ARA H 1 PEPTIDE IMMUNOTHERAPY IN A MOUSE MODEL OF PEANUT-INDUCED ANAPHYLAXIS

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LAY ABSTRACT

Peanut allergy is a growing public health concern. Its prevalence has doubled in the past 10 years and currently stands at 2%. Reactions to peanut account for the majority of food-induced fatal allergic reactions, termed anaphylaxis. Currently, there are no treatments available for patients with peanut allergy. Healthcare workers can only offer peanut-allergic patients advice on peanut avoidance and rescue medications in case of accidental ingestion. This research project investigated the ability of a new treatment called peptide immunotherapy to prevent severe allergic reactions to peanut in a mouse model of peanut allergy. Peptide treatment uses small portions of the peanut allergen to shift the immune response from pro-inflammatory to anti-inflammatory. After peptide treatment, peanut-allergic mice were protected from severe allergic reactions in response to peanut and their immune cells produced lower levels of pro-inflammatory molecules.

ABSTRACT

Background: Despite the clinical severity and rising prevalence of peanut allergy, there is a marked absence of widespread, practical treatments available for peanut-allergic patients. Peptide immunotherapy, a disease-modifying treatment that uses short peptides recognized by T cells, has been shown to reduce allergic symptoms of allergic rhinoconjunctivitis. This project investigated the ability of peptides from the major peanut allergen Ara h 1 to protect against peanut-induced anaphylaxis and induce immunomodulatory changes in a mouse model.

Methods: Mice transgenic for the human leukocyte antigen DRB1*0401 were sensitized to peanut epicutaneously and treated with two intraperitoneal injections of peptides from Ara h 1. Mice were then challenged with intraperitoneal whole peanut and observed for signs of anaphylaxis. Flow cytometry was used to isolate peanut-specific $CD4^+$ T cells labelled with Ara h 1 peptide-loaded tetramers and additional Th1, Th2, and regulatory markers.

Results: Peptide-treated mice were protected from severe peanut-induced anaphylaxis. Control mice treated with a sham peptide experienced a mean maximum temperature drop of 3.2°C, while mice treated with Ara h 1 peptides experienced a drop of 1.6°C (p=0.067 vs control). Maximum clinical score was 2.5 in control mice, and 1.4 in treated mice (p=0.0097). Mean hematocrit for control mice was 52.5%, and 47% for treated mice (p=0.013). PD-1^+CD4^+ T cells were significantly increased in the mesenteric lymph nodes (p = 2.28e-0.05) and spleens (p = 0.014) of peptide-treated mice. MIP1-α^+CD4^+ T cells were significantly decreased in the peritoneal lavage (p = 0.008).

Conclusion: Ara h 1 peptide immunotherapy protected against severe peanut-induced anaphylaxis in a mouse model. Peptide-treated mice experienced significantly reduced drops in core body temperature, clinical signs of allergic reaction, and hemoconcentration. Clinical

protection was associated with decreased expression of the pro-inflammatory chemokine macrophage 1-α and increased expression of the surface marker programmed cell death protein 1.

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LIST OF ABBREVIATIONS

AGE: advanced glycosylated end product

CD: cluster of differentiation

CLA: cutaneous lymphocyte-associated antigen

CPE: crude peanut extract

DC: Dendritic cell

DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin

DR4 mice: mice transgenic for the human leukocyte antigen DRB1*0401

FAFH: food allergy herbal formula

FcεRI: Fc epsilon receptor I

FcγRIII: Fc epsilon receptor III

HLA: human leukocyte antigen

IFN: interferon

Ig: immunoglobulin

IL: interleukin

LAG3: lymphocyte-activation gene 3

MHC II: major histocompatibility complex type II

PAF: platelet activating factor

PBS: phosphate buffered saline

PD-1: programmed cell death protein 1

PLA: phospholipase A2

RAGE: receptor for advanced glycation endproducts

Th1: type 1 T helper

Th2: type 2 T helper

TSLP: thymic stromal lymphopoietin

SIT: specific whole-allergen immunotherapy

SEM: standard error of the mean

STAT-6: signal transducer and activator of transcription 6

DECLARATION OF ACADEMIC ACHIEVEMENT

The author performed all experiments described in this document. Assistance with mouse work was provided by Ms. Jennifer Wattie, animal technician. Ms. Wattie performed blinded cell counts of peritoneal lavage samples. Assistance with tissue harvesting and processing was provided by Dr. Christopher Rudulier, Dr. Daniel Moldaver, Ms. Tarandeep Singh, and Mr. Ivan Nayve. Peanut-specific antibody measurements were performed by Ms. Tina Walker.

Chapter 1: Introduction

Allergy

Allergy is an inappropriate inflammatory immune reaction to an otherwise innocuous antigen. Allergic disease encompasses the following conditions: asthma; rhinoconjunctivitis; anaphylaxis; drug, food, and insect allergy; eczema; urticaria; and angioedema[1]. The global burden of allergy is rising, and 30-40% of the world's population is affected by one or more allergic disease(s)[1]. Food allergy in particular now affects nearly 5% of adults and 8% of children[2]. Symptoms of allergic reactions range in severity from mild to life-threatening, but commonly include: itching, hives, angioedema, shortness, abdominal pain, and vomiting. In anaphylaxis, the most severe form of allergic reaction, hypotension and circulatory collapse can also occur. Anaphylactic reactions have a rapid onset, involve multiple organ systems, and can lead to death[3]. The lifetime risk of anaphylaxis in the United States population is 1.6%[4], and a recent Canadian study reported that the percentage of anaphylaxis cases out of all emergency department visits at the Montreal Children's Hospital more than doubled over the four-year period between 2011 – 2015[5].

Etiology

Although largely considered a modern epidemic, descriptions of allergic disease can be found in ancient texts and early medical writings. Pharaoh Menes of Egypt died from an anaphylactic reaction to a wasp sting in 2600 BC[6], Roman Emperor Claudius suffered from rhinoconjunctivitis and his son Britannicus developed rashes when exposed to horses[7], and the writings of Thomas More detail King Richard III's strawberry allergy[8].

However, outside of sporadic references, allergic disease did not feature prominently in the medical literature prior to the last 150 years.

Reports of increasing hay fever emerged in the 1870s, pediatric asthma in the 1970s, and food allergy in the 1990s[9]. This rapid rise in allergic disease excludes a primary genetic cause[10]. Rather, it is likely a result of changes in living conditions, as an upsurge in the prevalence of allergies is observed as societies become more affluent and urbanized[11]. Landmark studies of East and West German populations since the country's reunification in 1990 have shown a steep increase in allergy in East Germany corresponding to its adoption of a more westernized lifestyle[12-16]. This observation has been supported by migration studies[17] and regional studies[18] from other areas reporting higher rates of allergic disease among members of families and ethnic groups who live in modern, industrialized areas, in comparison to rural-dwelling relatives.

There is a large body of research dedicated to dissecting the modern lifestyle and teasing out the cause of allergic disease. No single culprit has emerged – rather, it appears that a complex constellation of factors contributes to the increasing prevalence of allergy in urban, westernized populations.

The "hygiene hypothesis" and the changing microbiome

Lack of exposure to microbes, especially during crucial windows of growth and development, has been identified as a likely cause of the aberrant immune responses that drive allergic disease. In fact, early advances in urban sanitation that separated sewage from drinking water and introduced drinking water chlorination correlated temporally with the initial surge in reported allergy[9].

The "hygiene hypothesis" was originally proposed in the 1980s by David Strachan[19], who noticed an inverse correlation between family size and number of children in the household who suffered from hay fever. Strachan was the first to suggest that the development of allergic disease may be linked to improvements in personal cleanliness and reduced exposure to childhood infections. The principle tenet of Strachan's original idea remains largely accepted today, but has evolved over time to recognize the role of commensal flora. Recent advances in the field of microbiology indicate that changes in early-life intestinal colonization patterns over recent decades in urban societies are hampering individuals' ability to induce and maintain immune tolerance. It is now understood that early gut microbiota establishment during critical developmental periods can influence a person's risk of developing environmentally-influenced diseases, and post-industrialization habits have resulted in a depletion of the ancestral microbiota that humans evolved to depend on for optimal health. This is often referred to as the "disappearing gut microbiota hypothesis"[20].

A Th2-skewed immunity is the primary cause of allergic disease, and newborns are born with a Th2-biased immune system that must mature to incorporate balancing Th1 and T regulatory elements[21,22]. Evidence suggests that immune interactions with commensal microflora may be a major driving force in this maturation. Hill *et al*[23] have reported higher levels of serum IgE and circulating basophils in germ-free mice. Sudo *et al*[24] found that oral tolerance protected against sensitization to OVA (determined by IgE, IgG1, IL-4 production) in germ-free mice only if the intestine was reconstituted with *Bifidobacterium infantis* during the neonatal period.

Defects in TLR-signaling have been associated with the development of an allergic

phenotype: *My D88-/-* mice, and wild-type mice with adoptively transferred *MyD88-/-* B and T cells, have higher serum IgE concentrations, and higher frequencies of blood basophils[23], TLR4 knockout mice are significantly more susceptible to peanut sensitization[25], and *Grp43-/-* mice exhibit more severe OVA- induced airway inflammation, as evidenced by increased inflammatory cell infiltrate and expression of eosinophil peroxide in lung tissue[26].

The composition of the ideal "healthy gut microbiome" that promotes immune tolerance has yet to be determined[27], but many studies have uncovered differences in intestinal bacterial composition between allergic and non-allergic individuals – some as early as the first month of life. Broadly, *Bifidobacteria, Bacteroides, Proteobacteria,* and *Actinobacteria* have been measured in greater abundance in healthy children, while *Clostridium difficile, Staphylococcus aureus, Fermicutes* phyla, and *Enterococcus* genera are more likely to predominate in allergic children[28-31]. Both clinical allergy status and specific biochemical markers, including IgE, basophils, Th2 cytokines, circulating IL-10, and *FOXP3* expression in the colon, differ according to patterns in microbiota[23,31,32]. These differences in bacterial colonization have been correlated with discrete lifestyle differences between allergic and non-allergic children. Specifically, allergic children are more likely to have been born via Cesarean section, predominantly formula-fed, lacked older siblings, and exposed to antibiotics in infancy[33-38].

Increased exposure to allergens

Increased time spent indoors is a hallmark of the modern lifestyle, and brings with it increased exposure to indoor allergens[9,39]. Dust mite, cockroach, and indoor pets are

among the most significant indoor allergens - Pollart *et al* identified sensitization to one of these three allergens as a major risk factor for asthma exacerbation and hospitalization[40]. Early reports of increasing asthma in the pediatric population emerged from countries where dust mite was the dominant indoor allergen[41,42], and exposure to dust mites in the home has been identified as an important driver of asthma[43]. Cockroach is the second most common indoor allergen sensitization, after dust mite[44,45]. Patients will often be sensitized to both cockroach and dust mite[46], as they co-exist in similar environments[47]. The growing frequency in keeping indoor furry pets has increased dog and cat allergy[39]. Cat allergy in particular is among the most common allergic sensitizations and is strongly associated with asthma[48].

Changes in farming practices and the earth's climate have increased exposure to outdoor allergens. Rye grass was introduced to England in the 1800s, and is known to pollinate more heavily than traditional grasses[49]. The growth of ragweed in the United States has increased with the rise in arable farming[9]. Both grass and ragweed are significant causes of seasonal rhinitis. Over the past 50 years, increased burning of fossil fuels has caused the earth's temperature to rise and the carbon dioxide in the atmosphere to increase[50]. This change in climate has impacted the onset, duration, and intensity of the pollen season. Elevated CO_2 levels stimulate plants to enhance photosynthesis, increase reproduction, and produce more pollen[51]. Significant increases in birch, oak, ragweed, mugwort, and grass pollens have been documented in North American over the past decade[52].

Pollutants

Exposure to high levels of vehicle emissions is a consequence of living in an urban environment. A recent study in Vancouver, Canada found that pre-school children exposed to higher levels of traffic pollution were at greater risk of developing asthma[53]. Nitrogen dioxide (NO_2), ozone (O_3), and particulate matter are the most abundant components of air pollution[51]. Heightened exposure to NO_2 increases an individual's risk of developing atopy[54], and Gauderman *et* al reported that close residential distance to a freeway and elevated exposure to NO_2 increased emergency room visits, wheezing, and medication use in asthmatic children[55]. Ozone inhalation can damage the epithelial barrier in the upper and lower airways and induce inflammation. Kim *et al* found that exposure to O_3 increased rates of allergic rhinitis as well as rates of novel sensitization to outdoor allergens[56]. High environmental levels of O_3 have been observed to enhance the allergenicity of aeroallergens such as birch pollen: increasing *in vitro* immune cell migration and *in vivo* skin prick test size[57]. Exposure to particulate matter increases an individual's risk of developing asthma and rhinitis[58], as well as moderate-severe eczema[59].

Summary

Overall, research has shown that no single factor can account for the rise in allergic disease. Even a switch from using aspirin to acetaminophen, the progressive increase in recommended childhood vaccinations, and rising obesity rates have all been pinpointed as contributors to the allergy epidemic[9]. The adoption of a modern lifestyle brings with it changes in hygiene and increased exposure to common allergens and pollutants, all of

which contribute to immune dysregulation and the development of allergy.

Pathophysiology

The allergic response evolved from immune reactions directed against worms and parasites that serve to prevent acute infection, protect against venoms, and stimulate tissue healing after inflammation and toxin exposure[60]. In allergy, this type of immune response is inappropriately directed towards otherwise innocuous environmental and food antigens.

Allergic immune hypersensitivity is characterized by: 1) a sensitization phase triggered by initial allergen exposure, and 2) a reaction phase triggered by subsequent allergen exposure.

Sensitization

During sensitization, tissue-resident dendritic cells (DC) become activated in the presence of epithelial-derived cytokines, danger signals, and allergen. Dendritic cells may encounter allergen by antigen-sampling the lumen of certain tissues, such as the airway or intestine, or allergen may enter through disrupted epithelium. Typical epithelial-derived cytokines are IL-25, IL-33, and TSLP. The alarmin uric acid has recently been identified as a DC activator in the context of allergy[61]. Activated dendritic cells upregulate co-stimulatory molecules and major histocompatibility complex II (MHC II), and migrate to regional lymph nodes to induce the polarization of naïve $CD4^+$ T cells to allergen-specific Th2 cells[62].

$CD4^+$ Th2 cells that produce the cytokines IL-4, IL-5, and IL-13 are a hallmark of allergy. These cells serve to orchestrate the allergic response by driving IgE production by B cells and recruiting inflammatory cells, such as eosinophils and basophils, to the site of allergen exposure.

In the presence of IL-4, Th2 cells initiate B cell class switching and somatic hypermutation in germinal centres found in secondary lymphoid tissues. Germinal centre B cells then differentiate into memory B cells and long-lived plasma cells, producing high-affinity peanut-specific IgE antibodies[63]. Long-lived IgE^+ memory B cells that replenish antigen-specific IgE titres have been identified in a mouse model of peanut-induced anaphylaxis[64]. These IgE antibodies bind Fc receptors on the surface of effector cells, such as mast cells and basophils[62], priming these cells to activate and degraulate in response to contact with allergen.

T cell cytokines also directly interact with mast cells. While the Th2 cytokines IL-5 and IL-9 stimulate stem cell factor-dependent proliferation of mast cells, the Th1 cytokine IFN-γ suppresses SCF-mediated differentiation of mast cell progenitors[65].

Reaction

Upon re-exposure, allergen cross-links IgE bound to Fc receptors on the surface of allergic effector cells, prompting the release of inflammatory mediators. These mediators are either pre-formed (histamine, heparin, tryptase, serotonin) or newly-synthesized (platelet activating factor, leukotrienes, prostaglandin D2, and cytokines such as IL-4, IL-5, and IL-13) and drive ensuing local (itching, swelling, nausea, vomiting, diarrhea) and systemic (hives, airway obstruction, hypotension, arrhythmias) symptoms[3].

Anaphylaxis

The National Institute of Allergy and Infectious Diseases defines anaphylaxis as a serious reaction that is rapid in onset and may cause death[66].

Classic symptoms of anaphylaxis involve the integumentary, gastrointestinal, pulmonary, and cardiac systems. The criteria for a clinical diagnosis of anaphylaxis is as follows: 1) acute onset of illness (minutes to hours) with involvement of the skin, mucosa or both and at least one of a) respiratory compromise, b) reduced blood pressure; 2) after exposure to a likely allergen (minutes to hours), two or more of a) involvement of skin or mucosal tissue, b) respiratory compromise, c) reduced blood pressure, d) persistent gastrointestinal symptoms; or 3) reduced blood pressure after exposure to a known allergen (minutes to hours)[67].

Common triggers of anaphylaxis are medications, insect venoms, and foods[3]. The most common anaphylactic-inducing foods are milk, eggs, seafood, peanuts, and tree nuts[68].

Peanut Allergy

Epidemiology

Peanut allergy is a growing public health concern: its prevalence has doubled in the past 10 years, and currently stands at approximately 2%[69]. Widening bans on peanut in community spaces, including schools and recreation centres, reflect the public's growing anxiety surrounding this particular food allergy.

Peanut allergy develops early in life and is unlikely to be outgrown, persisting into adulthood in 80% of allergic children[70]. This is in contrast to milk, egg, soy, and wheat allergies that commonly resolve during childhood[66]. Reactions to peanuts account for the majority of food-induced anaphylactic fatalities. Due to the widespread use of peanut in common foods, even the most careful patients cannot always avoid exposure to peanut, and accidental reactions are common[71].

Etiology

Why is peanut so allergenic?

Peanut contains seventeen major allergens: Ara h 1-17. The majority of these fall into six protein families that are related to structure, storage or defense: the prolamin superfamily (Ara h 2, 6, 7, 9, 16, 17), the cupin superfamily (Ara h 1, 3), the profilins (Ara h 5), the Bet v-1-related proteins (Ara h 8), oleosins (Ara h 10, 11, 14, 15), and defensins (Ara h 12,13)[72]. Sensitization to multiple peanut allergens is associated with a clinical history of more severe reactions[73]. Peanut allergens cross-react with allergens from tree nuts and other legumes, and it is common for peanut-allergic patients to exhibit sensitization to these other allergens, though, this cross-reactivity is not always clinically relevant. For example, 50% of peanut allergic patients will have positive skin prick tests to other legumes, but less than 5% of these individuals will be clinically allergic[74].

Peanut protein can survive digestive processing, increasing the likelihood of it reaching the intestinal mucosa intact and interacting with immune cells. It contains a high number of disulfide bonds that allow for high heat and pH stability. Ara h 1 is particularly resistant to breakdown: its homotrimeric structure reduces access to catalytic sites in the

interior of the protein, allowing large fragments to survive exposure to proteolytic enzymes[75]. Another allergenic feature of Ara h 1 is the clustering of its IgE-binding epitopes; the presentation of clustered epitopes to mast cells and basophils may cause more efficient degranulation of these effector cells[76]. Ara h 2 and 6 demonstrate similar resistance to protease-mediated digestive breakdown[77]. Additionally, Ara h 2 can act as a trypsin inhibitor, further protecting itself and other peanut allergens from digestion[78].

Plant allergens are frequently glycosylated, meaning that sugars become attached to their protein components. Mannose and xylose moieties are attached to Ara h 1[79], and these carbohydrate components have been shown to stimulate the DC-SIGN receptor on the surface of dendritic cells, activating DCs to preferentially prime naïve $CD4^+$ T cells towards a Th2 phenotype[80].

Plant proteins can also be modified by carbohydrates through a process called the Maillard reaction: this occurs when free amines on proteins react with sugars to produce advanced glycosylation end products (AGEs). Ara h 1 and 3 undergo the Maillard reaction and resulting AGEs have been shown to stimulate the dendritic cell receptors RAGE and SR-AI/II, leading to the generation of IL-4 and IL-5 secreting Th2 cells[81].

The process of dry roasting accelerates the Maillard reaction and increases the generation of advanced glycosylation end products. Maleki *et* al have demonstrated that roasted peanut proteins bind IgE more effectively than raw peanut proteins[82]. Dry roasting has become an increasingly popular way of processing peanuts, and geographical areas where dry roasting, instead of boiling, is the preferred method of peanut preparation report higher rates of peanut allergy[83].

Is the peanut really to blame?

There has been no significant change to the biochemical properties of peanut since its introduction to the human diet. So why has peanut allergy increased so significantly in the span of only one generation? A broad upsurge in the prevalence of allergic disease as a whole certainly contributes. However, additional factors are also important in the context of peanut allergy. These include the delayed introduction of peanut feeding and hygiene practices that cause skin barrier disruption.

Older clinical guidelines recommended the avoidance of common allergenic foods in early childhood as a means of preventing food allergy. These guidelines were largely based on consensus rather than direct evidence, and were in place in Canada until as recently as 2013[84]. Over the past decade, epidemiological evidence has begun to cast doubt on the protective benefit of delaying the oral introduction of peanut. Du Toit *et al* observed that the prevalence of peanut allergy in Jewish children in the United Kingdom was 10-fold higher when compared to Jewish children of similar ancestry that were living in Israel[85]. This discordance in peanut allergy prevalence correlated with differences in timing of peanut introduction into the diet: children in the U.K. generally did not consume peanuts in the first year of life, while Israeli children were routinely eating peanuts starting at 7 months of age.

Recent data from The Learning Early About Peanut Allergy (LEAP) Study has demonstrated quite clearly that early feeding of peanut can prevent the development of peanut allergy. This study enrolled children between the ages of 4 – 11 months at risk of peanut allergy, due to severe eczema and/or egg allergy, and randomized them to either

avoid or regularly consume peanut until sixty months of age. Children who regularly consumed peanut were significantly less likely to develop clinical peanut allergy by the age of five[86].

The LEAP study is the first randomized controlled trial to directly demonstrate the benefit of early oral exposure for preventing peanut allergy. These findings lend credence to the theory that the route of initial antigen exposure can shape the nature of the immune response, be it tolerance or allergy.

There is emerging evidence that peanut sensitization occurs through the skin as a result of a compromised cutaneous barrier. In fact, peanut-allergic individuals will often react on their first known oral exposure[87]. Early-onset atopic dermatitis is a known risk factor for food allergy[88], and a positive association has been found between application of topical products containing peanut oil and peanut allergy[89]. Peanut-specific effector T cells isolated from peanut-allergic children expressed the skin homing molecule CLA[90]. Several animal models have demonstrated that sensitization, and subsequent clinical reaction, can be induced by painting antigen on disrupted skin[61,91-93]. For example, Strid *et al* found that exposing mice to peanut epicutaneously before oral introduction prevented the development of normal oral tolerance and mice exposed to peanut on the skin produced high levels of IL-4 and peanut-specific IgE[94].

Carriers of a genetic mutation in the filaggrin gene (*FLG*) have increased rates of atopic dermatitis, contact allergy, asthma, hay, fever, and peanut allergy. Filaggrin is a protein expressed in the outer layers of the epidermis that maintains the integrity of the skin barrier[95]. Filaggrin haploinsufficiency, meaning a 50% reduction in expression of the

protein, confers an odds ratio of 5.3 for peanut allergy (defined as a positive food challenge). When corrected for the presence of atopic dermatitis, the odds ratio changes to 3.8, suggesting that, even in the absence of full-blown atopic dermatitis, a barrier defect can confer risk for peanut allergy[96].

Environmental peanut exposure in the first year of life is associated with an increased risk of peanut sensitization and allergy in children with *FLG* mutation[97]. A study of the effects of environmental peanut found that each unit increase in house dust peanut protein level translated to a 6-fold increase in the odds of peanut sensitization and a 3-fold increase in odds of clinical peanut allergy. Household consumption of peanut butter in particular is highly associated with peanut sensitization and clinical allergy, compared to other forms of peanut-containing foods[98]. It is hypothesized that its stickiness can facilitate transfer onto a baby's skin from other family members through hand-to-hand contact[99].

Aside from *FLG* mutations, there is evidence that modern hygiene practices are compromising the skin barrier. These include an increase in washing babies over the last few decades, and higher use of exfoliating and depilatory products[9].

Diagnosing Peanut Allergy

The diagnosis of peanut allergy includes a medical history focusing on the temporal association between ingestion of peanuts and appearance of symptoms, and testing for peanut-specific IgE, either by skin prick test or measurement of serum levels[62]. Component resolved IgE testing for Ara h 1, 2, and 3 has been identified as more predictive of clinical allergy than whole peanut-specific IgE, with Ara h 2 being

particularly discriminatory. However, there are certain limitations to component testing for peanut allergy. These include a lack of consensus on appropriate component testing cutoffs, sensitivity and specificity measures of different cutpoints vary widely between studies, the importance of individual components varies regionally, and testing lacks standardization across commercial kits[100,101]. The gold standard for peanut allergy diagnosis is an oral food challenge[62].

Following diagnosis, the management plan involves the prescription of injectable epinephrine and counseling the patient on its use, teaching patients to recognize the signs and symptoms of an allergic reaction, and education on peanut avoidance for the patient and their family[62].

Pathophysiology of Peanut-Induced Anaphylaxis: Lessons from Mouse Models

Experimental mouse models have revealed two pathways of systemic anaphylaxis: a classic pathway mediated by IgE, FcεRI, mast cells, histamine, and platelet activating factor (PAF) and an alternative pathway mediated by IgG, FcγRIII, macrophages and PAF. The classic IgE-mediated pathway is known to mediate human anaphylaxis, while the importance of the alternative pathway in humans is unknown. However, the structure and function of human macrophages, IgG, and FcγRIII receptors would theoretically allow for the initiation of anaphylaxis if a sufficiently large amount of antigen were present[102].

Barrier disruption

Barrier disruption is required for allergic sensitization and reaction. Mouse models demonstrate that this can be accomplished through either a chemical disruption of the intestinal barrier or a mechanical disruption of the skin. The epithelium-derived cytokines IL-25, IL-33, and TSLP are markers associated with barrier disruption and allergic sensitization[103-108].

Recently, uric acid has been identified as a particularly important molecule in peanut allergy. Uric acid is an alarmin that activates tissue-resident surveying cells, namely dendritic cells. Elevated serum uric acid levels have been detected in peanut-allergic children as well as mice undergoing peanut sensitization. Depletion of uric acid during sensitization can prevent the generation of peanut-specific IgE and IgG1. These findings are consistent in both a mouse model of gastrointestinal peanut sensitization, using gavage of peanut plus cholera toxin, and a model of epicutaneous sensitization, using mechanical disruption of the skin barrier. Uric acid can also be used in place of cholera toxin as an adjuvant in gastrointestinal sensitization. Sensitized mice demonstrated enhanced expression of MHC II and the co-stimulatory molecules CD86, CD80, and OX40L on dendritic cells[61].

The cellular contribution

The T cell is a central player in allergic disease, and peanut-induced anaphylaxis is no exception. In the absence of proper T cell function, robust sensitization and antibody production does not occur, and mice do not undergo clinical anaphylaxis. CD4-deficient animals do not undergo clinical anaphylaxis when exposed to peanut-sensitization and

challenge protocols[109]. Mice without IL-4 or its associated transcription factor STAT-6 do not produce IL-5 in response to peanut, cannot generate IgE and IgG1, and do not exhibit clinical anaphylaxis when challenged with peanut[103].

OX40 ligand is a protein expressed on dendritic cells that binds OX40 on the surface of activated T cells, downregulating the inhibitory CTLA-4 protein and allowing for T cell amplification. This interaction drives autocrine/paracrine production of IL-4 and the Th2 priming that is central to peanut sensitization[104]. Blocking OX40L during sensitization results in decreased IgE and IgG1 production, attenuated anaphylaxis, and decreased late-phase cellular infiltrate[103].

Just as complete sensitization cannot occur in the absence of the T cell, clinical anaphylaxis cannot occur in the absence of allergic effector cells: mast cells, basophils, and phagocytes.

Peanut-induced anaphylaxis is substantially mediated by mast cells, though data supporting the relative importance of these cells is strain-dependent. In mast-cell deficient $Kit^{W}/Kit^{W\text{-}v}$ mice, both clinical anaphylaxis and plasma histamine were absent despite elevated peanut-specific IgE and IgG1 levels and the presence of late phase inflammation at the site of allergen challenge[110]. Mast cell deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice developed robust peanut-specific antibodies, but demonstrated significantly attenuated anaphylaxis and undetectable plasma histamine levels in response to peanut challenge[109].

Studies have also demonstrated the importance of basophils and phagocytes as effector cells in clinical anaphylaxis. Both basophil-deficient mice and phagocyte-deficient mice were protected from severe anaphylaxis, and a combination of phagocyte and mast cell

deficiency abrogated anaphylaxis entirely[109]. Overall, experiments indicate that mast cells and macrophages mediate most of the clinical and physiological anaphylaxis to peanut, and basophils contribute to the far end of the spectrum of severity.

Allergic effector cells are activated primarily through Fc receptors. Sensitizing mice using peanut and cholera toxin oral gavage significantly increases the number of peritoneal c-kit$^+$FcεRI$^+$ cells and boosts FcεRI expression on their surface[111]. Deficiency in either FcεRI or FcγRIII significantly attenuates anaphylaxis despite circulating peanut-specific antibody levels comparable to wild type mice.[110]

B cells and antibodies

B cells and antibodies are necessary for activating allergic effector cells and generating clinical anaphylaxis upon allergen re-exposure. In their absence, mice will mount a "cellular sensitization", demonstrated by the production of inflammatory cytokines and recruitment of inflammatory cells, but cannot mount an anaphylactic reaction.

B cell-deficient mice do not undergo peanut-induced anaphylaxis, despite having an intact T cell response to allergen as evidenced by the production of IL-4, IL-5, and IL-13 and the late phase influx of inflammatory cells into the site of antigen challenge[110].

CD40 ligand is a protein expressed on the surface of T cell that promotes B cell maturation and class-switching when it binds CD40 on the surface B cells. CD40 ligand-deficient mice produce Th2 cytokines and demonstrate the late phase influx of inflammatory cells at the site of antigen challenge. However, in the absence of concomitant B cell activation, they do not exhibit clinical anaphylaxis[110].

As mentioned previously, robust anaphylactic responses in mice are dependent on both IgE and IgG1. Mice deficient in either of these antibodies demonstrated attenuated anaphylaxis, but mice who were deficient in IgE and also have the FcγRII/III system blocked were entirely protected. Mast cell-deficient mice with blocked FcγRII/III were similarly protected from anaphylaxis.[109]

Experiments blocking different combinations of Fc receptors and mast cells or phagocytes have demonstrated that, as a general rule, IgE activates mast cells and IgG1 activates phagocytes. IgE does not work to activate phagocytes, but IgG1 can work to activate mast cells through FcγRIII[109]. Both IgE and IgG1 pathways must be blocked to completely abrogate peanut-induced anaphylaxis.

A hallmark of peanut allergy is that it is usually lifelong, with allergic individuals continuing to have recurrent anaphylactic reactions over the course of their lifetimes. A recent study by Jiménez-Saiz *et al* demonstrated that IgE persistent peanut sensitivity is driven by repeat allergen exposure, consequent IL-4 production by Th2 cells, and activation of long-lived memory B cells that replenish the peanut-specific IgE+ plasma cell compartment.[64]

Cytokines and inflammatory mediators

T cell cytokines, notably IL-4, are important in the sensitization phase and the late-phase cellular response to peanut challenge, while downstream inflammatory mediators contribute to anaphylaxis.

Platelet activating factor (PAF) is a phospholipid that mediates inflammation through both paracrine and autocrine pathways. It is produced by a number of cells, including platelets, endothelial cells, neutrophils, monocytes and macrophages[112]. Plasma PAF is significantly increased in patients presenting to the emergency room with food-induced anaphylaxis, and levels correlate with severity of reaction. Levels of PAF acetylhydrolase, the enzyme that inactivates PAF, are inversely correlated with reaction, and were found to be significantly lower in patients who suffered fatal peanut-induced anaphylaxis[113]. Intravenous injection of PAF into mice can induce hemoconcentration, thrombocytopenia, and death[114].

Experimentally, blocking platelet activating factor prior to peanut challenge in a mouse model prevented severe reactions and increased recovery time to a normal core body temperature of 37^{o}C. Protection against severe anaphylaxis increased when both histamine and PAF were blocked, with only mild reactions noted.

Histamine is an amine that, in the context of allergy, mediates systemic and local inflammation. The injection of histamine itself has been shown to drop body temperature in animal models[115] and induce tachycardia, flushing, and drops in diastolic blood pressure in human volunteers[116]. Blocking histamine alone before peanut challenge in a mouse model had no effect on anaphylaxis[111]. This is not an unexpected result, as allergen-activated mast cells are thought to be a primary source of histamine during anaphylaxis.

Anaphylatoxins, or activated complement peptides, stimulate cytokine and histamine release from inflammatory cells[117]. Serum C3a, C4a, and C5a levels have been found to

correlate with severity of anaphylaxis in human patients[118]. Peanut-induced anaphylaxis is prevented in C4-deficient mice[119], and peanut challenge can induce C3a, stimulating macrophages, basophils, and mast cells to produce PAF and histamine[120].

Anaphylaxis is a complex physiological process that requires the cooperation of the innate and adaptive arms of the immune system, and the interaction of various effector cells, antibodies, and inflammatory mediators. A comprehensive mechanism of action has yet to be elucidated, but extensive studies in animals have shed some light on the important players in this life-threatening disease process.

Treating Peanut Allergy

Whole Allergen Immunotherapy

Specific whole-allergen immunotherapy (SIT) has been used to treat allergies for over 100 years. It is clinically effective and disease-modifying, meaning that the clinical benefits last beyond the treatment period. SIT has been shown to prevent the progression of rhinitis to asthma and prevent sensitization to new allergens.

The earliest immunotherapy studies by Dunbar[121] and Noon[122] used subcutaneous injections of pollen extract to treat hayfever. Frankland and Augustin ran the first controlled immunotherapy trial in 1954 using grass pollen[123]. Whole-allergen immunotherapy is a routinely used in current clinical practice for many common allergens, including house dust mite, grass and tree pollen, insect venom, and animal dander[124]. It has also been investigated for use in peanut allergy.

Injection therapy

Despite its documented success in other allergies, early studies of whole-allergen injection immunotherapy for peanut allergy proved unsafe and unsuccessful. In the 1990s, two studies were conducted using subcutaneous injections of peanut extract.

Nelson *et al*[125] treated six adult peanut-allergic patients with a six-week rush protocol followed by one year of weekly maintenance injections. All patients experienced systemic reactions requiring epinephrine during both the rush and maintenance periods, and only three patients were able to reach the desired maintenance dose. One patient required thirty-nine injections of epinephrine over the course of the study. Serum peanut-specific IgE and IgG levels were measured one month after completion of the rush protocol. A significant increase in IgG was observed and there was no measurable change in IgE levels. Patients who were able to tolerate the maintenance dose had an increased oral challenge threshold dose after twelve months. The authors concluded that this treatment protocol would not be suitable for clinical use.

Oppenheimer *et al*[126] recruited eleven peanut-allergic subjects and treated them with either peanut injections or placebo once weekly for four weeks, followed by skin-prick testing and a double-blind placebo controlled peanut oral challenge. This study had to be terminated when a pharmacy error resulted in the accidental administration of a maintenance immunotherapy dose to a subject assigned to the placebo group, and the death of this individual from anaphylaxis. Data collected prior to termination indicated that subjects treated with immunotherapy had reductions in skin-prick size and symptom scores during the oral challenge. The authors also report a 13.3% incidence of systemic

reactions to immunotherapy injections. It is important to note that no statistical analyses were performed in this study.

Oral immunotherapy

Recent studies of oral whole peanut administration protocols have shown some promise in reducing sensitivity to peanut.

Vickery *et al*[127] conducted a non-placebo controlled trial where peanut-allergic children were treated for five years with peanut oral immunotherapy. Subjects with a history of severe anaphylactic reactions and asthma were excluded from the study. 62% of participants completed the full treatment regimen of 4 000 mg oral peanut per day and 15% withdrew because of allergic side effects. Of those who completed the full treatment course, 50% passed a 5 000 mg oral peanut challenge administered one month after stopping regular oral immunotherapy. These children were found to have smaller skin prick tests to peanut and lower IgE levels specific for peanut, Ara h 1, and Ara h 2, although they also had lower levels at baseline. Participants were encouraged to continue regular peanut oral intake after the study's conclusion, and the one patient who chose to eliminate peanut from his diet went on to have a "relapse", as defined by an increase in circulating peanut-specific IgE and skin prick size. 57% of parents reported difficulty in getting their child to willingly continue regular oral intake of peanut.

Many other oral tolerance studies have reported similar results[128-132]. All have used a period of gradual updosing followed by a prolonged maintenance phase of daily peanut ingestion and a final oral food challenge. Some included adjunct therapies, such as probiotics[133] and omalizumab[134]. All studies reported some degree of decreased

sensitivity to peanut, demonstrated by the ability of participants to tolerate larger doses of peanut at the final oral challenge. However, allergic side-effects were experienced by the majority of participants, length of effective desensitization is unknown, and the impact of these desensitization protocols on patient quality of life remains equivocal[135,136]. Immunotherapy using mutated peanut proteins, by means of amino acid substitutions, to decrease IgE-binding capability has been suggested[137] but not yet trialed.

Epicutaneous immunotherapy

Epicuteneous immunotherapy has been beneficial in treating environmental allergies[138,139], and has recently been studied in peanut allergy. To date, these studies have demonstrated a superior safety profile, when compared to injection or oral therapy, but limited efficacy. A multicenter study by Jones *et al*[140] applied a skin patch containing whole peanut extract to the upper arms of peanut allergic participants daily, increasing the duration of wear over fifty-two weeks. Patients with a history of severe anaphylaxis were excluded. 80% of doses resulted in patch-site reactions, and no anaphylaxis was reported. At the end of the study, treated participants were able ingest up to 130 mg more peanut (the equivalent of half a peanut) than they could at baseline, and none successfully passed the oral food challenge at week fifty-two. Most recently, Sampson *et al*[141] reported the results of a phase 2b randomized clinical trial that compared the efficacy of different doses of the peanut patch applied over twelve months. Participants were labelled as treatment responders if they were able to tolerate either 1 000 mg of peanut at the final oral food challenge, or 10x more peanut than baseline. The highest dose 250 μg patch was most effective, with 50% of participants meeting criteria for treatment response.

Summary

Whole allergen immunotherapy harbours several challenges that are particularly salient in the context of severe food allergy[142]. These include the requirement to reach (high) maintenance doses, long treatment duration, IgE-mediated allergic side effects (including the risk of anaphylaxis), and patient eligibility restrictions that exclude patients with more severe clinical allergy. Resulting difficulties with patient compliance make it difficult to complete immunotherapy regimens and achieve long-term clinical success. Unintended consequences of immunotherapy have also been reported, including eosinophilic esophagitis[143-145] in trials of oral immunotherapy for peanut. Additionally, uncertainty remains as to whether whole allergen protocols are in fact inducing only a temporary desensitization, and not true long-term clinical protection. The consequences of removing patients from daily maintenance regimens are largely unknown, and waning of tolerance has been reported after just one month of stopping regular therapy[127].

Other Approaches

Early mouse studies showed that the administration of Chinese herbal formula FAHF-2 ameliorated anaphylactic reactions in a model of peanut allergy. Treated mice showed significant improvement in clinical signs of allergic reaction, plasma histamine levels, vascular leakage; had reduced IgE levels; and suppressed cytokine production by splenocytes when cultured with peanut *in vitro*[146]. Unfortunately, a follow-up human study showed no affect in peanut-allergic patients. Subjects experienced no change in the amount of oral peanut they could tolerate and no change in any of the immunological markers assessed. One stumbling block was the impractically large amount of herbal

formula that volunteers were required to ingest, based on scaled-up dosing from mouse studies[147].

Some have suggested that post-transcriptional gene silencing techniques could be used to suppress the production of certain allergenic proteins, such as Ara h 2, and develop hypoallergenic transgenic plants[148]. However, there are so many allergenic peanut proteins that such agricultural modifications would render the resulting peanut no longer a peanut[62]. The significant agricultural overhaul required, and resulting uncertain payoff, render this approach largely impractical.

Summary

There remains a lack of widespread, practical, disease-modifying therapies available for peanut-allergic patients. Typical injection immunotherapy has proven unsafe, oral tolerance has a tenuous and largely uncharted period of efficacy, epicutaneous immunotherapy appears to be the safest trialed approach but a has questionable magnitude of efficacy, and outside-the-box ideas such as the agricultural development of a less-allergenic peanut seem far-flung and impractical. At present, healthcare workers can only offer peanut-allergic patients advice on allergen avoidance and rescue medication in case of accidental ingestion. Resulting anxieties surround food and social situations cause decreased quality of life for patients and their families[149]. Quality of life is significantly impaired in patients with peanut allergy, even in comparison to individuals with other chronic diseases, such as diabetes[150]. Given the measured psychological impact, along with increasing prevalence of this disease and its clinical severity, the medical community must do better for peanut-allergic patients. The safety,

tolerability, and long-term efficacy of whole allergen peanut immunotherapy remain ongoing concerns.

Peptide Immunotherapy

The primary challenges for peanut-specific immunotherapy development are safety, patient compliance, and duration of efficacy. A successful disease-modifying therapeutic for peanut allergy must minimize allergic side effects and be able to produce reliable, long-lasting tolerance to peanut.

Peptide immunotherapy is a newly emerging immunomodulatory treatment that makes use of short peptides representing major allergen T cell epitopes. Short peptides are unable to cross-link allergen-specific antibodies bound to allergic effector cells, and peptide immunotherapy avoids the potentially lethal side effects of traditional whole allergen specific immunotherapy[151]. In peptide immunotherapy, the immune system is presented with allergen in a quiescent environment and learns to switch from a pro-inflammatory allergic response to a tolerant one. Compared to whole allergen therapy, peptide immunotherapy has a greatly improved safety profile, requires fewer doses to achieve tolerance, and is amenable to standardization and regulation[152]. Studies of peptide immunotherapy in other allergic diseasess demonstrate reduced pro-inflammatory cytokine production, induction of tolerogenic regulatory T cells, and improvement of clinical symptoms[152-154].

Due to the clinical severity of peanut allergy, the development of a human peptide immunotherapeutic must be preceded by careful study in animal models to characterize

its safety, efficacy, and mechanism of action. This project investigated the use of Ara h 1 peptide immunotherapy in a mouse model of peanut-induced anaphylaxis.

Conclusion

Despite the clinical severity and rising prevalence of peanut allergy, there is a marked absence of widespread, practical disease-modifying therapies available for peanut-allergic patients. Currently, the primary treatment for peanut allergy is avoidance of peanut and rescue medication in case of accidental ingestion[62]. The resulting anxiety surrounding social and food-related situations leads to an impaired quality of life for peanut-allergic patients and their families[155].

This project examined the ability of peptide immunotherapy to protect against severe anaphylaxis in peanut-sensitized mice and characterized immune changes in peptide-treated animals.

Chapter 2: Ara h 1 Peptide-Immunotherapy in a Wild Type C57Bl/6 Mouse Model

Introduction

Peanut allergy is a severe allergic disease that can lead to anaphylaxis and death[71]. Previous attempts to develop peanut-specific immunotherapy have led to serious side-effects and, in one unfortunate instance, the death of a study participant[126]. Therefore, the development of a human immunotherapeutic must be preceded by careful study in an animal model to characterize is safety, efficacy, and mechanism of action. This project used a mouse model of peanut-induced anaphylaxis that was developed by Dr. Manel Jordana's group at McMaster University[61]. Mice were sensitized to peanut epicutaneously, received a systemic peanut challenge, and were observed for signs of allergic reaction using a standardized scale. The model replicated several key aspects of human peanut allergy: rapid reaction upon peanut challenge, clinical signs of systemic allergic reaction, vascular leakage, and raised levels of circulating allergen-specific antibodies. The first step of this project was to ensure that the model was robust and reproducible our laboratory and animal housing environment.

The second step of this project was to develop a therapeutic peptide treatment protocol. This involved identifying relevant T cell epitopes from peanut and then determining the most protective peptide dose and route of administration. Epitope prediction software was used to identify a peptide from a major peanut allergen that would bind MHC II in C57Bl/6 mice with high affinity – immunotherapy using low affinity peptides is less effective[156]. The dose, route, and administration schedule of this peptide was then optimized. Mice were sensitized to whole peanut, given peptide therapy, and

subsequently challenged with whole peanut. The efficacy of the therapeutic regimen was measured by the ability of peptide treatment to protect peanut-sensitized mice from severe reactions to peanut.

Methods

Animals

Female C57Bl/6 mice aged 4 – 6 weeks old were purchased from Charles River Laboratory (Wilmington, MA), housed in ultraclean conditions, and allowed to acclimatize for one week before experimental use.

All experiments were carried out in accordance with the Guide for Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board of McMaster University (AUP 12-02-04 and AUP 16-03-08).

Peanut Sensitization

Epicutaneous Sensitization

A small patch of fur was shaved at the base of the tail and exposed skin was gently tape stripped 6 – 8 times using clear cellophane tape to disrupt the stratum corneum. 200 μg of crude peanut extract (CPE) (Greer Laboratories, Lenoir, NC) in 10 μL of phosphate buffered saline (PBS) was then applied directly to the disrupted skin. This process was repeated for ten days: peanut was applied daily for five days, a two-day rest was taken, and peanut was again applied daily for five days. Two weeks after the final day of peanut sensitization, mice were systemically challenged with CPE (Figure 1).

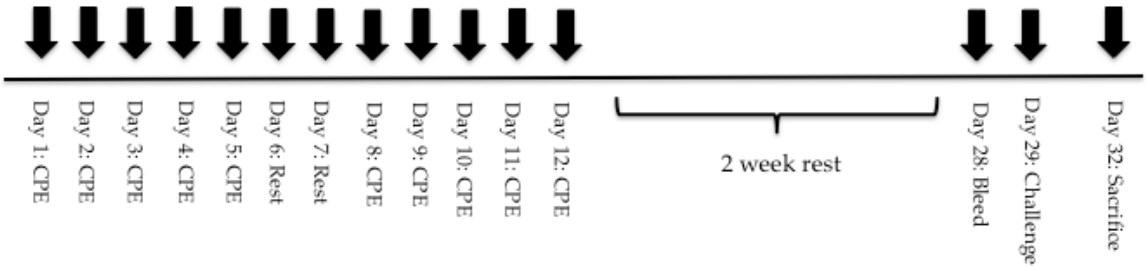


Figure 1. Timeline: epicutaneous peanut sensitization and challenge in wild type C57Bl/6 mice. Mice were exposed to crude peanut extract on disrupted skin over the course of twelve days. After a two-week rest period, mice were challenged with whole peanut and evaluated for anaphylaxis. They were sacrificed 72 hours after challenge.

Intraperitoneal Sensitization

Mice received one injection of 200 μg CPE and 1 mg aluminum hydroxide in 200 μL PBS weekly for four weeks. CPE and aluminum hydroxide were mixed on a rocker for six hours before injection to ensure adequate binding. One week after the final injection, mice were challenged with CPE (Figure 2).

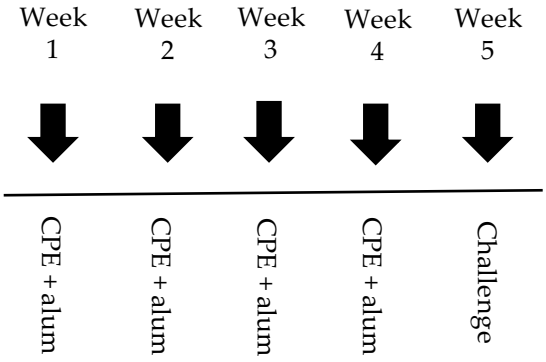


Figure 2. Timeline: intraperitoneal peanut sensitization and challenge in wild type C57Bl/6 mice. Mice were injected with crude peanut extract coupled with aluminum hydroxide weekly for four weeks. After a one-week rest period, mice were challenged with whole peanut and evaluated for anaphylaxis.

Peanut Challenge

Sensitized mice were challenged intraperitoneally with 5 mg of CPE in 500 μL of PBS and evaluated for signs of anaphylaxis over a period of 40 minutes. Temperatures were measured at baseline and every 10 minutes thereafter using a rectal thermometer (VWR, Radnor, PA). Mice were observed for clinical signs of allergic reaction that were then translated into numerical scores according to severity: 0 (no signs of allergic reaction), 1 (digging in the ear canal with hind leg), 2 (reduced movement, puffy eyes, increased respiratory rate), 3 (periods of motionless, lying flat for >= 1 minute), 4 (no response to whisker touch and/or prodding), 5 (tremor, convulsion, endpoint). Following the 40-minute observation period, 20 uL of blood was collected into heparinized micro-hematocrit capillary tubes via retroorbital bleeding and hematocrit was measured using a HemataSTATII microhematocrit centrifuge (Fisher Scientific, Waltham, MA). Mice then received 1 mL of saline subcutaneously and were placed on a heating pad to facilitate recovery.

Serum Collection

One day before peanut challenge, mice were anesthetized with isofluorane and 8 – 9 drops of peripheral blood were collected into heparinized micro-hematocrit capillary tubes (Fisher Scientific, Waltham, MA) via retroorbital bleeding. Fluids were replaced with 1 mL saline administered subcutaneously. Blood samples were spun at 700 rcf for 10 minutes at room temperature, and the serum layer collected and stored at -20^{o}C.

Peanut-Specific Immunoglobulins

Serum peanut-specific immunoglobulins were measured by an in-house sandwich enzyme-linked immunosorbent assay (ELISA).

IgG1

Maxi-Sorp 96-well plates (VWR, Radnor, PA) were coated with CPE at 2 mg/mL in carbonate-bicarbonate buffer (Sigma-Aldrich, St Louis, MO) at 4°C overnight. Plates were blocked with 1% BSA in PBS for 2 hours at room temperature, then washed and incubated with serum samples overnight at 4°C overnight. Biotinylated goat anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was added and incubated for 2 hours. Plates were washed and incubated with alkaline-phasophate-conjugated streptavidin (Sigma-Aldrich) for 1 hour at room temperature. *P*-nitrophenyl phosphate tablets were used to develop the assay, and H_2SO_4 (2M) was added to stop the reaction. Absorbance readings were taken at 450nm.

IgE

Maxi-Sorp 96-well plates (VWR) were coated with rat anti-mouse IgE (BD Biosciences, Mississauga, ON) at 2 μg/mL in PBS at 4°C overnight. Plates were washed and blocked for 1 hour at 37°C with Tween buffer made up of 10% bovine serum, 1% BSA, and 0.5% Tween in PBS. Serum samples were added and incubated for 2 hours at room temperature. CPE-digoxigenin-conjugate solution was added to induce coupling of IgE with CPE. Perixodase-conjugated anti-digoxigenin was added at 37°C for 1 hour. Tetramethylbenzidine solution at 0.1 mg/mL was added to develop the colour reaction,

and H_2SO_4 (2M) was added to stop the reaction. Absorbance readings were taken at 450nm.

Peritoneal Wash Collection and Processing

Mice were anesthetized and 10 mL PBS was injected into the peritoneal cavity using an 18G needle. The abdomen was massaged for one minute and the lavage removed from the injection site. The suspension was spun down at 400 rcf for 10 minutes at 4°C. The supernatant was decanted, stored at -20 °C, and sent to Eve Technologies (Calgary, AB) for multiplex cytokine analysis. The cellular component was resuspended in 1 mL PBS and total cells were enumerated. Cell isolates were diluted to an approximate concentration of 5×10^5 per mL and transferred to slides by centrifugation. The cells were Wright-Giemsa stained and differentiated by morphological criteria as one of: eosinophil, neutrophil, macrophage, lymphocyte, and basophil. Two slides from each sample were differentiated by a blind investigator, and the relative proportion of each cell type were multiplied by the total number of peritoneal wash cells obtained to determine absolute cell counts.

Peptide Identification

Amino acid sequences from the major allergens Ara h 1, Ara h 2, and Ara h 3 were obtained from www.allergen.org. These amino acid sequences were entered into the MHC binding prediction tool found at: http://tools.immuneepitope.org/mhcii/ to generate a list of epitopes predicted to bind the C57Bl/6-expressed MHC molecule I-A^b and to determine their relative affinities. NetMHCII version 2.2 was used to further analyze the affinity binding and predict the peptide core of this epitope with the highest affinity

binding. Water solubility was evaluated using Innovagen (pepcalc.com) and GRAVY score (web.expasy.org/protparam/).

Peptide Preparation

Lyophilized Ara h 1 peptides (GenScript, Piscataway, NJ) were reconstituted in acidic water (10^{-4} M HCl) and diluted to desired concentrations in sterile PBS. Peptide solutions were then aliquoted and stored at -20°C.

Peptide Treatment

Wild type C57Bl/6 mice were sensitized to peanut and after a two-week rest were treated with two injections of the peptide Ara h 1 (505-524), administered one week apart. Injections were either intradermal or intraperitoneal. Peptide doses ranged from 0.01 μg to 300 μg. Intradermal injections were administered in a volume of 10 μL and intraperitoneal injections were administered in a volume of 500 μL PBS. Treatments were well tolerated, with no local or systemic reactions occurring after peptide injection. No changes in behavior, body condition, or rectal temperature were seen. Control mice were treated with a sham peptide from influenza (amino acid sequence: PKYUKQNTLKLA). Mice were challenged with whole peanut extract one week after the last treatment injection and evaluated for signs of anaphylaxis (Figure 3).

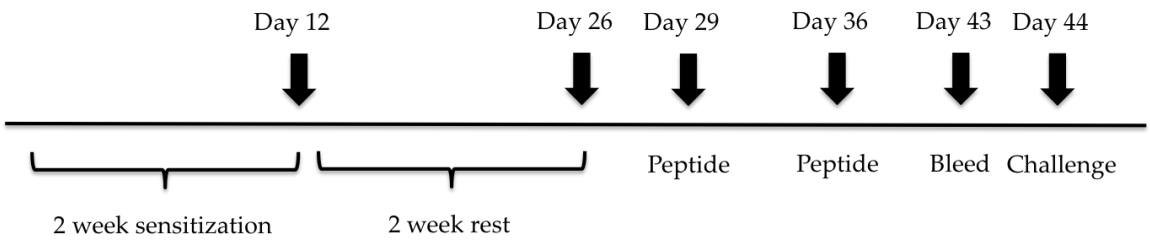


Figure 3. Timeline: Ara h 1 peptide treatment in wild type C57Bl/6 mice.

Statistical Analysis

Data were analyzed using GraphPad Prism 7.0a (La Jolla, CA) and expressed as mean ± SEM. Unpaired t-tests with Holm-Sidak corrections and one way ANOVA tests were used. Differences were considered significant when $p < 0.05$ (*), 0.01 (**), 0.001 (***), 0.0001 (****).

Results

Peanut Sensitization and Challenge in Wild Type C57Bl/6 Mice

Epicutaneous peanut sensitization resulted in anaphylaxis in response to subsequent peanut challenge

The mouse model of peanut skin sensitization and systemic challenge developed by Dr. Manel Jordana's group was replicated reliably. Mice were sensitized to peanut epicutaneously and challenged as described above. A control group received saline instead of peanut on the skin during sensitization. Mice sensitized with peanut experienced systemic anaphylaxis in response to peanut challenge, as demonstrated by significant drops in rectal temperature, clinical signs of allergic reaction, and increased hematocrit (Figure 4).

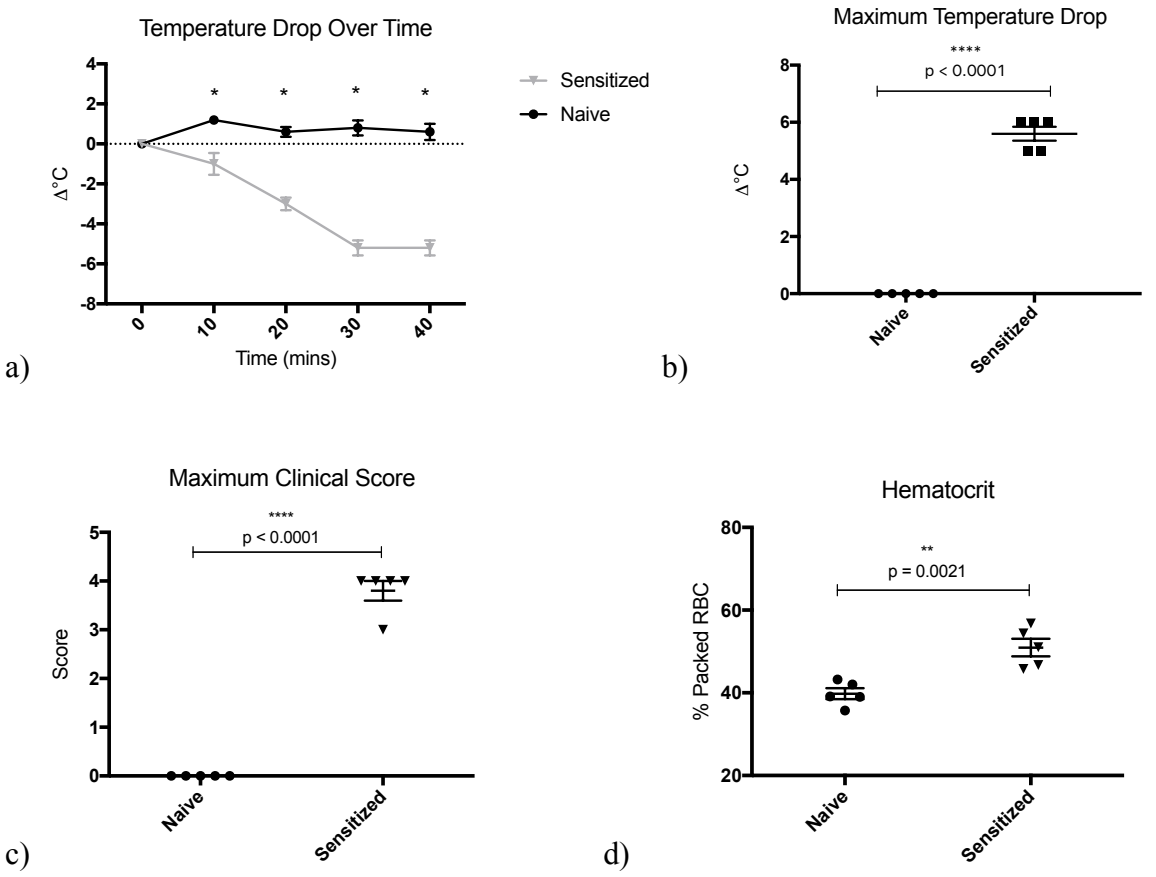


Figure 4. Female C57Bl/6 mice received either crude peanut extract peanut (sensitized) or saline (naïve) on shaved and tape-stripped skin and were subsequently challenged with whole peanut. Mice exposed to epicutaneous peanut during the sensitization phase experienced anaphylaxis in response to peanut challenge, as demonstrated by significant drops in rectal temperature (a,b), elevated allergic clinical scores (c), and hemoconcentration (d). Data from a representative experiment is expressed as mean ± SEM (n = 5 mice/group).

Peanut sensitization generated circulating peanut-specific immunoglobulins

Peanut-specific IgE and IgG1 antibodies were detected in sera of peanut-sensitized mice (Figure 5).

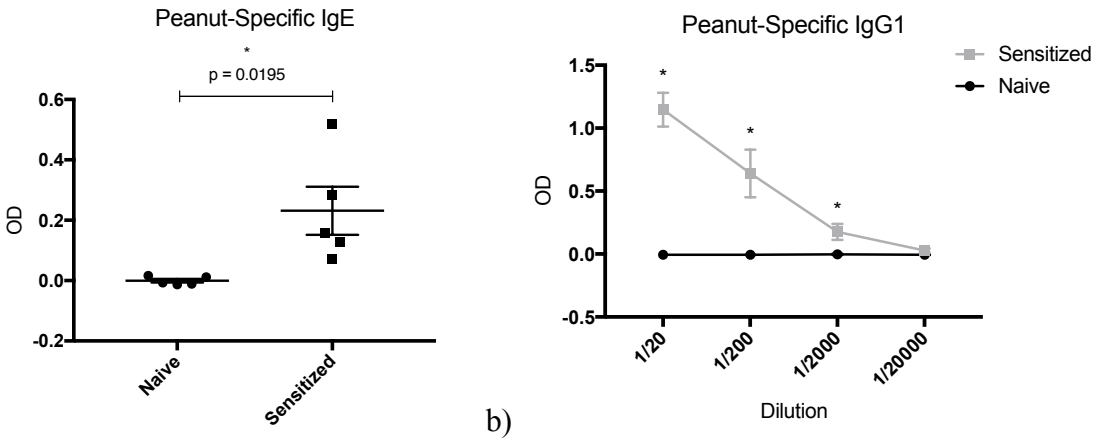


Figure 5. Peanut-sensitized mice had elevated levels of circulating peanut-specific IgE (a) and IgG1 (b), when compared to control mice. Data from a representative experiment is expressed as mean ± SEM (n = 5 mice/group).

Peptide Identification

The peptide Ara h 1 (505-524) was predicted to have high binding affinity for the C57Bl/6 MHC II molecule I-A^b

Immunotherapy with high affinity peptides is more effective than immunotherapy with low affinity peptides[156]. In this instance, affinity refers to the force that binds the peptide of interest to the MHC II molecule expressed by the animal. The major histocompatibility complex II molecule expressed in C57Bl/6 mice is I-A^b. Three major peanut allergens, Ara h 1, 2, and 3 were analyzed to identify peptides that would bind to I-A^b with high affinity.

The amino acid sequences of the major peanut allergens Ara h 1, Ara h 2, and Ara h 3 are listed in Table 1. Of the seventeen allergens present in whole peanut, Ara h 1 – 3 have

been identified as the most significant contributors to sensitization in humans[157] and mice[158].

TABLE 1: Amino Acid Sequences of Major Peanut Allergens

Ara h 1	**Ara h 2**	**Ara h 3**
MRGRVSPLMLLLGILVLASV SATHAKSSPYQKKTENPCAQ RCLQSCQQEPDDLKQKACES RCTKLEYDPRCVYDPRGHTG TTNQRSPPGERTRGRQPGDY DDDRRQPRREEGGRWGPAG PRERE REEDWRQPREDWRRP SHQQPRKIRPEGREGEQEWG TPGSHVREETSRNNPFYFPSR RFSTRYGNQNGRIRVLQRFD QRSRQFQNLQNHRIVQIEAKP NTLVLPKHADADNILVIQQG QATVTVANGNNRKSFNLDEG HALRIPSGFISYILNRHDNQNL RVAKISMPVNTPGQFEDFFPA SSRDQSSYLQGFSRNTLEAAF NAEFNEIRRVLLEENAGGEQE ERGQRRWSTRSSENNEGVIV KVSKEHVEELTKHAKSVSKK GSEEEGDITNPINLREGEPDLS NNFGKLFEVKPDKKNPQLQD LDMMLTCVEIKEGALMLPHF NSKAMVIVVVNKGTGNLELV AVRKEQQQRGRREEEEDEDE EEEGSNREVRRYTARLKEGD VFIMPAAHPVAINASSELHLL GFGINAENNHRIFLAGDKDN VIDQIEKQAKDLAFPGSGEQV EKLIKNQKESHFVSARPQSQS QSPSSPEKESPEKEDQEEENQ GGKGPLLSILKAFN	MAKLTILVALALFLLAAHAS ARQQWELQGDRRCQSQLERA NLRPCEQHLMQKIQRDEDSY GRDPYSPSQDPYSPSQDPDRR DPYSPSPYDRRGAGSSQHQER CCNELNEFENNQRCMCEALQ QIMENQSDRLQGRQQEQQFK RELRNLPQQCGLRAPQRCDL EVESGGRDRY	RQQPEENACQFQRLNAQRPD NRIESEGGYIETWNPNNQEFE CAGVALSRLVLRRNALRRPF YSNAPQEIFIQQGRGYFGLIFP GCPRHYEEPHTQGRRSQSQRP PRRLQGEDQSQQQRDSHQKV HRFDEGDLIAVPTGVAFWLY NDHDTDVVAVSLTDTNNND NQLDQFPRRFNLAGNTEQEFL RYQQQSRQSRRRSLPYSPYSP QSQPRQEEREFSPRGQHSRRE RAGQEEENEGGNIFSGFTPEF LEQAFQVDDRQIVQNLRGET ESEEEGAIVTVRGGLRILSPDR KRRADEEEEYDEDEYEYDEE DRRRGRGSRGRGNGIEETICT ASAKKNIGRNRSPDIYNPQAG SLKTANDLNLLILRWLGPSAE YGNLYRNALFVAHYNTNAHS IIYRLRGRAHVQVVDSNGNR VYDEELQEGHVLVVPQNFAV AGKSQSENFEYVAFKTDSRPS IANLAGENSVIDNLPEEVVAN SYGLQREQARQLKNNNPFKF FVPPSQQSPRAVA

Epitopes from Ara h 1 – 3 likely to bind the MHC II molecule I-A^b and their relative affinities, as identified by binding prediction software tools, are listed in Tables 2 – 4. The numbers listed in the "Range" column represent the specific sequence of amino acids from the whole protein. For example, Ara h 1 (506-520) refers to the string of 506^{th} – 520^{th} amino acids in the whole Ara h 1 protein sequence.

TABLE 2: Ara h 1 Epitope Binding Predictions		
Range	**Sequence**	**Percentile Rank (Lower = Good Binders)**
506-520	EGDVFIMPAAHPVAI	0.51
507-521	GDVFIMPAAHPVAIN	0.56
508-522	DVFIMPAAHPVAINA	0.56
505-519	KEGDVFIMPAAHPVA	0.69
504-518	LKEGDVFIMPAAHPV	0.92

TABLE 3: Ara h 2 Epitope Binding Predictions		
Range	**Sequence**	**Percentile Rank (Lower = Good Binders)**
10-24	LALFLLAAHASARQQ	6.84
11-25	ALFLLAAHASARQQW	6.87
9-23	ALALFLLAAHASARQ	7.07
12-26	LFLLAAHASARQQWE	7.48
8-22	VALALFLLAAHASAR	8.13

TABLE 4: Ara h 3 Epitope Binding Predictions		
Range	**Sequence**	**Percentile Rank (Lower = Good Binders)**
361-375	LLILRWLGPSAEYGN	0.95
360-374	NLLILRWLGPSAEYG	1.03
362-376	LILRWLGPSAEYGNL	1.06
359-373	LNLLILRWLGPSAEY	1.60

358-372	DLNLLILRWLGPSAE	1.89

Ara h 1 (505-524) was the epitope predicted to have the highest affinity binding to the C57Bl/6 MHC II molecule. Further analysis of the affinity binding and peptide core is listed in Table 5. Using NetMHCII binding prediction software, scores below 50 indicate strong binders. Scores between 50-500 indicate weak binders. The peptide core was identified as Ara h 1 (511-519) and was predicted to have high affinity binding.

TABLE 5: NetMHCII Predictions of Ara h 1 (505-524)

Sequence	**Core**	**Affinity (nM)**	**Binding Prediction**
LKEGDVFIMPAAHPVAINASS	FIMPAAHPV	16.4	High

Assessment of the solubility of Ara h 1 (505-524) is shown in Table 6. GRAVY scores in the negative range indicate greater solubility, while positive scores indicate poorer solubility.

TABLE 6: Solubility Predictions of Ara h 1 (505-524)

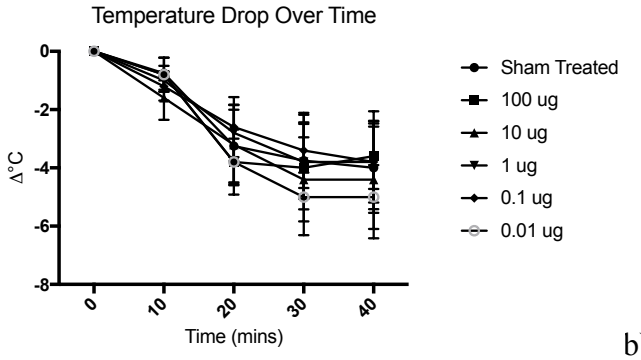
Peptide	**Sequence**	**Innovagen**	**GRAVY Score**
Ara h 1 (505-524)	KEGDVFIMPAAHPVAINASS	Poor water solubility	0.325

The peptide was predicted to be poorly soluble in water. Therefore, experiments required this peptide to be dissolved in acidic water (10^{-4} M HCl) before further diluting it in PBS to desired concentrations.

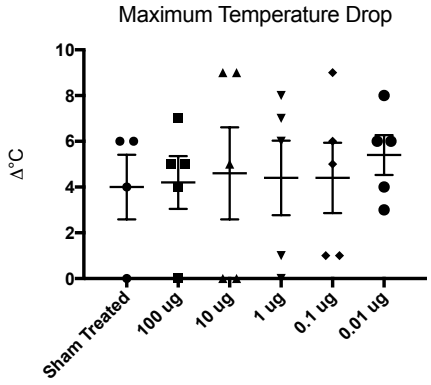
Ara h 1 Peptide Immunotherapy

Intradermal Ara h 1(505-524) peptide immunotherapy did not protect against anaphylaxis to whole peanut challenge

Mice were sensitized to peanut and received two intradermal injections of peptide according to the timeline outlined in Figure 2. This timeline and route of administration was based on previous experiments in a mouse model of Fel d 1 peptide immunotherapy for treatment of cat allergy[152,154]. Ara h 1 (505-524) intradermal peptide treatment did not confer protection against peanut-induced anaphylaxis in peanut-sensitized mice (Figure 6). Treated mice exhibited signs of anaphylaxis that were no different from sham treated controls.



a)



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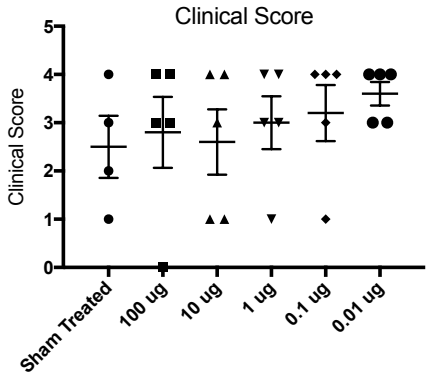

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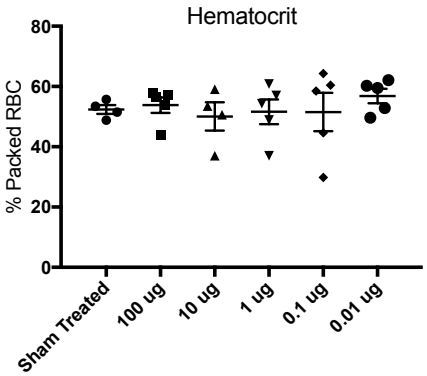


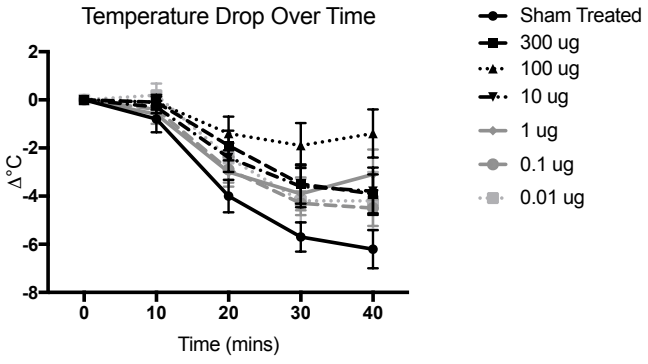
d)

Figure 6. Peanut-sensitized mice that received intradermal Ara h 1 (505-524) exhibited anaphylaxis upon peanut challenge that was comparable to control mice treated with sham peptide. Data from a representative experiment is expressed as mean ± SEM (n = 5 mice/group).

Intraperitoneal Ara h 1 (505-524) peptide immunotherapy protected against severe anaphylaxis to whole peanut challenge

Intraperitoneal administration of Ara h 1 (505-524) ameliorated peanut-induced anaphylaxis in peanut-sensitized mice. Mice that received 100 ug of peptide exhibited the highest level of protection. Control mice treated with saline experienced a mean maximum temperature drop of 6.3°C, while mice receiving 100 ug of peptide experienced a drop of 2.3°C (p=0.003 vs control). Maximum clinical score was 3.9 in control mice and 2.0 in treated mice (p<0.001). Mean hematocrit for control mice was 56.1% and 49.3% for treated mice (p=0.02) (Figure 7).

a)



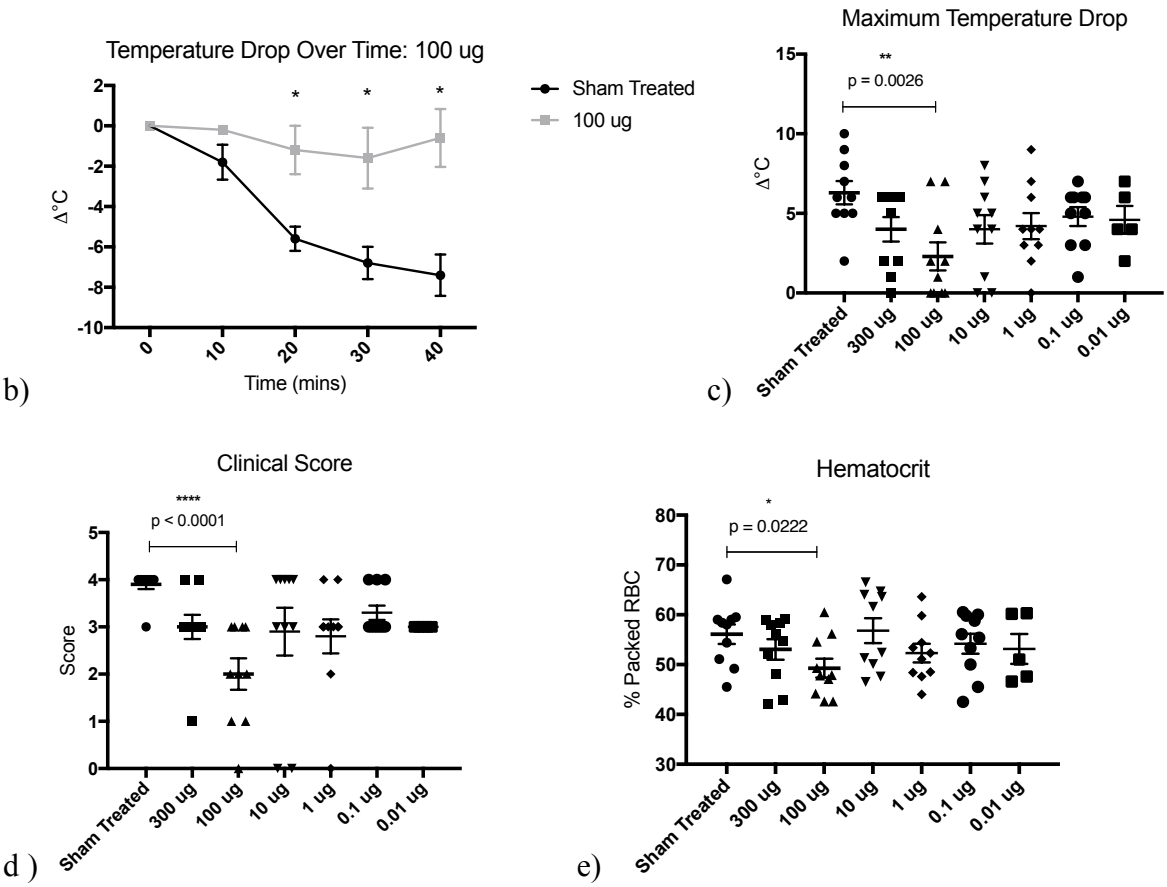


Figure 7. Peanut-sensitized mice treated intraperitoneally with 100 ug Ara h 1 (505-524) experienced attenuated anaphylaxis when challenged with whole peanut. Mice exhibited protection against severe temperature drops (a, b, c), clinical signs of reaction (d), and hemoconcentration (e). Data is expressed as mean ± SEM and is representative of two independent experiments (n = 10 mice/group).

Intradermal Ara h 1 (505-524) peptide administration in intraperitoneally sensitized mice did not protect against anaphylaxis

Previous studies of peptide immunotherapy in allergic mouse models have produced successful clinical protection using the intradermal route of administration[152]. Intradermal peptide treatment may not have been effective in the epicutaneously sensitized mice used in this project due to the inflammatory milieu created in the skin

during the sensitization process. With this in mind, intradermal peptide immunotherapy with Ara h 1 (505-524) was attempted in a model of intraperitoneal sensitization.

Intradermal peptide treatment in intraperitoneally sensitized mice did not protect against anaphylaxis (Figure 8). Peptide-treated mice experienced drops in temperature, clinical signs of reaction, and hemoconcentration similar to control mice treated with sham peptide.

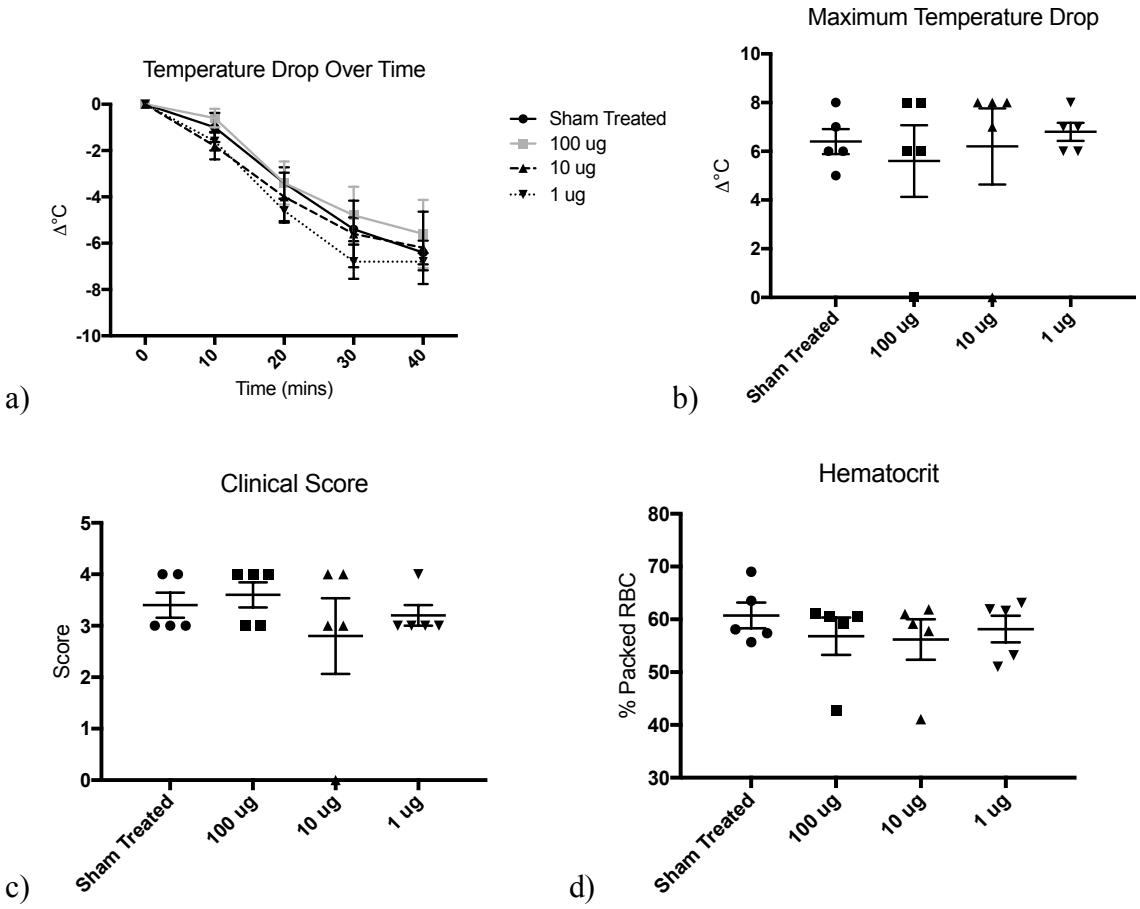


Figure 8. Mice sensitized to peanut intraperitoneally that received intradermal Ara h 1 (505-524) exhibited anaphylaxis in response to peanut challenge that was comparable to control sham-treated mice. Data is expressed as mean ± SEM (n = 5 mice/group).

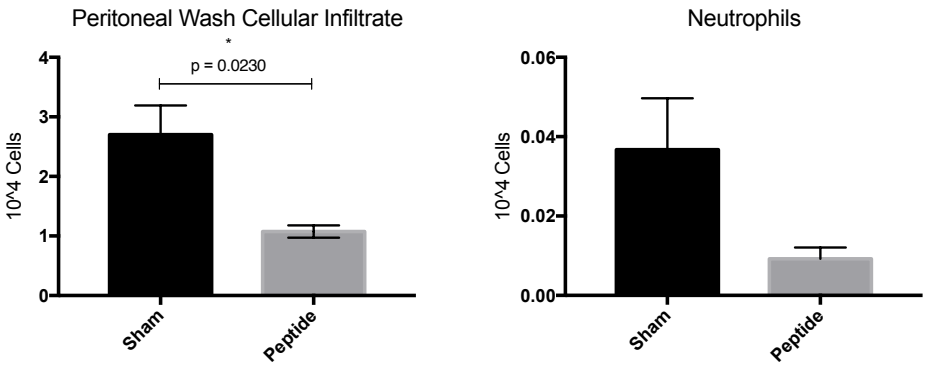
The project therefore moved forward with further studies using the epicutenaous method of sensitization and intraperitoneal route of peptide delivery.

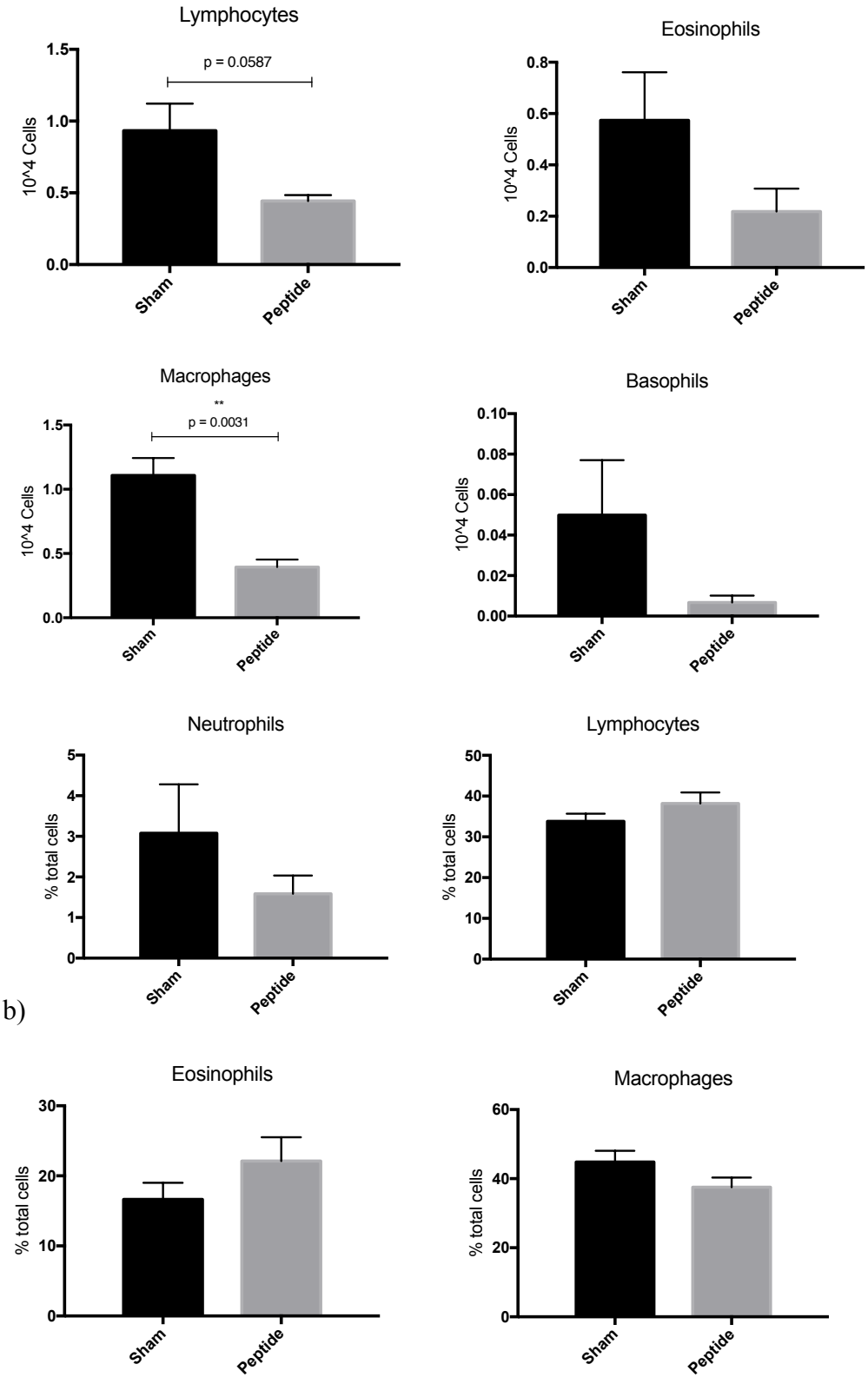
Peritoneal Wash Data

Ara h 1 (505-524) peptide treatment reduced cellular infiltrate at the site of peanut challenge

The peritoneal lavage of control mice and peptide-treated mice was examined 72 hours after challenge to characterize cellular infiltrate at the site of peanut challenge. Mice were sensitized to peanut epicutaneously, treated with intraperitoneal peptides, and challenged intraperitoneally with whole peanut. Previous experiments using intraperitoneal peanut challenge have shown the 72-hour time-point to represent the height of cellular infiltration[110]. Total cellular infiltrate was significantly reduced in peptide-treated mice. There was a reduction in cell numbers across all cell types measured: neutrophils, lymphocytes, eosinophils, macrophages, and basophils. Looking at the data proportionally, the composition of the cellular infiltrate shifted away from neutrophils and basophils towards lymphocytes and eosinophils (Figure 9).

a)




Lymphocytes
p = 0.0587
10^4 Cells
1.5
1.0
0.5
0.0
Sham
Peptide
Eosinophils
10^4 Cells
0.8
0.6
0.4
0.2
0.0
Sham
Peptide
Macrophages
**
p = 0.0031
10^4 Cells
1.5
1.0
0.5
0.0
Sham
Peptide
Basophils
10^4 Cells
0.10
0.08
0.06
0.04
0.02
0.00
Sham
Peptide
Neutrophils
% total cells
5
4
3
2
1
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Sham
Peptide
Lymphocytes
% total cells
50
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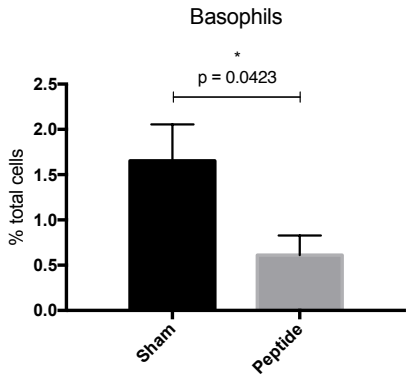


Figure 9. Peritoneal lavage cellular infiltrate expressed as cell number (a) and proportion of total cells (b). Data is expressed as mean ± SEM and is representative of two independent experiments (n = 10 mice/group).

Ara h 1 (505-524) peptide treatment reduced inflammatory cytokine production at the site of peanut challenge

Cytokines were measured in the supernatant of peritoneal lavage samples. A panel of thirty-two cytokines was measured using multiplex analysis. Cytokines that were reduced, though not significantly, in peptide-treated mice function to attract and activate innate inflammatory cells, namely monocytes, neutrophils, and basophils (Figure 10). Low levels of measured cytokine may have been a consequence of degradation that occurred during sample processing or transportation. Alternatively, it is possible that a time-point of 72 hours was optimal for cellular collection but did not represent peak cytokine release in the peritoneum. The cytokine data shown in Figure 10 was used to inform later experiments described in Chapter 5.

MCP-1, or CCL2, is a chemoattractant for monocytes and basophils. MIP-1α, or CCL3, recruits and activates neutrophils. KC, or CXCL1, and MIP-2, or CXCL2, are also neutrophil chemoattractants.

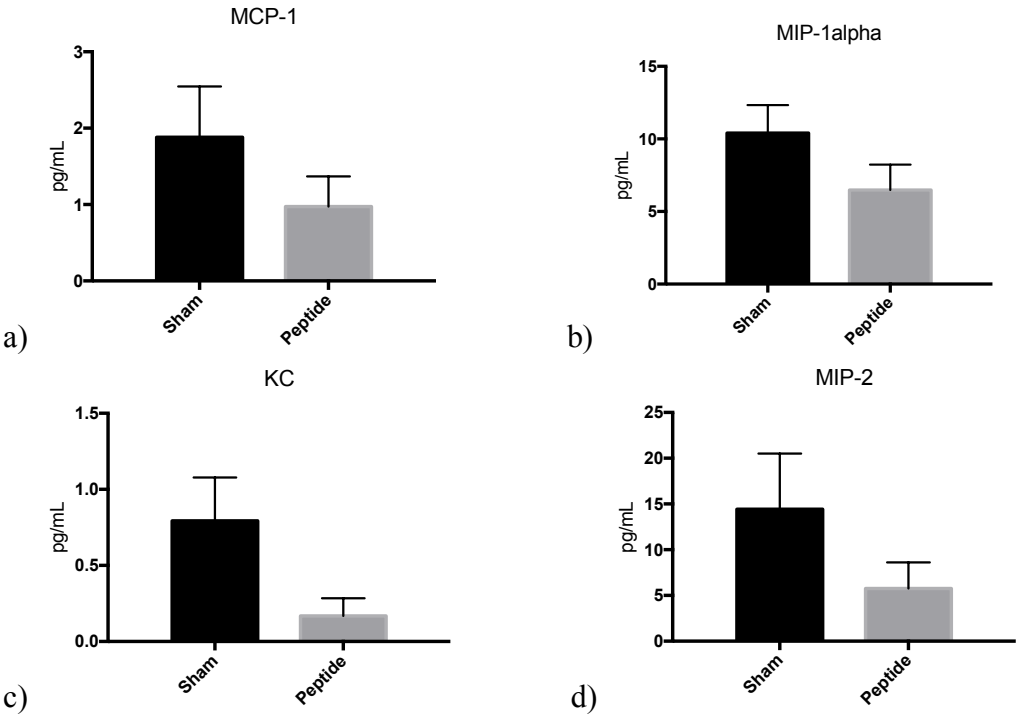


Figure 10. Cytokines involved in the chemotaxis of monocytes, neutrophils, and basophils were reduced in peptide-treated mice. Data is expressed as mean ± SEM and is representative of two independent experiments (n = 10 mice/group).

Antibodies

Ara h 1 (505-524) peptide treatment reduced peanut-specific antibody production

Peanut-specific IgE and IgG1 antibodies were measured in the sera of sham- and peptide-treated mice. Mice treated with 100 ug Ara h 1 (505-524) peptide had a non-significant reduction in peanut-specific serum antibodies (Figure 11).

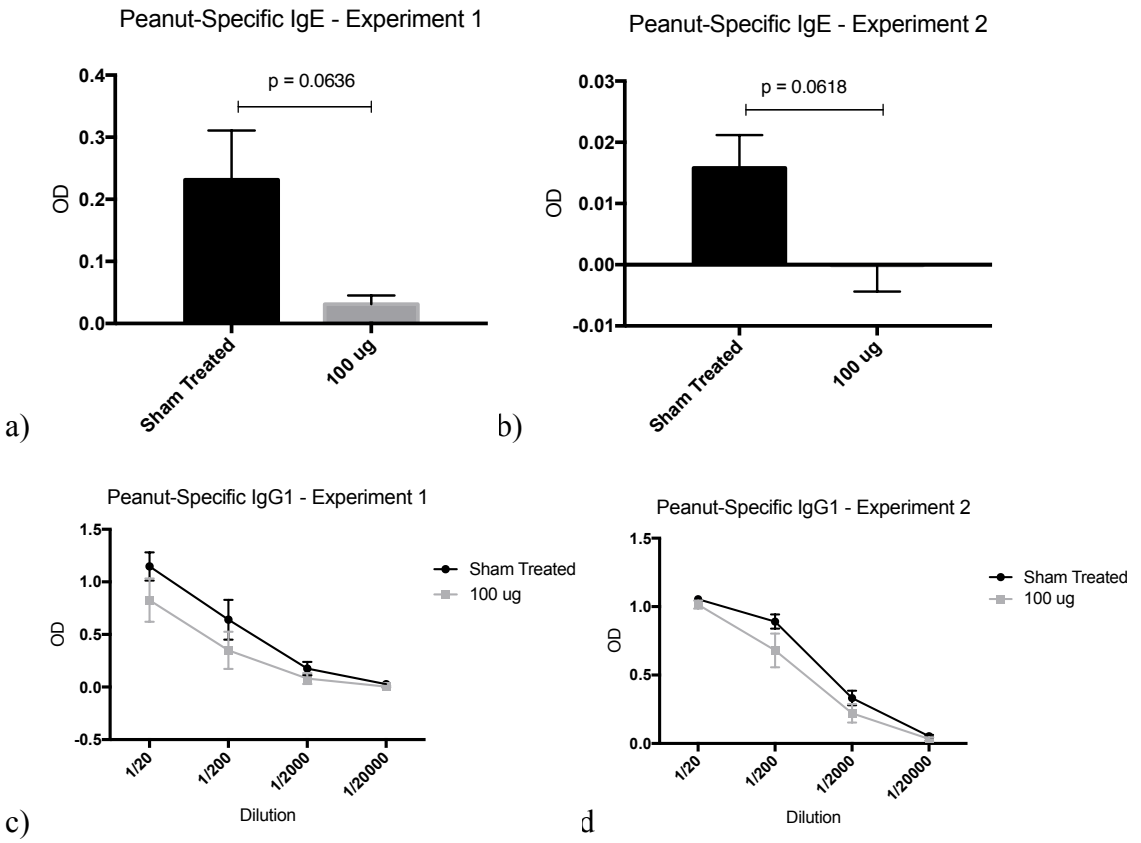


Figure 11. Serum peanut-specific IgE (a, b) and IgG1 (c, d) antibodies. Data is expressed as mean ± SEM and is representative of two independent experiments. Experiments are expressed separately, as OD ratings were obtained on separate days (n = 5 mice/group/experiment).

Discussion

C57Bl/6 mice treated with Ara h 1 (505-524) displayed marked clinical protection from anaphylaxis, as indicated by significant reductions in: core body temperature drops, clinical signs of allergic reaction, and hemoconcentration. Two intraperitoneal injections of 100 μg Ara h 1 (505-524) given one week apart was the most effective regimen. This effective dose was higher than the 1μg peptide doses that have been used in previous mouse models of Der p 1 and Fel d 1 treatment.

In contrast to whole allergen exposure, peptides can be presented to T cells by non-professional antigen presenting cells: endothelial cells, epithelial cells, keratinocytes, other T cells, and inactive dendritic cells. In this context, antigen is presented to the immune system in a non-inflammatory, quiescent environment[151]. Peptide immunotherapy has produced significant clinical benefit in the context of other allergic diseases, in both animal studies and human trials.

Mice with established cat allergen-induced airway disease demonstrated markedly reduced airway inflammation after treatment with a T cell epitope peptide from the major cat allergen Fel d 1. A single dose of Fel d 1(29-45) induced a significant improvement in lung function accompanied by significantly lower perivascular eosinophilic inflammatory infiltrates, goblet cell hyperplasia, and mucus hypersecretion[152].

Fel d 1 peptide immunotherapy has also been studied in human cat-allergic patients. Worm *et al*[156] identified relevant peptides based on their ability to bind MHC II molecules and their inability to induce histamine release from circulating basophils. A mixture of seven peptides specific for a variety of MHC II molecules was administered to participants at different doses by either intradermal or subcutaneous injection. Side effects of peptide treatment were recorded, and three weeks later an intradermal whole cat extract challenge was given. Following challenge, the late-phase skin response of peptide-treated participants was compared to their baseline. The late-phase skin response was used as a marker of the cat-specific T cell response. No serious adverse effects were reported and no patients withdrew from the study because of side effects. In patients who received intradermal peptide, the most common side effects reported were nasopharyngitis, cough, and headache. In patients who received subcutaneous peptide,

the most common side effects were nasal congestion and respiratory symptoms: two patients in this group experienced drops in FEV_1 up to 29% and mild to moderate asthma symptoms. The most effective dose was 35 μg Fel d 1 peptide administered intradermally. This treatment resulted in a 40% decrease in the late-phase skin response. This result from a single dose of peptide mixture is comparable to that seen after one year of subcutaneous immunotherapy with birch extract, and greater than that seen after twelve to eighteen months of sublingual therapy with grass pollen. In current cat dander whole allergen immunotherapy treatment, maintenance doses are recommended to contain approximately 15 μg Fel d 1, which the patient must build up to over a period of dose escalations. Therefore, larger doses of Fel d 1 in peptide form can be administered safely without a lengthy build-up period.

Follow-up studies have expanded on this original work and tracked the long-term effects of Fel d 1 peptide immunotherapy. A three-month course of treatment with Fel d 1 peptides was shown to protect against cat-induced rhinoconjunctivitis two years post-therapy[153]. Participants had significant reductions in rhinoconjunctivitis symptom scores after exposure to cat allergen in a controlled environmental exposure chamber, compared with baseline symptom scores.

Current research indicates that peptide immunotherapy using T cell epitopes is a safe and clinically promising treatment for cat allergy. Similar evidence exists in animal models for dust mite[159] and birch allergy[160], and a recent human trial of grass pollen allergy[161]. However, peanut hypersensitivity differs from these allergies in that it can induce severe, systemic reactions with only small amounts of antigen. Therefore, it is important to note

that peptide immunotherapy has been investigated in bee venom allergy, which can also cause systemic anaphylaxis.

One group investigated the safety and efficacy of a bee venom peptide vaccine in five patients with a history of IgE-mediated, systemic allergic reactions to honeybee venom[162]. Patients were treated with a mixture of three previously discovered peptides[163] representing T cell epitopes of phospholipase A2 (PLA), the major bee venom allergen. Peptides were administered subcutaneously in increasing weekly doses over two months, building up to a maintenance dose of 100 μg. One week after the last dose of peptides, patients were challenged with 10 μg PLA, the equivalent of one bee sting. One week after PLA challenge, patients were challenged with a live bee sting. No local or systemic allergic side effects occurred during peptide treatment. Following PLA challenge, two out of the five patients experienced local reactions at the injection site, but no systemic reactions occurred. Following whole bee sting challenge, two of the five patients experienced mild systemic reactions (one of these patients had also reacted locally to the PLA challenge). These systemic bee sting reactions were characterized by solitary wheals on the chest and mild swelling of the lips 15 minutes after challenge in one participant, and erythema of the face, mild angioedema of the eyelids, and a few solitary wheals on the chest 25 minutes after challenge in the second participant. In both cases, the participants reported that their symptoms were less severe than those experienced during reactions to bee stings prior to peptide immunotherapy treatment.

The clinical efficacy of peptide immunotherapy has been demonstrated in several allergies, including animal dander, environmental pollens, and insect venom. The results

of this project are exciting because they show a significant clinical benefit in severe, peanut-induced anaphylaxis. One criticism of human peanut immunotherapy studies to date has been their exclusion of patients with a history of severe reactions. If the end goal of peanut peptide immunotherapy is to help improve disease severity and quality of life in severely allergic patients, careful study in animal models of anaphylaxis is necessary. Peptide therapy was well tolerated in the mouse model used in this project, with no signs of local or systemic reaction after treatments, and was effective in significantly ameliorating anaphylactic responses.

Fewer inflammatory cells were recruited to the site of antigen challenge, and peritoneal lavage samples showed reduced expression of the cytokines MCP-1, MIP-1α, MIP-2 and KC at the site of antigen challenge. These cytokines all serve to attract and activate neutrophils. Whether these results point to a significant role for these particular cytokines in this model of anaphylaxis and peptide immunotherapy, or are simply representative of a reduction in general inflammation, cannot be determined from the data presented in this chapter. Further studies in subsequent chapters will attempt to address this by analyzing the cytokine profiles of peanut-specific T cells.

Peptide immunotherapy is a T cell targeted treatment, while peanut-induced anaphylaxis has been shown to be primarily driven by the effects of antibodies on mast cells and macrophages[109,110]. This project measured peanut-specific IgE and IgG1 antibodies in peptide-treated and sham-treated mice one week after administering the final peptide treatment. Though there were lower antibody levels in peptide-treated mice, no significant differences were found.

Other studies of peptide immunotherapy have looked for modulating effects on B cells and antibodies. Some changes in antigen-specific antibody profiles have been reported, but results have been inconsistent. A study by Tarzi *et al* found that PLA2 peptide immunotherapy induced allergen-specific IgG antibodies in patients with honeybee allergy, but this effect was transient[164]. Fellrath *et al* found no change in anti-PLA2 IgE antibodies in peptide-treated patients, but did detect a steady increase in IgG4 levels that became significant 80 days post-therapy[165]. The ratio of PLA-specific IgG4:IgE increased in peptide treated individuals in a study by Müller *et al*, but only after allergen challenge[162]. Hoyne *et al* found that culturing lymphocytes with Der p 1 in the presence of splenocytes from mice treated with Der p 1 peptide eliminated the production of Der p 1-specific antibodies[159]. Fel d 1-treated mice with cat-induced allergic airway disease had a significant reduction in total serum IgE and a moderate decrease in Fel d 1-specific IgE[152].

This study showed a reduction in circulating peanut-specific antibodies in treated mice that was not statistically significantly, potentially due to a lack of statistical power. Based on the transient nature of antigen-specific antibody changes reported by other groups, the fact that significant changes were not found in this study might also be because antibodies were measured at one time-point only. Additionally, serum antibodies were measured and no data on antibodies bound to effector cells was collected.

Conclusion

This project identified a novel peanut peptide that binds to the I-A^b MHC II molecule expressed by C57Bl/6 mice with high affinity: Ara h 1 (505-524). This peptide was used

to develop a therapeutic treatment protocol that protected against severe, systemic anaphylaxis in peanut-sensitized mice. Treated mice demonstrated a significant reduction in metrics of anaphylaxis across all measured parameters: body temperature, clinical signs of allergic reaction, and hemoconcentration. Peptide-treated mice had a significant reduction in inflammatory cell infiltrate at the site of peanut challenge, and reduced pro-inflammatory cytokines and chemokines. IgE and IgG4 were reduced but, at the time-point used in this study, this did not reach statistical significance.

Chapter 3: Generating a Model of Peanut-Induced Anaphylaxis in Mice Transgenic for the Human Leukocyte Antigen DRB1*0401

Introduction

The mouse model of peanut-induced anaphylaxis described in Chapter 2 was adapted for use in mice transgenic for the human leukocyte antigen DRB1*0401. The epicutaneous route of sensitization and intraperitoneal route of systemic challenge were used. Transgenic mice were bred on a C57Bl/6 background but lacked endogenous murine I-A^b MHC II and only expressed HLA DRB1*0401: mice with HLA-DRA-IEα and HLA-DRB1*0401-IEβ chimeric genes were backcrossed to MHC class II-deficient mice[166]. Hereafter, these mice will be referred to as "DR4 mice".

Using DR4 mice for this project conferred two important benefits. First, these mice could be treated with peanut peptides that had previously been found to bind T cells from human peanut-allergic patients[167]. Second, MHC class II tetramer reagents could be used to enumerate and characterize antigen-specific T cells using flow cytometry. This technique allowed for the measurement of specific surface markers and cytokines expressed by individual peanut-specific T cells and provided an additional metric of comparison between peptide-treated and sham-treated control mice.

Adapting the wild type C57Bl/6 model to work in the DR4 mice was the most significant challenge of this project. In order to generate robust, reliable peanut-induced anaphylaxis, the model had to be adjusted to incorporate more time for the mice to mature and a longer sensitization period. The 29-day protocol that was successful in C57Bl/6 mice had to be stretched to a 43-day protocol in DR4 mice.

Methods

Animals

Male and female mice transgenic for the human leukocyte antigen DRB1*0401 were obtained from Taconic Biosciences (Hudson, NY).

All experiments were carried out in accordance with the Guide for Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board of McMaster University (AUP 12-02-04 and AUP 16-03-08).

Peanut Sensitization and Challenge

Mice were initially sensitized to whole peanut epicutaneously as described in Chapter 2. Mice were systemically challenged with peanut as described in Chapter 2.

Peanut-Specific Immunoglobulins

Peanut-specific IgE and IgG1 were measured as described in Chapter 2.

Results

Applying the C57Bl/6 Sensitization Protocol to DR4 Mice

The epicutaneous peanut sensitization protocol used in C57Bl/6 mice did not produce anaphylaxis in DR4 mice in response to whole peanut challenge

The sensitization protocol utilized in C57Bl/6 mice described in Chapter 2 was applied to female 4 – 6-week old DR4 mice. Mice were subsequently challenged with peanut and observed for signs of anaphylaxis. Control mice received either saline during the

sensitization phase and peanut during the challenge phase, or peanut during sensitization and saline during challenge (Table 1). Mice that received peanut during the sensitization phase displayed no signs of anaphylaxis when challenged with peanut: rectal temperatures, clinical scores, and hematocrit measurements did not differ from control mice (Figure 1).

Group	Sensitization	Challenge
1	Peanut (CPE)	Saline (PBS)
2	Saline (PBS)	Peanut (CPE)
3	Peanut (CPE)	Peanut (CPE)

Table 1. Mice received either crude peanut extract or phosphate buffered saline during the sensitization and challenge phases. Groups 1 and 2 served as controls.

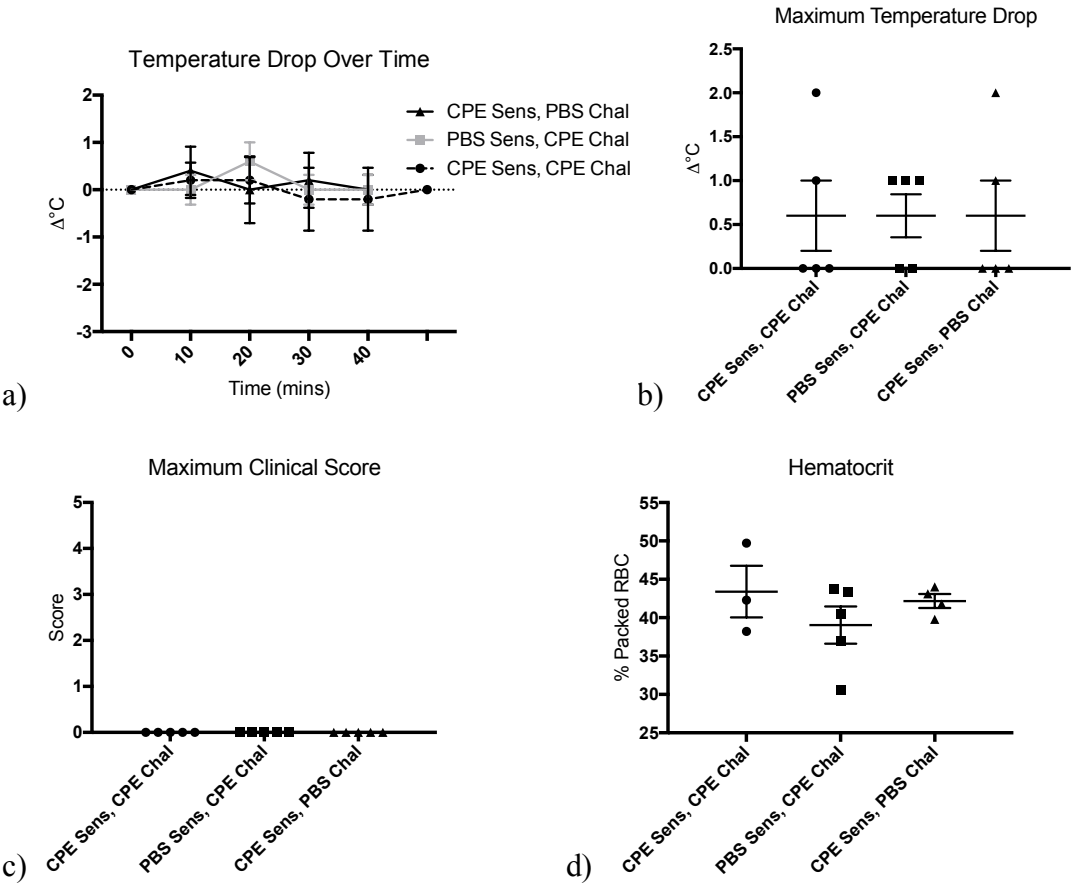
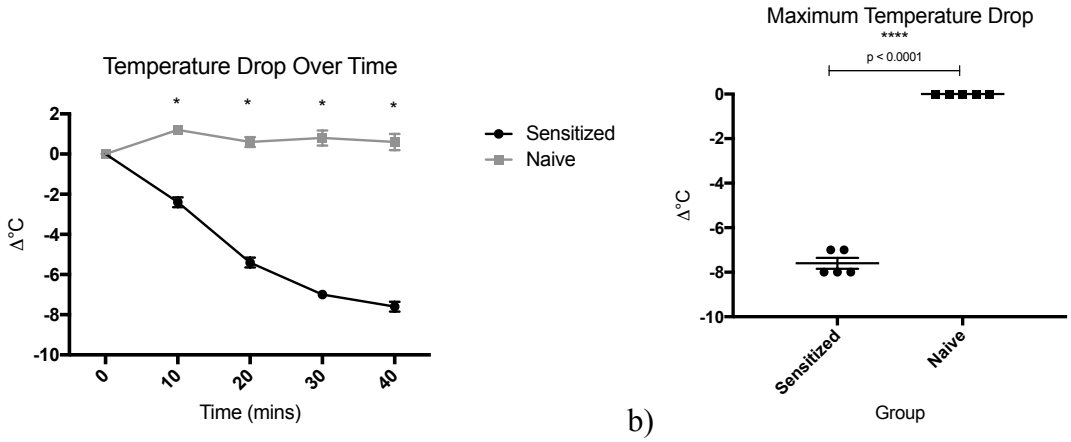


Figure 1. Female DR4 mice aged 4 – 6 weeks old received peanut on shaved and tape-stripped skin and were subsequently challenged with peanut. Control mice were sensitized with saline and challenged with peanut, or sensitized with peanut and challenged with saline. Mice sensitized and challenged with peanut did not undergo anaphylaxis, as demonstrated by an absence of significant changes in rectal temperature (a,b), allergic clinical scores (c), or hematocrit levels (d). Data from a representative experiment is expressed as mean ± SEM (n = 5 mice/group).

This experiment was repeated and similar results obtained. It was concluded that the original protocol for modeling peanut-induced anaphylaxis in C57Bl/6 mice was not suitable for use in DR4 mice.

The intraperitoneal sensitization protocol used in C57Bl/6 mice produced anaphylaxis in DR4 mice in response to whole peanut challenge

DR4 mice were sensitized using intraperitoneal injections of crude peanut extract coupled with alum, as described in Chapter 2. Mice sensitized and challenged with peanut exhibited significant temperature drops, clinical signs of reaction, and hemoconcentration (Figure 2).



a) b)

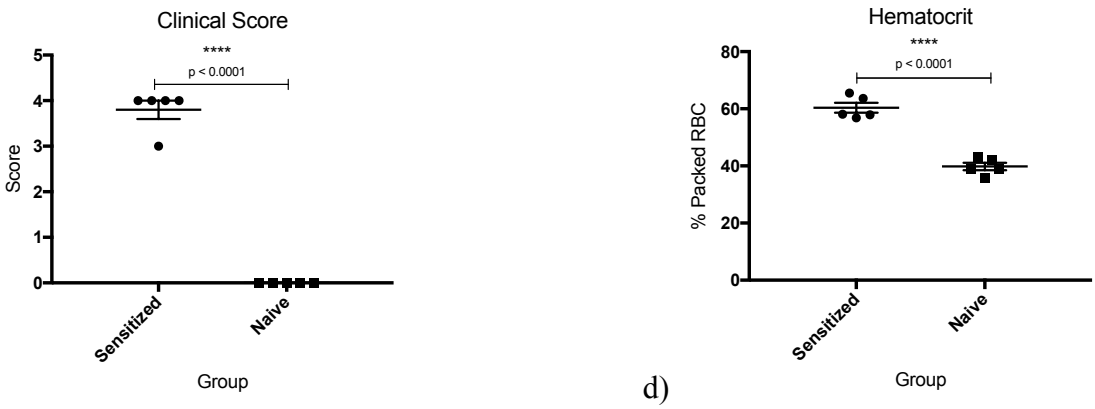


Figure 2. DR4 mice sensitized with peanut and alum exhibited significant drops in body temperature (a,b), clinical signs of allergic reaction (c), and hemoconcentration (d) in response to peanut challenge. Data is expressed as mean ± SEM (n = 5 mice/group).

This method of sensitization produced anaphylaxis in DR4 mice. Therefore, DR4 mice were capable of recognizing and responding to peanut antigen. However, an epicutaneous method of sensitization was preferable in order to align the DR4 work with the C57Bl/6 work, and the evidence presented in Chapter 1 that human peanut sensitization may occur through the skin. Therefore, troubleshooting the epicutaneous model in DR4 mice became a substantial portion of this project.

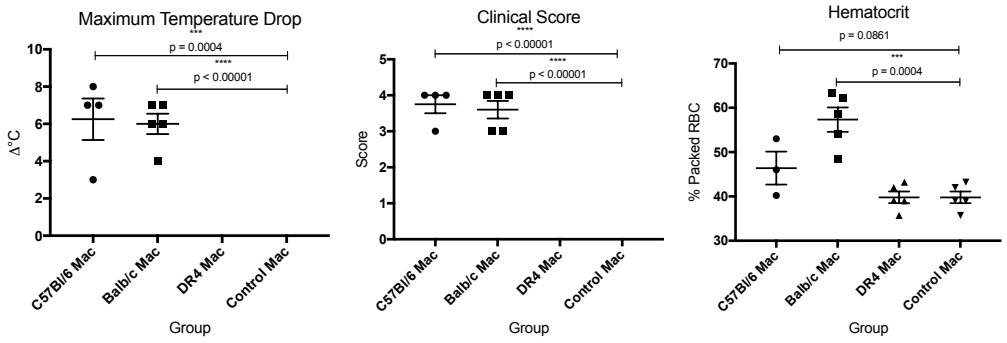
Troubleshooting the Epicutaneous Model in HLA DRB1*0401 Transgenic Mice

Several possible explanations for this discrepancy in response to the peanut sensitization and challenge protocol between C57Bl/6 and DR4 mice were investigated.

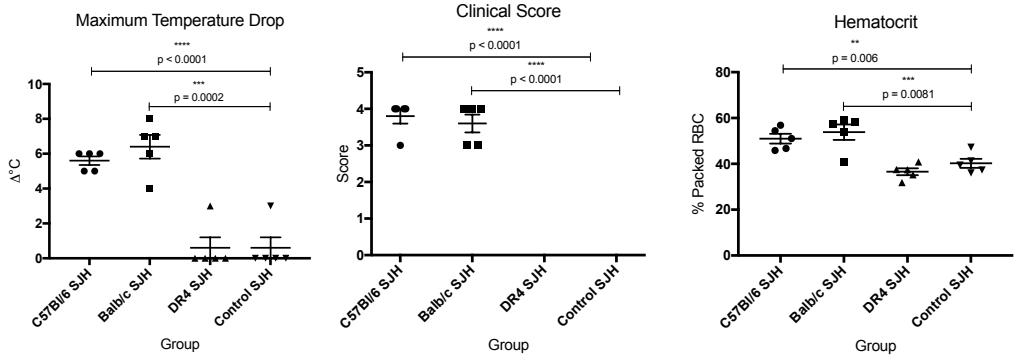
The animal housing environment

The model of peanut-induced anaphylaxis was originally developed at the McMaster Central Animal Facility (CAF), and this project was conducted at the St Joseph's Hospital Animal Facility. It was reasoned that different environments could influence the

protocol's ability to induce anaphylactic reactions to peanut. The peanut sensitization and challenge protocol was attempted on DR4 mice housed at both McMaster and at St Joseph's (Figure 3). Neither group of DR4 mice demonstrated peanut-induced anaphylaxis upon peanut challenge. Additionally, wild type mice (C57Bl/6 and Balb/c strains) housed at both McMaster CAF and St Joseph's AF were successfully sensitized and exhibited anaphylaxis. It was concluded that the animal housing environment was not responsible for the protocol's failure to induce anaphylaxis in DR4 mice.



a)



b)

Figure 3. Female C57Bl/6, Balb/c, and DR4 mice aged 4 – 6 weeks old received peanut on shaved and tape stripped skin and were subsequently challenged with peanut. Control mice were sensitized with saline and challenged with peanut. Wild type C57Bl/6 and Balb/c mice exhibited significant anaphylaxis in response to peanut challenge at both McMaster (a) and St Joseph's Hospital (b). DR4 mice did not undergo anaphylaxis in response to peanut challenge at either animal housing site. Data is expressed as mean ± SEM (n = 5 mice/group).

The animal supply company

Wild type C57Bl/6 mice were being sourced from Charles River, and DR4 mice from Taconic. At the time, Taconic was the exclusive supplier of DR4 mice. It was reasoned that mice born and raised in different environments could have microbiome variations that influenced their susceptibility to peanut sensitization. Therefore, wild type C57Bl/6 mice were sourced from Taconic and sensitized and challenged at St Joseph's Hospital according to our protocol. C57Bl/6 mice sourced from Taconic demonstrated robust peanut-induced anaphylaxis that was comparable to C57Bl/6 sourced from Charles River (Figure 4). It was concluded that variations in animal source companies was not responsible for the failure to induce anaphylaxis in DR4 mice.

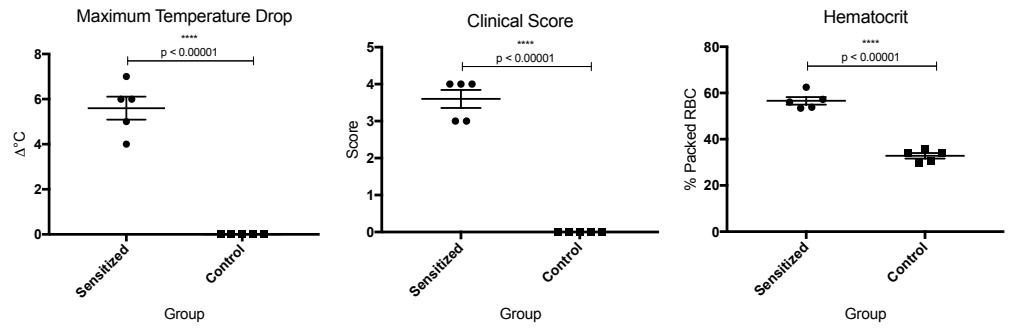


Figure 4. Female C57Bl/6 mice aged 4 – 6 weeks old sourced from Taconic received peanut on shaved and tape stripped skin and were subsequently challenged with peanut. Control mice were sensitized with saline and challenged with peanut. Mice sensitized and challenged with peanut experienced anaphylaxis in response to peanut challenge, as demonstrated by significant drops in rectal temperature, elevated allergic clinical scores, and hemoconcentration. Data is expressed as mean ± SEM (n = 5 mice/group).

Mouse size

It was observed that DR4 mice were smaller in size than age-matched wild type C57Bl/6 mice. Therefore, DR4 mice were allowed to reach a weight of 20 g before beginning the

peanut sensitization protocol. Weight-standardized DR4 mice did not demonstrate peanut-induced anaphylaxis in response to peanut challenge (Figure 5). It was concluded that animal size alone was not responsible for the failure to induce anaphylaxis in DR4 mice.

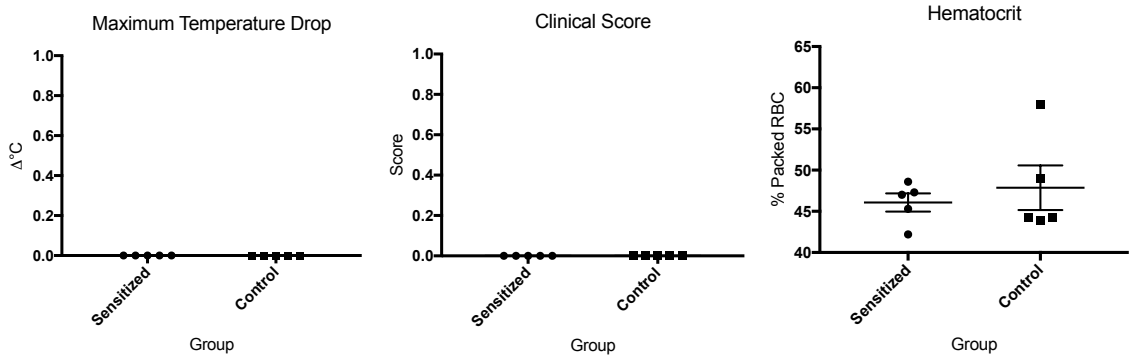


Figure 5. Female DR4 mice aged 4 – 6 weeks old and weighing 20 g received peanut on shaved and tape stripped skin and were subsequently challenged with peanut. Control mice were sensitized with saline and challenged with peanut. Mice did not undergo anaphylaxis in response to peanut challenge. Data is expressed as mean ± SEM (n = 5 mice/group).

Sensitization protocol

In addition to being smaller than their wild type counterparts, DR4 mice were also more prone to developing skin lesions. It was surmised that DR4 mice may not mount immune responses that are entirely comparable in strength to C57Bl/6 mice. Therefore, the possibility existed that the DR4 mice would require a more aggressive sensitization protocol in order to develop anaphylactic reactions to peanut. In developing the model, the Jordana group had validated a longer sensitization protocol that exposed mice to peanut over a greater period of time (Figure 6). Peanut was applied to tape-stripped skin daily for five days, after which the mice receive no peanut exposure for one week. This

two-week sequence was repeated three times for a total of six weeks. Mice were then challenged with whole peanut as described in Chapter 2. When this sensitization protocol was trialed in DR4 mice, it did produce anaphylaxis in response to peanut challenge. However, anaphylactic reactions were less robust than those seen in C57Bl/6 mice and could not be reproduced reliably. The longer sensitization protocol improved the project's ability to produce peanut-induced anaphylaxis in DR4 mice. However, additional work was needed to create a robust, reliable model.

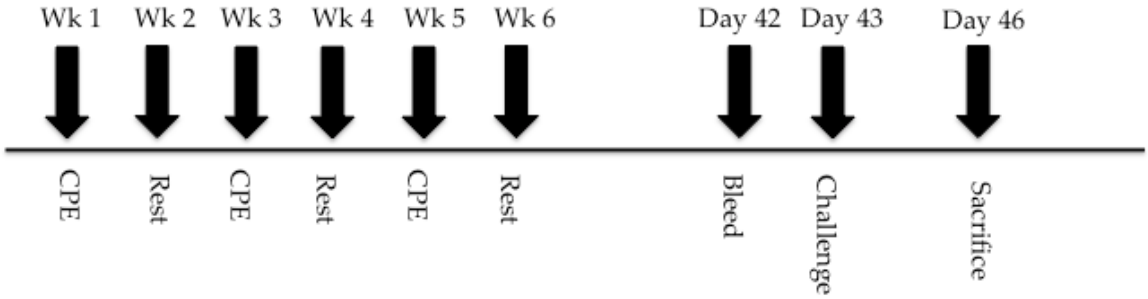


Figure 6. Timeline: peanut sensitization and challenge in DR4 mice. A small patch of skin above the base of the tail was exposed by shaving, the stratum corneum disrupted by tape-stripping and 200 ug of CPE in 10 uL of PBS applied directly to the skin. This process was repeated daily for 5 days. Mice received no intervention during the following week. This two-week cycle was repeated three times for a total length of six weeks. Mice were then challenged intraperitoneally with 5 mg of CPE in 500 uL of PBS and monitored for signs of anaphylaxis.

Mouse age

While troubleshooting the model in DR4 mice, it was observed that anaphylactic reactions most often occurred in mice older than 4 – 6 weeks that were subjected to the longer sensitization protocol. It was found that allowing by DR4 mice to age to a minimum of 9 – 10 weeks before beginning the sensitization protocol, anaphylaxis upon

peanut challenge was reliably observed. These results were validated and reproduced in both male and female DR4 mice (Figure 7).

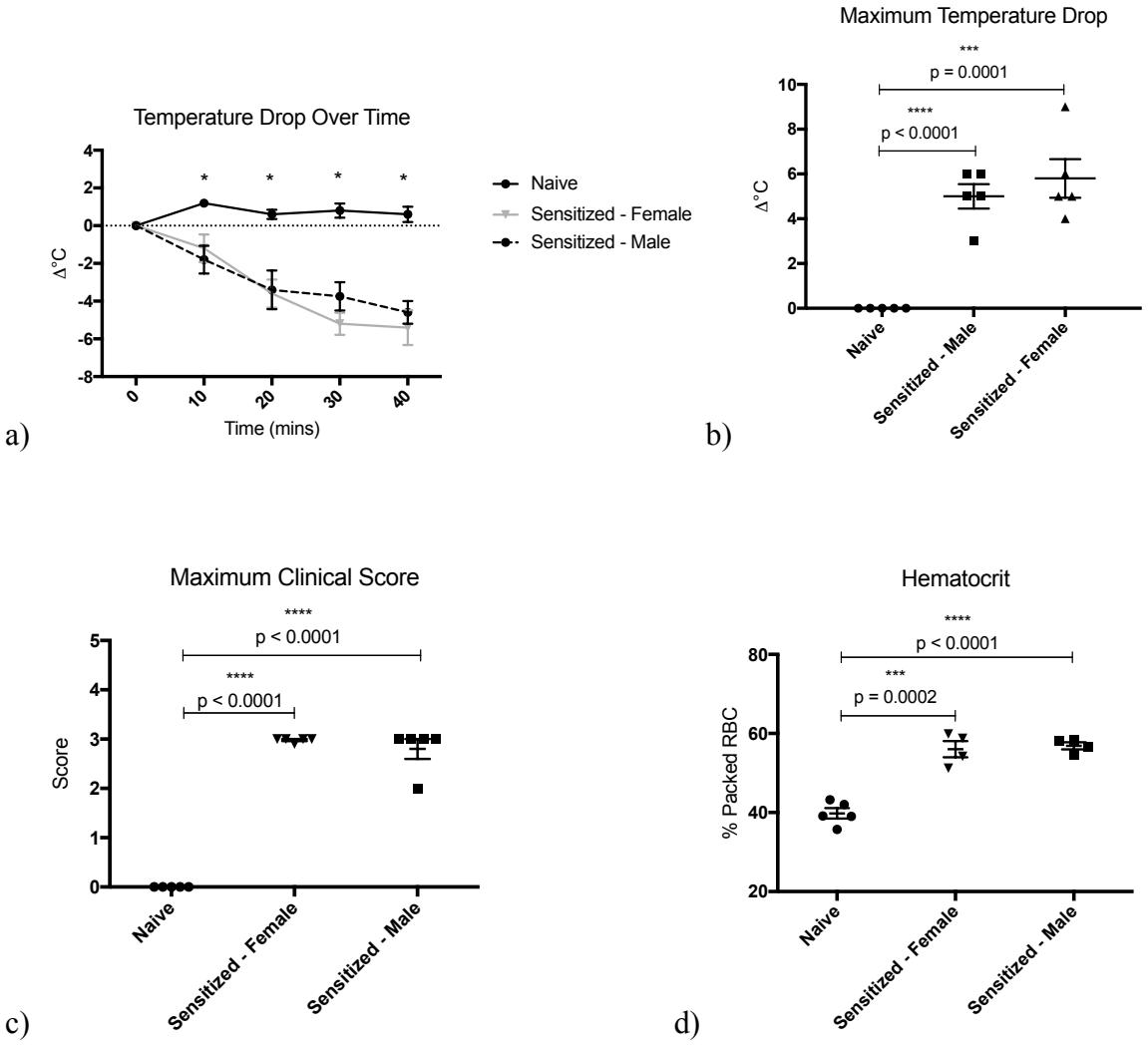


Figure 7. Male and female DR4 mice aged 9 – 10 weeks old received peanut on shaved and tape stripped skin and were subsequently challenged with peanut. Naïve control mice were sensitized with saline and challenged with peanut. Mice sensitized and challenged with peanut experienced anaphylaxis in response to peanut challenge, as demonstrated by significant drops in rectal temperature (a,b), elevated allergic clinical scores (c), and hemoconcentration (d). Data from a representative experiment is expressed as mean ± SEM with (n = 5 mice/group).

DR4 mice aged 9 – 10 weeks sensitized using the extended protocol exhibited circulating peanut-specific IgE and IgG1 immunoglobulins (Figure 8).

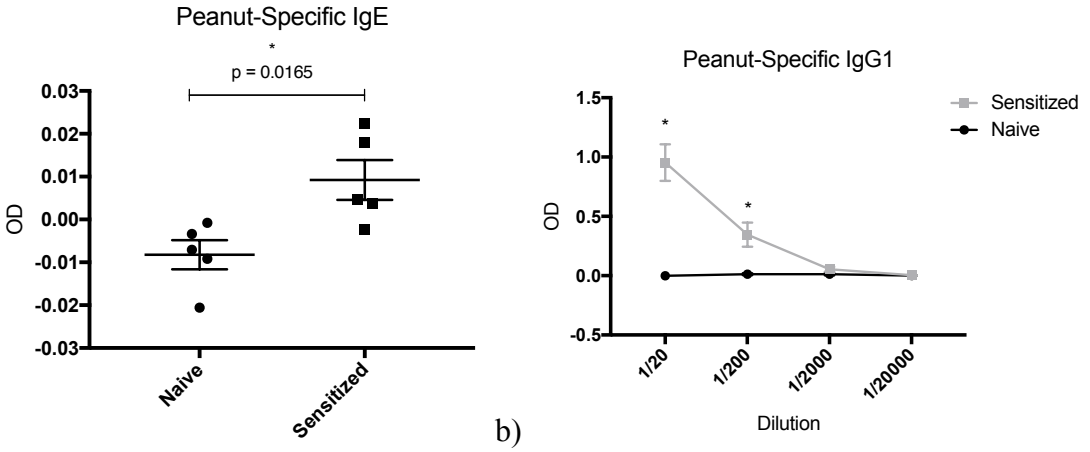


Figure 8. Peanut-sensitized mice had elevated levels of circulating peanut-specific IgE (a) and IgG1 (b), when compared to control mice. Data is expressed as mean ± SEM (n = 5 mice/group).

Sensitizing peanut dose

In addition to an extended timeframe, the 43-day protocol detailed in Figure 6 also allowed for a larger total dose of peanut over the course of sensitization. In order to determine if simply a larger total dose of peanut, independent of timeframe, was necessary for successful sensitization of DR4 mice, animals aged 9 – 10 weeks were subjected to the shorter sensitization protocol described in Chapter 2, but with a larger dose of peanut applied during each application. Increasing the dose of peanut administered during the shorter sensitization protocol did produce measurable reaction in response to peanut challenge, however this was not as robust as that seen with the extended timeline (Figure 9).

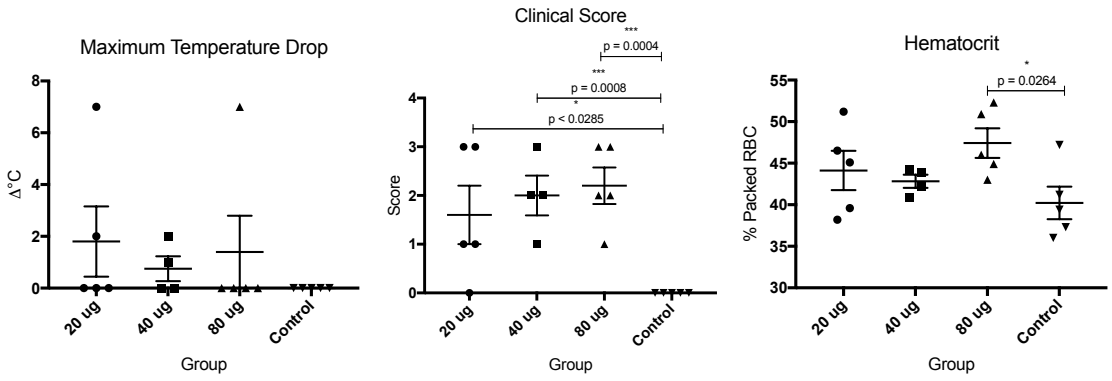


Figure 9. Female DR4 mice aged 9 – 10 weeks old received varying doses of peanut on shaved and tape stripped skin and were subsequently challenged with peanut. Control mice were sensitized with saline and challenged with peanut. Mice did not undergo full-blown anaphylaxis in response to peanut challenge. Data is expressed as mean ± SEM (n = 5 mice/group).

Summary

The most robust, reliable sensitization protocol in DR4 mice proved to be the extended 43-day protocol (Figure 6) used in mice 9 – 10 weeks old. Therefore, this protocol was used at the St Joseph's Hospital animal facility for the remainder of this project's experiments.

Discussion

Adapting the epicutaneous model of peanut sensitization developed in wild type C57Bl/6 mice to work in mice transgenic for the human leukocyte antigen HLA DRB1*0401 was a significant challenge faced by this project.

DR4 mice have been used in other models of immune-mediated disease, including collagen-induced arthritis[168], allergic encephalomyelitis[166], and allergic airways disease[152]. Published studies have not described the adaptation of a wild type model for

DR4 mice, so it is unclear whether the challenges faced by this project are unique to peanut as an antigen, anaphylaxis as a read-out, or if they are due to the nature of the DR4 mouse strain itself.

Optimal function of MHC II molecules requires efficient interaction with T cell receptors: the transmembrane glycoprotein CD4 binds the β_2 domain of MHC II. In HLA DRB1*0401, the region of amino acid residues 134 – 148 is the major contact point with CD4[169]. The interaction between these two binding domains is diminished when substitutions are made in this region[170]. Some studies have suggested that there exists species barrier between mice and human MHC II-CD4 interactions. Substituting the β_2 domain from murine H2-E MHC II into human DR transfected cell lines reduced their ability to induce proliferation of human CD4 T cells[171,172]. Similar findings were observed in reciprocal studies when murine MHC II was substituted with a human β_2 domain[173]. It is important to note that $CD4^+$ T cell responses to interspecies MHC II were blunted, rather than completely ameliorated. Indeed, studies in mice transgenic for human leukocyte antigen molecules have demonstrated that these animals are capable of generating T cell responses to specific antigens[166,174,175].

The purpose of this project was not to investigate interspecies interactions between human and murine MHC II and CD4. Rather, the goal was to develop and study a useful model of peanut-induced anaphylaxis in mice transgenic for the human leukocyte antigen HLA DRB1*0401. As such, any discussion regarding the mechanisms as to why older mice subjected to a longer sensitization protocol were required in this context is purely speculative. Based on the literature, the need for prolonged antigen exposure, and also

the heightened susceptibility to skin lesions, observed in DR4 mice may have been a consequence of less efficient MHC II-CD4 interactions when compared to their wild type C57Bl/6 counterparts.

Conclusion

A reliable, robust model of peanut-induced anaphylaxis was generated in mice transgenic for the human leukocyte antigen DRB1*0401. In comparison to their C57Bl/6 wildtype counterparts, DR4 mice needed a longer 43-day protocol and were required to reach at least 9 – 10 weeks of age before beginning sensitization. This DR4 model was generated to allow for the use of peanut peptides identified as human T cell epitopes and the use of tetramer reagents to identify and characterize peanut-specific T cell populations using flow cytometry.

Chapter 4: Characterizing Peanut-Specific $CD4^+$ T Cells in Sensitized Mice

Introduction

Peptide immunotherapy is an intervention that targets $CD4^+$ T cells via presentation of antigens bound to MHC II molecules. Therefore, examining peanut-specific T cells in the context of peptide immunotherapy was an important objective of this project. Tetramers were used to identify and characterize Ara h 1-specific $CD4^+$ T cells in peanut-sensitized DR4 mice. Tetramers are reagents comprised of four MHC II molecules loaded with a peptide of interest and labeled with a PE fluorophore. Tetramer-labeled cells can then be stained with other fluorescent-labeled antibodies and analyzed using flow cytometry.

B cells and monocytes were identified by staining for CD19 and CD14, respectively, and excluded from analysis. $Tetramer^+$ antigen-specific cells were then separated from the pool of $CD4^+$ T cells and further characterized according to markers of Th1 (IFN-γ), Th2 (IL-4, IL-5), and regulation (IL-10). MIP-1α was also included in the panel to follow up on the results of cytokine levels measured in the peritoneal lavage of wild type C57Bl/6 mice in Chapter 2.

MIP-1α, or macrophage inflammatory protein 1α, is produced by macrophages, dendritic cells, and lymphocytes. This cytokine reversibly forms rod-shaped polymers, with binding sites hidden, that is rapidly broken down into active monomers by heparin. Active MIP-1α monomer acts as a chemokine and binds CCR-1 expressed on a wide range of inflammatory cells, including neutrophils, eosinophils, monocytes, T cells,

basophils, and mast cells[176]. It is also a rapid histamine-releasing factor for basophils and mast cells, and has been shown as necessary for optimal mast cell degranulation and IgE cross-linking[177]. To date, this cytokine has not been examined in the context of severe allergy and anaphylaxis.

Methods

Animals

Male and female mice transgenic for the human leukocyte antigen DRB1*0401 were obtained from Taconic Biosciences (Hudson, NY).

All experiments were carried out in accordance with the Guide for Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board of McMaster University (AUP 12-02-04 and AUP 16-03-08).

Peanut Sensitization and Challenge

Mice were sensitized to peanut epicutaneously and challenged intraperitoneally with peanut as described in Chapter 3.

Tissue Collection and Processing

Mice were anesthetized with sodium pentobarbital and euthanized by exsanguination 72 hours after peanut challenge.

Spleen

Spleens were dissected out and pulverized using the plunger of a 1 mL syringe and a 40 μm sterile sieve (BD Biosciences) under sterile conditions. The cellular suspension was then washed in 10 mL RPMI 1640 culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich) by centrifugation at 400 rcf at 4°C for 10 minutes. The supernatant was decanted, the cell pellet resuspended in 20 mL of supplemented media, and centrifuged at 400 rcf at 4°C for 10 minutes. If cells were being frozen, they were resuspended in equal parts supplemented RPMI media and freezing media composed of 40% RPMI 1640 media, 20% FBS, and 20% DMSO at a maximum concentration of 10^6/mL. Samples were transferred to 1.5 mL CryoVials (Sigma-Aldrich) and placed in a Mr. Frosty (Thermo Fisher, Waltham, MA) at -80°C for 24 hours before being transferred to liquid nitrogen storage.

Lymph Nodes

Inguinal and mesenteric lymph nodes were dissected out and pulverized using the plunger of a 1 mL syringe and a 40 μm sterile sieve (BD Biosciences) under sterile conditions. The cellular suspension was then washed in 1 mL RPMI 1640 culture media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Sigma-Aldrich) by centrifugation at 400 rcf at 4°C for 10 minutes. The supernatant was decanted, the cell pellet resuspended in 5 mL of supplemented media, and centrifuged at 400 rcf at 4°C for 10 minutes. If cells were being frozen, they were resuspended in equal parts supplemented RPMI media and freezing media composed of 40% RPMI 1640 media, 20% FBS, and 20% DMSO at a maximum concentration of 10^6/mL. Samples

were transferred to 1.5 mL CryoVials (Sigma-Aldrich) and placed in a Mr. Frosty (Thermo Fisher) at -80^{o}C for 24 hours before being transferred to liquid nitrogen storage.

Peritoneal Wash

Peritoneal wash samples were collected and processed as described in Chapter 2. If cells were being frozen, they were resuspended in equal parts supplemented RPMI media and freezing media composed of 40% RPMI 1640 media, 20% FBS, and 20% DMSO at a maximum concentration of 10^6/mL. Samples were transferred to 1.5 mL CryoVials (Sigma-Aldrich) and placed in a Mr. Frosty (Thermo Fisher) at -80^{o}C for 24 hours before being transferred to liquid nitrogen storage.

Tetramer and Antibody Staining for Flow Cytometry

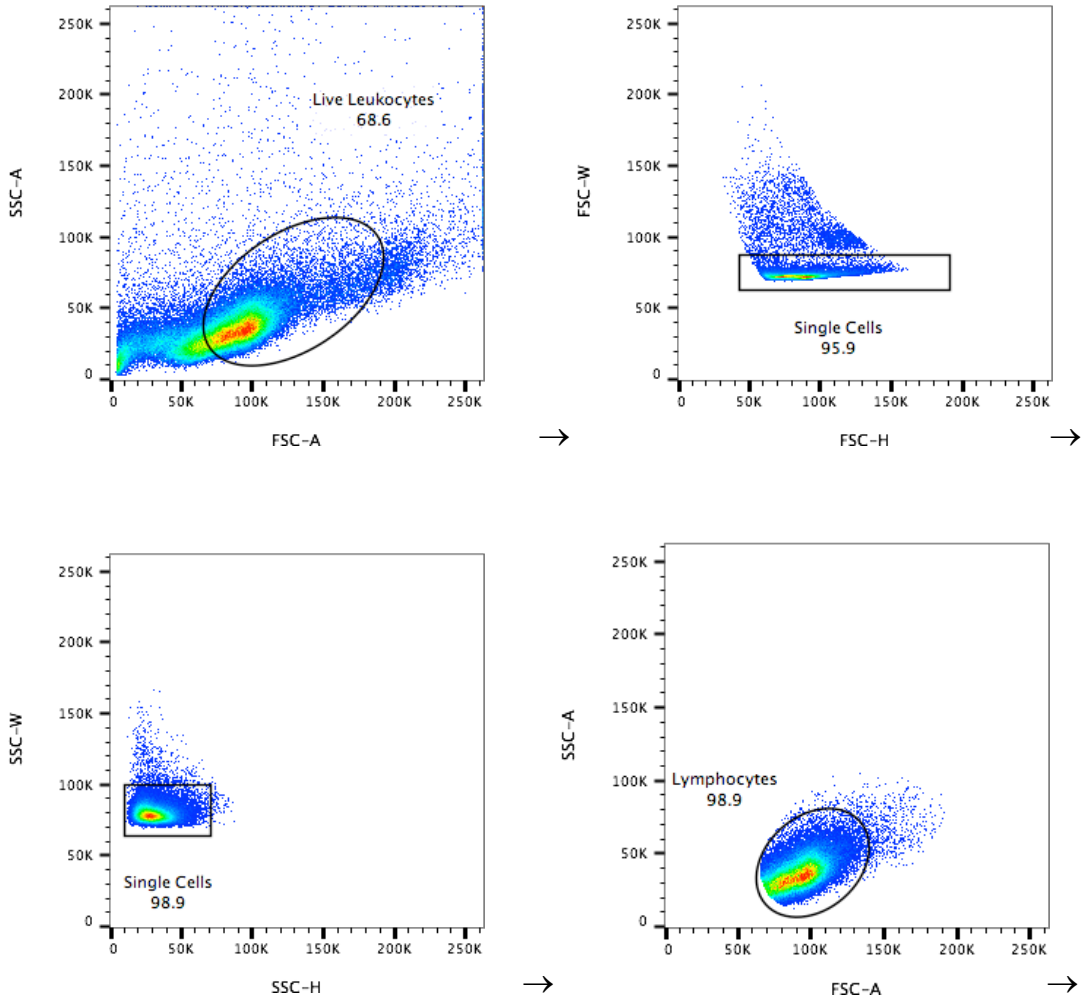
Cells were resuspended at a concentration of 10^7/mL and 500 000 – 1 000 000 cells were incubated with 0.2 uL of Leukocyte Activation Cocktail with GolgiPlug (BD Biosciences) in media in V-bottom plates (Thermo Fisher) for 3 hours at 37^{o}C. Plates were spun down at 400 rcf for 10 minutes at room temperature. Cells were resuspended and incubated with 50 nM dasatanib with 0.2 uL Leukocyte Activation Cocktail with GolgiPlug in media for 20 minutes at 37^{o}C to prevent T cell receptor downregulation in response to tetramer binding. Plates were spun down at 400 rcf for 10 minutes at room temperature. Cells were resuspended and incubated with 0.8 uL/well of 3 Ara h 1 PE-labeled tetramers (201-220), (329-348), and (577-596), plus 0.2 uL Leukocyte Activation Cocktail with GolgiPlug, and 50 nM dasatanib in a volume of 20 uL for 2 hours at 37^{o}C. Tetramers were sourced from Dr. William Kwok's lab at the Benaroya Institute, Seattle, WA. 150 uL media was added to wash, and plates were spun down at 400 rcf for 10

minutes at room temperature. Cells were resuspended in 100 uL of surface marker fluorophore-labeled antibody cocktails in staining buffer (BioLegend, San Diego, CA) and incubated for 30 minutes at 4°C. APC-Cy7 CD4 and PerCP-Cy5.5 CD8, CD14, CD19 were used for all panels. PE-Cy7 LAG3 and FITC CD49b were used in the regulatory panel, PE-Cy7 PD-1 in the Th1 panel, and FITC ST2 in the Th2 panel. 100 uL staining buffer was added to wash and cells were spun down at 400 rcf for 10 minutes at 4^0C. Cells were resuspended in 100 uL of 4% PFA and incubated for 20 minutes at 4°C. 100 uL staining buffer was added to wash and cells were spun down at 400 rcf for 10 minutes at 4^0C. Cells were resuspended in 200 uL perm/wash (BD Biosciences) and incubated for 20 minutes at 4°C. Plates were spun down at 400 rcf for 10 minutes at 4°C. Cells were resuspended in 50 uL of intracellular fluorophore-labeled antibody cocktails in perm/wash. IL-10 was used in the regulatory panel, IFN-γ and MIP-1α in the Th1 panel, and IL-4 and IL-5 in the Th2 panel. FMO and isotype controls were used for all antibody panels and all antibodies were sourced from BD Biosciences or eBiosciences. 150 uL of perm/wash was added to wash and plates were spun at 400 rcf for 10 minutes at 4°C. Cells were washed two more times with perm/wash and resuspended in 200 uL of cells staining buffer. Samples were transferred to 5 mL polystyrene cell-strainer cap tubes (Corning Inc, Tewksbury, MA) immediately before being run on the cytometer.

All samples were run on the FACS CANTO cytometer (BD Biosciences) and analyzed with FlowJo Version 10.0.8. (FlowJo, Ashland, OR).

Enumerating Tetramer Positive $CD4^+$ Cells

The following gating strategy was used to identify $tetramer^+$ $CD4^+$ T cells: live leukocytes → single cells → lymphocytes → $CD8a^-CD14^-CD19^-$ cells → $CD4^+$ cells → PE^+ cells (Figure 1). Each gate was determined using FMO samples, and a representative tetramer FMO plot from peritoneal wash is shown in Figure 2.



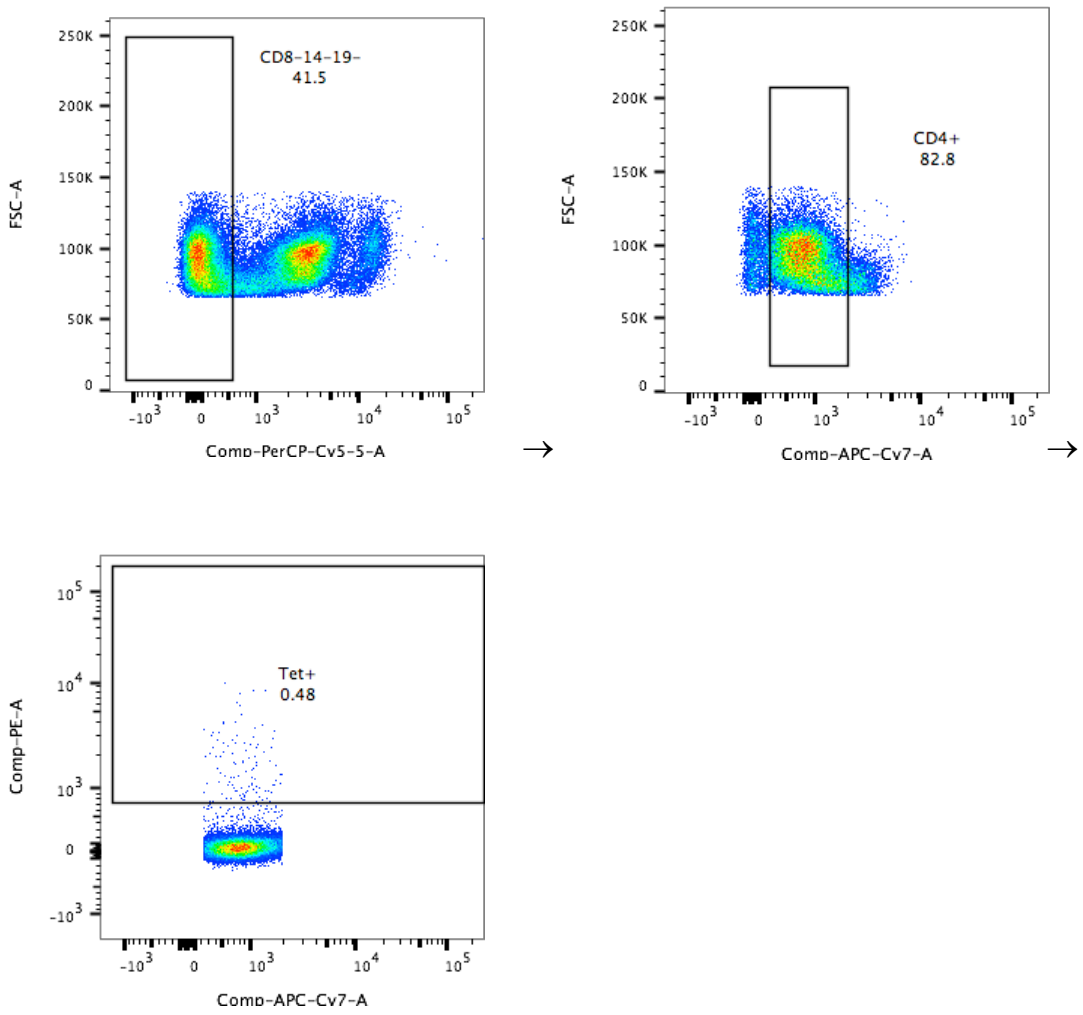
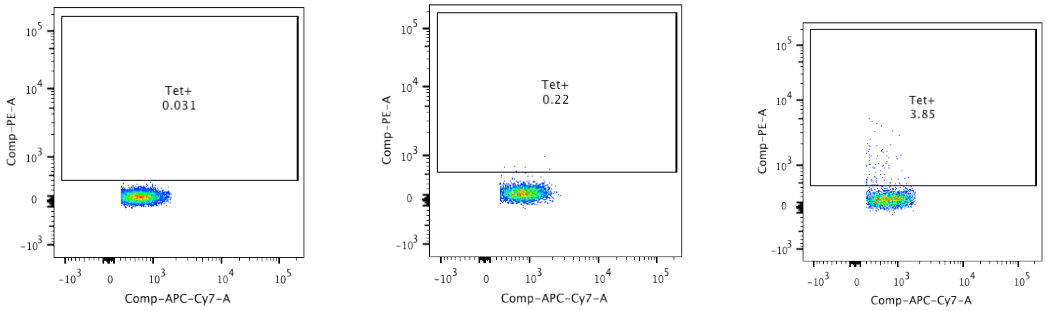


Figure 1. Gating strategy to identify tetramer$^+$ CD4$^+$ T cells.



a) b) c)

Figure 2. A tetramer FMO plot is shown in (a). This gate was generated using a sample that was not stained for tetramers and applied to all other tetramer-stained samples. The

application of the FMO gate to representative tetramer-stained samples from naïve (b) peanut-sensitized (c) mice is also shown.

Results

Peanut-sensitized mice had a larger Ara h 1-specific CD4$^+$ T cell population

Mice were sensitized to peanut epicutaneously, challenged systemically with peanut, and sacrificed 72 hours after challenge. Tetramer staining was used to enumerate Ara h 1-specific CD4$^+$ T cells in the spleen, peritoneal wash, and inguinal lymph nodes, with the peritoneal wash being the richest site of tetramer$^+$ cells (Figure 3). Mice sensitized to peanut had significantly larger Ara h 1-specific CD4$^+$ T cell populations than naïve mice in all three compartments. The peritoneal wash was the site of antigen challenge, the spleen was taken as a representative sample of systemic T cell populations, and the inguinal lymph nodes drained the site of dorsal skin sensitization.

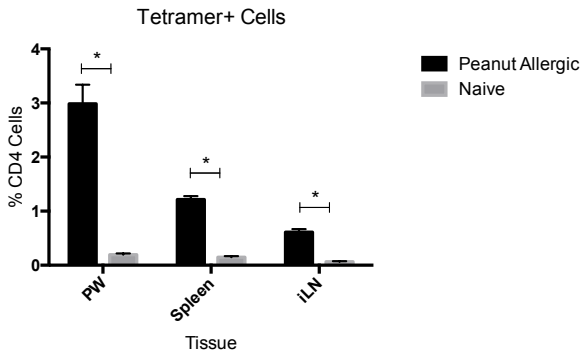
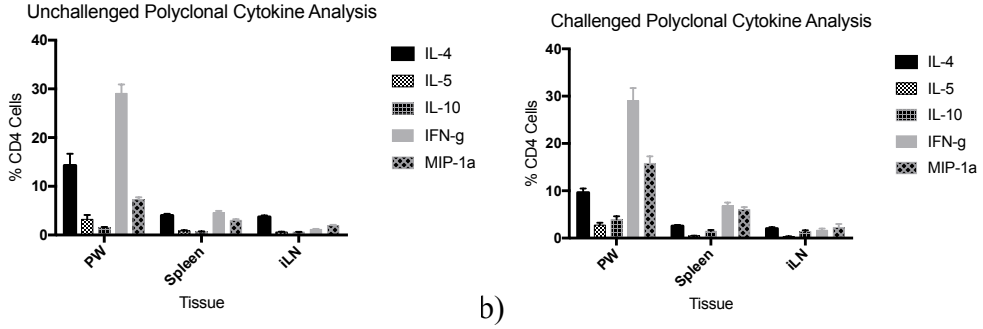


Figure 3. Ara h 1 tetramer-positive cells enumerated from three different compartments: peritoneal wash, spleen, and inguinal lymph nodes. Naïve, and peanut-sensitized groups are shown. Data is expressed as mean ± SEM (n = 5 mice/group).

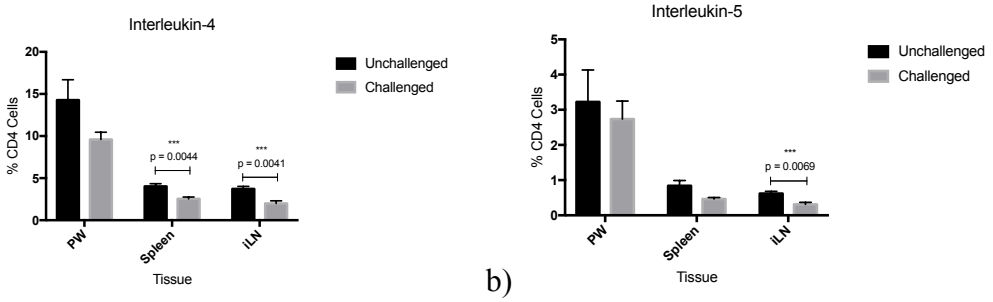
Ara h 1-specific T cells from peanut-sensitized, challenged mice expressed high levels of IL-4 and MIP-1α

DR4 mice were sensitized and challenged with peanut as described in Chapter 3. Polyclonal $CD4^+$ T cells were examined for cytokine expression using fluorophore-labeled antibody staining and flow cytometry. These samples were not gated to select for cells bound to Ara h 1 tetramers. In peanut-sensitized and unchallenged mice, Ara h 1-specific T cells predominantly produced IL-4 and IFN-γ. Following peanut challenge, the proportion of $CD4^+$ T cells producing MIP-1α and IL-10 significantly increased (Figures 4 and 5).



a) b)

Figure 4. Analysis of cytokine production by $CD4^+$ cells in peanut-sensitized unchallenged (a) and challenged (b) mice. Data is expressed as mean ± SEM (n = 5 mice/group).



a) b)

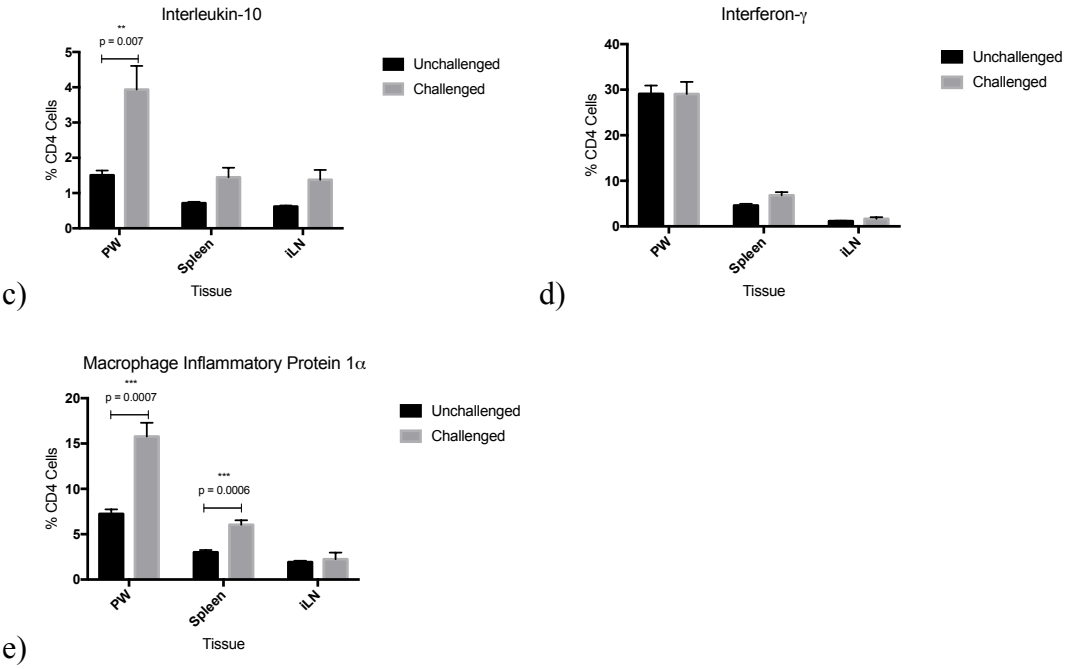
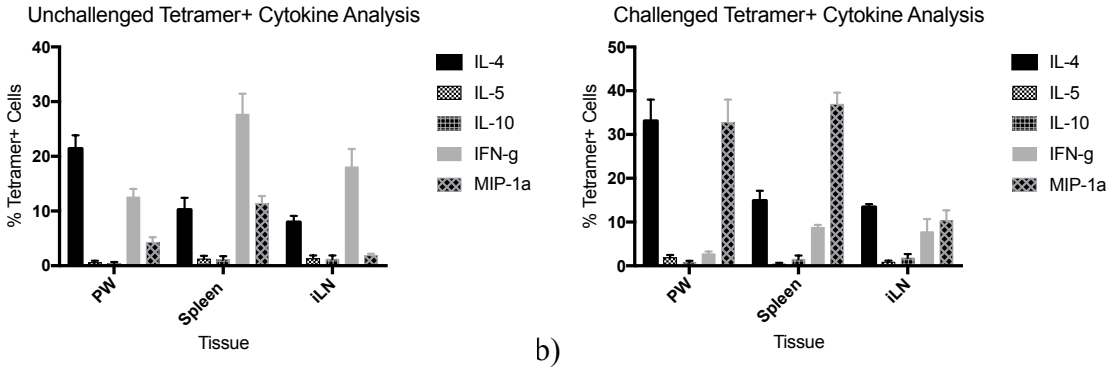


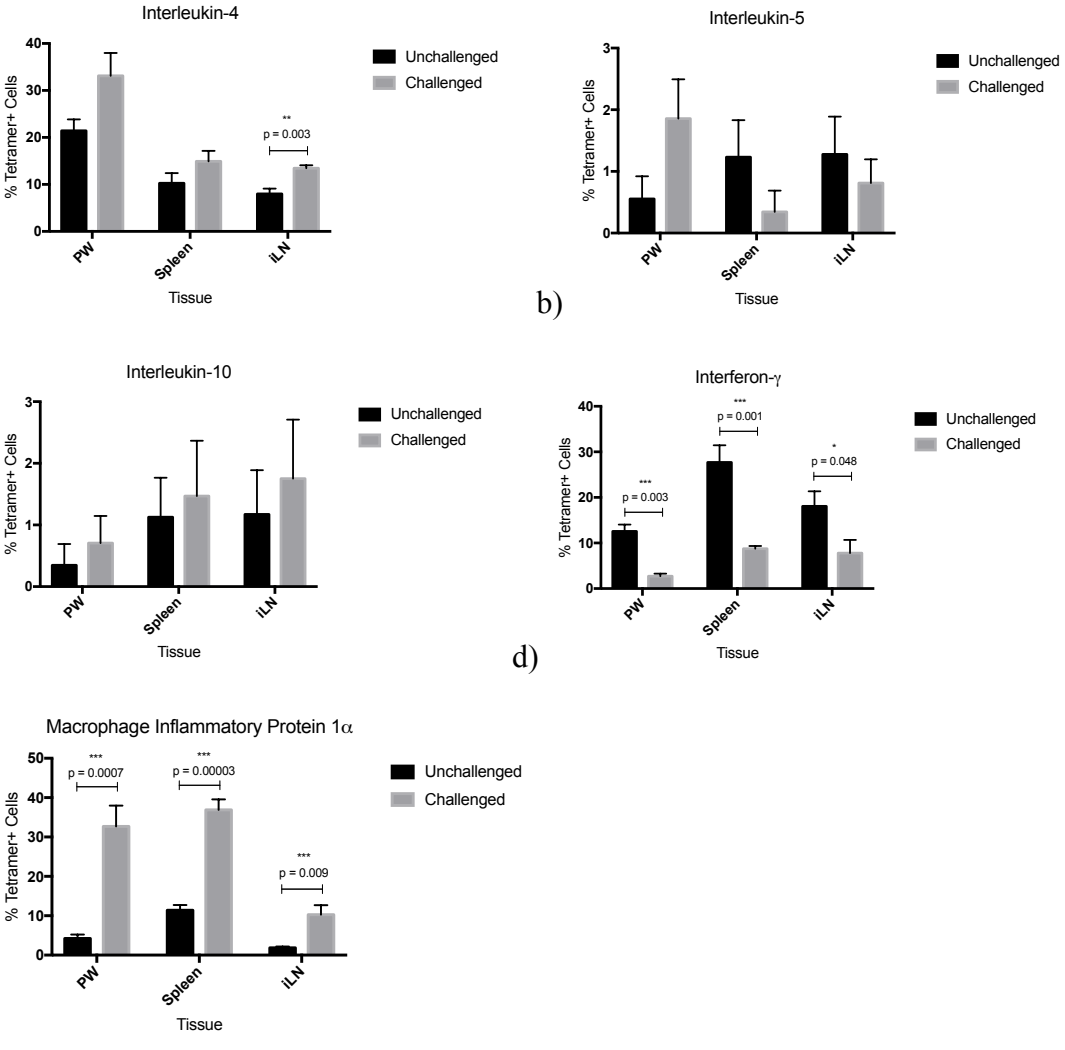
Figure 5. Analysis of cytokine production by $CD4^+$ cells in peanut-sensitized unchallenged and challenged mice. Peanut challenge increased the proportion of $CD4^+$ T cells in each tissue expressing IL-10 (c) and MIP-1α (e). Data is expressed as mean ± SEM (n = 5 mice/group).

Cytokine production by Ara h 1 $tetramer^+$ cells was then examined. In peanut-sensitized and unchallenged mice, Ara h 1-specific T cells predominantly produced IL-4 and IFN-γ. Seventy-two hours after peanut challenge, Ara h 1-specific T cells across all three compartments displayed increased expression of IL-4 and MIP-1α (Figures 6 and 7). Data from $tetramer^+$ $CD4^+$ T cells showed that there was not simply a broad jump in inflammatory cytokine production after challenge, rather that these two cytokines were preferentially upregulated. Interestingly, $CD4^+tetramer^+IL\text{-}10^+$ T cells were not increased. It is likely that a non-peanut specific population of T cells increased production of the anti-inflammatory cytokine IL-10 in response to inflammation induced by antigen challenge.



a) b)

Figure 6. Analysis of cytokine production by tetramer$^+$ cells in peanut-sensitized unchallenged (a) and challenged (b) mice. Data is expressed as mean ± SEM (n = 5 mice/group).



a) b)

c) d)

e)

Figure 7. Analysis of cytokine production by tetramer$^+$ cells in peanut-sensitized unchallenged and challenged mice. Peanut challenge increased the proportion of Ara h 1-specific tetramer$^+$ CD4$^+$ T cells in each tissue expressing IL-4 (a) and MIP-1α (e). Data is expressed as mean ± SEM (n = 5 mice/group).

Heparin alone did not induced anaphylaxis

Activated mast cells and basophils produce heparin, a known activator of MIP-1α polymers. Based on the high expression of MIP-1α in peanut-challenged mice, we conducted a small pilot to investigate the ability of heparin to induce anaphylaxis in peanut-sensitized mice.

C57Bl/6 mice were sensitized to peanut epicutaneously and then challenged with intraperitoneal heparin. Five mice were used, and each received a different dose of heparin going down by log scales. Mouse 1 received 2.94 units of heparin, mouse 2 received 0.294 units, mouse 3 received 0.0294 units, mouse 4 received 0.00294 units, and mouse 5 received 0.000294 units. This dosing was based on the recommended initial dose for human IV anticoagulation, scaled down for a 20g mouse. This dosage was selected in order to avoid inducing systemic or intracranial bleeds that would complicate outcome measurements.

Peanut-sensitized mice challenged with heparin did not experience any signs of systemic anaphylaxis (Figure 8). Some reduced movement was noted, but this was mild and not accompanied by temperature drops or hemoconcentration. Heparin alone did not induce anaphylaxis in peanut-sensitized mice.

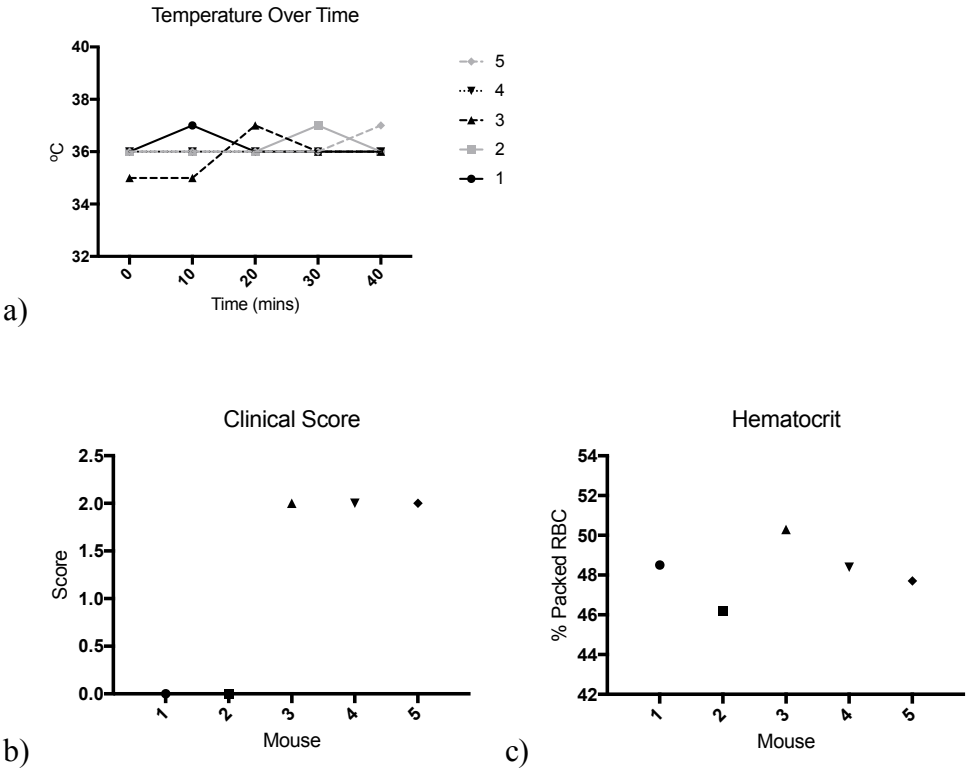


Figure 8. Peanut-sensitized mice challenged with heparin did not exhibit signs of anaphylaxis as measured by temperature drop (a), clinical score (b), or hemoconcentration (c). Data is expressed as mean ± SEM (n = 5 mice).

Discussion

Tetramer reagents loaded with Ara h 1 peptides were used to enumerate and characterize Ara h 1-specific $CD4^+$ T cells from peanut-sensitized mice. The frequency of these cells varied according to tissue, making up approximately 3% of $CD4^+$ cells in the peritoneal wash, 1% in the spleen, and 0.7% in the inguinal lymph nodes that drained the dorsal skin. These proportions were much higher than detectable Ara h 1 cells found in the peripheral blood of human peanut-allergic subjects, where their frequencies can be as low as nine per million[167]. One advantage of working in an animal model is the ability to obtain all tissues of interest and manipulate experimental protocols to induce robust

immune responses prior to cellular harvest. As in healthy control non-allergic human subjects, Ara h 1-specific T cells in unsensitized mice were nearly undetectable.

The cytokines selected for analysis in this chapter have previously been associated with peanut allergy. In animal models, splenocytes from peanut-sensitized mice expressed IL-4, IL-5, and IL-13 when cultured with peanut[103,104,178] and dendritic cells from peanut-sensitized animals, when co-cultured with peanut, induced naïve $CD4^+$ cells to produce IL-4, IL-5, IL-13[179]. Peanut-specific cells isolated from peanut-allergic subjects expressed the Th2 cytokines: IL-4, IL-5, IL-9, IL-13, and the IL-25 receptor IL-17RB[180]. Peripheral blood cells from peanut-allergic children stimulated with Ara h 1 and Ara h 2 expressed IL-4, IL-5, and IL-13. Children with high Ara h 1- and Ara h 2-specific IgE titers, defined as > 15 kU_A/L demonstrated increased Th2 skewing, based on the ratio of activated $IL4^+$ T cells to IFN-γ T cells[181]. In their study of Ara h 1 peptides in peanut-allergic subjects that led to the development of the tetramer reagents used in this project, DeLong *et al* found that these antigen-specific $CD4^+$ T cells secreted IL-4, IL-5, IL-10, IL-17, and IFN-γ[167].

This project found that in peanut-sensitized and unchallenged mice, Ara h 1-specific T cells predominantly produced IL-4 and IFN-γ. Seventy-two hours following peanut challenge, Ara h 1-specific T cells across all three compartments were predominantly expressing IL-4 and MIP-1α. To our knowledge, this study is the first to identify altered MIP-1α expression in the context of anaphylaxis.

Other published studies have described the association between elevated MIP-1α, hayfever, and asthma. Ragweed-allergic subjects had higher levels of serum MIP-1α

than healthy controls, and these levels peaked during pollen season. This was accompanied by an elevation in circulating IL-8[182]. In a study of children hospitalized with a first episode of wheeze, MIP-1α levels in nasopharyngeal aspirates were significantly higher in participants who went on to have recurrent wheezing[183]. $CD4^+$ T cell expression of MIP-1α is elevated in both obese and non-obese patients with asthma, and increases with disease severity[184]. When incubated with latex major allergen, the peripheral blood cells of latex-allergic individuals produced high levels of MIP-1α[185]. This cytokine has also been implicated in the development of atopic dermatitis in infants: MIP-1α was more likely to be higher in the colostrum and breastmilk of mothers with babies who would go on to develop AD[186].

One technological limitation of this study was the ability to use only six-colour staining panels. It was therefore challenging to assess co-expression of surface markers and cytokines on $CD4^+$tetramer$^+$ cells. The rarity of antigen-specific tetramer$^+$ cells also made it difficult to analyze co-expression. Future studies may be able to examine co-expression with the use of more advanced flow cytometry machines.

Conclusion

Sensitization with peanut expanded Ara h 1-specific $CD4^+$ T cells in the spleen, peritoneal wash, and inguinal lymph nodes. Ara h 1-specific $CD4^+$ T cells from peanut-sensitized mice produced high levels of IL-4 and MIP-1α after peanut challenge. IL-4 is traditionally associated with anaphylaxis and allergy, while MIP-1α may be a newly identified important player in peanut-induced anaphylaxis.

Chapter 5: Ara h 1 Peptide Immunotherapy in Mice Transgenic for HLA DRB1*0401

Introduction

The therapeutic efficacy of Ara h 1 peptides was tested in the model of peanut-induced anaphylaxis in DR4 mice. The peptides used in these studies were originally identified as T cell epitopes in human peanut-allergic patients. DeLong *et al* used MHC II tetramers to detect Ara h 1-specific cells in the peripheral blood of peanut-allergic individuals and identify dominant epitopes restricted by HLA class. Four Ara h 1 peptides were found to be recognized by allergic subjects expressing the MHC II molecule HLA DRB1*0401. DR4 mice are devoid of endogenous mouse MHC II and express human HLA DRB1*0401. Therefore, these four Ara h 1 epitopes would theoretically be recognized by DR4 mice. Epitope affinity prediction software was used to determine which of the four epitopes would be suitable for peptide immunotherapy in this model, based on their strength of binding affinity to HLA DRB1*0401.

Following immunotherapy, changes in Ara h 1-specific T cells in DR4 mice were characterized using tetramer staining. B cells and monocytes were identified by staining for CD19 and CD14, respectively, and excluded from analysis. Tetramer$^+$ antigen-specific cells were then separated from the pool of CD4$^+$ T cells and further characterized according to markers of Th1, Th2, and regulation: IFN-γ, PD-1, IL-4, IL-5, ST2, IL-10, LAG3, and CD49b. Studying the phenotype of antigen-specific CD4$^+$ T cells following peptide immunotherapy provided some insight into the mechanism of this treatment

modality and may lead to a greater understanding of the differences between tolerant and pro-inflammatory immune responses in the context of anaphylaxis.

Methods

Animals

Male and female mice transgenic for the human leukocyte antigen DRB1*0401 were obtained from Taconic Biosciences (Hudson, NY).

All experiments were carried out in accordance with the Guide for Humane Use and Care of Laboratory Animals and were approved by the Animal Research Ethics Board of McMaster University (AUP 12-02-04 and AUP 16-03-08).

Peanut Sensitization and Challenge

Mice were sensitized to peanut epicutaneously and challenged intraperitoneally with peanut as described in Chapter 3.

Peptide Identification

DeLong *et al* used tetramer guided epitope mapping to identify four Ara h 1 peptides that bind HLA DRB1*0401 in peanut-allergic individuals: Ara h 1 (201-220), (329-348), (505-524), and (577-596)[167]. We used the software tools Epitool kit and Sturniolo to predict affinity binding and the peptide core for peptides Ara h 1 (201-220), (329-348), (505-524), and (577-596) and select appropriate peptides for our treatment protocol.

Peptide Treatment

Therapeutic Peptide Administration

DR4 mice were sensitized to peanut and treated with two intraperitoneal injections of the peptides Ara h 1 (201-220), (329-348), and (505-524), administered one week apart. Peptide doses ranged from 0.01 μg to 100 μg. Treatments were well tolerated, with no local or systemic reactions occurring after peptide injection. No changes in behavior, body condition, or rectal temperature were seen. Control mice were treated with a sham peptide from influenza. Mice were challenged with whole peanut extract one week after the last treatment injection and evaluated for signs of anaphylaxis (Figure 1).

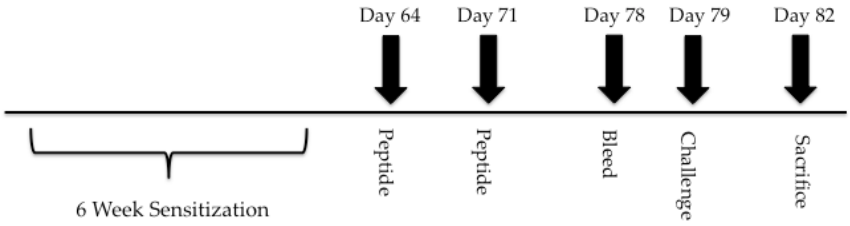


Figure 1. Timeline: Ara h 1 peptide treatment in DR4 mice.

Prophylactic Peptide Administration

DR4 mice received two intraperitoneal doses of three Ara h 1 peptides (201-220), (329-348), (577-598), one week apart. Dosing ranged from 1 μg to 300 μg. Ten days after the second dose, mice were sensitized to peanut epicutaneously. They were subsequently challenged with peanut an assessed for anaphylaxis (Figure 2).

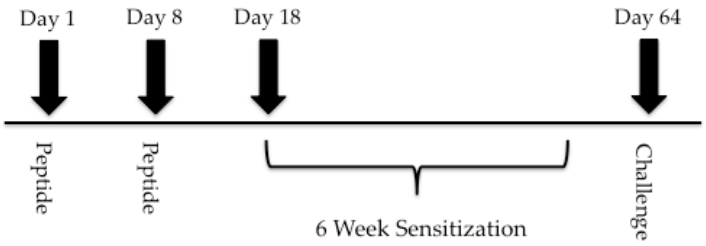


Figure 2. Timeline: Prophylactic Ara h 1 peptide immunotherapy in DR4 mice.

Tissue Processing and Cell Staining

Tissue processing, cell staining, and flow cytometry were conducted as described in Chapter 4.

Results

Peptide Identification

Ara h 1 (201-220), (329-348), (577-596) were predicted to have high binding affinity

Epitope affinity prediction tools Epitool and Sturniolo found high affinity cores in three of the four Ara h 1 peptides: Ara h 1 (201-220), (329-348), (577-596).

TABLE 1: Epitool Kit Predictions

Peptide	**Core**	**Affinity (nM)**	**Binding Prediction**
Ara h 1 (201-220)	FQNLQNHRI	3.5	High
	LQNHRIVQI	1.48	Potential
Ara h 1 (329-348)	VLLEENAGG	2.8	High
Ara h 1 (505-524)	VFIMPAAHP	0.9	Poor
Ara h 1 (577-596)	FVSARPQSQ	2.8	High

Table 1. Affinity binding and peptide core predictions. Scores >= 2 indicate strong binders. Scores between 1-2 indicate potential binders.

TABLE 2: Sturniolo Predictions			
Peptide	**Core**	**Affinity (nM)**	**Binding Prediction**
Ara h 1 (201-220)	FQNLQNHRI	3.5	High
Ara h 1 (329-348)	VLLEENAGG	2.8	High
Ara h 1 (505-534)	VFIMPAAHP	0.9	Poor
Ara h 1 (577-596)	FVSARPQSQ	2.8	High

Table 2. Affinity binding and peptide core predictions. Scores >= 2 indicate strong binders. Scores between 1-2 indicate potential binders.

These peptides were evaluated for water solubility using Innovagen (pepcalc.com) and GRAVY score (web.expasy.org/protparam/) tools (Table 3).

TABLE 3: Solubility Predictions			
Peptide	**Sequence**	**Innovagen**	**GRAVY Score**
Ara h 1 (201-220)	QRSRQFQNLQNHRIVQIEAK	Good water solubility	-1.39
Ara h 1 (329-348)	FNEIRRVLLEENAGGEQEER	Good water solubility	-1.245
Ara h 1 (577-596)	QKESHFVSARPQSQSQSPSS	Good water solubility	-1.455

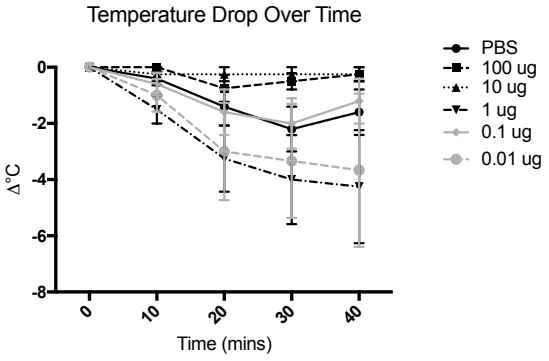
Table 3. Solubility predictions for Ara h 1 peptides (201-220), (329-348), (577-596). GRAVY scores towards the negative range indicate greater solubility.

All peptides were found to have good water solubility and it was not difficult to dissolve these peptides directly in PBS.

Ara h 1 Peptide Immunotherapy

Ara h 1 peptide immunotherapy protected against severe anaphylaxis in response to peanut challenge

Intraperitoneal administration of Ara h 1 peptides ameliorated peanut-induced anaphylaxis in peanut-sensitized mice. Mice that received 10 μg of each peptide exhibited the highest level of protection. Control mice treated with saline experienced a mean maximum temperature drop of 2.2°C, while mice receiving 10 μg of each peptide experienced a drop of 0.25°C (p=0.0750 vs control). Maximum clinical score was 2.8 in control mice and 0.5 in treated mice (p = 0.0023). Mean hematocrit for control mice was 55.14% and 46.83% for treated mice (p=0.0314) (Figure 3).



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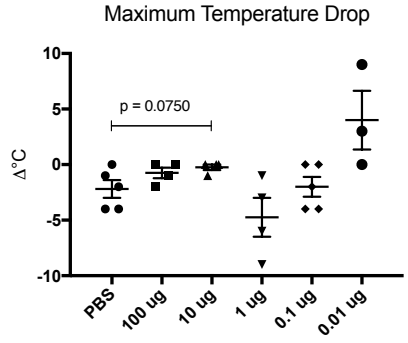

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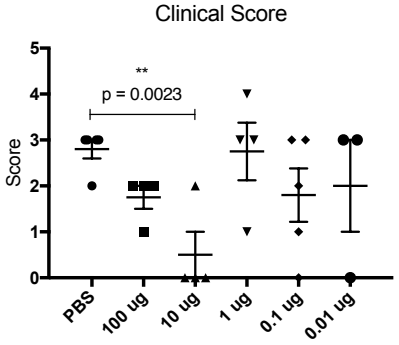

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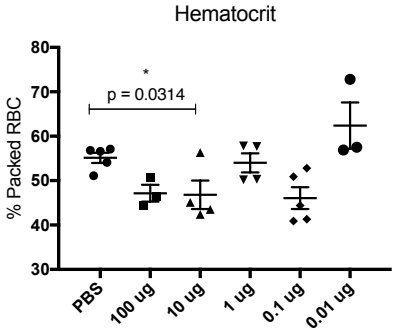


d)

Figure 3. Peanut-sensitized mice treated intraperitoneally with 10 μg Ara h 1 peptides experienced attenuated anaphylaxis when challenged with peanut. Mice exhibited protection against severe temperature drops (a, b, c), clinical signs of reaction (d), and hemoconcentration (e). Data is expressed as mean ± SEM (n = 5 mice/group).

Intraperitoneal peptide therapy with 10 μg each of Ara h 1 (201-220), (329-348), (577-596) reliably showed protection against anaphylaxis in repeat experiments (Figure 4).

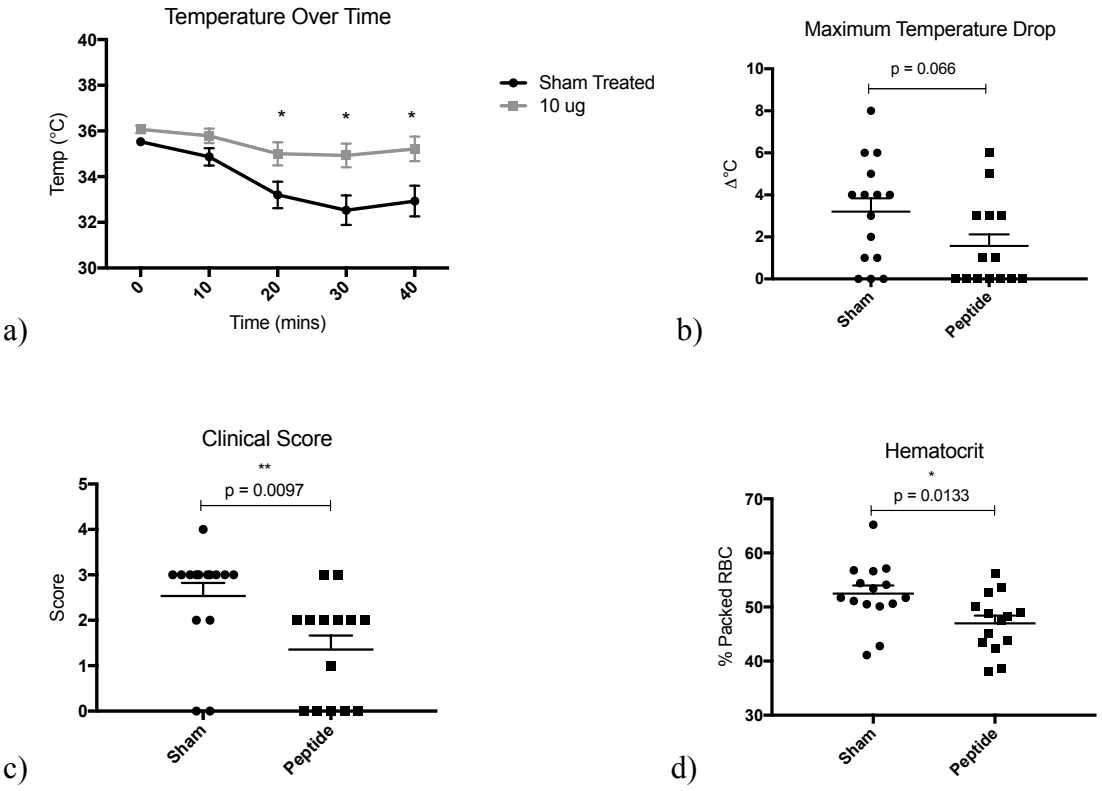
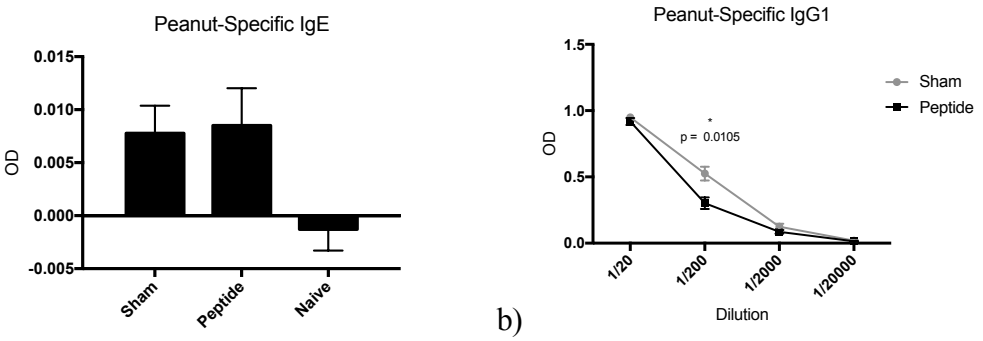


Figure 4. Peanut-sensitized mice treated intraperitoneally with 10 ug Ara h 1 peptides experienced attenuated anaphylaxis when challenged with peanut. Mice exhibited protection against severe temperature drops (a, b), clinical signs of reaction (c), and hemoconcentration (d). Data is expressed as mean ± SEM and is representative of three independent experiments (n = 15 mice/group).

No marked differences in peanut-specific IgE or IgG1 were measured at one week after the final peptide treatment (Figure 5).

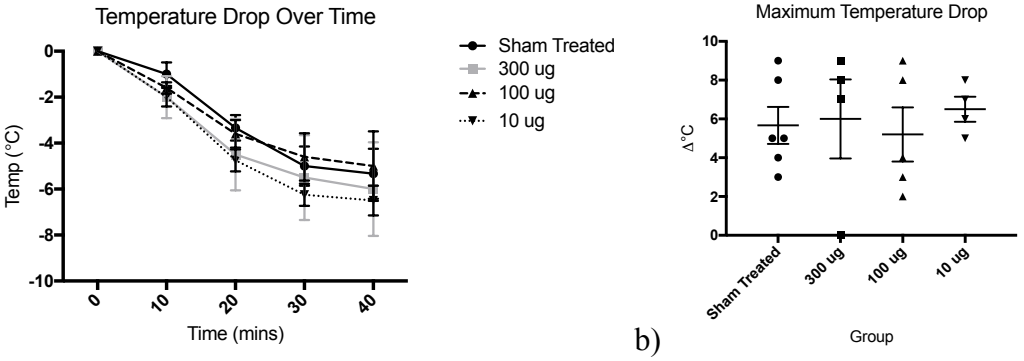


a) b)

Figure 5. Serum peanut-specific IgE (a) and IgG1 (b) antibodies. Data is expressed as mean ± SEM and is representative of three independent experiments (n = 15 mice/group).

Alternate peptide treatment protocols did not protect against anaphylaxis in response to peanut challenge

In Chapter 2, Ara h 1 (505-524) was identified as a strong binder in C57Bl/6 mice, and immunotherapy with this peptide induced clinical protection from peanut-induced anaphylaxis. This peptide was identified as a weak binder for DR4 mice. Ara h 1 (505-524) was given to DR4 mice in order to assess the importance of strong binding and to act as an additional control to confirm that clinical protection is peptide-specific. Treatment with Ara h 1 (505-524) did not protect peanut-sensitized DR4 mice from anaphylaxis in response to whole peanut challenge (Figure 6).



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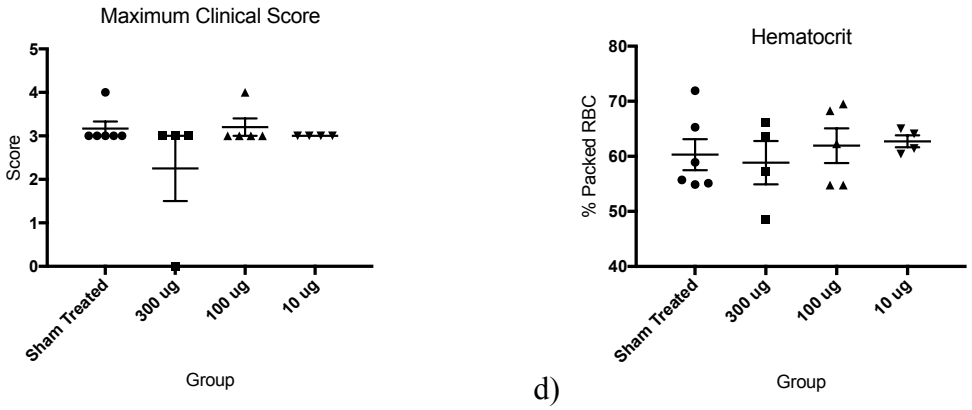


c) d)

Figure 6. Peanut-sensitized DR4 mice treated with Ara h 1 (505-524) were not protected against anaphylaxis when challenged with peanut. Treated mice exhibited temperature drops (a, b), clinical signs of reaction (c), and hemoconcentration (d) similar to control mice. Data is expressed as mean ± SEM (n = 5 mice/group).

In the process of optimizing this peptide treatment protocol, intradermal peptide administration was trialed. As in the wild type C57Bl/6 mice, this route of administration did not consistently protect against severe anaphylaxis: treatment with 100 μg of Ara h 1 peptides demonstrated protection in one experiment (Figure 7), but this finding could not be replicated (Figure 8).

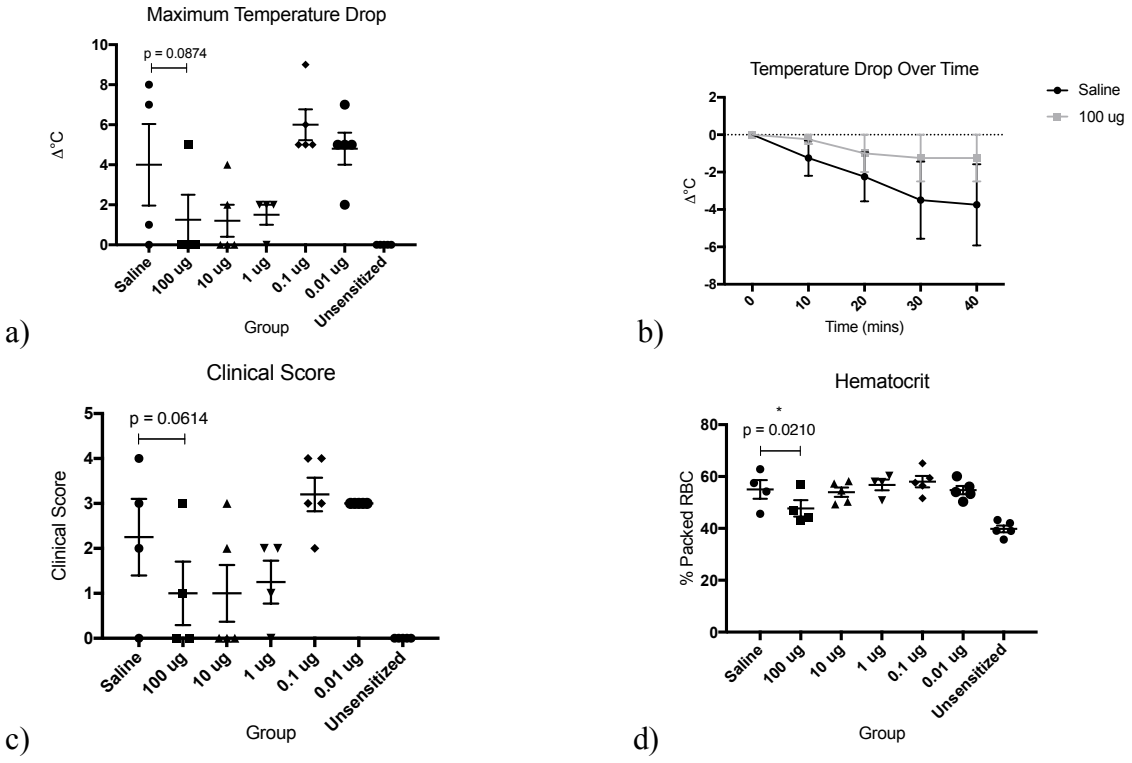
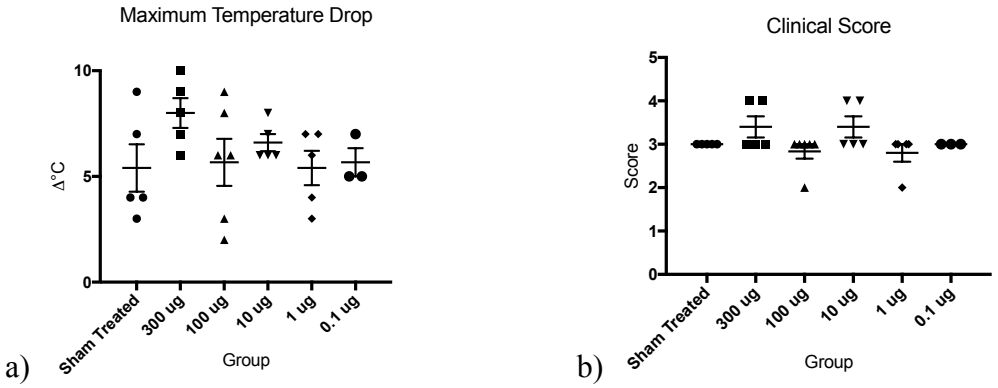
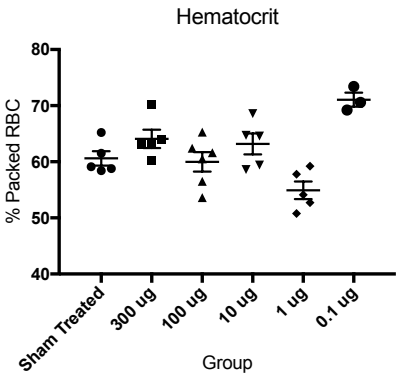


Figure 7. Peanut-sensitized mice treated intradermally with Ara h 1 peptides experienced protection against anaphylaxis when challenged with peanut. Treated mice exhibited attenuated temperature drops (a, b), clinical signs of reaction (c), and hemoconcentration (d). Data is expressed as mean ± SEM (n = 5 mice/group).

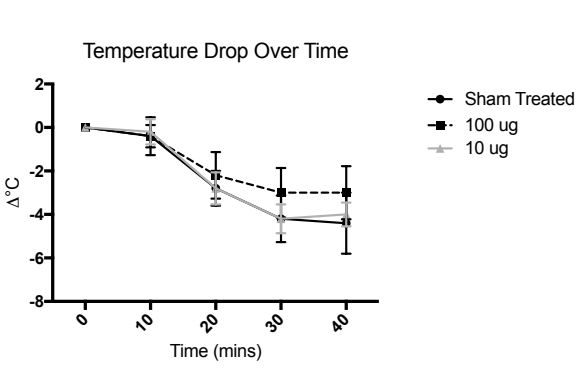




c)

Figure 8. Peanut-sensitized mice treated intradermally with Ara h 1 peptides were not protected against anaphylaxis when challenged with peanut. Control mice and peptide-treated mice exhibited similar temperature drops temperature drops (a), clinical signs of reaction (b), and hemoconcentration (c). Data is expressed as mean ± SEM (n = 5 mice/group).

During optimization, a shorter compressed treatment timeline was used. The shorter timeline was eight weeks in length, with one week between the last day of skin sensitization and the first intraperitoneal peptide injection. This protocol was also not protective (Figure 9). In contrast, the successful protocol was eleven weeks long, with four weeks between the last day of skin sensitization and the first peptide injection.



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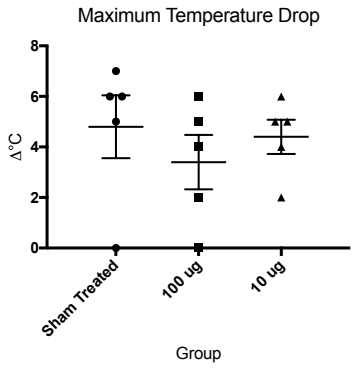

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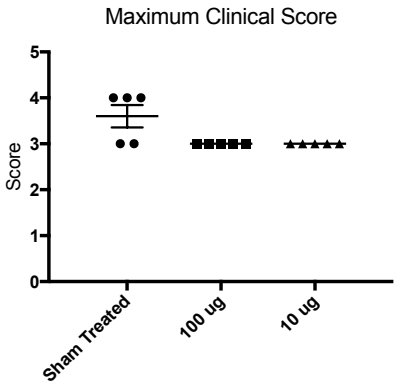

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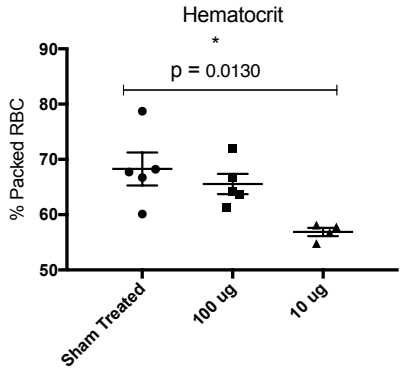

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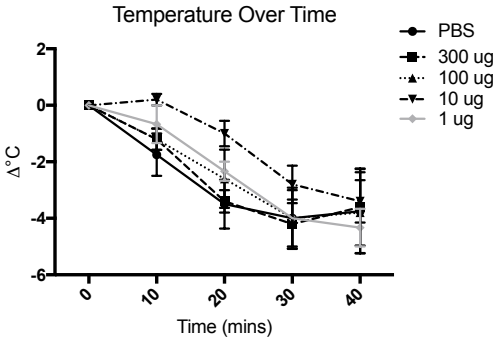
Figure 9. Peanut-sensitized mice treated intraperitoneally with Ara h 1 peptides one week after sensitization were not protected against anaphylaxis when challenged with peanut. Control mice and peptide-treated mice exhibited similar temperature drops temperature drops (a) and clinical signs of reaction (b). There was protection from hemoconcentration only (c). Data is expressed as mean ± SEM (n = 5 mice/group).

Prophylactic peptide immunotherapy did not protect against anaphylaxis in response to peanut challenge

Mice treated with Ara h 1 (201-220), (329-348), (577-596) were not protected from peanut-induced anaphylaxis at any dose (Figure 10).



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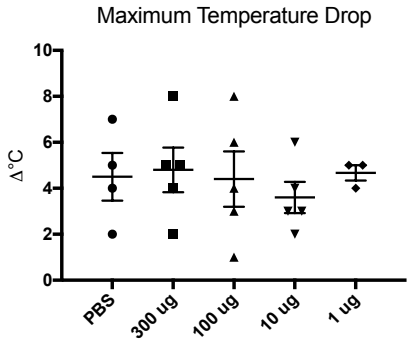

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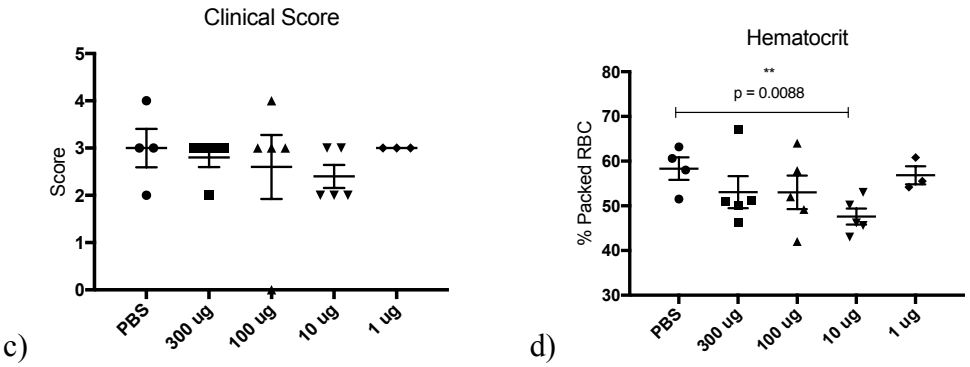
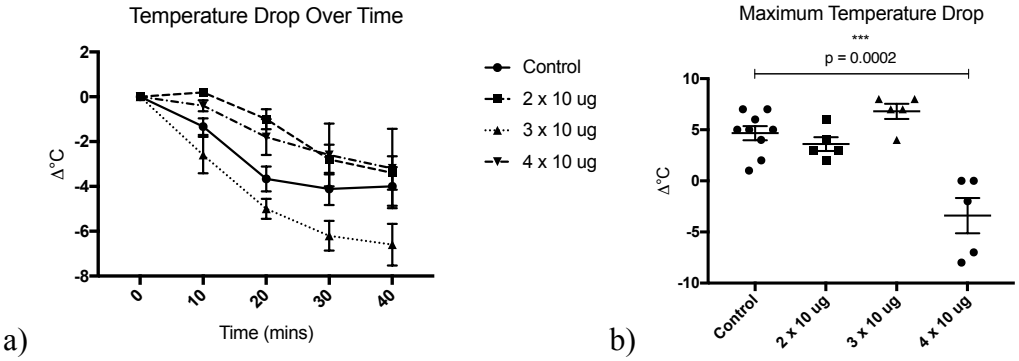


c) d)

Figure 10. Mice treated prophylactically with Ara h 1 peptides were not protected against peanut-induced anaphylaxis. Data is expressed as mean ± SEM (n = 5 mice/group).

This experiment was repeated with a different number of doses of 10 μg peptide: mice received either two, three, or four doses. Peptide doses were administered once weekly prior to sensitization. No significant protection emerged at any dose number (Figure 11). However, further study of prophylactic peptide treatment in this model may be warranted.



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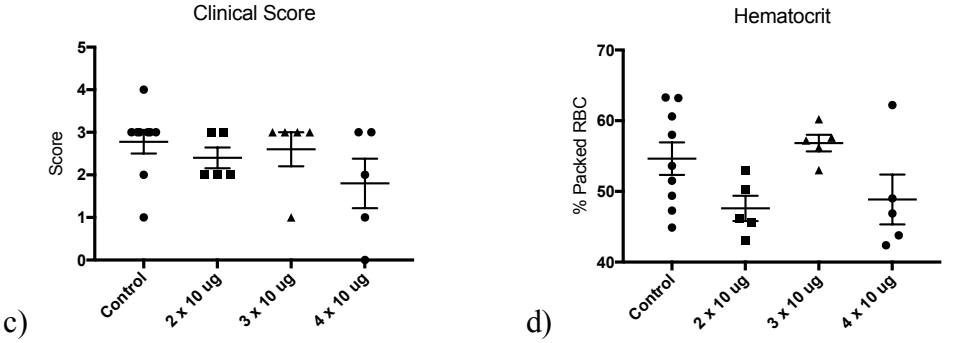

c) d)

Figure 11. Mice treated prophylactically with Ara h 1 peptides were not protected against peanut-induced anaphylaxis. Data is expressed as mean ± SEM (n = 5-10 mice/group).

Characterizing Ara h 1-specific $CD4^+$ T Cells

Mice treated with intraperitoneal injections of Ara h 1 were sacrificed before peanut challenge and 72 hours after challenge.

Peritoneal wash was collected to represent the site of peptide administration and antigen challenge, the spleen to represent systemic circulation, the mesenteric lymph nodes because they drain the peritoneum, and the bone marrow as a homing site for memory T cells. Tissues were then stained with Ara h 1 tetramers and fluorophore-labeled antibodies and analyzed using flow cytometry as described in Chapter 3.

The following surface markers were stained for: ST2, PD-1, LAG3, CD49b and the following cytokines: IL-4, IL-5, IFN-γ, MIP-1α, IL-10.

Peanut-sensitized, unchallenged mice treated with Ara h 1 peptides had reduced MIP-$1\alpha^+$ Ara h 1-specific $CD4^+$T cells and increased $CD49b^+$ Ara h 1-specific $CD4^+$T cells

Mice were sacrificed one week after peptide treatment without being challenged with whole peanut. No significant differences in IL-4- or IL-5-secreting cells were found in treated mice. There was also no difference in the Th2 surface marker ST2. No changes in the regulatory cytokine IL-10 was observed, or the regulatory marker LAG3. IFN-γ-producing $tetramer^+$ cells were also unchanged between peptide-treated and sham-treated mice. There was a significant decrease in MIP-$1\alpha^+$ $tetramer^+$ cells in the spleens of peptide-treated animals. There was also a significant increase in $CD49b^+$ $tetramer^+$ cells

in the mesenteric lymph nodes of treated mice. These lymph nodes drain the peritoneum, the site of peptide administration (Figure 12). CD49b is a marker of type 1 regulatory T cells (Tr1 cells).

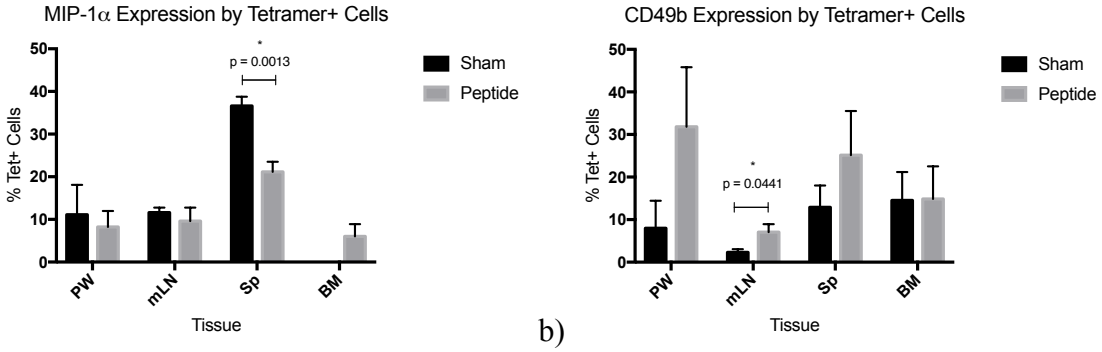

Figure 12. Unchallenged, peptide-treated mice expressed lower levels of MIP-1α$^+$ Ara h 1-specific T cells in the spleen (a), and higher levels of CD49b$^+$ Ara h 1-specific T cells in the mesenteric lymph nodes that drain the site of peptide administration (b). Data is expressed as mean ± SEM (n = 5 mice/group).

Peanut-sensitized, challenged mice treated with Ara h 1 peptides had reduced MIP-1α$^+$ Ara h 1-specific T cells and increased PD-1$^+$ and CD49b$^+$ Ara h 1-specific CD4$^+$T cells

DR4 mice treated with Ara h 1 peptides had reduced numbers of Ara h 1-specific CD4$^+$ T cells in the spleen, peritoneal lavage, and lymph nodes. There was a significant decrease in MIP-1α$^+$ tetramer$^+$ cells found in the peritoneal wash of peptide-treated mice after challenge. The peritoneal wash also displayed decreased ST2$^+$ tetramer$^+$ cells. PD-1$^+$ Ara h 1-specific T cells were significantly increased in the draining mesenteric lymph nodes and spleen. LAG3 and CD49b expression were significantly increased as well (Figure 13).

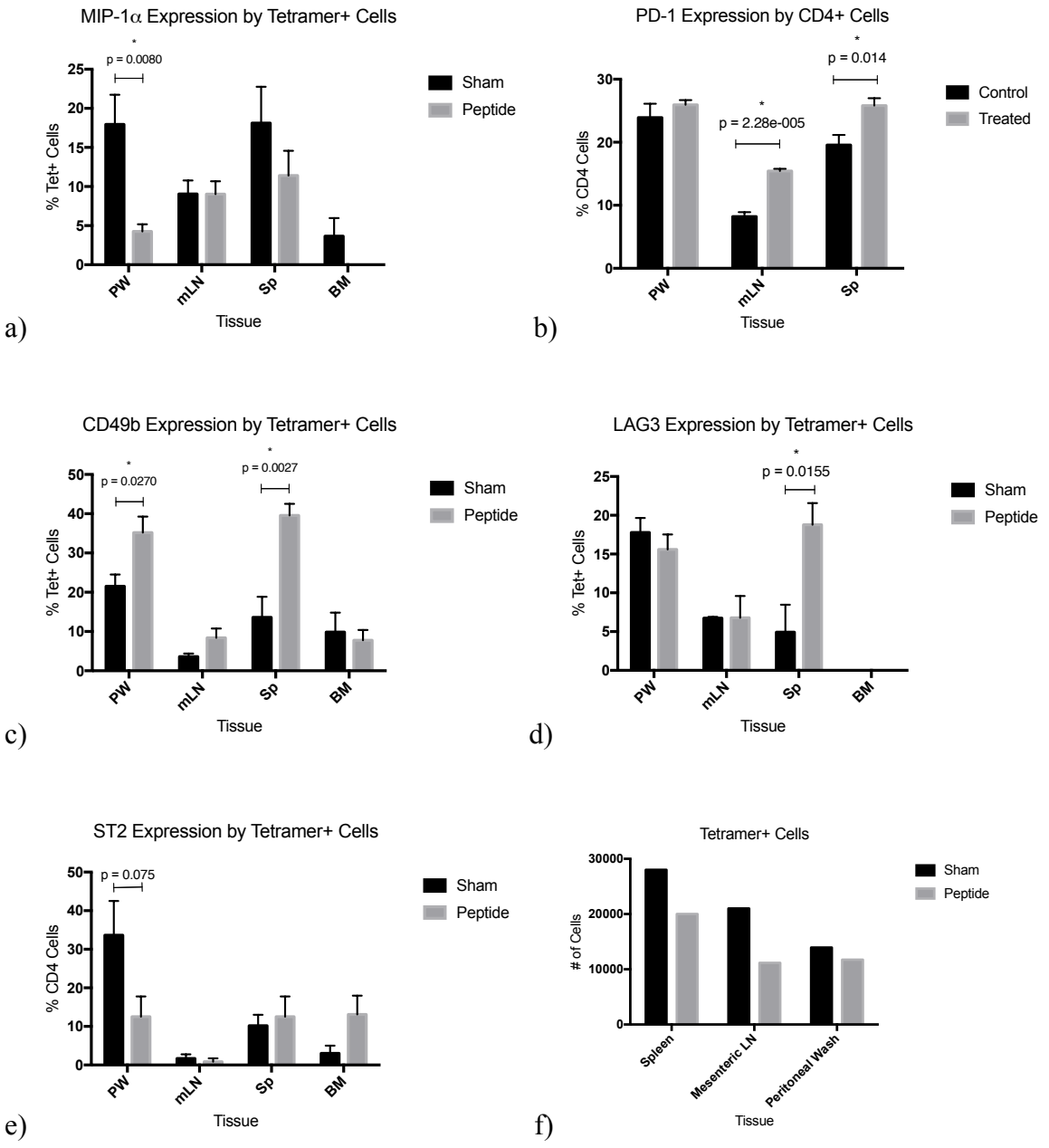

Figure 13. Challenged, peptide-treated mice expressed altered levels of the cytokine MIP-1α (a), the immunoregulatory molecule PD-1 (b), the regulatory markers CD49b and LAG3 (c, d), and the Th2 marker ST2 (e). Total tetramer$^+$ cells were reduced in the peptide group – this data is representative of pooled samples from 5 mice in each group (f). Data is expressed as mean ± SEM (n = 5 mice/group).

Programmed cell death protein 1 (PD-1) inhibits T cell activation by promoting apoptosis of antigen-specific effector T cells and inhibiting apoptosis in regulatory T cells.

Discussion

The mechanisms of action of peptide immunotherapy are still being elucidated, and many studies have examined both the cellular and humoral changes accompanying treatment. Conformational B cell epitopes are not present in short peptides[187], and the immunological effects of peptide immunotherapy have been identified as largely cellular. A down-regulation of inflammatory T cell responses to allergen has been consistently documented, as has an increase in immune regulatory signals, such as the production of IL-10.

Verhoef *et al* found that $CD4^+$ memory T cells isolated from the blood of Fel d 1-treated, cat-allergic patients exhibited reduced proliferation and IL-5 production in response to cat allergen. Additionally, $CD4^+$ T cells from treated patients produced increased IL-10 in response to cat, and these cells were able to suppress proliferation of pre-treatment $CD4^{neg}$ cells[188]. Other studies have documented a reduction in cat-stimulated proliferation, IL-4, IL-13, and IFN-γ production from $CD4^+$ cells isolated from Fel d 1-treated patients[189-191]. PLA2 peptide treatment of honeybee-allergic patients has produced similar reductions in allergen-specific $CD4^+$ T cell proliferation, IL-13, and IFN-γ production and increases in IL-10[164,165]. Decreased T cell proliferation to whole allergen has also been documented in Bet v 1 peptide treatment for birch allergy[160].

Fel d 1 peptide treatment in a mouse model of cat-induced allergic airways disease was associated with the induction of IL-10+ T cells and reduced recruitment, proliferation,

and effector function of allergen-specific Th2 cells. Numbers of $CD4^+$ T cells secreting IL-4 and IL-5 in lung tissue were significantly reduced; levels of IL-4, IL-5, and IL-13 were decreased in bronchoalveolar lavage and lung tissue; and Th2- and eosinophil-attracting chemokines CCL11, CCL17, and CCL22 were decreased. This treatment effect was reversed by anti-IL-10 receptor administration[152]. Amelioration of ovalbumin-induced allergic airways disease following Der p 1 peptide immunotherapy produced similar T cell changes, as well as increased Foxp3 expression among IL-10-producing T cells[154].

Although some studies of peptide immunotherapy have demonstrated reduced IFN-γ production associated with treatment, others have measured significant increases in $CD4^+$IFN-γ^+ T cell recruitment to the site of allergen challenge, suggesting that immune deviation (Th2 to Th1) may contribute to the efficacy of peptide immunotherapy in allergic diseases. This project did not find any differences in IFN-γ expression between treatment and control groups.

Interestingly, this project found no difference in IL-10 production in peptide-treated DR mice. Elevated LAG3 and CD49b expression was present in treatment groups, but a solid population of Ara h 1-specific $CD49b^+LAG3^+IL\text{-}10^+$ cells that would be representative of a Tr1 population could not be isolated. Anergic peanut-specific $CD49b^+LAG3^+$ cells have been isolated from non-peanut allergic healthy control subjects, while peanut-specific T cells expressing these markers produced high levels of Th2 cytokines[192]. Traditional Th2 markers, namely IL-4, IL-5, and ST2, did not differ significantly from control animals. Significantly increased expression of programmed cell death 1 (PD-1) was found on Ara h 1-specific $CD4^+$ T cells from peptide-treated mice. This molecule

inhibits T cell activation by promoting apoptosis in antigen-specific effector T cells and inhibiting apoptosis in regulatory T cells.

Experiments in C57Bl/6 mice outlined in Chapter 2 found a trend towards reduced expression of the cytokines MCP-1, MIP-1α, MIP-2, and KC at the site of antigen challenge. These cytokines all serve to attract and activate neutrophils. Initially, this data was believed to be reflective of a reduction in general inflammation. Interestingly, the data from DR4 mice pinpointed a specific reduction in MIP-1α expression by Ara h 1-specific $CD4^+$ T cells. This was in the absence of reductions in other inflammatory cytokines, including IL-4, IL-5, and IFN-γ, potentially indicating a more prominent role for the neutrophil in anaphylaxis pathophysiology.

Programmed cell death 1

PD-1 is a cell surface receptor expressed on T cells and pro-B cells. It is a member of the CD28/CTLA-4 family and has been identified as a key factor in the development and maintenance of peripheral tolerance. While CTLA-4 signals are required early in the lymph node during an initial immune response to antigen, PD-1 pathways act later during inflammation at specific tissue sites to limit T cell activity[193]. PD-1 has also been pinpointed as a negative regulator of B cell responses, as PD-1 deficient mice exhibit increased numbers of B cells and high levels of circulating IgG and IgA antibodies[194,195].

PD-1 binds two ligands: PD-L1 (B7-H1) and PD-L2 (B7-DC), both transmembrane glycoproteins. The expression of these ligands increases during inflammation and tissue insult[196]. PD-L1 is expressed on the surface of $CD4^+$ T cells, dendritic cells, macrophages, and B cells. PD-1-PD-L1 interaction delivers an inhibitory signal that

decreases IL-2 production, reduces cell proliferation, and triggers apoptosis[197]. PD-1-PD-L2 interaction inhibits T cell proliferation and cytokine production by inhibiting B7-CD28 interactions[198,199]. PD-L2 is also expressed on the surface of T cells, dendritic cells and macrophages[200].

PD-1 acts as a negative counterregulator of the immune system and is known to be important in immune regulation. Mice deficient in PD-1 exhibit a breakdown in peripheral immune tolerance and develop multiple autoimmune problems, including lupus-like peripheral arthritis, glomerulonephritis[194], and dilated cardiomyopathy[195]. Injection of anti-PD-1 or anti-PD-L1 accelerates the development of spontaneous diabetes in NOD mice[201]. PD-L2 blockade accelerates experimental autoimmune encephalomyelitis[202].

While PD-L1 plays a large role in tolerance to self and autoimmunity, PD-L2 has been shown to be important in establishing tolerance to environmental antigens. In a model of oral tolerance, PD-1 was found to be required for the development of oral tolerance to OVA[203]. PD-L2-deficient mice exhibit significantly increased airway hyperreactivity and airway inflammation in a model of OVA-induced airway disease. IL-4 production was increased in this model, and the authors also found significantly increased IL-4 in wild type mice when PD-L2 was blocked[204]. Matsumoto *et al* found that blocking PD-L2 during allergic sensitization significantly increased airway hyperreactivity and increased IL-5 and IL-13[205]. In a recent human study, PD-1 expression by $CD4^+$ T cells is inversely related to total and allergen-specific IgE in patients with allergic asthma[206].

There is some evidence to suggest that PD-1 may boost the function of T regulatory cells. PD-L1-deficient antigen-presenting cells minimally convert $CD4^+$ cells to induced Tregs, and PD-L1-coated beads induce Tregs *in vitro*. PD-L1-Ig can enhance Foxp3 expression and the suppressive function of established induced Tregs[207].

In summary, PD-1 can inhibit inflammatory responses by both downregulating inflammatory T cell proliferation activity and inducing and maintaining T regulatory cells. To our knowledge, this study is the first to identify an association of PD-1 expression and peptide immunotherapy or food allergy, though the specific role of PD-1 in this model of Ara h 1 peptide immunotherapy remains to be elucidated.

The neutrophil

This project identified significant increase in peanut-specific MIP-1α tetramer+ cells in sensitized mice after peanut challenge. This was significantly reduced with peptide treatment from the major peanut allergen Ara h 1. MIP-1α molecule is known to act as a chemoattractant for inflammatory cells implicated in the pathogenesis of allergic disease.

The roles of mast cells, basophils, and macrophages in allergy and anaphylaxis have been widely studied, as discussed in Chapter 1, while the contribution of the neutrophil remain less well understood. Neutrophils are innate immune cells that are abundant in peripheral blood and can be rapidly activated to trigger both local and systemic inflammation. When activated, these cells release elastase, collagenase, gelatinase, and lactoferrin; molecules that serve to degrade the extracellular matrix, upregulate adhesion molecules, and delay inflammatory cell apoptosis[208-210]. Activated neutrophils also release a host of pro-inflammatory cytokines, including IL-1, IL-6, IL-8, IL-12, and TNF-α that serve to

activate other inflammatory cells and potentiate a cascade of inflammation[211]. Recently published studies have shed some light on the potential ability of the neutrophil to initiate allergic inflammation and enhance the effects of other inflammatory cells to amplify systemic anaphylactic reactions.

Jonsson *et al*[212] found that neutrophil depletion in mice could inhibit both passive and bovine serum antigen-mediated active anaphylaxis. Replenishing murine or human neutrophils restored the anaphylactic response, and neutrophils were immediately and systemically activated during anaphylaxis. A study in a mouse model of casein-induced anaphylaxis found that reaction severity correlated with peripheral neutropenia and the extravasation of neutrophils into tissue[213]. Most recently, Francis *et al*[214] measured the neutrophil product myeloperixodase (MPO) and adhesion molecule CD62L in the serum of patients presenting to the emergency department with anaphylaxis. MPO levels in patients with moderate anaphylaxis were 2.9-fold higher than healthy controls, and 5.0-fold higher in patients with severe anaphylaxis. Soluble CD62L was significantly reduced in patients with anaphylaxis. These differences were not associated with serum concentrations of the mast cell markers of histamine or mast cell tryptase, and were present upon immediate presentation and sustained after 300 minutes of symptom onset.

This project found that peptide immunotherapy reduced the expression of pro-neutrophilic cytokines, but it did not provide direct evidence of neutrophil activation in anaphylaxis, or an immunotherapy-induced tempering of the neutrophil response. However, this data, in combination with the data of others, should prompt further study of the neutrophil in anaphylaxis and immunotherapy. The link between local inflammatory cell activation and the rapid development of systemic reactions remains

unknown. Data has shown that neutrophils are rapidly and systemically activated during early anaphylaxis. These cells may serve to potentiate systemic reactions in response to early IgE- and IgG1-mediated activation of mast cells, basophils, and macrophages.

Conclusion

Ara h 1 peptide immunotherapy protected against severe peanut-induced anaphylaxis in this model of peanut-induced anaphylaxis in mice transgenic for the human leukocyte antigen DRB1*0401. Protection was associated with decreased expression of the inflammatory cytokine MIP-1α by Ara h 1-specific $CD4^+$ T cells in the spleen prior to challenge, and at the site of antigen exposure after peanut challenge. Expression of ST2 by Ara h 1-specific $CD4^+$ T cells at the site of antigen challenge was reduced, and there was a concurrent increase in expression of PD-1.

These findings indicate that peptide immunotherapy may modulate allergen-specific T cell responses towards regulatory and/or exhausted phenotypes. The cytokine MIP-1α may play a role in this model of anaphylaxis.

Chapter 6: General Discussion

Project Accomplishments

This project identified a novel peptide from the major peanut allergen Ara h 1 that is recognized by the MHC II I-A^b molecule expressed by C57Bl/6 mice. Ara h 1 (505-524) was used to develop an immunotherapy protocol that protected peanut-sensitized mice from severe anaphylaxis when challenged with whole peanut. These studies demonstrated that peptide immunotherapy could induce clinical protection in a mouse model of peanut-induced anaphylaxis. This model can now be used to perform further studies of cellular and humoral changes in the context of Ara h 1-specific immunotherapy.

The model of peanut-induced anaphylaxis and peptide immunotherapy developed in wild type C57Bl/6 mice was adapted to work in mice transgenic for the human leukocyte antigen DRB1*0401. The use of these transgenic mice allowed for the study of major peanut peptides recognized by human peanut-allergic patients and the use of tetramer reagents to enumerate and characterize Ara h 1-specific $CD4^+$ T cells using flow cytometry. The feasibility of antigen-specific T cell identification using tetramer reagents was demonstrated.

The 20th century American oncologist Dr. Howard E. Skipper once stated: a model is a lie that helps you see the truth[215]. This project established a new experimental system that subsequent studies can use to further examine antigen-specific T cell changes induced by peptide treatment in the context of peanut-induced anaphylaxis.

Ara h 1 peptide immunotherapy was associated with an increased frequency of peanut-specific $CD4^+$ T cells expressing the programmed cell death surface receptor that inhibits inflammatory responses by downregulating inflammatory T cell proliferation activity and inducing and maintaining T regulatory cells. The frequency of peanut-specific $CD4^+$ T cells expressing the pro-inflammatory cytokine macrophage inflammatory protein 1-α decreased. These results may shed light on previously unidentified mechanistic components of anaphylaxis and immunotherapy.

Peanut-Specific Peptide Immunotherapy: The Wider Context

Despite its increasing prevalence and clinical severity, there remains a lack of widespread, practical, disease-modifying therapies available to treat peanut allergy. Presently, the only management options available for peanut-allergic patients are allergen avoidance and rescue medication in case of accidental ingestion. Dean *et al* argue that, as a consequence of this, individuals with severe peanut allergy must limit their spatial and social contact in order to ensure their own safety and wellbeing. This may take the form of modification of their local environment, such as removing peanuts from their homes, and limiting contact with outside environments where their risk of encountering peanut is heightened by avoiding certain restaurants or social events. As a result, peanut-allergic individuals experience heightened levels of risk in their daily lives, have feelings of social isolation, and are anxious in public spaces and social settings. These fears and worries extend to the family members of allergic patients, especially parents of young children with life-threatening peanut allergy [216].

Peanut allergy has been described as a growing public health concern since the 1990s. Cultural shifts in food allergy awareness, and concern regarding peanut allergy in particular, have prompted the introduction of peanut-free food products, bans on peanut in public schools, the removal of peanuts as a routine snack food on airplanes, and strict food-labeling requirements that flag any products that contain peanut or may have come into contact with it during preparation or packaging[217].

Concern surrounding peanut allergy has contributed to the formation of national advocacy organizations, such as Food Allergy Canada, the introduction of epinephrine auto-injectors in public places such as recreation centres and shopping malls in the city of Hamilton[218], and the enacting of legislation to ensure that children with fatal food allergies have increased protection while at school. Sabrina's Law was signed into effect by the Ontario government in 2006 and mandates that schools must have a strategy to reduce the risk of students being exposed to anaphylactic causative agents, provide training to educators on how to respond to anaphylaxis, and have an action plan for each allergic student in case of emergency[216].

The medical community has responded to the mounting burden of peanut allergy with attempts to modify the allergic response in sensitized individuals – the ultimate goal being the reduction of allergy symptoms and eventual long-term tolerance to peanut. The hope, for both patients and clinicians, is to usher in a new standard of care whereby targeted therapeutics effectively redirect, or eliminate, the atopic immune response to peanut. These studies have generated a flurry of public interest, with prominent news outlets frequently carrying pieces outlining the latest "breakthrough" or "cure" for peanut

allergy. Stories have been featured by ABC, the Los Angeles Times, NBC, USA Today, CBC, and BBC, among others.

As discussed in Chapter 1, injection, epicutaneous, and oral peanut-specific immunotherapy have been investigated. At this time, the most widely trialed peanut allergy treatment is oral immunotherapy. This involves the ingestion of increasing doses of whole peanut in regular intervals over several months. Protocols involve an initial build-up phase followed by a longer maintenance phase. The first open-label trial of peanut oral immunotherapy was published in 2009[219,220]. Twenty-nine peanut-allergic subjects were able to tolerate larger quantities of oral peanut after six months of whole peanut oral immunotherapy. Allergic side-effects were experienced by 93% of study participants during therapy, and epinephrine was required in 11% of cases. Since, then over a dozen peanut oral immunotherapy studies have been published, including local trials like STOP II[221] and multi-centre trails like ARC00[129].

The safety, tolerability, and long-term efficacy of whole allergen oral peanut immunotherapy remain ongoing concerns. Many commentary pieces have appeared in the literature, urging the medical community to approach peanut oral immunotherapy with caution and often featuring witty titles such as "More work needed to crack the nut"[222].

The risk of IgE-mediated side effects, including anaphylaxis, during oral immunotherapy treatment is significant. Almost all patients undergoing oral immunotherapy experience allergic side-effects: most occur in the build-up phase, but persistence into the maintenance phase is also seen. These are most commonly oral pruritus, hives, and gastrointestinal upset. GI symptoms, including abdominal pain, nausea, and vomiting are

the most common reason for participant drop-out and have been known to persist in the form of eosinophilic esophagitis[223]. Quality of life assessments conducted during oral immunotherapy have not demonstrated any measurable improvements[135].

It is unclear whether oral immunotherapy is able to merely induce desensitization, or if it can achieve true sustained immune unresponsiveness. Desensitization is a lack of clinical reactivity to antigen and requires regular, ongoing antigen exposure to be maintained. Sustained unresponsiveness is a long-term and potential permanent loss of reactivity to an antigen that does not require antigen exposure to be maintained[224]. This gives rise to a number of important practical questions. How long must patients continue with regular (often daily) oral peanut regimens? What happens when a patient stops regular peanut exposure? Is it safe to stop? Will the allergy return, and could it return with greater severity?

With peptide immunotherapy, short segments of allergen are presented to the immune system in a quiescent environment. These short peptides are unable to cross-link allergen-specific antibodies bound to allergic effector cells, and peptide immunotherapy avoids the potentially lethal side effects of traditional whole allergen specific immunotherapy[151]. Compared to whole allergen therapy, peptide immunotherapy has a greatly improved safety profile, requires fewer doses to achieve tolerance, and is amenable to standardization and regulation[152]. Studies of peptide immunotherapy in other allergic conditions demonstrate reduced pro-inflammatory cytokine production, induction of tolerogenic regulatory T cells, and improvement of clinical symptoms[152-154].

This project has demonstrated that peptide immunotherapy may be a viable option for treating peanut allergy. Ara h 1 peptide immunotherapy was safe, clinically effective, and had immunomodulatory effects in a mouse model of peanut-induced anaphylaxis. Further study of this treatment is warranted.

Future Directions

Future experiments should focus on the mechanisms whereby Ara h 1 peptide immunotherapy protects against anaphylaxis in response to whole peanut challenge. A key question of this project has been: how does peptide immunotherapy protect against an anaphylactic response? In general, the understanding of the pathogenesis of anaphylaxis remains incomplete, making the answer to this question that much more challenging to tease out.

In this project, short peptides from one peanut allergen, Ara h 1, were able to significantly temper the allergic response to whole peanut allergen challenge. This phenomenon has also been observed with Fel d 1 and Der p 1 peptide immunotherapy. Campbell *et al*[152] isolated peripheral blood mononuclear cells (PBMCs) from twenty-four cat-allergic patients treated with a mixture of twelve Fel d 1 peptides. PBMCs were incubated with the twelve treatment peptides plus four others from within Fel d 1. Post-treatment PBMCs displayed significantly reduced proliferative, IL-4, and IL-13 responses to all peptides, not just the twelve treatment peptides. In the same study, both Fel d 1-specific and non-specific $CD4^+$ cells from cat-sensitized mice exhibited reduced proliferative responses. Moldaver *et al*[154] demonstrated that treating mice with Der p 1 peptides in a model of dual-allergen sensitization could ameliorate OVA-induced allergic

airway disease. OVA-induced airway hyperresponsiveness, tissue eosinophilia, and goblet cell hyperplasia was suppressed, and these clinical changes were associated with a reduction in the recruitment, proliferation, and effector function of Th2 cells.

These findings are mediated by a process called intramolecular epitope suppression, whereby nonresponsiveness to one epitope of a molecule confers nonresponsiveness to other epitopes within the same molecule[225]. A similar process called bystander tolerance can occur with adjacent molecules in the immediate microenvironment[226]. Data from these peptides studies indicate that immune tolerance to an entire allergen may be achieved through treatment with just a few key peptides, with important implications for immunotherapy development. Therapeutics need not contain all T cell epitopes of an allergen to be clinically effective, and simpler mixes will be more efficient to formulate and manufacture. This study used tetramers loaded with the treatment peptides Ara h 1 (201-220), (324-348), and (577-596). Future experiments could investigate the effects of peptide immunotherapy on T cells specific for other peanut antigens by using tetramers loaded with non-treatment peptides.

Further *in vivo* studies to characterize the clinical impact of peptide-induced T cell changes are warranted. These would involve transferring T cells from peptide-treated mice into untreated, sensitized mice before peanut challenge. This study could include two arms, with one group receiving polyclonal T cells and the other receiving peptide-specific T cells.

Further *in vitro* experiments could involve culturing $CD4^+$ T cells isolated from peptide-treated mice with cells from untreated, sensitized mice and assessing suppression of proliferation and cytokine secretion in response to peanut.

To further expand on the antigen-specific T cell characteristics identified by this project, it would be interesting to trial the model in PD-1-deficient mice, or block PD-L1 and PD-L2, respectively. It would also be helpful to study the mouse model in a strain of MIP-1α-deficient mice, or mice in which MIP-1α was blocked. Ara h 1-specific T cells from peanut-allergic human subjects have been shown to express the memory T cell marker CD45RO and the Th2-associated T cell trafficking marker CCR4 [167]. Wambre *et al* found that allergen-specific Th2 cells isolated from subjects undergoing pollen immunotherapy were $CD27^-$ and expressed low levels of Bcl-2, increasing their susceptibility to activation-induced cell death[227,228] – interesting results in light of the PD-1 data generated by this project. Future studies of Ara h 1 tetramer-positive cells could also incorporate these makers in their staining panels to further tease out the differences and similarities between peanut-specific T cells in peanut-allergic human subjects and experimentally sensitized mice. Additional tissues that harbor secondary lymphoid tissue, such as the gut, could also be explored.

This study did not find any significant changes in peanut-specific IgE and IgG1 antibodies after peptide treatment. Other immunotherapy studies have demonstrated that antibody changes are often detected transiently, and may not be detectable at all time-points. Therefore, it would be useful to look at peanut-specific antibodies in this model at different time points during peptide treatment, before peanut challenge, and after peanut challenge.

B cells contribute more than just immunoglobulins to the immune response. Regulatory B cells (BREGS) are immunoregulatory cells that protect against chronic inflammation by producing IL-10, TGF-beta, and IL-35. They can suppress effector Th2 responses, inhibit dendritic cell maturation, and promote production of IgG4. These cells are increasingly being recognized as a key player in inflammation and tolerance in the allergic context. BREG numbers increase during venom immunotherapy[229], and future studies in this model of peanut-induced anaphylaxis could use flow cytometry to enumerate and characterize these cells after peanut peptide treatment.

Based on the initial findings of this project, it would be worthwhile attempting to optimize a protocol for prophylactic peptide immunotherapy, as mentioned in Chapter 5. As part of this, T cells from peptide-treated mice could be transferred into unsensitized mice prior to peanut sensitization and before challenge.

One key feature of a clinically useful immunotherapeutic for peanut allergy is persistence of treatment efficacy. Future studies in this model of peptide immunotherapy should address the longevity of clinical protection. Mice should be sensitized, treated with peptides, and then challenged with whole peanut at increasing intervals. The ability of peptide therapy to protect against multiple, sequential whole peanut challenges should also be assessed.

Translation to a Human Therapeutic

As discussed above, peptide immunotherapy for allergic disease has already been successfully trialed in humans. It has been found to be safer and more effective that whole allergen immunotherapy, conferring a longer clinical benefit with fewer doses.

Additionally, it is amenable to standardization and regulation, while whole allergen immunotherapy preparations may differ widely between protocols.

Some allergens have only one major component, such as Fel d 1 in cat dander. Peanut (*Arachis hypogaea*) is more challenging to tackle because it has seventeen known major allergens. There exist differences in geographical sensitization patterns, though these are by no means strict demarcations. North American patients are more likely to be sensitized to Ara h 1, 2, and 3, and sensitization to Ara h 6, 8, and 9 is more common in Europe[230,231]. There is also no MHC class II associated with peanut allergy[232], meaning that a human therapeutic would have to take into account peptide promiscuity as well as regional sensitization patterns.

As with other allergens, selected peanut peptides would first be evaluated for safety using *in vitro* histamine-release and basophil-activation studies. Low dose intradermal injections in non-allergic and allergic volunteers would be used to test for *in vivo* safety and tolerability. Finally, careful study in peanut-allergic volunteers would be used to develop the ideal therapeutic protocol that would first reduce reactions to skin-prick challenge and ultimately oral food challenge.

Conclusion

Ara h 1 peptide immunotherapy protected against severe peanut-induced anaphylaxis in a mouse model. Mice were sensitized epicutaneously to whole peanut, treated with Ara h 1 peptides, and challenged with whole peanut. Peptide-treated mice experienced significantly reduced drops in core body temperature, clinical signs of allergic reaction, and hemoconcentration.

Clinical protection was associated with decreased expression of the pro-inflammatory cytokine macrophage 1-α and increased expression of the surface marker programmed cell death 1.

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RESEARCH

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Prediction of clinical peanut allergy status among children in Hamilton, Ontario using chart review data collected during 2012–2015

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Abstract

Background: Peanut sensitization does not necessarily indicate clinical peanut allergy, and uncertainty as to whether or not there is true peanut allergy can lead to increased anxiety and decreased quality of life for patients and their families. The gold standard for diagnosing clinical peanut allergy is the oral food challenge, but this method is time-consuming and can cause severe allergic reactions. It would therefore be beneficial to develop a tool for predicting clinical peanut allergy in peanut-sensitized individuals whose peanut allergy status is unknown so as to better determine who requires an oral food challenge for diagnosis.

Methods: Two separate studies were conducted. In Study 1, we recruited 100 participants from the allergy clinic at McMaster University and community allergy outpatient clinics in the greater Hamilton area. We examined 18 different variables from participants and used univariate and multivariable logistic regression analysis to determine how well these variables, singly and in combination, were able to predict clinical peanut allergy status. In Study 2, we conducted a retrospective chart review of a second cohort of 194 participants to investigate the reproducibility of our findings. This was a matched case–control study where 97 peanut-allergic participants were gender- and age-matched to 97 non-allergic control participants.

Results: Peanut skin prick test wheal size was the best predictor of clinical peanut allergy in both study cohorts. For every 1 mm increase in wheal size, the odds ratio of an individual having clinical peanut allergy was 2.36 in our first cohort and 4.85 in our second cohort. No other variable approached the predictive power of wheal size.

Conclusions: Peanut skin prick test wheal size is a robust predictor of clinical peanut reactivity. The findings of this study may be useful in guiding clinician decision-making regarding peanut allergy diagnostics.

Keywords: Peanut allergy, Skin prick test, Wheal size

Background

Peanut allergy is a serious public health concern, especially in westernized countries. Its prevalence has doubled in the past 10 years and currently stands at approximately 2% [1].

Peanut allergy is diagnosed by combining clinical history with diagnostic methods that may include skin-prick tests (SPT) and serum IgE measurements to peanut [2]. Many parents have avoided feeding their children peanut in an attempt to prevent peanut allergy, yet many children have developed sensitization to peanut, as demonstrated by a positive peanut SPT or peanut-specific IgE, and continue to avoid peanut. However, peanut sensitization does not necessarily mean clinical peanut allergy, and uncertainty as to whether or not there is true peanut

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allergy can lead to increased anxiety and decreased quality of life for patients and their families. The gold standard for diagnosing clinical peanut allergy is the oral food challenge, but this method is time consuming and requires proper set-up and personnel to manage potential severe allergic reactions [3]. Therefore, it would be beneficial to develop a tool for predicting clinical peanut allergy in peanut-sensitized individuals whose peanut allergy status is unknown so as to better determine who requires an oral food challenge for diagnosis.

The goal of this study was to use clinical and laboratory data from patients of known peanut allergy status to develop a statistical model to predict clinical peanut allergy in peanut-sensitized individuals. To determine its predictive merit, the model was applied to a group of patients with positive skin prick tests, but unknown clinical reactivity because they had never knowingly ingested peanut. These patients of unknown clinical status then underwent an oral peanut challenge to determine their true peanut allergy status and this outcome was compared to their model-predicted peanut allergy status.

Methods

Study 1: patient recruitment and data collection

100 participants were recruited from the allergy clinic at McMaster University and community allergy outpatient clinics in the greater Hamilton area.

All participants were at least 6 years of age and of either sex. Exclusion criteria for the study were uncontrolled or severe asthma, receipt of allergy injections in the past, and use of injectable epinephrine 1 month prior to the start of the study. Individuals taking daily antihistamines, leukotriene receptor antagonists, or nasal, inhaled, or oral corticosteroids were also excluded. These interventions may have interfered with our study measurements, particularly cytokine secretion.

We collected the following data on each participant: age, sex, peanut SPT wheal size, clinical peanut allergy status, peanut ImmunoCAP, total IgE, supernatants from peripheral blood mononuclear cells (PBMC) under unstimulated and peanut-stimulated conditions, immediate family history of peanut allergy, asthma, rhinitis, and eczema status.

Participants were divided into 4 groups according to their peanut allergy status based on history and peanut skin prick test.

Group 1 consisted of peanut allergic individuals. These individuals had a prior history of an allergic reaction to peanut on ingestion and a positive SPT to peanut. Allergic symptoms included, but were not limited to, urticaria, angioedema, dyspnea, cough, wheeze, nausea, vomiting, lightheadedness, rash, and/or shock.

Group 2 consisted of individuals who had a positive skin prick test to peanut, but could tolerate peanut ingestion without difficulty. Thus, these individuals were not allergic to peanut and their skin test results were designated as "false positives".

Group 3 consisted of individuals who had a positive skin prick test, but no known history of peanut ingestion. Many of these individuals may have avoided peanut for specific reasons, such as a family history of peanut allergy. It was therefore uncertain whether they would react to peanut on ingestion and they were considered to be at risk of clinical reactivity based on the presence of sensitization.

Group 4 consisted of individuals who had a negative skin prick test to peanut and had previously ingested peanut without problems. Consequently, they served as a negative control group. This group did not have any other food or environmental allergies.

This study was approved by the Research Ethics Board at McMaster University and all participants, or their guardians, provided written informed consent.

Skin prick test measurements

The forearm was prepped with alcohol and peanut extract (ALK-Pharmaceuticals, Mississauga, ON, Canada) was applied to the skin of the dorsal forearm. A sterile metal lancet (HollisterStier, Spokane, WA, USA) was used to puncture the skin below the allergen droplet. Skin prick test wheal size was measured after 15 min. Tape was placed on the dorsal forearm and an outline of the wheal was traced. The widest diameter of the wheal was measured by two different study nurses.

Peanut and total IgE plasma measurements

Total IgE was measured using the Immage 800 (Beckman Coulter, Mississauga, ON, Canada) and peanut-specific IgE antibodies were measured using the Phadia 250 (Thermo Scientific, Waltham, MA, USA).

Cytokine measurements

Mononuclear cells were isolated from 30 to 40 ml of blood by density gradient centrifugation after red blood cells were lysed with AKC lysis buffer. Cells were re-suspended in RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, 55 μM 2-mercaptoethanol (Thermo Scientific), 1 mM sodium pyruvate, 10 mM HEPES and 0.1 mM MEM NEAA (Thermo Scientific). Viable cells were counted via Trypan Blue (Thermo Scientific) exclusion and re-suspended at 8×10^6 cells/mL. 125,000 live cells per well were cultured in triplicates in medium alone or with 50 μg/mL/well of crude peanut extract in flat-bottom 96-well plates (BD

Biosciences, Mississauga, ON, Canada). After 5 days of culture at 37 °C and 5% CO_2, the triplicates were pooled, spun down and cell-free supernatants harvested and stored at −80 °C until further analysis. Cytokines in cell-free supernatants were quantified using Luminex (Millipore Canada Ltd, Etobicoke, ON, Canada) following the manufacture's instructions.

Statistical analysis

Each predictor was entered into a univariate logistic regression analysis to determine if it was associated with the primary outcome—clinical peanut allergy status. We then generated cumulative models composed of multiple predictors using multivariable logistic regression. All univariate and multivariable analyses included the 69 study participants from Groups 1, 2, and 4.

For all models, parameter estimates were obtained for each predictor and expressed as odds ratios with corresponding 95% confidence intervals and associated *p* values. *p* values are reported to 4 decimal places.

Hierarchical models were compared to determine if the model with the greater number of predictors was statistically significantly better at predicting the primary outcome than the model with fewer predictors. This was done by comparing the models' −2 Log Likelihood statistics. For each model, the area under the Receiver Operating Characteristics (ROC) curve was reported as a measure of discriminability. The best model was used to predict the peanut allergy status of participants in Group 3 and to determine the predicted probability (Pr) of each participant having clinical peanut allergy. Using Pr, we classified each individual as having a peanut allergy or not based on a specific cutpoint. We chose this cutpoint to eliminate false negatives and maximize true positives in the data set.

All analyses were conducted in SAS version 9.4.

Peanut challenges

All individuals in Group 3 underwent a peanut challenge to determine peanut allergy status. The food challenge took place in the Allergy Clinic at McMaster University Medical Centre under the supervision of a study physician. A research/Critical Care nurse and study physician were present at all times with the appropriate set-up to deal with any and all allergic reactions.

All subjects had baseline vital signs taken, body weight measured, and an intravenous inserted prior to oral food challenge.

Each subject was given either 1 mg of peanut or placebo orally mixed with grape jelly or applesauce. Peanut flakes were the source of peanut and cracker crumbs were used as the placebo. The dose of peanut was increased to 5 mg and increased every 15–30 min to 10, 25, 50, 100, 250, 500 mg, 1, and 2.5 g until the maximum dose of 2.5 g was reached or objective findings of allergic reaction were observed. 2.5 g is the equivalent of 5 peanuts.

Subjects were carefully observed for the following signs of allergic reaction: rash (erythema, morbilliform rash, urticaria, angioedema), ocular (conjunctival swelling, scleral edema, tearing), nasal (congestion, rhinorrhea, sneezing), respiratory (wheezing, cough, drop of PEF or FEV1 by >20%), gastrointestinal (vomiting, diarrhea, abdominal pain), systemic (blood pressure drop by >20%).

Vital signs (oxygen saturation, blood pressure, heart rate, respiratory rate) were assessed before each dose, with every new symptom reported, and when objective findings were observed.

If a subject developed any two mild symptoms (generalized itchiness or flushing, runny nose, watery eyes, or sneezing) or any one severe symptom (persistent cough, significant abdominal pain, nausea, vomiting, diarrhea, swelling of the lips or face, difficulty breathing, wheezing, or fainting) the challenge was immediately stopped and the subject was considered to be peanut allergic [4].

Subjects who experienced allergic reactions were treated with appropriate medications, namely intramuscular epinephrine, intravenous antihistamines, and corticosteroids (1 mg/kg for 3 days). The subjects were observed for 4–8 h after an allergic reaction to ensure that it had been adequately treated and resolved [5].

If 2.5 g of peanut was tolerated, 10 g was administered in an open challenge and subjects were monitored for signs of allergic reaction. In the event of a reaction, each subject received appropriate medication and monitoring.

The results of the oral peanut challenges were then compared to patients' predicted peanut allergy status.

Study 2: patient recruitment and data collection

We conducted a retrospective chart review of a separate cohort of 194 subjects: 97 with confirmed clinical peanut allergy, and 97 sex- and age-matched controls without clinical peanut allergy. Peanut allergy was defined as: the participant had consumed peanuts in the past and displayed peanut allergy-compatible symptoms, as described earlier, and had undergone confirmatory testing. For each participant, we collected date of birth, sex, peanut skin prick test wheal size, allergic rhinitis, asthma, and eczema status. We also recorded food allergy status for milk, egg, wheat, individual nut, and nut mix.

Statistical analysis

The predictive value of each variable was analyzed using exact conditional logistic regression. All analyses were conducted in SAS version 9.4.

Results

Study 1: participant characteristics

A total of 100 subjects participated in this study and a summary of their characteristics is displayed in Tables 1, 2. Half of the participants were female, 14% had an immediate family member (parent or sibling) with a peanut allergy, and 59% had a comorbid allergic condition (asthma, rhinitis, or eczema). The median age in years was 15.5, but there was an imbalance in age between Groups 1, 2, and 4 versus Group 3. In Group 3 the median age was 9, whereas the median age of Groups 1, 2, and 4 was 21.

Predictive values of individual variables

For the 69 participants in Groups 1, 2, and 4, each of 18 predictor variables was entered as a single predictor of the primary outcome—clinical peanut allergy status. Nine variables were selected for entry into the multivariable model based on a univariate p value <0.1 (Table 3). All other variables evaluated had a p value >0.1. Using area under the ROC curve (AUC) as a measure of discriminability between peanut allergic and non-peanut allergic participants, peanut SPT wheal size was the best univariate predictor, with AUC = 0.927. In other words, peanut SPT wheal size was the variable most able to accurately predict true clinical peanut allergy. A larger wheal size was associated with a stronger risk of clinical peanut allergy: the odds ratio for each 1 mm increase in wheal size was 2.36.

Table 1 Study groups

Variable	Group 1 (n = 30)	Group 2 (n = 17)	Group 3 (n = 31)	Group 4 (n = 22)
Clinical peanut allergy status	Y	N	U	N
Positive skin prick test to peanut	Y	Y	Y	N

Y = confirmed clinical peanut allergy; N = confirmed not allergic to peanut; U = unknown

Table 3 Univariate logistic regression analysis of peanut allergy predictors

Variable	OR [95% CI]	p value	AUC
Wheal size	2.362 [1.533, 3.639]	<0.0001	0.927
Peanut IgE	1.083 [1.021, 1.149]	0.0080	0.812
Total IgE	1.001 [0.999, 1.002]	0.0749	0.822
Gender (M)	2.286 [0.859, 6.082]	0.0978	0.600
Asthma (Y)	10.199 [3.105, 33.511]	0.0001	0.736
IL-13 (P)	1.002 [1.000, 1.003]	0.0138	0.729
IL-5 (M)	1.000 [0.999, 1.002]	0.4921	0.539
IL-5 (P)	1.002 [1.001, 1.004]	0.0040	0.696
IL-9 (P)	1.0012 [0.9999, 1.003]	0.0621	0.650

n = 69

Generation of predictive models

The results of the univariate analyses revealed that peanut skin prick test wheal size was the strongest single predictor of peanut allergy status among all variables assessed. The area under the ROC curve for wheal size was significantly better than any other single predictor. Next, we entered wheal size into the model and added each of the remaining variables one at a time in a stepwise process. We found that rhinitis significantly improved the model by increasing the area under the ROC curve and improving the model's ability to predict true peanut allergy. Following the same strategy, we found that asthma was the only remaining predictor that was statistically significant when added to the model. We called this combination of peanut wheal size, rhinitis, and asthma "Model 1". No other predictors were significant ($p < 0.1$) when added to Model 1. In Model 1, wheal size and asthma were positively related to peanut allergy status, but rhinitis was

Table 2 Clinical characteristics

Variable	Groups 1 (n = 30)	Group 2 (n = 17)	Group 3 (n = 31)	Group 4 (n = 22)
Age (y)[a]	14.17 (6.20)	25.82 (16.00)	10.16 (3.12)	36.23 (10.82)
Wheal size (mm)[a]	7.50 (3.35)	3.97 (1.15)	5.48 (1.91	0 (0)
Sex (F)	14 (47)	11 (65)	10 (32)	15 (68)
Father with allergy	0 (0)	0 (0)	1 (3.2)	0 (0)
Mother with allergy	2 (2.9)	0 (0)	1 (3.2)	0 (0)
Sibling with allergy	2 (7)	1 (6)	8 (26)	0 (0)
Asthma	18 (60)	5 (29)	17 (55)	0 (0)
Rhinitis	11 (37)	12 (71)	8 (26)	0 (0)
Eczema	9 (30)	0 (0)	13 (42)	0 (0)

All other entries are n (%)

[a] Entries are mean (standard deviation)

curiously protective. The AUC for model 1 was 0.962, demonstrating an improvement in prediction over any single variable analyzed above. Table 4 shows the results of this model. The ROC curve for Model 1 is shown in Fig. 1.

Using model 1 to predict clinical peanut allergy status in group 3

Applying Model 1 to Group 3 generated a probability of peanut allergy (Pr) for each of the 31 participants with a positive skin prick test but unknown clinical peanut allergy status. We chose a cutpoint value for Pr that would minimize false negatives and maximize true positives, thereby maximizing the model's ability to predict true peanut allergy. Using a cutpoint of 0.35 for Pr, we classified participants into two groups: if Pr $\geq$ 0.35, the individual was predicted to have a peanut allergy, and if Pr < 0.35, the individual was predicted to not have a peanut allergy. When predicted peanut allergy status was cross tabulated with known peanut allergy status based on peanut challenge, Model 1 predicted Group 3 peanut allergy status well, but made four errors. It predicted a negative result (NOT allergic to peanuts) for two participants whose oral food challenge indicated they did have clinical peanut allergy. One of these subjects had a wheal size equal to 5 mm, and the other had a wheal size equal to 6 mm. It also predicted a positive result for two participants who did not have an oral food challenge reaction.

We then removed asthma from the model in order to examine whether sensitivity would improve. In our study, we defined sensitivity as the proportion of patients with a known peanut allergy who the model correctly predicts as having a peanut allergy. We called this new model comprised of only wheal size and rhinitis "Model 2" and used it to predict Group 3 peanut allergy status. Model 2 proved to have a sensitivity of 100%, as it correctly predicted every participant with a known peanut allergy as having clinical peanut allergy. However, only four of seven participants with known negative peanut allergy status were correctly predicted as having no clinical allergy, indicating a specificity of 57.1% (Table 5). We defined specificity as the proportion of patients with a known negative peanut allergy status who the model correctly predicts as not having a peanut allergy. Three participants with known negative peanut allergy status were incorrectly predicted to have a peanut allergy. We believe this is an acceptable type of error, as it does not carry the same risk as classifying an allergic subject as non-allergic. The wheal sizes of these three participants were 3, 4 and 5 mm.

When applied to Group 3, Model 2 (using a cutpoint of 0.35) correctly predicted the clinical peanut allergy status of 24/24 allergic individuals, indicating a sensitivity of 100%.

Table 4 Multivariable logistic regression results for model 1

Variable	OR [95% CI]	*p* value
Wheal size	2.606 [1.517, 4.477]	0.0005
Rhinitis (Y)	0.084 [0.010, 0.688]	0.0209
Asthma (Y)	6.278 [0.962 40.975]	0.0549

n = 69

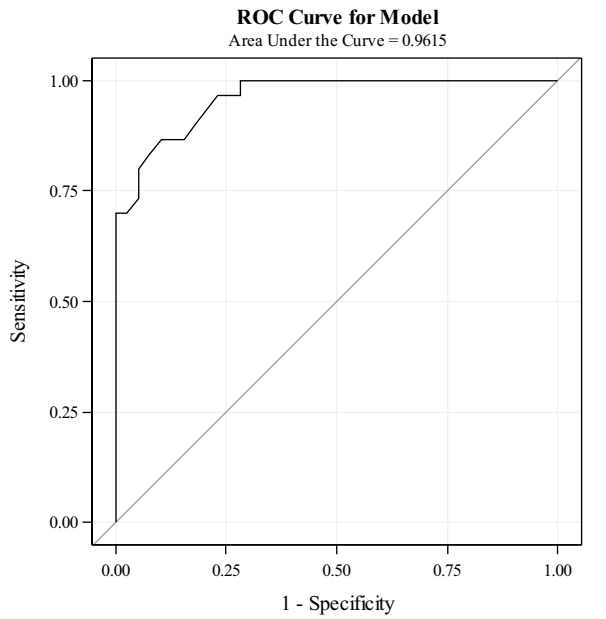

Fig. 1 Model 1 receiver operating curve

Study 2: evaluating the predictive value of wheal size and allergic rhinitis status in a second patient cohort

We conducted a retrospective chart review of 97 participants with confirmed clinical peanut allergy and 97 sex- and age-matched control participants without clinical peanut allergy. Their clinical characteristics are summarized in Tables 6, 7.

We classified allergic and non-allergic subjects according to wheal size (Table 8).

Table 5 Sensitivity and specificity of Model 2

Predicted allergy status	Known allergy status		
	Positive [n (column %)]	Negative [n (column %)]	Total [n (column %)]
Positive	24 (100)	3 (42.9)	27 (87.1)
Negative	0 (0)	4 (57.1)	4 (12.9)
Total	24	7	31

We analyzed the predictive value of each variable using exact conditional logistic regression. This analysis revealed a linear dependency among variables when wheal size was entered into the model. Because of this, we were unable to obtain a parameter estimate for wheal size.

We then analyzed the predictive value for each of the predictor variables using exact simple logistic regression (Table 9). We found wheal size to be the best predictor of clinical peanut allergy, with an odds ratio of 4.85 for every 1 mm increase in wheal size. The area under the ROC curve was 0.995, with a p value of <0.0001. The predictive value of wheal size was so dominant that no other variable was statistically significant when added to a model that included wheal size.

We then examined the sensitivity and specificity of wheal size at different cutpoints, ranging from 1 mm to 5 mm (Table 10). Sensitivity reached 100% at 1 mm, while specificity reached 100% at 5 mm.

Table 6 Participant demographics

Variable	Peanut allergic	Non-peanut allergic
Age in years [mean (SD); min, max]	9.87 (4.46); 3.00, 20.90	9.86 (4.45); 3.10, 20.80
Wheal size in mm [mean (SD); min, max]	8.62 (4.00); 2.00, 20.00	0.28 (0.97); 0.00, 5.00
Female sex [n (%)]	47 (48.5)	47 (48.5)

Table 7 Clinical characteristics

Variable	Peanut allergic	Non-peanut allergic
Allergic rhinitis status	57 (58.8)	54 (55.7)
Non-allergic rhinitis status	4 (4.1)	7 (7.2)
Asthma status	43 (44.3)	22 (22.7)
Eczema status	27 (27.84)	26 (26.80)
Egg sensitization	9 (9.28)	7 (7.22)
Milk sensitization	2 (2.06)	10 (10.31)
Wheat sensitization	1 (1.03)	0 (0)
Nut sensitization	33 (34.02)	13 (13.40)
Nut mix sensitization	19 (19.59)	7 (7.22)

All entries are n(%) for affirmative

Table 8 Peanut skin prick test wheal size by peanut allergy status

Wheal size (mm)	Clinical peanut allergy status		
	No	Yes	Total
0	89	0	89
1	0	0	0
2	1	1	2
3	4	4	8
4	2	7	9
5	1	8	9
≥6	0	77	77
Total	97	97	194

Table 9 Exact simple logistic regression univariate analysis

Variable	Odds ratio [95% CI]	p value	−2 Log L	Area under ROC
Wheal size	4.85 [2.859, 11.44]	<0.0001	33.306	0.995
Rhinitis	1.13 [0.618, 2.086]	0.7717	268.752	0.515
Non-allergic rhinitis	0.55 [0.115, 2.270]	0.5368	268.063	0.515
Eczema	1.05 [0.533, 2.082]	1.0000	268.915	0.505
Egg allergy	1.31 [0.415, 4.345]	0.7950	268.668	0.510
Milk allergy	0.18 [0.019, 0.901]	0.0329	262.767	0.541
Nut allergy	3.31 [1.548, 7.444]	0.0012	257.237	0.603
Nut mix allergy	3.11 [1.176, 9.248]	0.0191	262.329	0.562

Discussion

In Study 1 we analyzed the ability of eighteen different variables, alone and in combination, to predict clinical peanut allergy in peanut-sensitized individuals. Our results show that peanut SPT wheal size is by far the best predictor of peanut allergy. While the univariate analysis identified peanut-specific IgE, total IgE, male sex, asthma, and IL-5, IL-9 and IL-13 responses to peanut as being significant predictors of clinical reactivity, subsequent multivariable analyses found these variables to be related to peanut SPT wheal size and thus when entered into a model with wheal size were non-significant.

The analysis of our initial patient cohort revealed peanut SPT wheal size as the best univariate predictor, with an AUC of 0.927. For every 1 mm increase in wheal size, the odds ratio of an individual having a clinical peanut allergy was 2.36.

In this cohort, wheal size was positively associated with peanut allergy status while the presence of rhinitis was

Table 10 Sensitivity and specificity for wheal size cut-offs

Wheal size (mm)	Sensitivity (%)	Specificity (%)
>1	100	91.8
>2	99.0	92.8
>3	94.9	96.9
>4	87.6	99.0
>5	79.4	100

curiously protective. Using Model 2, that included both wheal size and rhinitis, we were able to successfully predict the clinical peanut allergy status of 100% of allergic subjects. However, the model misclassified three non-allergic subjects as allergic.

Interestingly, rhinitis was found to be protective against clinical peanut allergy in our first group of subjects. However, we were unable to reproduce this finding in our second patient cohort. To our knowledge, there are no other studies in the literature reporting a similar protective effect of allergic rhinitis in peanut allergy. This unexpected finding may have been an anomalous result caused by a small patient cohort size.

The strong predictive value of wheal size emerged in the analysis of our much larger second patient cohort. We found that for every 1 mm increase in wheal size, the odds ratio of an individual having clinical peanut allergy was 4.85. No other variable approached the predictive power of wheal size.

Other models of clinical peanut allergy prediction, such as The Cork Southampton Predictive Index [6], have used peanut SPT, serum specific IgE, total IgE, sex, and age to predict clinical peanut allergy. However, our data sets did not identify any variables that could reproducibly improve on the predictive ability of SPT wheal size in our patient cohorts.

The strong association between SPT wheal size and clinical peanut allergy has been described elsewhere. The HealthNuts longitudinal food allergy study in Melbourne, Australia, reported that a wheal size of 8 mm had 95% positive predictive value for clinical peanut allergy in 1-year-old infants [7]. Decreasing wheal size was associated with peanut allergy resolution in these patients at age 4, while increasing wheal size was associated with persistence [8]. Other groups have reported wheal size cut-points from 4 to 15 mm reaching 100% specificity when used to predict clinical peanut allergy [9, 10].

The skin prick test does produce false positive results that can lead to misclassification of non-allergic patients as allergic. False positive results emerged in our study, and have been reported previously [9, 11, 12]. Food allergy misdiagnosis negatively impacts the quality of life of patients and their families to the same degree as true peanut allergy. Heightened anxiety associated with eating, disruption of daily activities, and the need to carry an epinephrine auto-injector is common to both groups [13]. However, it is our strong belief that it is never acceptable to misclassify an allergic patient as non-allergic, especially in the context of a potentially severe diagnosis such as peanut allergy. A sensitive screening test, such as the SPT, is preferable to one that sacrifices sensitivity in favour of specificity.

One potential weakness of this study is the restricted range of variables it examined. Specifically, we did not include component testing or the basophil activation test, both of which are emerging tools in the field of food allergy research [14–19]. At the time of the study these tests were not available to us. Additionally, they are not without limitations and are not currently the standard of care in peanut diagnostics.

Component resolved IgE testing for Ara h 1, 2, and 3 has been highlighted as more predictive of clinical allergy than whole peanut-specific IgE, and sensitization to Ara h 2 emphasized as particularly discriminatory. However, there is a lack of consensus on appropriate component testing cutoffs and sensitivity and specificity measures of different cutpoints vary widely between studies. Reported sensitivity for a commonly used cutpoint of 0.35 kU/L ranges from 60 to 100% and specificity ranges from 72 to 96%. Beyer et al. [15] described a 90% probability for positive peanut challenge at 14.4 kU/L, and a cutpoint of 42.2 kU/L was required to reach a 95% probability. Additionally, the importance of individual components varies regionally, testing lacks standardization across commercial kits, and individual patient outcomes can deviate from component-associated correlations [20].

The basophil activation test has been proposed as a useful diagnostic tool for peanut allergy, but its broad utility is limited by its requirement for fresh blood and variability in basophil activity between individuals [21]. As with component testing, the cost of the basophil activation test limits its use in routine clinical practice.

The comprehensive statistical analyses used in this study consistently highlighted the superior ability of peanut SPT wheal size to predict clinical reactivity to peanut. SPT wheal size emerged as the dominant predictor of peanut allergy in both univariate and multivariable analyses in two separate patient cohorts. Our statistical analyses also determined that the predictive power of IgE laboratory measurements, both total and peanut-specific, were not independent of wheal size. This was also true for peanut-induced Th2 cytokine production from peripheral blood mononuclear cells. When added to any statistical models containing wheal size, the predictive power of all laboratory measurements became statistically non-significant. As such, the power of peanut SPT wheal size to predict clinical peanut allergy was dominant and reproducible.

Conclusions

Peanut skin prick test wheal size is a robust predictor of clinical peanut reactivity. We have found that patients with a wheal size of <1 mm do not display clinical peanut allergy and patients with wheal sizes ≥6 mm are clinically reactive. In patients with wheal sizes between 1 and 5 mm inclusive, oral food challenge may be appropriate

to determine allergic status if history is indeterminate. Further studies of a large cohort of patients with wheal sizes in this range may be warranted. The findings of this study may be useful in guiding clinician decision-making regarding peanut allergy diagnostics.

Abbreviations
SPT: skin prick test; PBMC: peripheral blood mononuclear cells.

Authors' contributions
ES prepared the manuscript for publication, GF conducted all statistical analyses, KA, TW performed laboratory experiments, ML provided guidance on study design and manuscript preparation, TF was the study research nurse, SG performed laboratory experiments, AM, AF was a study physician, MJ, SW designed and oversaw the studies. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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All data generated during or analyzed during this study are included in this published article.

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