich has played a prominent role in research into the kinetics of an allergic immune response. Measured by reductions in forced expiratory volume in 1 second (FEV_1), allergen challenge of an asthmatic individual can induce airflow obstruction over a prolonged period of time. Early phase asthmatic responses tend to begin approximately 10 minutes after allergen inhalation, peaking between 20-30 minutes post and waning 1-3 hours post while late phase responses begin approximately 4 hours post inhalation, peak between 6-8 hours[116]. The late response, especially in severe disease, can take days to completely resolve[117]. These two distinct reactions, termed early asthmatic response (EAR) and late asthmatic response (LAR), indicate there to be disparate immune mechanisms involved in the generation of an allergic reaction. Early research into the EAR and LAR identified the ease of triggering a LAR to be a biomarker of more severe disease, however, an important caveat to this finding was that a LAR could be induced in nearly any patient, when

a high-enough dose of antigen was administered[118]. This work not only described patient reactions to allergen, but also exemplified the immunological roots of allergic asthma.

The EAR is an IgE-mediated phenomenon[119]. Briefly, following allergen sensitization, the surface of mast cells becomes coated in allergen-specific IgE, through binding to the high affinity IgE receptor (FcεRI); allergen cross-linking of FcεRI induces the degranulation of mast cells[120]. The preformed granules of mast cells contain a variety of enzymes and mediators, including histamine, tryptase, chymase, arachidonic acid metabolites, cytokines and platelet-activating factor (PAF)[121]. These mediators efficiently stimulate smooth muscle cell contraction, increase mucus production, recruit lymphocytes and dilate the nearby blood vessels, facilitating edema[121]. Therefore, through these mediators, mast cells, once primed with IgE, can induce many of the hallmark symptoms of asthma. Importantly, these early events prime the airways for a subsequent LAR, upregulating adhesion molecules in the airways that facilitate the selective recruitment of eosinophils, basophils and Th2 T-cells[122]. The lack of a role for T-cells during the EAR could indicate that therapies targeting the T-cell, could ineffectively control the EAR.

LARs typically develop in ~50% of patients that demonstrate an EAR[123], however, most asthmatics can be forced to develop a LAR with sufficiently high doses of allergen. A LAR is typically defined as a 20% decrease in FEV_1[124] and has been associated with the recruitment of eosinophils, and evidence of eosinophil degranulation[41]. The robust effector cell recruitment and degranulation observed during the LAR has led to the suggestion that the profound, and repetitive inflammation observed during this response may be the root cause of lung remodeling and associated airway dysfunction[125]. Early research into the LAR suggested that it was an IgE mediated event[116], but subsequent research has implicated T-cells[126 127].

Identification of the immune mechanisms involved in the LAR has proven difficult. In mice and guinea pigs, the LAR is dependent upon IL-5 and eosinophils[128, 129]. However, when an allergen challenge was administered to asthmatic subjects receiving anti-IL-5 antibody therapy, LAR responses and histamine induced AHR were unchanged, despite reduced eosinophil numbers[77]. Similarly, mild asthmatic subjects treated with IL-12, an archetypal Th1 cytokine that antagonizes Th2 responses, had reduced sputum eosinophils but an unchanged LAR[130]. The involvement of adaptive responses was supported by the work of Don Cockcroft, when he examined the ability of a bronchodilator (salbutamol), mast cell inhibitor (sodium cromoglycate) and corticosteroid (beclomethasone dipropionate) to inhibit both the EAR and LAR in asthmatic patients. Sodium cromoglycate treatment abolished both EAR and LAR responses, but corticosteroid treatment only ameliorated the LAR[131]. Similar results have been found using Cyclosporin A (CsA), a drug believed to work primarily by inhibiting transcription in T-cells[132]. The ability of corticosteroid, and T-cell targeted therapies to affect the LAR, but not EAR supports the notion that the EAR is a process mediated by IgE sensitized mast-cells, but that the manifestation of a LAR involves other mechanisms.

The most direct evidence that the LAR may be mediated by T-cells comes from studies using synthetic peptides representing T-cell epitopes. In 1999, Haselden, Kay and Larché intradermally administered short Fel d 1 derived peptides that were unable to cross-link IgE, to cat allergic subjects. They observed that approximately 25% of cat-allergic asthmatic subjects developed an isolated LAR[127]. Similar results were obtained when subjects inhaled the T-cell epitopes[133], further supporting a role for T-cells during the elicitation of the LAR.

The LAR is of particular relevance to peptide immunotherapy. These studies indicated that peptide administration can induce airway dysfunction through the triggering of a LAR.

Importantly, the therapeutic potential of peptide administration was demonstrated through study of the LAR, as subjects were refractory to secondary peptide challenges[134].

1.7. Onset of allergic asthma

The majority of the population is not asthmatic. This begs the questions, what predisposes individuals towards atopy and asthma, and what are the mechanisms of disease onset? While a complete review of the risk factors and mechanisms of asthma onset are beyond the scope of this introduction, understanding precipitating events causing Th2 immune polarization will enable discussion of how immunotherapy can counteract these factors.

A common belief historically associated with the study of allergies was that allergens were innocuous proteins, therefore a healthy, appropriate response was to simply ignore the allergens presence. In the late 80s, strong evidence to the contrary was established as non-allergics were found to mount a low-grade IgG1 and IgG4 response to allergen, thereby implicating both B-cells and T-cells and ruling out immunological ignorance[135]. Subsequent reports confirmed that non-atopics possessed allergen-specific IgG, but possessed below-detectable levels of IgE[136]. As B-cells require T-cell help to antibody class-switch and produce IgG antibodies, a T-cell response to allergens in atopics was predicted. Indeed, purification of T-cells from non-atopic individuals after allergen challenge revealed that T-cells mounted a low grade IFN-γ response[136]; in contrast, atopics mounted a vigorous IL-4[136], IL-5 and IL-13, response, with little in the way of IFN-γ[137].

I. Normal responses to allergen

Animal models have been critical to our understanding of the establishment of a ‘normal’ response to an antigen. While many allergens contain adjuvant like properties, the use of ovalbumin (OVA), an innocuous protein allows the visualization of what the development of a

non-responsive immune phenotype to inhaled antigens consists of. A series of studies by Holt and colleagues in the mid-1990s examined the normal response of animals to repeated OVA administrations, establishing that animals undergo a biphasic response to inhaled allergen exposure that consists of: i) early generation of Ag-specific IgE, ii) a silencing of Th2 immunity, and iii) superimposition of a dominant Th1 response[138]. Later reexamination by Umetsu and colleagues revealed this process to be dependent upon IL-10-mediated CD4 T-cell hypo-responsiveness[139, 140]. These results were corroborated in humans, as T-cells derived from the PBMCs of non-allergic individuals were hyporesponsive to house-dust mite (HDM) and birch pollen provocation, in an IL-10 and TGF-β dependent manner[141] . The importance of IL-10 and TGF- β to the maintenance of peripheral tolerance and prevention of allergy has been demonstrated elsewhere as well. In an examination of atopic versus non-atopic individuals, the frequency of IL-10 producing CD4+CD25+ T-cells in the peripheral blood of non-atopics, was found to be enhanced, compared to atopic subjects[142]. These studies demonstrate that normal immunity to common aeroallergens comprises the overlapping of numerous, allergen-specific mechanisms of immune tolerance.

II. Sensitization: Environment and genetics

Three major factors contribute to the development of allergies: the environment, genetics and the allergen itself. The hygiene hypothesis, proposed in 1989 by David Strachan suggested that the increasing rates of allergy may be the result of increased levels of hygiene and reductions in early childhood infections[143]. Extensive research arose from this proposition associating many environmental factors with protection from allergic diseases. Data contradicting the hygiene hypothesis has arisen, such as raising rates of Th1 diseases[144] and the allergy-protective nature of parasitic worm infection[145]. Although the hygiene hypothesis is now known not to be entirely

accurate, the core tenants continue to guide our current understanding. Early life exposure to microbes likely educates the immune system, facilitating the development of robust anti-inflammatory networks capable of suppressing both Th1 and Th2 aberrant immunity[146]. Importantly, not all early-life exposures are protective by nature. Infection with respiratory syncytial virus (RSV) and rhinovirus (RV) prior to the age of 6 has been strongly related to the development of wheeze[147], and predicts the likelihood of asthma development later in life[148].

Much like early life exposures, genetic differences are a well-established predictor of allergies and asthma. It is easy to consider a wide range of genes that could be associated with allergic disease, as rationally, any gene alteration that induces Th2 cytokines/function, reduces regulation/Th1 responses or impairs barrier function, would plausibly induce asthma. For instance, guided genotyping of the IL-4, IL-13 and IL-4/13 signal transduction pathways in 1120 asthmatic children, revealed a robust association between altered Th2 cytokine genetics and the risk of asthma[149]. A review of the genetic polymorphisms that are associated with asthma is beyond the scope of this thesis, but it is important to acknowledge the importance of these factors.

III. Sensitization: Allergenicity & Th2 differentiation

Thus far, host factors conferring asthma susceptibility have been briefly discussed. Next, I will review what makes some antigens intrinsically allergenic. Some allergens, like papain are sufficiently potent to sensitize individuals in the absence of any notable genetic or environmental susceptibility. Papain is a proteolytic enzyme, used as a meat tenderizer, well known to induce sensitization following exposure to the respiratory tract, epithelium and digestive system[150]. In a study of workplace asthma, papain sensitization developed in 23% of individuals that lacked any

history of atopy[151], thereby demonstrating the ability of a potent allergen to override a healthy response. For other allergens, the root cause of intrinsic allergenicity isn't as obvious[152].

Sensitization to an allergen is critically dependent upon the uptake, processing and presentation of allergens by antigen presenting cells (APCs). Indeed, $CD11c^+$ DCs that extend dendrites into the airway epithelium have been tracked, using fluorescein isothiocyanate (FITC)-conjugated macromolecules and identified as the critical cell responsible for antigen uptake, transport to, and presentation within, the local thoracic lymph node T-cell area[153]. DCs antigen presentation is a complex process consisting of i) antigen uptake, via receptor-mediated endocytosis and phago-/pino-cytosis, ii) antigen degradation and processing for presentation by enzymes within the endoplasmic reticulum and lysosome, iii) loading of the peptide onto the MHC complex in lysosomes, and iv) shuttling of the peptide-MHC (pMHC) complex to the cell's surface for presentation[154]. Concurrently with antigen uptake and presentation, DCs integrate local signals through a series of pathogen-recognition receptors (PRRs), that collectively inform the DC to mature, migrate, or both[154]. DC maturation refers to the upregulation of co-stimulatory molecules and enhancement of MHC-dependent antigen presentation. How the DC, and other cells implicated in sensitization, sense the local environment is critical to what makes a protein allergenic.

The discovery of toll-like receptors (TLRs) was a critical step in establishing the ability of DCs to assess their local environment. In 1998, having identified the human homologue of the *Drosophila* TLR, Medzhitov demonstrated that TLRs conferred the ability to instruct the development of adaptive immunity to APCs[155]. Since this discovery, 10 TLRs in humans (13 in mice)[156] and numerous other receptor subfamilies[157-159] have been identified as mediators of

pathogen surveillance. The allergenicity of proteins is, in many cases, related to their ability to trigger these PRR receptors and induce DC maturation.

Since the demonstration that Toll-like receptor 4 detects LPS[160], this receptor has been intimately linked to allergenicity. In 2009, Germain and Lambrecht collaborated to demonstrate that HDM sensitization in mice is dependent upon TLR4. Interestingly, HDM sensitization was not dependent upon TLR4 on the surface of DCs, but rather, TLR4 activation on epithelial cells led to the secretion of DC activating cytokines[161]. TLR4 also regulates sensitization to non-proteolytic allergens. TLR4 ligation by LPS is capable of subverting ovalbumin tolerance[162]. Likewise, sequestration of LPS by ragweed may imbue the pollen with the ability to activate DCs in a TLR4 dependent manner[163]. These studies suggest that allergens can be intrinsically allergenic, or can acquire adjuvants to become allergenic.

A plethora of other molecular signals have been associated with allergenicity. For instance, molecular mimicry, solubility, size, complexity, stability and glycosylation are all determinants of allergenicity[164]. The major cat allergen, Fel d 1, is a potent allergen; however, the underlying mechanism is not known. The resolved crystal structure of Fel d 1 demonstrated a notable internal cavity, capable of transporting a ligand[165], that others[164] have termed a 'lipid pocket', capable of sequestering LPS. Alternatively, Fel d 1 may derive allergenicity from being notoriously 'sticky', as antigen complexity/heteromeric structure can confer the ability to potently induce Th2 differentiation[166]. Finally, uric acid may facilitate sensitization. In mice and humans, allergen challenge leads to accumulation of uric acid in the airways[167]. In mice, aluminum hydroxide (alum) is a commonly used Th2 polarizing adjuvant, a process found to depend upon uric acid[168].

The ability of an allergen to trigger PRRs, in combination with the dose of allergen, time of exposure, degree of foreignness, and other previously mentioned factors, are all important determinants of Th2 differentiation[169-171]. These signals are integrated by DCs and instruct maturation and presentation of allergen-derived epitopes to T-cells. It was established almost 50 years ago, that T-cell activation requires two signals[172], 1) T-cell receptor (TCR) recognition of its cognate ligand in the context of an MHC molecule (i.e.: pMHC), and 2) recognition of co-stimulatory molecules expressed by the APC; a third signal (APC produced cytokines) is known to assist T-cell activation, but is not required[173]. Co-stimulatory molecules, as the name implies, are activating cell-surface markers that stimulate T- and B-cells. Many costimulatory molecules have been identified in mice and humans, including, B7-1, B7-2, ICOS ligand, OX40 ligand, CD40, 4-IBBL and notch ligands[173]. Many of these molecules have been implicated as ***the*** co-stimulatory molecule critical to Th2 differentiation; however, others have described differentiation to be dependent upon the cumulative degree of stimulation induced by the collaboration of all-expressed co-stimulatory molecules[174]. Importantly, DCs do not hold exclusive sway over Th2 differentiation, as T-cells can integrate both DC and environmental signals and influence their own differentiation via cooperation.

To summarize, T-cell differentiation occurs when the TCR is ligated by a pMHC complex in the context of costimulatory molecules and cytokines. This concept was reviewed in detail, as it is important in two-regards, 1) Th2 T-cell polarization is a fundamental component of the development of allergic responses, and 2) immunotherapy induces immune deviation away from Th2, an occurrence limited by, and adhering to, the same principles as initial T-cell polarization.

1.8. Allergen specific immunotherapy

Thus far, the introduction has been shaped to invoke an inverted pyramid, with the heterogeneity of asthma and allergic pathologies being whittled down to a dependency upon a single event, the pMHC ligation of the TCR. This reductionist approach is simplistic, but it conveys how a therapeutic intervention, targeting this relationship, could topple the entire Th2 cascade, in a manner similar to knocking over a precariously balanced pyramid.

I. History

A criteria of atopy, established during the original definition, was that patients should benefit from allergic desensitization. Today, the process that Coca was referring to is known as allergen-specific immunotherapy (AIT)[20]. For over 100 years, AIT has consisted of administering increasing doses of allergen until a pre-determined, or symptom eliminating maintenance dose is achieved[175]. The establishment of AIT is commonly attributed to Leonard Noon, in 1911[176], however, many allergists (such as Dunbar, Besredka[177], and Curtis[178]) dabbled in the field prior to this demonstration. The work of Dunbar, in collaboration with Prausnitz, demonstrated that 'anti-toxin', generated by repeated allergen administrations to a horse, could protect from ocular symptoms of hay-fever, when co-administered with the allergen to a subject[23]. This is, to my knowledge, the first demonstration that allergen immunotherapy can exert allergen-specific tolerance through the generation of blocking antibodies.

These, and other studies inspired Leonard Noon to assess whether injection of low doses of purified allergens could protect patients from future allergen encounters. In 1911, hay-fever subjects were injected, subcutaneously, with increasing doses of grass-pollen derived allergen over periods of 3-14 days. Noon, through ocular allergen challenges, observed that this strategy led to subjects tolerating challenge doses 100 times larger than prior to treatment, thereby

demonstrating the induction of antigen-specific tolerance[176]. Noon's work also demonstrated the restrictive therapeutic window of immunotherapy, as use of doses too high for the individual *increased* the allergic responses. John Freeman expanded upon these results, demonstrating that 16/20 treated hay-fever subjects had markedly increased tolerance to subsequent ocular allergen challenges[179]. Interestingly, while not officially demonstrated, Dunbar refers to personal communications with Freeman, citing that therapy provided long term (>1year) protection to subjects[23]. Thus, the disease-modifying nature of allergen immunotherapy can be traced back to the very earliest clinical experiments.

Mechanisms of AIT were largely established in the 1960s and 1970s. The radioallergosorbent test (RAST), developed by Wide and Johansson[180], enabled quantification of serum IgE, a method leading to the demonstration that patients with hay fever, allergic asthma and atopic dermatitis had increased IgE levels[181]. These efforts rapidly led to the understanding that cell-bound, not free serum IgE was responsible for symptoms, and furthermore that increased IgE levels where predicative, but not direct evidence of the presence of allergies. To address these issue, Lichtenstein and Norman developed the *in vitro* histamine release assay to test leucocyte sensitivity. These assays facilitated early investigations into the mechanism of AIT, and are still commonly used today. Lichtenstein utilized these techniques to establish several key landmarks of AIT therapy, first that initiation of AIT enhances IgE production, second that years of AIT is required to reduce allergen-specific IgE and third, that the clinical benefit associated with AIT correlates with the magnitude of serum blocking antibodies, that out-compete IgE for allergen binding sites[182, 183]. Finally, Lichtenstein drove the field of AIT towards the use of purified allergens, rather than crude extracts. This is perhaps best illustrated by his trial of insect venom hypersensitivity, wherein subjects were treated with placebo, whole-insect-body

extract or insect venom. Patients treated with insect venom, but not placebo or whole-insect-body extract tolerated subsequent sting challenges[184].

These studies established many fundamental aspects of AIT, such as the requirement for high-dose therapy and the requirement for long-term therapy to change allergen-specific immunoglobulin titers. The following sections will briefly describe other important features of AIT, like the long-term protective effect, mechanism and safety. Through examination of these characteristics, the rational for peptide immunotherapy will become apparent.

II. Potency

The allure of AIT resides in the modification of underlying immunological processes that reduces patient sensitivity to allergens. Three major streams of evidence support the ability of AIT to modify disease, 1) AIT induces long-term efficacy that can persist years beyond the termination of treatment[185-187], 2) AIT can halt the development of asthma in those with rhinoconjunctivitis[188, 189], an effect that persists two[190] to seven[191] years post-therapy, and 3) AIT prevents *de novo* sensitizations during therapy[192-194], and for many years (12+) after the cessation of therapy[195, 196]. Thus, AIT provides long-term clinical benefit and slows disease progression.

The efficacy of AIT has been verified in over 12 meta-analyses, determining AIT to potently reduce symptoms of asthma and allergic rhinitis, when given subcutaneously or sublingually[197]. These analyses have confirmed that AIT enhances patient quality of life, reduces medication usage and typically offers potency at least on par with conventional pharmaceutical intervention. However, how does AIT compare to corticosteroid therapy, the current recommended therapy for GINA step 2+ asthma? Here, the relative value of AIT vs. pharmacotherapy becomes slightly more subjective. In fairly short trials (less than 1-year) inhaled corticosteroids (ICS) tends to outperform AIT[198, 199]. However, studies examining the

long-term effects of AIT vs ICS differ. A study of ICS vs 1 year of AIT, demonstrated that ICS therapy was more effective in the short term, but that with continued therapy AIT effectiveness caught up, and provided persistent benefit that ICS did not[200]. Elsewhere, the long-term (5 year) efficacy of ICS vs AIT was assessed. After 3 years, AIT was as effective as ICS at controlling airway symptoms and rescue medication use, while offering superior protection from AHR and rhinitis symptoms[201]. Thus, not only does AIT alter disease progression, but with persistent adherence, it rivals the potency of steroids. Problematically, the efficacy of AIT requires high-doses of allergen; doses known to be capable of inducing both local and systemic adverse events.

III. Mechanism of AIT

The clinical studies detailed above, and many others, have contributed many observations detailing changed immunological reactivity following AIT. While these observations do not provide a rigorous assessment of the mechanism of action through which AIT is functioning, they do provide many clues and common immunological shifts that are associated with antigen tolerance. While the mechanisms of peptide immunotherapy differ from the mechanisms of AIT, many similarities can be drawn; therefore a brief examination of the mechanisms of AIT is warranted.

AIT induces allergen specific tolerance. Many examinations of PBMCs post-therapy have revealed the induction of regulatory T-cell populations[202-205]. AIT also affects humoral immunity; indeed, the induction of IgG4 blocking antibodies may be required to modulate IgE-dependent events, and may represent a predictive factor that denotes efficacious AIT therapy[206, 207]. IL-10 secreted by regulatory T-cells, in the context of IL-4[208] facilitates B-cell isotype class switching, markedly enhancing the production of the protective IgG4 antibody[209]. Thus, regulatory T-cells may be critical to both the effector T-cell and B-cell responses. The role of

regulatory T-cells during AIT has been contested by some. Indeed, some studies have found no induction of regulatory cell number or regulatory function after AIT[210], instead noting deletion of allergen-specific Th2 cells[211, 212]. Others have noted no alteration in PBMC responsiveness after AIT[213]. In many of these studies citing a lack of a role for Treg cells, antibody isotypes shift towards IgG4 with therapy, implying a role for Tregs at some point during the tolerogenic cascade.

Several kinetic studies have tracked the development of tolerance during AIT. In a study of grass pollen SLIT, a series of sequential shifts in the predominant immune response to allergen were observed. At the onset of therapy, Th2 immunity became exaggerated, with increased specific IgE; by four months, a marked immune deviation away from Th2, towards Th1 and IgG4 was noted, and correlated with the induction of a regulatory T-cell population[214]. Similar results were found in a study that utilized HLA-peptide tetramers (HLA DRB1) to track antigen-specific T-cells during venom immunotherapy (VIT)[215]. The commencement of VIT induced an exacerbation of IL-4 production by antigen-specific T-cells that resolved by week six, and fell below baseline by week 12. In contrast, IL-10 production continuously increased until week 12, at which point IL-10+ antigen-specific T-cells were three times more common than baseline, and outnumbered IL-4 producing cells 3:1[215]. No change in antigen-specific IFN-γ was noted, although FoxP3+ T-cells increased with time. In a trial of birch pollen SLIT, opposite kinetics were noted as, 4 weeks of therapy increased the frequency of CD4+CD25+ T-cells, significantly enhanced expression of FoxP3 and IL-10 mRNA, and down regulated IL-4 and IFN-γ mRNA were reduced[216]. Re-examination of these subjects at week 52 revealed that neither IL-10 nor FoxP3 expression remained enhanced, but rather, IFN-γ was.

Collectively, these studies demonstrate the heterogeneity between treatment regimens and the phenotype of antigen-specific hyporesponsiveness that develops following AIT. Given the lack of consensus, it is possible that AIT works through both immune regulation and deviation. Intriguingly, evidence of contemporaneous production of IL-10 and IFN-γ has been noted previously, as VIT is known to induce IFN-γ+, IL-10+ and dual IFN-γ+IL-10+ T-cells[217].

To summarize, AIT utilizes numerous mechanisms to evoke tolerance. In general, the commencement of therapy exacerbates Th2 responses, which resolve by enhanced IgG4 production and immune deviation or regulation. Together, the altered T-cell response blunts granulocyte recruitment to the airways, limiting associated pathology. The event that triggers the development of regulatory T-cells is poorly understood. The leading hypothesis suggests that allergen administration in non-inflamed areas loads immature APCs with allergen-derived epitopes that fail to activate effector T-cells. In support of this hypothesis, a study evaluating the proteome of dendritic cells during a 4-month period of grass SLIT observed the induction of 'regulatory' dendritic cells in subjects that benefited from AIT[218].

IV. Safety

The uptake of immunotherapy is limited by both the length of treatment, and the demonstrated risk to patients. During AIT, increasing doses of allergen are administered to sensitized individuals. Injecting an allergen into a sensitized patient possess the risk of cross-linking IgE bound to FcεRI receptors on the surface of mast cells and causing degranulation. Moreover, injecting allergen bypasses protective barrier surfaces and provides direct entrance into the bloodstream. Thus, immunotherapy can induce systemic allergic reaction that can culminate in anaphylaxis. An estimated 5-35% of allergen injections induce an adverse event[219]. A retrospective analysis examining immunotherapy has indicated that the presence of poorly

controlled asthma is a risk for AIT related death[220]. As a result of this, severe asthma is almost viewed as a contraindication for AIT, thereby precluding the most in-need population.

Further compounding safety issues is the requirement for high-dose therapy. Frequently, AIT is only effective when administered at high-doses, yet these doses are often associated with notably more adverse events[221]. Thus, the development of adverse events can directly constrain the therapeutic dose-window, preventing efficacious treatment. In addition to the safety and dosing concerns, are issues associated with adherence. Efficacious AIT seems to develop following at least 3-years of therapy[222, 223], a time frame the leads to high patient drop-out rates[224]. Thus, the widespread uptake of AIT is impaired by dose requirements, a long duration of therapy and potentially life-threatening adverse events. The notable efficacy, and safety concerns of AIT, have given rise to many derivative therapies, that attempt to modify the allergen in a manner to maintain the immunogenicity of treatment, while removing the allergenicity. We have employed the use of synthetic, allergen-derived T-cell epitopes to replace traditional AIT, as this strategy retains T-cell immunogenicity, while circumventing IgE-related safety risks.

1.9. Regulatory T-cells

Tolerance induction by AIT & peptide immunotherapy depends upon many immune processes typically mediated by regulatory T-cells. Thus, understanding Treg function can facilitate understanding of AIT and peptide immunotherapy. Antigen-specific tolerance was first observed in 1953 by Medawar and colleagues, who demonstrated that *in utero* exposure to allogenic antigens protected mice exposed later in life to those same antigens[225]. Gershon and Kondo were the first to describe regulatory thymocytes, as they noted that adoptively transferred thymus-derived cells could suppress antibody production[226]. Today, numerous regulatory T-cells have been identified, and are broadly classified as either natural (nTreg) or inducible (iTreg)

cells. The most commonly discussed populations of iTreg are the IL-10 (FoxP3-), IL-35 and TGF-β producing subsets, known as Tr1, iTr35 and Th3 cells, respectively.

Natural Tregs were identified by Sakaguchi and colleagues as CD4+ T-cells that constitutively express CD25+, and possess enhanced immunosuppressive function[227]. Today, nTregs are now characterized by the transcription factor FoxP3. The role of FoxP3 in T-cell tolerance was established in the early 2000s, following observation of autoimmunity, severe allergy and fatal infection that accompanies the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) in mice with mutated FoxP3 (scurfy mice)[228]. Rudensky and colleagues demonstrated that the transfer of FoxP3 positive cells rescued scurfy mice from disease development, and that FoxP3 expression, not CD25, facilitated immune regulation[229] Shimon Sakaguchi's lab demonstrated that FoxP3 is specifically expressed in nTregs, and that retroviral gene transfer of FoxP3 into naïve T-cells confers a regulatory phenotype[230]. In humans, but not mice, FoxP3 can be transiently expressed upon activation of Teff cells[231], limiting its usefulness as a marker of human nTregs. Natural Tregs exert immunosuppression through a plethora of cell-contact dependent mechanisms[232-238], but tend to be unable to mediate regulation in a cell-contact independent manner[230, 231].

Inducible Tregs, specifically, Tr1 cells have been demonstrated as critical mediators of allergen-specific tolerance, in both normal individuals and following immunotherapy. In non-atopic individuals, provocation with common aeroallergens induced a population of regulatory T-cells that suppressed the function of Th1 and Th2 T-cells, through the secretion of IL-10 and TGF-β[141]. These cells were missing in allergic subjects, but were restored by immunotherapy[141]. Tr1 cell expansion may be the dominant response of non-allergic subjects to allergen. Direct evidence that Tr1 secreted IL-10 can ameliorate lung inflammation comes from a mouse model.

CD4+CD25+ OVA specific nTregs from IL-10 deficient animals were transferred into OVA-allergic mice. Transfer of Tregs ameliorated allergen induced inflammation and AHR, in an IL-10 dependent manner, indicating that nTregs facilitated the induction of disease modifying IL-10 producing Tr1 cells from recipient CD4+ cells[239].

Inducible regulatory T-cells develop *de novo,* in the periphery from mature CD4+ T-cells. While nTregs develop in the thymus and are exposed to ectopic expression of self-antigens in the thymus, via the AIRE gene[240], iTregs are not similarly targeted towards self-antigens. Thus, iTregs may primarily control tolerance to non-self-antigens. Expansion of iTregs occurs following exposure to tolerogenic stimuli, in a manner much like the decision to differentiate into Th1 or Th2 cells. Tr1 cells were first identified through the repeated exposure of C57BL/6 mice to a lympocytic choriomeningitis virus (LCMV) derived peptide, which resulted in antigen-specific tolerance towards LCMV that manifested as an inability to clear the virus[241]. These CD4+IL-10+ cells, expanded *de novo,* were sufficient to suppress Teffector (Teff) proliferation in response to antigen provocation and protect SCID mice from the development of colitis induced by the transfer of autoimmune, pathogenic memory T-cells[242]. These experiments established that iTregs could rapidly be expanded to self and non-self-antigens, and potently mediate immunosuppression.

Early studies of iTregs utilized experimental conditions to force expansion. Natural development of iTregs *in vivo* may rely on modified antigen presentation by APCs. In mice, airway challenged with ovalbumin induced IL-10 expression within mature pulmonary DCs, that in turn stimulated the development of Tr1 cells capable of responding to subsequent allergen challenges[140]. Similarly, *in vitro* examination of human DCs revealed that only immature DCs, as determined by a reduced costimulatory surface phenotype, could stimulate the development of

Tr1 cells[243]. Cumulatively, these and other studies[244-246], demonstrate that antigen-presentation by immature DCs leads to the development of iTregs capable of exerting antigen-specific tolerance. Natural Tregs may be the factor that limits DC maturation. Numerous studies have suggested that nTregs, following *in vivo* pMHC:TCR ligation, primarily target and regulate the APC[247-250].

The mechanism of tolerance effected by AIT is important to the resolution of disease symptoms. For instance, the paracrine release of suppressive cytokine by iTregs can regulate all nearby CD4+ T-cells, even CD4+ T-cells specific to other epitopes, a phenomenon known as bystander suppression. Similarly, the tolerogenic events occurring during AIT could affect the long-term efficacy of therapy. For instance, AIT that induces deletion of Th2 T-cells could provide short-term clinical benefit that would wane as Th2 polarized T-cells began to accumulate with subsequent allergen exposures. In comparison, the generation of a stable Treg population could lead to long-term clinical benefit, wherein each subsequent allergen exposure served to boost the efficacy and frequency of these cells. Taken together, these studies demonstrate the importance of both natural and inducible regulatory T-cells to the establishment of allergen-specific tolerance.

1.10. Peptide immunotherapy

The theme of the research to be presented is the examination of mechanisms of peptide immunotherapy, for treatment of allergic disease. This work is multidisciplinary, as it melds together asthma, allergy, AIT and peptide immunotherapy. The origins, clinical success and mechanism of peptide immunotherapy will be discussed to form the rationale for future studies, as well as highlight gaps in current knowledge. In 2011, we reviewed the field of peptide immunotherapy (Appendix 2[251]), here, relevant studies will be discussed.

I. Origins of peptide immunotherapy

The therapeutic benefit of peptide injection was first revealed by administering peptides to neonate mice, as this rendered peptide-specific T-cells unresponsive into adulthood[252]. The fact that peptide administration induced epitope-specific unresponsiveness encouraged study of peptide administration in diseases with well-defined pathogenic epitopes. Experimental autoimmune encephalomyelitis (EAE) is a murine model of multiple sclerosis, characterized by autoimmunity targeting myelin basic protein (MBP) that causes a chronic, relapsing disease & paralysis. In B10.PL mice, EAE pathology is driven by T-cell recognition of the amino-terminal MBP epitope (peptide: 1-9NAc). B10.PL mice injected with 1-9NAc or rat MBP, in complete Freund's adjuvant (CFA), develop a robust form of EAE; however, administration of 1-9NAc to neonatal and adult mice protected them from the development of EAE[253, 254]. Peptide therapy has been demonstrated to effectively regulate numerous murine autoimmunities[255-257]. Taken together, these studies demonstrate that administration of peptides can induce a form of T-cell mediated tolerance that prevents responses to entire proteins and protects from disease.

Autoimmune diseases are an excellent target for peptide immunotherapy because the genetic polymorphisms that predispose individuals to disease, tend to do so by altering antigen-presentation in a manner that breaks self-tolerance. Most frequently these genetic risk factors reside within human leucocyte antigens (HLA) such that many autoimmune diseases have HLA susceptibilities that map to particular HLA gene products. Thus, the very nature of disease progression specifies the epitopes that need to be silenced by therapy. Peptide therapy for allergic disease is not as straightforward. While immunodominant epitopes exist in allergens, the profound genetic diversity of antigen-presenting molecules precludes the possibility of a single, dominant allergen-derived T-cell epitope. Yet, this strategy has proven to be quite efficacious.

In 1993, peptide therapy was utilized to ameliorate mouse allergy to HDM and Fel d 1. In mice sensitized to HDM, intranasal administration of a peptide derived from the major HDM allergen (Der p $1^{(111-139)}$) inhibited the production of antibodies, lymphocyte proliferation during *in vitro* culture with HDM, and IL-2 production by T-cells[258]. This study established that peptide administration could ameliorate allergen-specific responses, while also demonstrating that intranasal peptide administration was effective in mice. In a similar set of experiments, treatment of mice with two Fel d 1 derived peptides protected Fel d 1 immunized mice from T-cell activation, immunoglobulin production, and conferred T-cell hyporesponsiveness to the entire Fel d 1 molecule[259]. Similar results were demonstrated in a murine model of Bet v 1 allergy[260]. Intriguingly, peptide immunotherapy was first demonstrated to ameliorate allergic airways disease in Bet v 1 allergic mice, inhibiting the recruitment of eosinophils to the lungs following birch allergen challenge[261]. Collectively, these murine experiments confirmed that peptide immunotherapy could ameliorate allergic disease, providing preclinical evidence to bring peptide immunotherapy to the clinic.

These early studies raised some questions that remain unanswered. How does treatment with a single T-cell epitope induce tolerance to the entire allergen? Early studies suggested that T-cell tolerance could spread between epitopes within an allergen, however, no study demonstrated efficacy in regulating responses to other allergens. Thus, how does therapy with Fel d 1 derived peptides affect Fel d 2 responses? These questions will be returned to later.

II. Trials of peptide immunotherapy

The first-in-man assessment of peptide immunotherapy for treatment of allergic disease utilized two Fel d 1 derived, 27-amino acid long peptides that spanned several dominant Fel d 1 epitopes. Ninety-five cat-allergic subjects were treated with four subcutaneous (SC)

administrations of peptides, or placebo. Patients treated with high doses of peptides (750µg) developed significantly fewer nasal and lung symptoms upon exposure to a live cat, compared to placebo treated individuals[262]. Treatment with 7.5 µg of Fel d 1 derived peptides elicited no clinical benefit, while treatment with 75 µg elicited moderate protection. Another trial of the same peptides confirmed the inability of low-dose peptides to confer clinical benefit[263].The efficacy of high-dose (750µg) peptide therapy was later linked to reduced IL-4 production by peptide specific T-cells[264]. Two other trials of the same peptides were performed. In a trial of 7.5, 75 and 750 µg of peptide or placebo, mixed clinical results were observed as high dose therapy failed to reduce bronchial sensitivity compared to placebo, but significant within subject changes pre-/post- therapy were noted[265]. The fourth, and largest, trial of these peptides examined 133 cat allergic subjects in a DBPC trial. Here, subjects that received 750 µg of the Fel d 1-derived peptides possessed significantly reduced symptom scores during cat-exposure[266]. Furthermore, in subjects with pre-existing airflow dysfunction, high-dose peptides significantly enhanced FEV_1 (increase of 0.28 L/6.27% predicted) compared to placebo, three weeks after the termination of therapy. All of these trials documented high levels of adverse events, with frequent induction of LAR symptoms. Early adverse events were also noted after therapy, indicating that these peptides were of sufficient length to engage circulating IgE.

In light of the high frequency of adverse events caused by peptide therapy with 27-amino long T-cell epitopes, a strategy to utilize shortened peptides was proposed, on the basis that shorter peptides should be less able to cross-link IgE. Indeed, analysis of shortened Fel d 1 derived peptides in a histamine release assay, using basophil-enriched PBMCs from allergic donors, confirmed that these peptides were unable to induce histamine release[127]. Injection of high doses (80µg) of these short, Fel d 1 derived peptides induced isolated late phase asthmatic

responses in some asthmatic subjects (9 of 40); the lack of early asthmatic response further supports the hypothesis that these peptides lack the ability to cross-link IgE[127]. Intriguingly, individuals receiving a second injection two-weeks after the first displayed no discernible LAR, demonstrating the rapid establishment of epitope-specific unresponsiveness[134]. These short Fel d 1 derived peptides were assessed in a DBPC-RCT of 24 cat allergic and asthmatic subjects. Significant immune modulation was observed following therapy, as PBMC secretion of IL-10 was significantly upregulated, while IL-4 and IFN-γ, were down regulated[267]. Furthermore, Fel d 1 skin prick testing revealed a reduced early and late phase skin responses, in treated subjects compared to placebo, although early skin responses to whole cat allergen persisted. Quality of life was improved, but AHR was not affected by therapy. Notably, the peptide selection criteria may have limited efficacy. Therapeutic peptides were generated without regard to TCR registers, as the Fel d 1 molecule was simply broken into 16 linear epitopes, and subjects were treated with 12 of them. As such, peptides may have inefficiently targeted key T-cell epitopes.

A recent double blind, placebo-controlled phase II study addressed the concern of peptide register, and safety. PBMCs from 100 cat-allergic subjects were used to screen the immunogenicity of 16 T-cell epitopes, derived from Fel d 1, and seven immunodominant peptides were identified by proliferative and cytokine responses. MHC binding analysis of these peptides, revealed that these 7 peptides were promiscuously recognized by 10 of the most common human HLA antigen presenting molecules, and failed to cross-link IgE from sensitized individuals, indicating that these peptides should be recognized by a large proportion of the population[268]. Indeed, the safety of these peptides was confirmed, as adverse events did not differ between the placebo and treatment groups.

The efficacy of this 7-peptide vaccine (known as Cat protein allergen desensitization; Cat-PAD) is currently being evaluated in a Phase III trial. Other studies using Cat-PAD have provided encouraging results. In a 1-year follow-up study, 89 cat-allergic subjects were examined to evaluate the long-term efficacy of peptide immunotherapy. In this DBPC trial, 4 injections of 6nmol (4x6) of Cat-PAD significantly reduced the development of nasal and ocular symptoms, as assessed by the total rhinoconjunctivitis symptom score, during exposure to cat allergen in an environmental exposure challenge[269]. This cohort was reassessed for rhinitic symptoms 2-years after the commencement of therapy. Efficacy, defined as reductions in the total rhinoconjunctivitis symptoms score, was maintained in the high-dose treatment group (4x6)[270]. These studies indicate the long-lasting therapeutic effect of peptide immunotherapy, as 4 injections over the course of 3 months resulted in disease-modification for at least 1.66 years post termination of therapy. In contrast, studies examining the long-term efficacy of subcutaneous and sublingual AIT have indicated that therapy for less-than two years was unable to confer long-term efficacy[222, 271] or persistently alter allergen-specific IgE, IgG1 or IgG4 levels[223].

Peptide immunotherapy for other allergens (and antigens) is also under development. Three contiguous overlapping peptides derived from the major birch allergen (Bet v 1) were assessed for their ability to treat birch-allergic subjects. In subjects receiving low dose peptide immunotherapy, the combined symptom and medication score improved, compared to placebo[272]. Some efficacy was also noted in high-dose treated subjects, although this was associated with an increased frequency of adverse events. Peptides derived from the honeybee venom Api m 1, administered to 5 bee-allergic subjects with documented systemic reactivity protected 3/5 patients from local wheal and flair development following PLA 2 challenge[273];

peptide therapy also inhibited the development of systemic symptoms following live bee sting in 3/5 patients. In a study with similar peptides, treatment of 12 bee-allergic patients lead to a significant reduction in late-phase skin response, compared to untreated controls[274]. In contrast, treatment of bee-allergic patients with long Api M 1 derived peptides failed to alter skin responses to PLA2 challenge[275]. Peptide immunotherapy for autoimmune diseases has also been investigated. A recent first-in-man trial of MBP derived peptides in patients with MS has provided encouraging results[276]. This follows demonstrations in the mid-1990s that treatment with MBP derived peptide could alter MBP-specific T-cell responses, lower autoantibody production and slow clinical progression, in some patients[277, 278]. Peptide immunotherapy for the treatment of type-1 diabetes has also been attempted. In a trial of 48 subjects, low-dose therapy with an HLA-DR4 restricted epitope derived from proinsulin, enhanced peptide specific IL-10 production[279]. Cumulatively, these studies demonstrate that peptide immunotherapy may become a safe, effective treatment strategy for difficult-to-treat immunological disorders.

III. Mechanism of action of peptide immunotherapy

The specific mechanisms that allow peptide therapy to control disease are not fully understood. Like AIT, the first step towards the induction of tolerance is believed to be the presentation of peptide by immature DCs.

Evidence from a study examining peptide immunotherapy in EAE elegantly indicated the importance of tolerogenic DCs during elicitation of epitope-specific tolerance. Following the elicitation of EAE, disease can be abrogated through a single injection of the $MBP^{(1\text{-}9NAc)}$ peptide. McPherson and colleagues examined epitope presentation post 1-9NAc administration and observed that splenic DCs, but not B-cells nor macrophages, maintained stable MHC restricted presentation of the tolerogenic peptide; immunofluorescence staining of spleens

revealed the predominant co-localization of tolerized pathogenic T-cells with CD11c+ cells[280]. The T-cell:DC relationship during peptide immunotherapy has also been examined in an allergic milieu. In OVA sensitized mice, OVA peptide administration was shown to induce tolerance, likely through the inability to induce co-stimulatory molecules expression on DCs, although peptide administration was linked to T-cell upregulation of PD-1, CD154, OX40 and RANKL[281]. In contrast, administration of OVA peptide with LPS to OVA sensitized mice led to robust costimulatory molecule up-regulation by DCs, and the development of enhanced immunity, rather than tolerance. Taken together, these studies indicate that the presentation of peptides by immature DCs may be critical to the initiation of epitope-specific tolerance by peptide immunotherapy.

Despite the involvement of immature DCs, peptide administration is known to induce T-cell mediated adverse events in human subjects. Intriguingly, the induction of adverse events and T-cell stimulation by peptide administration in both mice[282], and humans[134] does not impact the generation of tolerance. Therefore, it appears that TCR ligation in sensitized animals and human subjects is enough to stimulate T-cell activity[283], but that the lack of co-stimulatory molecules expressed by DCs[281] may limit this reaction, and give rise to tolerance.

DCs play a key role facilitating the development of tolerance, but it is the induction of regulatory T-cells that provides persistent, dominant tolerance following immunotherapy. Some evidence supporting the expansion of regulatory T-cells in humans after the administration of peptides exists. It is important to note that the expansion of these cells and their effectiveness during *in vitro* suppression does not directly support their role *in vivo.* Direct evidence of Treg involvement comes from murine models.

A series of mechanistic studies examining the T-cell repertoire of Fel d 1 peptide treated cat-allergic subjects has suggested distinct roles for nTregs and iTregs during the establishment, and persistence of tolerance. Cat allergic subjects treated with Fel d 1 derived peptides developed CD4+ T-cell unresponsiveness to *in vitro* culture with allergen, marked by reductions in proliferation and IL-13 production. However, the regulatory capacity of CD4+CD25+ T-cells did not change before and after therapy[284], suggesting that persistent tolerance is not associated with a modified nTreg population. In contrast, skin biopsies taken 24-hours after the injection of Fel d 1 derived peptides revealed the accumulation of CD4+CD25+ T-cells and CD4+IFN-γ+ T-cells, but not T-cells producing IL-10[285]. These phenotypes, while not conclusive for nTreg versus iTreg, suggest that nTreg function, or perhaps Th1 mediated immune deviation at the site of injection allows for the development of tolerance. Lending plausibility to this hypothesis, are the previously introduced studies the demonstrated that nTreg regulation of DCs can facilitate the induction of iTregs.

Peptide immunotherapy is believed to function through the induction of iTregs. Indeed, PBMCs donated by cat allergic subjects before and after Fel d 1 derived peptide immunotherapy tend to produce increased levels of IL-10 upon *in vitro* allergen stimulation, but possess unaltered CD4+CD25+ T-cell populations[286267]. Direct evidence supporting the expansion of iTregs following peptide immunotherapy comes from a mouse model of cat allergy. In cat-sensitized mice, treatment with a single Fel d 1-derived peptide protected mice from the development of AHR and airway inflammation upon intranasal challenge with CDE. In this model, effective peptide immunotherapy was accompanied by the induction of IL-10 expression in polyclonal T-cells recognizing Fel d 1, and also in treatment specific cells, but no changes in FoxP3 or TGF-β expression were noted[287]. Moreover, peptide immunotherapy was dependent

upon IL-10, as blockade of the IL-10 receptor abrogated peptide induced tolerance. In agreement with these results, prevention of EAE by peptide therapy has also been demonstrated to be IL-10-dependent[288, 289]. Similar evidence of enhanced antigen-specific IL-10 secretion by T-cells has been observed in mice following treatment of bee allergic individuals with peptides derived from PLA2[274, 275]. In these trials, and a previous trial of PLA2 derived peptides[273], treatment with peptides epitopes induced moderate amounts of IgG4, an antibody isotype produced by B-cells after exposure to IL-10. These studies indicate that successful peptide immunotherapy modulates immune responsiveness to whole allergen provocation through the induction of iTregs that regulate allergen specific T and B cells.

IV. pMHC:TCR

The immunogenicity of peptide immunotherapy resides in the ability of an injected peptide to be presented by the MHC class II complex of an APC, a process discussed in section 1.7 Onset of allergic asthma. The endocytosis, processing and presentation of an allergen is a fairly slow process (~2-6 hours[290]) as is the migration of a DC from the skin to the skin draining lymph nodes (>6 hours[291]). Therefore, the kinetics of adverse events following peptide administration is suggestive of an alternative pathway of peptide presentation.

An interesting set of experiments may explain this time discrepancy and emphasize the importance of immature DCs. In 1999, Santambrogio and colleagues demonstrated that empty MHC Class II molecules are abundant on the surface of immature DCs[292]. Moreover, these empty MHC Class II molecules could acquire peptide ligands from the extracellular space, and present them to T-cells. Indeed, further investigation revealed there to be MHC Class II antigen-processing machinery present on the surface of APCs, such as HLA-DM, an MHC Class II molecule with enzymatic activity that facilitates peptide exchange within the binding cleft of

presentation molecules[293]. These data suggest that, on the surface of cells, MHC Class II acts like a typical receptor. As such, peptide binding to surface MHC molecules should adhere to typical receptor binding properties, implying that the number of pMHC molecules should be dependent upon the affinity of the peptides to the MHC complex, and the dose of the peptide. The importance of peptide dose and affinity are further accentuated by kinetic examinations. For a given peptide of moderate-to-low affinity, the half-life of binding can be quite short, less than 15 minutes[294]. In contrast, intranasal administration of a peptide that binds MHC class II with high affinity yields APCs in the spleen and lymph node capable of stimulating T-cells for 5 days after peptide administration[295].

In 1993, Wraith examined the role of peptide affinity for the MHC molecule. Mice were treated with peptides with increasing affinity to the mouse MHC class II molecule (H-2 A^u), and only mice treated with the highest affinity peptides were completely protected from EAE[296]. Protection from EAE was later linked to the induction of co-IL-10 and IFN-γ producing T-cells[297]. These observations led to the conclusion that immune tolerance and the expansion of IL-10 regulatory T-cells were dependent upon a phenomenon termed, 'high signal strength'. In nearly all receptor mediated phenomenon, there are at least three defining characteristics, 1) affinity, 2) dose and 3) intrinsic efficacy. Given the work of Santomobrogio, these features should apply to the concept of high pMHC signal strength. Therefore, these experiments in the EAE model provide clear evidence that increased affinity *can* play a pivotal role during induction of tolerance, however, they fail to consider a role of antigen dose.

In a subsequent study, Wraith and colleagues investigated the role of dose in the context of a high-affinity peptide. In peptide therapy of EAE, higher doses of a peptide that bind MHC with high affinity, better protected mice from the development of disease[298]. Extensive

proteomics and transcriptomics throughout the treatment phase revealed that only low dose therapy with the high affinity peptide was able to induce FoxP3 expression. Interestingly, it is plausible that low-dose therapy was less efficacious than high-dose therapy due to the model used. In this study, a single pathogenic T-cell clone was responsible for ~90% of EAE. Therefore, ablating responses to this single epitope effectively treats disease. In contrast to this, responses to allergens are not dominated by a single epitope to this degree. Moreover, peptide immunotherapy for cat allergy has been shown to induce linked-epitope suppression[287], a process dependent upon sustained FoxP3 expression in some models[299]. Therefore, it is intriguing to consider that low-dose peptide immunotherapy may outperform high-dose therapy, in a disease that is not reliant on a single epitope.

The essential role of antigen dose to the generation of tolerance has previously been demonstrated. Chronic, low-dose (0.1µg) peptide administration is known to induce a Treg population capable of exerting antigen specific tolerance[300]. Similar observations have been made in a model of autoimmunity. Protection against the development of systemic lupus erythematosus in lupus-prone mice (SNF_1) was dependent upon the dose of synthetic histone-derived peptide that was administered, with 20µg outperforming treatment with 100µg[301]. These studies contrast the work of Wraith, emphasizing the importance of dose rather than affinity.

The role of peptide dose and affinity to the MHC molecule have been examined exclusively in mouse models of autoimmunity. Interpretation of these results must be considered in the context of disease etiology. While autoimmune disease is marked by the breakdown of tolerance to self-antigens, this is typically not caused by highly autoreactive T-cells, these being previously deleted in the thymus. In contrast, T-cell clones highly reactive to exogenous epitopes, such as allergens, do not undergo thymic regulation. Thus, the pathogenic T-cell

repertoire varies between autoimmune diseases and allergic disease. These variations in clonal behaviour are an important consideration for strategies trying to abrogate the function of pathogenic Teff. In light of these concerns and discordant results, a study examining the role of peptide dose and MHC affinity during the treatment of allergic responses is required. This question is clinically relevant, as the extensive genetic diversity in antigen presentation molecules, means that a high affinity peptide in one individual, is likely not a high affinity peptide in another.

1.11. The spread of tolerance

Thus far, it has been established that treatment with synthetic allergen-derived CD4 T cell epitopes can induce antigen-specific tolerance. How that epitope-specific tolerance leads to protection against challenge with complex allergens is poorly understood. Two distinct mechanisms are plausible: 1) iTregs specific for the treated epitope impair responsiveness to the whole allergen, or 2) peptide therapy induces iTregs specific for the treated epitope, and other epitopes within the allergen. If iTregs are induced only to the treated epitope, how does tolerance develop to the numerous untreated allergens contained within danders and pollens? Specifically, if peptides from Fel d 1 are used to treat allergic individuals, do Fel d 2 specific responses persist, unaffected? The second scenario suggests that tolerance to a single epitope within Fel d 1 could lead to the spread of tolerance, wherein treatment specific Tregs confer regulatory capacity onto T-cell clones specific to other epitopes. Early attempts to resolve how tolerance spreads after peptide immunotherapy were inconclusive. The treatment of EAE in rats with the 1-9NAc peptide, has been demonstrated to only induce epitope specific tolerance by some[253], while others noted the spread of tolerance to T-cells specific for other MBP epitopes[302].

In the field of allergy, therapy with peptide epitopes facilitates the spread of tolerance to other, untreated epitopes within the same allergen. Treatment of HDM allergic mice with Der p1$^{(111\text{-}139)}$ inhibited T-cell responsiveness to Der p 1$^{(111\text{-}139)}$ and two additional epitopes (Der p 1$^{(78\text{-}100)\ \&\ (21\text{-}29)}$)[258]. Intriguingly, this tolerance could not be spread to OVA upon dual-allergen sensitization[303]. Fel d 1 peptide immunotherapy has been demonstrated to induce linked-epitope suppression in both mice and humans. Treatment of cat allergic subjects with a mixture of 12 peptides induced PBMC hyporesponsiveness to the treatment peptides and an additional 4 Fel d 1 peptides that were absent from the treatment cocktail[287]. In cat allergic mice, the use of tetramers to track antigen-specific cells revealed that therapy with Fel d 1$^{(29\text{-}45)}$ suppressed proliferative responses in both treatment specific T-cells and T-cells specific for other epitopes[287]. These data demonstrate the development of linked-epitope suppression, as treatment with a single epitope impacted the response to another epitope in the same molecule (hence the 'linked' moniker), but the authors did not comment on whether tolerance can spread beyond the treated allergen.

Regulation likely spreads through bystander and infectious tolerance. Bystander tolerance is the ability of Tregs specific to one antigen to impair Teff responses to other antigens. Infectious tolerance is the ability of one Treg population to confer regulatory capacity to another T-cell population, typically this refers to T-cells specific for distinct epitopes on the same antigen. Finally, bystander suppression can be infectious, wherein bystander regulation gives rise to Tregs specific for distinct antigens. Thus, how peptide immunotherapy regulates responses to complex allergens is a question of whether bystander, infectious or infectious bystander tolerance is elicited.

Bystander tolerance was first demonstrated in EAE, as mice fed OVA prior to priming with MBP+OVA in CFA, failed to develop EAE or MBP delayed type hypersensitivity (DTH) responses, while sham-fed mice mounted robust disease[304]. Indeed, OVA pre-feeding regulated MBP specific responses as efficaciously as MBP pre-feeding. Thus, infectious bystander tolerance is an efficacious suppressive mechanism that instills lasting regulation. The ability of peptide immunotherapy to regulate responses to untreated antigens has been suggested in the past. Recently, the ability of bystander tolerance to regulate airway disease was investigated. Feeding mice OVA, after sensitization to β-lactoglobulin (BLG)-conjugated OVA (BOG), inhibited both OVA- and BLG-induced airway inflammation[305]. Thus, oral immunotherapy has been demonstrated to induce bystander suppression that inhibits allergic airways disease.

"Infectious transplantation tolerance", was coined to describe the "virulent" spread of tolerance between T-cell clones observed establishment of transplantation tolerance, by Cobbold and Waldmann. Indeed, the study of skin grafts from donors with minor (B10.D2, B10BR, etc) and major (BALB/c, C57BL/6) MHC mismatches onto recipient mice (CBA) has revealed many key mechanisms associated with infectious, and bystander tolerance. Indeed, in this model, a tolerogenic antibody depletion cocktail, prior to engraftment, facilitated the generation of CD4+CD25+ and CD4+CD25- cells, that combined to regulate ensuing grafts[306-308]. Following the cessation of therapy, graft acceptance was shown to depend upon the infectious expansion of Tregs (both nTreg and Tr1 cells) that modulate the behaviour of host and graft APCs[309]. In this model, FoxP3 was required to imbue subsequent generations of T-cells, and T-cells recognizing other epitopes, with the ability to become suppressive[299]. While not directly studied, the leading hypothesis describes bystander and infectious tolerance as a phenomenon of multiple epitope presentation by APCs. Specifically, the presentation of an epitope recognized by the TCR of a

Treg, leads to the regulation of the presenting DC and T-cells recognizing antigens on the surface of that APC, leading to the *de novo* induction of iTregs to untreated epitopes[310]. Together, these experiments describe a process wherein the induction of tolerance to a single epitope can facilitate the spread of tolerance to the parent molecule (or allergen) and other antigens presented contemporaneously.

In a particularly relevant and complex experiment, Waldmann and colleagues demonstrated the importance of context and how it can facilitate bystander tolerance. A simplified rendition of their observations noted that, a mouse (strain "a"), tolerized to a second strain (stain "b") accepts grafts from strain "b", but not a distinct strain ("c"). However, following the manifestation of tolerance within strain "a" to "b", strain "a" can tolerate grafts from F1(b x c) mice[311]. Therefore, the act of providing antigens in the context of epitopes that are the target of potent immune regulation enables the spread of tolerance from the regulated epitopes to the new antigens.

Given the ability of bystander tolerance to regulate robust, Th1 mediated allogeneic immunity, the potential clinical benefit of such a strategy for treatment of allergic disease is profound. For instance, peptide immunotherapy for a single allergy could be harnessed to modulate responsiveness to all of an individual's allergies. Consequently, this strategy would make identifying all allergens that a person is hypersensitive towards, irrelevant, as a limited number of peptide immunotherapy therapeutics could provide clinical benefit to nearly all allergies.

1.12. Animal models

This review of peptide immunotherapy has highlighted two major questions, 1) how do dose and affinity of peptide for the MHC class II molecule affect the efficacy of peptide

immunotherapy? and 2) does peptide immunotherapy induce epitope-specific regulation, or through a bystander effect give rise to infectious tolerance wherein regulation spreads to other, untreated epitopes?

To date, the Larché lab has extensively used clinical samples, predominantly PBMCs, to study the mechanism and efficacy of peptide immunotherapy[127, 267, 274, 286, 312]. More recently, a mouse model was employed to examine the spread of tolerance between epitopes, following treatment with peptides derived from Fel d 1[287]. The questions pertaining to peptide affinity and tolerance spread outlined above cannot be addressed using human samples because of the co-dominant expression of HLA haplotypes. Since the vast majority of people express two distinct antigen-presentation systems, it is impossible to dissect which pMHC molecule was responsible for therapeutic efficacy. By using inbred strains of mice, this problem is circumvented.

We chose to utilize humanized, transgenic mice that express human HLA molecules, while lacking endogenous murine MHC Class II[313]. Indeed, commercially available HLA-DR4 transgenic mice express HLA-DRA-IE alpha and HLA-DRB1*001-IE beta chimeric genes and lack endogenous IA^b and IE^a. These antigen presentation molecules utilize the peptide binding domain of the human HLA-DR4 molecule (HLA-DRA and HLA-DRB1*0401) transgenically linked to the murine IE^d. In this manner, human peptide recognition has been conferred to mice, while maintaining proper MHC function. The HLA DR4 transgenic mouse is an attractive model to study peptide immunotherapy, as the DR4 binding affinity of numerous allergen derived peptides has been established elsewhere[268], a feature enabling comparison between clinical and preclinical data.

The use of small animals to model peptide immunotherapy, asthma and major immune paradigms is well established. For instance, the use of guinea pig models of allergy date back to

the early 1900s[23]. Using mouse T-cells clones, the Th1 vs Th2 paradigm was established[314]. Mouse models led to the identification of ILC2s[98], nTregs[227], and iTregs[239]. Moreover, peptide immunotherapy was first demonstrated in a mouse model[252], as was peptide immunotherapy for HDM[258] and Fel d 1[259] allergy. Therefore, small animal models can provide extensive insight into immune responses, particularly when considering cellular function and interactions. The criticism levied against the use of mouse models to study asthma, is the numerous 'false-positives' generated, wherein a molecule successfully treated mouse allergic airways disease, but failed in clinical trials. This narrative is slightly simplistic, as molecules like anti-IL-5 and anti-IL-4 antibodies had failed to treat airway dysfunction in mice, long before their clinical failures[74, 315]. Thus, mouse models, when used appropriately, can yield pertinent, valuable results that can inform patient care.

Bystander tolerance could be studied in humans by treating polysensitized patients for one allergy, then later assessing responsiveness to another. The development of bystander tolerance is believed to happen at the surface of an APC, while that APC is presenting both the tolerated and third-party antigen. To replicate this scenario, individuals would need to receive the tolerated and non-tolerated antigen, simultaneously. The presentation of a tolerated antigen in the context of a not-tolerated antigen could lead to bystander *inflammation*, capable of subverting the tolerance instilled by immunotherapy. Thus, preclinical studies examining the interaction between treated and untreated allergens is required to ensure patient safety.

Thus, it is important to carefully structure mouse studies. For example, if T-cell mediated tolerance is being examined, the primary outcome measure of the study needs to be a disease index that T-cells can regulate, preferably in both mice and humans. This concept entails tailoring the outcome measures to the intervention. As peptide immunotherapy is expected to

expand Tregs, examining the inflammatory infiltrate after therapy will reveal the establishment of regulation.

1.13. Objective and hypothesis of the thesis

The burden of allergic asthma in developed countries continues to increase and current therapeutics are failing to properly control disease. Peptide immunotherapy is an emerging therapeutic option with the ability to modify the course of allergic disease. Peptide immunotherapy is a fast, safe, efficacious alternative to conventional pharmacotherapy. Research into the mechanism of peptide immunotherapy will aid its ascension to the forefront of allergic asthma management.

Individual experiments were performed under the aegis of the common hypothesis: "*Peptide immunotherapy is a disease modifying therapy that can protect mice from allergen induced inflammation".* From this starting point, we set out to examine several mechanistic several aspects of peptide immunotherapy with the extended hypothesis of, "*The induction of immunosuppression by peptide immunotherapy involves the infectious spread of tolerance beyond the treatment epitope, and is dependent upon treatment peptide dose and affinity to MHC"* .The strategy employed to address these claims was to, a) utilize and develop murine models of allergen sensitization, challenge and treatment, b) study the spread of tolerance in dual allergen models of allergy, and c) identify key molecular determinants (affinity, dose) that confer immunosuppressive potency to treatment with a peptide.

The first two chapters will examine the spread of tolerance following peptide immunotherapy. In our first manuscript, Chapter 2 – AMELIORATION OF OVALBUMIN-INDUCED ALLERGIC AIRWAY DISEASE FOLLOWING DER P 1 PEPTIDE IMMUNOTHERAPY IS NOT ASSOCIATED WITH INDUCTION OF IL-35, we employed a

dual allergen murine model of sensitization, wherein mice allergic to HDM and OVA, received therapy with two Der p 1-derived peptides, then were challenged with OVA to assess whether tolerance could be spread from HDM to OVA. Here, we demonstrated that Der p 1 immunotherapy could be utilized to suppress OVA-specific lung inflammation.

The first manuscript demonstrated that peptide immunotherapy could induce bystander tolerance that was capable of suppressing allergen induced inflammation. In this study, mice were treated with peptides intranasally, a milieu known to facilitate the development of tolerance in mice[258], but not in humans[133]. Moreover, in this demonstration, peptides from HDM were used, whereas the most clinically advanced product uses peptides derived from the primary cat allergen, Fel d 1. In the second manuscript, Chapter 3 -- INDUCTION OF INTER-MOLECULAR TOLERANCE FOLLOWING FEL D 1 PEPTIDE IMMUNOTHERAPY, we utilized another model of dual allergen sensitization, wherein mice were primed to Fel d 1 and OVA, treated intradermally with Fel d 1 derived peptides, given a series of injections to co-localize the responses, then recalled with ovalbumin or cat dander extract. Here, we demonstrated that intradermal therapy with Fel d 1 derived peptides could suppress both cat and ovalbumin-specific responses. Indeed, our observations indicate that peptide immunotherapy gave rise to a Treg population that could facilitate the development of protective immune deviation to other allergens. Together, Chapter 2 and 3 demonstrate that peptide immunotherapy can enable bystander tolerance that protects mice from challenge with both the treated and an unrelated allergen.

To study the role of peptide affinity and dose during the establishment of tolerance we set out to develop a mouse model of allergic airways disease in HLA DR4 transgenic mice. In Chapter 4 -- TISSUE, BUT NOT BAL EOSINOPHILS, CORRELATE WITH AHR IN A

TRANSGENIC MOUSE MODEL OF CAT DANDER ALLERGIC AIRWAYS DISEASE, we developed a model of cat allergy in HLA-DR4 transgenic mice. In this model, through a titration of the allergen challenge dose, we demonstrated that challenge with increasing doses of allergen induced a switch from eosinophilic, towards neutrophilic inflammation in the lung. Examination of the high-dose allergen induced neutrophilic inflammation revealed it to be refractory to both peptide immunotherapy and corticosteroid therapy.

In Chapter 5 – EPICUTANEOUS EXPOSURE TO CAT DANDER EXTRACT SENSITIZES MICE IN A MYD88 DEPENDENT MANNER, we developed a protocol to sensitize mice to cat dander following epicutaneous exposure to allergen. Here, we demonstrated that 'intermediary' doses of CDE applied to disrupted patches of mouse skin induced robust allergen sensitization, but that mice exposed to 'high' doses of allergen did not respond to subsequent allergen challenge. Studies utilizing adoptive transfers and transgenic mice were used to probe whether high-dose allergen exposure induced cat-specific tolerance, or impaired sensitization. **Chapter 5 (and 6) have not yet been submitted for publication.**

In Chapter 6 -- THE ROLE OF PEPTIDE AFFINITY AND DOSE DURING PEPTIDE IMMUNOTHERAPY FOR TREATMENT OF CAT ALLERGY, we utilized the model developed in Chapter 3 to examine the role of peptide affinity and dose during peptide immunotherapy. Here, we demonstrated that peptide affinity alone did not predict the maximal tolerogenic capabilities of a peptide, rather the ability to induce allergen-specific tolerance was a result of peptide affinity, dose and intrinsic properties.

The link connecting these studies is the goal of establishing and utilizing murine models of allergic disease to examine features of the tolerance manifest by the administration of allergen derived peptide.

Chapter 2: Amelioration of Ovalbumin-Induced Allergic Airway Disease Following Der P 1 Peptide Immunotherapy is not Associated with Induction of IL-35

2. Declaration of Academic Achievement

Chapter 2. Amelioration of ovalbumin-induced allergic airway disease following Der p 1 peptide immunotherapy is not associated with induction of IL-35

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- Rights for publication in thesis were retained by the authors (2013).

Daniel Moldaver's contributions

As co-primary author, I was responsible for conceiving, planning, managing and contributing to all aspects of the study. I treated animals, performed mouse dissections, fixed tissues, performed histology, prepared cells for flow cytometry and proliferation assays, & performed ELISAs. M. Bharhani was responsible for flow cytometry. J. Wattie was responsible for mouse tracheostomies, and performing the nebulized methacholine challenge. M Larché, R. Ellis, H. Neighbour, and M. Inman provided guidance on study design. Analyses, statistics and figure generation were performed by myself for all outcomes, except flow cytometry, which was done by M. Bharhani. Manuscript preparation was performed by myself and M. Bharhani, with guidance from M Larché.

Amelioration of ovalbumin-induced allergic airway disease following Der p 1 peptide immunotherapy is not associated with induction of IL-35

DM Moldaver[2,3], MS Bharhani[1,3], JN Wattie[1], R Ellis[1], H Neighbour[1], CM Lloyd[2], MD Inman[1] and M Larche´[1]

In the present study, we show therapeutic amelioration of established ovalbumin (OVA)-induced allergic airway disease following house dust mite (HDM) peptide therapy. Mice were sensitized and challenged with OVA and HDM protein extract (Dermatophagoides species) to induce dual allergen sensitization and allergic airway disease. Treatment of allergic mice with peptides derived from the major allergen Der p 1 suppressed OVA-induced airway hyperresponsiveness, tissue eosinophilia, and goblet cell hyperplasia upon rechallenge with allergen. Peptide treatment also suppressed OVA-specific T-cell proliferation. Resolution of airway pathophysiology was associated with a reduction in recruitment, proliferation, and effector function of T_H2 cells and decreased interleukin (IL)-17^+ T cells. Furthermore, peptide immunotherapy induced the regulatory cytokine IL-10 and increased the proportion of Fox $p3^+$ cells among those expressing IL-10. Tolerance to OVA was not associated with increased IL-35. In conclusion, our results provide in vivo evidence for the creation of a tolerogenic environment following HDM peptide immunotherapy, leading to the therapeutic amelioration of established OVA-induced allergic airway disease.

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2.2. Introduction

Allergen-specific immunotherapy, which targets the underlying mechanisms of allergic inflammation, is disease-modifying and remains the only curative approach for allergic diseases.[1,2] However, administration of whole-allergen molecules in current specific immunotherapy products carries the risk of IgE-mediated adverse events, which may be local or systemic and may include life-threatening anaphylaxis.[3,4] Many novel approaches are being designed to reduce the allergenicity of immunotherapy preparations while maintaining immunogenicity. One approach is the use of short synthetic peptides that represent dominant T-cell epitopes of the allergen. Short peptides exhibit markedly reduced capacity to cross-link IgE and activate mast cells and basophils, owing to a lack of tertiary structure. Studies in mouse models have established the feasibility of this approach, and clinical studies are currently in progress in both allergic and autoimmune diseases.[5,6] Peptide immunotherapy induces immunological tolerance mediated by regulatory T cells and interleukin (IL)10, which suppresses allergen-specific T-cell proliferation and production of T_H2 cytokines.[5,7–10]

Allergen proteins contain multiple T-cell epitopes restricted by a wide range of human leukocyte antigen (HLA) molecules. Furthermore, many allergen sources contain multiple allergenic proteins. For peptide immunotherapy products, the diversity present in both allergen molecules and HLA molecules requires that multiple immunodominant T-cell epitopes be represented to achieve population coverage. Peptide solubility and, in some cases, regulatory requirements (such as the ability to resolve individual peptide components from a mixture) constrain the numbers of individual peptides that can be formulated for treatment. Therefore, ideally, an effective peptide therapy should be capable of generating a local environment (e.g., through the induction of regulatory T cells) that can facilitate the induction of tolerance to multiple allergen proteins encountered contemporaneously. The demonstration that such a ‘‘tolerogenic environment’’ can be achieved in vivo within the respiratory mucosa will inform development of future interventions in allergic diseases.

In a recent study in the context of allergic asthma, we provided evidence of an association of peptide immunotherapy-induced immunological tolerance with linked epitope suppression.[5] In this case, tolerance was induced via intradermal, rather than airway, delivery of peptides. Others have shown that tolerance to an allergen (induced via the airways) can prevent sensitization (i.e., prophylaxis) to an unrelated antigen.[11] However, to be effective in the field, such an approach must be applicable to existing allergic airway sensitizations (i.e., therapeutic). This issue has yet to be addressed in the literature.

In the current study, we hypothesized that mice co-sensitized to house dust mite (HDM) and ovalbumin (OVA), treated by mucosal delivery of T-cell epitopes from the HDM allergen Der p 1 to create a tolerogenic environment and then concomitantly exposed (via the airways) to both allergens, would exhibit reduced allergic airway responses to OVA upon inhaled OVA challenge.

We provide evidence that peptide immunotherapy reduced airway hyperresponsiveness (AHR), OVA-specific T-cell proliferation, T_H2-cell numbers, and pro-inflammatory cytokines. Modulation of the response to OVA was associated with induction of IL-10 production and Fox p3 expression, but not increased IL-35 production.

2.3. Results

Der p 1 peptide immunotherapy improves lung function and reduces inflammation in the lungs of OVA-exposed, dual allergen-sensitized mice

To investigate whether peptide immunotherapy decreased airway inflammation, mice were killed and airway inflammation was investigated in lung tissue and in the airway (bronchoalveolar lavage (BAL) cell counts). Hematoxylin and eosin and periodic acid-Schiff stainings of fixed lung tissue sections were performed to identify eosinophils and mucous-secreting goblet cells. Histological observations of lung tissue showed that mice that were sham-sensitized, but challenged with allergen, did not exhibit any pulmonary inflammation (Figure 1a,d). Animals sensitized and treated with vehicle demonstrated extensive peribronchial eosinophilic inflammatory infiltrates (Figure 1b) with airway luminal hyperplasia of goblet cells (Figure1e). Peptide immunotherapy significantly reduced both eosinophilia (Figure 1c) and goblet cell hyperplasia (Figure 1f).

Total lymphomononuclear cell numbers in lung tissue digests were modestly, but significantly, decreased with peptide immunotherapy (Figure 1g). OVA airway challenge was associated with a significant increase in peribronchial eosinophil numbers from vehicle-treated mice. Treatment with Der p 1 peptides significantly reduced eosinophilia (Figure 1h). Similarly, peptide treatment significantly reduced the number of goblet cells (Figure 1i). Total and differential inflammatory BAL-fluid cell counts were performed by Wright–Giemsa staining of cytospin slides. In the airways of vehicle-treated mice, total BAL inflammatory cells were increased compared with unsensitized mice (Figure 1j). Of these cells, eosinophils constituted 56.08±11.8%, with the remaining cells being neutrophils (7.1±2.5%), lymphocytes (5.9±1.1%), and macrophages (41.3±9.3%). Peptide immunotherapy had no significant effect on BAL cell counts (total or differential).

To investigate whether peptide immunotherapy improved lung function in dual allergen- (HDM/OVA) sensitized mice, we measured airway responsiveness to MCh challenges using the FlexiVent system (Scireq, Montreal, QC, Canada) 48 h after the last OVA rechallenge. Allergen-sensitized mice treated with vehicle demonstrated MCh dose-dependent increases in total respiratory system resistance (R_{RS}), which were significantly reduced in mice treated with the Der p 1 peptides (Figure 1k, lung resistance; Figure 1l, area under the curve).

Der p 1 peptide immunotherapy reduces lymphocyte IL-5 production and T_H2-lymphocyte recruitment to thelungs of OVA-exposed, dual allergen-sensitized mice

Although (like eosinophils) airway luminal lymphocyte numbers were not modified by peptide immunotherapy (Figure 1j), total $CD4^+$ cell numbers in lung tissue digests were significantly reduced compared with vehicle-treated mice (Figure 2a). The numbers of tissue $CD8^+$ cells and tissue B cells ($CD19^+$) were also significantly reduced with peptide treatment compared with vehicle treatment (Figure 2b,c). Given the pivotal role T_H2 cells are believed to have in allergic disease, we performed flow-cytometry analysis of lung tissue digest cells to quantify T_H2 cells along with inflammatory cytokine-producing cells. $CD4^+$ cells were gated and further analyzed for T1/ST2 expression to identify T_H2 cells ($T1/ST2^+$ $CD4^+$). Total $T1/ST2^+$ $CD4^+$ cell numbers were significantly reduced in peptide-treated, compared with vehicle-treated, dual allergen-sensitized mice (Figure 2d,f). Furthermore, IL-5 producing T_H2 cells ($IL\text{-}5^+$ $T1/ST2^+$ $CD4^+$) were decreased by approximately 50% (Figure 2e,f). No decrease in both percent and total $IL\text{-}4^+$ $CD4^+$ cell numbers were observed in this study after peptide treatment. IL-13 levels were not measured.

Der p 1 peptide immunotherapy decreases $IL\text{-}17^+$ cell recruitment to the lungs of OVA-exposed, dual allergen sensitized mice

Previous studies have implicated the pro-inflammatory cytokine IL-17 in the development of airway inflammation and AHR. Therefore, we investigated intracellular expression of IL17 in this model by flow cytometry and assessed the influence of peptide treatment. Peptide immunotherapy reduced the number of $IL\text{-}17^+$ cells in lung tissue digests (Figure 3a). To examine IL-17-producing $CD4^+$ cells, gated $CD4^+$ cells were analyzed for IL-17 expression. Both the percentage of $IL17^+$ $CD4^+$ cells and the total number of $IL\text{-}17^+$ $CD4^+$ cells in lung tissue digests were decreased in peptide-treated mice, compared with vehicle-treated mice (Figure 3b,c). Peptide treatment also reduced total $IL\text{-}17^+$ $CD4^+$ cell numbers ($3.27\pm0.49\ \ 10^4$ vs. $2.04\pm0.23\ \ 10^4$, Po0.05) in draining lymph node (DLN) compared with vehicle treatment.

Der p 1 peptide immunotherapy reduces OVA-specific T-cell proliferation

To assess the effect of Der p 1 peptide immunotherapy on functional T-cell responses to allergen, OVA- and HDM specific T-cell proliferation was measured using two independent methods: (a) [^{3}H] thymidine assay and (b) flow cytometry following labeling with the cell division-tracking dye carboxyfluorescein diacetate succinimidyl ester (CFSE). DLN cells isolated from vehicle-treated and peptide-treated mice were cultured with different concentrations of OVA and HDM. In both sets of experiments, Der p 1 peptide treatment significantly reduced OVA-induced proliferation (Figure 4a,b). HDM induced less proliferation than OVA, and although peptide treatment was associated with smaller proliferative responses, the difference between peptide and vehicle was not significant using either method. Representative data plots of CFSE-labeled DLN cell cultures stained for CD4 demonstrate that both OVA and HDM induced substantial proliferation of CD4 cells isolated from vehicle-treated mice (Figure 4b). Peptide immunotherapy markedly reduced OVA-driven CD4 cell proliferation, while also resulting in a more modest reduction in HDM-driven CD4 cell proliferation.

Der p 1 peptide immunotherapy increases IL-10, but not IL-35, in the lungs of OVA-exposed dual allergen-sensitized mice

IL-10 and IL-35 protein levels were examined in BAL fluids and supernatants of lung tissue digests (and serum; IL-35 only) using enzyme-linked immunosorbent assay (ELISA). Peptide immunotherapy was associated with a significant, although modest, increase in IL-10 protein in supernatants of lung tissue digest (SLD; Figure 5a). The concentration of IL-10 was significantly lower in BAL fluids than in lung tissue digest and did not change after peptide immunotherapy (Figure 5a). As IL-10 can be produced by many cell types, we performed intracellular IL-10 analysis in lung tissue digest cells to investigate which lung cells produced IL-10. Peptide immunotherapy was associated with a significant increase in both percentage (Figure 5b, representative plots) and total (Figure5c) IL-10$^+$ lung tissue digest cells. To determine which cells produced IL-10, positive cells were gated and further analyzed for expression of CD4 (T-helper cells) and CD19 (B cells). In vehicle-treated mice, CD4$^+$ and CD19$^+$ cells constituted 26±2.6% and 39±6.5% of IL-10$^+$ cell population, respectively. In peptide-treated mice, the percentage of IL-10$^+$CD4$^+$ cells, but not IL-10$^+$CD19$^+$ cells, increased significantly following peptide immunotherapy (Figure 5d).

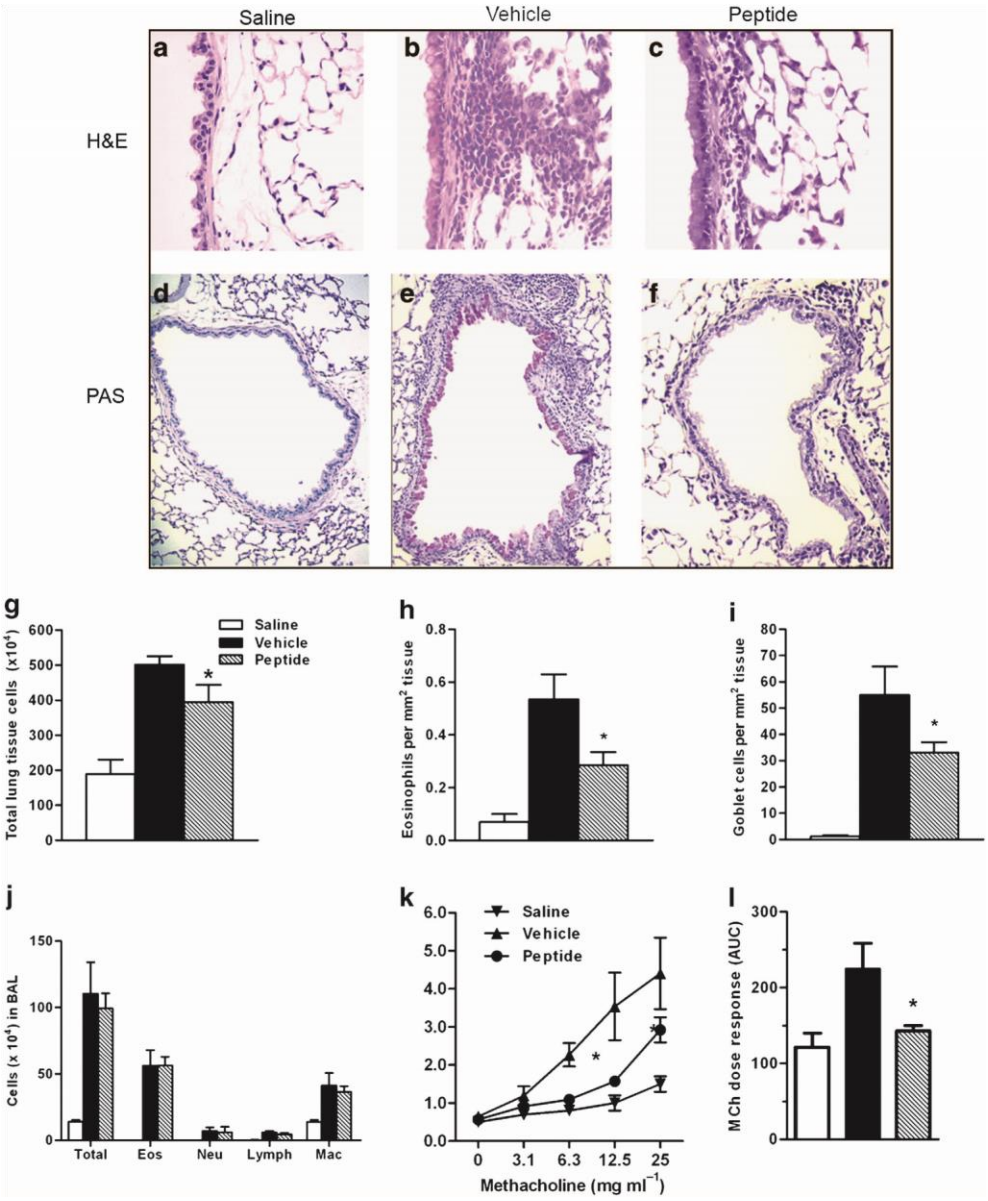

Figure 1. Der p 1 peptide immunotherapy reduces cell recruitment to the lung and airway hyperresponsiveness in ovalbumin-challenged, dual allergen sensitized mice. Mice were sensitized and challenged as described in Methods. (a–f) Representative lung histology from control, vehicle-, and peptide treated mice showing inflammatory infiltrates and goblet cells. Lung sections were stained with hematoxylin and eosin (H&E; a–c) or periodic acid-Schiff (PAS; d–f). (b) Single lobes of lung tissue were enzymatically digested to generate cell suspensions for the enumeration of total numbers of cells in lung tissue digest (g). Tissue eosinophils (h) and mucin$^+$ cells (i) in the submucosa were enumerated using a standardized protocol. (j) Total and differential cell counts were performed on bronchoalveolar lavage (BAL) fluids using Wright–Giemsa-stained cytospin preparations to obtain absolute numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lymph), and macrophages (Mac). Percentages were multiplied by the total number of cells obtained in the lavage fluid. On day 58, airway responsiveness was measured using the FlexiVent small animal ventilator system. (k) Total respiratory system resistance (cm H_2O/mL/s) in response to increasing doses of nebulized methacholine was assessed (n = 8). (l) The area under the methacholine dose–response curve (AUC) is shown. *P<0.05 vs. vehicle-treated mice.

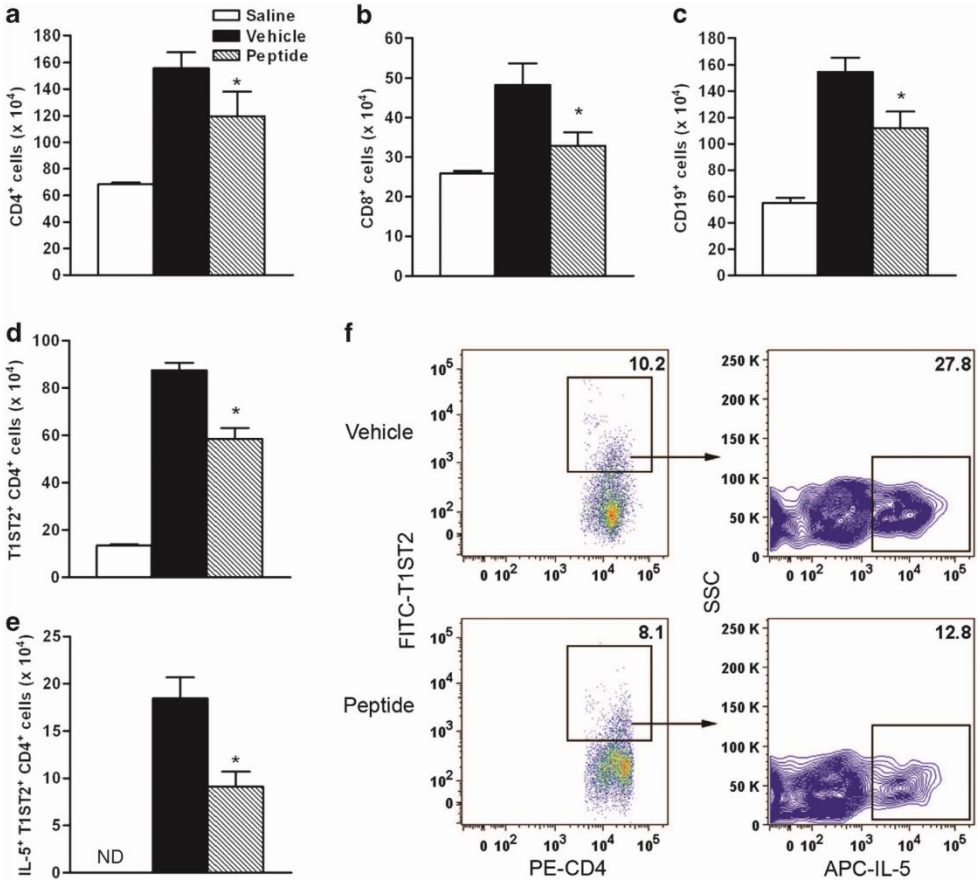

Figure 2. Der p 1 peptide immunotherapy reduces recruitment of lymphoid effector cells to the lung following OVA challenge. Mice were killed 48 h after ovalbumin rechallenge (i.e., day 58), and cells were isolated from lung tissue digest as described in Methods. Cells were stained for surface markers and intracellular cytokines. For intracellular interleukin (IL)-5 staining, cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. (a) Total $CD4^+$ cells. (b) Percentage of $T1ST2^+$ $CD4^+$ cells (left panels) and percentage of $IL\text{-}5^+$ cells in $T1ST2^+$ $CD4^+$ cells (center panels), total $T1ST2^+$ $CD4^+$ cells (top right panel), and total $IL\text{-}5^+$ $T1ST2^+$ $CD4^+$ (bottom right panel) are shown. (c) Total $IL\text{-}4^+$ $CD4^+$ cells, (d) total $CD8^+$ cells, and (e) total $CD19^+$ cells are shown. (f) Flow-cytometry analysis showing percent $T1ST2^+$ $CD4^+$ cells (left panels) and percent $IL\text{-}5^+$ cells in $T1ST2^+$ $CD4^+$ cells (right panels). $T1ST2^+$ $CD4^+$ cells shown in left panels are after gating $CD4^+$ cells. Data are expressed as mean cell numbers ± s.e.m. of each population by multiplying the percentage expression on total live cells acquired by the total cell counts. Cells of lung tissue digest from 2–3 mice were pooled; each bar shows the mean ± s.e.m. of 3–4 data points from eight mice. ND: intracellular cytokines in saline group were not quantified due to insufficient cell numbers in lung tissue digests. *P<0.05 vs. vehicle-treated mice.

$IL\text{-}10^+$ cells also increased significantly in the DLN of peptide treated mice (3.4±0.84% vs. 1.3±0.15%, Po0.05). In DLN of vehicle-treated mice, $CD4^+$ and $CD19^+$ cells constituted 52.6±2.7% and 27.9±3.4%, respectively, of the $IL\text{-}10^+$ cell population. Peptide treatment also significantly increased the $CD4^+$ population within $IL\text{-}10^+$ cells (63.2±4.1%, Po0.05, vs. vehicle-treated mice) without any significant change in $CD19^+$ cell population (30.9±1.4%).

Peptide immunotherapy was not associated with an increase in IL-35 in BAL, lung tissue digest, or serum (Figure 5e).

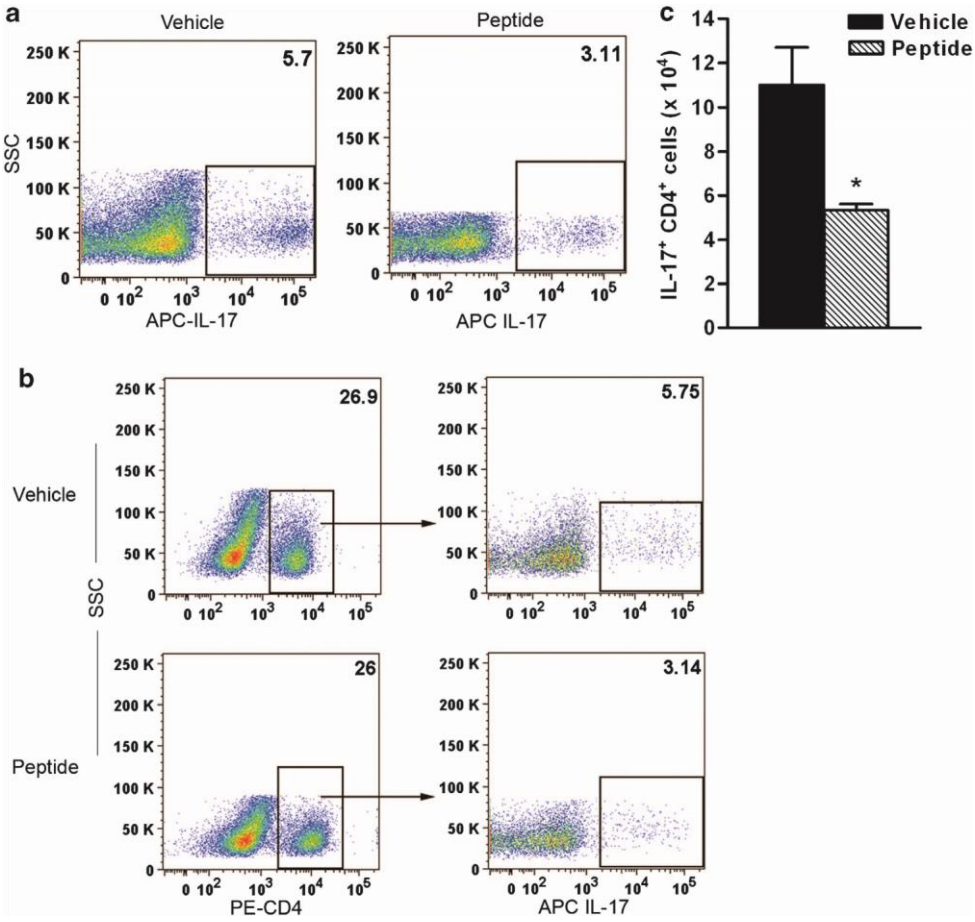

Figure 3 Der p 1 peptide immunotherapy decreases interleukin (IL)-17$^+$ cells in lung following ovalbumin challenge. Mice were killed 48 h after OVA rechallenge. Cells isolated from lung tissue digest as described in Methods were cultured with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. (a) Representative examples of IL-17 staining in lung digest lymphocytes. (b) CD4 gating (left panels) and percentage IL-17$^+$ cells within the CD4$^+$ population (right panel). (c) Total numbers of IL-17$^+$ CD4$^+$ cells. Data are expressed as mean ± s.e.m. by multiplying the percentage expression on CD4$^+$ cells in total live cells acquired by the total CD4$^+$ cell counts. *P<0.05 vs. vehicle-treated mice.

Increased expression of Fox p3 after Der p 1 peptide immunotherapy

Peptide therapy increased IL-10 expression in CD4$^+$ cells in lung tissue digest (Figure 6a). As expression of IL-10 can be associated with Fox p3$^+$ CD4$^+$ Treg cells and their function, we further investigated the Fox p3$^+$ subpopulation within IL-10$^+$ CD4$^+$ cells. IL-10$^+$ CD4$^+$ lung digest cells were gated and analyzed for Fox p3 expression. A significantly greater proportion of IL-10$^+$ CD4$^+$ cells were Fox p3$^+$ in peptide-treated mice, compared with vehicle-treated mice (Figure 6a,b). A similar effect was seen in the DLN where a significantly lower percentage

of Fox $p3^+$ $CD4^+$ cells (12.4±1.8%) was present in the $IL\text{-}10^+$ population from vehicle-treated mice compared with peptide-treated mice (28.45±6.67%, Po0.05). Fox p3 has been described as a marker of regulatory T cells, and approximately 15–20% of the $CD4^+$ cells in lung tissue digest and DLN of vehicle-treated mice expressed Fox p3. Interestingly, examination of the $CD4^+$ population irrespective of IL-10 expression yielded no difference in the percentage of Fox $p3^+$ between the treatment groups (Figure 6c). As determined by quantification of mean fluorescence intensity, the intensity of Fox p3 expression within lung digest $CD4^+$ cells was significantly increased in lung tissue digest cells from peptide treated mice compared with vehicle-treated mice, indicating that individual cells expressed more Fox p3 following peptide immunotherapy (Figure 6d). Similarly, peptide treatment enhanced mean fluorescence intensity of Fox p3 in DLN (485.5±19.6 vs. 804.7±63.2 in vehicle- and peptide-treated mice, respectively, $P<0.05$).

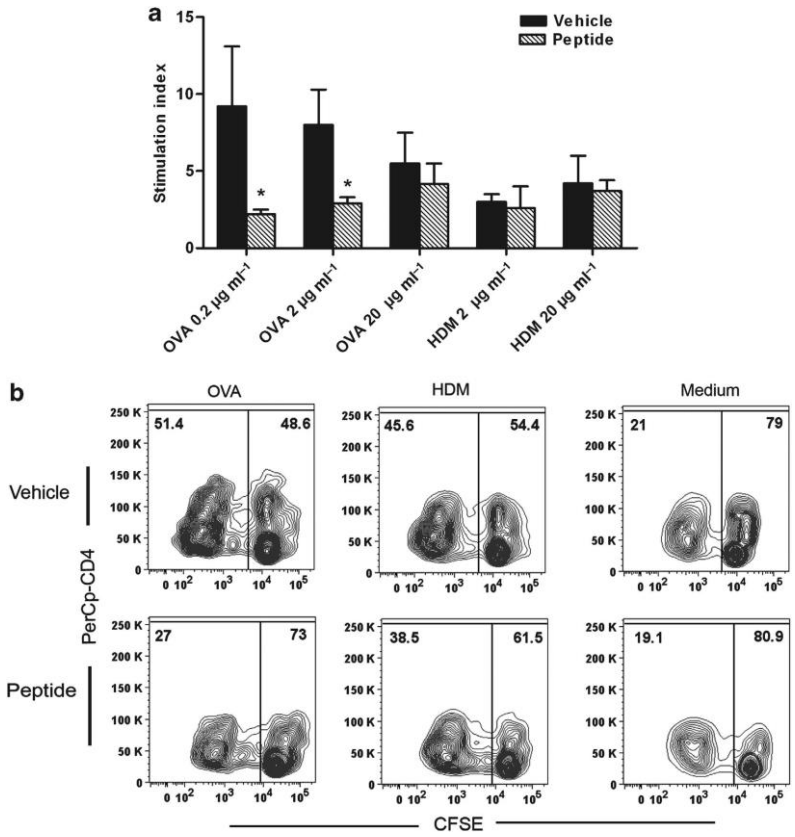

Figure 4. Der p 1 peptide immunotherapy decreases ovalbumin (OVA)-stimulated proliferation of draining lymph node (DLN) cells isolated from dual allergen-exposed mice. Mice were killed 48 h after OVA rechallenge. In vitro proliferation assays of DLN cells were performed by [^{3}H] thymidine incorporation and carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells were cultured in the presence of OVA, house dust mite (HDM), or medium alone for 6 days. (a) Proliferation of DLN cells was cultured with OVA or HDM extract, as correlated with [^{3}H] thymidine incorporation; data are expressed as mean stimulation index ± s.e.m. (n = 8). (b) Proliferation of DLN cells was cultured with OVA or HDM extract, as correlated with CFSE dilution in CD4+ cells. Plots show the proliferation of CD4+ cells. Numbers in the plots indicate percentage of CD4+ cells proliferated following treatment. Representative plots are from one of the eight mice with similar results. *P<0.05 vs. vehicle-treated mice.

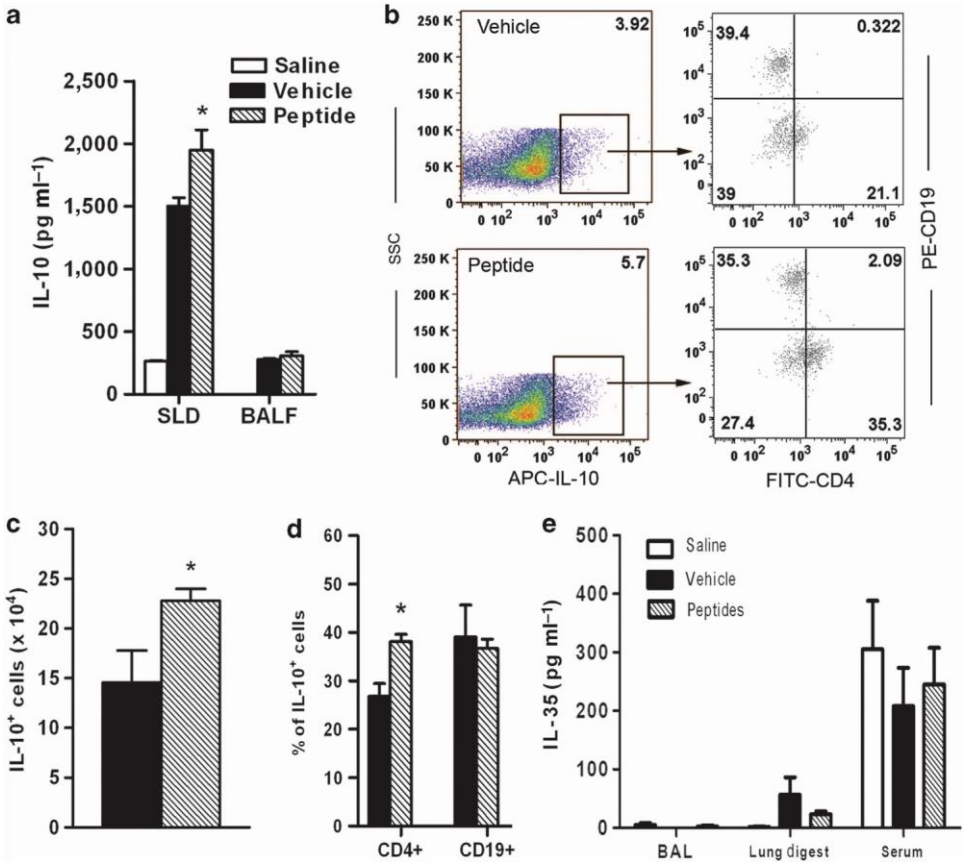

Figure 5. Der p 1 peptide immunotherapy increases interleukin (IL)-10 production in lung. Mice were killed 48 h after ovalbumin rechallenge, and lung tissue was digested as described in Methods. IL-10 was quantified in supernatants of lung tissue digest (SLD) and bronchoalveolar lavage fluid (BALF) by enzyme-linked immunosorbent assay. Intracellular expression of IL-10 in lung digest cells was evaluated by flow cytometry. (a) IL-10 protein levels in SLD and BALF. Data are expressed as mean ± s.e.m. (n= 8). (b) Intracellular IL-10 expression in $CD4^+$ T cells and $CD19^+$ B cells. Cells were cultured with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. Left panels show percent $IL\text{-}10^+$ cells and right panels show percentage of IL-10 contributed by $CD4^+$ and $CD19^+$ cells. Numbers in each quadrant indicate percent cells in that quadrant. (c) Total $IL\text{-}10^+$ cells in lung issue digest. Data are expressed as mean total $IL\text{-}10^+$ cell numbers ± s.e.m. by multiplying the percentage expression on total live cells acquired by the total cell counts. (d) Percentage of $CD4^+$ and $CD19^+$ cells in $IL\text{-}10^+$ population. Cells of lung tissue digest from 2–3 mice were pooled. Each bar shows the mean ± s.e.m. of 3–4 data points from eight mice. (e) IL-35 protein levels in the BAL, supernatants of lung tissue digest and serum. Data are expressed as mean ± s.e.m. (n=8). *$P<0.05$ vs. vehicle-treated mice.

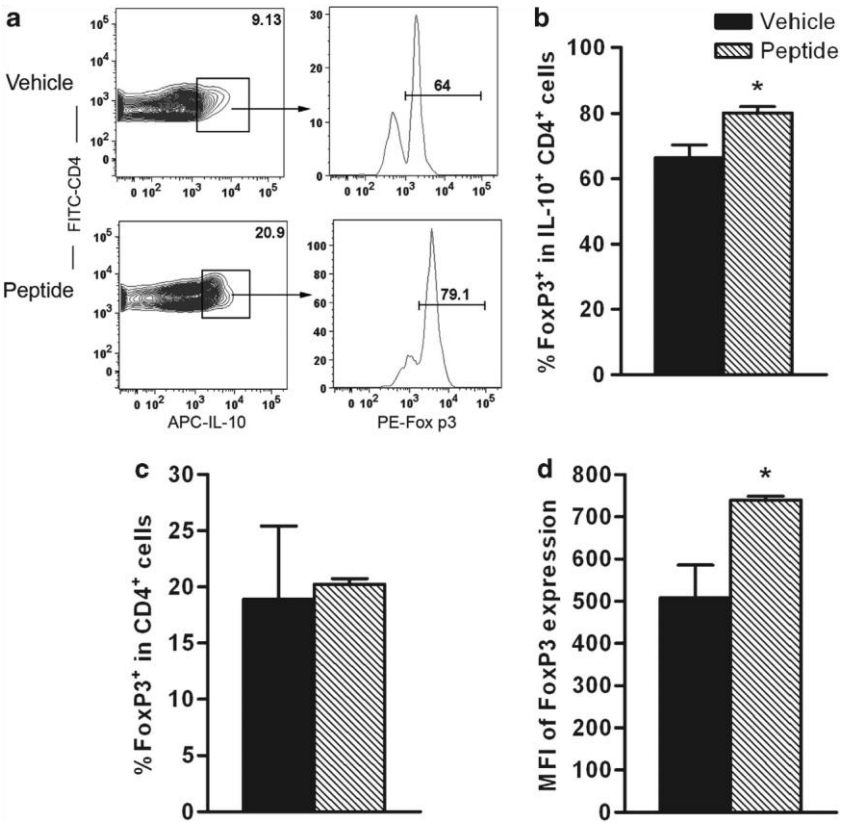

Figure 6. Der p 1 peptide immunotherapy induces interleukin (IL)-10 expression in Fox p3$^+$ CD4$^+$ cells in the lung following ovalbumin (OVA) rechallenge. Mice were killed 48 h after OVA rechallenge, and cells were isolated from lung tissue digest as described in Methods. Cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of brefeldin A for 6 h. (a) Representative plots of flow-cytometry analysis showing expression of Fox p3 in IL-10$^+$ CD4$^+$ cells. Gated CD4$^+$ cells were analyzed for IL-10$^+$ cells (left panels) that were further analyzed for Fox p3 expression (right panels). Numbers adjacent to outlined areas indicate percent cells in each gate. (b) Fox p3$^+$ cell population in IL-10$^+$ CD4$^+$ cells in lung tissue digest. (c) Fox p3$^+$ CD4$^+$ cells in lung tissue digest. (d) Mean fluorescence intensity of Fox p3 expression in CD4 cells in lung tissue digest. Each bar in panels b–d shows mean ± s.e.m. (n = 8). *P<0.05 vs. vehicle-treated mice.

2.4. Discussion

The ability to confer tolerance to a protein through creation of a local tolerogenic environment, after treating with one or more epitopes from another protein, has significant clinical potential, particularly in allergic and autoimmune diseases. To be effective in clinical practice, this approach must work in established disease. However, to date, published studies have only demonstrated prevention of de novo sensitization to unrelated antigens. For example, Van Hove et al.[11] induced enduring tolerance to OVA by chronic administration (8 weeks by aerosol). Re-immunization to OVA (with adjuvant) after this time was unable to elicit a recall response to OVA. Furthermore, immunization with an unrelated antigen (hen egg lysozyme) after the induction of tolerance with OVA was unable to elicit a primary T_H2 response to hen egg lysozyme. Thus, the induction of prophylactic tolerance (through the airways) with one protein can prevent subsequent T_H2 sensitization to another.

In a murine model of established, OVA-induced allergic airway disease, we show that peptide immunotherapy, with T-cell epitopes from the unrelated HDM allergen Der p 1, can reduce T_H2 inflammation and the consequences of such inflammation in vivo. Peptide immunotherapy reduced AHR, total lung cell numbers, tissue eosinophilia, goblet cell hyperplasia, T_H2 cells, pro-inflammatory cytokines IL-5 and IL-17, and OVA-specific T-cell proliferation. These decreases in inflammatory outcomes were associated with increased Fox p3 expression and increased IL-10 production by T cells, but not with increased levels of IL-35, suggesting that IL-35independent mechanisms of mucosal tolerance exist. Der p 1 peptide immunotherapy also reduced recruitment of B cells to the lung following OVA challenge. Although modulation of B-cell antibody production has been reported in some models of peptide immunotherapy,[5,9,12,13] this is the first demonstration of modulation of B-cell recruitment to the airways.

Peptide immunotherapy with CD4 T-cell epitopes has been extensively evaluated in mouse models to prevent and treat a variety of antigen-specific inflammatory responses,[14–20] and several clinical trials have been, or are currently being, performed in allergy and autoimmunity.[21–36] Ideally, effective peptide therapies should generate a limited local network of tolerance that would allow expansion of T-cell tolerance beyond the treatment epitopes to other antigens encountered contemporaneously. Such a phenomenon is hypothesized to occur through the dominant action of regulatory T cells on effector T cells encountering cognate ligand at the surface of the same antigen-presenting cell (APC).[37,38] Although expansion of tolerance to include antigens from pathogens present in the local environment at the time of treatment is a

potential outcome of this phenomenon, our clinical studies to date indicate that systemic recall responses (to purified protein derivative from Mycobacterium tuberculosis in an European population and Candida albicans extract in a North American population) are not compromised through peptide therapy (ref. 5 and unpublished data).

In the present study, mice were sensitized with both OVA and HDM extract and challenged intranasally (i.n.) with OVA and HDM to create established allergic airway disease driven independently by both allergens. Sensitization with OVA generated OVA-specific cells and i.n. challenged OVA-induced localization of OVA-specific T cells to the lung. Similarly, HDM-specific cells were also generated and localized to the lung through repeated intranasal challenge. Conceptually, inhaled challenge with OVA immediately after treatment with inhaled Der p 1 peptides resulted in presentation of T-cell epitopes from both proteins on the same local APCs, leading to the recruitment of both tolerant HDM-specific T cells and OVA-specific effector cells, the latter coming under the influence of the former resulting in suppression of responses to OVA.

Peptide treatment reduced AHR, a cardinal feature of asthma. It also reduced total cell numbers, goblet cell hyperplasia, and eosinophilic inflammatory infiltrates in lung tissue. Despite the reduction in inflammatory cells in lung tissue, no change in cellularity was observed in the airway lumen. This dissociation between airway inflammatory cells and parameters of lung function has been observed previously. For example, in patients with allergic asthma, the number of inflammatory cells in BAL did not correlate with the degree of AHR.[39,40] Furthermore, the failure of an anti-IL-5 intervention to improve clinical outcomes in human asthmatic subjects was associated with a failure to fully deplete parenchymal, rather than BAL, eosinophils.[41] In contrast, Lemiere et al.[42] demonstrated that high sputum eosinophil counts, rather than numbers of eosinophils in bronchial biopsies, were associated with higher rates of disease exacerbation.

OVA has been widely used in murine models of allergic airway disease, inducing AHR, T_H2 responses, and inflammatory airway infiltrates.[43] Earlier studies in a dual HDM/OVA sensitization model demonstrated that responses to OVA in such a system are not dependent on responses to HDM and that the two responses are independent and additive in nature.[44] Thus, merely reducing responses to HDM would not account for reductions in the responses to OVA. Reduction in T_H2-cell responses and AHR in the present dual allergen-sensitization model shows that immunotherapy with HDM peptides can, under the appropriate conditions, suppress established OVA induced allergic responses. These results are indicative of active regulation.

Peptide therapy with HDM peptides reduced T_H2-cell (T1/ $ST2^+$ $CD4^+$) recruitment after OVA airway challenge. These cells are thought to contribute to AHR by release of proinflammatory cytokines and are critical effectors of allergic responses.[45,46] A recent study also suggested that the T1/$ST2^+$ pathway, rather than the eosinophil, is involved in the induction of AHR,[47] supporting earlier studies by Foster and colleagues.[48] Therefore, modulation of T_H2-cell function could be of therapeutic benefit in allergic disease. We are currently investigating how peptide immunotherapy modulates allergen-specific T-cell function by using HLA-DR4 tetramers in two experimental models of allergic disease performed in HLA-DR4 transgenic mice. These studies will allow us to further define frequency, phenotype, gene expression, and epigenomic modification of the cells targeted during therapy.

Peptide treatment also reduced IL-5-producing T_H2 cells in the lung. However, we could not detect any difference in the numbers of IL-4-producing $CD4^+$ cells in the lung between peptide-treated and vehicle-treated mice. We did not measure IL-13 in this study. A previous study showed that treatment with anti-IL-4 and IL-13 antibody did not completely abrogate AHR, T_H2 cells, and mucous production in OVA-sensitized challenge mice.[47] Thus, murine mechanisms of AHR independent of IL-4 and IL-13 exist (e.g., IL-5-dependent accumulation of eosinophils in the lung), which can be modulated by peptide therapy.

IL-17, another pro-inflammatory cytokine produced by $CD4^+$ effector cells, is thought to potentiate allergic inflammation. IL-17-producing cells (T_H17) are increased in patients with allergic asthma.[49] The absence of AHR in airway sensitized IL-17ra $^{-/-}$ mice, and induction of AHR via airway delivery of exogenous IL-17 has demonstrated that IL-17 is required for AHR.[50] In the present study, we also detected IL-17-producing cells in lung tissues of dual allergen-sensitized mice, and these were significantly decreased with peptide immunotherapy.

Peptide immunotherapy reduced not only T_H2 cells but also $CD8^+$ T and B cells. Previous studies suggest that not only $CD4^+$ cells but also $CD8^+$ cells, particularly a subset of $CD8^+$ cells that produce IL-4, IL-5, and IL-13 (but not interferon-g), may be essential for the development of AHR and allergic inflammation.[51–53] A recent study demonstrated increased numbers of $CD8^+$ cells in the lung tissue of asthmatics.[54] Thus, reduction in $CD8^+$ cells via peptide immunotherapy may contribute to efficacy and should be evaluated as a mechanism in human studies. In T_H2 conditions such as asthma, IL-4 stimulates proliferation of B cells that can act as APC for the development of primary CD4 + T-cell responses.[55] Reduction in B cells through peptide immunotherapy may also have indirectly diminished T-cell-driven inflammation, thereby contributing to the amelioration of AHR and lung inflammation.

It has been demonstrated previously that generation of allergen-specific Treg cells and increased production of IL-10 regulate allergic responses following specific immunotherapy.[56] Subjects with allergic asthma, rheumatoid arthritis, and insulin-dependent diabetes treated with allergen peptides, heat shock protein peptides, or pro-insulin peptides, respectively, demonstrated increased levels of IL-10 production in antigen stimulated PBMC in culture.[26,33,34] In addition to increased IL10 production in PBMC culture,[26] our previous studies of peptide immunotherapy in cat-allergic mice have also demonstrated increased levels of IL-10 in BAL fluid and IL-10$^+$ cells in lung tissue.[5] In the present study, we also found a marked increase in IL-10 level in lung tissue digest supernatant, and IL-10$^+$ cells in both lung tissue and lung-associated DLN. Further analysis of IL-10$^+$ cells demonstrated an increased proportion of Fox p3$^+$ CD4$^+$ T cells in the IL-10$^+$ population from peptide-treated mice. In addition, the mean fluorescence intensity of Fox p3 staining increased, indicating increased levels of Fox p3 within individual cells. However, there was no significant difference in Fox p3$^+$ CD4$^+$ cell numbers between vehicle- and peptide-treated mice, in agreement with our earlier studies.[5] This may be due to transient induction in Fox p3 expression in activated effector cells in vehicle-treated mice.[57]

IL-35 is a recently described member of the IL-12 family of cytokines that has a critical role in the development and suppressive function of Treg.[58,59] The ability of IL-35 to convert conventional human T cells into IL-35-secreting iTr35 cells suggests that these cells may contribute to infectious tolerance.[59] Recently, in a murine model of OVA-induced allergic airway disease, Whitehead et al.[60] demonstrated that repeated oropharyngeal administration of OVA with lipopolysaccharide resulted in loss of AHR that was dependent on expansion of a population of Treg secreting IL-10, TGFβ, and IL-35. Loss of AHR was shown to be dependent on IL-35 alone. To evaluate the contribution of IL-35 to the tolerance observed in our model, we measured the cytokine in BAL, lung tissue digests, and serum. In contrast to IL-10, IL-35 levels did not change after peptide treatment, suggesting that in this particular form of immunological tolerance, IL-35 does not have a major role in our model of allergic airway disease. Our model was primarily eosinophilic in nature, in contrast to that of Whitehead et al.,[60] which was neutrophilic. An additional important difference between the models may have been the aerosolization of allergen in the neutrophilic model, which may have resulted in oral exposure to the allergen after grooming.

Apart from CD4$^+$ cells, other cells such as B cells, dendritic cells, and macrophages can also produce IL-10. B cells producing IL-10 have been shown to prevent allergic airway inflammation via inducing pulmonary infiltration of Fox p3$^+$ CD25$^+$ CD4$^+$ regulatory T cells.[61,62] We also detected IL-10 expression in B cells in allergic

mice; however, at the time point selected for outcome measurements in this model, these cells did not demonstrate increased expression of IL-10 after peptide treatment. Similarly, we also detected very small percentages of IL-10-producing $CD11b^+$ and $CD11c^+$ cells in allergic mice, which did not change after peptide treatment. Interferon-g that is produced by T_H1 cells is generally considered to antagonize T_H2 responses and exerts inhibitory effects on T_H2-cell differentiation. In our study, interferon-g-producing cell numbers in the lung did not increase after peptide treatment. This suggests that peptide therapy did not result in deviation from a T_H2 to a T_H1 response.

In conclusion, our data indicate that peptide immunotherapy can, under the appropriate conditions, modulate established allergic responses to a third-party immunogen. Treatment with two peptides containing CD4 T-cell epitopes from the HDM allergen Der p 1 reduced AHR and T_H2 immune responses induced by OVA airway challenge. Amelioration of allergic responses was associated with reduction in OVA specific T-cell proliferation, the induction of IL-10 and Fox p3, together with a reduction in T_H2-cell numbers, IL-5-, and IL-17-producing cells. Levels of IL-35 remained unchanged. Exploiting immunological tolerance induced by treatment with a limited number of T-cell epitopes may be important in the potentiation of clinical responses to peptide (and other forms of) immunotherapy in allergic, autoimmune, and transplantation-related diseases. The results of this study will inform future clinical study design.

2.5. Methods

Animals. Female BALB/c mice (8–10 wk of age) were purchased from Charles River (Montreal, QC, Canada), housed in specific pathogen free conditions and allowed to acclimatize for 1 week before experimental use. All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board at McMaster
University.

Peptides. For in vitro and in vivo studies, Der p 1 peptides were synthesized by standard Fmoc chemistry, purified (495%) by HPLC, and presented as lyophilized solid (GL Biochem, Shanghai, China). Peptides sequences were Der p $1^{55–69}$: RNQSLDLAEQELYDSASQH and Der p $1^{149–167}$: DEFKNRFLMSAEAFE (amino acid numbering including Der p 1 pro-peptide but exclusive of signal sequence). Lyophilized peptides (10 mg) were reconstituted in a small volume of 10^4M HCl, dilution to 1 mg ml^1 in phosphate-buffered saline (PBS) and frozen storage (80 1C), before use.

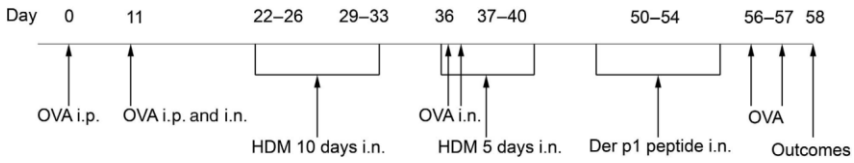

Figure 7. Schematic protocol flow chart for allergen-induced airway disease and treatment with Der p 1 peptides. Airway inflammation was induced in BALB/c mice sensitized intraperitoneally (i.p.) with 10 mg ovalbumin (OVA) in alum (d 0 and 11) followed by inhalation of 1.5 mg house dust mite (HDM) (days 22–33 and 36–40) and 100 mg OVA (days 36 and 37). To induce tolerance, a mixture of HDM peptides Der p $1^{55–69}$ and Der p $1^{149–167}$ (1 mg each) was administered intranasally (i.n.) for 5 consecutive days (days 50–54). After rechallenge with 100 mg OVA (days 55 and 56), lung function was analyzed and the mice were killed (day 58) for tissue harvest.

Allergen sensitization and peptide treatment. Allergic airway disease was induced in mice by sensitization to OVA (Sigma-Aldrich, St. Louis, MO), followed by inhaled challenges with HDM extract from Dermatophagoides pteronyssinus (Greer Laboratories, Lenoir, NC) and OVA to localize inflammation to the lung tissues (Figure7). Mice were sensitized by intraperitoneal injections of 10mg OVA in 50ml PBS with 150 ml of alum (Au-Gel-S; Serva Electrophoresis, Heidelberg, Germany) on days 0 and 11. Mice were challenged i.n. with HDM (1.5mg protein weight in 25ml PBS) on days22–26, 29–33, and 36–40 and OVA (100mg in 25ml PBS) on days 11, 36, and 37, during light anesthesia (isoflurane). To induce tolerance in mice with established airway disease, a mixture of Der p $1^{55–69}$ and Der p $1^{149–167}$ peptides (1mg each in 25ml PBS) was administeredi.n.for5days(days50–54).Mice were recall challenged i.n. with OVA (100mg in 25ml PBS) on days 55–56. Forty eight hours after the last challenge, AHR was measured, and BAL fluid, lung tissue, and DLNs were collected, and mice were euthanized via exsanguination.

Measurement of AHR. Airway responsiveness was measured using the FlexiVent small animal ventilator system. The single compartment model was used, and total respiratory system resistance (R_{RS}) in response to nebulized methacholine (MCh) was assessed. Mice were anaesthetized by an intraperitoneal injection of xylazine hydrochloride (Bayer, Toronto, ON; 10 mg kg^{1}) and sodium pentobarbital (Ceva Sante Animale, Leneka, KS; 30 mg kg^{1}). A tracheotomy was performed, into which a blunted 18-gauge needle was inserted and secured. The other end of the needle was inserted into the Y-adaptor of the FlexiVent apparatus. Mechanical ventilation was commenced at a rate of 150 breaths per minute with a volume of 10 ml kg^{1}. Pancuronium bromide (Santoz, Boucherville, QC; 20 mg kg^{1}) was administered intraperitoneally to achieve paralysis and prevent respiratory effort during measurement. Sequential increasing doses (0, 3.1, 6.3, 12.5, and 25 mg ml^{1}) of MCh were nebulized for inhalation by each mouse,

and the resultant R_{RS} was determined through 13 0.4-s perturbations over a 3-min period. Before each dose of MCh, an inflation to total lung capacity was initiated to normalize the data. A dose–response curve to MCh was generated. Heart rate and oxygen saturation were monitored throughout the procedure using a Biox 3700 infrared pulse oximeter (Ohmeda, Boulder, CO) with the probe placed on hind limb of the mouse.

Bronchoalveolar lavage. Immediately after measurement of AHR, mice were killed and the airways were lavaged twice with 0.25 ml PBS as previously described.[15] BAL fluids were centrifuged at 150 g for 10 min, and the supernatants were stored at 20 1C for cytokine analysis in future. Cell pellets were suspended in PBS and total cells enumerated. BAL cell isolates were diluted to an approximate concentration of 5 x 10^5 per ml and transferred to slides by cytocentrifugation. The cells were Wright–Giemsa stained and differentiated by morphological criteria as one of the following: eosinophil, neutrophil, macrophage, and lymphocytes. Two slides of 200 cells per slide were differentiated by a blinded investigator, and the relative proportions of each cell type were determined and multiplied by the total number of BAL cells obtained to determine absolute cell counts.

Lung cell isolation. After perfusion with PBS containing heparin (10 U ml^{-1}), the right lung was harvested, diced, and incubated on an orbital shaker for 90 min at 37 1C in digestion medium (Sigma Aldrich, RPMI-1640 medium containing 10% fetal bovine serum; Invitrogen, Burlington, ON, Canada; 300 U ml^1 collagenase type 1; Worthington Biochemical Corporation (Lakewood, NJ), 50 U ml^1 DNase; Sigma-Aldrich, 100 U ml^1 penicillin; Invitrogen, and 100 mg ml^1 streptomycin; Invitrogen). After vortexing for 10–15 s, the product of digestion was passed through 70 mm cell strainer (BD Falcon, Franklin Lakes, NJ). Supernatant collected after centrifugation was stored at -80°C for later cytokine analysis, and the cell pellet was resuspended. Cells were further purified by histopaque density gradient (histopaque-1083; Sigma-Aldrich).

DLN cell isolation. Mediastinal and peribronchial lymph nodes were removed, and cells were dissociated by applying gentle pressure with a syringe plunger over a 40-mm cell strainer. After washing twice, the cells were suspended in complete culture medium (RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 100 U ml^1 penicillin, and 100 mg ml^1 streptomycin).

Histology. Left lungs were inflated with 10% buffered formalin to a pressure of 20 cm H_2O, fixed for at least 24 h, and following dehydration paraffin-embedded. Sections of 3 mm were stained with hematoxylin and eosin and

periodic acid-Schiff to quantify eosinophils and mucin-containing goblet cells, respectively. Sections were examined under light microscopy in a blinded fashion, as described previously.[63]

Flow cytometry. Cells were pre-incubated with anti-CD16/32 monoclonal antibody (2.4G2; BD Biosciences, San Jose, CA) to block FcgR. For surface marker staining, FcgR-blocked cells were incubated with PE-anti-CD4 (RM4-5; BD Biosciences), PerCp-anti-CD8 (53-6.7; BD Biosciences), APC and PE-anti-CD19 (1D3; BD Biosciences), and FITC-anti-T1/ST2 (DJ8; MD Biosciences) monoclonal antibody for 30 min at 4 1C. After washing, cells were resuspended in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA).

For intracellular cytokine staining, cells were stimulated in 24-well plates with phorbol 12-myristate 13-acetate (20 ng ml^1; Sigma Aldrich), ionomycin (1 mM; Sigma-Aldrich), and brefeldin A (10 mg ml^1; Sigma-Aldrich) for 6 h at 37 1C. Cells were stained for surface markers as described above, fixed with 4% paraformaldehyde, and permeabilized in Perm/Wash buffer (BD Biosciences) before intracellular staining with anti-cytokine monoclonal antibody. Cells were stained for intracellular cytokines by incubating the cells in Perm/ Wash buffer containing a predetermined optimal concentration of APC-anti-IL-10 (JES5-16E3; BD Biosciences), APC-anti-IL-17A (ebio17B7; eBiosciences, San Diego, CA), and APC-anti-IL-5 (TRFK5; BD Biosciences) monoclonal antibody for 30 min at 4 1C.

Intracellular Fox p3 staining was performed using mouse regulatory T-cell staining kit (88-8111; eBiosciences) according to the manufacturer's instructions. Flow-cytometry analysis was performed using FACSCanto II (BD Biosciences) and Flowjo software (TreeStar, Ashland, OR).

Measurement of IL-10 protein levels. Levels of IL-10 present in BAL fluids and lung tissue digest supernatants were measured using an ELISA kit (sensitivity, 15 pg ml^1; eBiosciences) according to the manufacturer's instructions.

Measurement of IL-35 protein levels. Levels of IL-35 present in BAL fluids, lung tissue digest supernatants, and serum were measured using an ELISA kit (sensitivity, 5.7 pg ml^1; USCN Life Science, Wuhan, China) according to the manufacturer's instructions.

Cell proliferation. Proliferation assays of cells isolated from lung associated DLN were performed with [^{3}H] thymidine and CFSE. For the [^{3}H] thymidine assay, cells were cultured in triplicate at 2 10^4 cells per well, in U-bottom 96-well plates, in the presence of OVA, HDM, or complete medium alone at 37 1C in 5% CO_2 in air. After 6

days of culture, 0.5 mCi [^{3}H] thymidine per well was added, and the cells were cultured foranother18 h. [^{3}H] thymidine incorporation data in counts per minute (cpm) was converted into stimulation index by dividing cpm obtained from stimulated cells by cpm values from unstimulated wells. CFSE assay of proliferation was performed to identify the CD4 T-cell proliferation. Cells were stained with CFSE (CellTrace CFSE Cell Proliferation Kit; Invitrogen) at the final concentration of 1 mM as described previously[17]. CFSE-stained cells were cultured at4 10^5 cells per well in flat-bottom 96-well plate in the presence of 20 mg ml^1 OVA, HDM, or medium alone at 37 1C in 5% CO_2 in air. After 6 days, cells were harvested and surface stained with PE-anti-CD4 antibody as described previously. Flow-cytometry analysis was performed using a FACSCanto II cytometer, and data were analyzed with Flowjo software.

Statistical analysis. Data were expressed as mean ± s.e.m. and analyzed for statistical significance using Student's t-tests. Differences were considered statistically significant at a P-value p0.05.

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Disclosure
M. Larché is a founder, stockholder, and consultant of Circassia Ltd. and a founding scientist of Adiga Life Sciences Inc. and has received research support from both of these companies. The remaining authors declared no conflict of interest.

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Chapter 3: Induction of bystander tolerance and immune deviation following Fel d 1 Peptide Immunotherapy

3. Declaration of Academic Achievement

Chapter 3. Induction of bystander tolerance and immune deviation following Fel d 1 Peptide Immunotherapy

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- Two rounds of review at the Journal of Allergy and Clinical Immunology. Four of five reviewers have approved the study for publication while the fifth has not, and has returned a series of further comments. A rebuttal is being generated for this reviewer. The manuscript presented here is the most up-to-date, as of October 2017.
- Rights for publication in thesis were retained at submission (2017).

Daniel Moldaver's contributions

As primary author, I was responsible for conceiving, planning, managing and contributing to all aspects of the study. I treated animals, performed mouse dissections, fixed tissues, performed histology, prepared cells for flow cytometry and proliferation assays, performed flow cytometry and ELISAs. M. Bharhani assisted with analysis of flow cytometry. J. Wattie assisted with mouse treatments and was responsible for mouse tracheostomies, and performing the nebulized methacholine challenge. M. Larché & M. Inman provided guidance on study design. Analyses, statistics and figure generation were performed by myself. Manuscript preparation was performed by me, with guidance from C. Rudulier and M Larché.

Induction of inter-molecular tolerance following Fel d 1 Peptide Immunotherapy

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Running title: Inter-molecular tolerance after peptide immunotherapy

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Disclosure

M. Larché is a co-founder, and consultant of Circassia Ltd., a stockholder of Circassia Pharmceuticals PLC and a founding scientist of Adiga Life Sciences Inc. and has received research support from both of these companies. The rest of the authors declare no conflict of interest

3.1. Abstract

Treatment of cat allergic subjects with peptides derived from Fel d 1 (the major cat allergen) ameliorated symptoms of cat allergy in phase 2 clinical trials. Here, we demonstrate that the tolerance induced by Fel d 1 peptide immunotherapy can be exploited to reduce allergic responses to a second allergen, ovalbumin (OVA), in mice dually sensitized to OVA and Fel d 1. The induction of tolerance to OVA was achieved via a simultaneous exposure to both allergens following peptide treatment. Functional tolerance to each allergen was assessed in a model of allergic airways disease, wherein treated mice were protected from eosinophilia, goblet cell hyperplasia and Th2 cellular infiltration. Suppression of allergic responses to cat allergen challenge was associated with significant increases in CD4+CD25+Foxp3+ T cells, IL-10+ cells and CD19+IL-10+ B cells, whereas the response to OVA was associated with a marked reduction of Th2 cytokine-secreting T cells and less prominent changes in outcomes associated with immune regulation. These observations suggest that immune tolerance induced by peptide immunotherapy can be used experimentally to treat an allergic response to another allergen, and that the molecular mechanisms underlying the induction of tolerance to a treatment-specific allergen and a bystander allergen may be different.

3.2. Introduction

Sensitivity to cats is one of the most common forms of allergy and has been associated with severe asthma, wheezing in children and morbidity[1-3]. Allergic disease is typified by the presence of allergen specific IgE in the sera of patients [4], the presence of elevated Th2 cytokines and Th2 cells[5, 6],as well as compromised immune regulation[7-10]. By modifying the underlying immunological cause of allergic disease, specific immunotherapy (SIT) is capable of preventing the development of asthma in children with seasonal allergic rhinoconjunctivitis[11], reducing sensitizations to other allergens[12, 13], and is known to exhibit therapeutic benefit extending beyond the treatment period[14]. Despite the clear benefits of SIT, an estimated 5-35% of patients receiving SIT experience systemic adverse events[15] due to the ability of allergens to crosslink allergen-specific IgE on the surface of mast cells and basophils.

A recent double blind, placebo-controlled phase II study has shown that immunotherapy performed with Cat-SPIRE (Synthetic Peptide Immuno Regulatory Epitopes; peptides derived from Fel d 1 the major cat allergen), significantly improves symptoms in cat allergic subjects[16] for two years after treatment[17]. Importantly, the incidence of treatment related adverse events was no different to placebo [18], which likely reflects that the peptides employed lack the size and requisite three dimensional structure to cross-link IgE bound to FcεRI receptors[19, 20].

The mechanisms by which treatment with allergen-derived CD4 T cell epitopes can induce antigen specific tolerance to the entire allergen is poorly understood. It has been postulated that peptide treatment modifies allergen-specific T cell responses to generate cells with inhibitory or regulatory function that can then be exploited to confer tolerance on other cells in the same local environment; a so called “tolerogenic environment” [21]. Indeed, peptide immunotherapy has been reported to induce linked-epitope suppression in this way. Treatment of

cat allergic individuals with Fel d 1 derived peptide epitopes induced suppression of PBMC responses specific for not only the treatment peptides, but also other Fel d 1 epitopes that were not included in the treatment preparation [22]. A period of simultaneous presentation of both treatment and non-treatment epitopes by the same antigen presenting cell is likely required to facilitate linked-epitope suppression, which may occur by default when the two epitopes are within the same allergen[23]. We previously demonstrated that suppression/regulation can also occur between two different allergens if they are co-administered[24]. Specifically, mice dually sensitized to OVA and house dust mite (HDM) were intranasally treated with synthetic peptides derived from Der p 1, the major house dust mite allergen. Der p 1 peptide immunotherapy reduced airway hyperresponsiveness, eosinophilia and goblet cell hyperplasia, during a subsequent OVA allergen challenge[24].

Individuals with cat allergy typically suffer from allergic sensitization to multiple allergens. Thus, it would be therapeutically advantageous if the suppression generated by Fel d 1 peptides in cat allergic subjects could be exploited to suppress responses to other allergens. Although our finding that Der p 1 peptide therapy could suppress the OVA response in dually sensitized mice lends support to this idea, HDM is a very different allergen from Fel d 1, as evidenced by its ability to sensitize mice in the absence of adjuvant. Another key difference between the clinical administration of Fel d 1 peptides and HDM peptides in the previous study is the route used to administer the peptides. Cat allergic subjects receive intradermal peptide therapy while the HDM and OVA allergic mice inhaled Der p 1 peptides. The route of peptide administration is important because mice tend to mount tolerogenic responses to inhaled peptides, while humans do not[25, 26]. Thus, given the differences between clinical studies with Fel d 1 and the HDM/OVA model of therapy, it remains unclear if the tolerance to cat dander

induced by Fel d 1 peptide immunotherapy, given intradermally, can be used therapeutically to ameliorate sensitization to other allergens. We have addressed this outstanding question in the present study by treating with intradermal (ID) Fel d 1 peptides followed by subcutaneous (SC) co-administration of OVA and Fel d 1 proteins, to mice dually sensitized to cat and OVA, and assessing their impact on both the cat and OVA response. Importantly, the ID Fel d 1 peptides together with the SC OVA and Fel d 1 proteins constituted the treatment regimen in this study.

3.3. Methods and Materials

Animals

Female BALB/c mice, aged 6-8 weeks were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and housed in specific pathogen free conditions. Environmental control, a 12-hour light/dark schedule and *ad libitum* access to food and water were maintained throughout the study. Animals were acclimatized to the facility for one week prior to the beginning of studies. All procedures were approved by the Animal Research Ethics Board at McMaster University, and performed in accordance to the Guide for Humane Use and Care of Laboratory Animals.

Allergen materials and peptides

Lyophilized cat dander extract (CDE; Greer Laboratories, Lenoir, NC, USA) and Grade V Ovalbumin (OVA; Sigma, St. Louis, MO, USA) as well as purified Fel d 1 protein (Indoor Biotechnologies; Charlottesville, VA, USA) were purchased, and diluted in Dulbecco's phosphate buffered saline to the required concentrations. Allergens were stored, frozen, at -20°C. Treatment peptides were Fel d $1_{23\text{-}38}$ (EQVAQYKALPVVLENA) and Fel d $1_{29\text{-}45}$ (KALPVVLENARILKNCVDAK). These two peptides have been shown to elicit recall responses in BALB/c mice (unpublished data). These peptides do not have homology with the amino acid sequence of OVA. Peptides were synthetically produced (GL Biochem, Shanghai, China) by standard Fmoc chemistry and purified via HPLC (>95% purity). Lyophilized peptides were reconstituted in acidified water (10^{-4}M HCl) and frozen (-80°C) prior to use.

Allergen sensitization, challenge and peptide therapy

BALB/c mice (*n* = 4-5/group, performed twice) received an intraperitoneal (IP) prime and boost dual allergen sensitization to ovalbumin and Fel d 1 on D0 & 14 (1µg Fel d 1 and 10µg OVA in alum, per IP). Mice were treated three times, once per week, with peptides derived from Fel d 1 (D28, 35 and 42). Control groups (positive and negative) received PBS (peptide vehicle), while treatment groups received either 1µg or 0.1µg of both peptides, ID. Following a one-week rest, mice were simultaneously exposed to Fel d 1 (0.5µg) and ovalbumin (0.01µg), at sub-immunogenic doses (data not shown), over a series of three subcutaneous injections (D49, 56 and 63). Following the sensitization and treatment protocols, examination of lymphocyte proliferative responses and humoral responses were performed (Figure 1A), or the functional response to allergen was investigated through an intranasal allergen challenge (Figure 1B). Cat dander extract (25µg protein), or ovalbumin (100µg) airway challenge was performed via three intranasal (IN) instillations of 25µL. Negative control mice received PBS, rather than allergen throughout. Triple allergen sensitized mice received, in addition to 1µg Fel d 1 and 10µg OVA, 10µg Timothy grass allergen, in alum, on day 0 and 14. Twenty-four hours post final challenge, tissues and samples were collected to assess the inflammatory response.

Sample collection and Cell Isolation

A bronchoalveoloar lavage (BAL) was performed by injecting two aliquots (250µL) of PBS into the lungs of tracheotomised mice. Aliquots steeped for 5 seconds then were removed and pooled. BAL was subsequently centrifuged (150x *g),* cells enumerated then prepared for cytology by cytocentrifugation (Cytospin 3 centrifuge; Shandon scientific, Sewickley, PA, USA). BAL cells were differentiated by morphological criteria and classified as one of: eosinophil, neutrophil, macrophage/monocyte or lymphocyte, by a blinded investigator following Wright-Giemsa staining. Blood was collected form a cardiac puncture, allowed to clot and

centrifuged (400x *g*) to collect serum. BAL supernatants and serum were stored at -20°C.

Draining lymph nodes (dLN; mediastinal & peribronchial lymph nodes) and lungs were collected following exsanguination. Cells were released from the tissues by physical disruption (dLN) or a 1.5-hour enzyme digestion at 37 °C (lung) utilising 300units/mL of Type 1 collagenase (purified from *C. histolyticum*; Worthington Biochemical Corp., NJ, USA) and 50 units/mL of DNase I (Sigma). Resultant cell suspensions were washed, red blood cells lysed (using ACK lysis buffer), washed twice and suspended in RPMI media (10% FBS, 1% Pen/Strep, 0.1% 2-mercaptoethanol) for enumeration.

Lung Histology

Lung histology and morphometry were performed and quantified as described previously[27, 28]. Briefly, three-micron transverse sections of the paraffin embedded left lung lobe were taken, following dehydration, formalin fixation. Haematoxylin and Eosin (H&E) and periodic acid Schiff (PAS) stains were performed to determine tissue eosinophilia and goblet cell hyperplasia, respectively. Images of first generation airways were collected at 40*x* or 20*x,* and analyzed for eosinophils per mm^2 and goblet cells per mm, respectively, using a digital image analysis system (Northern Eclipse; Empix Imaging, Inc., Mississauga, ON, Canada).

Enzyme-linked immunosorbent assay (ELISA)

An ELISA MAX™ Mouse IgE kit (Biolegend Inc., San Diego, CA, USA) was used to assay serum IgE levels. A BD OptEIA™ Mouse IgG2a ELISA set (BD Biosciences, San Diego, CA, USA) was used to assay serum IgG2a concentrations. Kits were performed as per manufacturer's instructions. Allergen specific assays were created by coating assay wells with 10µg of allergen protein in coating buffer, rather than capture antibody.

Flow Cytometry

Cells were stained with antibody for 30 minutes, following washing in staining buffer and blockade of Fc receptors (FcγR). Following staining, cells were fixed in 1% paraformaldehyde prior to analysis. Cells to be stained for intracellular cytokines were stimulated for six hours in PMA (20ng/mL; Sigma-Aldrich), ionomycin (1mM/mL Sigma-Aldrich) and brefeldin A (10 µg/mL; Sigma-Aldrich), surface stained, permeabilized, then stained for intracellular cytokines for 30 minutes. Foxp3 intracellular staining was performed using eBiosciences' Treg staining kit, as per manufacturer's instructions. Antibodies used included: FITC-T1ST2 (CDJ8; MD Biosciences), PE and PerCP-CD4 (RM4-5; BD Biosciences), PerCp-CD8 (53-6.7; BD Biosciences), APC-CCr4 (2G12; BioLegend), PE-Cy7-CD69 (H1.2F3; eBiosciences), APC-Cy7-CD3 (17A2; BD Biosciences), APC and PE-CD25 (PC61.5; eBiosciences), PE-Cy7-CD19 (1D3; eBiosciences), PE-Siglec-F (E50-2440; BD Biosciences), APC-Gr1 (RB6-BC5; eBiosciences).APC-IL-10 (JES5-16E3; BD Biosciences), APC-IFN-γ (XMG1.2; BD Biosciences), A488-IL-13 (eBio13A; eBiosciences), PE-IL-4 (11B11; BD Biosciences), APC-IL-5 (TRFK5; BD Biosciences), PE-Cy7-IL-17A (eBio17B7; eBiosciences).Flow cytometric analysis was performed using FACSCanto II (Becton Dickinson) and Flowjo (TreeStar).

^{3}H-Thymidine incorporation assay

Proliferation to pre-titrated allergen (cat dander extract, ovalbumin or timothy grass) concentrations was assessed in triplicate. Splenocytes (2.5×10^5) and draining lymph node (2×10^4) cells were cultured (37°C, 5% CO2, 95% relative humidity), in a flat bottom 96-well microtitre plate (BD Falcon) for five days. On day six, the supernatant was harvested and replaced with fresh culture media (RPMI supplemented with 10% Fetal bovine serum and 1%

pen/strep; Invitrogen). On culture day seven, cells were pulsed with 0.05 µCi of tritiated ([m*ethyl*-^{3}H])- thymidine (General Electric Healthcare, Bale d'Urfe, QC, Canada). Sixteen hours later, plates were frozen (-20°C), then harvested (FilterMate Universal Harvester; Perkin Elmer, Ontario, Canada) onto glass-fibre filtermats (Wallac; Turku, Finland). Scintilation fluid (BetaPlate Scint; Perkin Elmer) was added to the filter mats and resultant light emissions were counted, for one-minute, using a MicroBeta Trilux (Perkin Elmer).

Data Analysis

Data are presented as mean ± standard error of the mean (SEM). All comparisons were two-tailed and *P* values less than 0.05 were considered significant. ANOVAs and post-hoc Bonferroni corrected *t*-tests were used to determine significance.

3.4. Results

Co-administration of Fel d 1 and OVA are required for Fel d 1 peptide immunotherapy to suppress the OVA response.

Having previously demonstrated that peptide therapy can facilitate the induction of tolerance beyond the treated allergen[24], we sought to develop a systemic model of dual allergen sensitization to Fel d 1 and ovalbumin (OVA), to assess whether treatment with Fel d 1-derived peptides could suppress responses to OVA. Briefly, mice were sensitized to OVA and Fel d 1 in alum using a prime-boost IP injection series, and were treated with three subcutaneous administrations of two synthetic Fel d 1 derived peptides (at either 0.1 or 1µg) to induce tolerance to Fel d 1. After peptide therapy, mice were exposed to OVA and Fel d 1, subcutaneously, to induce tolerance to OVA. Sham sensitized and treated animals had low levels of IgE (Figure 2A) and IgG2a (Figure 2B, C). Dual allergen sensitized, sham treated mice exhibited significant Th2 immune polarization observed as elevated total serum IgE (Figure 2A).

Treatment of dual sensitized animals with 1µg of Fel d 1 derived peptides significantly reduced serum IgE (Figure 2A), while also increasing cat specific IgG2a (Figure 2B). Importantly, the administration of both allergens (CDE and OVA) elicited bystander tolerance, as OVA-specific IgG2a was significantly increased (Figure 2C).

We next sought to assess whether the bystander tolerance was due to a specific immune response, or non-specific immune suppression arising from Fel d 1 peptide therapy. In animals that received subcutaneous OVA but not Fel d 1, after Fel d 1 peptide therapy IgE levels and OVA-specific IgG2a levels remained the same as untreated mice (Figure S1A and S1B). Additionally, dual allergen sensitized animals that received peptide therapy but a sham subcutaneous injection (diluent alone), possessed an unaltered proliferative response to OVA, *in vitro* (Figure S1C). The specificity of tolerance generated by therapy with synthetic Fel d 1 derived peptides and simultaneous allergen exposure was further assessed using triple sensitized animals. Splenocytes derived from animals sensitized to timothy grass, Fel d 1 and ovalbumin, retained a significant proliferative response to *in vitro* stimulation with timothy grass, following treatment with Fel d 1 derived peptides and simultaneous exposure to OVA/CDE (Figure S1D). Therefore, the induction of bystander tolerance was restrained to the allergens administered

Fel d 1 peptide immunotherapy followed by dual SC allergen administration ameliorates CDE induced lung inflammation, in dual allergen sensitized mice.

Following our finding that Fel d 1 therapy followed by dual SC allergen administration exposure reduces both cat and OVA specific humoral immunity, we investigated whether this was associated with functional tolerance to both allergens by performing an *in vivo* allergen challenge.

Mice were sensitized to both allergens, peptide treated followed by dual administration of allergen proteins and then intranasally challenged with CDE. The resultant cellular infiltrate in the bronchoalveolar lavage (BAL), peri-bronchial tissue (histology), and whole lung (flow cytometry) were assessed (Figure 1B). Intranasal challenge with CDE, in dual allergen sensitized and sham treated animals, induced marked cellular infiltration into the BAL (Figure 3A), that was primarily composed of eosinophils and neutrophils (Figure 3B). Low-dose peptide therapy significantly reduced both total BAL cell infiltrate and eosinophilia by up to 50%. Following CDE IN challenge, lung tissue eosinophilia was assessed by flow cytometry (Siglec-F+CD11c+)[29], and histology (Haematoxylin and Eosin staining. Sensitized mice receiving a sham treatment possessed significantly elevated levels of eosinophils in the lung tissue (Figure 3C) and peribronchial space (Figure 3D-H). In contrast, low-dose Fel d 1 peptide treated mice were protected from peri-bronchial eosinophil accumulation (Figure 3D).

Previously, it has been demonstrated that peptide immunotherapy can reduce goblet cell hyperplasia, a common feature of allergic airways disease[22]. CDE challenge of sham treated, allergen sensitized mice demonstrated a greater than 100-fold increase in goblet cell density compared to saline mice (Figure 3I-M). High-dose peptide therapy reduced goblet cell hyperplasia by 30% (Figure 3I-M).

OVA induced allergic airways disease can be regulated by Fel d 1 peptide immunotherapy and dual allergen administration.

Having determined that Fel d 1 peptide immunotherapy could protect mice from challenge with CDE, we proceeded to investigate whether tolerance could be conferred to OVA induced responses. To address this question, dual allergen sensitized, Fel d 1 peptide treated

mice were given three IN OVA challenges, after simultaneous subcutaneous exposure to OVA and CDE.

In dual allergen sensitized, sham-treated mice, three IN OVA challenges induced robust cellular infiltration into the lumen of the lungs (Figure 4A); the infiltrate consisting primarily of eosinophils (Figure 4B). Peptide immunotherapy with 1µg of Fel d 1 derived peptides significantly reduced total BAL cellular infiltration, and a trend towards a reduction in BAL eosinophils was observed ($p=0.07$; Figure 4A &B).

Analysis of whole lung eosinophilia after ovalbumin challenge confirmed that sham treated, allergen sensitized mice developed robust lung tissue eosinophilia. Peptide therapy induced a dose dependent reduction in lung tissue eosinophilia of up to 50% (Figure 4C). Further examination of the eosinophilic response demonstrated that peribronchial eosinophilia induced by OVA challenge was significantly reduced with 1 µg of peptides and dual allergen administration (Figure 4D-H). Thus, the dose of peptides that significantly improved responses to CDE (Figure 3D) differed from the dose required to suppress OVA responses (Figure 4D). Ovalbumin challenge in sensitized, sham treated mice, induced robust goblet cell hyperplasia, that was significantly inhibited in peptide/dual SC allergen treated mice (Figure 4I-M).

Fel d 1 peptide immunotherapy and dual allergen administration reduces OVA-induced inflammatory cytokine production.

Successful clinical studies of peptide immunotherapy in allergic subjects have routinely been associated with reduced inflammatory cytokine production by CD4+ T-cells[30-36]. We sought to assess whether Fel d 1 immunotherapy could similarly reduce Th2 polarization of CD4+ T-cells in OVA challenged mice, as assessed by flow cytometry.

Examination of the Th2 polarization of lung T-cells (assessed as T1/ST2$^+$ T cells [37-39]) following therapy, revealed that OVA challenge of dually sensitized, sham treated mice induced a significant Th2 polarization of lung T-cells (Figure 5A). Immunotherapy with Fel d 1 peptides and dual allergen administration significantly reduced the number of T1/ST2$^+$ T cells by approximately 50% (Figures 5A & 5B). Following assessment of Th2 polarized cells in the lungs of treated mice, the ability of T-cells to produce archetypal Th2 cytokines was assessed. CD4+ T-cells derived from the lungs of sham treated mice contained a significantly larger number of cells staining positive for IL-4, IL-5 and IL-13, by flow cytometry (Figures 5C-E). Peptide/dual allergen treatment significantly reduced the number of IL-4+ and IL-5+ T-cells, and resulted in a non-significant ~33% reduction in IL-13+ T-cells. Together, these data indicate that Fel d 1 immunotherapy and dual allergen exposure modulated the Th2 immune response to OVA. To corroborate these data, we examined the prototypical Th2 antibody IgE to assess if the reductions in Th2 polarity had affected the B-cell compartment. Sham treated mice possessed elevated levels of ovalbumin specific IgE that was significantly reduced by peptide therapy (Figure 5F).

Interestingly, in sensitized and treated mice that were rechallenged with CDE, rather than OVA, reductions in Th2 polarization were less notable. While CDE challenge of peptide treated, dual allergic mice reduced Th2 T-cell polarization (Figure S2A and S2B), modulation of cytokine production was less apparent, with no significant reduction in T-cells producing IL-4+ (Figure S2C), IL-5+ (Figure S2D) or IL-13+ T cells (Figure S2E).

Reduction of inflammatory responses to OVA, is associated with increased numbers of IL-10+ T cells and B cells

We have previously shown a dependency of peptide immunotherapy upon the presence of IL-10 in a murine model of cat allergy[22], and an association between IL-10 production and induction of bystander tolerance. We investigated whether IL-10 production was increased following treatment. Lung and draining lymph node (dLN) cells were analyzed for IL-10 expression following ovalbumin allergen challenge. The total number of IL-10+ lung digest cells was modestly (although not significantly) reduced following therapy, compared to sham treated animals (Figure 6A). In contrast to lung derived cells, the number of IL-10 producing CD4+CD25+ cells in draining lymph nodes was significantly elevated after peptide/dual allergen immunotherapy ($p>0.05$; Figure 6B).

We further characterized the regulatory phenotype of treated mice by quantifying FoxP3, a transcription factor that controls the development of regulatory T-cells[40]. Analysis of FoxP3 expression in T cells from digested lungs of OVA challenged mice revealed that both sensitized mice and treated mice had elevated numbers of FoxP3+ cells compared to unsensitized controls (Figure 6C& 6D). Finally, the intensity of FoxP3 staining within CD4+ T cells, as quantified by the mean fluorescence intensity (MFI), significantly increased with peptide therapy (Figure 6E).

In addition to assessing IL-10 and FoxP3 expression following OVA challenge, we assessed the regulatory phenotype of mice receiving a CDE challenge. In the lungs and draining lymph nodes of CDE challenged mice, peptide/dual allergen immunotherapy dose-dependently and significantly increased IL-10 expression (Figure S3A, S3B) compared to sham treated mice. Moreover, quantification of FoxP3 expression after peptide immunotherapy revealed that CDE challenged mice had significantly more FoxP3+ T-cells (Figure S3C, Figure S3D), and an increased abundance of FoxP3 in those cells (Figure S3E).

Regulatory B-cells (Bregs) have been identified in murine models as key mediators of peripheral tolerance[41] and have recently been demonstrated to be capable of preventing and/or resolving allergic airway inflammation[42]. We investigated whether Fel d 1 peptide immunotherapy was capable of inducing Bregs. Treatment with 1µg of Fel d 1 derived peptides followed by dual SC allergen administration led to a significant increase in the CD19+IL-10+ cell population within the lungs of mice, compared to sham treated animals (Figure S3F). However, the accumulation of Bregs following OVA challenge was also assessed and found to be unchanged compared to sham treated animals (data not shown).

Fel d 1 peptide/dual allergen administration induces immune deviation.

In addition to our investigation of the induction of regulatory T cell and B cell responses, we assessed whether peptide immunotherapy had induced immune deviation towards Th1, by examining the production of IFN-γ. Sensitized, sham treated mice developed a modest increase in CD4+IFN-γ+ cells following OVA challenge. In mice receiving low dose peptide therapy, CD4+ IFN-γ+ cells were significantly elevated by nearly two-fold, while therapy with 1µg of peptides induced a non-significant ($p=0.06$), 33% increase in CD4+IFN-γ+ cells (Figure 7A). Furthermore, both tested doses of peptides doubled the percentage of T-cells producing IFN-γ+ ($p < 0.05$; Figure 7B). As these data support the expansion of a Th1 phenotype following peptide immunotherapy, we determined whether a similar effect could be observed in mice challenged with CDE. Indeed, treatment of cat allergen-sensitized mice with Fel d 1 peptides induced a Th1 phenotype as evidenced by a significantly increased abundance of IFN-γ+ CD4+ T-cells (Figure S4A) and proportion of IFN-γ producing CD4 cells (Figure S4B).

IL-17 is a pro-inflammatory, non-Th2, cytokine commonly expressed by T-cells, that has been linked to the inflammatory cascade observed in allergic asthma[43], severe asthmatic responses[44] and Th1 immunity[45]. We investigated the production of IL-17 after challenge with OVA. Challenge of sham treated mice with OVA induced a significant 8-fold increase in IL-17 production by activated lung derived T-cells; however, the absolute numbers of IL-17+ cells was small (Figure 7C). Treatment reduced numbers of IL-17+ T-cells by approximately 50%. In CDE challenged mice, numbers of IL-17+ T cells appeared to be reduced but this change did not achieve statistical significance (Figure S4C).

3.5. Discussion

In the present study, we have demonstrated that therapy with Fel d 1 derived peptides, followed by a period of dual allergen exposure (CDE and OVA), can facilitate the induction of bystander tolerance and inhibit OVA-induced inflammation. Treatment protected dual allergen sensitized mice from developing eosinophilia, goblet cell hyperplasia or Th2 cellular infiltration, upon airway challenge with either CDE or OVA. Interestingly, the immune responses to these two allergen sources were subtly different. Fel d 1 peptide-induced modulation of the response to cat allergen extract was associated with significant increases in CD4+CD25+Foxp3+ T cells, IL-10+ cells and CD19+IL-10+ B cells. In contrast, the response to OVA, which was achieved through the Fel d 1 peptide therapy combined with subcutaneous co-administration of OVA and cat allergen extract, resulted in a marked reduction of Th2 cytokine-secreting T cells (immune deviation) and less prominent changes to outcomes associated with immune regulation. An important implication of these observations is that the mechanisms that give rise to bystander tolerance may be self-limiting (down-regulation of Th2 responses in this case), whilst peptide modulation of the specific response (characterized by induction of regulatory pathways; IL-10,

IL-10+ B cells) may be capable of dominant suppression of non-specific responses. Thus, the immune response to antigen-specific therapy appears to have dominant regulatory capacity, whereas the immune response to the bystander protein is a terminal process. Taken together, these data indicate that different elements of immune regulation induced by peptide immunotherapy can be used, experimentally, to treat an allergic response to another allergen.

In on-going clinical studies, cat allergic subjects have been treated by intradermal injection with an equimolar mixture of seven Fel d 1 derived peptides. Phase 2 studies of this approach have demonstrated the ability of peptide immunotherapy to reduce responsiveness to provocation with complex mixtures of cat allergens contained in extracts [16, 17]. The ability of peptides derived from one allergen protein to reduce responses to mixtures of multiple allergens raises the question of whether treatment with peptides from one molecule is able to create a localized tolerogenic environment in which tolerance, or hyporesponsivessness, can be induced to proteins from other simultaneously administered allergen sources. The results of this study demonstrate that peptides of Fel d 1, containing dominant T cell epitopes, can be used to create an environment that can be exploited to modulate responses to OVA.

Despite extensive investigation of the pathogenesis of allergic diseases and improved understanding of the mechanisms of action of allergen immunotherapy, novel, safe, rapid and effective therapeutics for allergic diseases have rarely reached the clinic[46]. Clinical trials have demonstrated that peptide immunotherapy for the treatment of cat allergic rhinoconjunctivitis is well tolerated[47], effective[16]and offers long-lasting protection [17]. We have previously demonstrated that linked-epitope suppression develops after peptide immunotherapy, as individuals treated with a mixture of twelve Fel d 1 peptides displayed reduced proliferative and cytokine responses, not only to the twelve treatment peptides, but also to an additional four Fel d

1 peptides not included in the treatment phase [22]. Furthermore, in accompanying murine studies, this phenomena was determined to be dependent upon IL-10, in agreement with other models of peptide-induced, T-cell mediated regulation[48]. We previously described an example of bystander tolerance in which treatment with peptides from one protein elicited tolerance, not only to the treatment protein, but to an unrelated protein. We observed a reduction in inflammatory responses to inhaled OVA challenge in OVA and house dust mite (HDM) dually sensitized mice that had been treated with intranasal HDM peptides [24]. To our knowledge, no clinical studies have directly and prospectively studied such phenomena. However, in a placebo-controlled study of twenty-nine multiple-allergen sensitized children, pollen immunotherapy significantly reduced responsiveness to allergen challenge with house dust mite or cat allergen[49].

The current studies were designed to specifically ask if treatment with peptides from a single allergen protein create a local tolerogenic environment that could be exploited to suppress inflammatory responses to a second, unrelated allergen. In addition, we wanted to see if this effect could be generated when treating animals intradermally, the same treatment route used in current clinical studies. The route of administration is relevant to clinical studies, as asthmatics treated with intradermal peptides[34], but not inhaled peptides[26], were protected from subsequent challenges. Furthermore, as allergic individuals are commonly poly-sensitized, we set out to use a model wherein sensitization to both allergens was fully established prior to therapy, rather than using a prophylactic treatment regimen that may misrepresent the clinical setting. This study focused on outcomes associated with allergic airways disease such as airway eosinophilia and inflammatory Th2 infiltration of the airways, since these have been historically linked to allergic asthma[50].

In our model of dual allergen sensitization, challenge with either allergen induced airway eosinophilia, goblet cell hyperplasia and other cardinal features of allergic airways disease. Treatment with Fel d 1 derived peptides and the SC administration of both allergen proteins significantly suppressed both cat dander extract-induced and ovalbumin-induced lung inflammation, as illustrated through reductions in total BAL cellularity, peribronchial eosinophilia, whole lung eosinophilia and goblet cell hyperplasia. Concomitant reductions in lung Th2 cell surface markers and numbers of cytokine expressing T cells were associated with the resolution of ovalbumin-specific (but not Fel d 1-specific) allergic responses, while increased expression of the prototypical T_H1 cytokine, IFN-γ, may implicate immune deviation as a means of regulation. Parameters related to regulatory responses such as numbers of CD4+CD25+Foxp3+ T cells, IL-10+ cells and CD19+IL-10+ B cells were all significantly increased following CDE challenge but not OVA challenge.

Our findings are in accordance with, and expand upon, two recent studies that examined both the antigen-specific and non-specific effects of immunotherapy. Bouchaud *et al* (2015)[51] prophylactically treated mice with large peptides (>50 amino acids) derived from the house dust mite allergen, Der p 2. Peptide treated mice were protected from challenge with HDM, thereby demonstrating the ability of a single peptide to block inflammation induced by challenge with a complex allergen, that contains multiple immunogenic epitopes. Furthermore, mice prophylactically treated with a Der p 2 derived peptide were moderately protected from Der f 2 induced airway hyperresponsiveness, as assessed by a methacholine challenge. This demonstrates that Der p 2 derived peptide therapy was able to impact the immune response to a distinct allergen, although whether this was via infectious tolerance or simply T-cell cross-reactivity is unknown. Our data expand upon this study by demonstrating that short peptides,

administered after sensitization and coupled with co-administration of whole allergen proteins (OVA and Fel d 1) can prevent the development of airway inflammation induced by a distinct, non-cross-reactive allergen. Chien *et al.,* (2015) also investigated the non-antigen-specific effects of immunotherapy, by demonstrating that feeding mice OVA, after sensitization to β-lactoglobulin (BLG)-conjugated OVA (BOG), ameliorated airway inflammation induced by challenge with either OVA or BLG. Our data corroborate the notion that immunotherapy can modulate both specific and non-antigen-specific effects, while demonstrating that peptide immunotherapy, a clinically relevant therapy administered in a clinically relevant route can perform this function. Furthermore, our study provides novel insight into the different immunologic mechanisms that underlie tolerance induced directly in an antigen-specific fashion, versus indirectly through bystander tolerance.

Fel d 1 peptide therapy has been linked to the expansion of regulatory T-cell activity within human and mouse CD4+ T cell populations[36]. However, the expression of FoxP3 by such populations has been an unreliable surrogate marker, although in those studies the intensity of FoxP3 expression per cell was not evaluated. In a previous study, we noted increased FoxP3 expression in T-cells following peptide treatment[24]. In another model, sustained suppression by FoxP3+ T cells was required in order to observe the related phenomenon of infectious tolerance in which tolerant T cells direct other T cells to become regulators [22, 52]. Thus, induction of FoxP3 expressing populations, or increased expression of FoxP3 per cell, is often associated with the induction of tolerance, but is not a pre-requisite.

In addition to the expansion of T cell populations with regulatory phenotypes following CDE challenge, we observed a three-fold increase in IL-10 producing B-cells which have been described as regulatory B cells (Br1/B10/Bregs). This substantial population of B cells was not

observed after OVA challenge and may be a key component of the dominant tolerance induced by peptide immunotherapy. Bregs have recently been acknowledged as potentially important modulators of peripheral tolerance, but have not previously been described in the context of peptide immunotherapy. Van der Veen and colleagues described the induction of IL-10 secreting B cells following whole allergen immunotherapy for bee venom allergy. These cells exhibited potent allergen-specific suppressive function and were particularly associated with the production of IgG4 antibodies which are believed to have "blocking" activity by virtue of their ability to sequester allergen and prevent contact with IgE-laden mast cells and basophils [53].

Evidence suggests that the dose of allergen (and likely peptide as well) during therapy impacts the mechanism of suppression, as low dose immunotherapy induces a profile of active suppression, while high doses therapy relies upon clonal deletion and anergy[54-56]. Relative to other murine models of immunotherapy, the current study employed low doses of Fel d 1 peptides and was able to reduce the Th2 pathology associated with OVA challenge, perhaps via active immune suppression associated with FoxP3, IL-10 and immune deviation. The impact of peptide dose during treatment requires further study.

In summary, using a model of dual allergen sensitization and peptide immunotherapy, we have demonstrated that Fel d 1 peptides, when coupled with co-administration of both Fel d 1 protein and an unrelated protein (OVA) are capable of attenuating Th2 inflammation within the murine allergic lung following inhaled allergen challenge with the unrelated protein allergen. Furthermore, we have confirmed that the local tolerogenic environment generated by peptide therapy can be exploited to confer tolerance to unrelated molecules. We show that treatment allergen-specific tolerance is associated with a mixed phenotype of enhanced regulatory profiles (CD4+CD25+Foxp3+ T cells, IL-10+ cells and CD19+IL-10+ B cells), while tolerance to the

unrelated allergen is associated more closely with reduction in Th2 cytokine secreting cells. These results may enhance our understanding of how allergen-specific (peptide) immunotherapy appears to result in the down regulation of immune responses to allergens beyond those employed for treatment. The potential therapeutic advantages of such phenomena remain to be fully evaluated.

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3.7. Figures

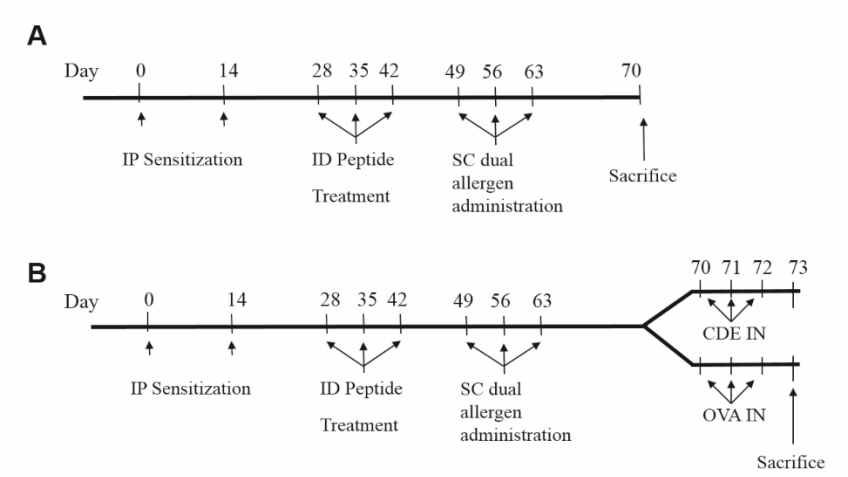

Figure 1. Allergen exposure and peptide therapy protocol. Mice were sensitized to ovalbumin (100 µg) and Fel d 1 (1µg), via two intraperitoneal injection (Day 0 & 14). Three intradermal treatments of Fel d 1 derived peptides were administered (Day 28, 35, 42), which was followed by three subcutaneous exposure to both allergens (Fel d 1 & Ovalbumin, Day 49, 56, 63). Sham treated mice received peptide vehicle (PBS) ID. Mice were sacrificed, [A] one week later to assess the humoral and proliferative immune response, or [B] after a series of IN allergen (Day 70, 71, 72) challenges with either OVA or CDE to assess the functional response. Mice were sacrificed 24-hour post final challenge for measurement of airway responsiveness and cell procurement.

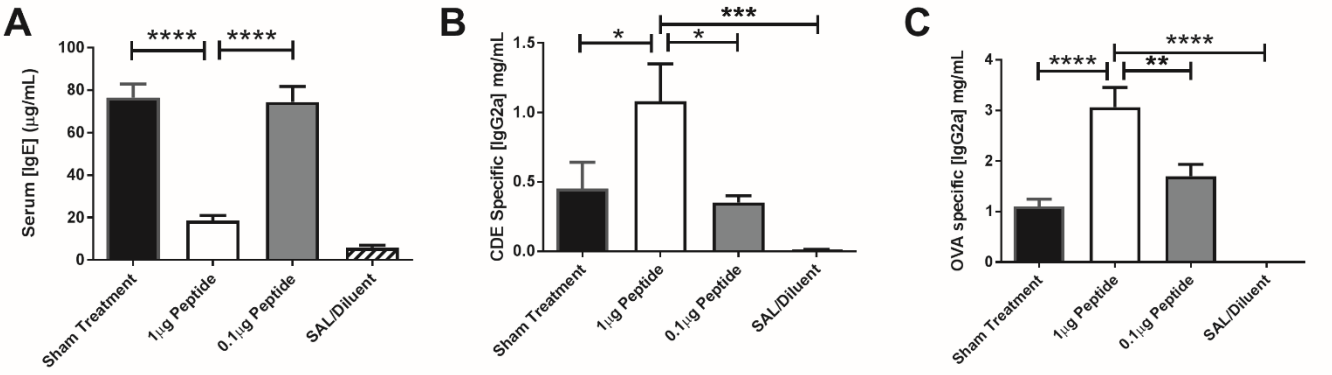

Figure 2. Fel d 1 derived peptide immunotherapy reduces antibody and lymphocyte proliferative responses. The concentration of serum [A] IgE, [B] Cat Dander Specific-IgG2a, and [C] Ovalbumin-specific IgG2a were assessed by ELISA, as described in *Methods*. Mice were sensitized to both Fel d 1 and Ovalbumin, and treated with Fel d 1 derived peptides prior to exposure to both allergens subcutaneously. Groups are as follows: sensitized & sham treated (Sham treatment); sensitized & treated with 1µg of each peptides (1µg Peptide); sensitized & treated with 0.1µg of each peptides (0.1µg Peptide); and sham sensitized and treated (Sal/Diluent). All data are presented as mean ± SEM, of two independent experiments pooled together (n=10/group. * = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$;**** = $P \leq 0.0001$ compared to 1µg Peptide via an ANOVA and post-hoc Bonferronni corrected Student's *t*-test. $ = $P \leq 0.05$,0.1µg Peptide compared to sham treatment, and # = $P \leq 0.05$,1µg Peptide compared to sham treatment via an ANOVA an individual, post-hoc Student's *t*-test.

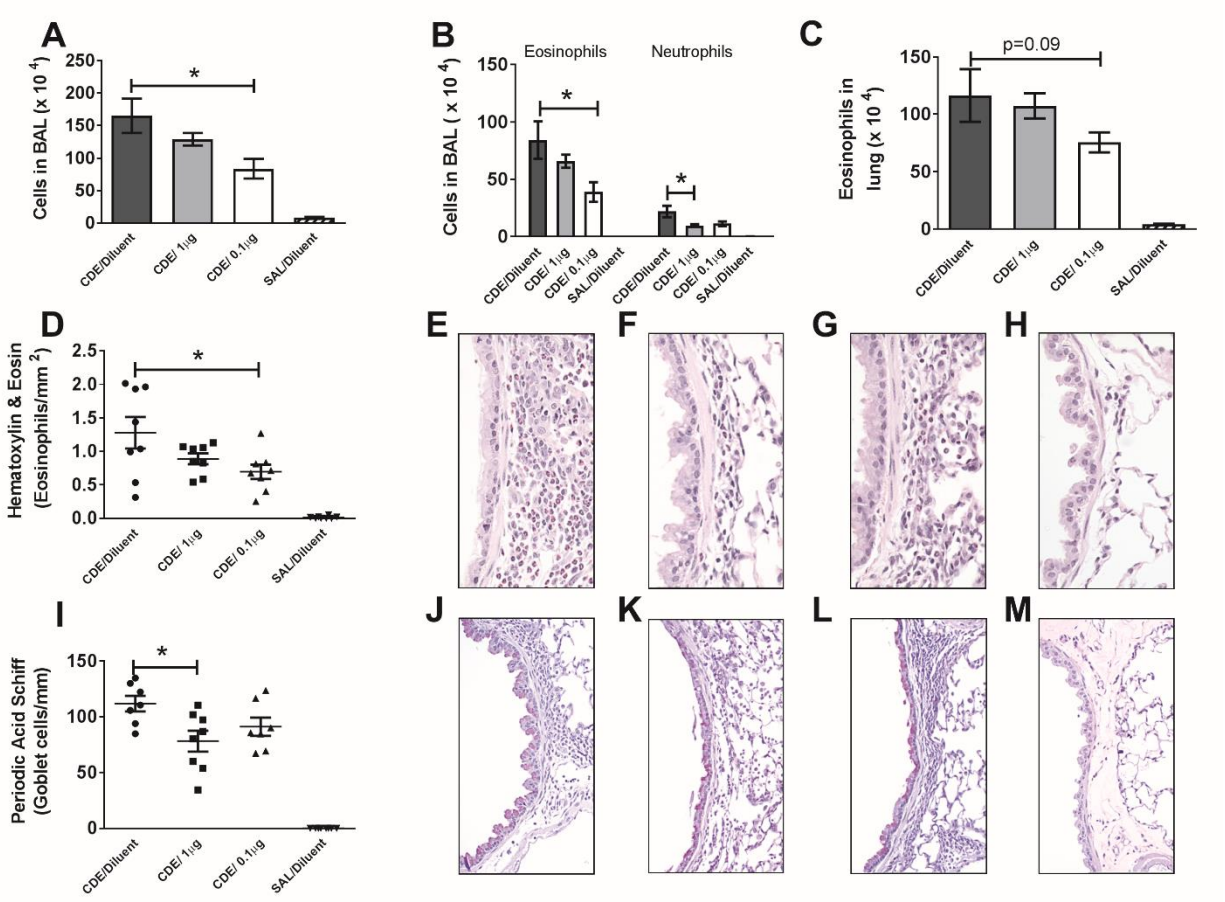

Figure 3. Fel d 1 peptide immunotherapy ameliorates CDE-challenge induced cellular recruitment to the lung. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with 25 µg cat dander extract (CDE/--) or saline(SAL/--). Total BAL cells were enumerated [A] and differentiated via Wright-Giemsa staining. BAL eosinophils and neutrophils are depicted [B]. [C] Lung cells were isolated, and eosinophilia was determined by flow cytometry (SIGLEC-F+Gr1-) after recall challenge. Peribronchial eosinophilia was determined by Hematoxlin and Eosin staining of formalin fixed lungs [D]; representative histological images of stained sections at 20x are shown: CDE/Diluent [E], CDE /1µg [F], CDE/0.1µg [G] and SAL/Diluent [H]. Goblet cell hyperplasia was determined by periodic acid Schiff (PAS) staining of formalin fixed lungs [I]; representative images of stained lung sections at 10x magnification are shown: CDE /Diluent [E], CDE /1µg [F], CDE /0.1µg [G] and SAL/Diluent [H]. Data presented as mean ± SEM, with or without individual points. * = $P \leq 0.05$ assessed by an ANOVA and post-hoc Bonferronni corrected Student's t-test.

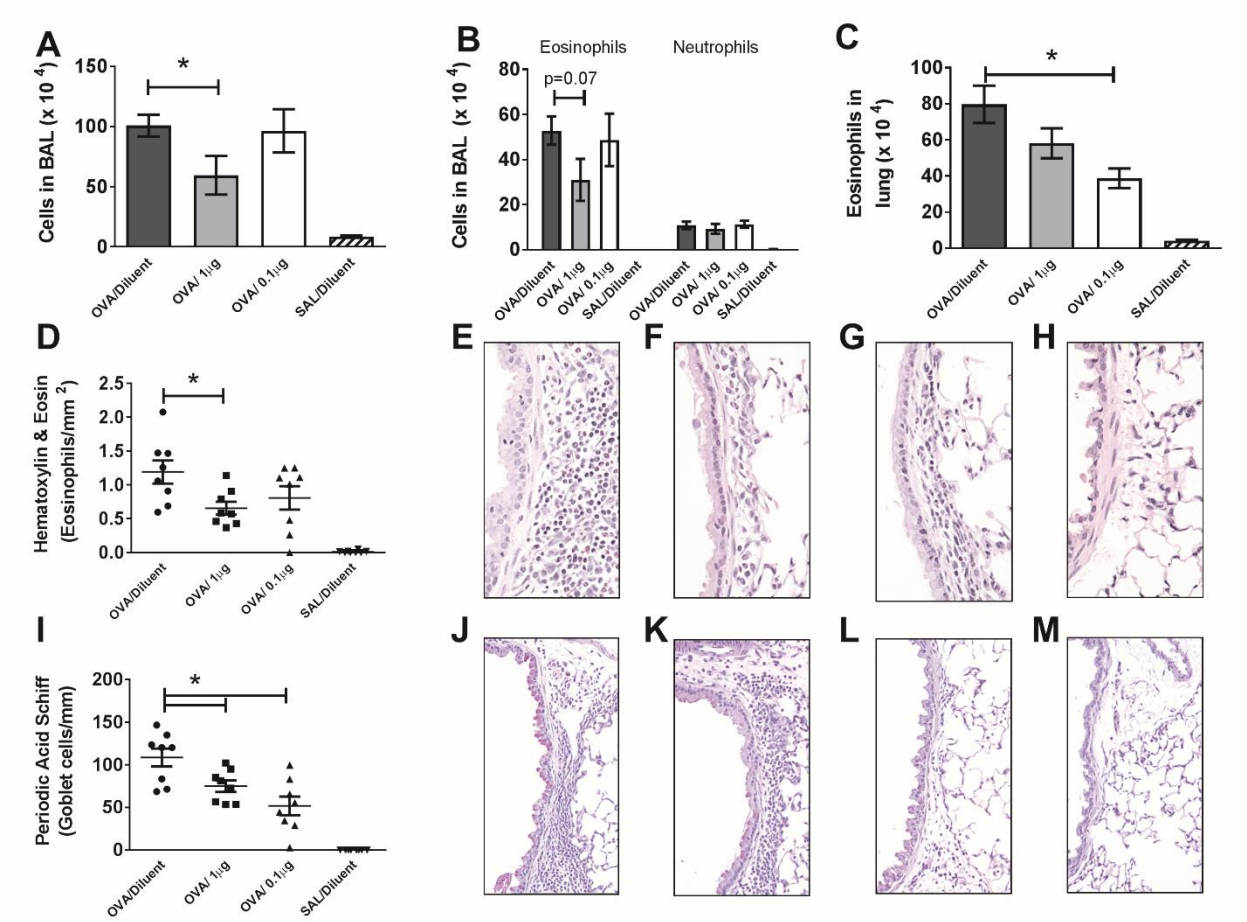

Figure 4. Fel d 1 peptide immunotherapy prevents OVA-induced lung inflammation. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1µg) or 0.1µg (--/0.1µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with100 µg ovalbumin (OVA/--) or saline(SAL/--). Total BAL cells were enumerated [A] and differentiated via Wright-Giemsa staining. BAL eosinophils and neutrophils are depicted [B]. [C] Lung cells were isolated, and eosinophilia was determined by flow cytometry (SIGLEC-F+Gr1-) after recall challenge. Peribronchial eosinophilia was determined by Hematoxlin and Eosin staining of formalin fixed lungs [D]; representative histological images of stained sections at 20x are shown: OVA/Diluent [E], OVA/1µg [F], OVA/0.1µg [G] and SAL/Diluent [H]. Goblet cell hyperplasia was determined by periodic acid Schiff (PAS) staining of formalin fixed lungs[I]; representative images of stained lung sections at 10x magnification are shown: OVA/Diluent [J], OVA/1µg [K], OVA/0.1µg [L] and SAL/Diluent [M]. Data presented as mean ± SEM, with or without individual points. * = $P \leq 0.05$ assessed by an ANOVA and post-hoc Bonferronni corrected Student's *t*-test.

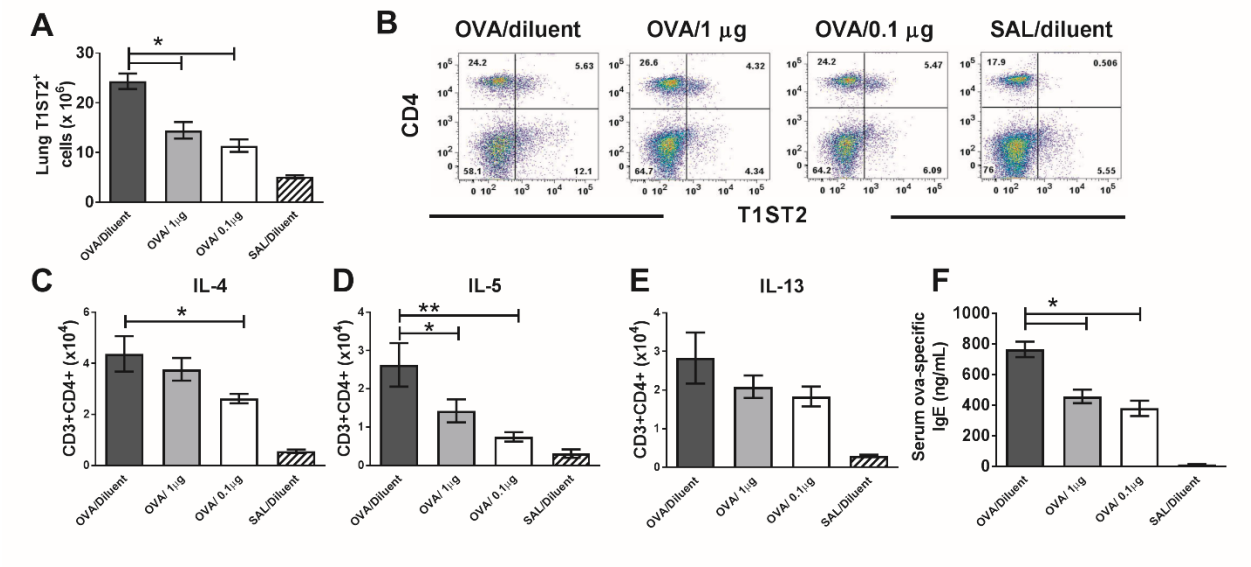

Figure 5. Reduced T_H2 cell recruitment and cytokine expression, following ovalbumin rechallenge, in Fel d 1-derived peptide treated mice. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with100 µg ovalbumin (OVA/--) or saline(SAL/--). The Th2 phenotype of cells derived from the lungs of allergen challenged mice was assessed. Expression of T1ST2 in lymphocytes [A]. The average percentage of CD4+ T1ST2+ cells is depicted within representative dot plot images, of a typical flow cytometric experiment [B], groups ordered as: OVA/Diluent, OVA/1µg, OVA/0.1µg, and SAL/Diluent. Lung cells were stimulated for 6 hours with PMA and ionomycin, in the presence of brefeldin A, at which point the expression of IL-4 [C], -5 [D], 13 [E] were examined within the T-cell (CD3+CD4+) population. The concentration of Ovalbumin-specific IgE was assessed by ELISA, as described in *Methods* [F]. Data presented as mean+SEM.* = $P \leq 0.05$; ** = $P \leq 0.01$, as assessed by an ANOVA and post-hoc Bonferronni corrected Student's *t*-test.

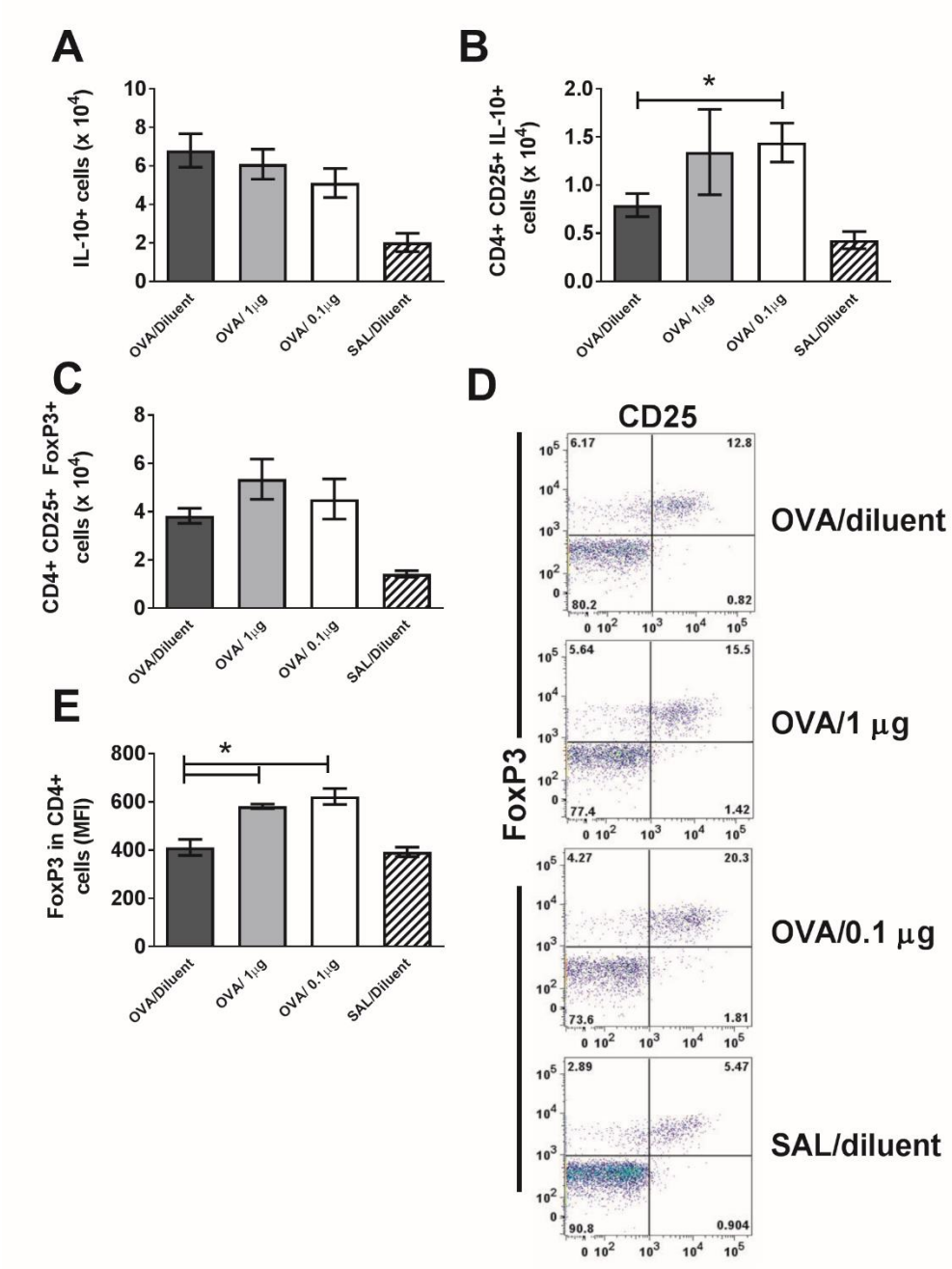

Figure 6. Mice challenged with OVA following Fel d 1 immunotherapy mount a moderate regulatory response. 24 hours post intranasal ovalbumin challenge, mice were sacrificed and isolated dLN and lung cells were stained for surface markers and intracellular cytokines expression. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with100 µg ovalbumin (OVA/--) or saline(SAL/--). [A] Total IL-10+ lung cells. [B] total IL-10+CD25+CD4+ dLN cells. Total FoxP3+CD25+CD4+ cells within the lungs [C]. Lung lymphocytes, gated on CD4, were further analyzed for their FoxP3 and CD25 expression, average percentage of positive cells is depicted within representative dot plots, with the panels in descending order: OVA/Diluent, OVA/1µg, OVA/0.1µg, and SAL/Diluent[D]. Magnitude of FoxP3 expression within lung CD4+ cells, assessed by Mean Flourescent Intensity [E]. Number of

CD19+IL10+ cells present in the lungs of dual allergen sensitized, peptide treated mice challenged with CDE (CDE/--) [F]. Data presented as mean+/- SEM. * = P ≤ 0.05 assessed by an ANOVA and post-hoc Bonferronni corrected Student's t-test.

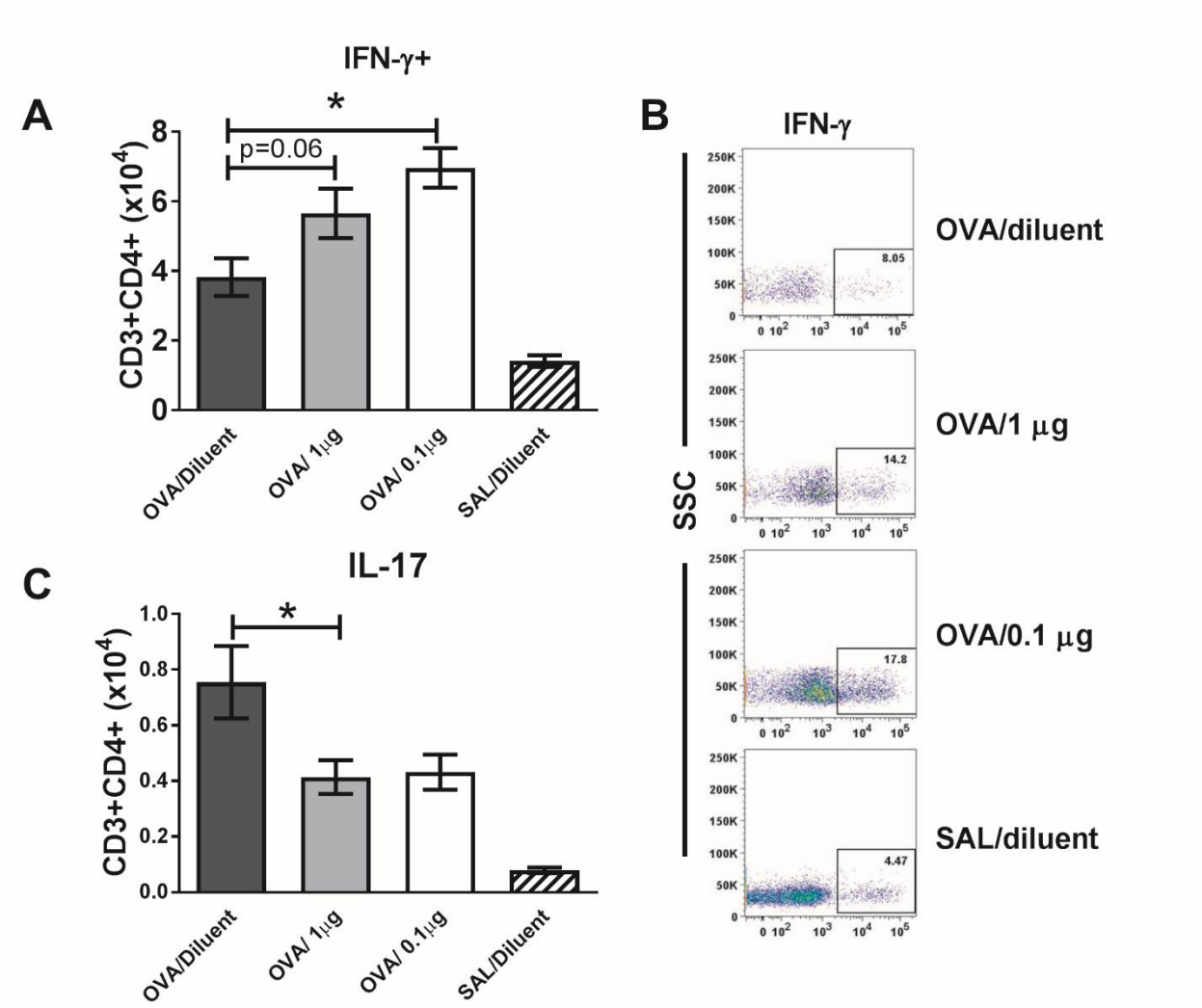

Figure 7. Fel d 1 immunotherapy induces immune deviation in OVA challenged mice. 24 hours post intranasal ovalbumin challenge, mice were sacrificed and isolated dLN and lung cells were stained for surface markers and intracellular cytokines expression. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1μg (--/1 μg) or 0.1μg (--/0.1 μg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with100 μg ovalbumin (OVA/--) or saline(SAL/--). [A] Total CD4+ T-cells producing IFN-γ, and the percentage of lung lymphocytes, gated on CD4, producing IFN-γ is depicted in representative dot plots, with the panels in descending order: OVA/Diluent, OVA/1μg, OVA/0.1μg, and SAL/Diluent[B]. Enumeration of lung T-cells producing IL-17 [C]. Data presented as mean ± SEM. * = P ≤ 0.05 assessed by an ANOVA and post-hoc Bonferronni corrected Student's *t*-test.

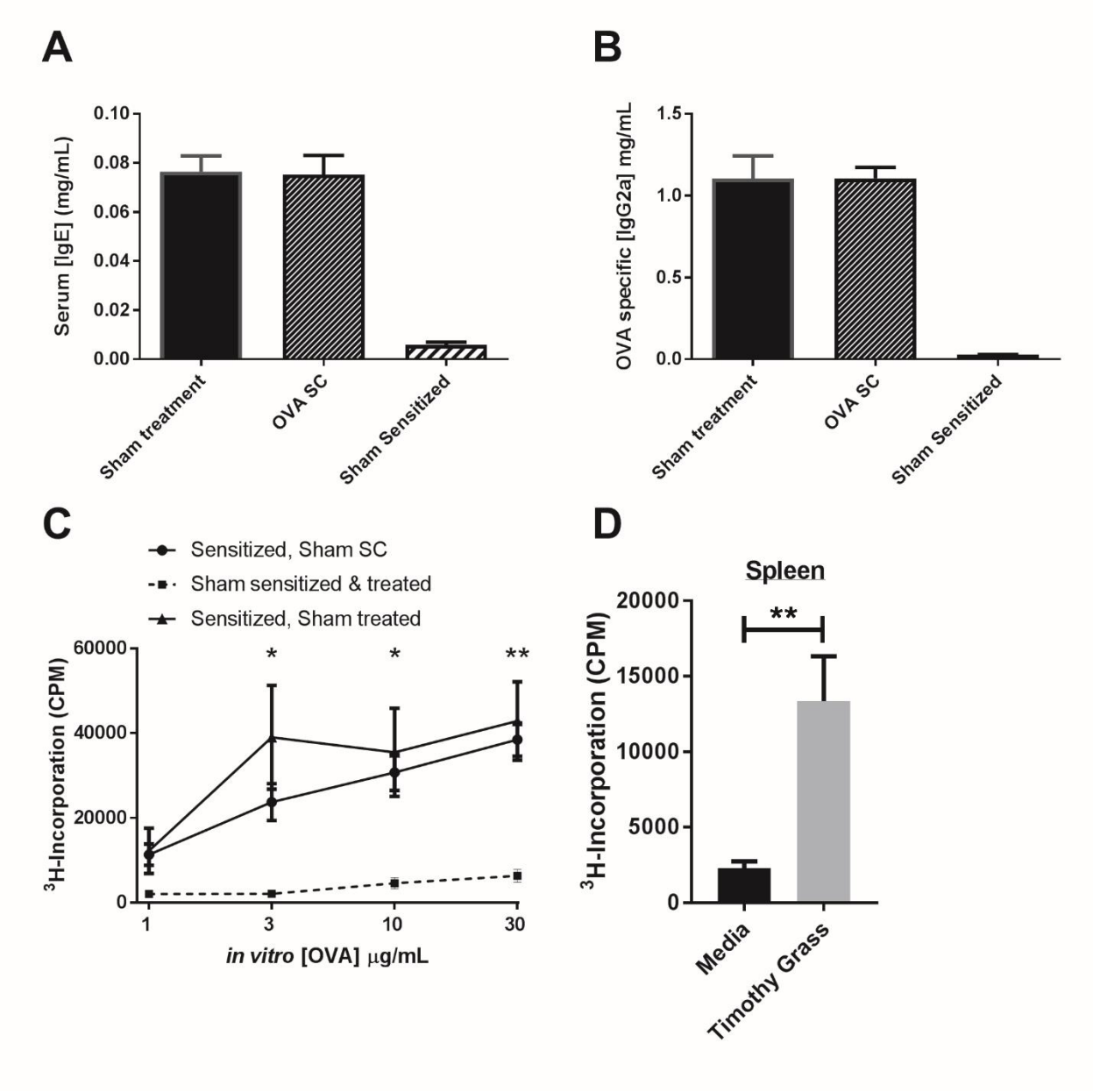

Figure S1. Fel d 1 derived peptide immunotherapy reduces antibody and lymphocyte proliferative responses. The concentration of serum [A] IgE, and [B] Ovalbumin-specific IgG2a were assessed by ELISA, as described in Methods. [C] The proliferation of splenocytes to ovalbumin, in vitro from sham sensitized, sham treated and sham subcutaneous mice. [D] The proliferation of splenocytes from triple allergen sensitized animals (Timothy grass, Fel d 1 and ovalbumin) that received therapy with 0.1μg of Fel d 1 derived peptides and simultaneous ovalbumin and cat dander, subcutaneously. Proliferation was assessed by a 3H-thymidine incorporation assay. Data are presented as mean ± SEM, of two independent experiments pooled together (n=10/group). * = $P \leq 0.05$; ** = $P \leq 0.01$, by student's t-test.

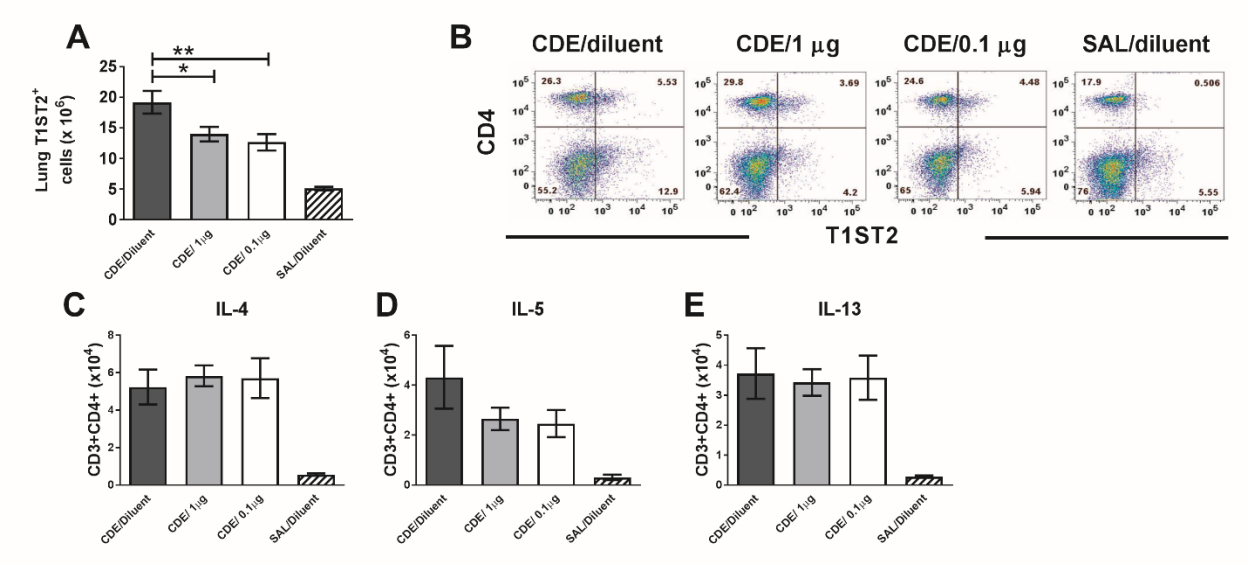

Figure S2. Fel d 1 peptide therapy prevents the recruitment of Th2 effector cells, but not the expression of Th2 cytokines in the lungs of cat challenged mice. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with 25 µg cat dander extract (CDE/--) or saline(SAL/--). The Th2 phenotype of cells derived from the lungs of allergen challenged mice was assessed. Expression of T1ST2 in lymphocytes [A]. The average percentage of CD4+ T1ST2+ cells is depicted within representative dot plot images, of a typical flow cytometric experiment [B], groups ordered as: CDE/Diluent, CDE/1µg, CDE/0.1µg, and SAL/Diluent. Lung cells were stimulated for 6 hours with PMA and ionomycin, in the presence of brefeldin A, at which point the expression of IL-4 [C], -5 [D], 13 [E] were examined within the T-cell (CD3+CD4+) population. Data presented as mean + SEM. Data presented as mean ± SEM. * = $P \leq 0.05$; ** = $P \leq 0.01$, as assessed by an ANOVA and post-hoc Bonferronni corrected Student's *t*-test.

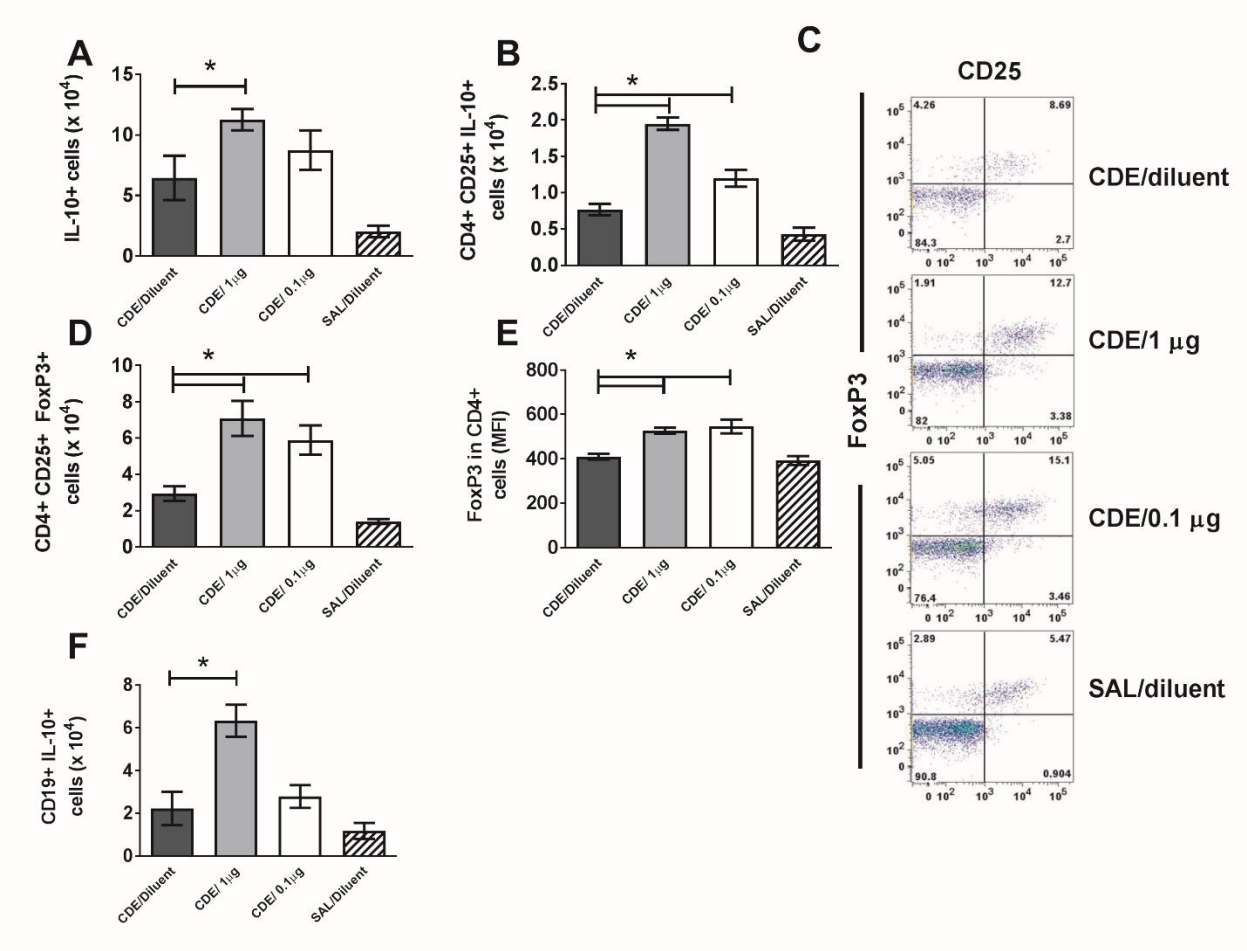

Figure S3. Fel d 1 immunotherapy allows the manifestation of a regulatory response following CDE challenge. 24 hours post intranasal ovalbumin challenge, mice were sacrificed and isolated dLN and lung cells were stained for surface markers and intracellular cytokines expression. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with 25 µg cat dander extract (CDE/--) or saline(SAL/--). [A] Total IL-10+ lung cells. [B] total IL-10+CD25+CD4+ dLN cells. Lung lymphocytes, gated on CD4, were further analyzed for their FoxP3 and CD25 expression, average percentage of positive cells is depicted within representative dot plots, with the panels in descending order: CDE/Diluent, CDE/1µg, CDE/0.1µg, and SAL/Diluent[C]. Total FoxP3+CD25+CD4+ cells within the lungs [D], the mean flourescent intensity FoxP3 expression within lung CD4+ cells [E] and the number of CD19+IL-10+ cells, in the lung [F]. Data presented as mean ± SEM. * = $P \leq 0.05$ assessed by an ANOVA and post-hoc Bonferronni corrected Student's *t*-test.

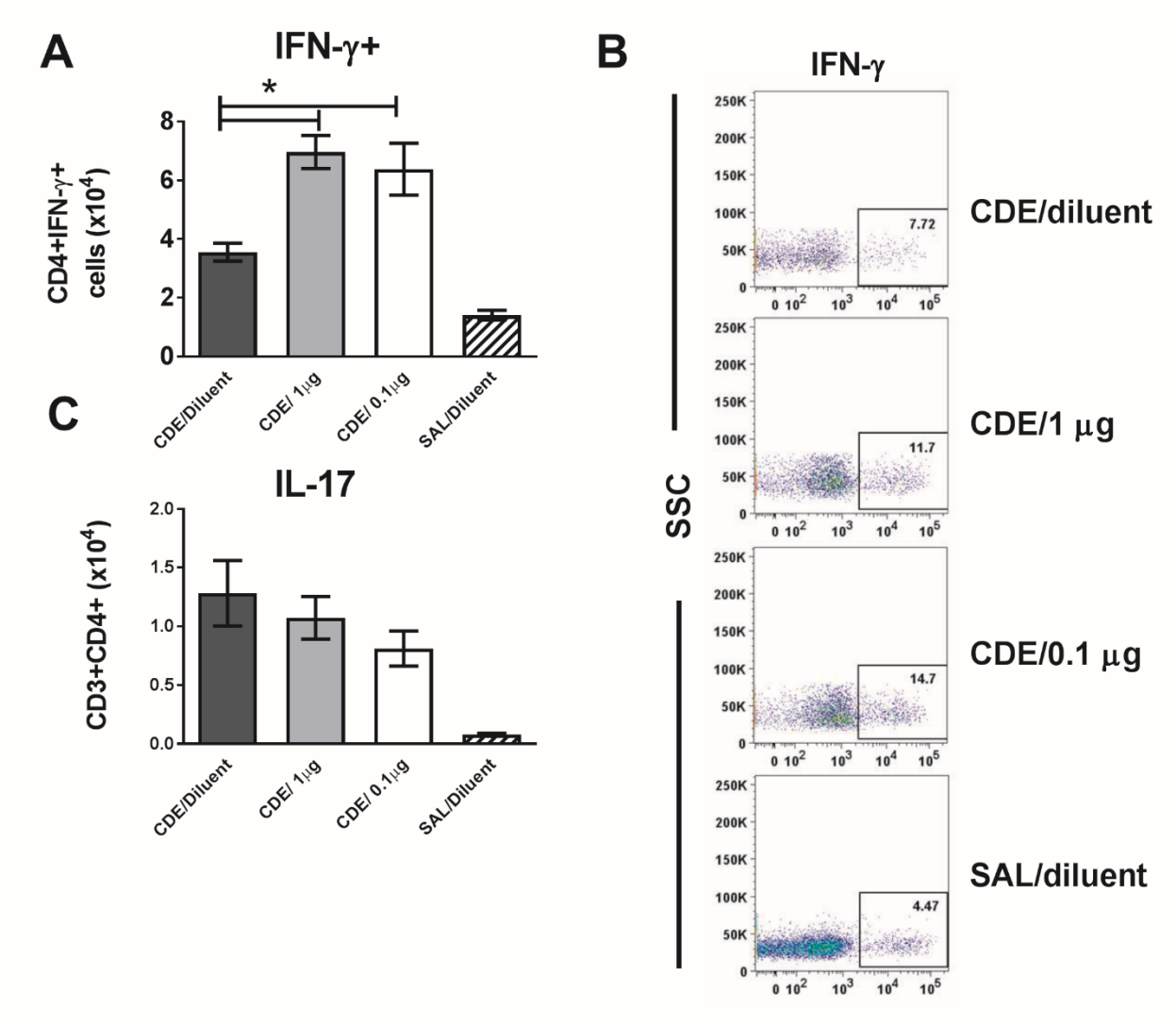

Figure S4. Fel d 1 immunotherapy allows the manifestation of a regulatory response following CDE challenge. 24 hours post intranasal ovalbumin challenge, mice were sacrificed and isolated dLN and lung cells were stained for surface markers and intracellular cytokines expression. Mice (n=8/group from two pooled experiments) were sensitized to Fel d 1 and ovalbumin then treated with 1µg (--/1 µg) or 0.1µg (--/0.1 µg) of Fel d 1 derived peptides, or PBS (--/Diluent). Following the therapeutic protocol, mice were intranasally challenged with 25 µg cat dander extract (CDE/--) or saline(SAL/--). The total CD4+ T-cells producing IFN-γ [A], and the percentage of lung lymphocytes, gated on CD4, producing IFN-γ is depicted in representative dot plots, with the panels in descending order: CDE/Diluent, CDE/1µg, CDE/0.1µg, and SAL/Diluent[B]. IL-17 expression within T-cells was probed, following PMA and ionomycin stimulation [C]. Data presented as mean ± SEM. * = $P \leq 0.05$ assessed by an ANOVA and post-hoc Bonferronni corrected Student's t-test.

Chapter 4: Dose-Dependent Changes in Lung Inflammation and Response to Therapy in a Humanized Model of Allergic Airways Disease.

4. Declaration of Academic Achievement

Chapter 4. Dose-dependent changes in lung inflammation and response to therapy in a humanized model of allergic airways disease.

D. M. Moldaver[1], T. Singh[1], M. S. Bharhani[1], I. Nayve[1,2], M. Babra[1], C. Rudulier[1,3], J. Wattie[1], M. van Hage[4], M. D. Inman[1] and M. Larché[1]†

- The following study has been submitted to Disease Mechanisms and Models.
- Rights for publication in thesis were retained at submission (2017).

Daniel Moldaver's contributions

As primary author, I was responsible for conceiving, planning, managing and contributing to all aspects of the study. I treated animals, performed mouse dissections, fixed tissues, performed histology, prepared cells for flow cytometry and proliferation assays, performed flow cytometry and ELISAs. T. Singh, I. Nayve, and M. Babra assisted with mouse treatments and cell processing. M. Bharhani assisted with analysis of flow cytometry. C. Rudulier provided guidance on manuscript generation. J. Wattie assisted with mouse treatments and was responsible for mouse tracheostomies, and performing the nebulized methacholine challenge. M. van Hage provided the cat dander extract. M. Larché & M. Inman provided guidance on study design. Analyses, statistics and figure generation were performed by myself & M Larché.

Dose-dependent changes in lung inflammation and response to therapy in a humanized model of allergic airways disease.

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Running title: Allergen dose controls lung response

Key words: mouse model, allergic asthma, allergen dose, neutrophils, eosinophils, steroid refractory

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Summary statement: In a humanized mouse model of allergic airways disease, the dose of inhaled allergen challenge determines the degree of airway dysfunction, type of inflammation and responsiveness to therapeutic intervention.

Disclosure

ML is a co-founder, stockholder, and consultant of Circassia Ltd. and a founding scientist, consultant of Adiga Life Sciences Inc. and has received research support from both of these companies. ML was a consultant for Aravax Pty and UCB in the 12 months prior to the submission of this manuscript. The rest of the authors declare no conflict of interest.

4.1. Abstract

Allergic asthma is a common disease with a significant socio-economic impact. In allergic asthma, and mouse models of allergic asthma, it remains unclear how lung inflammation, dysfunction and responsiveness to therapy relates to the dose of allergen exposure. We set out to determine how the dose of inhaled allergen exposure determines disease phenotype and response pharmacological intervention. A model of cat allergy was developed in humanized transgenic mice that express the HLA-DR4 MHC class II complex and lack endogenous I-A^b. HLA-DR4 transgenic mice were sensitized to Fel d 1 via intraperitoneal injection of Fel d 1 in alum. Inflammatory responses were localized to the lung via nasal allergen challenge with cat dander extract (CDE). Patterns of lung inflammation, airway hyperresponsiveness (AHR) and response to treatment (corticosteroids) were assessed following recall challenge with CDE at a range of doses. Intranasal recall challenge with CDE induced hallmark features of asthma, including AHR, eosinophilia and lung resident Th2-polarized T-cells. The dose of allergen recall challenge exquisitely controlled disease phenotype, as recall challenge with low-dose CDE (1 or 5µg) induced AHR and airway lumenal eosinophilia, while high-dose CDE (20 or 30µg) challenge induced more severe airway dysfunction and airway lumenal neutrophilia. Corticosteroids failed to modulate airway dysfunction in high-dose rechallenged mice. Thus, in a murine model of allergic airways disease, the dose of inhaled allergen challenge determined the degree of airway dysfunction, inflammation and responsiveness to therapeutic intervention.

4.2. Introduction

Clinically, asthma is defined as the presence of reversible airflow obstruction that typically manifests with airway hyperresponsiveness (AHR) and airway inflammation (Bateman et al., 2008). Allergic asthma has historically been viewed as a disease resulting from an aberrant T-helper type 2 (Th2) immune response directed towards common environmental proteins. Multiple cell types associated with Th2 immunity have been implicated in the pathogenesis of asthma, including: Th2 T-cells (Bentley et al., 1993; Robinson et al., 1992), B-cells (Burrows et al., 1989; Lundgren et al., 1989; Sears et al., 1991), eosinophils (Humbles et al., 2004; Lee et al., 2004; Nair et al., 2009), and more recently, type 2 innate lymphoid cells (ILC2s) (Barlow et al., 2012; Chang et al., 2011; Mjosberg et al., 2011).

Stratification of therapeutic intervention based upon disease phenotype/endotype, at the time of clinical presentation, has proven to be a successful treatment strategy for allergic or Th2 asthma, as numerous biologic interventions targeting Th2 cytokines or IgE have shown efficacy. However, some individuals remain unresponsive to Th2-targeted biologic intervention despite fulfilling phenotypic and endotypic criteria of Th2-high asthma. Few treatment options are available for subjects that present with other phenotypes/endotypes, such as neutrophilic asthma which tends to be refractory to corticosteroids.

Understanding how clinical and laboratory parameters are modulated by environmental factors, such as the dose of allergen exposure, may provide insight into the interrelationship between asthma phenotypes/endotypes and their differing susceptibility to therapeutic intervention. Experimental in vivo models of asthma have been employed in numerous studies to evaluate the clinical efficacy of pharmacological interventions (e.g. glucocorticosteroids, leukotriene receptor antagonists), since many pharmacological agents are functionally active in

pre-clinical models. Immunological interventions (e.g. biologics, allergen-specific immunotherapy), however, frequently cannot be effectively modelled in conventional mouse strains, due to lack of cross-species biological activity. To address our need to evaluate allergen-specific immunological interventions in the pre-clinical setting, we developed a murine model of cat allergen (Fel d 1)-induced allergic airways disease, in mice that express human MHC class II (HLA-DRA/DRB1*0401) in the absence of murine MHC class II(Ito et al., 1996). Expression of human MHC class II molecules allows binding and presentation of human, rather than murine, T cell epitopes and could, for example, be used to model T cell epitope peptide immunotherapy, as we have previously demonstrated (Campbell et al., 2009). During model development, we investigated the effect of cat allergen dose on the elicitation phase of the model. We found that the degree, location and quality of inflammatory responses, and airway hyperresponsiveness (AHR), were determined by intranasal allergen challenge dose. Furthermore, intranasal challenge with high doses of allergen elicited AHR that was refractory to intervention with corticosteroids.

4.3. Methods and Materials

Animals

Six-eight weeks old female HLA-DR4 transgenic mice (B6.129S2-*H2-Ab1*^{tm1Gru} Tg(HLA-DRA/H2-Ea, HLA-DRB1*0401/H2-Eb)1Kito; C57BL/6 background) expressing the human MHC Class II molecule HLA-DR4 were purchased from Taconic (Hudson, NY,USA). These mice lack endogenous MHC presentation molecules but interactions with murine CD4 co-receptors are maintained (Ito et al., 1996). Mice were housed in specific pathogen free conditions and acclimatized for one week prior to experimental use. All experiments were approved by the McMaster University Animal Research Ethics Board, and performed according to the Guide for Humane Use and Care of Laboratory Animals.

Animal allergen and treatment exposures

HLA-DR4 transgenic mice were injected with 2µg (in 200µl) recombinant Fel d1 (rFel d 1) in alum (Au-Gel-S; Serva Electrophoresis), intraperitoneally on Days 0 and 14. Subsequently, mice received a series of intranasal (IN) challenges (25µl) with cat dander extract (CDE; 10µg) to initiate lung inflammation (day 22, 24, and 26). After a rest period of three weeks, the allergic response was recalled to the lungs of mice via IN CDE challenges on day 51 and 52 (Figure 1). Mice were rechallenged with 1, 5, 10, 20 or 30 µg CDE. Sham sensitized mice received saline in alum IP, and saline IN challenges. Sham sensitized/CDE rechallenged mice received saline in alum IP, and saline during the D22, 24 and 26 challenges but CDE (1µg) during the final recall challenges. IN challenges were performed while the animal was under light anesthesia (isoflurane). Mice were sacrificed forty-eight hours post final challenge.

Corticosteroid treatment consisted of administering 0.075 mg of budesonide in saline, intraperitoneally to sensitized mice on day 47, 48, 51, 52 and 53. On days where mice received both allergen challenge and budesonide injections (D51, 52), budesonide was given 2-hours prior to the allergen challenge.

Measurement of airway responsiveness

Airway responsiveness was measured via nebulizing doubling-doses (0, 3.125, 6.25, 12.5, 25, 50 mg/mL) of methacholine (MCh) to mice. Mice were anaesthetized with pentobarbital (Ceva Sante Animale, Leneka, KS; 30 mg/kg) and xylazine (Bayer inc., Toronto, ON; 10 mg/kg). Total respiratory system resistance (R_{RS}) was quantified in tracheotomized mice by the FlexiVent ventilator (SCIREQ, Montreal, Canada) as described previously (Hirota et al., 2006) with an adjusted measurement pattern of 7, 0.4 second perturbations over a 45 second period. Prior to each dose of MCh nebulisation, a six second TLC inflation was performed to

normalize lung function. Once automated breathing had commenced mice received 20mg/kg of the paralytic pancuronium (Santoz, Boucherville, QC, Canada) to prevent respiratory effort. The provocative concentration inducing a 200% increase in baseline resistance (PC_{200}) was calculated by doubling baseline resistance and interpolating the corresponding concentration of methacholine through a linear regression between the neighboring resistance values. In mice failing to double baseline resistance by 50mg/ml MCh, PC_{200} was extrapolated using a linear regression fit to the 25 & 50 mg/mL doses. If the extrapolation generated an implausible value (defined as >75mg/mL), to enable conservative analysis, the highest dose tested (50mg/mL) was assigned as the PC_{200}.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was obtained from tracheostomized mice through two instillations of PBS (250 µL) into the lungs and allowing each too steep for five seconds. Aliquots were retrieved, pooled and centrifuged (150 x *g*, 10 min). BAL supernatants were stored for cytokine quantification and cell pellets resuspended in PBS, enumerated by trypan blue staining and transferred to microscope slides by cytocentrifugation (Cytospin 3 centrifuge; Shandon scientific, Sewickley, PA, USA). BAL cells were cytologically differentiated, following Wright-Geimsa staining by a blinded investigator, into eosinophils, neutrophils, macrophages or lymphocyte based upon staining and morphological criteria. Mice were sacrificed by exsanguination.

Lung histological preparation and cell isolation

Mouse lungs were perfused with PBS containing heparin (10U/ml) and the right lung was harvested, minced into 1x1x1mm pieces and incubated on an orbital shaker for 90 min at 37^{o}C in RPMI-1640 media (Sigma-Aldrich), containing 10% FBS (Invitrogen), 300 units/ml type 1

collagenase (Worthington Biochemical Corporation), 50 units/ml DNase (Sigma-Aldrich) and 1% pen/strep (Invitrogen). The lung digestion was passed through a 70 µm cell strainer (BD Falcon) and centrifuged (300 x *g*, 10 min, 4°C). Lung digest supernatant was stored (-80°C) and the cell pellet suspended in culture medium (RPMI-1640, 10% FBS, 1% Pen/strep, 0.1% 2-mercaptoethanol (Sigma)). Lung digest cells were washed twice using culture media and enumerated using the Countess automated cell counter (Invitrogen).

Following perfusion, the left lung was kept intact and prepared for histology as previously described(Moldaver et al., 2013). Briefly, the left lung was formalin fixed, dehydrated and embedded in paraffin. Three-micron transverse sections of the paraffin embedded left lung lobe were taken. Haematoxylin and Eosin (H&E) and periodic acid Schiff (PAS) stains were performed to determine tissue eosinophilia and goblet cell hyperplasia, respectively. Images of first generation airways were collected at 40*x* or 20*x*, and analyzed for eosinophils per mm^2 and goblet cells per mm, respectively, using a digital image analysis system (Northern Eclipse; Empix Imaging, Inc., Mississauga, ON, Canada) as described previously (DiGiovanni et al., 2009; Hirota et al., 2006).

Flow cytometry

To block Fc receptors, lung cells were incubated with anti-CD16/32 mAb (2.4G2; BD Biosciences, Mississauga, ON) in FACS staining buffer (PBS + 2% fetal calf serum + 0.05% sodium azide). Surface marker phenotyping consisted of incubating Fc blocked cells with pre-titrated antibodies for 30 minutes at 4°C. Antibodies used include PerCp-anti-CD4 (RM4-5), APC-Cy7-anti-CD44 (IM7), APC-Cy7-anti-CD11b(M1/70), PE-anti-Siglec-F (E50-2440), PerCp-anti-CD11c (N418; BD Biosciences), FITC-anti-T1/ST2 (DJ8; MD Biosciences, St Paul, MN, USA), PE-anti-CD69 (H1 2F3), APC-anti-CD103 (2E7; all eBiosciences). Lineage

assessment was performed with PE labelled antibodies for CD4, CD19, CD11b, CD11c, GR1 and NK1.1 (all BD Biosciences). Following surface staining, cells were washed twice with FACS staining buffer and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA).

Cells to be stained for intracellular cytokines were stimulated for 6 hours in culture media supplemented with PMA (20 ng/ml; Sigma-Aldrich), ionomycin (1 mM; Sigma-Aldrich) and brefeldin A (10 µg/ml; Sigma-Aldrich) at 37ºC. Cells were collected, washed in FACS buffer and surface stained then fixed with 4% paraformaldehyde and permeabilized in Perm/Wash buffer (BD biosciences). Permeabilized cells were intracellularly stained with pre-titrated anti-cytokines antibodies diluted in Perm/Wash buffer for 30 min (4°C). Antibodies used consisted of PerCp-cy5.5-anti-IL-4 (11B11; BD Biosciences), APC-anti-IL-5 (TRFK5; BD Biosciences), PE-Cy7-anti-IL-17A (ebio17B7, ebiosciences) and A486-anti-IL-13(eBio13A, ebiosciences). Stained cells were washed twice with Perm/Wash buffer then suspended in FACS staining buffer. Cells were quantified on using a FACSCanto II (Becton Dickinson) and analyzed using Flowjo (TreeStar).

Enzyme-linked immunosorbant assay (ELISA)

Ready-Set-Go! ELISAs (eBiosciences; San Diego, CA, USA) were used to quantify IL-4, -5 and -13 in the BAL. Kits were performed as per manufacturers' instructions.

Statistical analysis

Data presented as mean ± standard error of the mean (SEM). Statistical significance was determined through ANOVA and post-hoc Bonferroni corrected *t*-tests. Comparisons were two-tailed and a p value ≤ 0.05 was considered significant.

4.4. Results

Airway challenge with increasing doses of CDE induces greater airway dysfunction in sensitized mice

Measurement of airway dysfunction has long been a mainstay in asthma diagnosis. Since the 1970s, techniques have evolved to assess airway reactivity to enable diagnosis of airway hyperreactivity to allergens and chemical stimulants(Benson, 1975; Juniper et al., 1978). To assess the development of airway dysfunction induced by intranasal CDE challenge of Fel d 1 sensitized animals (Figure 1), a nebulized methacholine (MCh) challenge was performed. Moreover, to estimate airway sensitivity to MCh, the dose of MCh capable of inducing a doubling in baseline resistance (PC_{200}) was calculated, by linear regression. Unsensitized mice challenged with saline demonstrated minimal increase in R_{RS} upon MCh challenge (Figure 2A) and failed to double baseline airway resistance by the highest tested dose of MCh, 50 mg/mL (Figure 2B). In contrast, challenging Fel d 1 sensitized mice with increasing concentrations of CDE was associated with marked AHR and airway sensitivity. Mice responded to increasing challenge doses of CDE with a clear dose-response effect, as recall challenge with 1µg CDE generated modest AHR (above 25 mg/mL MCh) while a robust curve shift was noted at 5µg CDE recall and above (Figure 2A). This shift is supported by a significantly increased sensitivity to MCh in mice challenged with 5, 10, 20 or 30 µg CDE. Through PC_{200}, a potency order of $30 > 20 > 10 > 5 >> 1 \geq$ SAL was observed, indicating that higher doses of CDE are capable of inducing greater degrees of airway dysfunction. To establish that the observed AHR was due to recalling an established adaptive immune response, unsensitized animals were challenged with CDE (recall challenge only) and found to be unresponsive to MCh, like unsensitized mice challenged with saline (Figure 2A and B).

Peripheral airway resistance has been demonstrated to be exquisitely sensitive to underlying disease activity, commonly revealing exacerbations that other methods fail to identify (Little et al., 1978). We sought to further examine the lung mechanics associated with the observed increase in R_{RS}, by analyzing tissue damping (G) and tissue elastance (H), which are measures of energy dissipation and conservation by lung tissue, respectively (Bates, 2009). Analysis of changes in G and H reveal that challenge with increasing doses of CDE is associated with increasingly dysfunctional lungs. Fel d 1 sensitized mice, challenged with CDE demonstrated increased tissue damping and elastance, with a potency order similar to that observed for R_{RS} (Figure 2C and E). Examination of the dose of MCh that doubles baseline G and H, revealed a similar potency order to PC_{200}, indicating that higher challenge doses significantly affected peripheral airways (Figure 2D and F). The observed differential increase in the degree of tissue damping and elastance is indicative of increased tissue hysteresivity, calculated as the ratio of G to H and is indicative of increased energy loss. The potency order established during examination of $R_{RS,}$ G and H ($30 > 20 > 10 > 5 >> 1 \geq$ SAL) was reaffirmed during examination of tissue hysteresivity (data not shown). Interestingly, the highest tested dose of MCh (50 mg/ml) was frequently incapable of doubling baseline tissue damping or elastance in control mice, whereas sensitized mice consistently doubled both G & H by 5-20 mg/mL MCh (Figure 2D & F).

Allergen challenge dose determines the nature and location of inflammation

The induction of AHR, by allergen challenge, has been associated with an influx of cells into the lumen of the lung (Wardlaw et al., 1988). Specifically, recruitment of eosinophils to both the airway lumen (de Monchy et al., 1985; Metzger et al., 1987) and lung tissue (Bousquet et al., 1990) is a cardinal feature of allergic asthma. To determine if our model mirrored clinical

allergic asthma, we sought to characterize the allergen challenge induced inflammatory lung infiltrate. Forty-eight hours post final allergen challenge, mice were sacrificed and cells from BAL were enumerated. The intranasal challenge of sensitized animals with 5µg of CDE doubled the number of cells in the BAL, compared to unsensitized control animals (Figure 3A; $p<0.05$). Allergen doses above and below 5µg showed smaller elevations in total cell number creating a bell-shaped dose response curve. BAL cells were differentiated into eosinophils, neutrophils, lymphocytes and macrophages, by Wright Giemsa staining and morphological features. CDE challenge with 1, 5 or 10 µg allergen induced robust airway eosinophilia in Fel d 1 sensitized animals compared to both saline challenged and unsensitized/CDE challenged mice (Figure 3A). The accumulation of lymphocytes and macrophages induced by challenge with increasing doses of CDE varied little. Interestingly, cat sensitized mice challenged with high doses of CDE (20 and 30µg) failed to develop BAL eosinophilia, but rather, developed BAL neutrophilia ($p<0.05$); these same doses of allergen were also associated with the most striking increases in measurements of AHR and related lung physiology outcomes (Figure 2).

Next, we assessed the accumulation of eosinophils in the airway tissues after allergen challenge. Examination of histologically identified tissue eosinophils revealed that challenge of cat sensitized mice with increasing doses of CDE (>10µg) induced significant accumulation of eosinophils (Figure 3B). Therefore, the magnitude of the allergen challenge dose appears to control how the Th2 response manifests, as evidenced by an inverse relationship between BAL and tissue eosinophilia.

In addition to airway eosinophilia, a common clinical symptom of allergic asthma is the induction of mucus production and expansion of goblet cells during exacerbations (Aikawa et al., 1992; OrdoÑEz et al., 2001). Cat sensitized animals challenged with CDE underwent significant

goblet cell hyperplasia, as assessed by PAS staining of the major airways (Figure 3C). Goblet cells were rare in CDE challenged/unsensitized animals and saline challenged, unsensitized animals revealing that goblet cell hyperplasia was induced by a memory response to cat allergen. Although no significant differences in goblet cell numbers were detected between the rechallenge doses, the lower doses of 1μg and 5μg appeared to be associated with greater goblet cell numbers (Figure 3C). Muc5b and Muc4 are expressed by lung epithelial cells and have been implicated as regulators of eosinophil accumulation through their ability to induce eosinophil apoptosis by ligating Sialic acid-Binding, immunoglobulin-like F (Siglec-F) (Kiwamoto et al., 2015). Therefore, Muc4 and 5b levels in the BAL were quantified to determine whether allergen dose was differentially inducing eosinophil regulation mechanisms. We observed that the eosinophil regulating mucins, Muc4 and 5b were readily detectable by ELISA in the BAL of unsensitized/saline challenged mice (Figure 3D). Unsensitized mice exposed to CDE presented with significantly reduced levels of Muc4 and 5b, compared to challenge with saline. Surprisingly, this reduction was not present in cat sensitized mice, as challenge with CDE, regardless of dose, induced levels of Muc4/5B similar to levels observed in unsensitised, sham challenged animals. Therefore, it appears that Muc4/5b production appears to be relatively high at homeostasis as unsensitised mice, sham challenged mice had relatively high Muc4/5b in the BAL, despite low numbers of goblet cells. Allergen sensitized animals responding to allergen challenge with goblet cell hyperplasia readily produced Muc4/5b. It appears as though production of Muc4/5B was downregulated upon de novo exposure to CDE, in the sham sensitized animals.

AHR correlates with eosinophil accumulation in airway tissues, but not the lumen

In Fel d 1-sensitized animals, challenge with increasing doses of CDE led to increased AHR and tissue eosinophilia, but reduced BAL eosinophilia. Therefore, cursory analyses suggested that AHR may correlate with tissue eosinophils but not BAL eosinophil counts. This relationship was formally examined through mathematic modeling. A linear regression analysis between the severity of AHR, quantified using PC_{20}, and the number of eosinophils in the tissue (Figure 4A) revealed a strong, significant relationship (r^2=0.7) wherein increasing numbers of tissue eosinophils correlated with heightened airway dysfunction (i.e. a lower PC_{200}). A similar analysis seeking to define the relationship between BAL cellular infiltrate and degree of airway dysfunction revealed that BAL eosinophilia (Figure 4B, solid line) did not correlate with AHR, but that BAL neutrophilia did correlate with AHR (Figure 4C). In summary, tissue eosinophils and BAL neutrophils correlated with AHR, while BAL eosinophils did not. It should be noted that a weak trend towards a positive relationship between reduced airway dysfunction and increasing number of BAL eosinophils was noted, when control mice animals were removed from the analysis (Figure 4B, dashed line). Figure 4D depicts the effect of recall challenge dose on the inflammatory infiltrate. Increasing the CDE challenge caused a bell-shaped dose-response induction of BAL eosinophilia that peaked at 5µg recall and waned by 20µg CDE. In contrast, BAL neutrophilia and tissue eosinophilia demonstrated linear dose-response curves, increasing with increasing challenge dose. Indeed, analyzing the BAL cell differentials as a ratio of eosinophils to neutrophils (Figure 4E) revealed that low dose recall challenge with 1 or 5 µg CDE induced a primarily eosinophilic BAL, while higher challenge doses (10, 20, 30µg CDE) induce a progressively more mixed neutrophilic/eosinophilic infiltrate. Thus, in this model of cat allergy, AHR severity was approximated by tissue eosinophil counts and BAL neutrophilia, but not BAL eosinophilia.

Sensitized HLA DR4 transgenic mice mount a robust Th2 polarized T-cell response to challenge with cat dander

Challenging CDE sensitized mice with 10µg of CDE induced all salient features of allergic airways disease (lumenal, tissue eosinophilia and airway dysfunction), while lower doses failed to some of these features and higher doses induced neutrophilia. As we sought to develop a murine model of CDE induced allergic airways disease, that would enable study of allergen specific immunotherapies, we characterized the T cell response induced by allergen challenge with 10µg of CDE.

We performed flow cytometry on lung derived cells and examined the BAL for the presence of inflammatory cytokines. To verify histological assessment, lung tissue eosinophils were quantified by flow cytometric analysis of mouse lungs, after allergen challenge (eosinophils identified as Siglec-$F^{+}CD11c^{-}$) (Tateno et al., 2005). Challenging Fel d 1-sensitized mice with 10µg of CDE induced a significant, threefold increase in tissue eosinophilia, compared to unsensitized mice challenged with saline and unsensitized mice challenged with CDE (Figure 5A), thereby corroborating previous histological observations. Eosinophil recruitment is typically associated with an ongoing Th2 immune response. To confirm that Th2 polarized T cells were induced by challenge with 10µg of CDE, the surface expression of T1/ST2 (IL-33R) on T-cells was quantified, as T1/ST2 is preferentially expressed on Th2 T-cells (Löhning et al., 1998). Neither unsensitized and saline challenged animals nor unsensitized, CDE challenged animals had many CD4+T1ST2+ cells. In contrast, CDE challenge of Fel d 1 sensitized animals induced a significant fivefold expansion of Th2 polarized T cells (Figure 5B).

Analysis of CD4+ cells for markers of tissue resident memory T-cells (T_{RM}), by flow cytometry, revealed a marked increase in T_{RM}-like (CD4+CD63+CD103+) T-cells following

allergen challenge (Figure 5C; $p<0.05$), that was not present in sham challenged animals. Furthermore, combined analysis of CD69, CD103 and T1ST2 demonstrated accumulation of a Th2 polarized T_{RM}-like population of CD4+ T-cells in response to allergen challenge (Figure 5C; $p<0.05$).

Type 2 innate lymphoid cells (ILC2s) are a population of tissue resident cells that respond to tissue injury by proliferating and secreting large quantities of IL -5 and -13 (Bonilla et al., 2012; Koyasu and Moro, 2011; Lambrecht and Hammad, 2013; Moro et al., 2010). To determine if cat dander challenge increased the frequency of ILC2s, lung derived cells were obtained from mice after challenge and enumerated as CD45+ Lin-Sca-1+c-kit+ T1/ST2+. Fel d 1 sensitized animals challenged with 10µg CDE had a fivefold induction in ILC2s compared to unsensitized mice challenged with saline (Figure 5D). Due to cell number limitations, ILC2 enumeration was not performed in unsensitized mice challenged with allergen.

The allergic response is characterized by the induction of Type 2 cytokines, such as IL-4, -5 and -13. Th2 cytokines were quantified in the BAL of animals by ELISA. Sensitized mice, challenged with 10µg CDE had significantly elevated levels of IL-4, -5 and -13 in BAL compared to both unsensitized mice challenged with saline and unsensitized mice challenged with allergen (Figure 5E). We sought to define the cells producing these cytokines through flow cytometric analysis of lung derived cells. IL-17 production by lung cells was also investigated, as neutrophil recruitment has been linked to an ongoing Th17 response. In sensitized mice, allergen challenge significantly increased the number of cells in the lungs producing IL-4, -13 and -17 (Figure 5F). A trend indicating increased IL-5 production was also observed, but failed to achieve significance ($p=0.0567$). Analysis of CD4+ T-cells and memory T-cells ($CD44^{hi}$) (Gerberick et al., 1997) revealed that allergen challenge preferentially induced T-cell expression

of IL-4, -5, -13 and -17 in sensitized animals, and that cytokine producing T-cells were predominantly memory T-cells (Figure 5G).

Systemic corticosteroid therapy fails to attenuate AHR induced by high-dose CDE challenge

Corticosteroids are currently the gold standard, first line therapy for the treatment of asthma(Barnes, 2006). Resistance to corticosteroids and severe asthma are often associated with neutrophilic sputum (Wenzel et al., 1999; Wenzel et al., 1997). As Fel d 1-sensitized mice challenged with high doses of CDE had severe airway dysfunction accompanied by neutrophilic BAL, we set out to examine whether these mice were also refractory to treatment with corticosteroids.

We administered high doses of budesonide, systemically, prior to and concurrently with high-dose CDE challenge (Figure 6A). Sham treated, Fel d 1-sensitized mice challenged with 20 or 30 µg of CDE developed airway dysfunction, as demonstrated by increased sensitivity to MCh (Figure 6B; $p<0.05$). Corticosteroid therapy failed to attenuate the onset of airway dysfunction resulting from challenge with either 20 or 30 µg CDE (Figure 6B). Budesonide therapy attenuated both BAL eosinophilia (Figure 6C) and neutrophilia (Figure 6D) in animals challenged with CDE. Histological analysis of the lungs of sham- and budesonide-treated animals revealed that budesonide treatment also significantly attenuated tissue eosinophilia (Figure 6E). Thus, systemic corticosteroid therapy failed to ameliorate airway dysfunction induced by high-dose allergen challenge, but did prevent the accumulation of inflammatory cells. As a positive control, Fel d 1-sensitized mice challenged were given high dose budesonide, systemically, prior to and concurrently with low-dose CDE challenge. Budesonide therapy significantly attenuated AHR in Fel d 1-sensitized mice challenged with 10 µg CDE (Figure S1), when compared to a historical control group (group: 10 µg recall).

4.5. Discussion

Asthma causes a significant healthcare burden globally, in part due its heterogeneity (Agache et al., 2012). As diagnostic tools develop and allow the evaluation of asthma endotypes, novel therapies will be required to treat the underlying disease mechanisms. In the present study we describe the generation of a model of cat dander induced allergic airways disease that can mimic the heterogeneity seen in clinical asthma, by adjusting the final allergen challenge dose.

In our model, allergen dose exquisitely controlled the induced disease phenotype. Challenging Fel d 1-sensitized HLA-DR4 transgenic mice with low dose CDE (1, 5, 10 µg) induced a Th2 polarized model of allergic airways disease typified by eosinophil accumulation in the BAL and lung tissues, IL-4, -5 and-13 expression by T-cells, accumulation of ILC2s, and onset of AHR. In contrast, high dose CDE challenge (20, 30 µg) induced a different disease profile, one that is reminiscent of severe, steroid resistant asthma due to the neutrophilic inflammation and severe airway dysfunction. Our data are in agreement with those of Corry and colleagues (Porter et al., 2011), who reported that low-dose exposures to fungi gave rise to eosinophilic, Th2 polarized lung inflammation, whereas repeated high dose exposure converted the response to a neutrophilic/Th1 predominant inflammation. Our data corroborate these findings and lend credence to the view that Th2 and Th1/17 mediated forms of asthma may not be mutually exclusive, but rather, representative of the extreme ends of a spectrum of inflammatory responses that are controlled by the allergen exposure dose (Lambrecht and Hammad, 2015). While the importance of IL-13 and Th2 immunity in asthma have long been acknowledged, the importance of Th1/Th17 immunity during asthma pathogenesis (Hansen et al., 1999; Lambrecht and Hammad, 2015; Li et al., 2013) is now being recognized. This

phenomenon is highlighted clinically in asthmatic individuals who present with sputum neutrophilia and steroid resistant symptoms (Green et al., 2002).

Correlation of clinical phenotypes with improved mechanistic understanding of pathogenesis in different forms of asthma has led to the concept of asthma endotypes. Mechanistic insights have been gleaned through a variety of techniques including the examination of cells in sputum and blood, measures of airway dysfunction, genetic polymorphisms and circulating protein concentrations. Obtaining lung tissue samples from asthmatics, to assess tissue cellular infiltrate is too invasive to be routinely performed, thereby enforcing the reliance on surrogate measures of disease. Our data demonstrate the importance of understanding the immunological milieu within lung tissues, as BAL cell differentials were commonly not reflective of the degree of inflammation in the tissue. Moreover, tissue inflammation, but not BAL eosinophilia, correlated with the presence of airway dysfunction highlighting the importance of understanding both lung lumenal and tissue inflammation together. Interestingly, other experimental studies have also shown an association between tissue (but not lumenal) eosinophils and AHR. These studies identified IL-4(Cohn et al., 1998), ICAM-2 (Gerwin et al., 1999) and CD49b (Henderson et al., 1997) as regulators of eosinophil migration out of the tissues and into the airway lumen. Thus, it is conceivable that individuals with asthma could present without sputum eosinophilia (and thus be missed by laboratory sputum screening protocols) but have persistent lung tissue eosinophilia and AHR requiring clinical intervention. Furthermore, data generated by challenge with high dose CDE revealed the possibility that clinical presentation of 'neutrophilic' asthma may be accompanied by underlying tissue eosinophilia. Together, these observations may contribute to an improved understanding of the spectrum of disease in human subjects.

Corticosteroids are a major component of asthma management, making steroid resistant asthma difficult to manage. Research into therapies that can effectively reduce symptoms of disease in these patients are urgently needed. We examined whether the neutrophilic airway disease caused by challenge with high-doses of CDE was resistant to steroid therapy. Budesonide, like most corticosteroid therapies, can successfully prevent the onset of airway dysfunction and attenuate airway dysfunction when given concurrently with allergen (Green et al., 2002); however, delayed therapy cannot effectively treat airway dysfunction (Southam et al., 2008). Thus, we elected to commence treatment before the final allergen challenges to ensure that animals were systemically under the influence of the corticosteroid treatment prior to exposure to allergen. In contrast to previous research using budesonide to treat ovalbumin induced allergic airways disease in mice (Southam et al., 2008), we demonstrated that AHR induced by high doses of allergen was not reduced by prophylactic and concurrent corticosteroid therapy. Duechs *et al*., (Duechs et al., 2014), recently also developed a murine model of allergic airways disease that is resistant to corticosteroid therapy. In their model, mice were sensitized to ovalbumin, cockroach and house dust mite, and animals challenged with all three allergens together developed robust AHR, airway remodeling and inflammation that was resistant to therapeutic intervention with dexamethasone. The two models differ in two significant manners, (1) the relative abundance of airway neutrophils in our high dose challenges, and (2) a severe-asthma-like phenotype after sensitization and challenge with a single allergen, compared to three allergens. Thus, different experimental protocols may model different aspects of steroid-resistant, neutrophilic asthma.

Budesonide treatment reduced measures of inflammation (eosinophils, neutrophils) but was unable to completely resolve AHR associated with high dose allergen challenge. This

observation calls into question the causal association between tissue eosinophils and the development of AHR identified in this and other studies. Thus, how budesonide was able to attenuate inflammatory cell infiltration but not AHR in a brief model of allergen exposure, is intriguing and highlights the need for further research.

Much of the data presented here details airway dysfunction through assessment of AHR. It has been suggested that resistance measures may be skewed in mice suffering from severe lung disease as it is possible that the tissue component measures may dominate airway components (Larcombe et al., 2013). The dose-response effect and agreement between our airway and peripheral tissue mechanics data reflect that, if any such skewing is occurring, the effects are limited and likely inconsequential to the study's conclusions. Clinically, the provocative dose inducing a 20% drop in forced expiratory volume in one second (FEV_1) (PC_{20}) is a very important diagnostic tool. PC_{200} represents a surrogate diagnostic in mice, where the measurement of FEV_1 is impractical. In our model, PC_{200} proved to be a sensitive measure of airway dysfunction, as 50 mg/ml nebulized MCh was largely incapable of doubling baseline R_{RS}, tissue damping or elastance in control mice, whereas 5-20 mg/mL MCh consistently doubled all three measures of airway function in allergic mice.

Together, our data indicate that the dose of allergen used to recall the asthmatic phenotype may control the type of disease that manifests. In addition to this, we defined a protocol that establishes a typical Th2 polarized model of allergic airways disease. Challenging cat sensitized mice with a low dose of CDE (10µg) induced AHR, eosinophilia, Th2 cytokine accumulation in the BAL and Th2 cytokine production by memory T-cells. Interestingly, investigation into the memory T-cell population in the lungs revealed a putative, T_{RM}-like subset of T-cells. Resident memory T cells are cells that typically reside in peripheral tissues and

represent the first line of adaptive memory responses. While tissue resident memory T-cells (T_{RM}) are typically studied for their rapid anti-viral capacity, IL-4 and IL-13 producing T_{RM} have been identified in the lungs (Purwar et al., 2011). Therefore, it is plausible that T_{RM} may be involved allergic responses. As T_{RM} are best understood as rapid mediators of anti-viral immunity, markers for CD8 T cells are far better established than are markers for CD4+ T-cells. Extensive research has established the surface markers CD69 and CD103 as markers of T_{RM} CD8 T-cells (Masopust and Picker, 2012) as these molecules inhibit S1P dependent tissue egress (Shiow et al., 2006) and mediate interactions with the epithelial ligand E-cadherin (El-Asady et al., 2005), respectively. As we utilized surface markers known for CD8 T-cells (Masopust and Picker, 2012), and not markers defined for CD4 T-cells, the work presented does not rigorously demonstrate a role for CD4+T_{RM} cells in our model of allergic asthma. However, as the surface markers for CD4+ T_{RM} cells are unknown, we believe that the use of established CD8+T_{RM} cell markers is justifiable. These observations will encourage research into a potential role for Th2 polarized CD4+ T_{RM} cells as effectors of asthmatic responses.

To conclude, we have demonstrated that humanized transgenic mice expressing the human MHC Class II molecule HLA DR4 can respond to allergen challenge with a phenotype dependent upon the challenge dose. Through the use of a low allergen challenge dose we have described a model of asthma that readily replicates cardinal features of Th2 dominant allergic asthma. Through the use of a high-dose allergen challenge we report the development of a model of asthma characterized by neutrophilic BAL and steroid insensitive AHR. Expression of human MHC class II molecules makes this model suitable for evaluation of both pharmacological and immunological (T cell-targeted) interventions.

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4.6. Figures

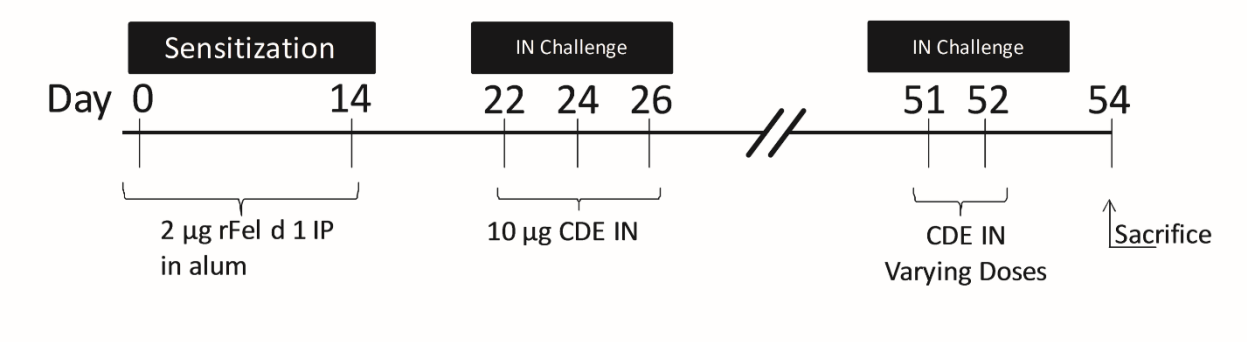
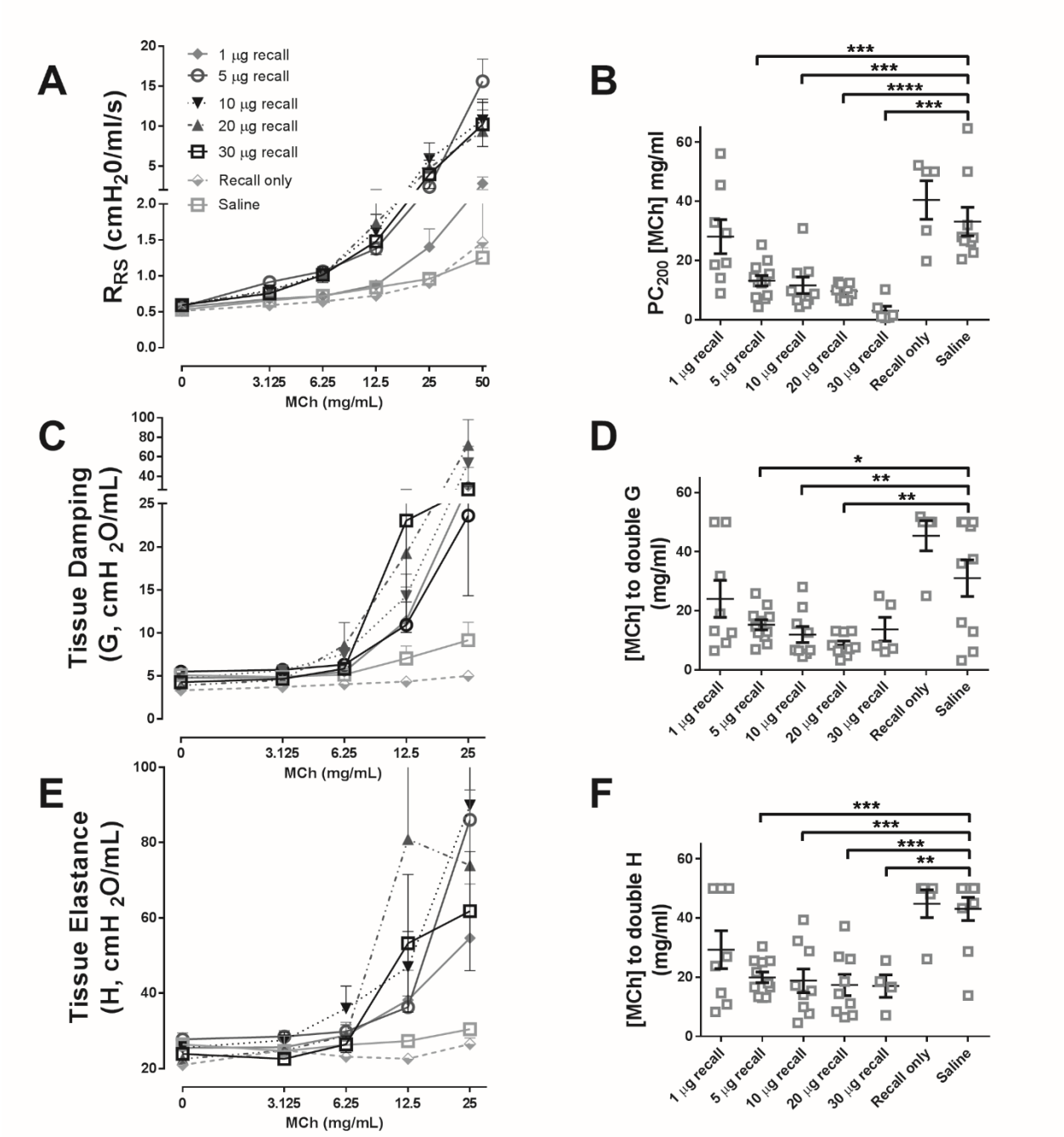


Figure 1. Fel d 1 sensitization and cat dander extract challenge protocol. Mice were sensitized as indicated and rechallenged with CDE on day 51/52 with 0, 1, 5, 10, 20 or 30µg CDE. IP, intraperitoneal; IN, intranasal.



Group	1μg Recall	5μg Recall	10μg Recall	20μg Recall	30μg Recall	Recall only	Saline
Sensitization	+	+	+	+	+	-	-
IN CDE (d22-26)	+	+	+	+	+	-	-
IN CDE (d51-52)	1μg CDE	5μg CDE	10μg CDE	20μg CDE	30μg CDE	1μg CDE	Saline

Figure 2. Challenge with CDE induces airway hyperresponsiveness and airway dysfunction. A) total respiratory resistance (R_{RS}), C) Tissue damping (G) and E) Tissue elastance (H) in response to increasing doses of methacholine (MCh). The provocative dose inducing a doubling in baseline airway: B) resistance, D) tissue damping or F) tissue elastance.

HLA DR4 transgenic mice sensitized to cat as previously described, and challenged intranasally with CDE (1, 5, 10, 20 or 30 µg). Control animals were sham sensitized and challenged with saline (Saline), or cat dander (1µg; group: Recall only). Data presented as individual points & mean ± SEM (n=5-9; pooled from two independent experiments). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$; ****$p<0.0001$ as determined by an ANOVA and post-hoc Bonferroni corrected t-tests.

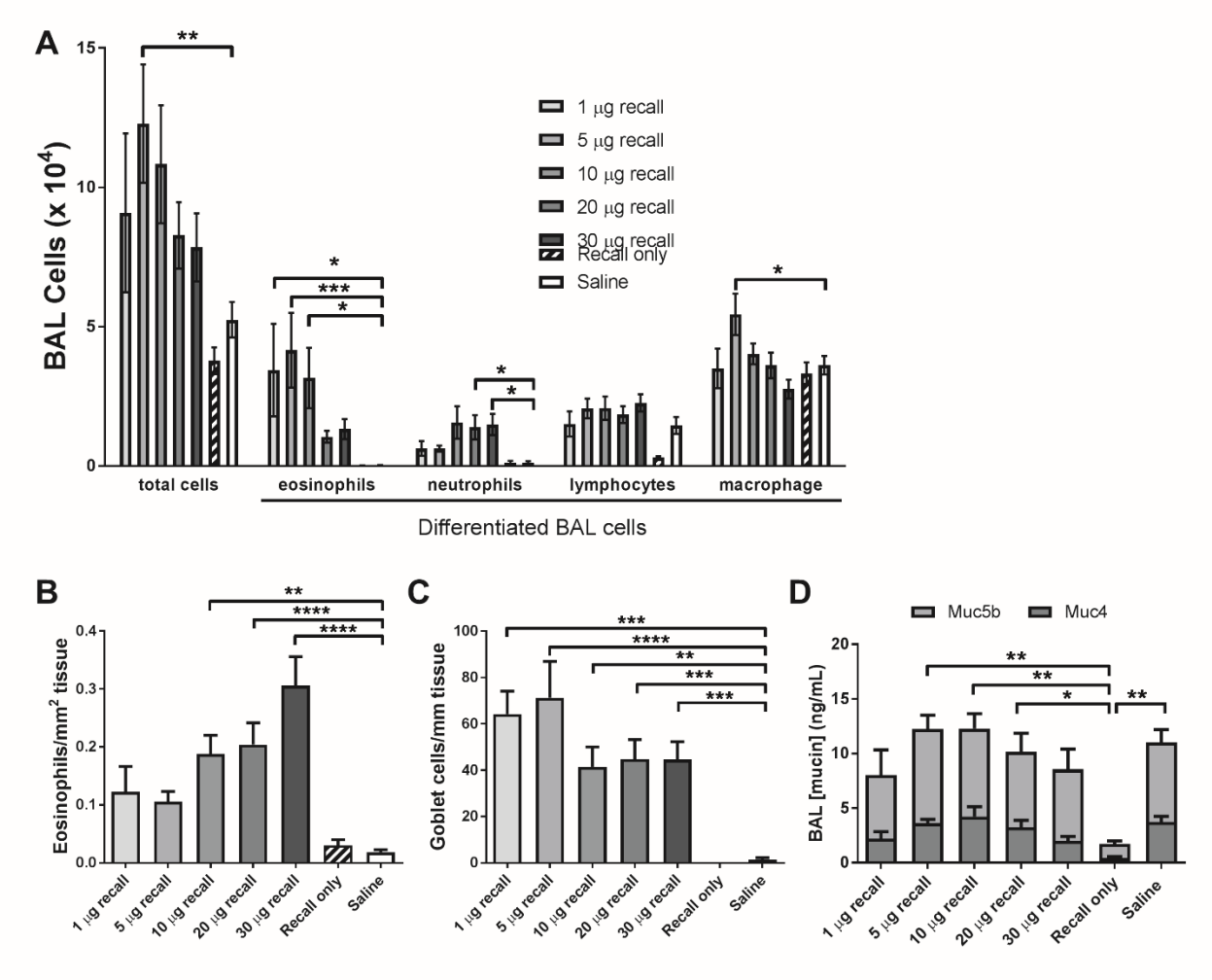


Figure 3. The magnitude of Cat Dander challenge dose controls the resultant inflammatory phenotype. HLA DR4 transgenic mice sensitized to cat were challenged with CDE (1, 5, 10, 20 or 30 µg) and bronchoalveolar lavage (BAL) cells were enumerated and differentiated as described in Methods (A). Lungs were collected and stained with Haematoxylin and Eosin or periodic acid Schiff for enumeration of eosinophils (B) and goblet cells (C), respectively. Muc5b and Muc4 BAL concentrations were determined by ELISA. Data presented as mean ± SEM (n=5-19/group; pooled data from three independent experiments). *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$; ****$p<0.0001$ as determined by an ANOVA and post-hoc Bonferroni corrected t-tests.

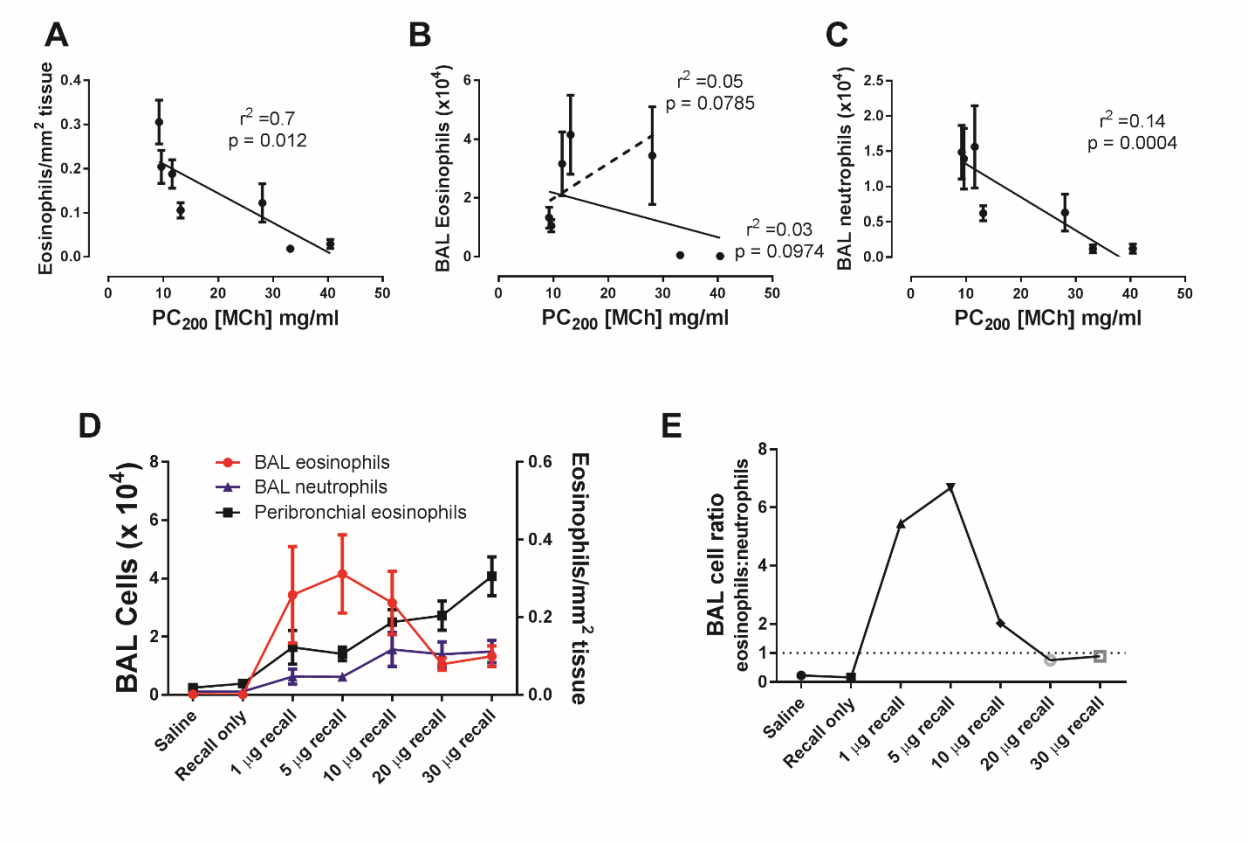


Figure 4. Tissue, not BAL eosinophil numbers correlate with airway hypersensitivity. Tissue eosinophils (A), BAL eosinophils (B) and BAL neutrophils (C) graphed versus the observed airway hypersensitivity, as estimated by PC_{200}. A linear regression was performed across all groups (solid line), or just within sensitized animals (dashed line) and the resultant goodness-of-fit (R^2) is displayed. $p < 0.05$ indicative of a significantly non-zero slope. Data presented as (X, Y) PC_{200} (n=5-19/group; pooled data from three independent experiments), cells ± SEM. BAL inflammatory infiltrate (graphed on the left y-axis) and tissue eosinophil accumulation (graphed on the right y-axis) are overlaid, and graphed versus the challenge dose (D). The preferential induction of neutrophils into the BAL by increasing challenge dose is depicted through the BAL neutrophil:eosinophil ratio (E).

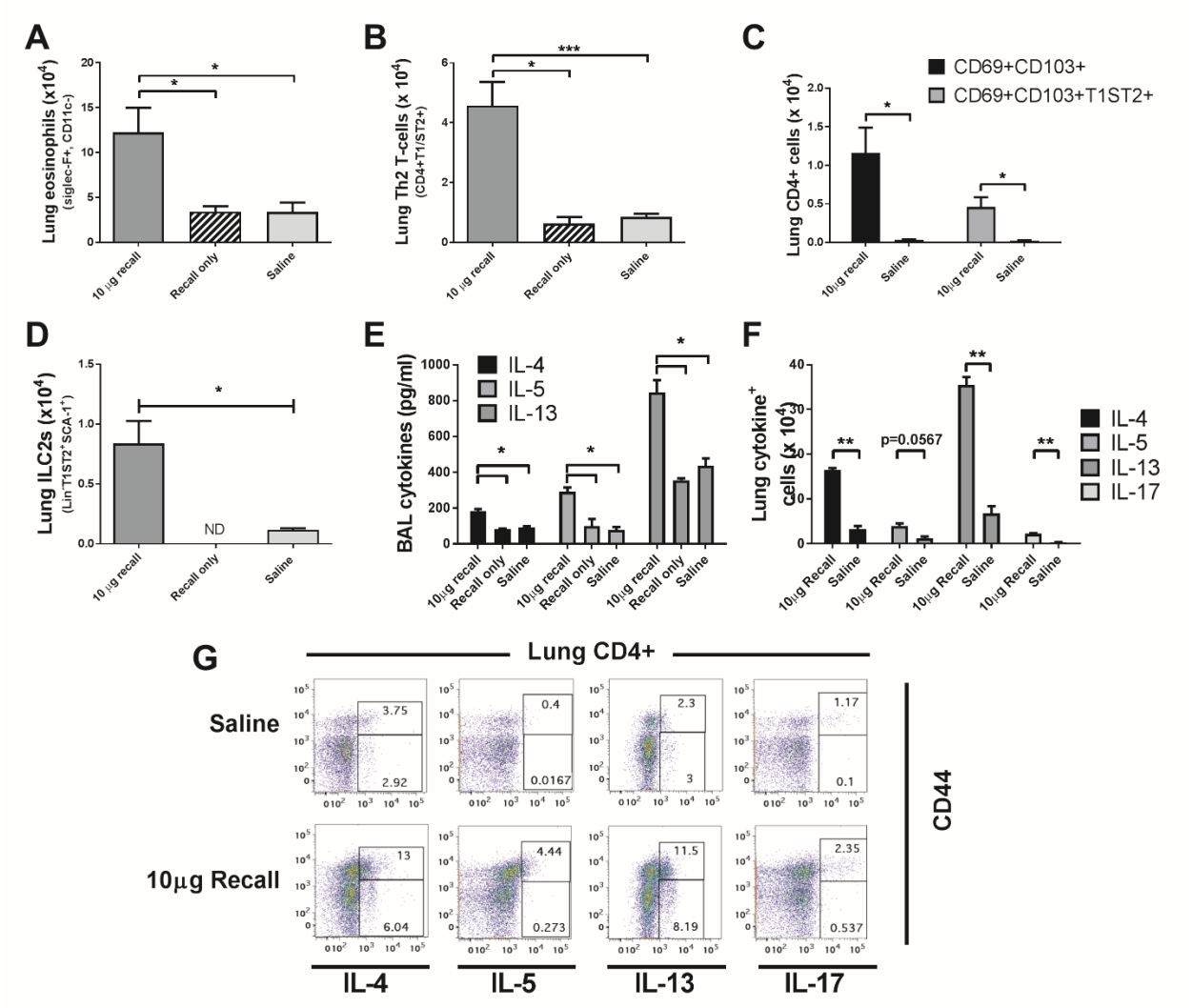


Figure 5. Challenge with 10µg CDE induces eosinophils, ILC2s and Th2 cytokines. The lungs of Fel d 1 sensitized, CDE challenged (10µg) HLA DR4 transgenic mice were examined for the presence of (A) eosinophils (Siglec-F+CD11c-), (B) Th2 T-cells (CD4+T1ST2+), (C) T_{RM}-like and Th2 polarized T_{RM}-like cells, (D) type 2 innate like lymphocytes (CD45+ Lin- Sca1+c-kit+ T1/ST2+). (E) BAL Th2 cytokines IL-4, IL-5 and IL-13 quantified by ELISA. Production of IL-4, -5, -13 and -17 by lung derived cells was assessed by flow cytometry (F). Lung CD4+ cells were assessed for their inflammatory cytokine production (IL-4, -5, -13 and -17) and memory status (CD44 expression) following PMA and ionomycin stimulation (G). Data presented as mean ± SEM (n=5-10/group; pooled data from two independent experiments). Representative flow plots present contour plots and average cell numbers within the indicated gate. *, $p < 0.05$; *** $p < 0.001$ as determined by an ANOVA and post-hoc Bonferroni corrected t-tests. ND = not determined due to insufficient cell numbers.

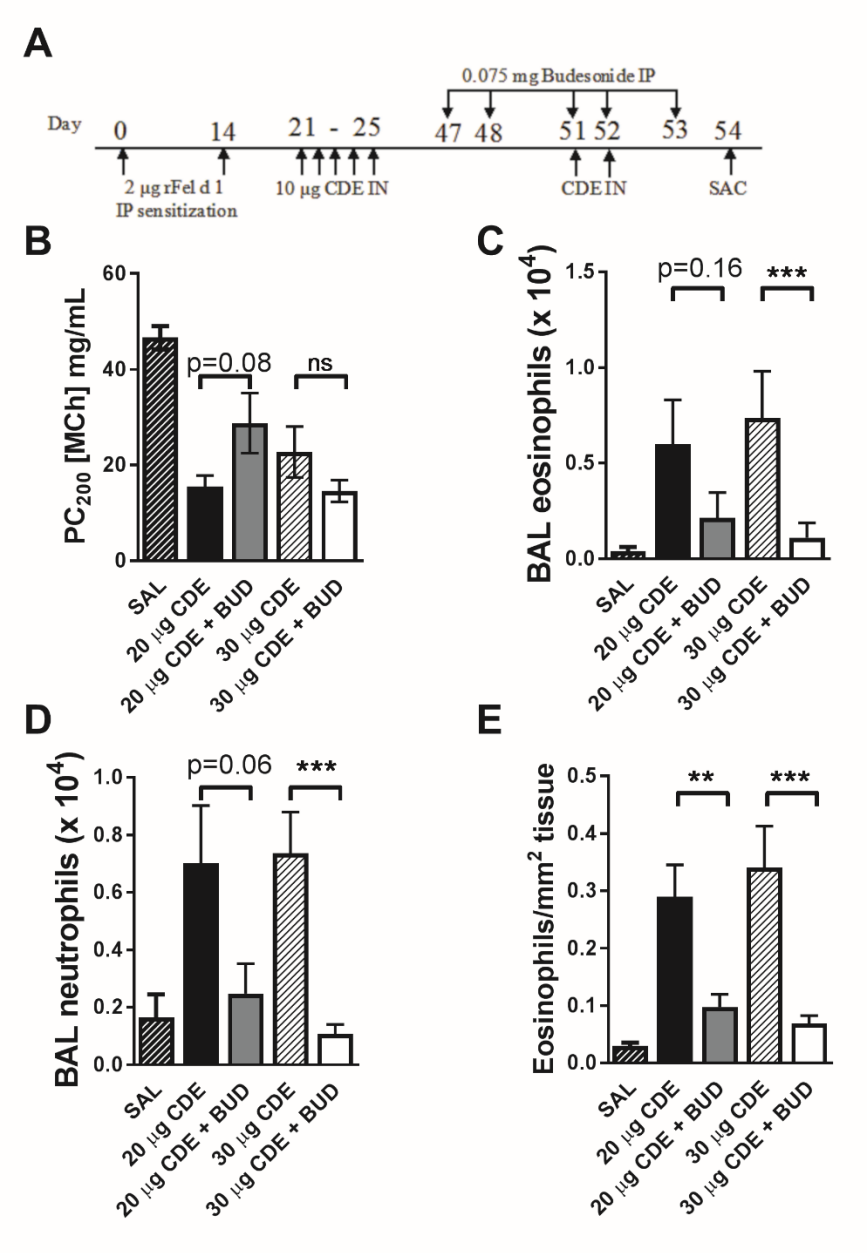
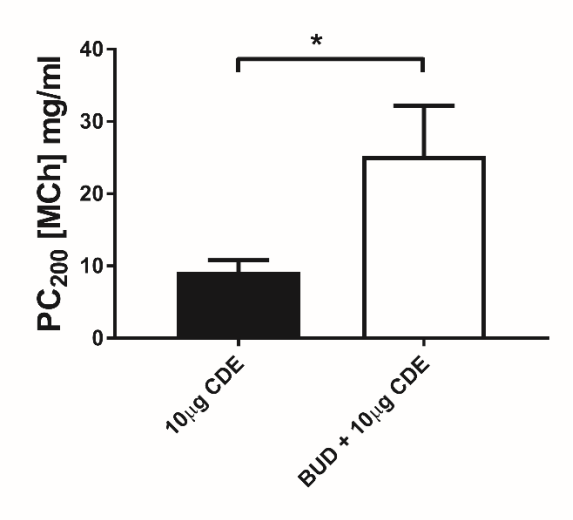


Figure 6. Budesonide therapy fails to attenuate high-dose CDE induced AHR. (A) Mice were sensitized to Fel d 1 & cat, then challenged with 20 or 30µg CDE in the presence of sham therapy (groups denoted as 20 or 30µg CDE) or with systemic budesonide therapy (treated groups denoted as challenge dose '+BUD'). (B) A nebulized MCh-challenge was performed, and the provocative dose inducing a doubling in baseline airway resistance determined. The BAL of challenged animals was assessed for eosinophils (C) and neutrophils (D). Tissue eosinophils (E) were assessed by Haematoxylin and Eosin staining of histologically prepared lungs. Data presented as individual points & mean ± SEM (n=9-10/group; pooled from two independent experiments). ns= $p>0.05$; **, $p < 0.01$; *** $p <0.001$ as determined by an ANOVA and post-hoc Bonferroni corrected t-tests



Supplementary Figure 1. Budesonide therapy fails attenuates low-dose CDE induced AHR. Mice were sensitized to Fel d 1 & cat, then challenged with 10µg CDE while receiving systemic budesonide therapy (group: BUD+10µg CDE). To enable comparison, historical data for Fel d 1 sensitized mice challenged with 10µg CDE is also shown (group: 10µg CDE). Data presented as mean ± SEM (n=5-10/group; pooled from two independent experiments). *, $p < 0.05$; as determined by t-test.

Chapter 5: Epicutaneous Sensitization to Cat Dander

5. Declaration of Academic Achievement

Chapter 5. Epicutaneous sensitization to cat dander

D. M. Moldaver[1,2], T. Singh[1,2], I. Nayve[1,2], J. Wattie[1,2], M. van Hage[3], Kwok, W[4], M. Larché[1,2]. C. Rudulier[1 2]

- The following study is to be submitted to Allergy or Clinical and Experimental Allergy, upon completion of follow-up experiments.
- Rights will be retained at submission.

Daniel Moldaver's contributions

As primary author, I was responsible for conceiving, planning, managing and contributing to all aspects of the study. I treated animals, performed mouse dissections, tracheostomies and MCh challenges, fixed tissues, performed histology, prepared cells for flow cytometry and proliferation assays, performed flow cytometry and ELISAs. C. Rudulier contributed to study design, and assisted with cell processing & flow cytometry. T. Singh and I. Nayve assisted with the blinded quantification of histology. J. Wattie assisted with mouse tracheostomies, and methacholine challenges. M. van Hage provided the cat dander extract. W. Kwok provided tetramers. Analyses, statistics and figure generation were performed by myself. Manuscript preparation was performed by me, with guidance from C. Rudulier and M Larché.

Epicutaneous sensitization to cat dander

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5.1. Introduction

Cutaneous exposure to an allergen can lead to allergic sensitization. In mice, epicutaneous exposure to ovalbumin (OVA) can induce atopic dermatitis, allergic airways disease[1] and anaphylaxis[2]. Similarly, application of aeroallergens (house dust mite[3], *Aspergillus fumigatus*[4]), or a food allergen (Peanut[5, 6]) to a patch of disrupted skin on a mouse induces allergic sensitization. Epicutaneous sensitization to an allergen occurs as the result of encountering allergen at a site with impaired epidermal barrier integrity[7,5]. In mice, barrier impairment is typically obtained through mechanical disruption with either tape stripping, or shaving. In humans, and in transgenic animals, genetic disorders giving rise to barrier impairment have been linked to epicutaneous sensitization[8-11].

Cat dander represents one of the most common indoor aeroallergens, with approximately 10-20% of atopic individuals being sensitive to cat[12, 13]. Cat allergy can manifest symptoms ranging from mild rhinoconjunctivitis to severe asthma. Moreover, allergic sensitization to cats (the presence of cat specific IgE) is a major risk factor for the diagnosis of asthma[14, 15]. While six cat allergens have been identified, Fel d 1 is the dominant allergen being recognized by IgE from 88% of cat allergic individuals[16] and T-cells from nearly all cat allergics individuals[17].

High prevalence of cat ownership has led to Fel d 1 being a nearly ubiquitous environmental allergen. For instance, high levels of Fel d 1 (1000ng/g dust) were found in 27-35% of dust samples taken from mattresses in houses that have never owned a cat[18]. High levels of Fel d 1 have been found on chairs in schools (953ng/g dust)[19], and even in allergen-avoidance day-cares (640ng/g dust)[20]. Thus, regardless of pet-ownership, children and adults encounter a large amount of Fel d 1 routinely. While some studies have linked cat-ownership with a risk for sensitization[21], numerous studies have failed to associate childhood exposure to cats with an

increased risk for sensitization[12, 14, 22, 23]. These differential findings could, perhaps, be attributed to the identification of a 'modified-Th2 response' wherein exposure to high-levels of Fel d 1 protects against the development of cat allergy[23]. The dominant route of allergen exposure, airways versus skin, however, has not been demonstrated. Given the preponderance of settled Fel d 1in the household and 'work' environment of children, the potential role of cutaneous exposure to cat is intriguing.

The atopic march is a theory describing the evolution of type 2 inflammatory diseases. Specifically, the atopic march refers to the natural history of atopic disease within children, suggesting that children that display symptoms of atopic dermatitis (AD) at a young age are at an increased risk to develop allergies later in life, and possibly asthma[24]. While cat dander has classically been considered an aeroallergen, high levels of cat-specific IgE have been documented in patients that exclusively have atopic dermatitis[25]. Indeed, cutaneous exposure to cat dander has been found to induce an eczematous reaction in approximately 15-30% of patients with atopic dermatitis[25]. Thus, in patients with no airway symptoms, cat allergy can manifest as an atopic disease, suggesting that cutaneous exposure to Fel d 1 may be a critical step in the development of cat allergy.

We sought to investigate whether cutaneous exposure to cat dander could facilitate sensitization. Here, we describe a model of epicutaneous sensitization to cat dander. Furthermore, data are presented indicating that high-dose allergen exposure impairs the development of a robust immune response. Finally, through the use of transgenic mice lacking the key signal transduction molecule MyD88, we demonstrate that epicutaneous sensitization is dependent upon the IL-1 /Toll-like receptor axis.

5.2. Methods

Mice

Female (4-6 week old) C57BL/6, Myeloid differentiation primary response gene 88 locus deficient mice (MyD88$^{-/-}$) and HLA-DR4 Transgenic mice were purchased from Jackson laboratories, and Taconic, respectively. HLA-DR4 transgenic mice expressed human HLA-DR4, and lack their endogenous MHC Class II[26]; both HLA-DR4 and MyD88$^{-/-}$ transgenic mice were on a C57BL/6 background.

Allergen exposure models

Mice were cutaneously exposed to allergen by applying cat dander extract (CDE; 0 to 150 µg; Greer, Lenoir, USA) to a small (1cm x 1cm) patch of shaved and tape-stripped skin. Cutaneous exposure lasted for 10 days total (Figure 1). Following cutaneous exposure, the development of a polarized immune response to CDE was probed with intranasal (IN) allergen challenges on D22, 24 and 26 and where appropriate, with a secondary round of recall challenges on D49 and 50. All CDE IN administrations were performed at a fixed dose, 10µg CDE. Mice were sacrificed 24 or 48 hours post final allergen administration (Figure 1). Where required, CDE for cutaneous application was suspended in PBS containing LPS (*Escherichia coli* 0111:B4; Sigma Aldrich).

Intranasal high-dose house dust mite (HDM) was assessed in female, 6-8 week old BALB/c mice (Charles River). Animals received 25, 75 or 225µg of HDM intranasally for 10 (day 0-4, 7-11). Sham sensitized animals received PBS for 10 days. Following early HDM exposure, animals were recall challenged on day 22, 24 and 26 with 15µg HDM. Mice were sacrificed on day 27 for assessment of lung inflammation.

Quantification of airway responsiveness

Mice were assessed for the development of airway inflammation and hyperreactivity (AHR) after allergen challenge. Airway responsiveness in mice was assessed via a nebulized methacholine (MCh) test, as described in Chapters 2 and 4. Airway responsiveness is shown alternatively as total respiratory system resistance (R_{RS}) or provocative concentration causing a 200% elevation in basal resistance (PC_{200}).

Primary cell acquisition and differentiation

Following the nebulized MCh test, bronchoalveolar lavage (BAL) was obtained from animals by instilling two aliquots of 0.25mL of PBS into the lungs of mice, massaging the thoracic cavity and retrieving the PBS. BAL cells were enumerated by trypan blue staining and differentiated by Wright-Giemsa staining, following cytocentrifugation.

Lung processing for quantification of cells and histology was described in Chapter 2, 3 and 4. Briefly, the purification of a single cell suspension from the lung consisted of: dissecting out the right lung, liberating cells from the tissue by physical disruption followed by enzymatic digestion with collagenase and DNAase at 37ºC for 1.5 hours, followed by washing the cells through a 40µm sieve to filter out debris. Histological assessment of the lung consisted of: dissecting out the left lobe of the lung, filling to 20cmH_2O, fixing in 10% buffered formalin, dehydrating a portion of the lung, paraffin embedding and finally sectioning (3µm thick) for assessment by hematoxylin and eosin (H&E) or periodic acid schiff (PAS) staining.

Flow cytometry was described in detail in Chapters 2, 3 and 4. Briefly, aliquots of one million cells were stained with titrated antibodies for 30 minutes (4°C) then fixed in 4% paraformaldehyde (PFA). Cells to undergo intracellular staining were first activated via a 6 hour

culture (37°C) with Leukocyte Activation Cocktail (BD Biosciences), then stained and fixed. Intracellular staining for cytokines and transcription factors was performed as per manufacturer's instructions. Stained cells were quantified using a FACSCanto II (Becton Dickinson) and analyzed using Flowjo (TreeStar).

Antigen-specific T-cells were assessed by staining with HLA-DR4 tetramer, loaded with a Fel d 1 peptide (Fel d $1^{(23-38)}$), labelled with Phycoerythrin (courtesy of Dr. Kwok, Benaroya Institute). Prior to tetramer staining, cells were incubated for 2 hours (at 37ºC) with 50 nmolar dasatinib. Dasatinib inhibits a tyrosine kinase critical to T cell receptor (TCR) function (Lck), a feature beneficial to tetramer staining as it renders TCRs unresponsive to ligation thereby preventing receptor internalization upon binding by the tetramer; importantly, dasatinib does not induce apoptosis[27]. Dasatinib-incubated cells were stained with tetramer (10µg/mL) for 30 minutes (at 37ºC), at a volume of 20µL that contained 50nmolar dasatanib. Following tetramer staining, cells were surface stained or intracellularly stained as previously described. Due to the rarity of tetramer positive cells, a 'dump-gate' strategy to identify these cells was employed wherein cells expressing CD8, CD14 and CD19, were excluded from analysis.

Adoptive Transfer

Donor mice, following the completion of a 52-day CDE exposure period, were sacrificed and a purified lung-cell suspension was obtained, as previously described. CD4+ cells were enriched using a negative selection CD4+ T cell isolation kit, as per manufacturer's instructions (Milteny Biotec, Auburn, California, USA). CD4+ enrichment yielded an average purity of 60-70% CD4+ Cells. Enriched cells were stained with carboxyfluorescein succinimidyl ester (CFSE), washed and suspended in sterile PBS. Cells were transferred via a 200uL, intraperitoneal injection. 3.8 million cells were transferred to recipient mice, as this was the

maximum amount of cells obtainable from each donor mouse, following enrichment and CFSE staining. A transfer ratio of 1 donor lung into 1 recipient mouse was used as a guideline. CFSE staining enabled tracking of donor cells in recipient mice.

Data Analysis

Comparisons of data were made using one-way ANOVAs with a post-hoc bonferroni corrected student's t-test (Graphpad Prism). All comparisons were two-tailed and a $p \leq 0.05$ was considered statistically significant.

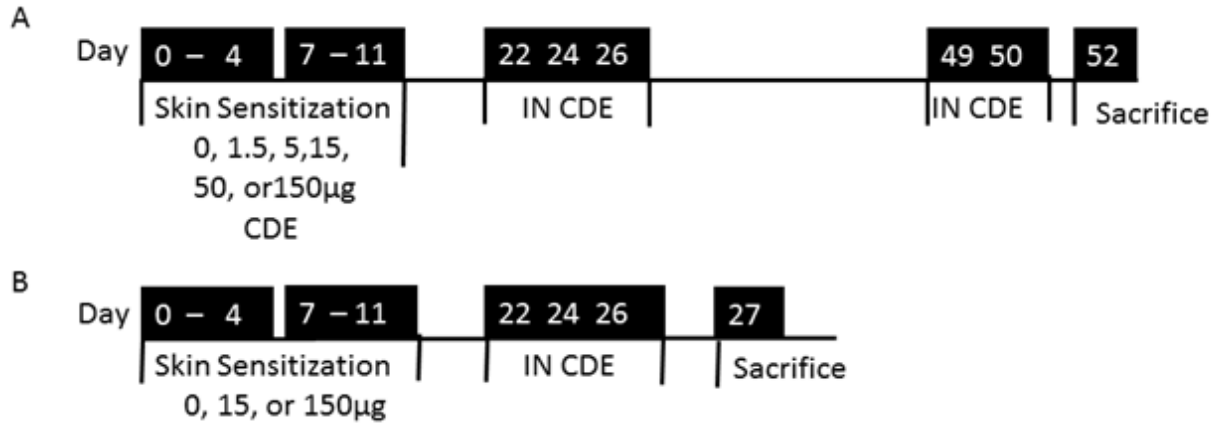

Figure 1. Schematic representation of the allergen exposure timelines. Cat dander extract (CDE) was applied to shaved, and tape stripped skin patches. Response to allergen provocation was assessed using intranasal (IN) CDE challenges, with 10µg of allergen. Animals were sacrificed on the indicated day.

5.3. Results

Cutaneous exposure to 15µg, but not higher doses, of CDE primes for allergic airways disease in mice

We set out to develop a model of epicutaneous sensitization using CDE. As we sought to assess antigen-specific T-cells responses, HLA-DR4 transgenic mice were employed. Mice were cutaneously exposed to a range of CDE doses between 1.5 to 150 µg, using the timeline illustrated in Figure 1a. The ability of cutaneous CDE exposure to polarize CDE-specific immune responses was assessed in the lungs of mice following challenge with 10µg CDE IN.

A defining feature of allergic asthma, that is replicated in mice, is the manifestation of an aberrant Th2 response typified by robust cellular, predominantly eosinophilic, infiltration[28]. To investigate if cutaneous application of CDE to the skin of mice had primed a CDE-specific immune response, mice were sacrificed after IN allergen challenge and inflammation in the BAL and lung were examined. Despite all mice receiving the same degree of barrier disruption, the dose of CDE applied uniquely controlled the development of CDE immunity. Mice exposed to low dose (1.5 & 5 µg) and high dose (50, 150 µg) CDE, failed to mount an inflammatory response to CDE when challenged intranasally. In contrast, mice exposed to 15µg of CDE on the skin mounted significantly increased BAL cell infiltration (Figure 2A) that, upon cellular differentiation, was predominantly eosinophilic (Figure 2B). Priming with 15 µg of CDE on the skin of mice did not affect the number of neutrophils responding to allergen challenge (Figure 2B), however, a significantly increased macrophage and lymphocyte population was noted (Figure 2B). Sham sensitization consisted of applying saline to a patch of shaved and tape-stripped skin. Challenging sham sensitized animals with CDE did not result in an enhanced cellular recruitment to the lung lumen.

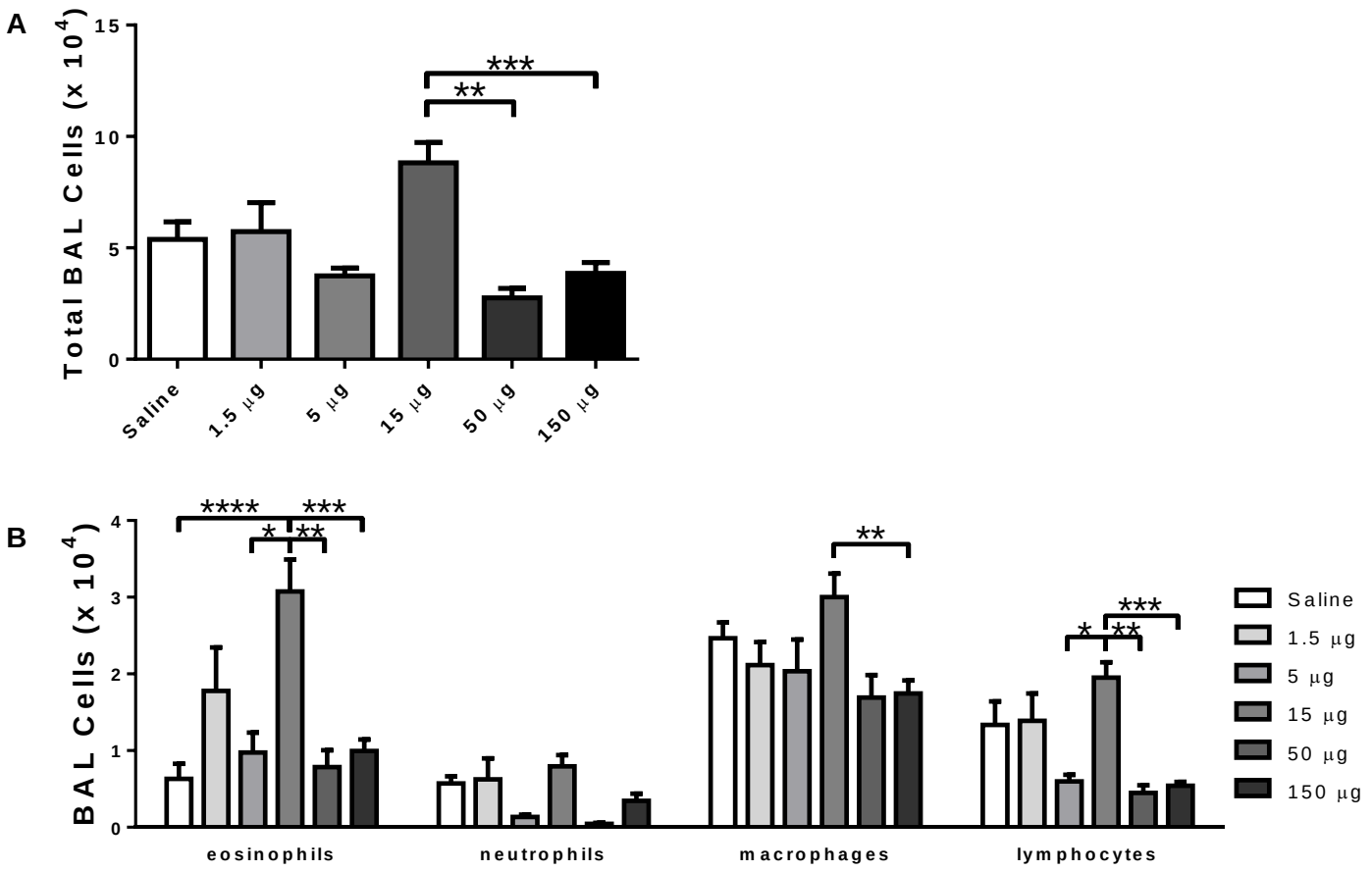

Figure 2. IN CDE challenge induces a Th2 skewed inflammatory infiltrate into the BAL of mice primed with intermediate, but not high-or low-doses of CDE. CDE (1.5 - 150 µg) or saline was applied to a patch of shaved, tape stripped skin of HLA-DR4 transgenic mice. Mice were challenged with 10µg CDE intranasally, and a BAL was performed 48 hours later. Total BAL cells were enumerated (A) by trypan blue staining, and differentiated into eosinophils, neutrophils, macrophages or lymphocytes (B) by Wright-Giemsa staining. Data from three independent experiments pooled, and presented as mean ± (SEM), n=8-24/group. *, $p < 0.05$; **, $p < 0.01$; ***$p<0.0001$, ****0.0001, as determined by a one-way ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

Eosinophilic inflammation in the lungs of allergen challenged animals, following cutaneous exposure was assessed by histology and flow cytometry. Peribronchial eosinophils were quantified following H&E staining. Similar to BAL samples, cutaneous application of 15µg, but not 150µg, of CDE primed mice to mount an eosinophilic response to subsequent CDE-exposure, as only mice sensitized with 15µg of CDE developed significant peribronchial eosinophilia upon challenge (Figure 3A). Total lung eosinophilia was assessed by flow cytometry by staining cells liberated from the right lung. Eosinophils were categorized as sialic

acid-binding immunolobulin-like lectin F (Siglec-F) positive[29], and CD11c negative cells[30]. The effects of cutaneous CDE exposure were consistent with previous examinations; however, a dose-response effect was apparent when examining whole lung eosinophilia. Cutaneous exposure to intermediate doses of CDE (5 and 15µg) primed mice to develop significant eosinophilia throughout the lung upon IN CDE challenge, whereas cutaneous exposure to higher (50, or 150 µg) or lower (1.5µg) doses of CDE did not (Figure 3B).Asthma is defined a reversible airflow obstruction that is commonly accompanied by airway inflammation and airway hyperreactivity (AHR). As cutaneous exposure to 15µg primed mice for the development of airway inflammation, allergen challenged animals were examined for the development of AHR using a nebulized methacholine challenge. Mice exposed to 15µg CDE cutaneously responded to an intranasal CDE challenge with significantly increased R_{RS}, and a decreased PC_{200} (Figure 4A and B); indicating that cutaneous allergen exposure both increased the maximum degree of airway narrowing in these mice, while also lowering the threshold to respond to bronchostimulatory agents. Surprisingly, IN challenge of mice cutaneously exposed to 150 µg CDE significantly decreased PC_{200}, indicating the development of airway hypersensitivity, despite no observed increase in cellular infiltration to the lung.

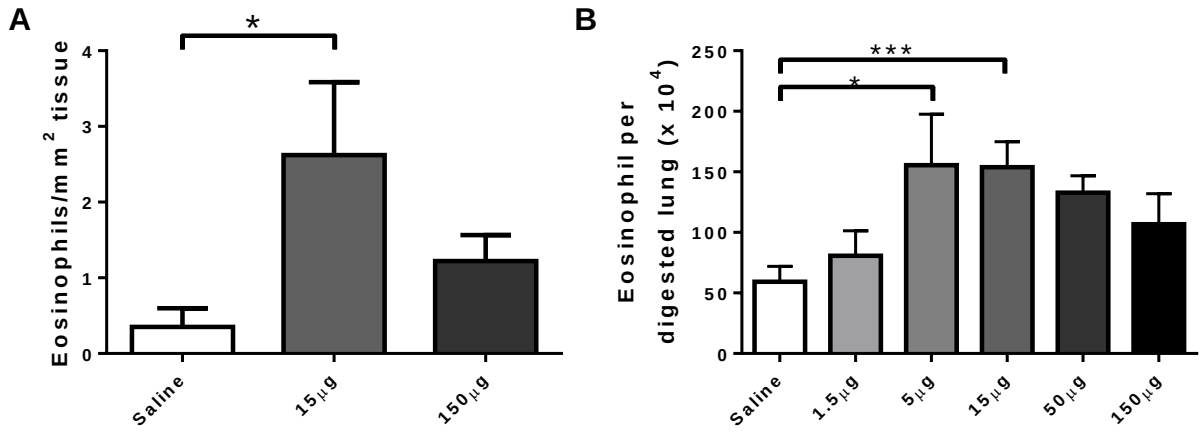

Figure 4. Cutaneous exposure with intermediate, but not high or low doses of CDE induces a Th2 polarization of the immune response.CDE (1.5 - 150 µg) or saline was applied to a patch of shaved, tape stripped skin of HLA-DR4 transgenic mice. The left lung from mice challenged with 10µg CDE intranasally, was fixed, sectioned and stained with heamatoxylin and eosin(H&E). Eosinophils within 50µm of the bronchi, were enumerated (A). (B) Total eosinophils in the right lung were enumerated (SIGLEC-F+CD11c-) by flow cytometry. Data from two independent experiments pooled and presented as mean ± (SEM), n=8-16/group. *, p <0.05; ***p<0.0001, as determined by a one-way ANOVA and post-hoc Bonferroni corrected Student's t-tests.

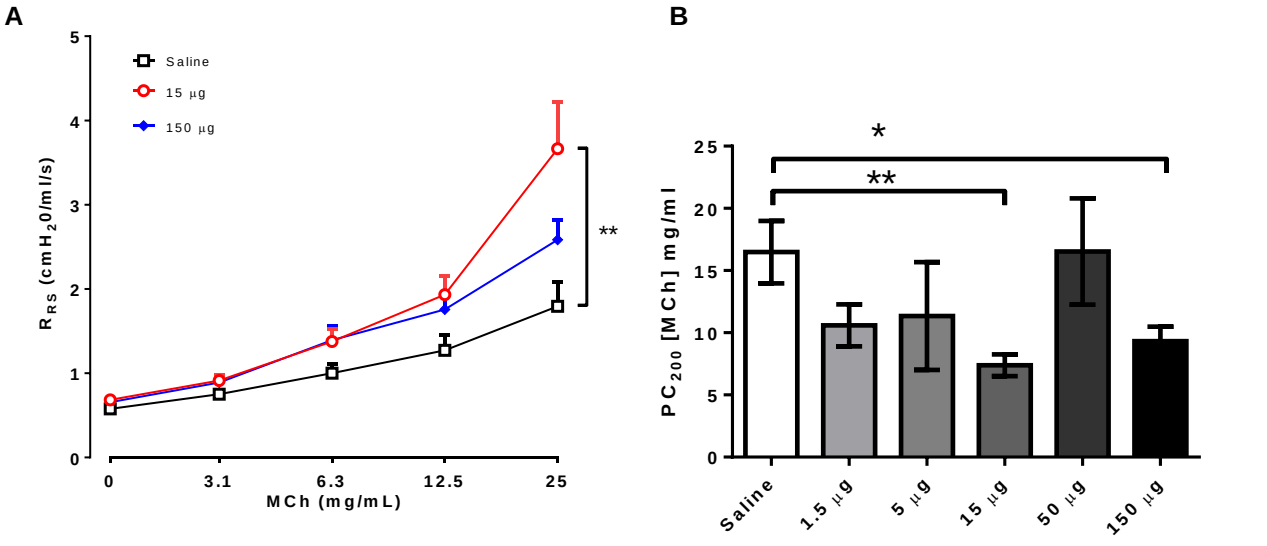

Figure 3. Cutaneous exposure with both intermediary and high doses of CDE induces aspects of AHR.HLA-DR4 transgenic mice cutaneously exposed to CDE (1.5 - 150 µg) were challenged with CDE intranasally, and the airway responsiveness was assessed by a nebulized methacholine (MCh) dose-response test. (A) Maximum respiratory system resistance induced by each dose of MCh and (B) dose of MCh inducing a doubling of baseline resistance (PC_{200}) Data from three independent experiments pooled, and presented as mean ± (SEM), n=8-24/group. *, p <0.05; **p<0.001, compared to saline, as determined by a one-way ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

Cutaneous exposure to CDE induces antigen-specific T-cells producing Th2 cytokines

Increased expression of the archetypal Th2 cytokines IL-4, -5[31, 32] and IL-13[33] have all been observed in the lungs of asthmatics. While these, and numerous other studies have demonstrated the role of IL-4, -5, and -13, in asthma, analyses have typically been limited to the examination of polyclonal T-cell responses. Efforts to examine antigen-specific T-cells during allergic responses have commonly been limited by reagent availability (lack of tetramers) and/or confounding factors (use of transgenic mice with a single T-cell receptor). Use of HLA-DR4 mice enabled us to analyze antigen-specific T-cells within a polyclonal T-cell response, through the use of PE-labeled HLA-DR4 tetramers loaded with a Fel d 1 peptide.

First, we sought to define the anatomical location of antigen specific T-cells after an allergen challenge. Following cutaneous CDE exposure and intranasal allergen challenge, lungs, lung draining lymph nodes, skin, skin draining lymph nodes, spleen, and bone marrow were dissected and assessed for the presence of antigen-specific T-cells. As antigen specific T-cells represent a rare population, a stringent gating strategy was used to reduce the risk of false positives; tetramer positive cells were identified as singlet CD4+, PE-tetramer+, CD8-CD14-CD19- cells. Intranasal allergen challenge preferentially induced the recruitment of antigen-specific T-cells to the lungs of mice (Figure 5A). Approximately 50% of all detected antigen-specific cells were in the lungs of allergen challenged mice, with 30% residing in the spleen, 15% in the bone marrow and the remainder in the skin and lung draining lymph. The dose of cat dander applied cutaneously, was a key determinant of the resulting immune response. A bell-shaped dose-response effect of CDE application was observed, with intermediate doses of CDE significantly, and robustly expanding antigen-specific T-cells, while both high and low doses of CDE were unable to induce expansion (Figure 5A).

As the majority of antigen-specific T-cells were found in the lung, these cells were probed for their ability to produce Th2 cytokines. The polyclonal T-cell response (singlet CD4+CD8-CD14-CD19-, tetramer- cells) was also investigated. All examined Th2 cytokines (IL-4, -5 and -13), revealed a similar bell-shaped dose response curve, with intermediate doses of CDE maximally enhancing Th2 cytokine production (Figure 5B, C, & D). Intermediate doses of CDE applied to the skin enhanced Th2 cytokine production by antigen-specific cells by 5-15 fold, in contrast, cutaneous exposure to high-dose CDE either failed to alter or induced comparatively minor changes (range: 0.7-2-fold change) in Th2 cytokine production. Intriguingly, this bell-shaped response was not resolved in polyclonal T-cell responses, with CDE application not having a consistent effect upon polyclonal IL-4, IL-5 or IL-13 expression.

Cutaneous application of both high and low dose CDE failed to induce a Th2 response in mice. We investigated if these doses had given rise to a Th1 or modified Th2 response (characterized by the production of IL-10 in addition to Th2 cytokines[34]). IFN-γ, the prototypical T-cell Th1 cytokine and IL-10 were quantified in lung T-cells, after allergen challenge. Antigen-specific production of IFN-γ followed the same bell-shape dose-response curve as observed for Th2 cytokines, with peak IFN-γ production noted in mice cutaneously exposed to 15 µg of CDE (Figure 5E). While the fold-change increase in IFN-γ expression was of a greater magnitude than the change in Th2 cytokines (30-fold, compared to 5-fold for IL-4), the absolute number of antigen-specific cells producing Th2 cytokines remained higher. High-dose CDE did not preferentially enhance IFN-γ production in antigen-specific nor polyclonal T-cells. Allergen challenge revealed that high dose cutaneous CDE application did not enhance antigen-specific nor polyclonal IL-10 expression, compared to exposure with a sensitizing (15µg) dose of CDE cutaneously (Figure 5F).

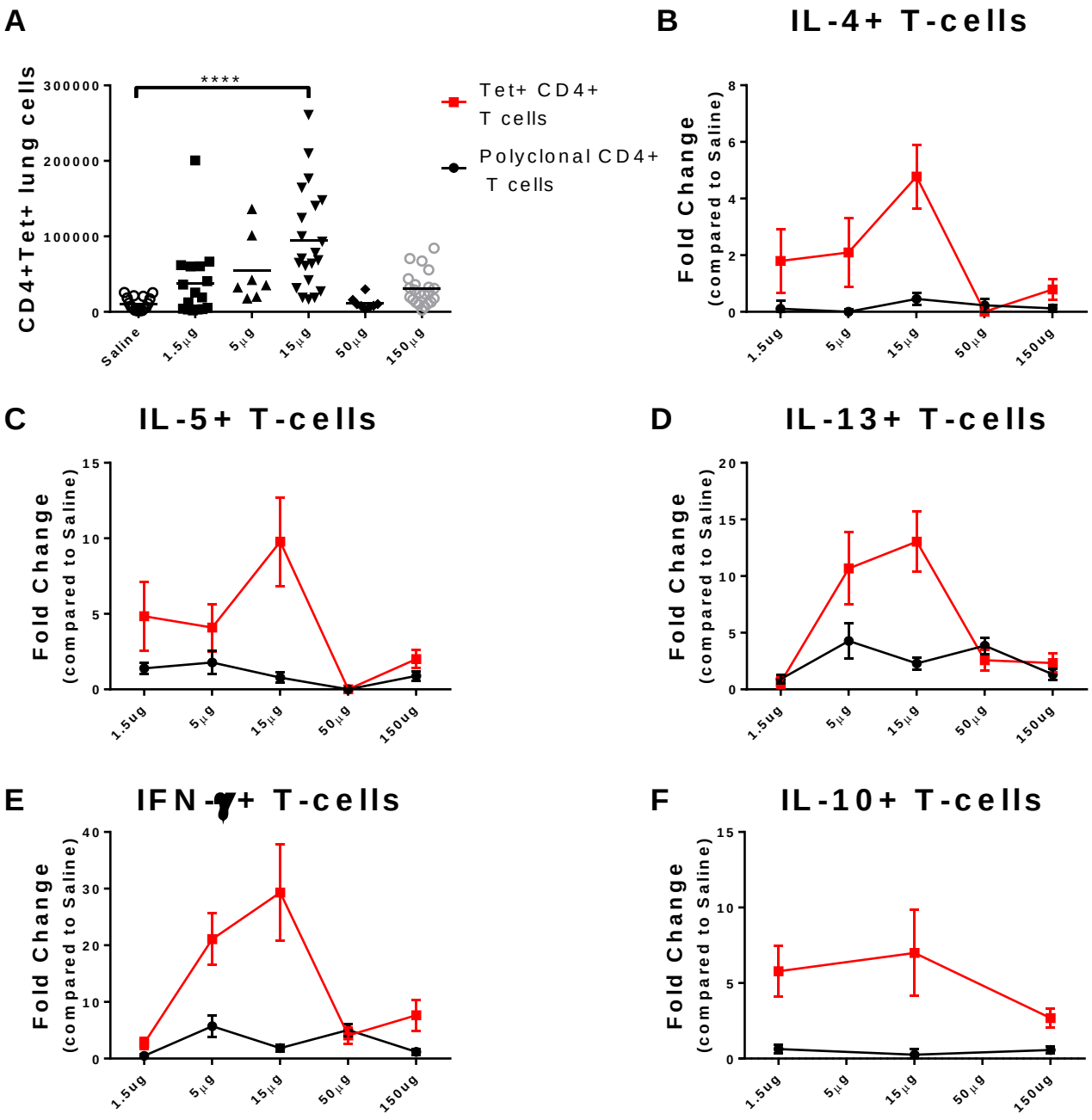

Figure 5. A bell-shaped dose response curve exists between cutaneous CDE application and Th2 cytokine production.HLA-DR4 transgenic mice cutaneously exposed to CDE (1.5 - 150 µg) were challenged with CDE intranasally, and lung derived T-cells were assessed for their antigen-specificity (A), and ability to produce cytokines, IL-4 (B), IL-5 (C), IL-13 (D), IFN-γ (E), and IL-10 (F). Antigen specific T-cells (red line and dot), and polyclonal T-cells (black line and dot) were defined as singlet CD4+PE-teramer+CD8-CD14-CD19- cells, CD4+PE-teramer-CD8-CD14-CD19- cells, respectively. Data from two independent experiments pooled, and presented as mean ± (SEM), n=8-16/group. Fold change calculated as (x-y)/y, where x is individual data points and y is the average number of t-cells/tetramer+ t-cells in sham sensitized animals. ****, p <0.00001; as determined by a one-way ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

Analysis of antigen-specific T-cells revealed that 15µg of CDE applied to a patch of disrupted skin primed a Th2-polarized response to cat. We next sought to assess whether this polarized response was accompanied by increased IgE, the prototypical Th2 antibody. To our surprise, HLA-DR4 mice exposed to 15µg of CDE epicutaneously failed to develop increased IgE titers (Figure 6A). Of note, baseline antibody levels in HLA-DR4 mice were nearly undetectable; thus, we elected to verify these results in wild-type C57BL/6 mice. C57BL/6 mice also develop a Th2-polarized response following epicutaneous exposure to CDE (Figure 7). C57BL/6 mice epicutaneously exposed to sensitizing doses of CDE also failed to develop enhanced IgE levels (Figure 6A). Cat-specific IgE was also not increased by epicutaneous exposure to CDE (Figure 6B). Like IgE, the production of IgG1 depends upon B-cell exposure to the Th2 cytokine, IL-4[35]. We quantified IgG1 in allergen exposed mice, to determine if this alternative pathway had been engaged. Like IgE, the epicutaneous exposure of C57BL/6 and HLA-DR4 mice to CDE (15µg or 150µg) failed to enhance production of IgG1 (Figure 6C).

As the modified Th2 response to cat, in humans, is associated with enhanced titers of IgG4, a blocking antibody, we sought to assess whether a similar mechanism of action was protecting high-dose CDE exposed mice. Mice lack IgG4, but IgG2a, a Th1-associated antibody, is considered to be a surrogate marker in mice. High-dose CDE exposure (150µg) did not enhance IgG2a levels in C57BL/6 or HLA-DR4 mice, compared to animals receiving a sensitizing dose of CDE or saline (Figure 6D). Importantly, C57BL/6 mice may lack the gene required to produce IgG2a, and instead may produce IgG2c[36]. Investigation of total and CDE-specific IgG2C (Figure 6E and F) again revealed no difference between high-dose CDE exposed mice versus mice receiving a sensitizing dose of CDE epicutaneously. Taken together, despite

the induction of a Th2 polarized immune response by epicutaneous CDE exposure, no alterations in IgE, IgG1, IgG2a or IgG2c could be detected.

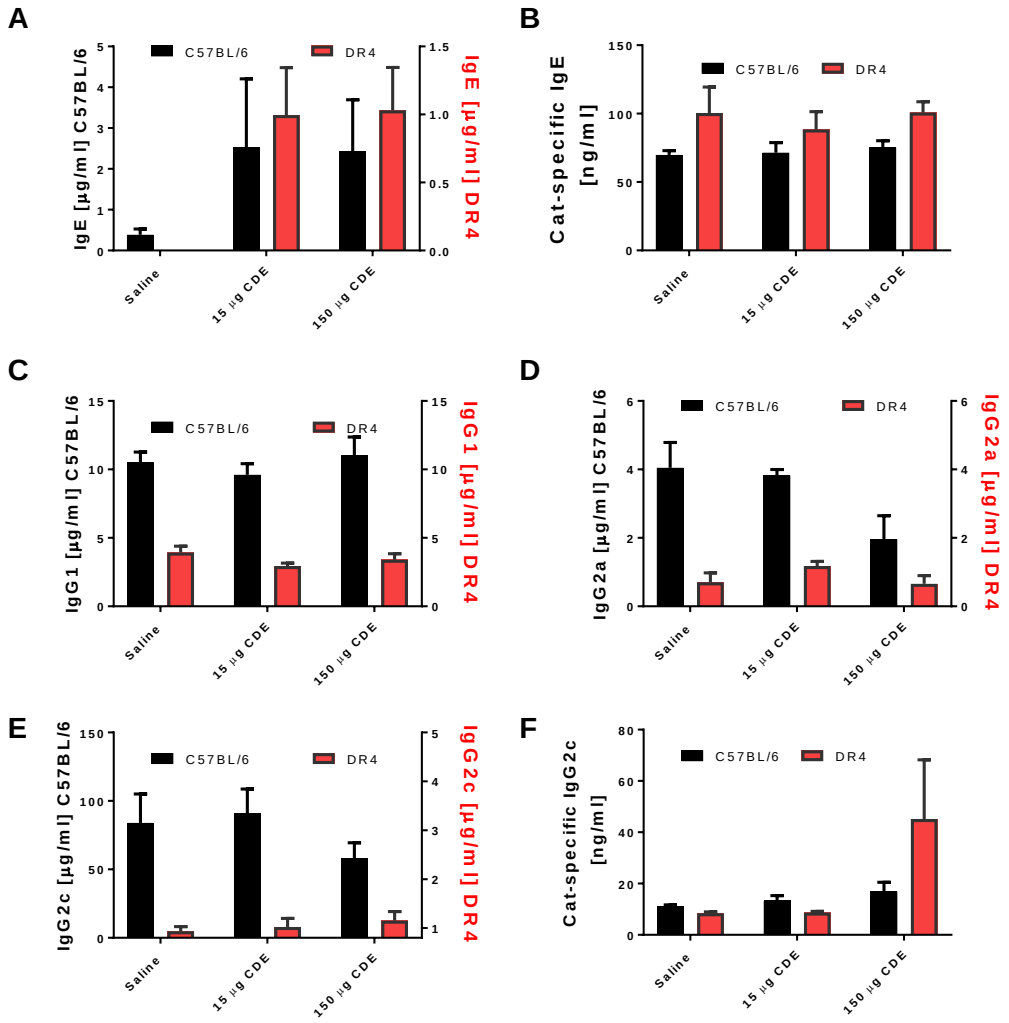

Figure 6. HLA-DR4 and C57BL/6 mice exposed to a cat dander epicutaneously do not develop humoral immune responses.C57BL/6 (black bars) and HLA-DR4 mice (red bars) were cutaneously exposed to saline or CDE (15 or 150 µg). Following an intranasal CDE challenge, blood was collected, and total serum IgE (A), cat-specific IgE (B), IgG1 (C), IgG2a (D), IgG2c (E) and cat-specific IgG2c (F) were quantified by ELISA. Antigen-specific antibodies were quantified by pre-coating ELISA plates with CDE. Data from two independent experiments pooled, and presented as mean ± (SEM), n=8/group.

Kinetic examination of cutaneous exposure to CDE

Since our analysis of fully sensitized and challenged mice revealed a reduced number of Fel d 1-specific CD4 T cells, we next assessed whether CDE dose affected T cell activation during epicutaneous sensitization. To this end, cells from the inguinal lymph node (the draining lymph node) were isolated from mice sensitized with saline, 15µg or 150µg of CDE half-way through the allergen exposure protocol (Day 4), at the end of the protocol (Day 11) and 10 days post the protocol (Day 21; Figure 1B).

While all mice (Saline, 15 and 150µg) were devoid of antigen-specific cells at day 4 (Figure 7A), by day 11 antigen-specific T-cells were detected in the inguinal lymph nodes of mice exposed to both the 15 and 150µg dose. A trend indicating that mice exposed to high-dose CDE had more cat-specific T-cells was observed, but did not achieve significance. By day 21, antigen-specific T-cells were no longer detectable in the lymph nodes.

To assess whether high-dose CDE was impairing Th2 differentiation, inducing Th1 differentiation or regulatory T-cells, IL-4, IFN-γ, and IL-10 production by polyclonal and antigen-specific T-cells were quantified. IFN-γ production by polyclonal (Figure 7B) and antigen-specific T-cells (Figure 7C) peaked at day 11, the end of the cutaneous exposures. A trend towards increased IFN-γ production in the 15µg CDE exposed group was noted, but failed to attain significance. IFN-γ production by cat-specific T-cells was low in mice exposed to 15 or 150µg CDE. IL-4 production was undetectable at day 4. At the completion of the cutaneous exposures, IL-4 production by polyclonal T-cells from high-dose CDE exposed mice was significantly elevated (Figure 7D). IL-4 production returned to baseline by day 21. A trend towards increased IL-4 production was noted in antigen-specific T-cells from high-dose CDE exposed mice (Figure 7E). Analysis of IL-10 production by polyclonal T-cells (Figure 7F) and

antigen-specific T-cells (Figure 7G) revealed there to be no discernible difference in production at any assessed time point. Thus, only IL-4 expression was altered by high-dose CDE exposure. The expansion of antigen-specific T-cells in the high-dose exposed group and significantly increased IL-4 production suggests that impaired priming may not be the mechanism protecting these mice from the development of allergic airways disease.

To better characterize the model, the kinetics of airway inflammation following epicutaneous allergen exposure and IN CDE challenge were assessed. Previously, it was established that mice epicutaneously sensitized to an intermediate dose of CDE, then intranasally challenged with CDE on days 22-26 were primed to develop eosinophilic lung inflammation upon subsequent rechallenge. Given the enhanced expression of IL-4 by polyclonal T-cells in the inguinal lymph nodes of mice exposed to 150ug, we sought to define whether the differential effects of intermediate versus high-dose cutaneous exposure to CDE were present after the initial CDE challenges, or whether this effect matured between the day 22-26 exposure and the subsequent rechallenge. Mice were cutaneously exposed to CDE (15 or 150µg) or saline, then challenged with CDE between 22-26. Animals were sacrificed at day 27, to assess the polarity of the immune response after the initial IN exposures and again at day 48, to establish whether inflammation persisted or resolved during this time. Finally, a parallel group of mice were rechallenged with CDE on day 49/50 and sacrificed on day 52 to enable a direct comparison between the inflammatory infiltrate that develops on day 52 with each of the other time points.

Mice cutaneously exposed too saline or high-dose CDE, sacrificed on day 27 (24-hours post initial IN CDE exposure) did not possess increased cellular infiltration into the BAL (Figure 8A). In contrast, by day 27, mice cutaneously exposed to 15 µg CDE mounted a significant Th2 polarized immune response, marked by significantly elevated cellular infiltration into the BAL,

that was predominantly comprised of eosinophils (Figure 8B) and lymphocytes (Figure 8C). Neutrophil recruitment to the lung lumen was enhanced in mice cutaneously exposed to 15 µg CDE (Figure 8D). By day 48 (22 days post allergen challenge), the majority of inflammation had resolved; however, a minor increase in the total number of cells in the lung lumen persisted in mice cutaneously exposed to 15µg. Following a second round of IN allergen challenges, a more robust Th2 response was observed in mice cutaneously exposed to 15 µg of CDE. Indeed, compared to day 27 inflammation, a 40% increase in the number of BAL eosinophils and a 50% reduction in BAL neutrophils was noted on day 52. In keeping with previous observations, mice exposed to high-dose CDE failed to mount an immune response that significantly differed from sham sensitized animals.

Data generated from the kinetic examination of CDE cutaneous sensitization failed to define a mechanism that could explain the differential effects of dose. However, the kinetic study established that, despite an early increase in IL-4 production in mice exposed to a high-dose of CDE, that by day 27 a diminished allergic response was present, compared to animals exposed to 15µg of CDE. Subsequent mechanistic studies attempting to delineate the effect of dose employed the 27-day model.

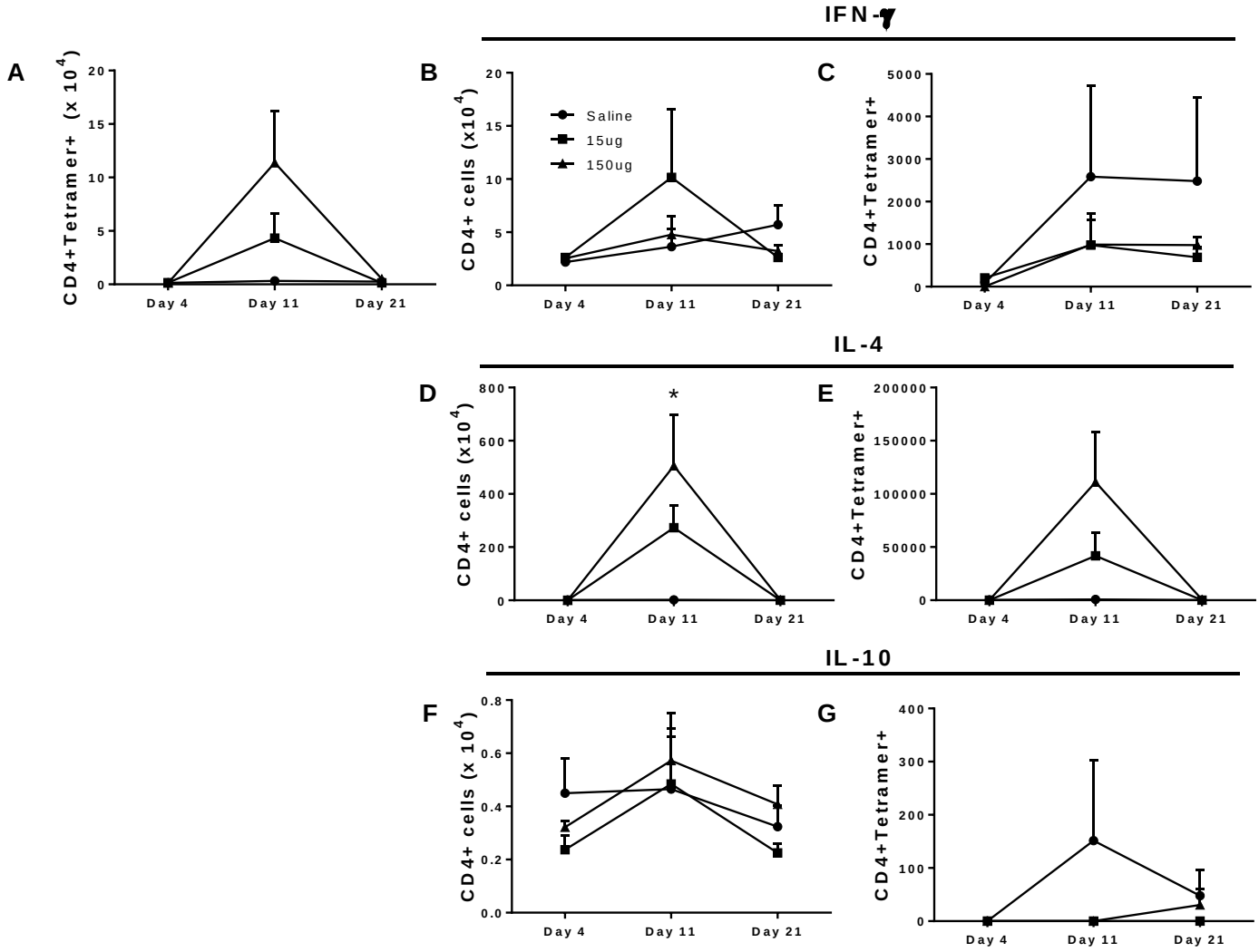

Figure 7. Kinetic examination of polyclonal and cat-specific T-cells in the inguinal lymph nodes of mice cutaneously exposed to CDE. Mice were exposed to saline (circle), 15µg CDE (square) or 150 µg CDE (triangle) for a 10-day period. Mice were sacrificed on the indicated day (Day 4, 11 or 21), and lymphocytes from the inguinal lymph nodes were assessed for the development of antigen specific cells (A), and cytokine expression by flow cytometry. Polyclonal T-cell expression of IFN-γ (B), IL-4 (D) and IL-10 (F) were assessed. Antigen-specific T-cell expression of IFN-γ (C), IL-4 (E) and IL-10 (G) were also assessed. Pooled samples from two independent experiments presented as mean ± (SEM), n=5/group. *, $p < 0.05$ vs saline mice, as determined by a one-way ANOVA and post-hoc bonferronni corrected *Student's* t-tests.

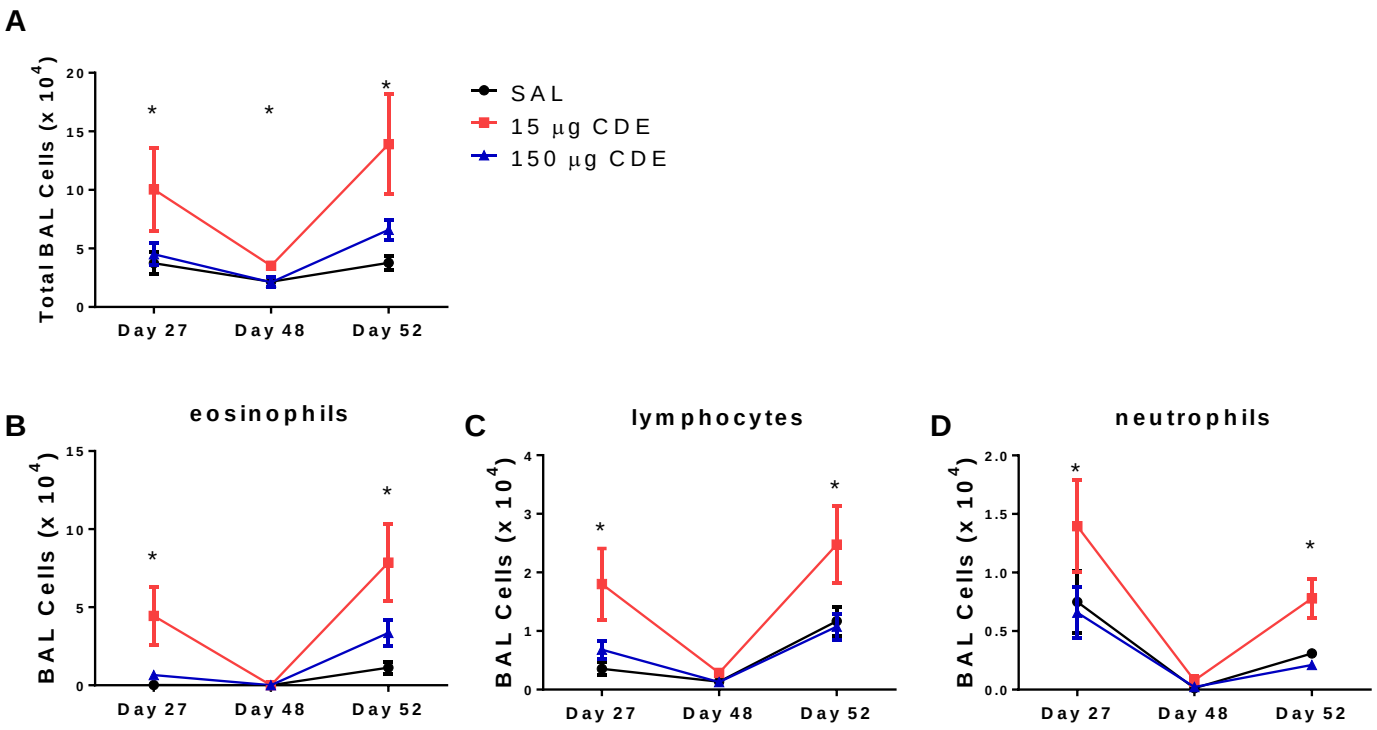

Figure 8. Kinetic examination of airway inflammation induced by CDE challenge, following exposure too intermediate or high-dose CDE. Mice were cutaneously exposed to saline (SAL, black line/dot) 15 (red line/dot) or 150 (blue line/dot) µg of CDE, and challenged with IN CDE (D22, 24 and 26). BAL was taken on D27,48 and 52 from parallel groups of mice. Total BAL cells were enumerated (A) by trypan blue staining. BAL cells were differentiated into eosinophils (B), lymphocytes (C), neutrophils (D), and macrophages (data not shown) by Wright-Giemsa staining. Representative data from one of four independent experiments presented as mean ± (SEM), n=5/group. *, $p < 0.05$ vs saline mice, as determined by a one-way ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

Role of LPS and T-cell mediated regulation during CDE epicutaneous sensitization

The kinetic examination of T-cells during exposure to CDE failed to identify a candidate mechanism of action that could explain the immune paralysis that high-dose CDE exposure invoked. Recently, a study by Bryant and colleagues indicated that cat dander may derive allergenicity from bound LPS, that can then ligate toll-like receptor (TLR) 4 and activate antigen presenting cells[37]. High dose exposure to LPS can drive a Th1, rather than Th2 response[38]; therefore, we hypothesized that high dose CDE application may be unable to induce a Th2 response due to high levels of LPS/TLR agonists that would contaminate cat dander.

To assess the role of TLR agonists and LPS during epicutaneous sensitization, we exposed wild-type (WT) mice and mice lacking a critical TLR signalling molecule (MyD88) to doses of CDE that alternatively sensitize (15µg) or impair sensitization (150µg). Mice were then challenged with CDE and lung inflammation assessed (Figure 1B). MyD88$^{-/-}$ mice possess a severely impaired response to provocation through Toll-like receptors (TLRs)[39] and cytokines from the IL-1 family[40]. While LPS signalling through TLR4 is commonly believed to be a requisite for the initiation of an immune response, studies using MyD88$^{-/-}$ mice have consistently demonstrated a preserved ability to mount a Th2 response, citing retained MyD88 independent maturation of DCs as a putative mechanism.[41-45]. While MyD88 is also a critical signal transducer for the IL-1 cytokine family[40], epicutaneous sensitization has been shown by some groups to be dependent upon thymic stromal lymphopoietin (TSLP) and not the IL-1/IL-33 family[46-48]. Finally, while LPS possesses MyD88-independent effects, the efficacy of LPS is severely diminished in the absence of MyD88[39].

To our surprise, despite a paucity of data citing a role for MyD88 and LPS during epicutaneous sensitization, MyD88 deficient mice completely failed to manifest AHR (Figure 9A), while WT (C57BL/6) mice developed significant, robust AHR as a result of cutaneous exposure to 15, but not 150 µg CDE (Figure 9B). Following exposure to 15, but not 150 µg CDE, WT, but not MyD88$^{-/-}$ animals, were primed to develop airway inflammation, highlighted by significantly enhanced total BAL cells and BAL eosinophils after IN CDE challenge (Figure 9C and D). Furthermore, MyD88 deficient animals failed to develop eosinophilic lung inflammation, while WT mice cutaneously exposed to 15µg CDE mounted significant eosinophilic inflammation of the lungs (Figure 9E). Analysis of lung Th2 cytokine production corroborated these observations. T-cells from WT, but not MyD88$^{-/-}$, mice exposed to 15µg of

CDE cutaneously possessed significantly elevated production of IL-5 (Figure 9F), and IL-13 (Figure 9G); IL-4 production did not change (Figure 9H).

Therefore, MyD88 deficient mice demonstrate a critical role for TLR ligation during epicutaneous sensitization. As MyD88 deficient mice could not be primed, these experiments do not define the contribution of the TLR/IL-1 axis to the effects of high-dose cutaneous CDE application.

Addition of LPS to a sensitizing dose of CDE fails impair sensitization

The MyD88 knock-out approach revealed that TLR ligation is a critical component of epicutaneous sensitization, however, this method did not identify whether increased levels of LPS/TLR ligands were driving the immune hyporesponsiveness associated with high-dose cutaneous CDE application. If, as we hypothesized, high doses of LPS/TLR ligands are sabotaging the development of a robust immune response to high-dose cutaneously applied CDE, the addition of LPS/TLR ligands to a sensitizing dose of CDE should protect mice, and attenuate allergic responses. Thus, we set out to investigate whether high-dose CDE is unable to induce a robust immune response because of high TLR ligands/LPS concentrations, by adding LPS to sensitizing doses of CDE. To instruct LPS dosing requirements, LPS levels in the CDE solutions used to prime mice was assessed by a chromogenic LAL assay. Aliquots of 'High-dose' CDE (150µg) contained 0.57µg LPS, while 'sensitizing' doses of CDE (15µg) contained 0.1µg LPS, indicating that mice receiving 'high-dose' CDE received approximately 0.5µg more LPS than did mice exposed to sensitizing doses.

To investigate the effects of adding LPS to sensitizing doses of cat dander, C57BL/6 mice were cutaneously exposed to 15µg CDE + no LPS, 15µg CDE + 1µg LPS, 15µg CDE + 10µg LPS, 15µg CDE + 100µg LPS. CDE hyporesponsiveness was established in control mice

through exposure to 150µg CDE. The CDE specific immune response was probed by challenging mice with CDE intranasally three times and sacrificing animals 24 hours later (Figure 10A). The cutaneous application of 15 µg, but not 150 µg, of CDE to the skin of mice primed them to develop AHR upon subsequent allergen challenge (Figure 10B). The addition of LPS to the 15µg sensitizing CDE dose failed to confer a protective effect that could inhibit the development of AHR. In contrast, upon examination of airway hypersensitivity to methacholine, a dose-response effect became apparent, with the addition of 10µg of LPS significantly elevating PC_{200}, raising it to the level of mice that had received 150µg CDE cutaneously (Figure 10C). The addition of LPS failed to modify the number of cells (Figure 10D) or eosinophils (Figure 10E) infiltrating the BAL; however, eosinophil recruitment to the lung tissue was impaired by the addition of 1µg LPS (Figure 10F).

To assess whether the addition of LPS impaired Th2 differentiation, the production of archetypal Th2 cytokines by T-cells and ILC2s were quantified. LPS dose-dependently affected IL-4 production by T-cells, as addition of low-dose LPS (1µg) to 15µg CDE significantly reducing IL-4 production, to levels equivalent to that induced by exposure to 150µg CDE (Figure 11A). A trend was also observed for IL-5 and IL-13, as addition of low-dose LPS non-significantly reduced production of these cytokines by 60% and 46%, respectively (Figure 11B &C). Addition of higher doses of LPS (10 or 100µg) to a sensitizing dose of CDE (15µg) had no discernible effect.

Type 2 innate lymphoid cells (ILC2s) are cells that rapidly respond to inflammatory stimuli by releasing large amounts of IL-5 and IL-13[49]. During allergen provocation of sensitized animals, ILC2s can act as effector cells, secreting IL-5 and IL-13, at levels equivalent to that of primed T-cells[50, 51]. Thus, the number of IL-5 and IL-13 producing ILC2s was assessed,

following antigen challenge. LPS addition failed to reduce the number of ILC2s producing IL-5 (Figure 11D), IL-13 (Figure 11E) or both IL-5 and IL-13 (Figure 11F). Of note, high-dose CDE also failed to reduce the number of Th2 cytokine producing ILC2s. Intriguingly, a trend was observed wherein lower doses of LPS were associated with reduced numbers of cytokine producing ILC2s.

Taken together, the addition of LPS may not be able to subvert a sensitizing dose of CDE, as observed differences in airway function were small and changes in inflammation were inconsistent. Our examinations of LPS yielded both unexpected, and inconclusive results. Examination of MyD88 deficient mice revealed LPS to be a critical mediator of epicutaneous sensitization; moreover, we demonstrated that high-dose LPS does not reduce the allergenicity of cat dander applied to the skin of mice. Intriguingly, a modest signal that low-dose LPS may subvert allergenicity did arise and warrants further study.

Assessment of T-cell mediated protection in high-dose CDE exposed mice

Both the epicutaneous CDE dose-response study and kinetic examination demonstrated that applying 15µg of CDE to barrier-disrupted skin induced epicutaneous sensitization, but that application of 150µg CDE did not. These studies failed to demonstrate a dose-dependent difference in T-cell behaviour that could account for the protection observed during high-dose exposure. However, the kinetic study demonstrated that cat-specific cells and IL-4 production are enhanced in the high-dose CDE group, suggesting that high-dose CDE does commence priming that is terminated shortly thereafter, leaving mice protected from subsequent challenge in a manner perhaps similar to the onset and development of OVA-tolerance[52]. We set out to directly test whether high-dose cutaneous exposure to cat dander induced protective immunity by

adoptively transferring CD4+ enriched cells from high-dose exposed animals into sensitized animals.

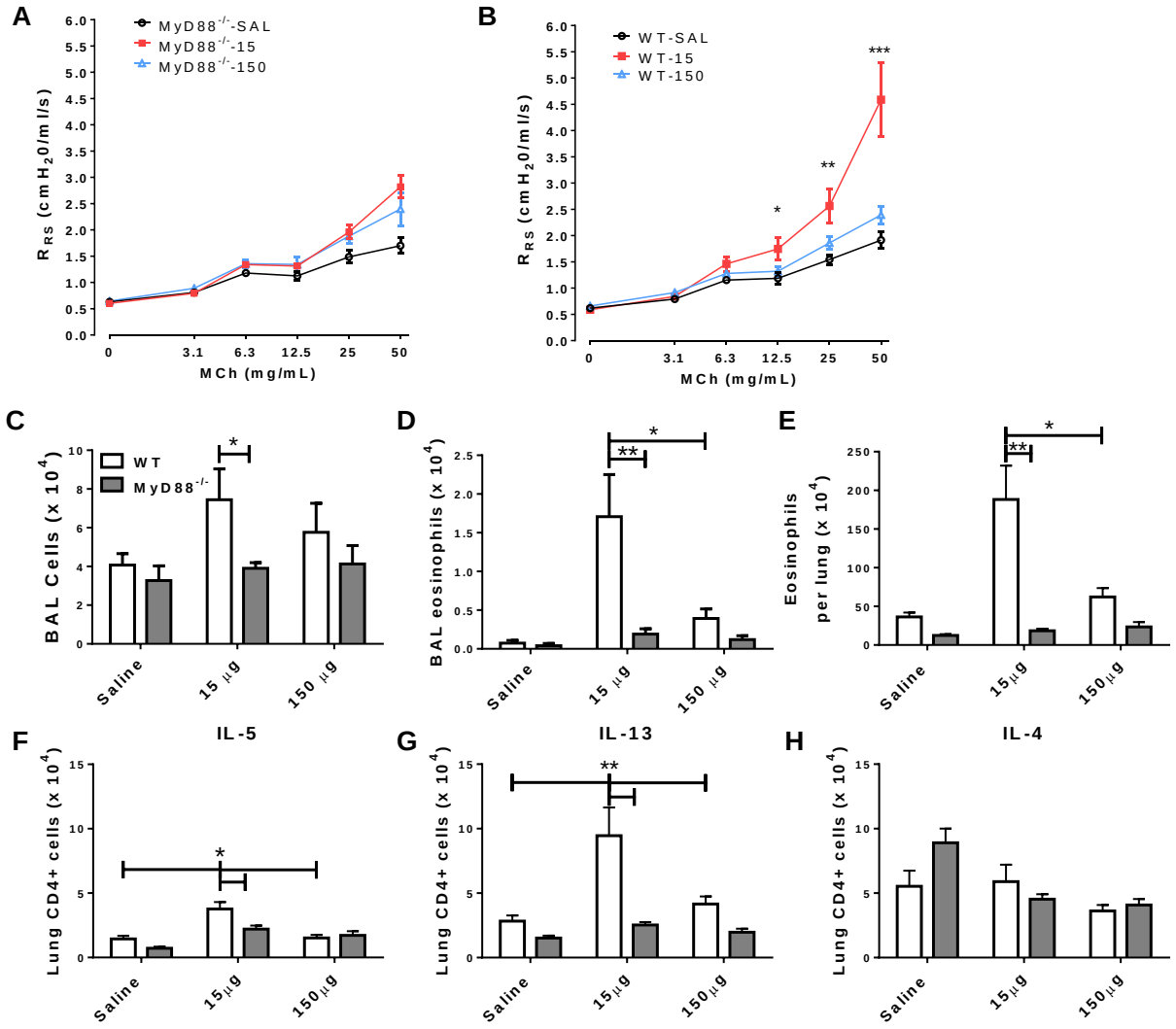

Figure 9. MyD88 is required for epicutaneous sensitization to cat dander. WT (white bars) and MyD88$^{-/-}$ mice (gray bars) were cutaneously exposed to saline (open circle, black), 15 (Square, red) or 150 µg (open triangle, blue) of CDE and challenged with IN CDE (10µg). Airway responsiveness was assessed in MyD88$^{-/-}$ (A) and WT mice (B) mice. BAL was performed on both WT and MyD88-/- mice and resultant cells were enumerated (C) and differentiated to determine eosinophils (D). Lungs from allergen challenged mice were analyzed by flow cytometry for the number of eosinophils (E), and CD4 cells producing IL-5 (F), IL-13 (G) and IL-4 (H). Pooled data from two independent experiments presented as mean ± (SEM), n=8-10/group. *, p <0.05; **, p <0.01; ***, p <0.001 compared to saline, or between indicated group, as determined by a *Student's* t-tests and ANOVA.

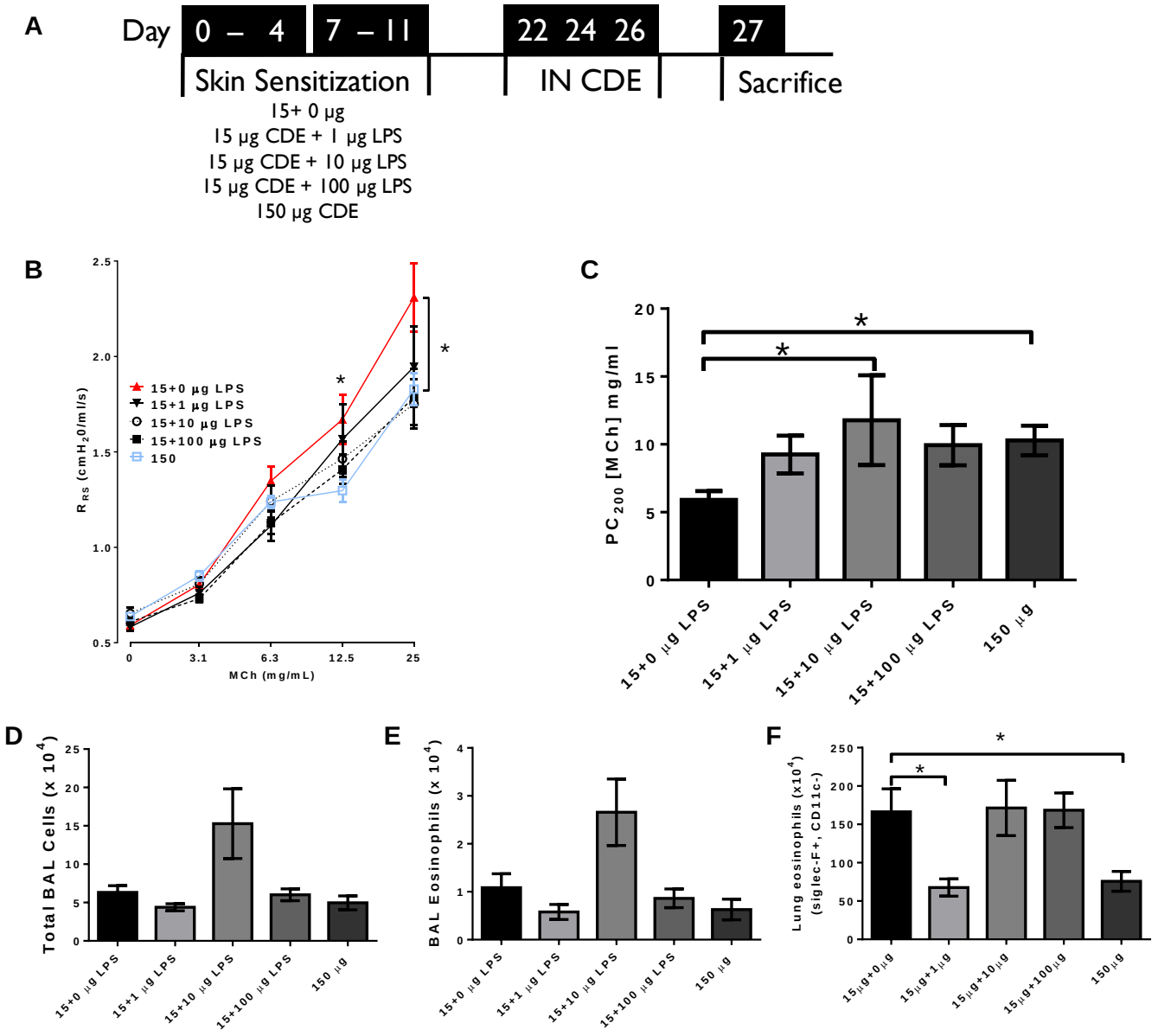

Figure 10. Addition of LPS does not prevent a Th2 polarized response.C57BL/6 mice were tape-stripped and cutaneously exposed to 15 µg of CDE, with additions of 0, 1, 10 or 100µg LPS, or 150µg CDE, then challenged with CDE intranasally (A). Airway responsiveness was examined using a nebulized MCh challenge (B), and provocative concentration causing a 200 percent increase in baseline resistance (PC_{200}) was calculated (C). Lungs from allergen challenged mice were analyzed for the number of eosinophils (D), by flow cytometry. Bronchoalveolar lavage (BAL) cells were enumerated by trypan blue staining (E) and differentiated, following Wright-Giemsa staining (F). Pooled data from two-four independent experiments presented as mean ± (SEM), n=10-20/group. *, $p < 0.05$; compared to 15+0µg LPS mice, as determined by ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

Donor mice were prepared by applying saline, 15 or 150 µg of CDE to a patch of disrupted skin for 10 days. Following allergen challenge, lung cells were obtained by physical disruption and enzymatic digestion (Figure 10), and prepared for transfer. CD4+ cells were enriched using negative separation kit (Miltenyi Biotec) to between 60-70% of cells (data not shown), and CFSE labelled. CD4+ enriched lung cells were transferred into mice primed with a sensitizing dose of CDE on the skin (15µg; Figure 12). After transfer and intranasal CDE challenge, a distinct population of CFSE+ CD4 T-cells were identifiable in the lungs of recipient mice (Figure 13A) indicating successful engraftment.

All recipient mice were epicutaneously sensitized to cat dander, via application of 15µg CDE. Each recipient received an entire donor mouse's worth of CD4+ enriched lung donor cells. The transfer of cells from mice receiving 150 µg CDE did not exacerbate, nor protect recipient mice from AHR (Figure 13B). In contrast, mice receiving cells from donor mice previously exposed to 15 µg CDE, possessed significantly increased AHR following allergen challenge, demonstrating that the transfers had led to successful engraftment capable of manipulating recipient mouse immunity. Inflammation following CDE IN challenge of recipient mice was quantified by flow cytometry. Recipient mice receiving naive (i.e.: saline exposed) CD4+ cells mounted a robust eosinophilic response. In fact, the transfer of naive CD4+ cells enhanced lung eosinophilia by 25%, compared to eosinophilia observed in sensitized HLA-DR4 mice without additional transferred cells (Figure 13B). Recipient mice receiving cells from mice epicutaneously sensitized with 15µg did not mount a significantly enhanced eosinophilic response compared to recipient mice that received naive cells, although a trend towards a 50% increase was noted. The transfer of cells from mice that were exposed to high dose CDE (150 µg), did not prevent the development of eosinophilia after airway allergen challenge (Figure

13C). IL-4 production by lung derived T-cells was reduced by transfer of cells from 150 µg CDE primed mice (Figure 13D).

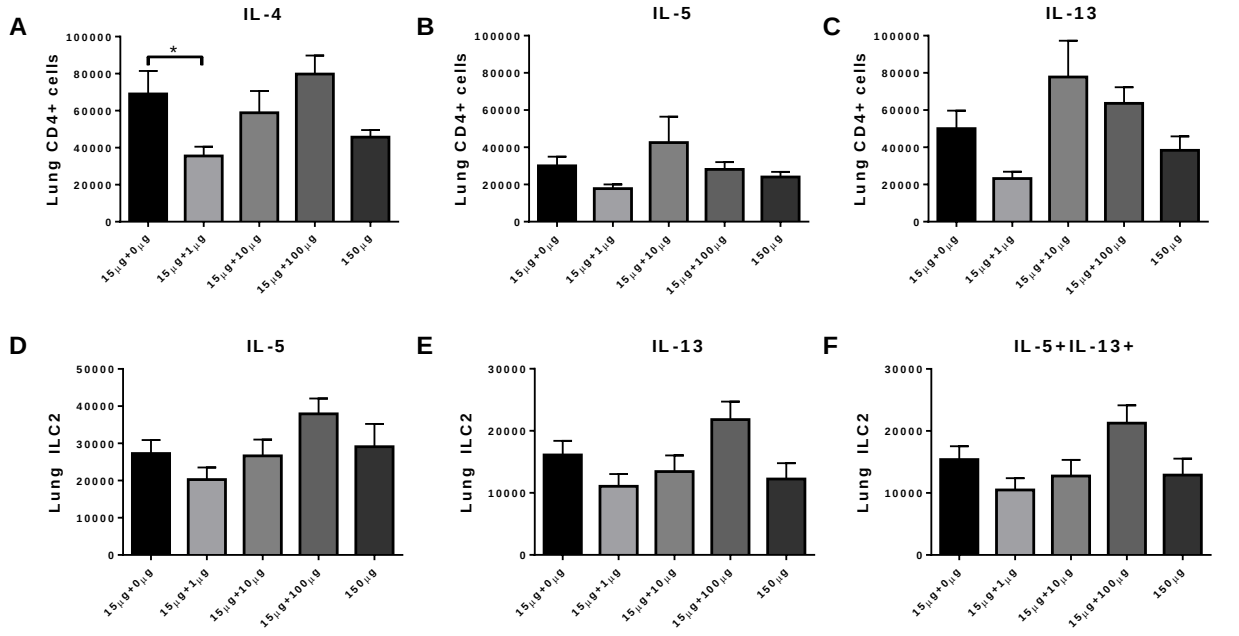

Figure 11. Addition of LPS to cutaneously applied CDE modestly lessens Th2 cytokine expression in T-cells.C57BL/6 mice were cutaneously exposed to 15 µg of CDE, with additions of 0, 1, 10 or 100µg LPS or 150µg CDE. Following intranasal CDE challenge, animals were sacrificed and a single cell-suspension was purified from the lungs. IL-4(A), IL-5(B) and IL-13(C) expression by lung CD4+ T-cells was assessed by flow cytometry. ILC2, identified as lineage negative, T1ST2 positive cells, were assessed for the production of IL-5(D), IL-13(E) and coexpression of IL-5 and IL-13(F) expression, by flow cytometry. Cytokines were assessed after a 6 hour stimulation with leukocyte activation cocktail (BD Biosciences. Pooled data from two independent experiments presented as mean ± (SEM), n=10/group. *, p <0.05; between indicated groups, as determined by ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

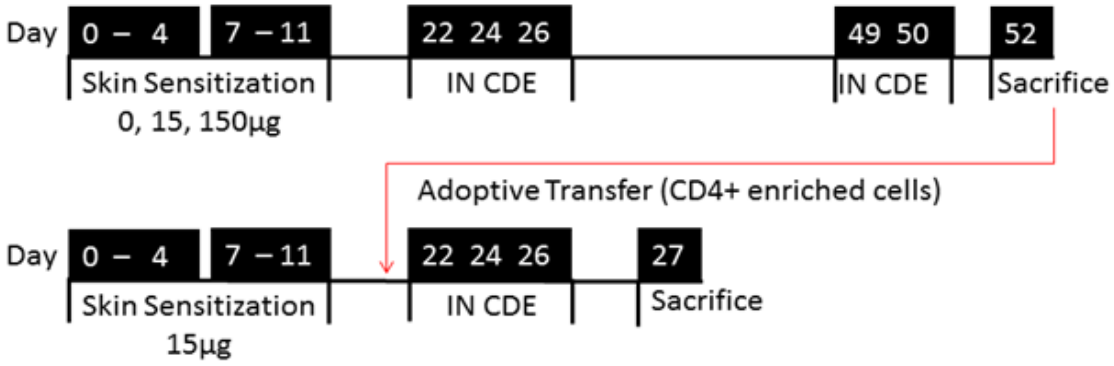

Figure 12. Schematic representation of the adoptive transfer experiment. HLA-DR4 transgenic mice were utilized

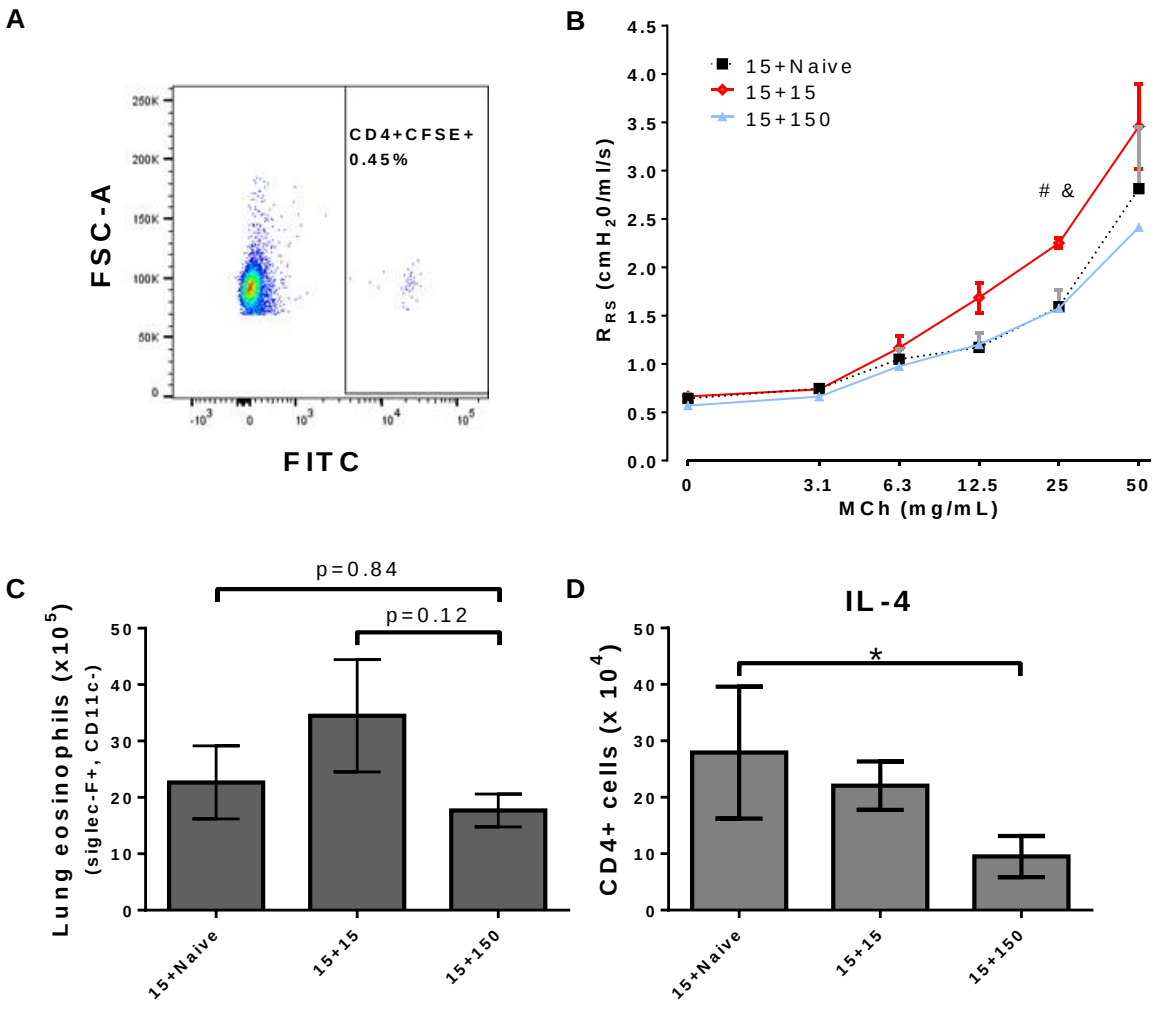

Figure 13. Transfer of CD4+ enriched lung lymphocytes from high-dose CDE exposed mice does not protect against the development of a Th2 polarized response.Donor mice were cutaneously exposed to saline, 15 or 150µg of CDE, then challenged intranasally with CDE. Lungs were collected, CD4+ cells enriched by negative depletion and stained with CFSE prior to transfer into recipient mice. Recipient mice were sensitized with 15 µg of CDE and received a transfer of one donor mouse's worth of CD4+ enriched lung cells prior to a series of IN CDE challenges. CD4+ CFSE+ donor cells identified in the lungs of recipient mice (A). Airway responsiveness of recipient mice, post transfer and allergen challenge, as assessed by a nebulized MCh challenge (B). Resultant eosinophil recruitment (C) and IL-4+ production by CD4+ cells (D), in the lungs of recipient mice after challenge, assessed by flow cytometry. Data from a single experiment presented as mean ± (SEM), n=5/group. *, p <0.05, between indicated groups; #, p <0.05 between 15+15 vs 15+Naïve; &, p <0.05 between 15+15 vs 15+150, as determined by ANOVA and post-hoc Bonferroni corrected *Student's* t-tests.

High-dose house dust mite does not impair the development of Th2 responses

We sought to examine whether high-dose induced immune paralysis was a unique feature of CDE, or was a feature of high-dose exposure to any allergen. Thus, we exposed mice to a high dose of another common aeroallergen, house dust mite (HDM). We hypothesized that, if high-dose exposure to an any allergen is sufficient to impair sensitization/induce antigen-specific tolerance, that high-dose HDM would not sensitize mice. In 2008, Llop-Guevara *et al*., administered increasing intranasal doses of HDM to mice for 10 days, noting that allergic inflammation, as assessed by eosinophilic BAL, plateaued at exposure to 25µg HDM, failing to increase with higher doses (50, 100µg)[53]. In contrast to eosinophilia, analysis of IgG1 revealed that increasing-doses of HDM increased greater sensitization, as mice receiving 50µg HDM possessed greater levels of IgG1. Intriguingly, IgG1 titers also indicated the possibility that high-dose IN HDM could be protective, as mice exposed to 100µg HDM had a slight reduction in antibody titers.

To test our hypothesis, we administered high doses of HDM (0, 25, 75 and 225µg) to animals for 10 days, then recalled the allergic response two-weeks later with a normalized concentration of HDM (15µg; Figure 14A). Sham sensitized animals challenged with HDM failed to develop eosinophilic lung inflammation (Figure 14B). In contrast, all animals exposed to HDM intranasally were primed to develop airway eosinophilia upon subsequent recall challenge. Similarly, while sham sensitized animals develop minor goblet cell hyperplasia upon HDM airway challenge, all animals previously exposed to HDM developed markedly increased mucin production (Figure 14C). Thus, early exposure to high-doses of HDM (75, 225µg) did not protect mice from the development of profound allergic inflammation upon subsequent recall challenge.

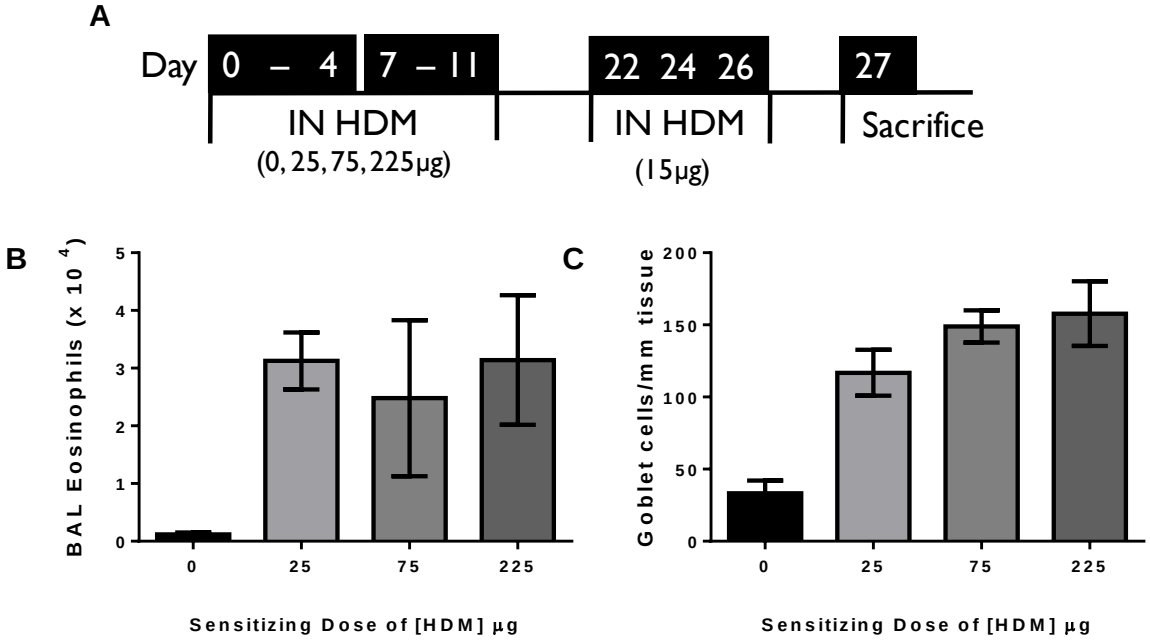

Figure 14. Mice exposed to high-dose house dust mite do not possess impaired airway inflammation upon allergen rechallenge.BALB/c mice received 0, 25, 75 or 225µg of house dust mite (HDM) intranasally for 10 days, then were rechallenged with 15µg HDM intranasally on day 22, 24 and 26 (A). 24-hours post final challenge, animals were sacrificed and eosinophils in the bronchoalveolar lavage (BAL) were enumerated following Wright-Giemsa staining (B). Goblet cell hyperplasia was assessed by Periodic Acid Schiff staining of histologically prepared lungs (C). Data from a single experiment presented as mean ± (SEM), n=5/group.

5.4. Discussion

We set out to determine whether application of CDE to barrier-disrupted skin could sensitize mice. We demonstrated that cutaneous exposure to an 'intermediate' dose (15µg) of CDE induced robust Th2 polarization in both HLA-DR4 and C57BL/6 mice. Intriguingly, we also observed that the dose of CDE was critical to sensitization, despite animals receiving the same degree of shaving and tape-stripping (i.e. 'danger' stimuli). Application of high-dose CDE to a patch of barrier impaired skin failed to induce an allergic response. The mechanism of high-dose CDE induced immune paralysis was probed using a kinetic study, removal of TLR ligand responsiveness, addition of LPS and transfer of CD4+ T-cells. These experiments were unable to

define the precise mechanism through which high-dose CDE functions; however, several conclusions can be drawn from these experiments: i) Epicutaneous sensitization is dependent upon MyD88 signaling, ii) it is unlikely that high-dose CDE induces allergen specific tolerance and iii) allergic hyporesponsiveness following high-dose exposure may not develop during exposure to highly allergenic allergens such as house dust mite.

The application of allergen to a patch of skin disrupted by shaving and/or tape-stripping has been shown to cause epicutaneous sensitization for many allergens. For instance, application of the model allergen ovalbumin (OVA) to shaved patches of skin on BALB/c mice has been demonstrated to induce potent OVA-specific Th2 responses, comprised of allergen-specific IgE production and IL-4 and -5 production by T-cells[54]. Upon intranasal challenge, animals epicutaneously sensitized to OVA mount eosinophilic inflammation in the BAL and lung parenchyma, as well as airway hyperresponsiveness to methacholine[55, 56]. Epicutaneous sensitization has also been demonstrated following the application of HDM to the intact-skin of mice[57] and dogs[58] as well as peanut to a patch of skin lacking an intact stratum corneum[5, 6]. Presumably the enzymatic activity of HDM, through degradation of tight junctions[59], sufficiently impairs stratum corneum function to facilitate sensitization. Consistently with this, we have, for the first time, demonstrated that application of cat dander to a patch of disrupted skin induces epicutaneous sensitization capable of priming mice to develop allergic airway inflammation and AHR upon nasal allergen challenge.

It has previously been suggested that high-dose exposure to an allergen may be protective. A cross-sectional study of 12-14 year old children demonstrated that children exposed to high doses of cat (defined as above >23µg Fel d 1 at home) failed to become sensitized[60]. Corroborating evidence was later provided by the German Multicentre Allergy Study (MAS

cohort), as the majority of children exposed to a high-dose of cat dander possessed cat specific IgG, but not IgE and had no history of wheeze or AHR[23]. Clinical tolerance to cat, following high-dose exposure, is referred to as a 'modified-Th2 response' has been attributed to an exaggerated IL-10 response that is superimposed upon a typical Th2 cytokine response, together resulting in IgG1 and 4 production, rather than IgE[34]. Evidence indicating that IL-10 mediates the modified-Th2 response is derived from a subset of patients, those with the HLA DR7 allele, as T-cells from these patients produced exaggerated amounts of IL-10 during *in vitro* stimulation with a Fel d 1 derived T-cell epitope (Peptide 2:1), compared to allergic controls[34]. In the same study, however, peripheral blood mononuclear cells (PBMCs) from cat allergics and modified allergics, with undefined HLA haplotypes, were compared and PBMCs from modified allergic patients were found to predominantly respond to Fel d 1 *in vitro* provocation with increased production of IFN-γ, decreased IL-5 and surprisingly, decreased IL-10 production. Thus, while the clinical presentation of a modified-Th2 response is consistent with a perceived role of IL-10 (increased IgG, reduced IgE production, reduced T-cell responsiveness), the direct evidence supporting a role for IL-10 is restricted to a single HLA haplotype. In light of the importance of HLA-DR7, it is unsurprising that our HLA-DR4 mice did not possess enhanced IL-10 production, following high-dose CDE exposure, despite developing CDE hyporesponsiveness. It is intriguing to consider that 'modified' type 2 responses may be heterogenous, with the dominant mechanism evolving as a result of MHC presentation of Fel d 1 derived epitopes.

While little work has been done investigating the effect of epicutaneous allergen dose on airway inflammation, some studies have examined the effect of allergen dose given directly to the airways. In 1999, Saxon and colleagues demonstrated that diesel exhaust particles (DEP), administered nasally, could act as an adjuvant and drive *de novo* sensitization to nasally

administered keyhole limpet hemocyanin (KLH)[61]. The provision of DEP with KLH induced KLH-specific IgE and increased IL-4 in the nasal lavage. In contrast, when the dose of KLH was titrated upwards, in the presence of DEP, sensitization was inhibited. Indeed, administration of 0.1, 10, 1000 or 100,000µg of KLH in the presence of DEP, led to allergic sensitization in 0, 100, 57 and 11% of subjects, respectively[62]. Taken together, our results align with the previous literature as we have demonstrated that an allergen applied at high-enough doses to the skin can inhibit the development of sensitization, furthering our understanding of high-dose allergen exposure. Importantly, high-dose allergen trumped the presence of inflammatory 'danger' stimuli evoked by tape-stripping.

Not all allergens appear to function in this manner. For instance, it has been demonstrated that increasing the dose of HDM administered intranasally by 100-fold (two log units) induces more robust Th2 polarization[63, 64]. Similar results have been found by others[53, 65]. Our preliminary work agrees with this, as high-dose HDM did not inhibit type 2 polarization, despite employing doses 2.25 times as high as those previously reported. Furthermore, unlike high-dose exposure to CDE, high dose exposure to dust mites does not correlate with the development of a modified-Th2 response[66], rather it correlates with increased mite specific IgE, asthma and wheeze[67]. Similarly to HDM, increasing the amount of cockroach allergen administered to guinea-pigs directly correlates with the degree of sensitivity[68]. The possible presence of high-dose 'tolerance' or impairment is less-clear for other, less potent, allergens. The administration of OVA precipitated with alum to C57BL/6 mice revealed that low-dose OVA induced marked Th2 polarization, while administration of >1mg OVA induced Th1 polarization[69]. In contrast, titrating the dose of OVA in alum above 1mg, in BALB/c mice, yielded only exaggerated type 2 responses[69]. Similarly, cutaneous application of OVA to a patch of disrupted skin on BALB/c

mice has demonstrated a dose-dependency of the magnitude of the Th2 responses. Titration of cutaneously applied OVA from 1µg to 10mg revealed that higher doses of OVA induced significantly greater amounts of IgE, T-cell derived IL-4 and T-cell proliferation, with low-doses (below 10µg) of OVA failing to discernibly sensitize mice[70]. Together, these studies indicate the inability of high-dose OVA to mimic the effects of high-dose CDE. Titration of fungal allergens in mice revealed that low-dose exposure induced Th2, eosinophilic lung inflammation but that high-doses of fungal allergen deviated the response towards Th1/neutrophilic inflammation[71]. Interestingly, while this conversion of the immune-response through upwards dose-titration does mimic our findings, we found that high-dose exposure yielded allergen hyporesponsiveness, not Th1-skeweing. Taken together, it appears as though cat dander may be unique amongst common allergens in its ability to impair sensitization at high-doses, although further studies are needed to investigate other aeroallergens, such as birch, grass, ragweed, etc. and routes of exposure.

In our efforts to identify the mechanism of high-dose Th2 impairment, the role of MyD88 was investigated. MyD88 is a signal transduction molecule of the toll/IL-1 receptor (TIR) adaptor family that is critical to the induction of innate immune responses following exposure to microbial components, such as LPS[39]. To date, the role of MyD88 during epicutaneous sensitization has not been thoroughly investigated. In 2007, Geha and colleagues proposed that TLR ligation may play a role in epicutaneous sensitization to OVA, as MyD88$^{-/-}$ mice had reduced skin IL-17 expression compared to wild type mice; however, inflammation in allergen exposed MyD88$^{-/-}$ mice was significantly higher than in saline challenged animals[55]. Recently, preliminary data were presented by Ziegler and colleagues suggesting that MyD88 is responsible for ~70% of epicutaneous sensitization following cockroach allergen application to the skin of mice (American Academy of Allergy, Asthma and Immunology 2016 annual meeting, session 2803). Here, we corroborate and

extend these findings, through the demonstrating that MyD88 is required for epicutaneous sensitization to cat dander extract.

We hypothesized that contamination of CDE with bacterial components, such as LPS, may be the critical factor that impaired immune polarization at high-dose exposure. Previously, it has been demonstrated that low-dose LPS exposure facilitates Th2 responses, whereas high-dose LPS predisposes towards Th1 immunity[38]. LPS, and other TLR ligands, are known to induce TSLP release by dendritic cells and keratinocytes, a cytokine required for epicutaneous sensitization[72-75]. In our experiment, doses of LPS demonstrated by others[76, 77] to induce skin inflammation, were we applied to the skin of mice. The doses chosen were also in-line with the measured amount of LPS in the CDE extract. While skin inflammation due to LPS was not assessed, a notable reddening and increased number of lesions on the skin of mice receiving high-dose LPS was noted at the site of application. In contrast to studies of intranasal administration of allergen plus LPS[38], the addition of high-dose LPS to cutaneous allergen exposure did not abrogate the development of type 2 polarized, allergen specific responses. Whether other TLR ligands implicated in skin inflammation (TLR3, TLR5, etc.) are important to the observed high-dose hyporesponsiveness warrants further investigation.

The ability of high-dose CDE to induce immune hyporesponsiveness was investigated by adoptively transferring T-cells, from donor mice cutaneously exposed to 150µg CDE into CDE allergic recipients. We confirmed successful engraftment by labelling donor cells, and identifying them in the lungs of recipient mice following allergen challenge. Moreover, cells from donor mice exposed to a sensitizing dose of CDE (15µg) exacerbated allergic responses in recipient mice, demonstrating that the engraftment seeded sufficient donor cells in the lungs of recipient mice to alter the immune response to CDE. If high-dose CDE induced regulatory T-

cells, the transfer of these CD4+ CDE-specific regulatory cells should suppress subsequent type-2 responses in recipient mice. In our study, CDE induced eosinophilia in recipient mice was not ameliorated by transfer, suggesting that regulatory T-cells may not evolve following high-dose CDE exposure. A limitation of our approach was that the number of transferred cells was not titrated, thus we may have simply transferred insufficient cells to observe the development of tolerance. With this limitation in mind, our approach was to transfer as many cells into recipient mice as possible, so as not to lose cells during a titration, this approach led to the transfer of a relatively high number of cells that, in other studies, has been sufficient to establish tolerance in the recipient mouse. For instance, in 2005, the transfer of $5x10^5$ OVA-specific Tregs was found to ameliorate OVA-specific allergic airway responses[78]. Elsewhere, the transfer of 10^6 Treg enriched CD4 cells abrogated OVA-induced T-cell activation and IgE upregulation, following OVA challenge of recipient mice[79]. While our study did not transfer exclusively CDE-specific T-cells, we transferred nearly 4-8 times as many T-cells. Regarding the transfer of non-antigen specific T-cells, the transfer of $2x10^6$ CD4 cells derived from donor mice that received killed *Mycobacterium vaccae*, a treatment that induced IL-10 producing Tregs, protected sensitized recipient mice from the development of OVA-induced allergic airway inflammation[80]. Taken together, we are confident that the methods employed yielded a successful engraftment of sufficient T-cells to have seen a protective response, had one been present as i) donor cells were found in the lungs of recipient mice, ii) the number of CD4+ cells transferred was capable of exacerbating existing CDE immunity and, iii) the number of cells transferred was in line with previous adoptive transfer experiments that successfully demonstrated a protective effect.

Interestingly, the results from our kinetic analysis reveal that high-dose CDE exposure is not ignored by the immune system, as CDE-specific T-cells and IL-4 production were enhanced.

Thus, high-dose CDE may be inducing an immune response that is self-terminating, perhaps in a mechanism like OVA-tolerance. IL-10 expression by DCs has been demonstrated to be critical to the evolution of OVA inhalation tolerance[81]. Thus, future attempts to examine the mechanism of high-dose CDE immune hyporesponsiveness should investigate the production of IL-10 by structural cells and DCs in the area of allergen exposure.

The atopic march is a theory that has evolved recently to explain the evolution of type 2 inflammatory diseases in children and the general population. Specifically, the atopic march refers to the natural history of atopic disease within children, suggesting that children that display symptoms of atopic dermatitis (AD) at a young age are at an increased risk to develop allergies later in life, and possibly asthma[24]. Clinical, epidemiological and basic research have coalesced to substantiate the validity of the atopic march. A longitudinal prospective birth cohort followed 1,314 infants and observed that 50% of children with AD by 2 years of age, would go on to develop asthma or rhinitis[82]. Elsewhere, a progression rate from AD to asthma/rhinitis of 35.8% has been determined, translating to an odds ratio of 2.14[83]. A direct link between AD and asthma was demonstrated by Flavell and colleagues. Mice over expressing Th2 cytokines spontaneously developed AD and airway inflammation comprised of eosinophilic infiltration and goblet cell hyperplasia[84], thereby highlighting the shared aetiology of these diseases.

The aetiology of the atopic march is poorly defined. A common hypothesis suggests that early barrier impairment in children (by soap, inherent skin deficiencies, etc) facilitates allergen entry through the skin, epicutaneous sensitization and eventually atopic dermatitis. As previously discussed, patients with atopic dermatitis, but not asthma, commonly have IgE-specific to cat[25]. Cat, while commonly considered an aeroallergen, is found at high-concentrations within dust

samples from beds, day-cares and schools[18-20], thus, people likely encounter CDE through the skin in addition to through the airways. Here, we show that CDE exposure at a sight of impaired barrier function can lead to allergic sensitization. Given that CDE is measured in dust samples, and HDM can degrade barrier function, it is intriguing to consider a series of events wherein house dust degrades barrier function, perhaps enhanced by the frequent use of detergents on the skin, allowing CDE entry through the skin that causes sensitization, atopic dermatitis and eventually, asthma. Moreover, our observations provide a putative mechanism of allergen-exposure that could explain why high-dose exposure to cat, in people, is linked to a 'modified' Th2 response and not the development of allergies.

To conclude, the application of CDE to barrier impaired skin was observed to cause epicutaneous sensitization. In contrast, high-dose CDE exposure leads to a form of immune hyporesponsiveness, that develops after early immune activation. Finally, MyD88 is required for epicutaneous sensitization.

5.5. Reflection & Relation to the Remainder of the Thesis

Chapters 4 and 5 describe models of cat allergy in HLA DR4 transgenic mice. These models were developed to be utilized to investigate the role of peptide affinity to the HLA DR4 MHC Class II molecule. Intriguingly, neither prophylactic nor therapeutic administration of peptides could ameliorate allergic lung inflammation in epicutaneously sensitized animals (data not shown). As a result of this, the peptide affinity research (Chapter 6) was performed using the model developed in Chapter 4. The work presented in this chapter ties into the theme of the thesis, because it was a model designed to enable study of peptide immunotherapy.

During the development of the model, we observed that mice exposed to

high-dose CDE developed markedly less allergic airway inflammation compared to animals exposed to intermediate doses. We set out to assess whether this observation modeled the modified-Th2 response, as set forth by Thomas Platts-Mills. We hypothesized that, like the modified-Th2 response, high-dose CDE was inducing IL-10 production by regulatory T-cells. Early experiments supported this hypothesis, demonstrating increased IL-10 production by CD4 cells, following exposure to high levels of CDE, however, following several repeats, this signal dissipated (Figure 5F). Subsequent studies were designed to investigate whether high-dose exposure was instilling allergen specific tolerance. The kinetic study, adoptive transfer experiment, and original model development collectively provide evidence that exposure to high-dose CDE is not instilling a tolerogenic signal. During these investigations, the potential for contamination of CDE extracts with TLR ligands was considered, and experiments were designed to both 'knock-out' TLR responsiveness (via MyD88 deficient animals) and 'knock-in' additional TLR signaling, through the supplementation of additional LPS. Collectively, while these experiments demonstrated that TLR ligands contamination is likely not the root cause of the high-dose hyporesponsivness that has been demonstrated, while also demonstrating that epicutaneous sensitization, to CDE, is dependent upon MyD88. Thus, the studies described here evolved from addressing a core component of the thesis, to attempting to define the mechanism of high-dose hyporesponsiveness. While this mechanism remains unclear, several plausible mechanisms have been investigate and the experiments performed form a foundation for future studies.

5.6. References

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Chapter 6: Inhibition of Allergic Inflammation by Peptide Immunotherapy is not Solely Dependent upon a Peptide's Affinity to the Presentation Molecule

6. Declaration of Academic Achievement

Chapter 6. Inhibition of allergic inflammation by peptide immunotherapy is not solely dependent upon a peptide's affinity to the presentation molecule

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- The following study will be submitted to Allergy, once Chapter 3 is accepted.
- Rights will be retained at submission.

Daniel Moldaver's contributions

As primary author, I was responsible for conceiving, planning, managing and contributing to all aspects of the study. I treated animals, performed mouse dissections, tracheostomies and MCh challenges, fixed tissues, performed histology, prepared cells for flow cytometry and proliferation assays, performed flow cytometry and ELISAs. M. Inman & M. Larche contributed to study design. T. Singh and I. Nayve assisted with the blinded quantification of histology & processing of tissues for flow cytometry. C. Rudulier & M. Bharhani assisted with flow cytometry. J. Wattie was responsible for mouse tracheostomies, and methacholine challenges. M. van Hage provided the cat dander extract. Analyses, statistics and figure generation were performed by myself. Manuscript preparation was performed by me, with guidance from M Larché.

Chapter 6: Inhibition of allergic inflammation by peptide immunotherapy is not solely dependent upon a peptide's affinity to the presentation molecule

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6.1. Abstract

Peptide immunotherapy is an effective, disease modifying treatment option for allergic diseases that consists of administering synthetic allergen derived peptides to induce T-cell hyporesponsiveness. Peptide recognition by T-cells is dependent upon peptide presentation within major histocompatibility complex (MHC) class II molecules. To maximize the population coverage of clinical peptide based therapeutic strategies, peptides are chosen to promiscuously bind many MHC Class II molecules. This strategy has limited the ability to assess the role of a peptide's affinity to any given MHC Class II molecule. Studies of autoimmune disorders have demonstrated that peptides with a high-affinity to the MHC Class II molecule possess markedly greater suppressive capacity. Here, we have utilized a mouse model of cat allergy in mice transgenic for the human MHC Class II molecule, HLA-DR4 and a series of peptides with defined affinity to the HLA-DR4 molecule to investigate the role of peptide affinity during the treatment of allergic airways disease. We demonstrate that high and moderate affinity peptides significantly ameliorate airway hyperresponsiveness (AHR), and eosinophilic lung infiltration. In general, high-affinity peptides were efficacious at low doses, while moderate affinity peptides required higher dosing (≥1μg) to evoke similar degrees of efficacy. Moreover, while low affinity peptides could not resolve AHR, they could attenuate eosinophilic inflammation. Finally, we demonstrate that the suppressive mechanism engaged by a peptide is not dependent upon peptide affinity, while defining a role for PD-1 and CTLA-4 during the establishment of tolerance following peptide immunotherapy. Taken together, our findings alleviate concerns that low-affinity peptides may subvert clinical efficacy of peptide based strategies and support the selection of peptides to maximize population coverage.

6.2. Introduction

Allergic responses are driven by polarized T-cells that respond to common environmental allergens by producing exaggerated amounts of Th2 cytokines[1, 2]. Th2 cytokines are central to the progression of allergic diseases, such as asthma. IL-4 production from polarized T-cells can induce B-cell class-switching, leading to the production of allergen-specific IgE[3, 4] that primes granulocytes in the lungs. IL-5 and -13 are known to induce lung inflammation, airway remodeling and, perhaps as a result of these functions, airway dysfunction[5-9]. Pharmacotherapy of allergic disease attempts to alleviate the end result of the presence of these cytokines, ie.: the symptoms. In contrast, immunotherapies modulate the allergen-specific T-cell response in a manner that can modify underlying disease pathology and progression[10, 11]. Immunotherapy with synthetic, allergen derived T-cell epitope peptides induces T-cell hyporesponsiveness comprised of epitope specific regulation[12, 13] and reduced Th2 polarization[14, 15].

Recently, this approach has been employed for the treatment of patients with cat allergy. Intradermal administration of seven synthetic peptides derived from the primary cat allergen, Fel d 1 was found to be well tolerated[16] and induce a persistent[17], significant, reduction in patient symptoms[18]. The seven peptides employed in these trials were chosen for their ability to promiscuously bind many human major histocompatibility complex (MHC) class II, in an attempt to circumvent issues that arise from the profound genetic diversity found in antigen presentation molecules.

To date, the role of peptide dose and affinity to the MHC class II molecule during immunotherapy have been predominantly researched in models of models of autoimmunity. Indeed, peptide therapy using analogs of the encephalitogenic amino-terminal myelin basic

protein (MBP) peptide Ac1-9, revealed that analogs with increasing affinity to the mouse antigen presentation molecule (H-2^{u}) were better able to prevent the onset of and treat existing experimental autoimmune encephalomyelitis (EAE)[19]. In a later study revisiting treatment of EAE with peptides, high-affinity peptides given to animals at high-doses were best able to protect mice from disease progression[20]. Treatment of another autoimmune disease has revealed similar results. Nonobsese diabetic (NOD) mice are prone to the development of autoimmune type 1 diabetes. In these mice, disease progression is driven by a loss of T-cell tolerance towards the islet cell antigen p69 (ICA69) caused by the expansion of pathogenic T-cells recognizing the Tep69 T-cell epitope (peptide ICA69$^{(36\text{-}47)}$)[21]. Treatment of NOD mice with a the Tep69 epitope does not slow disease progression, rather exacerbating autoimmunity[22]. ABBOS is a T-cell epitope peptide, recognized by Tep69 specific T-cells that possess markedly higher efficacy to the NOD mouse MHC Class II complex (I-A^{g7}). Treatment of NOD mice with a high-dose of ABBOS peptide protects mice from the development of diabetes[22]. Therefore, examination of autoimmune diseases has suggested that peptide immunotherapy using peptides at a high-dose that possess high-affinity to the presentation molecule is critical to efficacy. Problematically, in these models, treatment with low-affinity peptides or high-affinity peptides at a low-dose has been linked to disease exacerbation or, at least, a paucity of efficacy[20, 22]. Clinically, the degree of genetic variation in human MHC molecules and co-dominant allelic expression precludes the ability to know whether a peptide administered to a person will have ‘high’ or ‘low’ affinity to the relevant MHC complex.

Interpretation of the relevance of using high-affinity peptides to treat autoimmune diseases, to the treatment of allergic diseases is clouded by important differences in disease etiology. In the aforementioned models of autoimmunity, upwards of 90% of disease progression

can be due to a single T-cell clone. Thus, deleting this T-cells with this idiotope, through high-dose peptide administration is an attractive, effective therapy. In contrast to this, T-cell recognition of allergen is rarely driven by a single T-cell clone. Indeed, in a study using T-cells from the peripheral blood of 53 cat-allergic patients, T-cell epitopes were found to span the entire Fel d 1 molecule[23]. Thus, nominating a single T-cell idiotope for deletion, that could provide protection to the entire population is likely impossible.

Efficacious peptide immunotherapy for the treatment of allergies has been linked to the evolution of regulatory T-cells, that can actively suppress allergen-specific responses[12, 13]. Moreoever, we have demonstrated that peptide immunotherapy induces active immune suppression mediated by regulatory T-cells, not deletional tolerance, that effect tolerance through the spread of regulation to both linked[24] and related antigens[25]. The spread of tolerance following peptide administration is likely the dominant mechanism that affords treatment with T-cell epitopes the ability to suppress responses to complex mixtures of allergens.

In light of the difference between allergic and autoimmune disease pathogenesis, lessons from autoimmunity advocating the use of high-dose, high-affinity peptides to delete specific T-cell clones may not apply to the treatment of allergies. Here, we sought to investigate the relationship between peptide affinity and dose with efficacious peptide immunotherapy for allergic airways disease. Using a humanized mouse model of cat allergy, we demonstrate that peptide affinity is not the critical determinant of efficacy, rather a combination of dose and affinity predicts efficacy. Evidence supporting low-dose therapy with high-affinity peptides is presented.

6.3. Methods

Mice

Female (4-5 weeks old, C57BL/6 background) transgenic mice ((B6.129S2-*H2-Ab1*^{tm1Gru} Tg(HLA-DRA/H2-Ea,HLA-DRB1*0401/H2-Eb)1Kito) expressing the human HLA-DR4 MHC class II molecule were purchased from Taconic (Hudson, NY, USA). These animals lack endogenous (murine) MHC Class II molecules[26]. Upon arrival, mice were allowed to acclimatize for one week prior to experimental use. Mouse were maintained in an ultraclean, specific pathogen free environment throughout experiments, and had *ad libitum* access to food and water. The McMaster University Animal Research Ethics Board approved all experiments. Procedures adhered to the Guide for Humane Use and Care of Laboratory Animals.

Cat allergen exposure and peptide therapy

HLA DR4 transgenic mice were sensitized to Fel d 1 via two intraperitoneal (IP) injections of recombinant Fel d 1 (2µg per 200µl) in alum (Au-Gel-S; Serva Electrophoresis), on days 0 and 14. Allergic airways disease was initiated through intranasal (IN) administration of cat dander extract (CDE; 10µg in 25µl PBS) on days 21-25. A single peptide of high affinity (Fel d 1$^{23-38}$; Fel d 1$^{39-55}$; Fel d 1$^{\text{Chain 2:56-71}}$), moderate affinity (Fel d 1$^{1-17}$, Fel d 1$^{29-45}$; Fel d 1$^{\text{Chain 2:40-55}}$) or low affinity (Fel d 1$^{54-69}$) to the HLA DR4 molecule was administered to mice on day 34, 41 and 48. Each peptides was titrated (administered at 100, 10, 1, 0.1 or 0.01 µg). Control mice received either 100µg of a control peptide derived from influenza virus hemagglutinin (HA$^{306-318}$) or sham (diluent, PBS). After the final peptide administration, animals were challenged intranasally on day 51 and 52 with 10µg CDE and resultant airway dysfunction and inflammation were assessed 48 hours post-challenge, upon animal sacrifice (Figure 1).

Measurement of airway responsiveness

Airway responsiveness was measured through the quantification of total respiratory system resistance (R_{RS}) by the FlexiVent ventilator (SCIREQ, Montreal, Canada), following a nebulized methacholine (MCh) challenge. Animals were anaesthetized via IP injection of Xylazine (10mg/kg; Bayer inc., Toronto, ON, Canada) and sodium pentobarbital (30 mg/kg; Ceva Sante Animale, Leneka KS, USA). A blunted 18G needle was inserted into the trachea of anaesthetized mice, following a tracheostomy, that was then connected to the FlexiVent small animal ventilator. Mice were maintained with a respiration rate of 150 breaths/min, 10 mL/kg (pressure limit 30 cmH_2O) and a positive end expiratory pressure of 2 cmH_2O. Upon mechanical ventilation, respiratory effort was halted to prevent uncontrolled interference, by pharmacological paralysis (20 mg/kg pancuronium; Santoz, Boucherville, QC, Canada) administration IP. Nebulized MCh doses (Saline, 3.125, 6.25, 12.5, 25 and 50 mg/mL) were administered to mice. Data was normalized by an inflation to total lung capacity prior to the commencement of the nebulized challenge and between each dose. The Flexivent ventilator collected airway resistance data via using "QuickSnap-150" perturbations. At the completion of the nebulized challenge, mice were sacrificed by exsanguination and tissues were collected.

Bronchoalveolar Lavage

Two aliquots of 250 μL of phosphate-buffered saline were injected into the lungs, with the thoracic cavity intact. Each aliquot was injected and retrieved through the 18G tracheal cannula and allowed to steep for ~5 seconds, while the chest was massaged. Bronchoalveolar lavage (BAL) fluid aliquots were pooled and centrifuged (150*g* for 10 minutes). BAL supernatant was stored (-20ºC). BAL cells were enumerated by trypan blue staining and spread

onto a microscope slide for differentiation, by cytocentrifugation (Cytospin 3 centrifuge; Shandon scientific, Sewickley, PA, USA). BAL cells were differentiated by Wright-Giemsa (Sigma Aldrich) staining and morphological characteristics by a blinded investigator, into: macrophages, eosinophils, neutrophils or lymphocyte. A total of 400 cells were counted per mouse.

Lung and Lymph node sample preparation

Mouse lungs were resected, following perfusion with PBS containing heparin (10 units/ml). The right lung was minced, and enzymatically digested for 90 minutes at 37^{o}C on an orbital shaker. Enzymatic digestion was performed using 50 units/ml DNase (Sigma-Aldrich) and 300 units/ml type 1 collagenase (Worthington Biochemical Corporation) in RPMI-1640 media (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) and 1% pen/strep (Invitrogen). Lung debris was removed by passing the digestion product through a 70 μm cell strainer (BD Falcon). The sample was centrifuged (300 x *g*, 10 min, 4°C), and the supernatant stored (-80°C) for later analysis. Lung cells were washed in buffered RPMI 1640 (10% FBS, 1% Pen/strep, 0.1% 2-mercaptoethanol (Sigma)), and enumerated using an automated cell-counter (Countess; Invitrogen). Lung draining lymph nodes (DLN), mediastinal and intrathoracic, were resected, pooled together, mechanically disrupted by mashing through a 40 μm sieve (BD Falcon), washed and stored in supplemented RPMI-1640 media. DLN were enumerated using the cell-counter.

The resected left lung inflated (20 cmH_2O) and fixed using 10% buffered formalin, and histologically prepared by dehydration and paraffin embedding as previously described[25]. Transverse sections (3μm thick) were cut and stained with haematoxylin and eosin (H&E) and periodic acid Schiff (PAS) stains. First generation airways were examined using a digital image

analysis system (Northern Eclipse; Empix Imaging, Inc., Mississauga, ON, Canada) as described previously[27, 28].

Flow Cytometry

Fc receptors on prepared lung and DLN cells were blocked via a 30minute incubation with Fc receptor blocking antibodies (anti-mouse CD16/32; BD Biosciences). FC-blocked cells were washed with staining buffer (PBS supplemented with 2% FBS 0.1% sodium azide). Cells were stained with pre-titrated antibodies specific for cell-surface antigens for 30 minutes (4°C). Antibodies used included: PE and PerCP-CD4 (RM4-5; BD Biosciences), PerCp-CD8 (53-6.7; BD Biosciences), PE-Cy7-CD19 (1D3; eBiosciences), FITC-T1ST2 (CDJ8; MD Biosciences), PE-Cy7-CD69 (H1.2F3; eBiosciences), APC and PE-CD25 (PC61.5; eBiosciences), PE-Siglec-F (E50-2440; BD Biosciences), APC-Gr1 (RB6-BC5; eBiosciences), FITC-CD11c (HL3; BD Biosciences), PerCP-cy5.5-CD11b (M1/70; BD Biosciences), PE labelled lineage markers (CD3, CD19, CD11c, Gr1, MAR-1, Ter-119, Dx5 and CD11b; BD Biosciences), PerCP-CD45(30-F11; BD Biosciences), PE-Cy7-CD117(c-Kit; ACK2; eBiosciences) and APC-cy7 labelled Ly6A/E (Sca-1; D7; Biolegend). Surface stained cells were fixed in paraformaldehyde (1% PFA, 20 minutes) and stored in cell staining buffer till acquisition.

Cytokine and transcription factor analysis was performed following a non-specific, 6-hour activation using PMA (20ng/mL), ionomycin (1mM/mL) and brefeldin A (10 µg/mL). Stimulated cells, following surface staining, were assessed for intracellular cytokine and Foxp3 expression were determined following manufacturer's instruction (BD Biosciences & eBiosciences respectively). Briefly, cells were fix in 4% PFA, permeabilized, washed twice, stained with pre-titrated antibodies for 30 minutes, washed twice and stored in cell-staining

buffer till acquisition. Antibodies utilized include: PE-IL-4 (11B11; BD Biosciences), APC-IFN-γ (XMG1.2; BD Biosciences), APC-IL-10 (JES5-16E3; BD Biosciences), APC-IL-5 (TRFK5; BD Biosciences), A488-IL-13 (eBio13A; eBiosciences), PE-Cy7-IL-17A (eBio17B7; eBiosciences) and PE-FoxP3 (NRRF-30; eBiosciences). Data was analyzed using Flowjo (Treestar) following acquisition using a FACSCanto II (Becton Dickinson).

Data Analysis

Data from two identical experiments has been pooled and are shown as mean ± standard error of the mean (SEM). Data was analyzed through one-way ANOVAs and post-hoc, Bonferroni corrected t-tests. A $p \leq 0.05$ value was considered statistically significant.

6.4. Results

High affinity peptides, administered at low doses ameliorate airway hyperreactivity

Airway dysfunction due to allergic lung responses is commonly characterized by the establishment of airway hyperresponsiveness (AHR) to non-specific stimuli, such as methacholine or histamine. This has long been a diagnostic feature of clinical allergic asthma[29], and is a conserved feature that reproducible in mice[30]. To determine the functional impact of peptide immunotherapy with peptides of varying affinity to the HLA DR4 molecule, airway function was quantified. Specifically, following allergen challenge mice received a nebulized methacholine challenge, the respiratory system resistance was determined and the provocative dose causing a doubling in baseline resistance (PC_{200}) was calculated. A lower PC_{200} indicates increased sensitivity to MCh and the presence of airway dysfunction.

Cat sensitized, sham treated animals challenged with CDE intranasally developed significant, robust AHR compared to sham sensitized/challenged animals (Figure 2A). Naive mice (sham sensitized/challenged) tended not to respond to nebulized MCh, as 7 of 10 mice failed to double baseline resistance. Treatment of cat sensitized animals with an irrelevant peptide ($HA^{306-318}$) failed to ameliorate CDE-induced AHR (Figure S1).

Cat sensitized mice received peptide immunotherapy with a series of high, moderate and low peptides. Intradermal administration of each high affinity peptide (Fel d $1^{39-55; 23-38; C2: 56-71}$) significantly ameliorated AHR at a single dose. High affinity peptides attenuated AHR at low doses (≤1µg), with significant attenuation noted at a dose of 1 µg for Fel d $1^{C2:56-71}$, 0.01 µg for Fel d 1^{23-38} and 0.1 µg for Fel d 1^{23-38} (Figure 2A). Intriguingly, dose-responsiveness was abnormal, adhering to a sigmoidal curve, rather than a more-typical hyperbolic model. R^2 values

for the fit of a sigmoidal curve to Fel d $1^{(C2: 56-71)}$, Fel d $1^{(23-38)}$, Fel d $1^{(39-55)}$, are 0.14, 0.06, and 0.23, respectively.

In contrast to high affinity peptides, moderate affinity peptides (Fel d 1^{1-17}, Fel d 1^{29-45}, Fel d $1^{Chain\ 2:40-55}$) and a low affinity peptide (Fel d 1^{54-69}) were markedly less capable of protecting sensitized mice from the development of AHR following intranasal CDE challenge. Indeed, only the moderated affinity peptide Fel d 1^{29-45} significant attenuated AHR. A notably higher dose of the moderate affinity peptide (10µg) was required to significantly reduce AHR. The remaining two moderate affinity peptides trended towards attenuating AHR, but only at doses ≥1µg. The moderate affinity peptide capable of suppressing AHR was also best fit by a sigmoidal curve (R^2 =0.22; data not shown). Therefore, of the seven tested peptides, all high affinity and one moderate affinity were capable of suppressing AHR. Treatment with high affinity peptides demonstrated a therapeutic effect at low doses (0.01 to 1µg) while the single efficacious moderate affinity required a much higher dose (10µg). Treatment with a low-affinity peptide, even at a high-dose of 100µg, failed to affect allergen-induced AHR. Interestingly, with titration, the maximal degree of AHR suppression does not appear to vary significantly by peptide affinity.

Peptide immunotherapy is hypothesized to effect tolerogenesis by recognition of an allergen fragment in a ‘quiescent’ immunological environment that favours subsequent epitope presentation by immature antigen presenting cells. To ensure that moderate and low-affinity peptides had not been sabotaged by a persistent inflammatory response arising from the day 21-25 intranasal CDE challenges, animals were sacrificed 7, 14 and 21 days after this period. These sacrifice dates correspond closely to the dates of peptide administration (Figure S2A). Compared to sensitized mice that are rechallenged with IN CDE, mice sacrificed 7, 14 and 21 days after

CDE exposure possessed no demonstrable AHR (Figure S2A) nor lung inflammation, as assessed by bronchoalveolar lavage eosinophilia (Figure S2C). Intriguingly, in addition to a lack of inflammation 7, 14 and 21 days after CDE challenge, serum IgE was not increased at these time points nor after sensitization (Figure S2D).

High, moderate and low peptides can equivalently suppress lung tissue inflammation

In addition to airway dysfunction, allergic asthma is typified by exacerbated Th2 cytokine secretion leading to eosinophilic inflammation of the airways[31]. Similarly, eosinophilic recruitment is a common feature of mouse models of allergic airways disease that, like human disease is mediated by enhanced production of IL-5[32]. The inflammatory status of mouse lung following peptide therapy and allergen challenge was investigated by examining peribronchial eosinophilia, by hematoxylin and eosin staining of histologically prepared lungs and eosinophils within the lumen of the lung, by examination of BAL eosinophilia.. Sham treated animals and challenged animals were nearly devoid of peribronchial eosinophils (Figure 3). In contrast, sham treated, cat sensitized and challenged animals possessed a significant 25-fold increase in peribronchial eosinophils. All seven peptides (high, moderate and low) could, in a dose-dependent manner, significantly ameliorate peribronchial eosinophilia. While the maximally efficacious dose varied between each peptide, each peptide possessed a similar protective capacity.

Upon examination of the BAL, sham treated/sensitized animals possessed few eosinophils. In contrast, sham treated animals that received either an irrelevant peptide or peptide diluent developed 6 fold greater BAL eosinophilia (Figure S3). While both sham treated groups had a similar magnitude of eosinophilic infiltration, animals receiving peptide diluent were marked by a high-degree of variability within the BAL data. To account for the unpredicted

variation in BAL cell counts, comparisons were performed against mice treated with the irrelevant peptide. Similar to examination of peribronchial eosinophilia, each of the seven tested peptides significantly ameliorated BAL eosinophilia, compared to mice receiving the sham peptide ($HA^{306-318)}$. Taken together, mouse lung and lumen eosinophilia indicates that all peptides, regardless of affinity, possess a similar maximal protective capacity. Intriguingly, the dose of peptide that elicits maximal protection seem to depend on both peptide affinity and other, peptide intrinsic characteristics.

High affinity peptides provide reliable, consistent attenuation of eosinophilia at lower doses.

The titration of high-affinity peptides revealed that they did not inherently possess a greater suppressive capacity than peptides with a lower affinity. Intriguingly though, titration of high affinity peptides revealed a dose-response curve wherein therapy suppressive capacity increased with lower-doses of peptide. Titration of the high affinity peptide Fel d $1^{(23-38)}$ demonstrated variable protection at high doses (>10µg) as suppression of eosinophilia only occurred in one compartment at each dose. Treatment with 1µg, no therapeutic effect was noted. In contrast, low dose therapy with this high affinity peptide (0.1 and 0.01µg) demonstrated consistent effects, suppressing the accumulation of eosinophils in both the lung and BAL (Figure 4A and D). Similarly, titration of the high-affinity peptide Fel d $1^{(C2:56-71)}$ possessed variable effects at high doses (>10µg), but suppressed eosinophilic accumulation consistently at 1µg (Figure 4B and E). The last high-affinity peptide examined, Fel d 1(39-55) revealed robust attenuation of eosinophil accumulation at both high (100 and 10µg) and low (0.1 and 0.01µg) doses (Figure 4C and F). Thus, while high-affinity peptides tend to offer a more uniform, consistent suppressive effect at low doses, there does seem to be a degree of intrinsic peptide activity that influences the dose-response curve. Intriguingly, all high-affinity peptides possessed

a bi-phasic activity profile. For instance, the high-affinity peptide Fel d $1^{(39\text{-}55)}$ significantly reduced eosinophilia when administered at 100, 10, 0.1 and 0.01, but not 1µg. The other high-affinity peptides performed similarly, although with less efficacy at higher doses.

Peptide function depends on an intrinsic factor in addition to affinity and dose

The mechanism of peptide immunotherapy is poorly defined. Some have suggested a preferential downregulation of Th2 antigen-specific responses, without evoking a concomitant rise in antigen specific regulation[33]. Alternatively, we[24] and others[34] have demonstrated peptide immunotherapy to expand a population of T-cells that possess potent suppressive activity. Finally, models of autoimmunity have indicated the accumulation of co-inhibitory molecules, such as PD-1, may limit immune responses[35, 36]. We hypothesized that peptide dose and affinity may critically determine which mechanism of tolerance arises.

The Th2 response, following peptide therapy, was assessed by quantification of T1ST2 expression on CD4+ cells. T1ST2 is a marker of murine Th2 T-cells[37]. Approximately 1% of lung derived T-cells from sham-treated, cat sensitized and challenged mice were T1ST2 positive. Therapy with the high affinity peptides Fel d $1^{(23\text{-}38)}$ and Fel d $1^{(39\text{-}55)}$ failed to suppress the number of T1ST2 positive T-cells (Figure 5A), compared to sham treated animals. In contrast, the high-affinity peptide Fel d $1^{(C2:56\text{-}71)}$ significantly suppressed the number of Th2 polarized T-cells in the lungs of allergen challenged mice, in a dose-dependent manner. In keeping with previous observations that high-affinity peptides were generally more efficacious when administered at lower doses, suppression of T1ST2 expression waned when Fel d $1^{(C2:56\text{-}71)}$ was administered at a high dose. Similar results were observed upon the expression of T1ST2 in the draining lymph node of peptide treated and sham treated animals. Thus, despite being of a similar affinity, these peptides did not possess a similar ability to suppress the accumulation of

Th2 T-cells, indicating that peptide may employ a unique mechanism of action. Some peptides with lower affinities (Fel d 1 $^{(C2:40-55)}$ & Fel d 1 $^{(54-69)}$) were able to similarly suppress T1ST2 expression in a dose-dependent manner (Figure S4), while others (Fel d 1$^{(1-17)}$ & Fel d 1$^{(39-55)}$) were not.

Programmed cell death protein-1 (PD-1) is a co-inhibitory receptor expressed on CD4+ cells that, upon ligation by PD-L1 or PD-L2, limits CD4+ cell accumulation and activation[38]. The expression of PD-1 on CD4+ cells following peptide immunotherapy was quantified in the lungs and draining lymph nodes. Approximately 0.5% of T-cells in the lungs of sham-treated, CDE challenged mice expressed PD-1. Peptide therapy failed to affect PD-1 expression in the lungs of mice. Intriguingly, expression of PD-1 was significantly and robustly upregulated in the draining lymph nodes of mice treated with the peptides Fel d 1$^{(23-38)}$ and Fel d 1$^{(39-55)}$, compared to sham treated animals (Figure 5B). A dose-dependent upregulation of PD-1 was noted. Intriguingly, the magnitude of PD-1 upregulation by Fel d 1$^{(23-38)}$ therapy was greater than treatment with Fel d 1$^{(39-55)}$. In contrast, Fel d 1$^{(C2:56-71)}$, the peptide that suppressed T1ST2 expression, was unable to impact PD-1 expression. Of the moderate and low-affinity peptides, only therapy with Fel d 1$^{(1-17)}$ was similarly capable of enhancing PD-1 expression (Figure S5).

Expansion of regulatory T-cells has been theorized to contribute to the efficacy of peptide immunotherapy. FoxP3 expression, a marker of natural Tregs[39], and expression of the suppressive cytokine IL-10, a marker of inducible regulatory T-cells (Tr1)[40] were quantified. In the lungs of peptide treated mice, neither IL-10 nor FoxP3 expression was increased (Data not shown). In the draining lymph nodes, FoxP3 expression was significantly enhanced following low-dose therapy with Fel d 1$^{(39-55)}$ (Figure S6). Therapy with Fel d 1 (23-38) and Fel d 1(C2:56-71) failed to upregulate FoxP3 in the DLN of allergen challenged mice, compared to sham

treated mice. Cytotoxic T lymphocyte antigen 4 (CTLA-4) is an inhibitor marker expressed on T-cells[38] that is critically linked to the functional capacity of regulatory, FoxP3+ T-cells[41]. While the total number of FoxP3 positive cells in the DLN was not changed following therapy, we investigated whether peptide administration enhanced the suppressive capacity of existing Tregs, through quantification of CD4+FoxP3+CTLA-4+ cells.

FoxP3 expression in the lung draining lymph nodes was also assessed. CTLA-4 was expression was low in sham treated mice. Peptide therapy with Fel d $1^{(23-38)}$ at any dose, and low-dose therapy with Fel d $1^{(39-55)}$ significantly and robustly enhanced the number of CTLA-4 positive FoxP3+ T-cells (Figure 5C). Therapy with the moderate affinity peptide Fel d $1^{(1-17)}$ at doses ≥1µg similarly increased the frequency of CTLA-4 expressing FoxP3+ Tregs in the DLN (Figure S7). Intriguingly, therapy with the high affinity peptide Fel d $1^{(C2:56-71)}$ significantly downregulated CTLA-4 expression at all tested doses.

Taken together, peptides of a similar affinity tend to possess a unique activity profile. Peptide affinity, nor dose, is an accurate predictor of which suppressive mechanism will be engaged by therapy, however, affinity and dose are predictors of the efficacy of therapy.

Peptide immunotherapy with a high affinity peptide can suppress the accumulation of ILC2s

Since the identification of type 2 innate lymphoid cells (ILC2s) in 2010[42], they have been implicated as important cells contributing to the elaboration, and potentiation of allergic lung responses[43-45]. ILC2s were identified as cells devoid of lineage markers (CD3, CD19, CD11c, Gr1, MAR-1, Ter-119, Dx5 and CD11b) that express T1ST2 and the lymphocyte marker, CD45. Sham sensitized mice, challenged with saline had few ILC2s in the lung (less than 1,000). ILC2s significantly accumulated in the lungs of sensitized, sham treated animals following CDE challenge. Treatment with the high-affinity peptide Fel d $1^{(23-38)}$, significantly attenuated ILC2

accumulation in a dose-dependent manner (Figure 6). The suppressive effects of Fel d $1^{(23\text{-}38)}$ waned at high doses, as mice treated with 100µg were not protected from ILC2 accumulation whatsoever. Surprisingly, no other peptide at any tested dose impacted the accumulation of ILC2s. Thus, peptide therapy appears to be capable of ILC2 suppression; however, this may be a feature unique to some peptides.

6.5. Discussion

Peptide immunotherapy has a long history of ameliorating immune responses through the induction of T-cell hyporesponsiveness. Here, we demonstrate that several peptides derived from Fel d 1 can each independently attenuate markers of allergic lung disease. Perhaps more importantly, these peptides, despite possessing vastly different MHC Class II binding properties, were all found to be equally capable of suppressing allergic inflammation. Finally, high-affinity peptides were demonstrated to effectively treat allergic inflammation and reduce airway dysfunction when administered at very low doses (0.01µg).

Peptide immunotherapy was originally discovered through the observation that peptides given to neonatal mice rendered them unresponsive to that peptide during adulthood[46]. This phenomenon was transitioned to a therapeutic by administering MBP derived peptides to regulate EAE[47, 48]. Peptide immunotherapy for the treatment of autoimmune diseases is intuitive, as autoimmune diseases commonly evolve from altered antigen presentation that fosters the development of autoreactive T-cells that avoided thymus regulation. Thus, the core progression of disease instructs peptide immunotherapy strategies.

Identifying therapeutic target epitopes for allergen derived peptide immunotherapy has proven more difficult, as allergy is not confined to specific HLA alleles, translating to a large, diverse population of putative T-cell epitopes responsible for allergic disease. To address this concern, early work with cat-derived peptides utilized long peptides (27 amino acids) that spanned several potentially dominant T-cell epitopes. Treatment with these peptides did possess some clinical efficacy, but was met with a high rate of adverse events[49-51]. To circumvent safety issues associated with longer peptides, we've successfully, and safely[16], employed several shortened CD4 T-cell epitopes to ameliorate allergen-specific responses in cat-allergic patients

and generate lasting protection from symptoms[17, 18]. The peptides utilized in these clinical studies were selected due to highly promiscuous MHC Class II binding, emphasizing population coverage, not the affinity of each peptide to MHC molecules. Studies of peptide immunotherapy for the amelioration of EAE and autoimmune diabetes have demonstrated that the tolerogenic capacity of a peptide is directly linked to that peptide's affinity to the MHC class II molecule[19, 20, 22]. Thus, the concern arises that emphasizing population coverage may sacrifice tolerogenic capacity. In contrast to previous studies of peptide affinity, our data demonstrate that, for the amelioration of eosinophilic airway inflammation, peptide affinity to the MHC Class II molecule is not the dominant factor. Rather, we demonstrate that peptides of 'high, moderate and low' affinity can equally suppress the accumulation of eosinophils, albeit with different dose-response characteristics. High-affinity peptides tend to function maximally when administered at low-doses, while peptides of lower-affinity exerted robust, consistent suppression at higher-doses.

To our surprise, the titration of peptides revealed an unorthodox dose-response, highlighted by a waxing and waning of peptide efficacy with dose, best characterized by a sigmoidal curve. Perhaps, the loss and return of tolerance with increasing doses may demarcate a conversion point wherein the dominant mechanism of immune tolerance engaged by peptide administration switches. Extensive evidence has demonstrated that the dose of peptide administered can control the suppressive mechanism elicited. For instance, low-dose peptide administration has been linked to the expansion of IL-10 producing regulatory T-cells[52]. High-dose peptide immunotherapy effectively deletes epitope specific T-cells[53], making this an effective strategy for eliminating well defined, pathogenic T-cells. Finally, peptides can also be utilized to induce hyporesponsiveness, indicative of T-cell anergy[48]. Thus, the sigmoidal dose-response relationship may represent the progressive switching through these tolerogenic

mechanisms. Moreover, the ineffective doses may represent inefficient engagement of any one mechanism. The current study primarily examined mechanisms of active tolerance, and not anergy nor deletion; thus, the presented data do not comment upon this hypothesis. However, that individual peptides preferentially induced a single mechanism of tolerance (ie: PD-1 or CTLA-4) does provide some confounding evidence. A study examining the immune response immediately following peptide administration, rather than after the completion of a therapeutic regimen and antigen challenge is required to further define these dose-response effects. It is plausible that the allergen challenges may have obfuscated the dominant mechanism of tolerance.

The mechanism of tolerance engaged may be relevant to why peptides of lower affinity to the MHC molecule may effectively treat allergic inflammation. To date, effective treatment of autoimmune diseases has primarily depended upon the deletion of autoreactive T-cells[22, 35]. In contrast to this, we and others, have demonstrated that effective peptide immunotherapy is associated with the establishment of IL-10 secreting Tr1 cells, and the spread of tolerance from T-cells specific for the treatment peptide, to T-cells specific to other epitopes[13, 24, 25]. Thus, the relative importance of peptide affinity during treatment of autoimmune diseases versus allergic diseases may arise from the differential mechanisms associated with the therapeutic resolution of each disease.

The discussion of dose and affinity thus far has ignored the importance of intrinsic peptide properties. The concept that T-cell epitopes may contain intrinsic properties that preferentially drive polarized immune responses has been suggested in the past[33]. Here, we demonstrate that peptides do not behave in a uniform fashion, rather they engage a unique tolerogenic mechanism, likely as the result of an undefined intrinsic factor. The suppressive

mechanisms engaged by peptide administration were: upregulation of PD-1 in the lymph nodes, reduction of Th2 polarized T-cells in the lungs and an increased frequency of CTLA-4 expressing FoxP3+ Tregs in the DLN. Previously, we have observed the increased expression of FoxP3 within positive cells[25], but this marks the first observation of an increased number of regulatory FoxP3+ cells following peptide immunotherapy with Fel d 1 derived peptides. In our earlier studies, FoxP3 expression has primarily been examined in the lungs of mice or in the peripheral blood of treated patients. Thus, these studies not observing a role for FoxP3 may stem from anatomical difference as here, FoxP3 positive cells have been implicated in the lung draining lymph nodes. Intriguingly, previous studies have found peptide immunotherapy to be dependent upon IL-10[24]. In the current study, we did not observe elevated levels of IL-10 producing cells in the lungs, nor draining lymph nodes following allergen challenge. It is plausible that mouse strain differences between this study (HLA DR4 transgenic mice on a C57BL/6 background) and previous work caused these divergent results.

The present work demonstrates that PD-1 may be increased following peptide immunotherapy with select peptides. PD-1 is a receptor that, upon binding the endogenous ligands, PD-L1 and PD-L2, suppresses T-cell activity through engagement of an immunoreceptor tyrosine-based inhibitory motif (ITIM)[54]. Indeed, PD-1 expression has been demonstrated to have a critical role in the maintenance of peripheral tolerance to allergens in healthy individuals[55], suppression of spontaneous autoimmune responses[56] and regulation of experimental autoimmune encephalomyelitis (EAE)[57]. We have not observed a role for PD-1 following peptide immunotherapy, although others have during whole-allergen immunotherapy[58]. A regulatory role for PD-1 has been demonstrated during peptide immunotherapy for amelioration of EAE using peptides derived from major basic protein

(MBP). Therapy with a high-affinity MBP derived peptide is known to suppress the establishment and progression of EAE. In this model PD-1 is markedly upregulated on T-cells[20] and critical to the establishment of tolerance, as PD-1 deficient T-cells are refractory to peptide immunotherapy[36]. However, PD-1 is dispensable for peptide immunotherapeutic regimens that rely on deletion of peptide-specific T-cells[35]. Our data are in accordance with these studies and indicate that PD-1 may play a role in the maintenance of allergen-specific tolerance. Intriguingly, PD-1 appears to be one of many mechanisms that peptide therapy can engage to induce peripheral tolerance, as only three of seven tested peptides affected PD-1 expression. Engagement of the PD-1 pathway appeared to be primarily a peptide-specific feature and not one primarily determined by peptide affinity nor dose.

CTLA-4 is a potent regulator of immune tolerance. Indeed, CTLA-4 deficient animals die within 3-4 weeks of birth, following the development of multiorgan tissue destruction as a result of lymphocytic infiltration[59]. Targeted deletion studies have demonstrated that CTLA-4 expression is an absolute requirement for Treg function, as FoxP3+ Tregs lacking CTLA-4 fail to sustain peripheral tolerance, despite the presence of fully-intact alternative suppressive mechanisms[41]. CTLA-4 has been identified as a critical mediator of normal responses to allergen, in healthy individuals, that acts distinctly from, but can synergize with, PD-1[55]. A role for CTLA-4 during tolerogenesis following peptide administration has been previously demonstrated. T-cells from DO11.10 mice specifically recognize the ovalbumin (OVA) peptide $OVA^{(323-329)}$. Treatment of BALB/c mice, following transfer of DO11.10 T-cells, with the $OVA^{(323-329)}$ peptide leads to the development of OVA-specific T-cell tolerance; the tolerogenic effect of $OVA^{(323-329)}$ administration is completely reversed by antibody blockade of CTLA-4 during peptide treatment[60]. CTLA-4 has also been implicated in development of venom tolerance

within bee keepers. Upon repeated exposure to bee-stings, bee keepers mount a predominant IL-10 secreting Treg response (Tr1) towards subsequent exposures. Intriguingly, through *in vitro* suppression assays, the suppressive capacity of these cells has been demonstrated to depend upon the presence of IL-10, PD-1 and CTLA-4[61]. In the current study, we have, for the first time, demonstrated that therapeutic administration of allergen derived peptides can induce the expression of CTLA-4 upon FoxP3+ T-cells in the lung draining lymph nodes of mice.

In summary, we have demonstrated that the affinity of allergen-derived peptides to the MHC Class II molecule does not exclusively predict the maximal suppressive capacity of therapy. Rather, a complex interaction between dose and affinity has been resolved, wherein maximal suppression was achieved with low-doses of high-affinity peptides or higher doses of moderate affinity peptides. Finally, the head-to-head comparison of seven Fel d 1 derived peptides has revealed that each peptide possesses intrinsic characteristics that influences which mechanism of suppression is accessed. Thus, these findings support the practice of selecting peptides for clinical use based upon MHC binding promiscuity and alleviate concerns that low-affinity interactions may sabotage clinical efficacy. In addition, the enhanced suppressive capacity of high-affinity peptides administered at low-doses warrants further investigation of ultra-low dose strategies.

6.6. References

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6.7. Figures

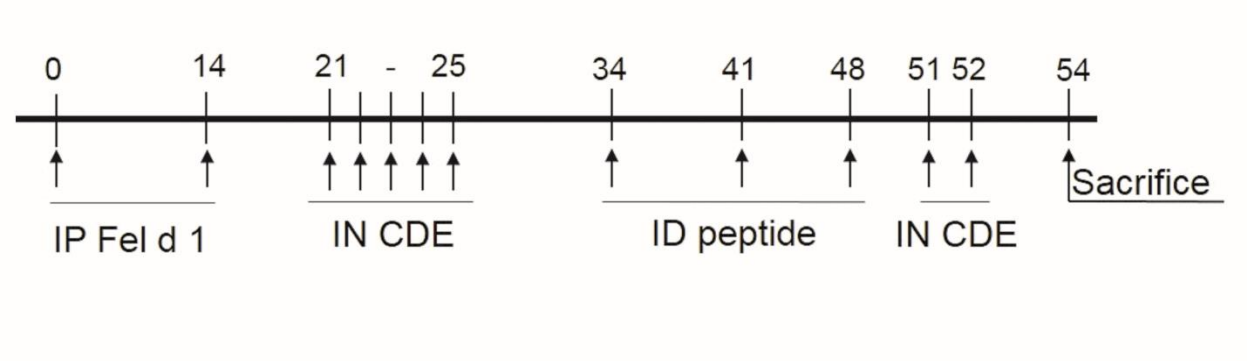


Figure 1. Schematic depicting cat sensitization and treatment with peptides. HLA DR4 transgenic mice were sensitized to Fel d 1 via intraperitoneal (IP) injections of recombinant Fel d 1 (2µg) in alum, on day 0 and 14. Mice were challenged with CDE (10µg) on day 21-25, intranasally (IN). Sensitized mice were treated with three intradermal administrations of a single peptide (Fel d $1^{23\text{-}38}$, Fel d $1^{39\text{-}55}$, Fel d $1^{\text{Chain 2:56-71}}$, Fel d $1^{1\text{-}17}$, Fel d $1^{29\text{-}45}$, Fel d $1^{\text{Chain 2:40-55}}$, Fel d $1^{54\text{-}69}$. Each peptide was titrated from 100µg to 0.01µg, by log doses, in parallel groups of mice. Sham treated mice received either peptide diluent (PBS) or an irrelevant peptide (hemagglutinin; $HA^{306\text{-}318}$). Following therapy, the immune response to CDE was recalled to the lung via a pair of CDE IN challenges (10µg). Mice were sacrificed 48 hours post challenge (day 54).

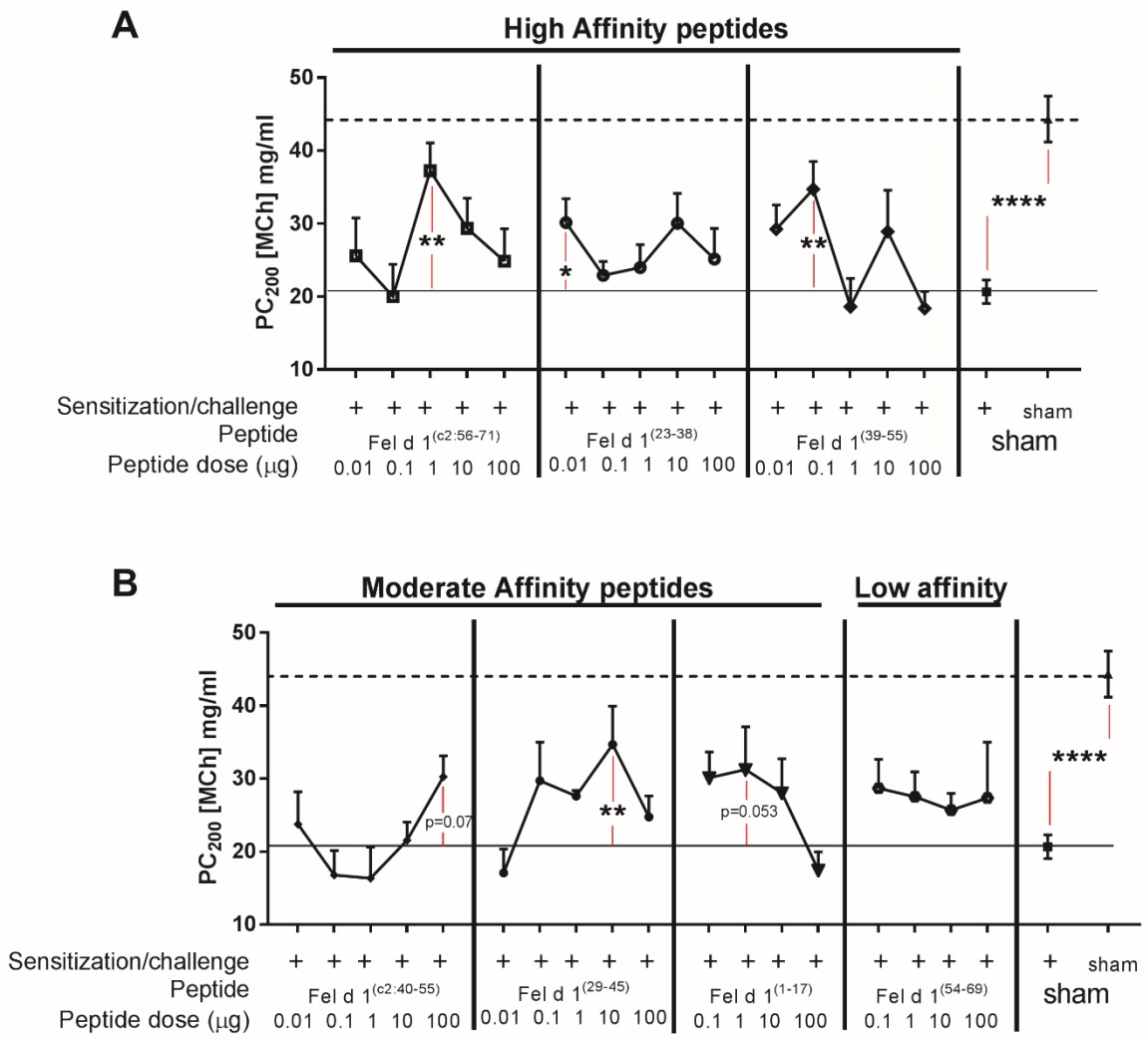


Figure 2. Low-doses of high affinity peptides ameliorate airway hyperresponsiveness. Mice were sensitized and challenged (denoted as '+'), following treatment with the indicated peptide/dose of peptide. Sham treated animals were sensitized and allergen challenged, but treated with PBS intradermally. Sham sensitized animals received saline throughout. Airway responsiveness was measured during a nebulized methacholine (MCh) challenge. From the total respiratory resistance, the provocative dose causing a 200% increase in baseline resistance was calculated (PC_{200}). PC_{200} in animals treated with a high affinity peptide (A) and moderate/low affinity peptides (B). Data presented as mean ± SEM (n=10-30; pooled from two-three independent experiments). The solid horizontal line demarcates the PC200 of sham treated mice and the dashed horizontal line demarcates the PC200 of sham sensitized/challenged mice. *, $p < 0.05$; **, $p < 0.01$; ****, $p<0.0001$ between the indicated groups, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.

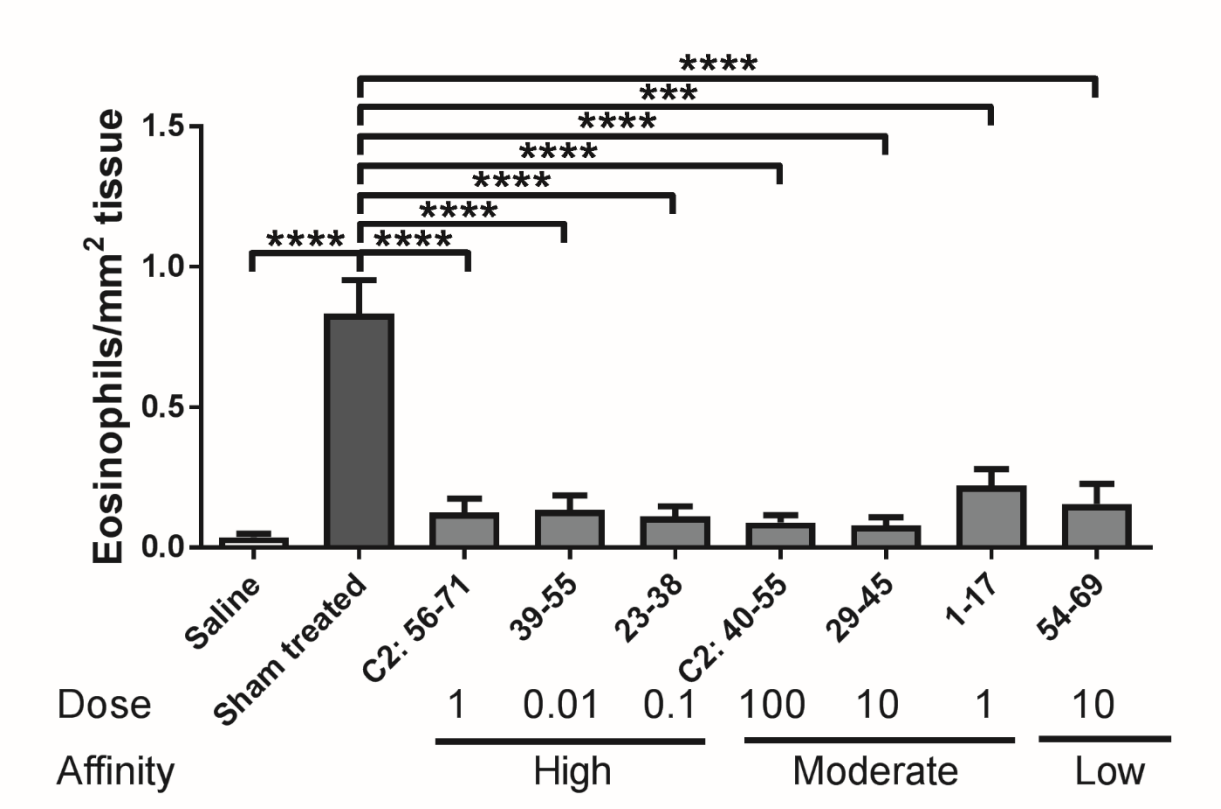


Figure 3. Affinity to the MHC molecule does not affect the suppressive capacity of Fel d 1 derived peptides. Peribronchial eosinophils, quantified histologically by haematoxylin and eosin staining, are shown. Saline mice were sham sensitized/challenged. Sham treated animals were sensitized and challenged with CDE, after treatment with PBS. The remaining groups are designated by the peptide that (C2:56-71, 39-55, 23-38, C2:40-55, 29-45, 1-17 and 54-69) the mice were treated with. The dose and affinity of the peptide are also indicated. Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). ****, $p<0.0001$ between the indicated groups, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.

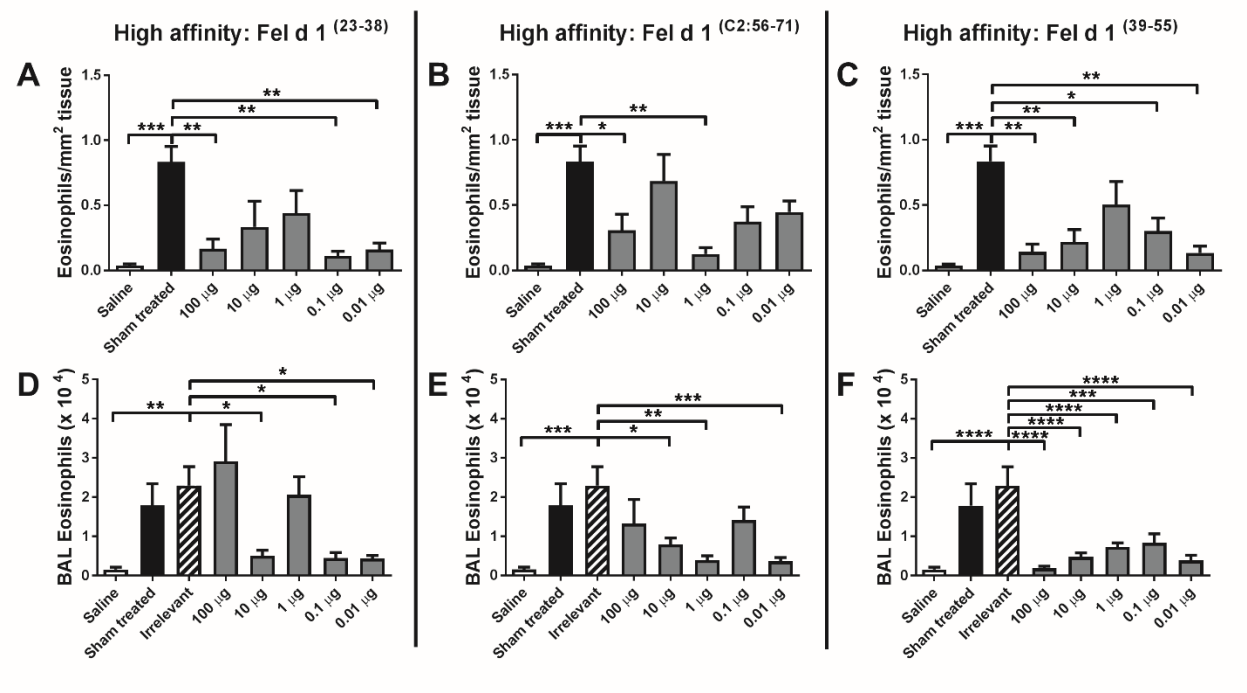


Figure 4. High-affinity peptides suppress eosinophilia in the lungs of allergen-challenged mice in a dose-dependent manner. Sensitized mice were treated with a high-affinity peptides Fel d $1^{(23-38)}$, Fel d $1^{(C2:56-71)}$ or Fel d $1^{(39-55)}$ over a range of doses (0.01-100µg), challenged with CDE and resultant eosinophilia in the lung tissue (A, B and C) and bronchoalveolar lavage (D, E, F) are shown, respectively. Tissue eosinophilia was assessed by haematoxylin and eosin staining of histologically prepared lungs. Bronchoalveolar lavage (BAL) eosinophils were enumerated by trypan blue staining and quantified by morphometric characteristics after Wright-Giemsa staining. Sham treated animals were sensitized and challenged with CDE, after treatment with PBS or an irrelevant peptide (group: irrelevant, peptide $HA^{(306-318)}$. Saline mice were sham sensitized, treated and challenged. the mice were treated with. The dose and affinity of the peptide are also indicated. Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$; ****, $p<0.0001$ between the indicated groups, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.

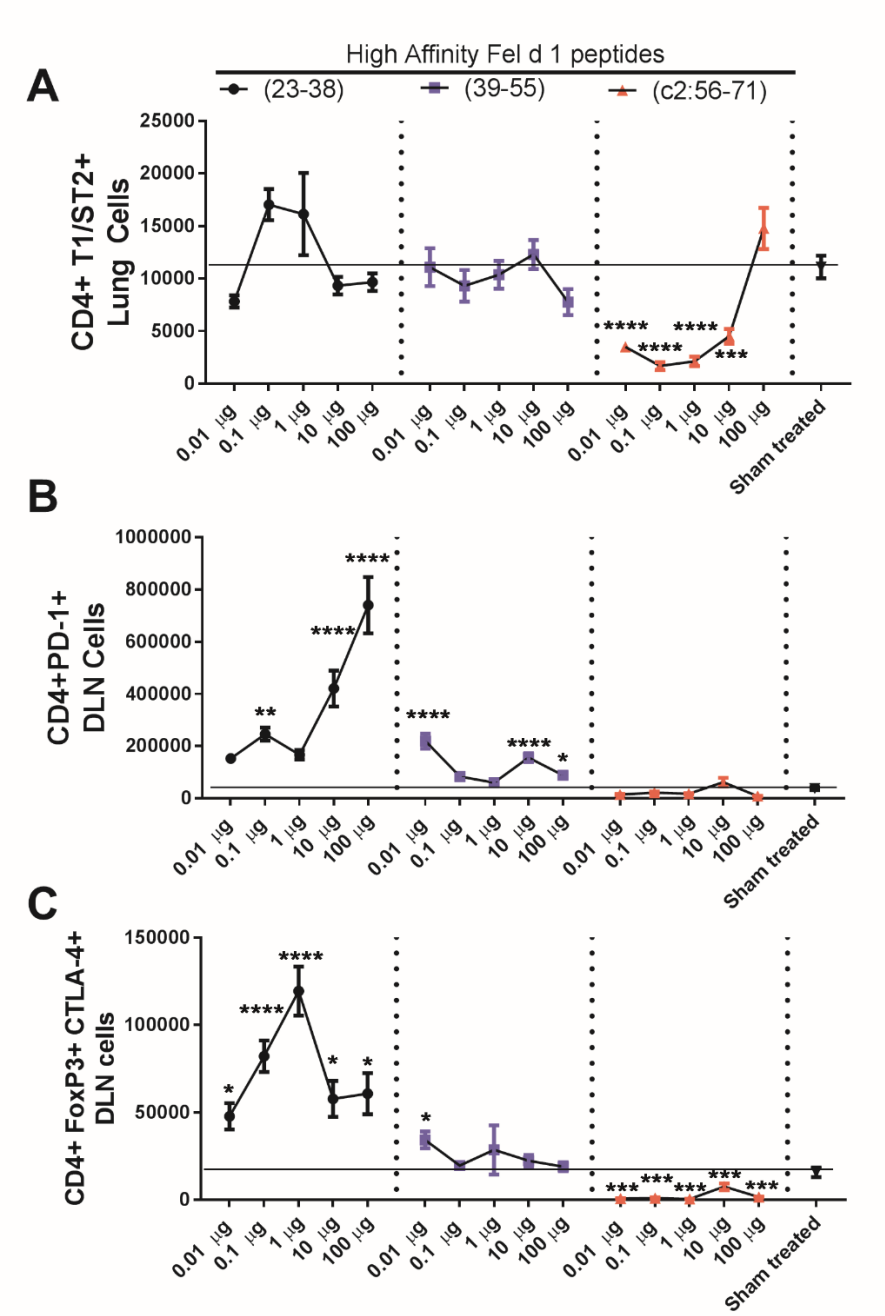


Figure 5. Intrinsic peptide characteristics influence the mechanism of suppression engaged. Following treatment with a high-affinity peptide (Fel d $1^{(23\text{-}38)}$ (black circle), Fel d $1^{(39\text{-}55)}$ (blue square) or Fel d $1^{(C2:56\text{-}71)}$ (red triangle)) over a range of doses (0.01-100µg), sensitized mice were challenged with CDE. Mouse lungs and draining lymph nodes (DLN) were collected for flow cytometric assessment of (A) lung expression of CD4+T1/ST2+ cells, (B) expression of PD-1 by CD4+ cells in the DLN and (C) expression of CTLA-4 by CD4+FoxP3+ cells in the DLN. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. The horizontal line indicates the number of cells staining positive in sham treated animals. Data presented as mean ± SEM (n=10-30; pooled from two-three independent experiments). *, $p < 0.05$; ***, $p<0.001$; ****, $p<0.0001$ between the indicated group and sham treated mice, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.

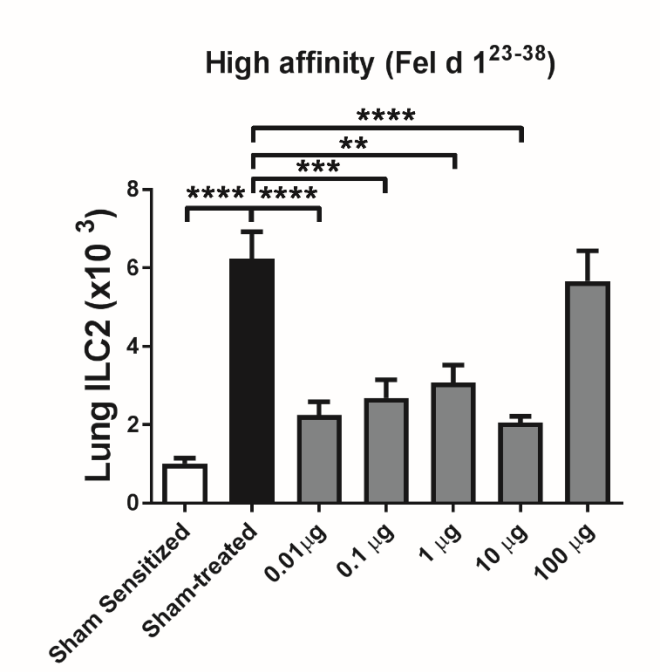
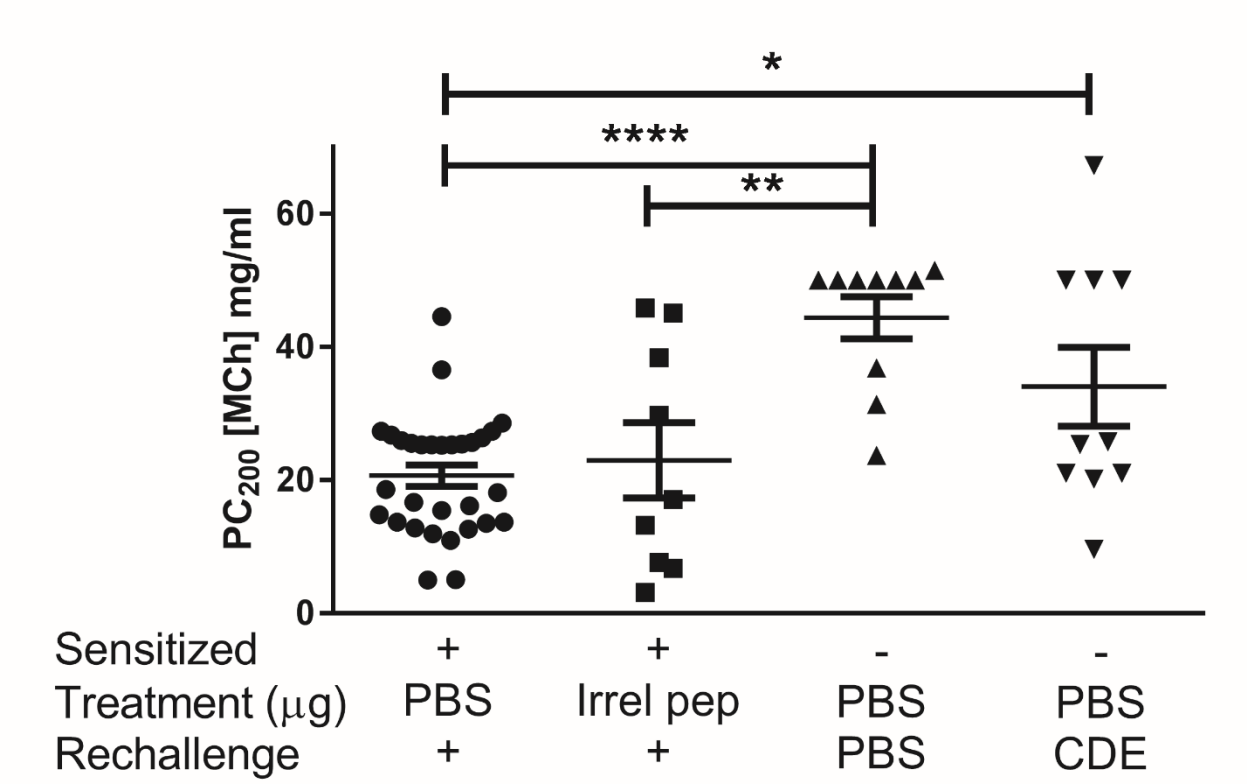
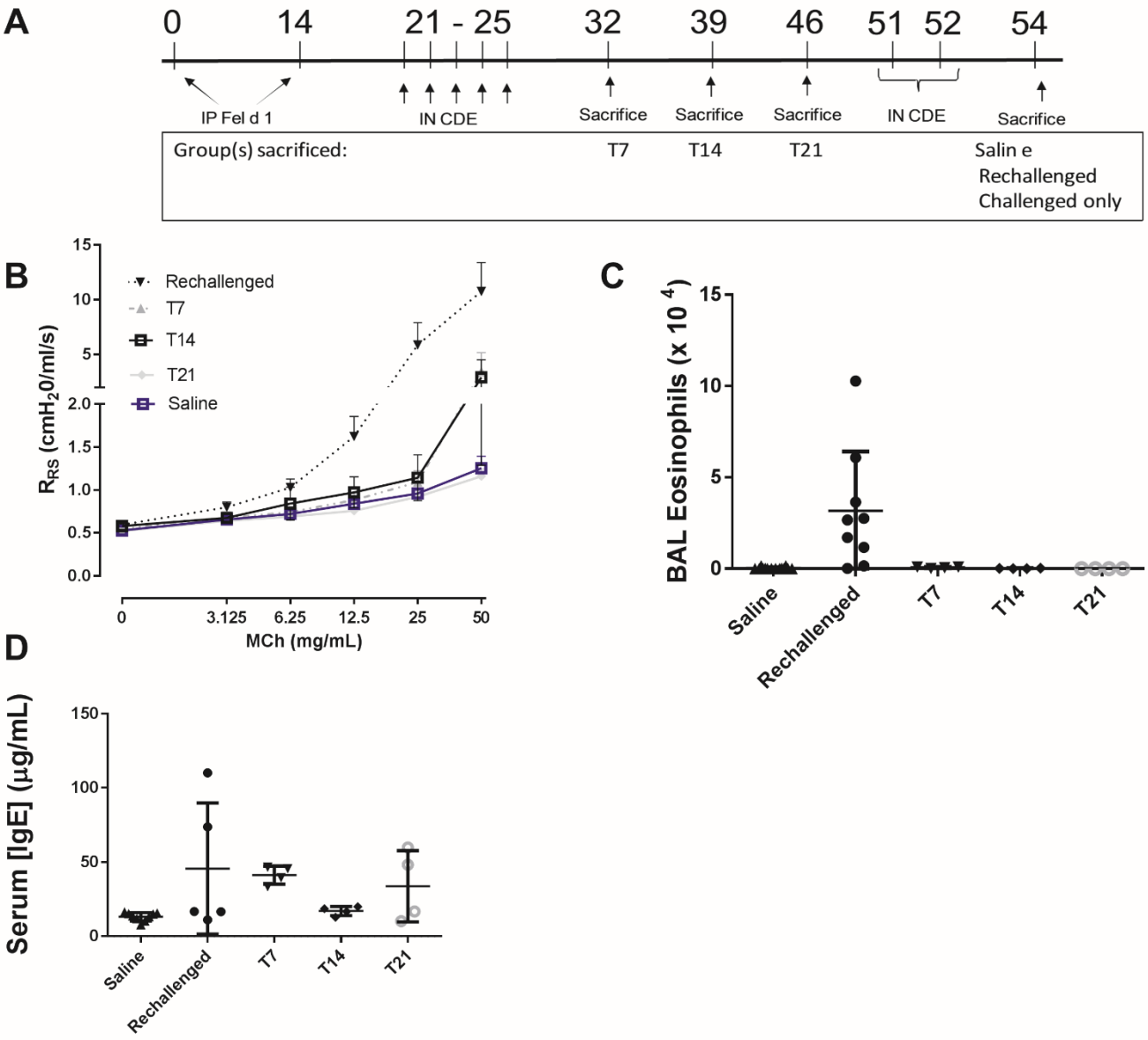


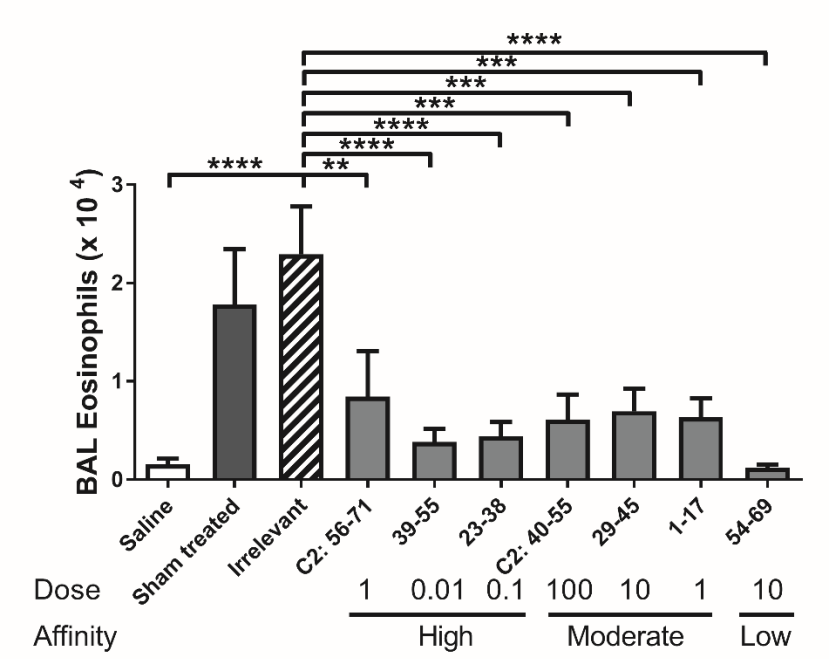
Figure 6. Treatment with Fel d 1$^{(23-38)}$ suppresses lung ILC2 accumulation following CDE challenge. Mice sensitized to cat were treated with the high-affinity peptide Fel d 1$^{(23-38)}$ over a range of doses then challenged with CDE. Mouse lungs were assessed by flow cytometry for the presence of type 2 innate lymphoid cells (ILC2s). ILC2s were characterized as cells lineage negative, and positive for T1/ST2 and CD45. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. Sham sensitized animals received saline throughout. Data presented as mean+ SEM (n=10-20; pooled from two independent experiments). **, $p < 0.01$; ***, $p<0.001$; ****, $p<0.0001$ between the indicated group and sham treated mice, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



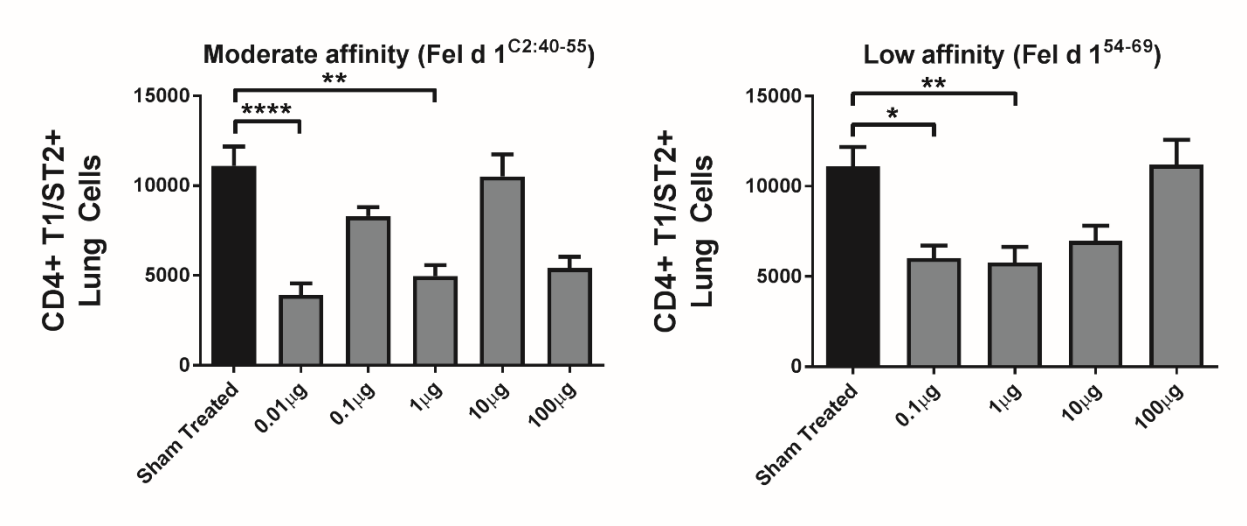
Supplementary Figure 1. Airway sensitivity of control animals. Mice were sensitized with Fel d 1 and challenged with CDE (denoted as '+') or sham sensitized with saline (Denoted as '-'). Sham treated animals were treated, as indicated, with PBS or an irrelevant peptide (group: irrel pep; $HA^{(306-318)}$). Airway responsiveness was measured during a nebulized methacholine (MCh) challenge. From the total respiratory resistance, the provocative dose causing a 200% increase in baseline resistance was calculated (PC_{200}). Data presented as individual points and mean ± SEM (n=10-30; pooled from two-three independent experiments). *, $p < 0.05$; **, $p < 0.01$; ***, $p<0.001$ between the indicated groups, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



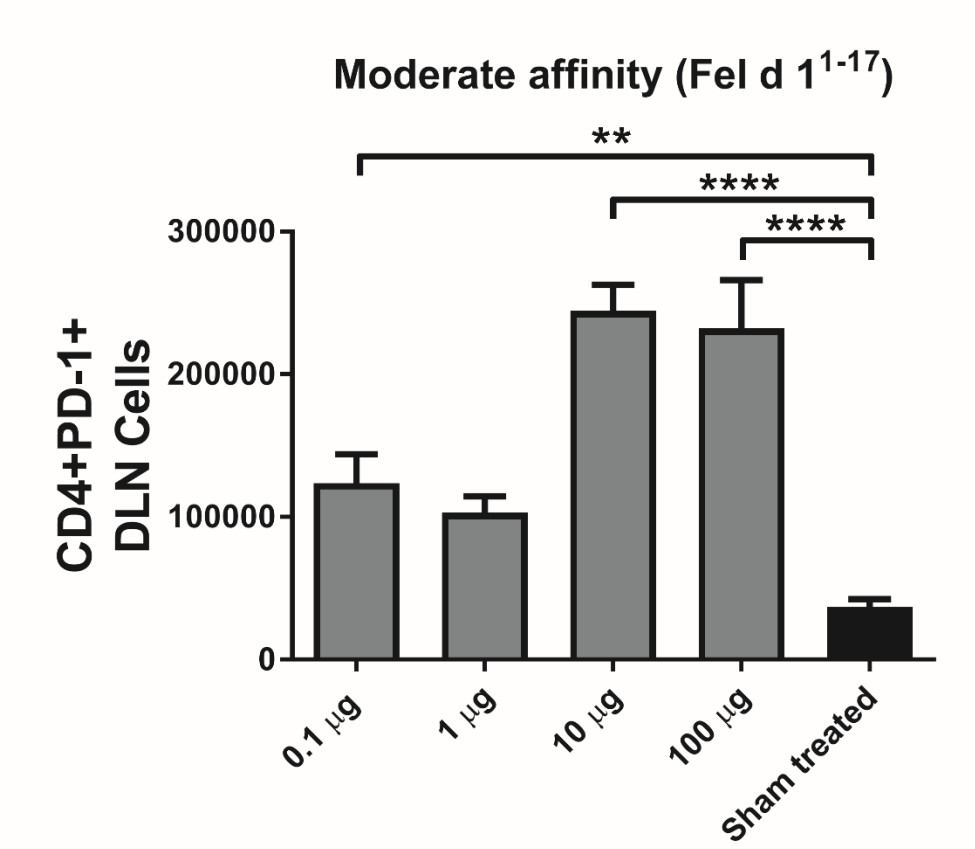
Supplementary Figure 2. Airway inflammation and hyperreactivity are resolved within seven days of intranasal challenge. HLA DR4 transgenic mice were sensitized to Fel d 1 via intraperitoneal (IP) injections of recombinant Fel d 1 (2µg) in alum, on day 0 and 14. Mice were challenged with CDE (10µg) on day 21-25. Parallel groups of mice were sacrificed 7, 14 and 21 days post CDE challenge (group: T7, T14 and T21, respectively). Control mice were sacrificed on day 54 after challenge with CDE (group: rechallenged) or saline (group: saline) on days 51 and 52. Airway responsiveness was measured during by a nebulized methacholine (MCh) challenge and resultant total respiratory system resistance (R_{RS}) was quantified (B). Eosinophils in the bronchoalveolar lavage (BAL) were enumerated by trypan blue staining and quantified by morphometric analysis following Wright-Giemsa staining. (C) Blood was obtained from sacrificed mice and IgE in the serum was quantified by ELISA. Data presented as mean ± SEM or individual points plus mean ± SEM (n=5-10 from a single experiment).



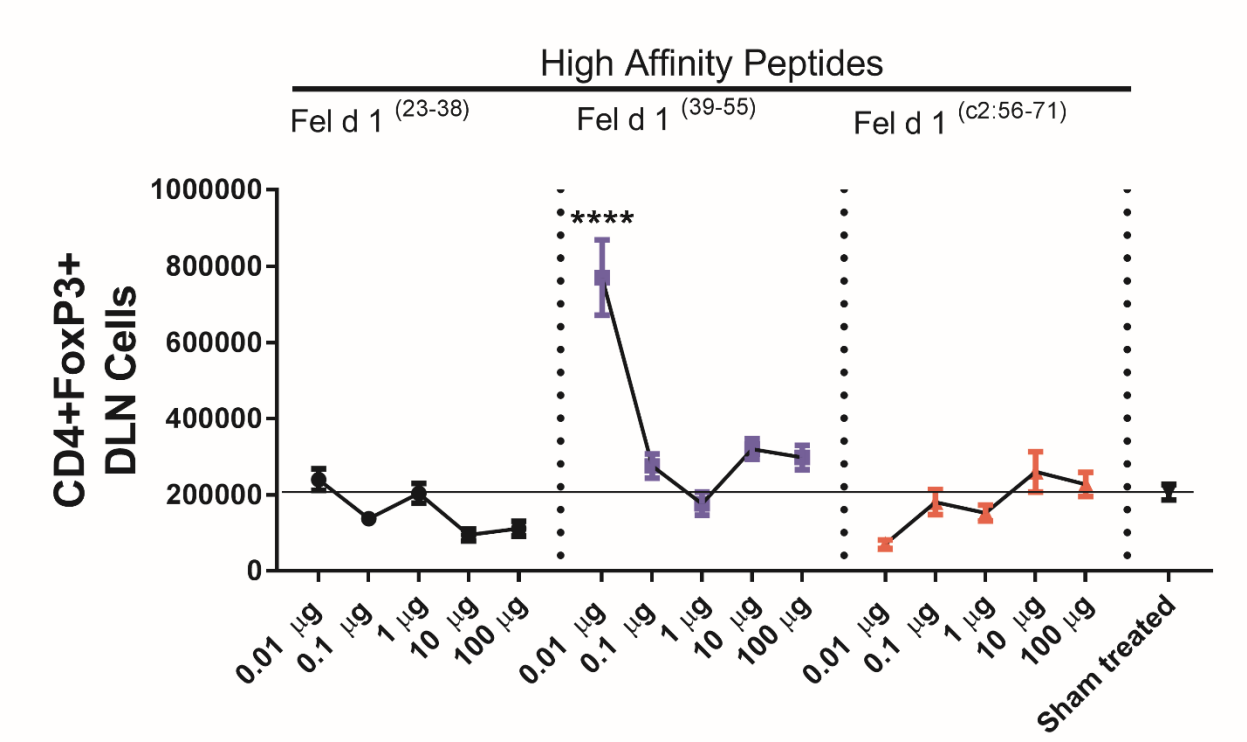
Supplementary Figure 3. Affinity to the MHC molecule does alter the ability of peptides to suppress eosinophil accumulation in the BAL of CDE challenged mice. Cat sensitized mice were treated with the designated peptide (C2:56-71, 39-55, 23-38, C2:40-55, 29-45, 1-17 and 54-69). The dose and affinity of the peptide are also indicated. 48-hours post CDE challenge, a bronchoalveolar lavage (BAL) was performed; eosinophils were enumerated by trypan blue staining and identified by morphometric characteristics after Wright-Giemsa staining. Saline mice were sham sensitized/challenged. Sham treated animals were sensitized and challenged with CDE, after treatment with PBS (group: sham treated) or irrelevant peptide (group: irrelevant, peptide: $HA^{(306\text{-}318)}$). Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). **, $p < 0.01$; ***, $p<0.001$; ****, $p<0.0001$ between the indicated groups, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



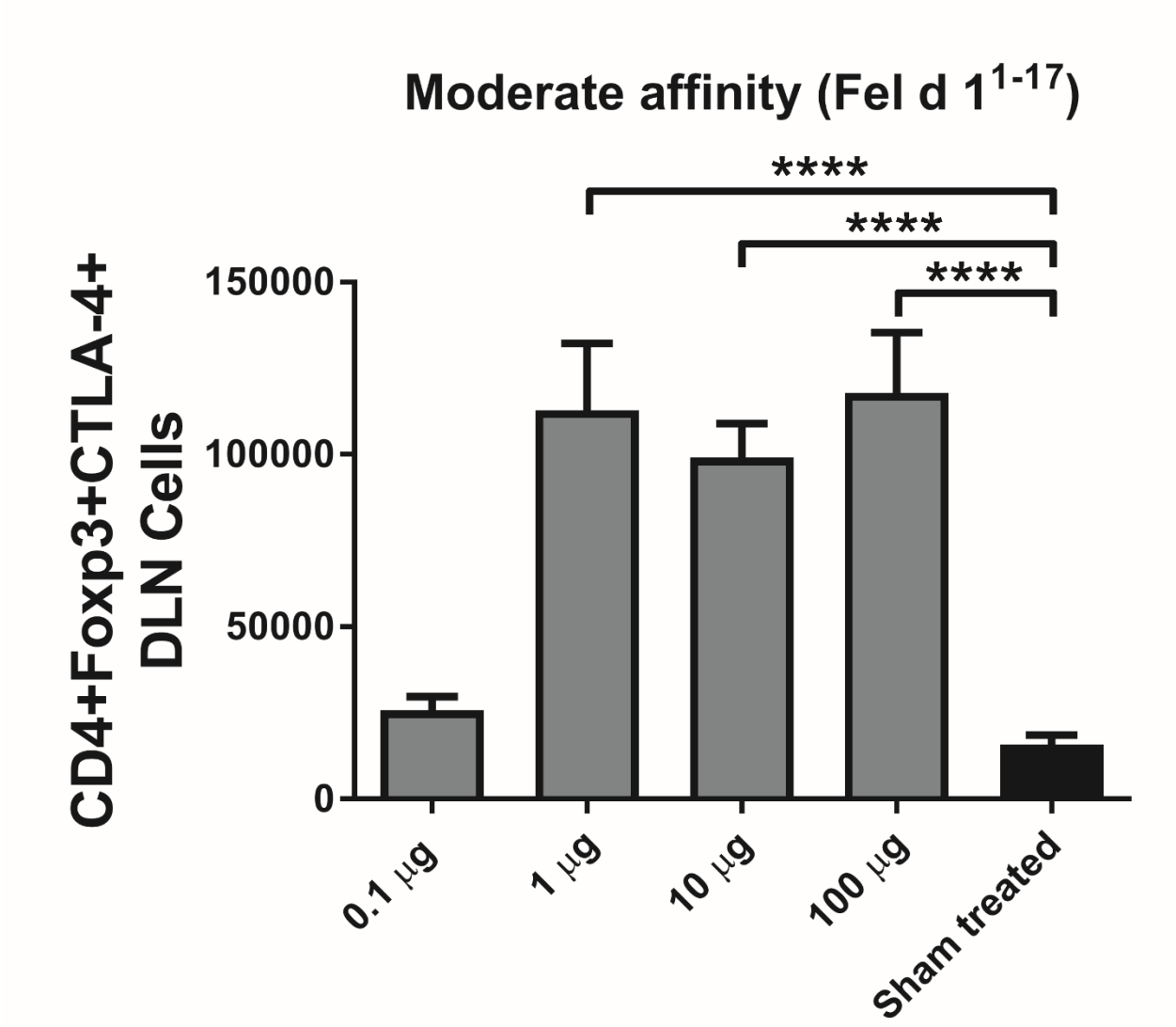
Supplementary Figure 4. Treatment of cat sensitized mice with a moderate or low affinity peptide can suppress Th2 polarized T-cells in the lungs. Following treatment with a moderate-affinity peptide (Fel d $1^{(C2:40-55))}$, or low affinity peptide (Fel d $1^{(54-69)}$) over a range of doses (0.01-100µg), sensitized mice were challenged with CDE. Mouse lungs were assessed for the presence of CD4+T1/ST2+ cells, by flow cytometry. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). *, $p < 0.05$; **, $p < 0.01$; ****, $p<0.0001$ between the indicated group, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



Supplementary Figure 5. A moderate affinity peptide can induce PD-1 expression in the draining lymph nodes of mice. Cat sensitized mice treated with a moderate-affinity peptide (Fel d $1^{(1\text{-}17)}$ over a range of doses (0.01-100μg), were challenged with CDE. Lung draining lymph nodes were resected and assessed for CD4+PD-1+ cells, by flow cytometry. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). *, $p < 0.05$; **, $p < 0.01$; ****, $p<0.0001$ between the indicated group, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



Supplementary Figure 6. Low-dose therapy with a high-affinity can, but does not always, induce FoxP3 expression in the lymph nodes. Following treatment with a high-affinity peptide (Fel d $1^{(23-38)}$ (black circle), Fel d $1^{(39-55)}$ (blue square) or Fel d $1^{(C2:56-71)}$ (red triangle)) over a range of doses (0.01-100µg), sensitized mice were challenged with CDE. Lung draining lymph nodes (DLN) were collected and analysed by flow cytometry for the presence of FoxP3 expressing CD4 cells. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. The horizontal line indicates the number of cells staining positive in sham treated animals. Data presented as mean ± SEM (n=10-30; pooled from two-three independent experiments). ****, $p<0.0001$ between the indicated group and sham treated mice, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.



Supplementary Figure 7. Intrinsic peptide characteristics influence the mechanism of suppression engaged. Following treatment with a moderate-affinity peptide (Fel d $1^{(1-17)}$ over a range of doses (0.01-100µg), sensitized mice were challenged with CDE. Lung draining lymph nodes were assessed for the presence of CTLA-4 expressing CD4+FoxP3+ cells, by flow cytometry. Sham treated animals were sensitized to cat, treated with PBS and challenged with CDE. Data presented as mean + SEM (n=10-30; pooled from two-three independent experiments). ****, $p<0.0001$ between the indicated group, as determined by an ANOVA and post-hoc Bonferronni corrected t-tests.

Chapter 7: Discussion

7. Thesis rationale & synopsis Asthma afflicts an estimated 300-million people globally[316], and approximately 1-in-3 Canadians will develop asthma over their lifetime[6]. Allergic asthma is the most common clinical phenotype of asthma, with prevalence estimates ranging from 40-to-80%[317, 318]. While many therapeutic options for the treatment of allergic asthma exist, the need for effective disease modifying therapies persists, as ~65% of Canadian asthmatics have poorly controlled disease[5].

Allergen-specific immunotherapy (SIT) is a well-established treatment option for allergic asthma, possessing efficacy and disease-modifying effects that provide benefit to patients well after the cessation of treatment, limits the development of new sensitizations and halts the progression from allergies to asthma[197, 319]. Peptide immunotherapy is a form of SIT that attempts to provide the same disease-modifying benefits while offering a better safety profile and an expedited treatment regimen. In this thesis, we sought to characterize mechanisms of peptide immunotherapy, thus the overarching hypothesis was, "*Peptide immunotherapy is a disease modifying therapy that can protect mice from allergen induced inflammation*". Early clinical research studies of peptide immunotherapy have demonstrated it to reduce allergic reactions, but the mechanism of action remains poorly defined. Here, we specifically sought to define important mechanistic aspects of peptide immunotherapy, as outlined within the extended hypothesis, "*The induction of immunosuppression by peptide immunotherapy involves the infectious spread of tolerance beyond the treatment epitope, and is dependent upon treatment peptide dose and affinity to MHC*". Through the definition of these mechanistic traits we hoped to expedite the design of future peptide based therapeutics, while also defining an untapped therapeutic avenue for future therapies to capitalize on, specifically, the induction of bystander tolerance. To address the hypothesis, we developed mouse models of dual-allergic sensitization

and allergic-sensitization in humanized, transgenic mice, that collectively enabled our investigation of infectious tolerance after peptide administration, and the role of peptide affinity for the MHC Class II complex.

Through two mouse models of dual-allergen sensitization, we demonstrated that peptide immunotherapy can evoke a bystander infectious tolerance effect, as we observed the amelioration of ovalbumin allergy following administration of either Der p 1 or Fel d 1 derived peptides. Through the treatment of HLA-DR4 transgenic mice with 7 peptides with known affinity to HLA-DR4, we demonstrated that the affinity of the peptide is irrelevant to the therapeutic potential, although, affinity did affect the dose-response relationship. Surprisingly, our development of the model of allergic sensitization in HLA-DR4 mice revealed a fascinating phenomenon, wherein we observed that application of high-doses of cat dander to a patch of disrupted skin failed to prime mice, while application of 'moderate' doses induced robust allergic disease. Through a series of investigations, we defined that epicutaneous sensitization is reliant upon TLR signaling, but were unable to define the mechanism of high-dose hyporesponsiveness.

In summary, mouse models of allergic airways disease were employed to examine the hypothesis; in this manner we demonstrated that the immunosuppressive effects of peptide immunotherapy are not limited to the treatment epitope, nor even the antigen from which peptides have been derived. Throughout this thesis, data presented have been discussed in the corresponding chapter. The remainder of this discussion will attempt to extend what has already been discussed by highlighting the relationship between the presented studies, discussing avenues for future studies and exploring concepts that augment the arguments made within the thesis.

7.1. Infectious bystander tolerance

Since the inception of peptide immunotherapy, attempts have been made to assess the ability of a single peptide to suppress responses to complex antigens. The first such study did not observe any spread of tolerance, as prophylactic treatment of mice with the 1-9 amino terminal peptide (1-9NAc) of major basic protein (MBP) could not prevent whole MBP induced EAE[253]. In contrast, subsequent studies demonstrated that tolerance could be spread beyond the treatment epitope. For instance, administration of the proteolipid protein (PLP) peptide 139-151 inhibited T-cell responses to both whole PLP and MBP in a model of EAE[302]. Similarly, HDM allergic mice treated with Der p1$^{(111\text{-}139)}$ became hyporesponsive to Der p 1$^{(111\text{-}139)}$, Der p 1$^{(78\text{-}100)}$ & Derp 1$^{(21\text{-}29)}$, although suppression could not be extended to a distinct antigen (OVA) in this model[258, 303]. More recently, the ability of Fel d 1 derived peptides to induce linked-epitope suppression has been demonstrated, as treatment of cat allergic patients with a cocktail of 12 synthetic Fel d 1 derived peptides attenuated T-cell responsiveness to these 12, and four additional Fel d 1 epitopes[287]. Thus, current evidence suggests that peptide immunotherapy may be able to induce a bystander suppressive effect, but the requirements to elicit this immune response are unknown.

Through the treatment of mice allergic to two allergens, we demonstrated that peptide therapy with peptides derived from either Der p 1 or Fel d 1, followed by a period of allergen dual exposure (HDM + OVA or CDE+OVA), can suppress inflammation induced by challenge with ovalbumin, the bystander allergen[320]. Moreover, we demonstrated that the elicitation of bystander tolerance is not dependent upon the route of peptide administration. These findings provide mechanistic insights into peptide immunotherapy, suggesting that treatment with a select number of T-cell epitopes may suppress responses to whole allergen through an infectious spread of tolerance to bystander epitopes. These studies were the first to describe the ability of allergen-

derived peptides to induce bystander tolerance, beyond the treated allergen. It is unlikely that peptide immunotherapy is uniquely able to trigger this mechanism of tolerance; thus, if this is a clinically relevant phenomena, it is likely that past clinical studies of similar immunotherapies (i.e.: allergen-specific immunotherapy) have unintentionally induced bystander tolerance. Indeed, a literature review of allergen-specific immunotherapy identified several clinical studies that reported a bystander effect; although these results were often discussed as 'erroneous' or 'unexpected'. The first rigorously demonstrated clinical example of bystander tolerance was in 1988, when Løwenstein and colleagues treated patients allergic to the tree pollens birch, alder and hazel with either birch immunotherapy or a mixture of birch, alder and hazel immunotherapy for three years[321]. In that study, subjects receiving mono-immunotherapy (birch alone) or the combination immunotherapy (birch, alder and hazel), were equally protected from skin-prick tests with each individual allergen and a nasal allergen challenge containing all three allergens[321]. While this was not termed a ‘bystander effect', a clear example of the phenomena was observed after birch immunotherapy. The development of a bystander effect without an explicit period of triple allergen exposure (ie: hazel and alder present in the context of birch) can be justified through the accumulation of environmental exposures over the 3-year treatment period. Retrospective analysis of the environmental pollen levels of hazel and alder during the treatment period (1980-83), if available, could shed light on the plausibility of this hypothesis and indicate possible tolerogenic threshold exposure levels.

Løwenstein and colleagues have observed several other instances of bystander effects, post immunotherapy. In 1986, a trial designed to investigate the efficacy of cat and dog immunotherapy demonstrated that individuals treated with cat dander tolerated bronchial provocation with either cat or dog dander[322]. Finally, the most robust demonstration of bystander

tolerance comes from a double-blind, placebo controlled trial of immunotherapy in allergic children also performed by Løwenstein and colleagues. Twenty-nine children allergic to both a perennial allergen (cat or house dust mite) and a pollen (birch or timothy) were randomized to receive either immunotherapy for both the perennial and pollen allergy, or placebo and immunotherapy for the pollen allergy. Following 3-years of treatment, bronchial sensitivity to the perennial allergen (cat or house dust mite) was assessed by a nebulized allergen challenge. Children in the pollen immunotherapy plus placebo group demonstrated significantly decreased sensitivity to provocation with the perennial allergen[323], thereby demonstrating a bystander effect of pollen immunotherapy in allergic asthmatic children.

Importantly, the Lowenstein lab is not the only research group to document a bystander effect after immunotherapy. In 2007, birch allergic subjects were given 52 week of birch pollen SLIT and T-cell responses were examined after 4 and 52 weeks of therapy. After 4 weeks of therapy, IL-10 and FoxP3 expression was enhanced in the peripheral blood, and *ex vivo* responses to the treated allergen (Bet v 1), a cross-reactive allergen (Mal d 1) and an unrelated antigen, tetanus toxoid, were all impaired[216]. This effect was T-cell mediated, and dependent upon IL-10 and CD25+ T-cells. Later examination of these same patients revealed that, Mal d 1 and tetanus toxoid responses returned as IL-10 and FoxP3 expression in PBMCs declined. Thus, as evidence of active regulation waned, so did the bystander effect. This study establishes a mechanistic link between the activity of regulatory T-cells and the development of bystander tolerance, while also providing supporting external clinical validation.

Cumulatively, these trials support the hypothesis that bystander tolerance may evolve following immunotherapy, but they are not conclusive. For instance, the trial of pollen immunotherapy in dual allergic children[323] represents the most direct assessment of a functional

bystander effect; however, this trial lacked a placebo group. Thus, whether the improved responsiveness to perennial allergen in children receiving pollen immunotherapy was due to a bystander effect or a natural resolution of the perennial allergy is unclear. To directly assess the ability of immunotherapy to induce bystander tolerance, a trial of immunotherapy in dual allergic-individuals that controlled exposure to one of the two allergens would be required. An ideal study of this nature could examine people with a dust and pollen sensitivity, in a climate largely devoid of dust (ie: Northern Sweden). A less costly, proof-of-concept study could consist of a retrospective analysis of human samples (blood and PBMCs) from a placebo controlled trial of immunotherapy (for example[205, 269]), wherein specific-antibody titers and T-cell responsiveness to other environmentally relevant allergens could be in investigated.

As discussed in chapter 6, during design of peptide based therapeutics, peptides are chosen based upon their ability to bind many HLA molecules. Our demonstration of bystander tolerance further justifies this practice, as our observations define a mechanism wherein a limited number of peptides can induce the spread of tolerance to a complex allergen, emphasizing that optimization of population coverage may indeed be the most important characteristic during the development of peptide based therapeutics.

In both our demonstrations of bystander tolerance, we observed increased IL-10 expression. In models of transplantation tolerance, bystander tolerance has been show to be dependent upon the persistent presence of FoxP3+ T-cells[299] that alter the phenotype of antigen-presenting cells in a manner that IL-10 producing T-cells cannot[309]. Thus, whether peptide immunotherapy of allergies is inducing a form of bystander tolerance with different mechanistic requirements (IL-10 vs. FoxP3), or whether both of these regulatory molecules are required, but at different time-points, remains to be investigated. The use of IL-10 neutralizing antibodies and

mice with FoxP3 linked expression of the diptheria toxin (DT) receptor could be utilized to transiently deplete IL-10 and FoxP3 activity, thereby enabling research into their relative roles during establishment of bystander tolerance in allergy without permanently compromising the ability of T-cell to regulate allergen challenge.

In Chapter 6, we observed increased CTLA-4 and PD-1 expression by T-cells after peptide immunotherapy, and that the expression of these molecules correlated with protection from subsequent antigen challenge. Intriguingly, exogenous CTLA-4 therapy has been demonstrated to induce infectious tolerance[324]. Thus, it is plausible that increased expression of CTLA-4, and perhaps PD-1, by peptide immunotherapy contributed to the development of bystander tolerance. To investigate this hypothesis, we could employ our models of bystander tolerance and administer neutralizing anti-CTLA-4 and PD-1 antibodies.

Taken together, we have demonstrated that Fel d 1 and Der p 1 derived peptides can evoke tolerance to both the allergen from which they were derived (cat and house dust mite) and also to an unrelated allergen. This phenomenon was demonstrated using peptides administered at very low doses, through a clinically relevant route. We believe that the employment of low-dose therapy was critical to our demonstration of bystander tolerance. Most clinical immunotherapy regimens stress the use of the highest dose possible, but high-dose peptide is known to delete specific T-cells. Thus, it is plausible that current clinical best practices may systematically prevent the development of bystander tolerance. It would be intriguing to assess the ability of high dose (100-400µg) peptide to induce bystander tolerance to potentially disprove this hypothesis. Further, performing such an experiment in HLA-DR4 transgenic mice would enable the accurate tracking of antigen-specific T-cell deletion through the use of commercially available PE-labelled DR4 tetramers.

7.2. Development of an adjuvant based model of cat allergic airways disease in HLA-DR4 transgenic mice

We set out to develop a model of cat allergy that replicated salient features of allergic asthma in humans, such as the development of Th2 inflammation and airway dysfunction in response to an allergen challenge. To enable investigation of mechanisms of peptide immunotherapy, humanized HLA DR4 transgenic mice were employed, as they enabled the tracking of antigen-specific T-cells (primarily performed in Chapter 5) and investigation of how peptide affinity for the presentation molecule affects the development of tolerance (Chapter 6).

We demonstrated that intraperitoneal injection of Fel d 1 in alum primes HLA DR4 mice to develop the cardinal features of asthma, such as AHR and eosinophilia, in response to subsequent intranasal CDE challenge. Development of this model revealed several potentially broadly relevant immunological trends, i) high-dose CDE IN challenge converted the induced disease phenotype from predominantly Th2 towards Th1, ii) high-dose CDE IN challenge induced airways disease refractory to therapeutic intervention and iii) eosinophilia in airway tissues but not the lumen, correlated with the development of airway dysfunction.

Our observation that the dose of allergen challenge controls the disease phenotype that manifests may be relevant to efforts attempting to ‘endotype’ asthmatic diseases. For instance, several recent studies have successfully utilized anti-Th2 therapeutics (anti-IL-5 and IL-13 antibodies) to attenuate symptoms of allergic asthma, in patients with a high-Th2 disease phenotype[325, 326]. Our results suggest that the disease phenotype apparent at clinical trial enrolment may simply be a result of whether the subject had recently encountered a large or small dose of allergen. This may be of considerable importance to the recruitment of patients to clinical trials, as subjects likely amenable to a therapy may be excluded simply based on their

recent exposure history, and vice versa. We propose that a patient screening process involving an allergen challenge titration may better stratify patients and predict therapeutic responsiveness.

Not only did high-dose CDE challenge induce a deviated immune response, but this response was also treatment-refractory, as therapy with either peptides or high-dose, systemically administered budesonide failed to resolve AHR. Several models of treatment-refractory allergic airways disease have been developed; however, our model is the first to demonstrate that this can be achieved simply through titrating the challenge dose upwards. Some have suggested that treatment refractory asthma may be due to enhanced involvement of ILC2s, as ILC2s are insensitive to corticosteroids[327, 328]. To test such a hypothesis, we could examine whether ILC2 depletion restored sensitivity to peptide therapy and corticosteroids, in our model of treatment refractory allergic airways disease. While ILC2 depletion is complex, we could employ a Thy disparate system to perform this experiment, wherein T and B lymphocytes from a Thy1.2 mouse are adoptively transferred into Thy1.1, RAG deficient mouse, thereby enabling the selective depletion of ILC2s through administration of a depleting Thy1.1 antibody.

Taken together, the adjuvant-based model of cat allergy in HLA-DR4 mice successfully generated a Th2-high model of disease, appropriate for study of peptide immunotherapy, while also incidentally creating a model of allergic disease consisting of airway neutrophilia and marked airway dysfunction that is reminiscent of severe, treatment-refractory asthma. The severe-asthma-like model warrants future study, as it may enable the evaluation and identification of biomarkers and therapeutics that could help patients with severe-asthma.

7.3. Epicutaneous CDE exposure

In parallel with our development of an adjuvant dependent model of cat allergy, we developed an adjuvant free model of cat allergy, that consisted of applying cat dander to patches

of disrupted skin. Inspiration to develop a model of epicutaneous sensitization to cat dander was drawn from the robust Th2 polarization elicited by epicutaneous exposure to peanut[329, 330] and ovalbumin[331], by others. Furthermore, we believed that this model, as it lacked an adjuvant, may better replicate Th2 responses in humans, potentially making epicutaneously sensitized animals better able to evaluate and predict the efficacy of therapeutics. We demonstrated that epicutaneous exposure to cat dander induced Th2 polarization in both HLA-DR4 and C57BL/6 mice, as intranasal CDE challenge induced eosinophil recruitment to the lungs and Th2 cytokine expression by T-cells. Through the use of HLA-DR4 tetramers, we demonstrated that allergen challenge preferentially induced cat-specific T-cells to increase the production of Th2 cytokines, while polyclonal T-cell responses were inconsistent.

To develop the protocol for epicutaneous sensitization of mice to cat dander, the dose of CDE applied to the skin was titrated. To our surprise, cutaneous application of high-dose CDE failed to prime a robust Th2 response, as intranasal CDE challenge in these mice failed to induce airway eosinophilia or enhanced Th2 cytokine expression. Experiments were designed to determine the mechanism through which high-dose CDE impaired inflammatory responses; specifically, we investigated whether high-dose CDE induced regulatory T-cells and whether high-dose hyporesponsiveness was due to TLR ligands in the allergen preparation.

Similarities between the inability of high-dose CDE to induce allergic responses can be drawn to the modified Th2 response, wherein people exposed to high-doses of Fel d 1 tolerate challenge with cat dander[332]. The modified Th2 response is characterized by the development of cat specific IgG, but not IgE, and in HLA-DR7 positive individuals, an increased production of IL-10 by T-cells stimulated with cat derived peptides[333]. A limitation of the HLA-DR4 mouse model is that we have consistently been unable to identify antibody responses. Indeed, we've

largely been unable to detect IgE in undiluted blood by ELISA. While IgG antibodies are detectable, they are several log-fold lower than in other mice, for instance, low µg levels rather than mg levels. In our model, an enhanced IgG response induced by high-dose cat dander would explain subsequent hyporesponsiveness, while also being congruent with the observed early peak in IL-4 production by T-cells in the skin draining lymph nodes 10 days after the commencement of cutaneous priming. Yet, quantification of IgE, IgG1, IgG2a and IgG2c revealed antibodies levels to be unchanged following cutaneous exposure to CDE at any dose, in both HLA-DR4 and wild type (C57BL/6) mice. These results indicate that a mechanism wherein high-dose CDE is impairing subsequent inflammation through the induction of IgG antibodies is unlikely; however, such a mechanism was not completely ruled out. A serum transfer, from animals receiving high-dose cutaneous CDE exposure into CDE-primed animals could conclusively ascertain whether blocking antibodies were responsible for the functional allergic hyporesponsiveness.

To generate clues that may reveal the mechanism of high-dose CDE induced allergen hyporesponsiveness, we performed a kinetic examination of animals before, during and after cutaneous exposure. Analysis of antigen-specific T-cells revealed that high-dose CDE induced more antigen-specific T-cells and production of IL-4 than did animals exposed to a sensitizing dose of CDE. We hypothesized that, perhaps like OVA tolerance, that a self-terminating immune response may be induced by high-dose CDE exposure, characterized by early immune activation that is rapidly quieted by immunosuppression[138, 140]. Previously, this subversion of early immune activation has been accredited to IL-10 production by dendritic cells[140], but altered APC behaviour could also explain the observed effects. To investigate whether high-dose CDE is altering APC behaviour, we propose an experiment wherein FITC-conjugated CDE is applied to the skin, thereby labeling all skin-derived cat presenting APCs for phenotypic analysis.

It is possible that high-doses of CDE fail to prime allergic responses because of correspondingly greater levels of TLR-activating contamination, that could subvert the development of Th2 responses[162]. We investigated this hypothesis by removing the ability of mice to respond to TLR ligands. To our surprise, MyD88 deficient animals could not be sensitized to CDE applied cutaneously. This unexpected finding impaired our ability to determine if contaminants in the high-dose CDE preparation were disrupting allergic sensitization. Further investigations into the role of TLR ligands during epicutaneous sensitization are warranted. Specifically, a more refined strategy, such as the use of TLR-4 deficient mice (ie: C3H/HeJ[160]), may allow for the development of allergic sensitization, thereby enabling determination of whether LPS concentrations are impairing the development of immune responsiveness. Results from this study would dictate whether examining the role of other TLR ligands was warranted.

The epicutaneous model of cat dander allergy was refractory to intradermal therapy with peptides, several potential mechanisms could account for this. For instance, disruption of the stratum corneum is known to induce APC activation[330] and epicutaneous sensitization has been linked to thickening of the dermis[334]. Thus, we hypothesize that intradermal peptide injection may have failed to treat mice due to the peptides being trapped within the altered skin structure. To investigate this, radiolabeled peptides could be injected into the skin of epicutaneously sensitized and sham exposed animals. Sequestration as a result of skin structural changes induced by sensitization would be revealed by impaired spread beyond the injection site. Alternatively, it is possible that epicutaneous allergen exposure leads to a inflammation in the skin and draining lymph nodes that persisted until the therapeutic intervention. The efficacy of immunotherapy is hypothesized to rely upon the presentation of peptides by immature/semi-mature DCs, and

induced activation of DCs (by agonistic CD40-antibody[335], or LPS[281]) has been shown to subvert the development of peptide induced tolerance. Studies proposed earlier to phenotype APCs during epicutaneous allergen exposure could also provide supportive evidence, by ascertaining whether the skin-draining lymph nodes were persistent inflamed.

To conclude, we demonstrated that epicutaneous exposure to cat dander extract primed mice to develop eosinophilic inflammation and airway dysfunction upon intranasal challenge with cat dander extract. Surprisingly, and out-of-keeping with current dogma, epicutaneous exposure to high-dose CDE failed to prime an allergic response. The mechanism by which high-dose CDE exposure acted through was investigated using several techniques, but could not be defined. During these investigations, we investigated the role of TLR ligands through exposing MyD88 deficient mice to sensitizing and high-dose CDE. These studies revealed that epicutaneous sensitization is dependent upon MyD88 activity, as MyD88 deficient mice could not be sensitized. Taken together, these studies contribute to the understanding of epicutaneous sensitization while also highlighting a novel, potentially therapeutic allergen-exposure strategy. Further studies comparing and contrasting the development of a 'modified-type 2 response' in humans, with our studies of high-dose induced allergen-specific hyporesponsiveness will shed light upon potential clinical applications of these findings.

7.4. The role of peptide affinity and dose during the establishment of immune tolerance

In Chapter 6 we investigated whether peptide affinity for the MHC complex predicted the efficacy of peptide immunotherapy. Heterogeneous, unexpected, results were generated. In contrast to previous studies examining the interplay between peptide affinity and the efficacy of peptide immunotherapy, we observed that peptide affinity for the MHC complex was not the sole determinant of the tolerogenic capacity of a peptide. Instead, our data indicate that the affinity of

the peptide shifts the dose-response curve, as high-affinity peptides protected mice from allergic disease at low-doses, while moderate/low affinity peptides were optimally tolerogenic at higher doses. Indeed, we observed that high affinity peptides were maximally effective at ameliorating eosinophilic responses at very low doses (0.01-to-1µg).

To date, studies examining the role of peptide dose and affinity have exclusively been performed in models of autoimmunity. In 1993, prophylactic administration of a high-dose, high-affinity peptide (100 µg, intranasally) to mice was found to block the progression of EAE[336]. Subsequent studies by the same group demonstrated that high-dose peptide administration was better able to modulate EAE clinical score than lower doses[298]; however, in this study, therapy with a low dose (0.8µg) delayed disease-on-set as well as high dose therapy and induced similar levels of IL-10 expression. In another model, peptide immunotherapy with a high-dose of high-affinity peptide ameliorated onset of diabetes in NOD mice, while high-dose administration of a low-affinity peptide exacerbated disease[337]. Intriguingly, low-dose therapy with a high-affinity peptide also exacerbated diabetes in NOD Mice[337]. Thus, to date, evidence from models of autoimmunity have predominantly supported the hypothesis that high-affinity, high-dose peptide administration is required to generate a therapeutic effect. We demonstrated that this dogma may not pertain to the treatment of cat allergy, as treatment with low/moderate affinity peptides or high-affinity peptides at low doses both inhibited subsequent immune responses. Lending further credence to the potential importance of low-dose therapy are our observations that bystander tolerance can be induced with low-doses of peptide (0.1 to 1µg). Taken together, our data indicate that peptide immunotherapy for the treatment of allergic disease likely does not require high-doses.

In an attempt to characterize the T-cell population during the induction of tolerance by peptide immunotherapy, Wraith and colleagues performed an elegant series of '-omics' analyses. Proteomic analysis of T-cells, following peptide immunotherapy, revealed the upregulation of co-inhibitory molecules, and the selective induction of FoxP3 following low-dose peptide strategies[298]. In some models, the elicitation of linked-epitope suppression is dependent upon FoxP3 expression[299]. As low-dose peptide immunotherapy of cat allergy induces linked-epitope suppression[287, 299], it is intriguing to consider whether high-dose therapy may subvert the ability of peptides to induce bystander tolerance.

In our studies of peptide immunotherapy, we have seen moderate changes in FoxP3 expression, commonly seeing enhanced amounts of FoxP3 expressed on a per cell basis, rather than an increased number of FoxP3 positive cells. Given the lack of clinical evidence supporting a role for FoxP3 expression during peptide immunotherapy for allergic disease, these results are difficult to interpret. To date, most trials of peptide immunotherapy have simply assessed FoxP3expression pre- and post- therapy. It is possible that FoxP3 is predominantly associated with early tolerance induction events, in which case these time points may be biased against identifying a role for FoxP3 in peptide induced tolerance induction. A kinetic examination of the T-cell compartment during peptide immunotherapy for treatment of allergic disease may reveal a role for FoxP3, and outline a process wherein early FoxP3 induction predisposes T-cells to up-regulate co-inhibitory molecules. In support of this potential sequence of events, we have observed increased co-inhibitory molecules (PD-1, CTLA-4) expressed on the surface of T-cells following peptide immunotherapy.

We observed that peptide immunotherapy can elicit tolerance by spreading regulation beyond the treatment epitope[320 & Chapter 3]; the ability of peptide administration to elicit bystander

suppression is likely to be dependent upon the mechanisms engaged by peptide administration. For instance, T-cell deletion or induction of anergy by high-dose immunotherapy may preclude the generation of bystander suppression, as these studies remove the cell populations that could exert active tolerance required for bystander suppression. Evidence from a murine model of OVA allergy supports this hypothesis. In a study of high-dose OVA peptide immunotherapy, no evidence of linked-suppression was observed[338], and likely as a result of this, these mice were not protected from subsequent challenge with OVA. In this study, a dose of peptide known to delete T-cells was utilized, suggesting that regulatory T-cells that could facilitate infectious tolerance and linked-epitope regulation were never established. A follow-up experiment, testing lower-doses of the same OVA peptide would reveal whether low-dose peptide administration could attenuate OVA specific responses. Our examination of peptide dose and affinity did not quantify T-cell deletion or anergy. For future studies, it would be intriguing to correlate the depletion of epitope specific T-cells and efficacy of therapy, as T-cell depletion may compromise the induction of tolerance to complex allergens.

Peptide affinity for the MHC Complex and the density of pMHC complexes are not the only variables determining the efficacy of a peptide. Several characteristics of the pMHC:TCR interaction are known to impact the elicitation of a response, for instance: stable TCR:pMHC interactions are believed to increase T-cell reactivity[339], with weaker interactions being associated with TCR antagonism[340]. Beyond this, weak TCR engagement has been linked to FoxP3 induction[341], while strong TCR engagement has been associated with IL-10 expression[342]. Alternative theories also exist, with some evidence indicating that pMHC potency, not avidity, governs the development of a response[343] or that beyond a minimum threshold number of pMHC copies, that the resultant response is primarily dependent upon co-stimulatory molecule

expression on the APC[344]. Taken together, these somewhat contradictory results render it difficult to predict the effect that a particular pMHC:TCR interaction will have upon T-cell activity, while also highlighting that peptide efficacy is likely dependent upon several features, and not just affinity for the MHC Class II molecule. Extrapolating from these studies, we should expect to observe differential efficacy between peptides of similar affinity for the MHC complex. In keeping with this prediction, in chapter 6 we demonstrated that peptides of low, moderate and high affinity for the DR4 MHC Class II molecule could equally suppress eosinophilia, thereby providing evidence that peptide affinity is not the sole predictive factor of efficacy. Lending further credence to the thesis that each peptide may possess a unique activity profile, our examination of T-cell phenotypes following immunotherapy revealed different peptides to stimulate differential regulatory pathways (FoxP3/CTLA4 induction, reduced number of Th2 cells, PD-1 induction). If these findings are to be transferred to the clinic, we must elucidate whether the unique activity profile of a peptide is consistent during presentation by distinct MHC Class II molecules. To assess the role of MHC presentation, we could repeat the studies performed in chapter 6 in HLA DR1 transgenic mice (commercially available from Jackson Laboratory), that possess a human-mouse chimeric HLA DR1 MHC Class II molecule and are deficient for endogenous MHC Class II. The affinity of the treatment peptides for the DR1 molecule is known[345], therefore, results could easily be compared to determine whether the intrinsic efficacy of a peptide is consistent across presentation molecules.

The concept of using peptides with a defined activity profile has been attempted in the clinic, but was met with mixed results. Altered peptide ligands (APLs) are peptide ligands with one (or more) amino acids changed that alter T-cell recognition. This strategy, in mice, successfully ameliorated diabetes, EAE and allergic disease [346-348349], but transition to clinical

settings has been fraught with safety concerns. Two trials of APLs for the treatment of MS have been halted due to the identification of unacceptably high rates of treatment-related disease exacerbation[350, 351]. The elicitation of tolerance by APL therapy is encouraging but the risk of exacerbating disease supersedes the observed benefits. Collectively, these data support the concept that treatment with peptides that possess defined efficacy profiles can be successful, while also highlighting potential safety issues. It is important to consider the root cause of the safety issues associated with APL therapy. The trials of APLs for the treatment of MS utilized doses of peptide ranging from 5-50mg per dose. We have demonstrated that ultra-low dose strategies, comprising therapy with sub µg quantities of peptide, can potently ameliorate allergic disease in mice[352]. Furthermore, we have provided evidence that therapy at low-doses induces bystander tolerance, a feature likely critical to the establishment of tolerance to complex antigens and, and that many peptides lose therapeutic efficacy at high doses (Chapter 6). Thus, the induction of adverse events by APL therapy may have been reflective of the dose used, rather than an inherent problem with the strategy. A small trial, wherein the previously tested APLs are administered at low-doses to subjects with relapsing-remitting MS, would be particularly intriguing, as I would hypothesize that this strategy may maintain the immunomodulatory effects of APL therapy, while averting safety concerns.

Our observations support two distinct strategies for future peptide based therapeutics. Firstly, the success of high affinity peptides at low-doses suggests that low doses of peptides may be efficacious in clinical research settings too, although this strategy would be predicated upon utilizing peptides with a known, high-affinity for human MHC molecules, a strategy that is difficult to employ in an outbred (i.e. human, not mouse) population. Secondly, our demonstration that moderate/low affinity peptides can inhibit allergic responses supports

selecting promiscuous peptides, that bind many MHC Class II molecules regardless of affinity, during development of peptide based therapies; although, this strategy may rely on higher doses of peptides to suppress allergic responses, a potentially dangerous proposition. Ultimately, both strategies may be employed, with current strategies employing option two, and advances in personalized medicine eventually enabling consistent, targeting with peptides of high affinity.

7.5. Limitations

Much of our current understanding of the pathogenesis of allergic immune responses has been derived from mouse models. The studies described in this thesis employed several murine models to examine both pathogenic allergic immune responses and immune modulating therapeutic strategies. This strategy offers two major benefits over the use of readily accessible clinical samples, 1) the ability to directly study the target organ (lung & draining lymph node) and 2) the ability to control allergen exposures & treatments. To interpret these data though, the limitations of mouse models must be acknowledged. Allergic responses are frequently described as an aberrant, exaggerated type 2 inflammatory response to a protein that non-allergic individuals view as innocuous. Similarly, allergen-specific immunotherapy has been hypothesized, by some, to function through modulation of immune responses away from type 2, towards type 1. This paradigm of T-cell responses being either Th1 or Th2 was originally determined using cloned murine CD4+ T-cells. Indeed, it was Mossman and colleagues demonstration that CD4+ T-cells, identical in surface antigen expression, could be influenced to produce unique, non-overlapping sets of cytokines[314, 353], that initiated this line-of-thought. While strong antigens can provoke similar behaviour in human T-cells[354], the division between Th1 and Th2 is not as clear in humans, as polarized human T-cells often retain the ability to simultaneously produce both Th1 and Th2 cytokines[355]. T-cell plasticity, in human asthma, is a

growing concern and may be driving severe disease. In allergic asthmatics, Th2 T-cells that also produce IL-17 have been identified[356, 357]. Similarly, some have hypothesized that the progression to severe asthma results from a superimposition of Th1 responses onto pre-existing, allergen-specific Th2 responses[358]. Taken together, the heterogeneity seen in human disease, and within human immune responses to allergens, may not be accurately replicated by mice. Thus, therapies developed in mice to deviate responses away from Th2 may be less efficacious when transferred to the clinic. In Chapter 3, we demonstrated that mice treated with Fel d 1 derived peptides were protected from challenge with OVA, and that this bystander effect was associated with the down regulation of OVA-induced Th2 responses. As human airways disease tends to be more heterogenous than the diseases induced in mice, whether an immune deviation of this sort would be clinically efficacious requires further testing

In mice and humans, activation of T-cells requires successful ligation of the TCR and ligation of co-stimulatory molecules, such as CD28. CD28, on the surface of T-cells, binds stimulatory B7 family proteins in addition to the inhibitory CTLA-4 glycoprotein[359]. CD28 is nearly ubiquitously expressed by murine T-cells, but is only present on approximately 80% of human CD4+ T-cells, and 50% of human CD8 T-cells[360]. The increased expression of CD28 in mice may indicate an increased reliance upon this pathway for both stimulatory and inhibitory stimuli, compared to humans. If mice and humans are differentially reliant upon the CD28-B7/CTLA-4 pathway, it becomes difficult to interpret the importance and relevance of our observation that mouse Tregs up-regulated CTLA-4+ after peptide immunotherapy. The success of CTLA-4-Ig therapy (abatacept) in rheumatoid arthritis[361] and type 1 diabetes[362] suggests that CTLA-4 does remain a dominant suppressive mechanism in humans, despite reduced expression of CD28. Thus, interspecies differences in CD28 expression may be unimportant.

We observed that low-doses of high-affinity Fel d 1 derived peptides significantly attenuated allergic disease in HLA DR4 mice. Moreover, we observed that low-doses of Fel d 1 and Der p 1 derived peptides suppressed allergic responses to HDM, OVA and CDE, in BALB/c mice. The term 'low-dose' to describe our strategies, while accurate compared to other studies of peptide immunotherapy, may be a misnomer. Indeed, our definition of 'low-dose immunotherapy' is likely not 'low-dose' to the immune system, nor in comparison to clinical strategies. At the level of the T-cell, live-imaging assays examining single APC-T-cell interactions via fluorescently labeled pMHC Class II complexes, have revealed that T-cells flux calcium in response to interaction with a *single* pMHC molecule[363], and that 25 pMHC molecules was a 'high' ligand density for T-cells, that rendered CD4 engagement superfluous[363]. Others have used markedly lower doses of peptide to induce tolerance in mice, for instance, diffusion of 65 picomoles of $HA^{(107-119)}$ peptide into mice each day for 14 days established regulatory T-cells that could suppress responses to the entire HA protein, *ex vivo*[300]. Thus, it becomes difficult to interpret whether our strategies tested a true 'low' dose. While the nomenclature between 'low' versus 'high' dose may seem somewhat arbitrary, rigorously defining the therapeutic window of peptide therapy may enable lower-doses in clinical studies.

In several of our studies, we initiated tolerogenic and inflammatory responses in the skin of mice. The reliance of these studies upon the immunological milieu of mouse skin poses a potential limitation, as several notable differences between mouse and human skin exist. For instance, the dominant T-cell subset in the skin of mice is γδT-cells, whereas αβT-cells dominate human skin[364]. Other critical immunological differences exist between mouse and humans, like the lack of IgG4 in mice. These are limitations of all mouse research but they are

particularly relevant to studies of allergen tolerance, where tolerance induction may rely upon modulating T-cell responses and/or inducing blocking IgG4 antibodies.

7.6. Reflection

The presented work has focused upon mechanisms of tolerance induction in mice. The general methods employed were to model human disease and administer clinically relevant treatments, that would provide immunological insights that cannot be achieved in clinical studies. This design allowed us to investigate mechanistic determinants of peptide immunotherapy, such as the role of peptide dose, dosing strategies and peptide affinity, and correlate them to functional outcomes of therapy. The consequence of this approach is that the majority of our mechanistic insights consist of observations and correlations. Thus, our data tend to highlight the plausibility of a mechanism rather than disprove alternative. As such, the work presented here primarily expands our foundation of knowledge and helps to form a frame-work that subsequent investigations can build upon. In contrast, this thesis does not provide a rigorous defined mechanism by which peptide immunotherapy functions in mice. While perhaps this represents an abnormal approach, we felt that this work was required for several reasons, 1) the ability of peptide immunotherapy to induce bystander tolerance had not been widely considered by the field and 2) the role of peptide affinity to the MHC complex had not been investigated, in allergic disease. Thus, we sought to highlight the relevance of these topics, as we felt that the value of this approach was greater than defining a tolerogenic cascade in mice, that may or may not be present in humans. Importantly, through this approach, we identified important immunological trends and biomarkers associated with efficacious peptide immunotherapy that will focus future mechanistic studies.

In contrast to the studies of peptide immunotherapy, our investigations into the role of allergen dose upon epicutaneous sensitization attempted to define precise molecular mechanisms. In this work, we took a methodical approach, iteratively performing a series of studies (kinetic studies, adoptive transfer, addition of LPS, knockout of MyD88) that each could have defined a molecular mechanism of action. To our surprise, these studies failed to define the mechanism and rather disproved our numerous hypotheses. Importantly, there is value to this approach as it elucidated pertinent immune mechanisms in the skin, such as the importance of MyD88 to sensitization. For this work to become clinically relevant, further efforts to define the mechanism of action of high-dose allergen induced allergen hyporesponsiveness will be required. On a personal level, it was exciting to research in a focused, mechanism and data driven manner.

7.7. Conclusion

The burden of asthma continues to rise in Canada, and other nations, throughout the world. While numerous therapeutic options exist, immunotherapy represents a disease modifying approach that can alter the progression of disease, prevent subsequent sensitizations and effectively treat symptoms well after the cessation of therapy[197, 319]. Despite a long history of peptide immunotherapy in clinical research, how treatment with a small number of T-cell epitopes can induce tolerance to complex allergens, in genetically diverse populations, is unknown. We hypothesized that peptide immunotherapy could induce tolerance to a complex allergen through the inducting the infectious spread of tolerance beyond the treatment epitope and that the efficacy of peptide therapy was dependent upon the peptide dose and affinity.

Through characterization of the effects of peptide immunotherapy in models of dual-allergen sensitization, we provide data indicating that peptide immunotherapy can induce

bystander tolerance, suppressing immune responses to distinct allergens. These findings support the current paradigm employed during rational peptide vaccine development, namely, that enhancing population coverage is the primary concern. Whether these data can further inform clinical practice, for example, through guiding the induction of bystander tolerance post-immunotherapy, remains to be determined. Beyond the ability of tolerance to spread beyond treatment epitopes, we characterized the importance of treatment epitope dose and affinity through examining seven peptides with defined binding affinity for the HLA DR4 MHC molecule. We observed that the maximum therapeutic potential of a peptide was not solely dependent upon affinity for the MHC complex, but rather that the affinity shifted the dose-response relationship. These data could be valuable for the design of future peptide based therapeutics, as they support emphasizing population coverage (i.e.: ability to bind many MHC molecules) over affinity to any single MHC complex.

Finally, during our development of mouse models, we found that high-dose allergen applied to the skin of mice failed to induce allergic responses. While the clinical relevance of these findings is currently unknown, our demonstration that MyD88 is required for epicutaneous sensitization could be relevant to the atopic march, a currently popular theory describing the progression of allergic disease.

In summary, the studies described in this thesis highlight immunological mechanisms that are relevant to the induction of tolerance in mice and humans. We provided evidence that expands the current understanding of peptide immunotherapy and may expedite and enhance the design of future peptide based immunotherapeutics.

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Appendix 1: Treatment with anti-Cytokine Monoclonal Antibodies can Potentiate the Target Cytokine Rather Than Neutralize its Activity

9. Declaration of Academic Achievement

Appendix 1: Treatment with anti-cytokine monoclonal antibodies can potentiate the target cytokine rather than neutralize its activity

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Daniel Moldaver's contributions

As the senior author, I was responsible for manuscript writing and development with C. Rudulier. C. Rudulier conceived the article. M. Larché provided guidance prior to submission.

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NEWS AND COMMENTARIES

Treatment with anti-cytokine monoclonal antibodies can potentiate the target cytokine rather than neutralize its activity

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Airway diseases such as allergic rhinitis and allergic asthma are due to a Th2 response to innocuous environmental antigens. Animal models have shown that the antibody-mediated neutralization of Th2 cytokines can greatly diminish airway inflammation (1–3); these results lead to the development and clinical investigation of humanized anti-Th2 cytokine antibodies for the amelioration of asthma (4–8). Unfortunately, clinical trials of these biologics have not been a resounding success, with inabilities to significantly improve clinical symptoms being common and successful trials requiring strict patient stratification (9). These failures have spawned numerous plausible explanations, such as a requirement to neutralize more than one Th2 cytokine to achieve a disease-modifying effect or that antibodies with a greater affinity for the target cytokine may be required. Alternatively, a well-documented phenomenon may be hampering clinical efficacy, namely the potentiation of cytokines through the formation of cytokine/anti-cytokine immune complexes.

The formation of cytokine/anti-cytokine immune complexes does not guarantee cytokine neutralization, as these complexes can actually increase the potency of a cytokine. Due to their bivalent nature, antibodies form immune complexes when neither the antibody nor the antigen is in excess. Murine studies have shown that cytokine/anti-cytokine immune complexes can potentiate the activity of numerous cytokines. For instance, IL-4/anti-IL-4 complexes are much more efficient than IL-4 alone at stimulating B cells, increasing their production of IgE and inducing the proliferation of CD8 T cells (10–12). Similarly, IL-2/anti-IL-2 complexes are much more potent in their ability to expand memory CD8 T cells, NK cells or regulatory T cells (Tregs) than IL-2 alone (12–15). An increase in the potency and bioactivity of IL-3, IL-6 and IL-7, when complexed with their respective antibodies, has also been reported (11, 16–22). Furthermore, recent studies have demonstrated the ability of IL-2/anti-IL-2 complexes to cause type 2 innate lymphoid cells (ILC2s) to expand, produce IL-5 and induce airway eosinophilia (23, 24). Together, these studies establish that the formation of immune complexes can potentiate the effects of the cytokine rather than neutralize its activity.

The ability of immune complexes to increase the potency of the cytokine has also been described in humans. Stein et al. reported the presence of IL-5/anti-IL-5 complexes in the circulation of subjects treated with mepolizumab and that these immune complexes facilitated increased production of IL-5 by CD4 T cells (25). Indirect evidence that immune complexes can worsen disease comes from a dose-finding study using lebrikizumab, an anti-IL-13 antibody, in subjects with moderate to severe asthma in which there was a direct correlation between the exacerbation rate and the dose of lebrikizumab in periostin-high subjects (26). Although the presence of IL-13/lebrikizumab complexes was not assessed, the seemingly paradoxical dose response of lebrikizumab is readily explained by the hypothesis that higher doses of lebrikizumab led to increased complex formation; this is also in line with the observations from the murine studies outlined above. The potentiation of cytokine activity is not restricted to antibodies but also extends to decoy receptors. For instance, when subjects with ankylosing spondylitis were treated with etanercept, which is a dimerized TNF receptor/Fc fusion protein designed to bind to and neutralize soluble TNF-α, a significant increase in the percentage of CD4 and CD8 T cells producing TNF-α and IFN-γ was reported (27). Collectively, these data extend the findings of murine studies to humans and emphasize that attempting to neutralize free cytokine with antibodies or decoy receptors may be an inefficient approach, as complexes can potentiate cytokine activity rather than neutralize it.

Treatment of subjects with neutralizing antibodies can not only increase the activity of the cytokine but may also increase its levels in the circulation. For example, subjects treated with mepolizumab displayed greater levels of IL-5 in the circulation with the majority of it bound in IL-5/anti-IL-5 complexes (17), while the treatment of asthmatic subjects with anti-IL-13 antibodies directly increased the levels of IL-13 in the serum (7). Similarly, the treatment of patients with metastatic breast cancer with etanercept increased TNF-α levels in the plasma, demonstrating that this effect is not restricted to Th2 cytokines and diseases (28). Studies in mice using antibodies to IL-2, 4, 6 and 7 have paralleled these clinical findings as greater bioactive levels of the targeted cytokine have been found following antibody treatment (12, 14, 16). Despite these observations, there appears to be little importance placed upon the formation of cytokine/anti-cytokine complexes in subjects treated with monoclonal antibodies as it is largely assumed that cytokines present in immune complexes are neutralized. However, as we have discussed, studies have demonstrated that the neutralization of a cytokine by its binding to antibody is not a foregone conclusion and further studies in humans are required.

The formation of immune complexes is prevented when the neutralizing antibody is present in excess. This concept is very clearly exhibited in early studies on the stimulatory potential of cytokine/anti-cytokine complexes where an

approximate 2:1 ratio of antibody to antigen is required to form cytokine/anti-cytokine complexes. Typically, the addition of greater amounts of neutralizing antibody prevents the formation of immune complexes. In line with these original observations is the ability of IL-6/anti-IL-6 complexes to stimulate an IL-6-dependent cell line which is then inhibited when additional anti-IL-6 is added to the assay (13, 19). Further complicating matters is evidence demonstrating that for certain cytokines, the addition of more antibody simply induces greater cytokine potentiation. For instance, titrations of an anti-IL-2 antibody (clone S4B6) in the presence of a fixed concentration of IL-2 have demonstrated that increasing levels of S4B6 induce greater CD8 T cell stimulation (12). Therefore, efforts to use biologics to target free cytokine might be sabotaged by the development of complexes, again highlighting a possible inefficiency of this approach.

The mechanisms by which anti-cytokine antibodies increase the circulatory levels and potency of the target cytokine are incompletely defined. The most obvious explanation is that cytokine ligation by an anti-cytokine antibody prevents cytokine degradation, thereby greatly increasing the half-life of the cytokine from a few hours to a few days (11, 13, 21). Indeed, an increase in the half-life of some cytokines has been shown, in mice, to be dependent upon the neonatal Fc receptor (FcRn), which rescues antibody/antigen complexes from endosomes and recycles them to the cell surface (12, 22). The fusion of targets to Fc domains, an analogue of immune complexes, has been used by the pharmaceutical industry for several years as it utilizes normal physiological mechanisms (namely, the FcRn receptor) to facilitate drug trafficking across membranes and enhances half-life (29). Evidence has begun to accumulate that indicates that immune complexes may directly potentiate cytokines independently of FcRn-mediated half-life prolongation. In studies investigating the mechanism of cytokine potentiation by FcRn, IL-2/IgG Fc fusion proteins (IL-2-FP) were constructed. Interestingly, at equivalent doses, IL-2–FP was significantly less effective than IL-2/anti-IL-2 complexes in its ability to induce expansion of CD8 T cells, despite similar *in vivo* lifespans (13). The co-administration of an anti-CD25 antibody with the IL-2–FP rescued the ability to robustly stimulate T-cell proliferation. These data indicate that the antibody portion of the cytokine/anti-cytokine complex may enhance proliferation by preventing cytokine binding to scavenger receptors. Importantly, this mechanism of potentiation is not limited to the relatively unique IL-2 receptor as similar findings have been noted for IL-7. Murine studies investigating the use of anti-IL-7 antibody have indicated that the presence of an immune complex may protect the complexed cytokine from receptor-mediated consumption (22). Clearly, cytokine potentiation by anti-cytokine antibodies is a complex process that spans numerous disciplines from pharmacology to immunology. These mechanisms of potentiation highlight the potential risks of treating subjects with anti-cytokine antibody and may indicate that a modification to the approach is required.

Given the large amount of neutralizing antibody that is required to effectively neutralize a cytokine, we propose that it may be more effective to focus efforts on developing humanized antibodies that target cytokine receptors. In contrast to experimental asthma models in inbred strains of animals, effective dosing of individuals is complicated by the heterogeneity of human asthma. The level of Th2 cytokines produced by an individual is determined by both the severity of their asthma and whether the subject is exposed to the allergen. Thus, a dose of neutralizing anti-cytokine antibody that is high enough to avoid the formation of immune complexes in one individual may form cytokine/anti-cytokine complexes in other individuals. Moreover, in a given patient, the ratio of cytokine to antibody will increase during exacerbations, thereby increasing the risk of immune complex formation.

Targeting the receptor avoids the complication of forming cytokine/anti-cytokine complexes while still preventing the target cytokine from initiating a signal. The efficacy of targeting the cytokine receptor is evident from the impressive results observed in the clinical trials of dupilumab, which targets the alpha subunit of the IL-4 receptor, in subjects with severe asthma (30) and atopic dermatitis (31). Similarly encouraging clinical trial results were obtained with benralizumab, an anti-IL-5 receptor antibody, in subjects with severe asthma (32).

We have outlined a series of observations that may explain why targeting cytokines may be clinically inefficient in some cases and suggest that much larger quantities of anti-cytokine antibodies be used in clinical trials, with this dose being guided by the assessment of immune complexes in the subjects. We also suggest that targeting of cytokine receptors, rather than the cytokine itself, may be a more fruitful avenue of research as it avoids the complication of immune complex formation and may require lower levels of antibody since a blocked receptor is insensitive to increases in cytokine levels that occur following exposure to allergen.

Author contributions

The idea for this manuscript was conceived by C.D.R. and developed by C.D.R, M.L and D.M. The manuscript was written by C.D.R, M.L. and D.M.

Conflicts of interest

C.D.R and D.M. have no conflict of interest to declare. M.L. is a cofounder, stockholder and consultant of Circassia Ltd. and a founding scientist of Adiga Life Sciences Inc. and has received research support from both of these companies.

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Appendix 2: Immunotherapy with Peptides

10. Declaration of Academic Achievement

Appendix 2: Immunotherapy with Peptides

D. Moldaver and M. Larché.

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Daniel Moldaver's contributions

M. Larché and I were both responsible for manuscript conception and writing. M. Larché generated the figures.

Allergy

REVIEW ARTICLE

Immunotherapy with peptides

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Abstract

Specific allergen immunotherapy is clinically effective and disease modifying. It has a duration of effect that exceeds the treatment period and prevents both the progression of allergic rhinitis to asthma and the acquisition of new allergic sensitizations. However, immunotherapy is associated with a high frequency of adverse events related to the allergenicity of vaccines. Allergenicity is conferred by the presence of intact B-cell epitopes that crosslink allergen-specific IgE on effector cells. The use of linear peptide sequences representing fragments of the native allergen is one approach to reduce allergenicity. Preclinical models of peptide immunotherapy have demonstrated efficacy in both autoimmunity and allergy. Translation of this technology into the clinic has gained momentum in recent years based on encouraging results from early clinical trials. To date, efforts have focused on two major allergens, but vaccines to a broader range of molecules are currently in clinical development. Mechanistically, peptide immunotherapy appears to work through the induction of adaptive, allergen-specific regulatory T cells that secrete the immunoregulatory cytokine IL-10. There is also evidence that peptide immunotherapy targeting allergen-specific T cells can indirectly modulate allergen-specific B-cell responses. Peptide immunotherapy may provide a safe and efficacious alternative to conventional subcutaneous and/or sublingual approaches using native allergen preparations.

Specific allergen immunotherapy is clinically effective and has benefit that extends beyond the treatment period (1). Unlike conventional pharmacotherapy, specific allergen immunotherapy is disease modifying and has been shown to prevent the development of asthma (2) and to reduce sensitizations to other allergens (3). Optimal treatment times vary based on allergen and therapeutic preparation, but in general terms, a course of 3 years therapy is regarded as optimal. Although there are no formal published data on the relationship between treatment time and duration of clinical response, it is widely held that clinical benefit will last for twice as long as the treatment period. Thus, treatment for 1 year results in additional benefit for the subsequent year, whereas treatment for 3 years will provide clinical benefit for a further 3 years. The lengthy duration of treatment, the frequent administration of allergen and the associated high frequency of allergic adverse events, which may be systemic and in some cases life-threatening, have limited uptake of this form of therapy. These shortcomings are the result of the allergenicity of the preparations employed for therapy. These contain whole allergen molecules with intact B-cell epitopes that are readily able to crosslink specific IgE molecules on the surface of effector cells (Fig. 1). A number of strategies aimed at reducing the allergenicity of treatment preparations, whilst maintaining immunogenicity, have been described. Physical modification of allergen molecules may reduce or eliminate IgE reactivity and thus allergenicity (4). Such approaches have taken many forms including chemical modification, conjugation with synthetic bacterial DNA motifs, point mutations in native allergen gene sequences and the use of allergen multimers, fragments and peptides of various lengths. The use of soluble, short synthetic peptides for the treatment of allergic disease allows the delivery of T-cell epitopes of the allergen in an adjuvant-free, tolerogenic form, whilst avoiding IgE-mediated allergic reactions (5, 6). Synthetic peptides have been developed and evaluated in both experimental animal models and human clinical studies. Synthetic peptides are defined active pharmaceutical ingredients (API) and can be produced in a reproducible and standardized fashion that cannot be achieved with allergen extracts. Further advantages include low production costs, ease of purification and good stability in lyophilized form without the need for cold storage.

Presentation of T-cell epitopes to T cells by nonprofessional antigen-presenting cells (APC) results in the induction of 'anergy', a term describing antigen-specific nonresponsive-

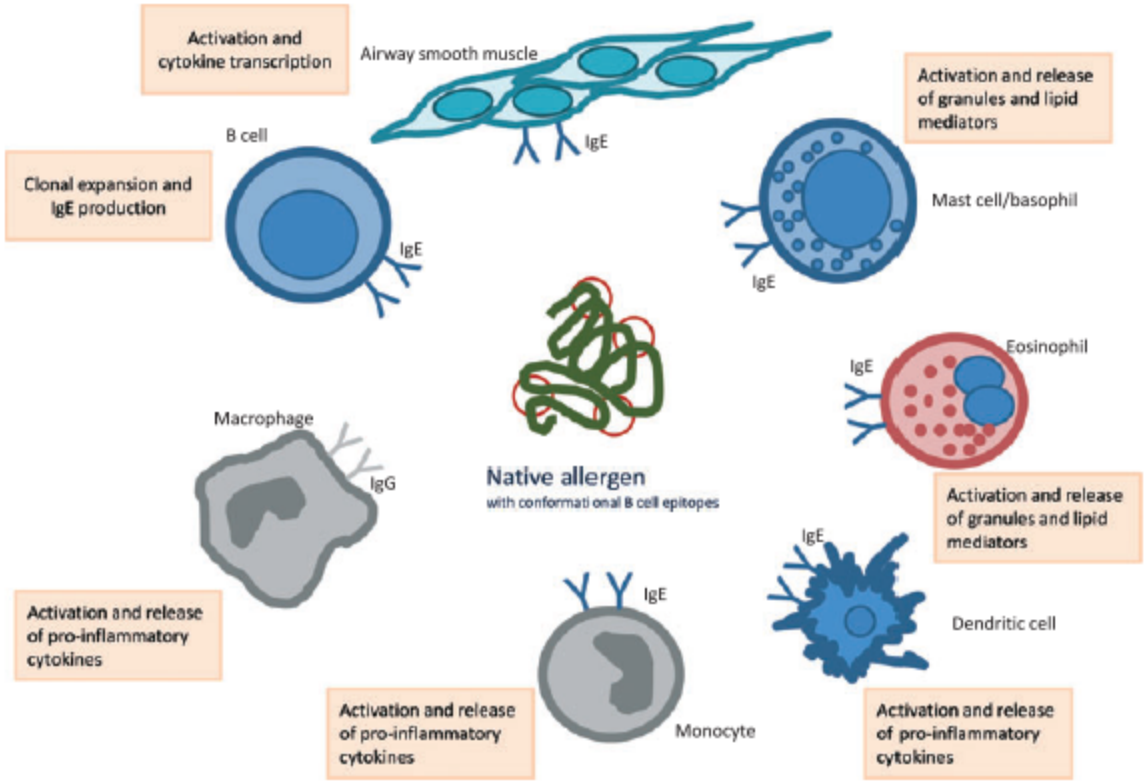

Figure 1 Encounter with native allergen activates effector cells, antigen-presenting cells and structural cells. Cells bearing Fc receptors for IgE and IgG bind allergen-specific antibodies. Crosslinking of cell-bound allergen-specific antibodies by different epitopes (conformational or linear) of the native allergen molecule results in cellular activation. Mast cells, basophils, B cells, dendritic cells, monocytes and airway smooth muscle cells all express IgE receptors, and macrophages express IgG receptors; all are thus capable of activation during allergen encounter.

ness. For example, when highly purified $CD4^+$ T-cell clones were cultured with supraoptimal concentrations of specific epitope, they were refractory to subsequent stimulation with an optimal antigen challenge (7). Similarly, when T-cell clones were cultured with epithelial cells, keratinocytes or myoblasts, expressing MHC class II and specific peptide, anergy ensued (8–10). Indeed, presentation of antigenic peptide by epithelial cells resulted in the generation of T cells with regulatory/immunosuppressive function (10).

Experimental *in vivo* studies of peptide-induced tolerance have been reported in a number of disease areas including allergy, autoimmunity and transplantation. Initially, tolerance was induced in models of autoimmune disease using high-dose, prophylactic administration of peptide in adult or neonatal mice (11–21). More recently, intranasal peptide delivery led to protection from disease, which required deletion of effector T cells and the presence of IL-10, in a murine model of multiple sclerosis (22, 23). Treatment of established disease has also been achieved in allergen sensitization models. Mice were immunized with the house dust mite allergen Der p 2 and tolerized with immunodominant peptides resulting in the down-regulation of both T-cell and antibody responses to Der p 2 (24). Similarly, mice sensitized to the birch allergen Bet v 1 were rendered tolerant by the administration of peptide-containing T-cell epitopes (25). Mice sensitized to insect venoms have also been treated both prophylactically and therapeutically with peptides to reduce allergic responses following allergen challenge (26, 27). Mice sensitized to cats through priming with the Fel d 1 allergen were treated with two polypeptides derived from the sequence of Fel d 1 chain 1. As a strong adjuvant was used during priming, only Th1 outcomes were assessed. Treatment resulted in the decreased production of IL-2 and allergen-specific IgG (28). More recently, mice lacking endogenous MHC class II but expressing a transgene encoding the human MHC molecule HLA-DRB1*0101 were sensitized with recombinant Fel d 1 and treated with a Fel d 1 peptide known to bind to HLA-DRB1*0101. A single, low-dose (1 μg) of peptide was able to modulate multiple parameters of allergic sensitization including significant reduction in nonspecific airway hyperresponsiveness (AHR), Th2 recruitment and cytokine/chemokine secretion, IgE production, mucus hypersecretion and airway eosinophilia. Tolerance induced by one T-cell epitope was found to confer tolerance to other T-cell epitopes of the same

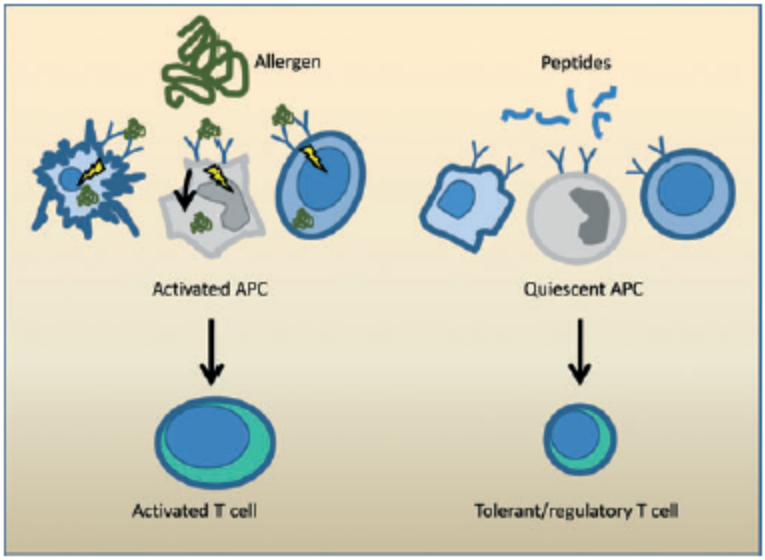

Figure 2 Native allergen, but not short-peptide epitopes, results in the activation of antigen-presenting cells (APC) and inflammatory T-cell responses. Crosslinking of allergen-specific antibodies bound to the surface of APC results in APC activation and upregulation of pro-inflammatory genes. The activated APC presents processed allergen epitopes in an inflammatory context to allergen-specific T cells. In contrast, short synthetic peptides are unable to crosslink allergen-specific antibodies, resulting in a quiescent encounter between peptide epitope and APC. Presentation of allergen epitopes to T cells in this context results in the induction of immune tolerance.

molecule, providing evidence of the induction of linked epitope suppression. Blocking studies with a monoclonal antibody directed against the IL-10 receptor demonstrated that the treatment effect in this model was IL-10 dependent (29).

Translation of peptide immunotherapy into the clinical setting has been slow but the approach is gaining popularity in the treatment of both allergic diseases and autoimmune diseases. Clinical development programmes are currently underway in multiple sclerosis (http://www.apitope.com), type I diabetes (30), rheumatoid arthritis (31, 32) and coeliac disease (http://www.nexpep.com). However, these programmes are beyond the scope of the current review. Treatment of allergic disease with peptides has, until recently, been limited to cat allergy, ragweed allergy (although these studies have only been published in abstract form) and bee venom. Clinical development programmes are currently underway with other allergens (http://www.circassia.co.uk).

Peptides from Fel d 1

Following preclinical studies described earlier (28), a series of clinical trials were performed to evaluate the safety and efficacy of two polypeptides from the major cat allergen Fel d 1. In the first of these to be published, an equimolar mixture of the peptides identified as IPC-1/IPC-2 (AllervaxCat®; ImmuLogic Pharmaceutical Corporation, Waltham, MA; equimolar combination of two 27-amino acid synthetic peptides) was compared with placebo in a treatment regimen involving four subcutaneous injections over 2 weeks. Cat-allergic subjects (with documented allergic rhinitis plus or minus asthma) were treated in 10-fold dose increments (7.5, 75 and 750 μg per injection) (33). Significant improvements in lung and nasal symptom scores were observed in the high-dose group. A related mechanistic study demonstrated reduced IL-4 production in peptide-specific T-cell lines following treatment given by Marcotte et al. (34). A large placebo effect was observed in common with many allergen immunotherapy trials. Despite the observed clinical efficacy, the vaccine was poorly tolerated in many subjects with treatment-emergent adverse events occurring acutely and up to several hours after peptide injection. Most frequently, adverse events were reported in the respiratory system and in particular, subjects with a history of asthma reported symptoms of chest tightness and shortness of breath several hours after dosing. Subsequent investigation identified MHC-restricted activation of allergen-specific effector T cells in the airways as being responsible for these manifestations, highlighting IgE-independent mechanisms for airway narrowing (35). In an inhaled allergen challenge study, small groups of cat-allergic asthmatic individuals were treated with variable doses of IPC-1/IPC-2. The provocative dose of inhaled cat allergen resulting in a 20% reduction (PD_{20}) in forced expiratory volume in one-second (FEV_1) was compared before and after dosing. A significantly higher dose of allergen was required to achieve a 20% reduction in FEV_1 after treatment in the higher dose groups. However, these differences were observed only when comparing pretreatment and posttreatment challenges in vaccine-treated individuals, but not when

these changes were compared with those in the placebo group (36).

Modest clinical improvements were reported in a multicentre study in which cat-allergic subjects received eight subcutaneous injections of high-dose vaccine (750 μg). There was a significant improvement in pulmonary function 3 weeks after therapy, but only in individuals with reduced baseline FEV_1 (37). In common with earlier studies, frequent, acute and delayed adverse events were reported during treatment. Delayed symptoms of asthma (isolated late asthmatic reactions) were reduced with successive doses of peptide suggesting the induction of immunological tolerance. A fourth study in which cat-allergic subjects received four weekly injections of an intermediate dose of vaccine (250 μg) failed to show any significant change in surrogate markers of efficacy or changes in mechanistic outcomes. In common with other studies, treatment was associated with delayed symptoms of rhinitis, asthma and pruritis (38).

More recently, a series of clinical studies have been performed using mixtures of shorter peptides from Fel d 1 (39–44). In a small pilot study, cat-allergic asthmatic subjects received a single intradermal injection of a mixture of 12 short synthetic peptides comprising the majority of the T-cell epitopes of Fel d 1 (45). Skin late-phase reactions (LPSR) to challenge with whole cat dander allergen extract were significantly reduced following peptide treatment. Modulation of skin responses was associated with reductions in allergen-specific proliferation and a reduction in both Th1 and Th2 cytokines (39). In a double-blind, placebo-controlled clinical trial, an identical mixture of Fel d 1 peptides was given in a multiple dose, incremental fashion (cumulative dose = 90 μg) to a small cohort (n = 24; 16 active, 8 placebo) of cat-allergic asthmatic subjects. In common with the earlier study, statistically significant reductions were observed in the LPSR to whole allergen challenge. Additionally, at later follow-up (3–9 months posttherapy), the magnitude of the early-phase skin response (EPSR) was also significantly reduced. Significant within-group changes in quality of life observed that these changes did not achieve statistical significance when compared with placebo. Changes in clinical surrogate markers were associated with reduced allergen-specific responses of peripheral blood mononuclear cells (PBMC) *in vitro*, including reduced proliferation and reduced secretion of both Th1 and Th2 cytokines. A concomitant and significant increase was observed in PBMC secretion of the regulatory cytokine IL-10. No acute, treatment-related adverse events were reported in this study, but isolated late asthmatic reactions were expected (based on the dose of peptides employed) and observed in some subjects. Induction of isolated late asthmatic reactions did not appear to be related to the induction of allergen-specific tolerance (40). Related mechanistic studies using PBMC isolated from subjects treated in the trial demonstrated that proliferative and cytokine responses to sequences within Fel d 1 that did not form part of a peptide vaccine were also significantly reduced. These findings are indicative of intramolecular tolerance (also known as 'linked epitope suppression') and imply that peptide vaccines need not necessarily contain all T-cell epitopes from a target antigen to achieve broad antigen-specific tolerance (29).

Changes in nonspecific AHR were addressed in two studies, with differing outcomes. In a small, open-label study using a similar peptide preparation delivered at 2-week intervals and using a lower dosing regimen, a significant reduction in airway hyperreactivity (measured by PC_{20}) was observed (41). In contrast, no change in AHR was observed in a slightly larger, double-blind, placebo-controlled study (40). The complex nature of AHR and the marked variability of longitudinal measurements within patients make this outcome difficult to assess in small, underpowered clinical studies.

Most recently, a seven peptide vaccine (Toleromune Cat®; Circassia Ltd., Oxford, UK) has been evaluated in a phase IIa safety and tolerability study (46). Peptide components were evaluated in direct MHC binding assays (10 commonly expressed MHC alleles) to determine which sequences would provide the best population coverage. Peptides displaying promiscuous binding to MHC class II were assessed in proliferation, cytokine (IFNγ, IL-10, IL-13) and histamine release assays, and vaccine components were selected. The resulting vaccine was administered, without adjuvant, to 88 cat-allergic individuals either intradermally or subcutaneously to evaluate safety and tolerability and, as a secondary outcome, the dose of peptide resulting in the greatest inhibition of the LPSR to whole allergen challenge as a surrogate of clinical efficacy. The vaccine was safe and well tolerated and, importantly, had an adverse events profile that was indistinguishable from placebo providing confidence that future clinical trials will be adequately blinded in contrast to current approaches. Cohorts of eight cat-allergic subjects received a single injection of vaccine (n = 6) or placebo (n = 2). Intradermal delivery gave a maximal (40%) reduction in the LPSR at a dose of 3 nmol of vaccine, similar to earlier findings with the prototype vaccine (39, 46). Phase IIb clinical studies are now underway to further evaluate the safety and tolerability of a vaccine and to begin to explore clinical efficacy.

Peptides from Api m 1

Only three studies of peptide immunotherapy in bee venom–allergic individuals have been reported. None of these were fully blinded, placebo-controlled studies, and each study was performed in small numbers of individuals. However, the data that emerge strongly support the findings of earlier clinical interventions with peptides from Fel d 1. Immunodominant T-cell epitopes of the major bee venom allergen Api m 1 were identified (47) and subsequently used in a pilot clinical study to treat five bee venom–allergic subjects who had documented (moderately severe) systemic allergic reactions to bee venom. Subjects received incremental doses (cumulative dose 397.1 μg) of an equimolar mixture of peptides at weekly intervals (48). Ten subjects treated with standard bee venom immunotherapy acted as a control group. All five subjects tolerated a subcutaneous challenge with 10 μg of whole Api m 1 following the last treatment injection. Subsequently, a wild bee sting challenge was performed, which was tolerated

by three of five subjects, with the remaining two developing mild systemic allergic reactions.

Texier et al. (49) used direct peptide-MHC binding assays to quantify the binding affinities of overlapping synthetic peptides spanning the Api m 1 sequence. Four immunodominant peptides were identified, three of which were similar to those used previously for therapy by Müller and colleagues. Tarzi et al. (50) used these peptides (formulated in saline) to perform an open-label, single-blind study of peptide therapy in subjects with mild bee venom allergy (subjects had not experienced systemic reactions following bee sting). The peptides were well tolerated, and no treatment-emergent adverse events were observed. In common with studies performed using Fel d 1 peptides, treatment resulted in reduced proliferative and cytokine (Th1 and Th2) responses in PBMC cultured with allergen. In contrast, IL-10 secretion was significantly up-regulated. Furthermore, LPSR to both whole bee venom and Api m 1 were significantly reduced.

Fellrath et al. (51) treated bee venom–allergic subjects with a RUSH desensitization protocol using three long synthetic peptides (LSP) encompassing the entire Api m 1 sequence. Starting at 0.1 μg, subjects were administered approximately 250 μg in incremental doses at 30-minute intervals. Maintenance injections of 100 or 300 μg were administered on days 4, 7, 14, 42 and 70. A transient increase in T-cell proliferation to the treatment peptides was observed, together with increases in IFNγ and IL-10 levels, but not Th2 cytokines. Allergen-specific IgG_4, but not IgE, levels increased throughout the study period, likely due to the presence of some intact B-cell epitopes within the polypeptides. Peptide-specific IgE was induced during treatment in some subjects, reminiscent of seasonal increases in IgE to allergens during the pollen season. In contrast to a number of studies with Fel d 1 peptides, no significant change in skin sensitivity to intradermal whole allergen challenge was observed. Peptide therapy was generally well tolerated, although local and disseminated erythema with hand (palm) pruritis was observed in two subjects.

Evidence for the induction of regulatory T cells during peptide immunotherapy

Reductions in the LPSR to allergen challenge were a consistent feature of several studies employing mixtures of short, synthetic peptides. Immunohistochemical analysis of skin biopsy sections following skin allergen challenge demonstrated a significant increase in $CD25^+$ cells and $CD4^+$/IFN-γ^+ cells after peptide therapy, suggesting that the recruitment of Th1 cells (and perhaps regulatory T cells) to sites of allergen encounter may contribute to the mechanism of action of these vaccines. In contrast to PBMC responses following culture with whole allergen, no increases in IL-10^+ cells were observed in allergen-challenged skin biopsies; however, the expression of TGFβ mRNA was increased suggesting that different mechanisms of tolerance may dominate at different anatomical locations (41).

Evidence of a role for the immune regulatory cytokine IL-10 and modulation of the allergen-specific B-cell response following peptide immunotherapy has also come from studies employing peptides from Api m 1. Increased IL-10 production in cultures of PBMC incubated with allergen was observed in studies employing both short and LSP from Api m 1 (50, 51). Both studies were also associated with an increase in allergen-specific IgG_4, although this was modest with the use of short peptides. Although no measurements of IL-10 were made in the earliest of these studies (48), changes in the isotype of allergen-specific antibodies indicative of an increase in IL-10 were observed. Initially, no change was observed in the levels of allergen-specific serum IgE or IgG_4 during the course of treatment in this study. However, following subcutaneous challenge with Api m 1, a week after treatment, the concentration of both isotypes, particularly IgG_4, increased. These data suggest that whilst therapy with short peptides may not directly stimulate allergen-specific B cells (because of the inability of the peptides to crosslink B-cell surface antigen receptors), encounter of whole allergen molecules following the induction of IL-10-secreting, allergen-specific T cells results in the redirection of B-cell responses to IgG_4 through the action of IL-10, which has previously been shown to potentiate the production of this isotope (52).

The effect of peptide immunotherapy on regulatory T-cell function was evaluated using PBMC obtained at baseline and following the completion of treatment in a double-blind, placebo-controlled trial (43). Treatment was associated with significant reductions in allergen-specific proliferative responses and IL-13 production from PBMC *in vitro*. Measurement of the functional regulatory activity of $CD4^+CD25^+$ cells to suppress allergen-specific proliferative and cytokine responses was assessed by mixing these putative regulatory T cells with autologous $CD4^+CD25^-$ cells. Peptide immunotherapy did not alter the suppressive activity of purified $CD4^+CD25^+$ cells suggesting that naturally occurring regulatory T cells may not play a significant role in the immunological changes associated with this form of immunotherapy. These experiments were performed prior to the identification of more specific markers of regulatory T cells such as the $CD4^+CD25^{hi}CD127^{lo}$ phenotype and it is thus likely that the putative regulatory population isolated in this study was a mixture of regulatory cells and recently activated T effector cells. However, recent data from murine studies of low-dose peptide immunotherapy support the conclusion that natural (thymus-derived) regulatory T cells may not play a significant role in peptide immunotherapy as no increase was observed in $Foxp3^+$ T cells in the lungs of mice after treatment (29).

A role of antigen-specific inducible regulatory T cells in the efficacy of peptide immunotherapy was addressed through the isolation of $CD4^+$ T cells (containing the putative regulatory cells) from PBMC samples obtained before and after peptide immunotherapy. $CD4^+$ cells were cultured with $CD4^{negative}$ cells that had been labelled with the cell division–tracking dye (44). $CD4^+$ T cells from posttreatment samples suppressed the antigen-driven proliferation of $CD4^{negative}$ cells from pretreatment samples, demonstrating that peptide immunotherapy induces a population of $CD4^+$ T cells with suppressive/regulatory activity. Assembling

the available data from a limited number of studies, it appears that low-dose peptide immunotherapy may induce IL-10-secreting adaptive regulatory T cells (akin to Tr1 cells) that are capable of down-regulating Th2 responses to allergen. However, in this form of treatment, there is little evidence of a major role for natural $CD4^+CD25^+Foxp3^+$ regulatory T cells.

Mechanisms of peptide-induced tolerance

The induction of T-cell anergy and T cells with immunosuppressive function following presentation of peptides by non-professional (such as myocytes and keratinocytes) or immature (dendritic cells) APC supports the notion that antigen recognized by T cells under noninflammatory conditions results in tolerance (53). Indeed, tolerance to both exogenous (54) and endogenous antigens (55) may be maintained, at least partly, by pools of regulatory T cells that secrete IL-10. Administration of highly soluble peptides in the absence of pro-inflammatory adjuvants may exploit this in intrinsic tolerance pathway (Fig. 2). Furthermore, administration of peptides lacking sufficient secondary structure to crosslink adjacent immunoglobulin molecules may further reduce the likelihood of creating an inflammatory milieu during T-cell recognition of peptide–MHC complexes.

Conclusions

Peptide immunotherapy represents one strategy to reduce the allergenicity of therapeutic preparations for the treatment of allergies. Short, synthetic peptides containing T-cell epitopes show markedly reduced ability to crosslink allergen-specific IgE. The resulting reduction in allergenicity has allowed more recent clinical trials to be performed in a truly blinded fashion in contrast to studies of subcutaneous and sublingual immunotherapy performed with intact allergen preparations. Peptide immunotherapy modifies numerous surrogate markers of allergen exposure such as skin responses to allergen challenge, nonspecific AHR (some studies), symptom scores following inhaled allergen challenge, quality of life and the ability to tolerate natural allergen exposure. Peptide immunotherapy appears to induce a population of functional regulatory/suppressive cells with characteristics of Tr1 cells. Peptide immunotherapy, in human subjects, is associated with the induction of IL-10. In experimental murine models, where such experiments are possible, tolerance has been shown to be dependent upon IL-10. Peptide immunotherapy has a variable effect on the B-cell compartment with larger peptides (which presumably retain more B-cell epitopes) being better able to induce allergen-specific IgG_4 responses than short peptides.

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Author contributions

DM and ML conceived and wrote this manuscript. ML produced the figures.

Conflict of interest

The author is a founder, shareholder and consultant of/to Circassia Ltd, a company developing peptide-based immunotherapy. The author is the scientific founder of Adiga Life Sciences Inc., a joint venture between Circassia Ltd and McMaster University.

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