ADVANCES IN TREATMENTS AND ANIMAL MODELS OF PEANUT ALLERGY

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ABSTRACT

Kelly A. Orgel: Advances in Treatments and Animal Models of Peanut Allergy
(Under the direction of A. Wesley Burks)

Food allergies are a growing health concern affecting approximately 6-8% of the US population. In particular, peanut allergy has an estimated prevalence of greater than 1% of the population and is uncommonly outgrown, making it a life-long disease. Ingestion of allergens can lead to a variety of allergic symptoms ranging from hives or gastrointestinal symptoms to constriction of the airways and anaphylactic shock. Because there is currently no FDA-approved treatment for food allergy, these patients are managed with education and strict allergen avoidance. However, even with the most careful avoidance, accidental ingestion does occur and can lead to life-threatening anaphylaxis. As a result, treatment options are needed. Treatments currently under investigation in clinical trials include peanut oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and epicutaneous immunotherapy (EPIT), though mechanisms of these therapies remain unclear. While results from these trials are promising, limitations include daily dosing, adverse effects, and limited long-term efficacy after therapy is discontinued. Thus, there remains an urgent need for improved therapy options. The work in this dissertation provides the foundation for future drug discovery. First, IgG-mediated basophil inhibition was elucidated as a mechanism of OIT and SLIT and was shown to be associated with long-lived protection. Understanding this mechanism further may result in a targeted therapy option. Separately, a therapy targeting inhibitory receptors on antigen-specific B cells was developed for the prevention of sensitization in a mouse model of peanut allergy. Unfortunately, understanding of food allergy etiology and advances in treatment options has been limited by the lack of an animal model that accurately recapitulates the human disease.

Here, we describe the use of the genetically diverse Collaborative Cross to identify CC027/GeniUnc as a more relevant mouse strain that exhibits a severe reaction following oral sensitization and challenge. Together, this work provides a platform for better understanding the mechanisms of food allergy and its treatments, as well as the development of new therapies.

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LIST OF ABBREVIATIONS

Alum	aluminum hydroxide
ARA-LAMP-vax	peanut Lysosomal Associated Membrane Protein DNA Plasmid vaccine
BCR	B cell receptor
CC	Collaborative Cross
CHILD	Canadian Health Infant Longitudinal Development
CoFAR	Consortium of Food Allergy Research
CRD	component resolved diagnostics
CT	cholera toxin
DBPCFC	double-blind, placebo-controlled food challenge
DO	Diversity Outbred
EPIT	epicutaneous immunotherapy
FAB	facilitated antigen binding
FAHF-2	Food Allergy Herbal Formula-2
FVIII	factor VIII
Gata3	Gata-3 gene
GRP	genetic reference panel
GWAS	genome wide association assay
HLA	human leukocyte antigen
HRP	horseradish peroxidase
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgG4	immunoglobulin G4
IFNγ	interferon γ

IL10	interleukin-10 gene
IL-10	Interleukin-10
IL12	interleukin-12 gene
IL-12p40	interleukin-12 subunit p40
IL-13	interleukin-13
IL-4	interleukin-4
IL-5	interleukin-5
IP	intraperitoneal
ITIMs	immunoreceptor tyrosine-based inhibitory motifs
IV	intravenous
LEAP	Learning Early About Peanut Allergy
MMCP-1	mucosal mast cell protease-1
NOD	non-obese diabetic
OFC	oral food challenge
OIT	oral immunotherapy
OVA	ovalbumin
Ox40L	Ox40 ligand gene
Ox40L	Ox40 ligand
PN-sIgE	peanut-specific IgE
PN-sIgG4	peanut-specific IgG4
PRROTECT	Peanut Reactivity Reduced by Oral Tolerance in an Anti-IgE Clinical Trial
SCID	severe combined immunodeficient
SEB	Staphylococcal Enterotoxin B
sIgE	specific IgE
Siglecs	sialic acid-binding immunoglobulin-type lectins
sIgG1	specific IgG1

SLIT	sublingual immunotherapy
SNP	single nucleotide polymorphism
SPDP	succinimidyl 3-(2-pyridyldithio) propionate
SPT	skin prick test
STAL	siglec-engaging tolerance-inducing antigenic liposome
SU	sustained unresponsiveness
Tfh	T follicular helper
Th	T helper
TIM-4	T cell immunoglobulin-domain and mucin domain-4
TLR	toll-like receptor
TNFα	tumor necrosis factor α
Treg	T regulatory cell
UK	United Kingdom

CHAPTER 1: INTRODUCTION

1.1 Public Health Concern

IgE-mediated food allergies occur in 6-8% of children under four years old and are estimated to affect 15 million Americans.[1-3] The foods most commonly associated with food allergy in the US include milk, egg, peanut, tree nuts, wheat, soy, fish, and shellfish.[4] Not only are food allergies common, but they are also increasing in prevalence.[5-7] In a 2013 report, the US Centers for Disease Control and Prevention estimated an increase in food allergy prevalence from 3.4% to 5.1% over a 14 year period.[8] A survey study estimated that peanut allergies in the US increased from 0.4% in 1997 to 0.8% in 2002 and then to 1.4% in 2008.[9] This increase in prevalence places a larger number of US children at risk for life-threatening anaphylaxis.

Food-induced anaphylaxis accounts for about one third of cases of anaphylaxis seen in hospital emergency departments.[10] It is estimated that approximately 30,000 food-induced anaphylactic events are seen in US emergency departments each year with about 200 of these reactions resulting in death.[11,12] The majority of fatal reactions are associated with ingestion of peanuts or tree nuts.[11,12] During an allergic reaction, symptoms can range from mild urticaria to life-threatening anaphylaxis, and most frequently include involvement of the skin, oropharyngeal, gastrointestinal, and respiratory systems. Unlike milk and egg allergies that are outgrown by nearly 70-80% of children in their early adolescence, peanut allergies are only outgrown in about 20% of those affected with the disease.[5] Thus, in most cases, peanut allergy is a lifelong disease that can negatively impact quality of life for these patients, as they have to carefully read food labels and often sacrifice participating in social activities centered around

food.[13] In fact, the stress and anxiety provoked by food allergies, reach beyond the affected patient and truly impact the entire family.[14] Despite the severe nature of the disease, large public health concern, and drastic lifestyle implications, the lack of FDA-approved treatments and understanding of the etiology of food allergy leave significant knowledge gaps.

1.2 Pathogenesis

The pathogenesis of food allergy can be divided into two phases which are summarized in Figure 1-1. In the first phase, the sensitization phase, an allergen, defined by the American Academy of Asthma, Allergy and Immunology as a typically harmless substance that is capable of triggering an allergic reaction, enters the body and leads to the production of antigen-specific IgE. In the second phase, the reaction phase, a later exposure to the allergen causes allergic symptoms including symptoms related to the skin, gastrointestinal and respiratory tracts. In the sensitization phase, antigen is taken up and presented by antigen presenting cells to naïve CD4+ T cells. Allergic individuals develop a CD4+ T helper (Th) 2 skewed immune response. During such a response, CD4+ T cells secrete Th2-type cytokines including IL-4, IL-5, and IL-13.[15] These cytokines trigger B cells to class switch and produce antigen-specific IgE.[16] This antigen-specific IgE binds to the high affinity IgE receptor, FcεRI, on effector cells including mast cells and basophils. At this point, the effector cells are primed for a future reaction. It is important to note that unlike B cells and T cells, mast cells and basophils are not specific for a single antigen. It is estimated that up to 500,000 IgE molecules can blanket the mast cell surface at one time, and these molecules have different specificities.[17]

The reactions elicited in a peanut allergy are classified as type 1 hypersensitivity reactions.[18] Upon subsequent exposure, antigen can bind to the Fab region of IgE molecules and result in cross-linking of the receptors. This cross-linking ultimately leads to activation of Syk kinase and an influx of calcium ions with subsequent degranulation by exocytosis.[19] Extensive work has been done to elucidate the signaling pathways responsible for this

degranulation event, and this work has been reviewed elsewhere.[19] During degranulation, mast cells release pre-formed granules containing mediators such as histamine, proteoglycans, and proteases including tryptase.[20] The most readily available mediator, histamine has several physiological effects including vasodilation, increased vascular permeability, bronchoconstriction, and mucus production that are responsible for the symptoms observed during an allergic reaction.[19] Because of the quick release of pre-formed histamine, the resulting symptoms of itching, swelling, throat tightness, and difficulty breathing have an acute onset, usually within minutes of ingestion of the antigen. Histamine has a short half-life, resulting in its quick clearance and resolution of symptoms. In addition to the release of pre-formed mediators, activated mast cells also synthesize and release cytokines, leukotrienes, prostaglandins and platelet activating factor, all of which further act on smooth muscle and vasculature.[17]

During mast cell development, progenitor mast cells migrate from the bone marrow to tissues, where they take up residence and complete their maturation.[20] As a result, human mast cells have proven difficult to study. Basophils are effector cells that, similar to mast cells, express FcεRI and release granules containing histamine.[21] Despite their many similarities, basophils also have many characteristics that distinguish them from mast cells. For example, basophils are short-lived, with a life span of days, while mast cells remain in tissues for months.[21] Basophils also have larger and fewer granules than mast cells and have low tryptase content.[21] Unlike mast cells, mature basophils circulate through the vasculature system, making them easier to obtain from subjects for research purposes. Because of their similarities in IgE-activation, basophil activation following *ex vivo* stimulation with antigen has been widely studied, as will be discussed in Chapter 2.

Some allergic patients experience a late-phase reaction, also referred to as a biphasic reaction, in addition to the immediate reaction described above. Biphasic reactions can be defined as recurrent symptoms without additional exposure to the allergen after symptoms of

the initial reaction have resolved.[22] These late-phase reactions peak 4-8 hours following allergen ingestion.[23] One study of emergency room records from two Canadian hospitals found that 14.7% of patients who were seen for an allergic reaction to food experienced a biphasic reaction.[24] In separate studies, examination of affected tissues including skin, nasal mucosa, and lung revealed infiltration by eosinophils, neutrophils, CD4+ T cells, and basophils during late-phase reactions.[23] It is not known which patients are likely to develop late-phase reactions and monitoring guidelines remain inconsistent.[25]

1.3 Etiology and Risk Factors

Much remains unknown about the initial phase of sensitization in food allergic patients, which is ultimately a failure of oral tolerance mechanisms. Oral tolerance, described as a state of active inhibition of immune responses to ingested food proteins, occurs in the majority of people.[26] While the mechanisms of oral tolerance are not completely understood, it is well accepted that T cell anergy, T cell deletion, or induction of T regulatory cells (Tregs) all mediate the induction of oral tolerance.[26] A breach in any of these mechanisms can result in the development of food allergy, yet the risk factors that make an individual susceptible remain unclear. Research on this topic has focused primarily on the potential routes of initial exposure, genetics, and the gut microbiome. Research in each of these areas is discussed briefly below.

1.3.a Routes of Sensitization

For unknown reasons, patients with food allergies have a defect in either their ability to develop or maintain oral tolerance to a food antigen. Interestingly, in most cases of food allergy, the first reaction is observed during the first consumption of the eliciting food. Thus, the timing and route of the first exposure remain an intriguing question. Some researchers have hypothesized that sensitization may happen *in utero*. One study of 503 infants with likely egg or milk allergy found that peanut consumption during pregnancy was a predictor for the development of peanut allergy.[27] Conversely, a 2003 study by Lack, *et al.* used data from the

Avon Longitudinal Study of Parents and Children, a large birth cohort study in which 14,000 pregnant women were enrolled, to investigate this question.[28] This study reported that of 23 preschool-aged children with confirmed peanut allergy, there was no evidence of sensitization from maternal diet and cord blood had no detectable peanut-specific IgE (PN-sIgE). A separate study showed that while IgE was detectable in cord blood samples, it appeared to be of maternal origin, as it matched the specificity of IgE in maternal blood, and was not found in blood samples from these infants at 6 months of age.[29] These reports suggest that sensitization does not occur *in utero*, though there is clearly conflicting data. Other studies have investigated whether peanut exposure occurs through breast milk, and the results are similarly inconclusive. Peanut proteins Ara h 1, Ara h 2, and Ara h 6 have been detected in breast milk as soon as one hour after consumption, making breast milk a potential source of allergens for sensitization.[30-32] Three large studies did not find any association between consumption of peanut in lactating moms and the development of peanut allergy in their infants.[27,28,33] Despite the recommendation that pregnant and lactating mothers of children at risk for developing atopic diseases avoid antigen consumption, a meta-analysis found that this avoidance did not protect against allergic diseases, including food allergy.[34]

There is a well-established link between atopic dermatitis, or eczema, and food allergy.[35] Reports have documented that about one third of patients with moderate to severe atopic dermatitis have a food allergy.[36] Because eczema results in a defective skin barrier, it has been proposed that children with eczema are sensitized to food allergens epicutaneously. Environmental peanut protein is detectable in house dust[37] as well as in schools,[38] and was found to increase the risk of peanut allergy in a dose-dependent manner,[37] further increasing the plausibility of this hypothesis. However, the results of several studies suggest that a disrupted skin barrier, as occurs in eczema is essential for this phenomenon to occur. Fillagrin, a filament aggregating protein, plays a role in maintaining skin integrity.[39] Fillagrin loss-of-function mutations are associated with the development of eczema, as well as the development

of food allergies.[40,41] However, because food allergies and eczema are so closely linked, it is hard to distinguish the contribution of fillagrin mutations to the development of food allergy, independent of its role in eczema pathogenesis. Several small animal models, which will be discussed further in Section 1.8.b, have demonstrated that mice can be sensitized to food allergens epicutaneously.[42,43] Together, this work suggests that environmental exposure to food allergens may ultimately lead to sensitization in a select, atopic population.

Recently, work has investigated whether peanut protein in the environment is capable of sensitizing children through the airway. Detecting peanut protein in air samples has proven difficult, but it was detectable by a sampler head placed directly above peanuts actively being shelled.[37,44] In a recent study, peanut-responsive Th cells (indicated by increased CD154 expression following *ex vivo* stimulation with peanut) from peanut-allergic subjects had increased expression of CCR4, a skin and airway chemokine receptor, compared to non-allergic subjects.[45] However, they did not have different expression of the skin chemokine receptor, CCR10, or the skin homing antigen, CLA. These results suggest that peanut sensitization may, at least partly, occur through the airways. Another study found that both BALB/c and C57BL/6 mice developed peanut allergy after four weeks of airway exposure to peanut.[46] Altogether, these findings suggest epicutaneous and airway exposures may result in sensitization in addition to presumed sensitization via oral exposure.

1.3.b Genetics

There is little dispute that a person's genetics are a contributing factor to the development of peanut allergy; however, knowledge about the specific genes and their relative contributions to the development of disease remain unknown. A 2009 study examined disease patterns of familial aggregation in 581 nuclear families.[47] Results showed that food allergy in one child was a direct and independent predictor of food allergy in a sibling. There were positive associations for antigen-specific IgE for the eight major food allergens in the following

pairs: mother-child, father-child, child-sibling. Later, a twin study, which found that there was a higher concordance of peanut allergy among monozygotic twins, reported the heritability of peanut allergy to be 81.6%.[48] Several studies have specifically evaluated the association of human leukocyte antigen (HLA) alleles with the development of peanut allergy, and these are thoroughly reviewed elsewhere.[49] Briefly, there is conflicting data on the association between HLA loci and peanut allergy. In one study by Howell, *et al*., the frequency of HLA-DRB1*08 and DQB1*04 were increased in peanut-allergic subjects compared to controls; however, no significant HLA class II associations were found when comparing peanut-allergic subjects to their non-allergic siblings.[50] As a result, these alleles are likely associated with atopy rather than specific to peanut allergy. Shreffler and colleagues were unable to replicate these findings and concluded that there is not an association between HLA alleles and the development of peanut allergy,[51] while a recent 2017 study was able to replicate the original findings.[52] Based on a similar study, Dreskin, *et al.*, concluded that HLA-DRB1*08 is a marker of families that have an increased propensity for developing peanut allergy.[53] In 2015, the first genome wide association assay (GWAS) of a food allergy cohort was performed to identify associations between genetic variables and food allergy.[54] This study found two single nucleotide polymorphisms (SNPs), one intergenic between HLA-DQB1 and HLA-DQA2 and one in the HLA-DRA gene product, to be associated with peanut allergy. Further, the association between these SNPs and peanut allergy was at least partially due to DNA methylation. Overall, these studies demonstrate that the relationship between HLA and peanut allergy is not straightforward.

Recent studies have identified candidate genetic risk factors outside of HLA. A large GWAS study on 850 cases of peanut allergy and 926 controls identified C11orf30/EMSY as a risk locus for both peanut allergy and food allergy.[55] Authors also identified numerous other loci whose gene products have functions ranging from histone modification to endothelial cell factors. A different 2017 GWAS study on peanut allergy identified five loci associated with

peanut allergy: clade B serpin gene cluster, the cytokine gene cluster at 5q31.1, the fillagrin gene, C11orf30, and HLA.[56] These loci are all involved in immune regulation and epithelial barrier function. Interestingly, this study found that the effect of the fillagrin mutation (discussed previously in Section 1.3.a) was independent from the development of eczema. In summary, the genetic component of food allergy is complex, but studies such as the ones mentioned above give rise to potential risk identifiers as well as insight into disease mechanisms.

1.3.c Gut Microbiome

The rise in food allergy prevalence observed over the last 50 years has led to immense speculation about potential causes, with special attention to changes in lifestyle and environment. The hygiene hypothesis, which says that lack of appropriate microbial exposure early in life increases the risk of allergic disease,[57] was the first to suggest an association between the microbiome and food allergy. Consistent with this hypothesis, children with older siblings as well as children raised in rural areas have a lower incidence of allergic diseases.[58-60] These factors are each known to influence the microbiome of an individual.[57] To further investigate relevance of the hygiene hypothesis, several clinical studies have investigated the possible link between gut microbiome and allergic diseases. In one such study, researchers performed 16s rRNA sequencing on feces from 34 infants with food allergy and 45 controls.[61] The overall microbiota diversity was not found to be different between these groups; however, increased levels of *Clostridium sensu stricto* was associated with disease and correlated with IgE levels. Additionally, decreased levels of *Bacteroides* and *Clostridium XVIII* were associated with food allergy. While the above findings are interesting, and suggest a role for microbiome in the development of food allergy, the data are limited because cross-sectional studies are not able to control for contributing factors such as dietary differences between groups. The only prospective study to investigate the role of microbiome in the development of food allergies was published by Azad, *et al.* in 2015.[62] This study followed 166 infants from the Canadian Health

Infant Longitudinal Development (CHILD) study, and fecal samples were collected at three months and 12 months for 16s rRNA sequencing. They found that low microbial richness at three months was associated with food sensitization (confirmed by skin prick test) at one year. Further, Enterbacteriaceae were overrepresented and Bacteroidaceae were underrepresented in samples from food-sensitized infants. The Enterbacteriaceae/Bacteroidaceae ratio was elevated both at three months and one year, suggesting that this colonization occurs early in infancy and persists. In addition to studies investigating microbiota associated with sensitization, one study has found that gut microbiome composition can predict whether subjects will outgrow a milk allergy.[63] Together, these findings from human studies demonstrate a clear association between gut microbiome and both the development and resolution of food allergies.

Mechanistic microbiome studies have been conducted in mouse models of food allergy. Initial studies demonstrated that animals lacking gut colonization through either antibiotic treatment[64] or germ-free housing,[65] were more susceptible to peanut allergy. These results suggest that microbes play a protective role in the development of food allergy. Germ-free mice were then specifically colonized with different microbes to identify the one(s) exhibiting a protective effect. Several mouse studies have now demonstrated that colonization with Clostridia species is sufficient to suppress peanut sensitization. One study demonstrated that colonization with Clostridia results in increased IL-22 expression and decreased Ara h 2 and Ara h 6 absorption into the blood stream following oral gavage with peanut.[65] Use of an IL-22 neutralizing antibody reversed this effect suggesting Clostridia influences intestinal barrier function in an IL-22-dependent manner. Other studies have found that colonization of germ-free mice with Clostridia leads to expansion of Foxp3+ Tregs, suggesting that induction of regulatory mechanisms may play a role in microbiome-induced protection.[66] Collectively, these reports demonstrate that microbes, specifically Clostridia, prevent mice from sensitization to food allergens. Further studies to elucidate the protective mechanisms of the microbiome and

identify other microbes involved may allow for future manipulation of the microbiome for the prevention and treatment of food allergy.

1.4 Peanut Allergens

Extensive work has been done to identify and characterize the peanut allergens responsible for eliciting the allergic response. The International Union for Immunological Societies recognizes 16 peanut allergens. These allergens are named Ara h 1-17, with the exception of Ara h 4, using the Genus and Species classification for the peanut (*Arachis hypogaea)* plant. It currently remains unknown what is responsible for the allergenicity of these proteins and why proteins with similar structures from other foods do not elicit an allergic response. A 2010 study showed that peanut-allergic individuals in different parts of the world mount an IgE response to peanut allergens to differing extents.[67] In this study, they demonstrated that IgE specificity differed in patients from Spain, the United States, and Sweden and hypothesized that the results were due to differences in dietary habits and pollen exposures. Each allergen contains several IgE-binding epitopes as well as T cell-binding epitopes. Epitopes can be linear (dependent on the neighboring amino acids) or conformational (dependent on the tertiary structure of the protein). Understanding these allergens and their epitopes has been the target of investigational diagnostics and therapeutics, discussed in Sections 1.5 and 1.7, respectively. Ara h 1, 2, 3, 6, and 9 are considered major allergens, accounting for 75% of peanut protein content,[68] and will be discussed further, along with Ara h 8, which has been implicated in Oral Allergy Syndrome.

Ara h 1 is a member of the vicilin seed storage family.[69] This family of proteins are typically disc-shaped and comprised of trimers.[70] The 63.5 kDa monomers combine to form a 180 kDa trimer.[70] As a glycoprotein, Ara h 1 has the potential to be post-translationally modified, and the types and extent of these modifications leads to variable contents of the trimer components. Several studies have shown up to 80% of peanut-allergic subjects have IgE

reactivity to Ara h 1.[71] Twenty-four linear IgE-binding epitopes, ranging from 6-25 amino acids in length, have been identified.[71,72] Interestingly, the IgE-binding epitopes have been shown to cluster in the contact points of the trimer components. The stability of Ara h 1 when subjected to enzymatic digestion remains unclear, as results have varied widely, perhaps due to differing digestion conditions.[73]

Ara h 2 and Ara h 6 are both 2S albumins that have gained a great deal of attention as major peanut allergens. Ara h 2 has two isoforms with molecular masses of 16.7 kDa and 18 kDa and contains 10 IgE-binding epitopes, whereas Ara h 6 is 15.0 kDa and contains 7 IgE-binding epitopes.[70] These glycoproteins contain four helices held together by four disulfide bonds.[70] Ara h 6 and Ara h 2 share 55% homology in their amino acid sequences and have known cross-reactivity.[74] Both Ara h 2 and Ara h 6 have been shown to be resistant to heat and enzymatic digestion.[75] Disruption of the disulfide bonds in either allergen has been shown to decrease their allergenicity, suggesting that the conformation of Ara h 2 and 6 are essential for eliciting an allergic response.[76] Most peanut-allergic individuals mount an immune response to Ara h 2 and 6. In one study, 100% of children with a peanut allergy had IgE to Ara h 2 and 80% had IgE to Ara h 6.[77] This response was exploited in a mouse study where immunotherapy of Ara h 2 and Ara h 6 was sufficient to desensitize peanut-allergic mice.[78]

Ara h 3 belongs to the legumin family and is a hexameric protein in which each subunit is 60 kDa and contains a disulfide bond.[70,79] Four IgE-binding epitopes have been shown to be important in peanut allergy.[80] In one study, 42% of peanut-allergic subjects were reported to have Ara h 3-specific IgE.[81] Cross-reactivity between Ara h 1, Ara h 2, and Ara h 3 has been noted.[82] Further, researchers believe that the similarities of Ara h 3 to other legumins in tree nuts may account for some cross-reactivity observed between allergens.

Ara h 8, a homologue of the birch pollen allergen, Bet v 1, is thought to be responsible for cross-reactivity between birch pollen and peanut.[83] Patients with isolated Ara h 8 sensitization typically experience oral cavity symptoms in the absence of a systemic reaction.

This local reaction is due to a phenomenon referred to as Oral Allergy Syndrome in which pollen-allergic individuals experience a local reaction confined to the mouth, lips, and throat upon ingestion of a cross-reacting food allergen.[84] Thus, identification of isolated Ara h 8 sensitization may be suggestive of a less severe allergy. Ara h 9, while still of significant importance, is less well-studied than the previously mentioned allergens. Ara h 9 has been of particular interest in peanut-allergic patients in the Mediterranean.[85] It is a nonspecific lipid transfer protein, thought to require sensitization to the peach allergen, Pru p 3, prior to the development of allergic symptoms to peanut.[86]

1.5 Diagnosis

The diagnosis of food allergies is based on a thorough clinical history that will guide any further studies. Questions regarding the possible triggers, symptoms of the reaction, timing of symptoms following ingestion of the eliciting allergen, and a family history of atopy can be useful in guiding testing. Two types of routine testing are typically done.[87] The first, skin prick testing (SPT), measures mast cell degranulation to a specific antigen. In this testing, an epicutaneous prick is used to place the antigen under the skin and within minutes, local mast cells may release their mediators resulting in wheal and flare formation. SPT has a negative predictive value of greater than 90%, making it a useful test for excluding food allergy.[88] However, this testing only has a 50% positive predictive value that has been shown to vary based on age.[89] Therefore, use of SPT needs to be chosen carefully to avoid unnecessary avoidance of foods. The second testing type involves measurement of serum antigen-specific IgE levels. Measurement of serum IgE has been shown to be sensitive but not specific in the diagnosis of allergy.[90] Together, SPTand serum IgE measurements only indicate sensitization.[88] There are many cases where people are sensitized, meaning they make IgE to an allergen, but can tolerate ingesting the allergen without any clinical response. Thus, these tests can be helpful only in conjunction with a clinical history.

Due to the discrepancy between sensitization and allergy, an oral food challenge (OFC) remains the gold standard for diagnosis of food allergies.[91] This test involves having the patient or research subject ingest gradually increasing doses of food containing the allergen while a supervising medical professional observes for signs of an allergic reaction. An OFC is typically done unblinded and without a placebo control. The OFC has many obstacles including the length of time required, cost, often anxiety for the patient and family, and potential risk of an allergic reaction, including anaphylaxis. Because of safety concerns, OFCs should only be performed in clinical settings that have experienced personnel with proper medications to treat potential reactions.

Research has led to several advancements in the diagnostics described above. Lately, a great deal of attention has been given to component resolved diagnostics (CRD). CRD measures serum IgE against individual peanut allergens. The hope of using CRD over conventional serum PN-sIgE levels is that identification of IgE to certain allergens may give rise to reactivity information. Ara h 2-specific IgE levels have been suggested to best correlate with reactivity.[92] Other studies have demonstrated that sensitization to a combination of Ara h 1-3 is useful in predicting more severe reactions.[81] CRD testing is particularly useful when results demonstrate sensitization to only Ara h 8. As discussed previously, Ara h 8 is a homologue of the birch pollen allergen, Bet v 1.[93] Many patients who only have Ara h 8-specific IgE are likely to experience the mild, local symptoms such as itching of the mouth, associated with oral allergy syndrome rather than a systemic allergic reaction.[94] While CRD testing seems promising, it is currently not widely used due to cost and lack of understanding if the values indicate allergy versus sensitization. Epitope analysis is currently being used as a research modality, but may be a future diagnostic tool. One group performed microarray immunoassays using patient sera to identify two epitopes in Ara h 2 that seemed to predict clinical reactivity.[95] Further research in Germany has developed a nanoallergen platform that displays separate epitopes from the

peanut allergen, Ara h 2.[96] They used patient sera to determine immunogenicity of these epitopes and hope that this will become a future diagnostic tool.

1.6 Prevention

Landmark studies completed in the last five years have drastically altered the American Academy of Pediatrics recommendations regarding the timeframe for introduction of peanut. Previously, parents were told to avoid feeding their children peanut until 3 years of age.[97] However, in 2008, researchers in the United Kingdom (UK) noted that the prevalence of peanut allergy amongst Jewish children in the UK was 10 times higher than in Jewish children from Isreal.[98] Further, their surveys indicated that peanut was introduced earlier and eaten in larger quantities in Israel than in the UK. These results suggested that the increased early consumption of peanut may lead to prevention of peanut allergy.

Du Toit, *et al.* tested this hypothesis in the Learning Early About Peanut Allergy (LEAP) trial. In this study, 640 infants at risk for developing peanut allergy due to a history of severe eczema and/or egg allergy, but skin prick test <4 mm were randomized to either early peanut consumption or peanut avoidance starting between four and 11 months of age.[99] The 60 month outcomes were broken down into subjects with a negative skin prick test and positive skin prick test at the start of the study. For the intention-to-treat analysis, of the infants that had negative skin prick tests, 13.7% of the avoidance group and 1.9% of the consumption group ($p<0.001$) developed a peanut allergy. Similarly, in the intention-to-treat analysis of infants with a positive skin prick test, 35.3% of the avoidance group and 10.6% of the consumption group developed a peanut allergy. These results confirm that early and regularly ongoing weekly exposure to peanut in a high-risk population prevents the development of a peanut allergy.

In a follow-up study, referred to as both the Persistence of Oral Tolerance to Peanut and the LEAP-ON study, researchers investigated whether the subjects who were randomized to early peanut consumption in the LEAP study described above would remain protected after

discontinuing peanut consumption for 12 months.[100] After avoiding peanut for 12 months, the intention-to-treat analysis revealed that the prevalence of peanut allergy was 18.6% in the peanut-avoidance group and 4.8% in the peanut-consumption group when both the skin prick positive and negative cohorts were combined ($p<0.001$). These results demonstrate that the protection conferred during the 60 months of peanut-consumption was long-lasting, even after consumption was discontinued. Immunologic assessment showed that PN-sIgE and Ara h 2-specific IgE were higher in the avoidance group and that peanut-specific IgG4 (PN-sIgG4) levels continued to be higher in the consumption group, despite a decline that started around 30 months, while subjects were still consuming peanut. As a result, an addendum was created to the 2010 Guidelines for the Diagnosis and Management of Food Allergy in the United States. This addendum now recommends introducing peanut into infants' diets as early as 6 months of age.

1.7 Treatment

Despite the number of people affected and the severe nature of the disease, there are currently no FDA-approved treatments for food allergy. Patients and their families are advised to avoid ingestion of the allergen, which requires extensive caution with reading food labels. However, even with the most careful avoidance, accidental ingestion is possible, and patients are prescribed an epinephrine auto-injector such as EpiPEN to treat potential reactions. Mild reactions can be treated with diphenhydramine, whereas severe, anaphylactic reactions require treatment with epinephrine. The diagnostic criteria for anaphylaxis is complex and patients are advised to call emergency responders following administration of epinephrine. As a result, many families and care providers are unsure and hesitant about the use of epinephrine in potentially emergent settings. Even when used correctly, epinephrine will only treat the current symptoms; there is still not treatment for the underlying disease.

Recent work in the field has focused on the use of peanut immunotherapies to prevent life-threatening reactions. These immunotherapies include oral immunotherapy (OIT), sublingual immunotherapy (SLIT), and more recently, epicutaneous immunotherapy (EPIT). Characteristics of OIT, SLIT, and EPIT are summarized in Table 1-1. While there are many differences between these therapies, the idea behind each of them is the same. The subject is exposed to escalating doses of allergen, with the goal being that after treatment, a peanut-allergic individual may tolerate an accidental ingestion of the allergen. Two clinical endpoints are often discussed: desensitization and sustained unresponsiveness (SU). A subject experiences desensitization when they are able to tolerate ingestion of a predetermined amount of allergen while still on therapy. During SU, however, the subject can still tolerate ingestion of the allergen after therapy has been discontinued for a period of time, suggesting that the immune changes induced by therapy are more long-lived. While these terms are useful in discussing clinical trial outcomes, it is important to note that the OFC doses used to determine desensitization and SU as well as the time off therapy before designating someone as experiencing SU are not consistent between studies. Numerous paramount, well-designed studies have been conducted on the use of OIT, SLIT, and EPIT to treat peanut allergy and these will be reviewed below.

1.7.a Oral Immunotherapy

Early reports by Patriarca and colleagues, dating back to 1998, showed that OIT could be used to successfully desensitize subjects to a wide variety of food allergens.[101] Since then, OIT has been used in clinical trials to desensitize subjects with allergies to cow's milk, hen's egg, and peanut. In one of the earliest landmark peanut OIT trials, Jones, *et al.* demonstrated that peanut OIT was both safe and effective at desensitizing allergic subjects.[102] In this 2009 study, peanut-allergic subjects aged 1 to 16 years old underwent an OIT regimen consisting of an initial day escalation, build-up and maintenance phases, and finally an OFC. Twenty-seven

of the 29 subjects successfully consumed 3,900 mg peanut with no more than mild symptoms. Only one subject required the use of epinephrine during the OFC. Interestingly, desensitization appeared to occur much earlier than expected. By 6 months of therapy, skin prick test reactivity was smaller and basophils were less reactive to *ex vivo* peanut stimulation. By 18 months of therapy PN-sIgE was decreased from baseline and PN-sIgG4 was increased. Additionally, the frequency of CD4+CD25+FoxP3+ Tregs was transiently increased in subjects on peanut immunotherapy, leaving changes in their function unknown.

In 2011, the first double-blind placebo-controlled OIT trial was reported by Varshney, *et al.*[103] In this study, subjects aged 1 to 16 years old were randomized 2:1 to receive peanut flour or placebo for 12 months. After an initial day escalation and build-up phase, subjects received a 4,000 mg maintenance dose for one month before undergoing a double-blind, placebo-controlled food challenge (DBPCFC) with a maximum cumulative dose of 5,000 mg of peanut protein. Three peanut OIT subjects withdrew from the study due to adverse symptoms while dosing, but all of the remaining 16 subjects in the treatment arm ingested the 5,000 mg of protein. Subjects in the placebo arm, however, ingested a median cumulative dose of 280 mg protein. In comparison to the placebo group, the immune changes in the treatment group were similar to those described previously by Jones and colleagues. This trial further showed that therapy resulted in decreased IL-5 and IL-13, and an increased ratio of $FoxP3^{hi}$:$FoxP3^{intermediate}$ CD4+CD25+ T cells. This study demonstrated that unlike placebo, peanut OIT safely results in the desensitization of most subjects.

Later, a study sought to compare the effect of peanut OIT to peanut avoidance, which is the current standard of care.[104] Peanut-allergic subjects between the ages of 7 and 16 years old were randomized 1:1 to active OIT or avoidance for the first phase of the study. Following 6 months, the subjects initially randomized to avoidance were crossed-over to 6 months of active treatment in the second phase of the study. The primary end-point of the study was desensitization, which was determined by successful ingestion of 1,400 mg peanut protein in a

DBPCFC. Of the 39 subjects in the active treatment group who completed the study, 69% were successfully desensitized while none in the control intervention group were desensitized. After the second phase of the study in which the avoidance group was crossed-over to active treatment, 54% of the subjects tolerated the 1,400 mg DBPCFC. Most side effects reported during OIT were mild, and affected the gastrointestinal tract, with only one subject requiring the use of intramuscular epinephrine. Importantly, this study also showed that OIT improved quality of life scores in subjects, suggesting that the positive effect of OIT reaches beyond immune changes.

Together, the previously mentioned studies show that peanut OIT can be used to desensitize subjects, but the longevity of this affect remained in question. In a follow-up study to the 2009 Jones paper described above, Vickery, *et al.* investigated the ability of OIT to induce SU, defined as the persistence of tolerance to an antigen after stopping therapy for a period of time.[105] To test this, eligible subjects from the previous study underwent two additional food challenges of 5,000 mg. The first, termed “desensitization food challenge”, was completed at the end of therapy, while the second, termed “SU food challenge” was completed one month after therapy was discontinued. The authors found that of the 24 subjects who completed the study, 50% achieved SU and peanut was subsequently re-introduced into their diet. These subjects had decreased skin prick test reactivity and lower levels of peanut-specific, Ara h 1-specific, and Ara h 2-specific IgE both at baseline and at completion of the study. PN-sIgG4 levels and function, as determined by facilitated antigen binding (FAB) assay, were not different between groups.

In response to the exciting new findings of the LEAP study discussed in Section 1.6, immunotherapy studies have shifted focus towards trying to treat a younger study population. In a trial published earlier in 2008, 40 children aged 9 to 36 months with confirmed peanut allergy were randomized to receive OIT ending in maintenance doses of either 300 mg or 3,000 mg peanut protein.[106] An average of 2.5 years later, subjects received a desensitization DBPCFC

of 5,000 mg, during which 81% of subjects on OIT were found to be desensitized. SU was assessed 4 weeks after discontinuing therapy by a second DBPCFC of 5,000 mg peanut protein. Results were compared to that of 154 standard of care controls retrospectively collected from a pediatric allergy clinic database. Authors found that in the intention-to-treat analysis, 81% of the study population passed the desensitization challenge, and 78% of the 300 mg arm and 85% of the 3,000 mg arm achieved SU. Adverse events were common in both arms of the study, affecting 95% of the subjects, though slightly more frequent within the group receiving the higher maintenance dose. However, side effects were mostly mild, with only one subject requiring treatment with epinephrine. Compared to baseline, PN-sIgE levels decreased while PN-sIgG4 levels increased following treatment with OIT. These results suggest that in younger peanut-allergic children, a shorter regimen of OIT with a lower maintenance dose is sufficient to induce desensitization and SU.

Other clinical studies have investigated the use of peanut OIT in combination with other treatments. In one such clinical trial, Tang, *et al.* determined the efficacy of probiotics in combination with OIT to desensitize subjects with peanut allergy.[107] In this 2014 double-blind, placebo-controlled trial, 62 children between the ages of 1 and 10 were enrolled in an 18 month study in which they were randomized to either OIT combined with probiotics or placebo. On the last day of treatment, a DBPCFC of 3,000 mg was conducted to assess desensitization. The subjects who were desensitized discontinued therapy for 2 to 5 weeks, and then underwent a second DBPCFC of 3,000 mg to determine SU status. Results showed that 82.1% of the treatment group and only 3.6% of the placebo group achieved SU. Similar to OIT studies reviewed above, treatment with probiotics combined with OIT led to decreased skin prick test reactivity and PN-sIgE levels but increased PN-sIgG4 levels compared to placebo-treated controls. Importantly, this study lacked an OIT-treated group that received peanut in the absence of probiotic. Thus, further clarification is needed to determine the relative contributions of OIT and probiotic in the outcome of this study.

Separately, a study published in 2016 investigated the use of omalizumab, an anti-IgE monoclonal antibody, in conjunction with OIT to treat peanut allergy.[108] In the Peanut Reactivity Reduced by Oral Tolerance in an Anti-IgE Clinical Trial (PRROTECT), 37 subjects between the ages of 7 and 25 with known peanut allergy were randomized to either omalizumab or placebo in combination with peanut OIT. Subjects received study drug (omalizumab or placebo) for 12 weeks before undergoing a one-day rapid desensitization with peanut OIT. They then continued weekly up-dosing until reaching 2,000 mg peanut protein. The omalizumab-treated group tolerated a median peanut dose of 250 mg while the placebo-treated group tolerated a median of 22.5 mg on the initial desensitization day, suggesting that treatment with omalizumab allows for rapid desensitization. After discontinuing study drug for 6 weeks, 79% of the subjects in the omalizumab arm and only 12% of the placebo arm were able to tolerate ingestion of 2,000 mg peanut protein, demonstrating that this desensitization is long-lasting in many subjects.

1.7.b Sublingual Immunotherapy

Unlike OIT, which involves the ingestion of the allergen, SLIT requires the allergic subject to place a liquid extract of the allergen under their tongue and hold it in place for a few minutes, allowing for antigen uptake by tolerogenic Langerhans cells in the oral mucosa. SLIT is FDA-approved to treat ragweed and grass pollen allergy but is still investigational for food allergy. In the past 10 years, several reports have investigated the ability of SLIT to desensitize peanut-allergic subjects. In 2011, Kim, *et al.* conducted the first double-blind, placebo-controlled trial on peanut SLIT.[109] In this study, 18 peanut-allergic subjects between the ages of 1 and 11 years old completed 12 months of peanut SLIT before undergoing a DBPCFC. On the day of the DBPCFC, the 11 subjects randomized to peanut treatment consumed a median cumulative dose of 1,710 mg of peanut protein whereas the 7 subjects on placebo consumed a median cumulative dose of 85 mg (p=0.011). Mechanistic studies on these subjects found that, compared to the placebo group, the treatment group had decreased skin prick test reactivity, *ex*

vivo basophil activation, PN-sIgE levels, and IL-5 levels but increased PN-sIgG4 levels. However, they did not find any changes in levels of IL-13, IFNγ, Tregs, or IL-10. Furthermore, this study demonstrated that the use of peanut SLIT to desensitize peanut-allergic subjects is safe. The study had no dropouts and symptoms reported for 11.5% of peanut doses and 8.6% of placebo doses. The symptoms observed on peanut SLIT were mild, mostly consisting of oropharyngeal itching, and none required treatment with epinephrine.

At the same time as the study discussed above, the Consortium of Food Allergy Research (CoFAR) conducted a randomized, double-blind, placebo-controlled trial in a larger, older study population to investigate the safety and efficacy of SLIT for the treatment of peanut allergy.[110] In this study, 40 peanut-allergic subjects between the ages of 12 and 37 were treated with 44 weeks of peanut SLIT or placebo before undergoing a DBPCFC of up to 5 g peanut powder (2.5 g peanut protein). Responders were defined by successful consumption of 5 g peanut powder or a 10-fold increase in the consumed dose compared to their baseline OFC. Seventy percent of subjects on active treatment were considered responders with the median consumption dose increasing from 3.5 mg to 496 mg. Eight of the 14 responders still consumed less than 500 mg at the 44 week OFC. Fifteen percent of the placebo group were classified as responders. After the 44 week challenge, the placebo group crossed over to receive 44 weeks of high dose peanut SLIT and then underwent a second OFC. Of the 16 subjects who were evaluated at this OFC, seven (44%) were responders, however, four responders still consumed less than 500 mg at the OFC. Similar trends in PN-sIgE, PN-sIgG4, basophil activation, and SPT reactivity to the Kim 2011 paper were observed. Also similar to the 2011 study, most dosing symptoms involved oral/pharyngeal itching; however, one subject required treatment with epinephrine and dosing was discontinued. Overall, these initial two studies demonstrated that SLIT is safe and moderately effective in desensitizing subjects with peanut allergy.

Based on these early studies, desensitization with peanut SLIT seemed promising, but as with OIT, the question of the longevity of these effects and the induction of SU remained

unanswered. To address this question, Burks and colleagues in the CoFAR network continued following the subjects from the 2013 cross-over study described above.[111] Subjects remained on peanut SLIT for a total of up to three years. By the two-year OFC with 10 g peanut powder, 4 out of 37 (10.8%) of the subjects were desensitized, with no difference in median consumed dose between the high dose SLIT cross-over group and the low dose group. All four of these subjects experienced SU as was determined by a 10 g challenge 8 weeks after discontinuing therapy. Again, this study demonstrated that peanut SLIT was safe with about 18% of the doses eliciting mild dose-related symptoms. The study was limited by a large number of subjects who chose to drop out, mainly due to difficulties with daily dosing, but showed that SLIT has a modest effect at inducing SU in peanut-allergic subjects.

Recent work has sought to directly compare OIT to SLIT in terms of both safety and efficacy in the treatment of peanut allergy. In a double-blinded study, Narisety, *et al.* randomized a total of 21 subjects to either active SLIT/placebo OIT or placebo SLIT/active OIT.[112] SLIT doses reached 3.7 mg while OIT doses reached 2,000 mg, and then subjects underwent two DBPCFCs after 6 months and 18 months of therapy. Both arms of the study experienced a greater than 10-fold increase in tolerated OFC dose, though the median tolerated dose in the active OIT group and active SLIT groups were 141-fold higher and 22-fold higher, respectively. More adverse events were reported in active OIT compared to active SLIT. A similar study was conducted to compare the safety and efficacy of OIT and SLIT in the treatment of cow's milk allergy.[113] All subjects underwent an initial SLIT escalation before being randomized to continuation of SLIT, low dose OIT or high dose OIT. Consistent with the findings of Narisety, *et al*., the OIT groups had a higher rate of desensitization and SU, but also a higher rate of systemic reactions during therapy.

1.7.c Epicutaneous Immunotherapy

EPIT is an emerging investigational treatment modality for peanut allergy. EPIT involves application of the antigen via a patch that is applied to the skin. Preclinical studies have shown that the antigen is taken up by dendritic cells in the dermis and does not enter the circulation, suggesting that EPIT may be a safer option for the delivery of antigen.[114] In clinical studies, the Viaskin patch, created by DBV Technologies, is 26 mm in diameter containing dried peanut extract.[115] In a Phase 1 clinical study, Jones, *et al.* demonstrated that the Viaskin patch is a safe delivery system for peanut in allergic individuals.[116] In this double-blind placebo-controlled study, 100 subjects were randomized to patches containing a range of peanut doses or placebo for two weeks. Eighty-four percent of the peanut-treated subjects experienced at least one local adverse event, though 60% of the placebo group did as well, suggesting that the symptoms may be due to the adhesive itself. Importantly, symptoms were mostly mild to moderate, including erythema and pruritus.

In a later trial, Jones, *et al.* investigated the efficacy of the Viaskin patch in desensitizing peanut-allergic subjects.[115] Seventy-four participants were randomized to placebo, Viaskin Peanut 100 µg, or Viaskin Peanut 250 µg. After 52 weeks of treatment, 12% of placebo, 46% of Viaskin Peanut 100 µg, and 48% Viaskin Peanut 250 µg subjects were desensitized. The largest median change in dose tolerated was 130 mg in the Viaskin Peanut 250 µg. Consistent with the previously published safety study, mild reactions at the site of patch application were common in the active treatment group. These results indicate that peanut EPIT is slightly effective, but has a good safety profile. The largest peanut EPIT trial to date was completed earlier this year by Sampson, *et al.*[117] In this trial, 221 subjects were randomized to treatment with three different doses of peanut or placebo for 12 months. Responders consumed a 10-fold increase in dose compared to baseline or at least 1,000 mg peanut protein. The response rate for the highest dose (250 µg) patch was 25%, with the mean cumulative reactive dose at month 12 being 1117.8 mg peanut protein. The patch seemed to have a bigger effect in the youngest

age group (6-11 years), in which the response rate was 34.2%. Adverse events in this study were common but mild, as was seen in the other EPIT studies. While peanut EPIT doesn't seem to be as effective as OIT or SLIT it is possible that a subset of allergic patients may benefit from this technology or that future adjustments in dose and/or duration of treatment may improve outcomes.

1.7.d Other Investigational Therapies

Allergen-specific immunotherapies have stolen the spotlight when it comes to investigational therapies for food allergies, especially peanut allergy. However, many other modes of treatment are under investigation both in clinical and pre-clinical studies. Food Allergy Herbal Formula (FAHF-2) is one such investigational treatment. FAHF-2 is a botanical investigational new drug consisting of 9 herbs based on a classical Chinese herbal formula.[118] Pre-clinical studies showed promising results in murine models of food allergy.[119] Peanut-sensitized mice were treated with FAHF-2 for 7 weeks and then challenged at several time intervals. FAHF-2 completely blocked anaphylaxis in the treated mice. Further, Th2 cytokine production and PN-sIgE levels were decreased in treated mice. Recently, a Phase 2 clinical study on the safety and efficacy of FAHF-2 in the treatment of food allergy was completed.[118] This study randomized 68 subjects with known allergies to peanut, tree nut, sesame, fish, and/or shellfish to treatment with FAHF-2 or placebo for 6 months of therapy before completing a DBPCFC. The treatment was safe with no serious adverse events, but the therapy was less successful than placebo at desensitizing subjects. In fact, 45.5% of the placebo-treated subjects and only 17.4% of the active treatment group had improvements in consumed allergen dose. Further, there was no significant difference between groups in terms of persistence of effect 3 months after discontinuing treatment. Overall, these findings imply that FAHF-2, at least at the dose and duration of therapy tested here, is not effective at treating food allergies in humans. Another recent animal study found that FAHF-2 combined with OIT is more effective,

resulting in longer protection than OIT alone in the treatment of food allergies.[120] These findings, though yet to be shown in human studies, provide another possible use of FAHF-2 in food allergy treatment.

Because food allergy is an IgE-mediated disease, researchers have tried to treat peanut allergic subjects with the anti-IgE monoclonal antibody, omalizumab. Sampson, *et al.* investigated the use of omalizumab independent of OIT to reduce the risk of peanut allergic reactions compared to before treatment.[121] Subjects were treated with either omalizumab or placebo for 20 to 22 weeks and then underwent an OFC. The study was stopped early because of several severe anaphylactic reactions during the entry OFC. Of the 14 subjects who completed the study prior to its discontinuation, the subjects on active treatment tended to have greater improvements in tolerated peanut dose. Omalizumab has since been studied in combination with peanut OIT as discussed previously in Section 1.7.a.

The use of recombinant peanut proteins that maintain their ability to bind T cells but lack the ability to bind IgE would provide a safer form of immunotherapy. To accomplish this, researchers mutated the IgE-binding epitopes while leaving the T cell epitopes intact.[122,123] As discussed in Section 1.4, the IgE epitopes of the major peanut allergens have been identified, but more specifically, the amino acids important for the binding have been identified. Moreover, multiple T cell epitopes that varied from patient to patient were identified.[122] Bannon, *et al.* performed site-directed mutagenesis on peanut allergens Ara h 1, Ara h 2, and Ara h 3.[123] They demonstrated that these modified proteins maintained their ability to induce T cell proliferation but were poor competitors for binding to PN-sIgE. To demonstrate the effect that the modified proteins would have on effector cell degranulation, a passively sensitized RBL-2H3 cell line was stimulated with different concentrations of peanut extract, wild-type Ara h 2, or mutated Ara h 2. Results confirmed that a higher dose of the modified protein than the wild-type protein was required to elicit 50% β-hexaminidase release by the cells.[122] In a mouse model of peanut allergy, mice were sensitized with wild-type Ara h 2 and desensitized with either PBS, wild-type

or mutated Ara h 2 and then challenged.[123] The mice treated with the mutated Ara h 2 had less severe symptom scores and lower plasma histamine levels following challenge than either of the other two groups. Surprisingly, human IgE-binding assays identified several peanut-allergic subjects in whom binding to IgE was not altered with mutated Ara h 2. The mutated recombinant allergens were tested in a Phase 1 trial of E. coli-encapsulated recombinant modified peanut proteins, Ara h 1, Ara h 2, and Ara h 3.[124] Five of the 10 peanut-allergic subjects enrolled in the trial experienced adverse reactions that prevented them from continuing dosing. These results suggest that either not all IgE-binding epitopes were identified or the binding in these subjects depends on more than just the amino acids mutated.

Similar in rationale to the use of mutated recombinant proteins, researchers have tried to develop peptide immunotherapy for the treatment of peanut allergy. This strategy uses short synthetic peptides that contain the sequences of T cell epitopes, but are not long enough to cross-link IgE, thus should not elicit allergic symptoms. Intranasal or subcutaneous administration of a vaccine containing 30 overlapping Ara h 2 peptides, 20 amino acids in length, reduced symptoms of anaphylaxis as well as serum levels of Ara h 2-specific IgE in a C3H/HeJ murine model of peanut allergy.[125] One study sought to develop short T cell epitope-based peptides that target Ara h 2-specifc CD4+ T cells but can't cross-link Ara h 2-specific IgE.[126] By using T cells from 16 HLA-diverse patients, five dominant CD4+ T cell epitopes were identified in Ara h 2. Three short peptide variants, each less than 18 amino acids long and containing these epitopes, were created. Peptides were modified to have serines replace cysteines in order to increase stability. Experiments using sera from these subjects confirmed that none of the peptides bound IgE. ELISPOT cytokine assays demonstrated that the peptides maintained their ability to stimulate T cells to produce IL-4, IL-5, and IFNγ. This methodology has yet to be developed further for treatment in human studies.

A great deal of pre-clinical work has been done investigating the use of DNA vaccines to treat type 1 hypersensitivities, including food allergies. It was shown over 25 years ago that

injection of mouse skeletal muscle with DNA or RNA expression vectors resulted in protein expression that was detectable in the muscle for up to 2 months after injection.[127] Additionally, it has been long known that such DNA vaccines produce a humoral response and that this response can be boosted with subsequent doses of the vaccine.[128] Of particular interest for the treatment of food allergy, a Th2-skewed process, DNA vaccines result in a Th1-skewed immune response.[129] The use of genetic vaccination and the rationale for its use to treat peanut allergy in pre-clinical models have been extensively reviewed elsewhere.[130] Briefly, the potential of an Ara h 2 DNA vaccine has been investigated in several mouse strains to treat peanut allergy.[131,132] A study where C3H/HeSn, AKR/J, and BALB/c mice received intramuscular injections with the vaccine demonstrated that the immune response induced varied by strain. These results suggest that there will be similar variability in humans. A mouse study found that treatment with a single multivalent peanut (Ara h 1, 2, and 3) Lysosomal Associated Membrane Protein DNA Plasmid Vaccine (ARA-LAMP-vax; Astellas Pharma Inc.) protected peanut-sensitized mice from allergic reactions following challenge.[133] Currently, a Phase 1 clinical trial is enrolling subjects to test the safety and tolerability of ARA-LAMP-vax for the treatment of peanut allergy in humans.

While several of the treatment modalities listed here have shown promise in animal and/or human studies, they have not been investigated in human studies at all or to the same extent as peanut OIT, SLIT, and EPIT discussed previously.

1.8 Animal Models

As with all diseases, animal models of peanut allergy have been paramount in understanding the pathophysiology of the disease as well as the development of investigational therapies, such as those previously discussed in Section 1.7.d. Extensive work on small animal models has been done, though models in larger animals including pigs, dogs, and sheep have also added to the field. A major challenge in the development of food allergy models is that the

default immune response to antigens in the gastrointestinal tract is tolerance. Thus, to induce an allergy, researchers first need to break oral tolerance. This has been done in numerous ways including the use of adjuvants and manipulation of the epithelial barrier. Because oral tolerance is the natural response, some researchers believe that sensitization occurs through the skin, and mouse models that are sensitized through this route have been developed. Further manipulation including the creation of humanized mice have been used to model food allergy. These models as well as large animal models will be discussed here.

1.8.a Th2-skewing Adjuvants

Th2-skewing adjuvants have been used to break oral tolerance in animal models of food allergy. Cholera toxin (CT), one such Th2-skewing adjuvant, has been used extensively in food allergy models. In models of both cashew allergy and peanut allergy, oral co-administration of CT and food antigen to either BALB/cJ or C3H/HeJ mice on days 1, 8, 15 and 22 induces allergen-specific IgE and IgG1 production.[134,135] C3H/HeJ mice contain a Toll-like receptor (TLR) 4 mutation, leaving the receptor defective, believed to be at least partly responsible for the Th2-skewing of these animals.[136] Upon subsequent challenge via intraperitoneal (IP) injection with the antigen, mice experience anaphylaxis. Reactions can be measured objectively by decrease in body temperature following challenge. Reacting mice also exhibit symptoms of allergic reactions including itching, puffiness, decreased activity, cyanosis, labored breathing or even death. Importantly, one group has shown that sensitizing C3H/HeJ mice through a similar protocol results in mice that show signs of a reaction following oral challenge with antigen.[137] Li, *et al.* showed that, as with the IP challenge model, mice sensitized with peanut extract and CT have increased PN-sIgE, exhibit symptoms including puffiness around the eyes and mouth and diarrhea as early as 10-15 minutes after challenge, have increased plasma histamine levels, and significant T cell proliferative responses to peanut stimulation. Unfortunately, these oral reactions have not been able to be recapitulated by other groups,

including our own. As a result, most groups have to challenge these mice by IP injection, leaving the need for an orally reacting mouse model of peanut allergy.

The mechanisms by which CT breaks oral tolerance have yet to be confirmed, though such findings may help to shed light on the break-down of oral tolerance in humans. Studies have shown that *in vitro* treatment with CT leads to maturation of macrophages and dendritic cells and upregulation of their costimulatory molecules as well as chemokine receptors.[138] Specifically, CT was shown to increase OX40L expression in CD11c+ dendritic cells located in the mesenteric lymph nodes, and neutralizing antibodies against OX40L abrogated the CT-induced Th2 response.[139] Several experiments have shown that treatment with CT results in increased migration of dendritic cells to lymph nodes and to the T cell area of the Peyer's patch.[139-141] Further, these antigen presenting cells were then able to prime naïve CD4+CD45RA+ T cells and drive their polarization towards a Th2 phenotype. Separately, studies have found that treatment of mice with CT results in increased levels of IL-1 in the gastrointestinal tract and that activating dendritic cells with IL-1 results in Th2 skewing.[142,143] Collectively, these findings suggest that alterations in dendritic cells may play an important role in the break-down of oral tolerance and resulting sensitization to foods.

Another model of food allergy uses Staphylococcal Enterotoxin B (SEB), a common food contaminant, as an adjuvant to break oral tolerance in mice.[144] BALB/c mice or C57BL/6 mice were orally gavaged with either Ovalbumin (OVA) and SEB, or peanut extract and SEB once a week for eight weeks. At nine weeks, they were challenged orally with antigen. Mice sensitized with OVA and SEB developed an IgE antibody response to the antigen. Oral challenge with OVA elicited allergic symptoms in mice sensitized with adjuvant as well as resulted in increased plasma histamine levels and hypotension. Similar results were observed for mice sensitized and challenged with peanut extract. In a study that sensitized mice by IP administration of SEB and OVA, Yang, *et al.* reported that T cell immunoglobulin-domain and mucin domain (TIM)-4 and costimulatory molecules were upregulated in intestinal mucosal dendritic cells.[145] Blocking

TIM-4 with a polyclonal antibody dampened the Th2 response in these mice, suggesting a potential mechanism for SEB-mediated sensitization. Similar to the unreproducible findings above in C3H/HeJ mice with CT, the oral reactions following this SEB sensitization scheme have proven unreproducible in unpublished work by our group and others. Overall, these data demonstrate that SEB exposure in mice can lead to allergic sensitization in some published reports.

The last adjuvant commonly used to break oral tolerance in mice is aluminum hydroxide (alum). Using this model, mice were successfully sensitized to tree nuts.[135] Tree nut extracts and alum were administered by IP injections over four weeks. Mice sensitized according to this protocol mount an IgE response to tree nuts. Following IP challenge, mice experienced hypothermia with body temperatures decreasing greater than five degrees compared to baseline. The use of alum to break oral tolerance has been expanded to sensitize mice to peanut in a similar manner.[146]

1.8.b Epicutaneous Sensitization

Food allergy is closely associated with eczema in humans. Often the development of eczema precedes the development of a food allergy, and with the impaired skin barrier present in eczema, it seems possible for patients to be sensitized through the skin. Strid, *et al.* developed a model of epicutaneous sensitization in which BALB/c mice were exposed, following the removal of the stratum corneum with tape-stripping, epicutaneously to peanut protein and then underwent an oral challenge.[43] They found that following sensitization, mice mounted a Th2 response characterized by the production of IgE and IL-4. Oral challenge resulted in further sensitization. In a recent study, Tordesillas, *et al.* exposed both C3H/HeJ mice and BALB/c mice to peanut on undamaged skin. Repeated application of antigen led to sensitization and anaphylaxis upon oral challenge.[147] Sensitized mice produced Ara h 1- and Ara h 2-specific IgE. By purifying dendritic cells from the draining lymph nodes of epicutaneously sensitized

mice and culturing them with CD4+ T cells, researchers showed that these cells were stimulated to produce Th2 cytokines, IL-4 and IL-5. These results suggest that the dendritic cells are sufficient to induce Th2 priming. Interestingly, this study also found that peanut acted as an adjuvant in the epicutaneous sensitization to OVA. These studies along with several others[148,149] that demonstrate epicutaneous sensitization give rise to a possible initial exposure site in humans.

1.8.c Airway Sensitization

Recently, the first animal model of peanut sensitization following airway exposure was developed.[46] In this study, BALB/c mice and C57BL/6 mice were exposed to peanut flour in the absence of an adjuvant for four weeks by inhalation. After the four week sensitization period, both strains produced PN-sIgE. Mice IP challenged with peanut extract elicited symptoms of an allergic reaction including hypothermia. In this model, airway exposure initiates peanut allergy by involving the IL-1 pathway and IL-4- and IL21-secreting T follicular helper (Tfh) cells. Further research is needed to determine if the contribution of Tfh cells is unique to airway sensitization.

1.8.d Humanized Mouse Models

While work is ongoing to improve mouse models of peanut allergy, the concern remains that differences between rodent and human physiology limit understanding of disease. Recently two humanized mouse models have been created to ameliorate this problem. In the first, published in 2016 by Bryce, *et al.,* a model was developed on the non-obese diabetic (NOD)-severe combined immunodeficient (SCID) IL2rg^{null} SCF/GM-CSF/IL3 (NSG-SGM3) strain engrafted with human thymus, liver, and hematopoietic stem cells (referred to as BLT) that supports human mast cell engraftment.[150] Mast cells were phenotypically similar to human mast cells, expressing FcεRI, CD117, and tryptase. Human, tryptase-positive mast cells could be found in the lung and spleen of these mice. Passive cutaneous and passive systemic anaphylaxis models were developed in these mice using administration of a chimeric IgE

containing human constant regions. Additionally, Burton, *et al.*, engrafted NOD-SCID mice carrying a human stem cell factor transgene with hematopoietic stem cells.[151] These mice were engrafted with functional human T and B lymphocytes and human mast cells. Humanized mice were sensitized and challenged intragastrically with peanut butter in sodium bicarbonate. Mice produced a PN-sIgE response and exhibited signs of anaphylaxis including hypothermia. These mice will provide a valuable tool for studying the immune system during sensitization, anaphylaxis and treatment, as well as enable the development and testing of targeted therapies.

Interestingly, all mouse models of food allergy are sex-specific. Only female mice are able to be reproducibly sensitized. Though it has been observed in humans that menses can affect reaction thresholds to foods, little is known about any other differences between males and females with the disease. There is a need to better understand these differences as well as a need to develop a mouse model of peanut allergy in which both males and females react, as future therapies should be tested in both sexes.[152]

1.8.e Large Animal Models

As discussed previously, one of the limitations in murine models of food allergy, is that the natural response to antigen in the gastrointestinal tract is oral tolerance. Dogs on the other hand are one of the few animals that naturally exhibit allergies to a range of antigens including food and environmental antigens. Thus, dogs provide a potentially valuable model in which to study allergy and anaphylaxis. In 2002, Teuber, *et al.* published a canine model of food allergy in which dogs were sensitized starting soon after birth to peanut, English walnut, Brazil nut, soy, wheat, and barley in the presence of alum.[153] By 6 months of age, SPTs were positive to these antigens and at two years of age, the four dogs sensitized to peanut reacted upon oral challenge. Symptoms included vomiting and lethargy, but all resolved spontaneously without intervention. This model has since been used to test the use of heat-killed Listeria monocytogenes as an adjuvant for immunotherapy.[154] These results demonstrate that dogs can

be a sensitized to peanut, and thus can be used to improve understanding of peanut allergy as well as test new therapeutics.

One group previously used sheep to developed an allergic asthma model, and in 2012 they sought to examine the allergic response following sensitization to peanut allergens.[155] Twenty sheep were sensitized separately to peanut extract and either OVA (experimental phase 1) or house dust mite (experimental phase 2) by four subcutaneous injections in the presence of alum. For the two phases of this study, 40-50% of sheep were sensitized to peanut, as was defined as greater than 50% increase in PN-sIgE levels compared to baseline. Nearly all sensitized sheep produced an IgE response to major peanut allergens, Ara h 1 and Ara h 2. Four out of the five sensitized sheep in Phase 1 also had a positive skin prick test to peanut.

A 2002 report demonstrated the usefulness of pigs as a model system in which to study food allergy and anaphylaxis.[156] Previously, it was shown that pigs could be used as a model for asthma.[157] Because asthma and food allergy are both Th2-skewed processes, Helm, *et al.* investigated the use of neonatal pigs as a model system for peanut allergy.[156] Newborn piglets were sensitized by IP injection with peanut extract and CT five times within 4 weeks after birth. Pigs underwent an intragastric challenge and skin prick testing alternating at weekly intervals. Reaction symptoms were observed in 11 out of 14 of the sensitized animals that underwent an oral challenge with peanut. Three reacting piglets had signs of respiratory distress and anaphylactic shock, requiring treatment with epinephrine. Skin prick tests were positive in peanut-sensitized animals, suggesting IgE and mast cell involvement. These large animal models provide useful tools for the future study of peanut allergy, although high costs and limited immunologic reagents available for these animals limits enthusiasm.

Despite all of these advances, a major goal in the development of food allergy models has been to create a model in which animals are sensitized in the absence of adjuvant and react upon oral exposure to the allergen. While this has been shown in models of some routes

of sensitization including epicutaneous, a model that reproducibly is both sensitized and reacts orally has yet to be identified.

1.9 Topics Addressed

The research presented in this dissertation addresses several knowledge gaps necessary to improve upon, and develop therapy options for food allergies, focusing specifically on peanut allergies. Chapter 2 will further explore the mechanism of peanut OIT and SLIT by determining the role that IgG plays in regulating the activation of basophils following therapy. Chapter 3 will introduce a new investigational treatment targeting antigen-specific B cells to prevent the development of peanut allergy. The lack of an orally reacting animal model of peanut allergy that closely mimics human disease has been extremely limiting to the development of treatments. Chapter 4 will present the development of such an animal model of peanut allergy, which will be invaluable to the development of therapies, as well as understanding of the disease.

1.10 Tables

Table 1-1. Immunotherapies under investigation for the treatment of peanut allergy.

	Oral Immunotherapy	Sublingual Immunotherapy	Epicutaneous Immunotherapy
Trial Phase (ongoing)	Phase 3	Phase 2	Phase 3
Form of Peanut	Powder	Liquid extract	Dried extract
Dose (protein quantity per day)	300-4,000 mg	2-7 mg	100-500 µg
Safety	Mostly oral or gastrointestinal; highest risk for systemic adverse effects	Local (oral or pharyngeal) effects	Local (skin) effects; lowest risk for serious adverse effects
Efficacy			
Desensitization	Large effect	Moderate effect	Variable effect
Sustained Unresponsiveness	Many subjects	Subsets of subjects	Not known

1.11 Figures

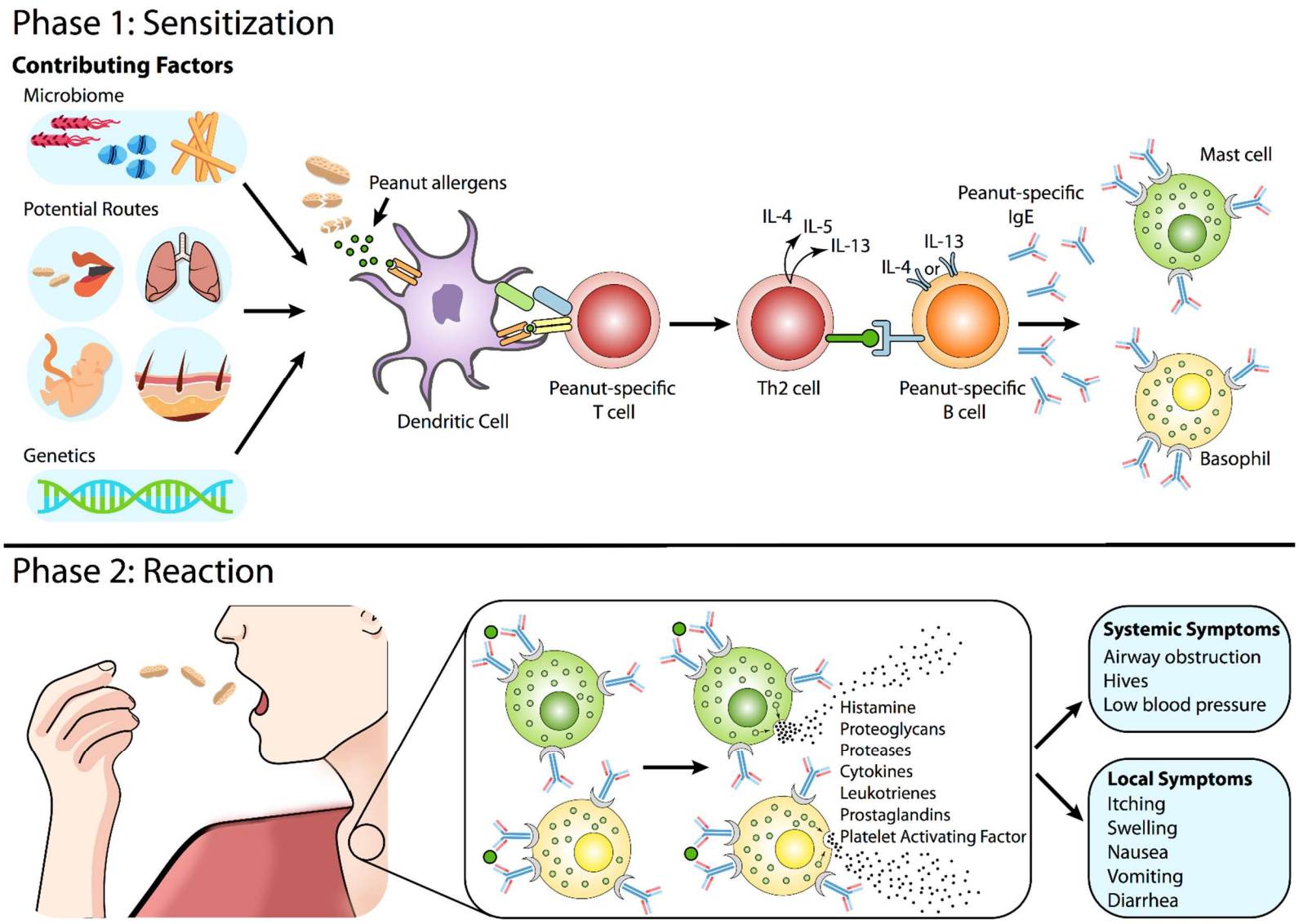
Phase 1: Sensitization
Contributing Factors
Microbiome
Potential Routes
Genetics
Peanut allergens
Dendritic Cell
Peanut-specific T cell
IL-4
IL-5
IL-13
Th2 cell
IL-13
IL-4 or
Peanut-specific B cell
Peanut-specific IgE
Mast cell
Basophil
Phase 2: Reaction
Histamine
Proteoglycans
Proteases
Cytokines
Leukotrienes
Prostaglandins
Platelet Activating Factor
Systemic Symptoms
Airway obstruction
Hives
Low blood pressure
Local Symptoms
Itching
Swelling
Nausea
Vomiting
Diarrhea

Figure 1-1. Schematic of peanut allergy pathogenesis. During the sensitization phase (Phase 1), there are several factors that are proposed to influence the development of food allergy. These factors include a person's gut microbiome, potential routes of exposure such as oral, airway, *in utero*, or epicutaneous, and a person's genetics. During sensitization, allergen is taken up by antigen presenting cells such as dendritic cells and presented to naïve peanut-specific T cells via MHC Class II molecules. This triggers the maturation of Th2 cells which secrete IL-4, IL-5, and IL-13. These cytokines, in addition to the binding of co-stimulatory molecules, results in IgE class-switching by peanut-specific B cells. IgE will then bind to FcεRI receptor on mast cells and basophils. The reaction phase (Phase 2) occurs following subsequent ingestion of peanut. Peanut allergens can bind and cross-link IgE on the surface of mast cells and basophils, triggering the release of mediators, which ultimately result in allergic symptoms.

CHAPTER 2: BLOCKING ANTIBODIES INDUCED BY PEANUT ORAL AND SUBLINGUAL IMMUNOTHERAPY SUPPRESS BASOPHIL ACTIVATION AND ARE ASSOCIATED WITH SUSTAINED UNRESPONSIVENESS

2.1 Introduction

Peanut allergy is a major public health concern affecting 1% of the US and European populations, rising in prevalence, and outgrown in only 20% of those affected with the disease.[5,9,158] Although there are no FDA- or EMA- approved treatments for peanut allergies, extensive investigation has focused on the use of several immunotherapy approaches. Emerging peanut allergy therapies include OIT,[102,105,106] SLIT,[109,111] and EPIT,[115,117] among others.[159] While routes, doses, and duration vary for each form of therapy, these therapies all expose the allergic subject to increasing quantities of peanut protein over a period of months to years. OIT and SLIT have been effective at inducing both short-lived desensitization (defined as an increased allergen threshold while taking therapy daily) and SU (defined as an absence of allergic symptoms during challenge after stopping therapy).[160,161] However, no reliable biomarkers exist to identify which subjects will achieve SU and which will be transiently desensitized.

Despite the promising clinical findings, the mechanisms by which OIT and SLIT alter the immune system and result in desensitization or SU remain unclear. Skin prick test data and mechanistic studies have previously shown that peanut immunotherapies promote mast cell and basophil hyporesponsiveness[102,103,162] as well as an increase in Tregs.[102,103,163] Extensive work by our group and others has demonstrated that PN-sIgE levels often transiently increase on OIT and SLIT within a few months of starting therapy but are significantly decreased after many

months or years of therapy.[102] It is important to note that PN-sIgE levels are uncoupled from desensitization as observed when PN-sIgE is increased from baseline but clinical reactivity and mast cell and basophil degranulation has diminished.[104] OIT and SLIT both cause a significant increase in levels of PN-sIgG4 though these quantities alone have not been shown to be predictive of SU versus desensitization.[105]

Because OIT and SLIT result in basophil hyporesponsiveness in the presence of persistent levels of IgE, it has been speculated that IgG4 plays a role in blocking the IgE-mediated activation of basophils and mast cells in allergic subjects on OIT and SLIT. Previous work has shown that serum from subjects on peanut OIT inhibits facilitated antigen binding, suggesting that a plasma factor has antigen-specific blocking capabilities.[105] Two recent studies have investigated the functional role of IgG in the context of effector cell inhibition. In the first, LAD2 mast cells passively sensitized with plasma from peanut-allergic subjects exhibited greater activation following peanut stimulation than those passively sensitized with plasma from peanut-sensitized but tolerant subjects, which had higher levels of PN-sIgG4.[164] Furthermore, plasma from subjects on peanut OIT was able to block mast cell activation and removal of IgG4 partially abrogated these findings. Similarly, a second study demonstrated that sera from mice on OVA OIT or humans on peanut OIT suppressed activation of sensitized bone marrow mononuclear cells or basophils, respectively.[165] Inhibition of these effector cells was further shown to be dependent on the inhibitory receptor FcγRIIb.

In our present study, we definitively demonstrate that OIT-induced changes in IgG lead to suppression of basophil activation to peanut and that there is a cellular-bound and unbound mechanism involved. We also compared the inhibitory effects of plasma from subjects on peanut OIT and SLIT and on different durations of therapy. Finally, we sought to determine whether basophil inhibition caused by plasma transfer can be used to distinguish subjects that experience desensitization from those that experience SU on peanut OIT or SLIT.

2.2 Materials and Methods

2.2.a Clinical trials

OIT and SLIT studies were IRB approved and study drug administered under INDs.[166] The details provided below are relevant to the present studies on blocking antibodies.

OIT 1 (Clinical trial #s: NCT00815035, NCT00597675): Peanut-allergic subjects were randomized to peanut OIT or placebo. Doses started at 0.1 mg and reached maintenance doses of 4,000 mg protein. Subjects on placebo crossed-over to active treatment after 12 months. After 48 months of active treatment, subjects underwent an oral food challenge (OFC) to assess desensitization. Subjects were taken off therapy for up to three months before undergoing an OFC to assess SU. For the purpose of these experiments, a 5,000 mg peanut cut-off was used to define SU.

OIT 2 (NCT01814241): Peanut-allergic subjects were given open-label peanut OIT up to 1450 mg. After six months of active treatment, subjects underwent an OFC to assess desensitization. Subjects were taken off therapy for up to one month before undergoing an OFC to assess SU. For the purpose of these experiments, a 3,750 mg peanut cut-off was used to define SU.

SLIT (NCT00597727): Peanut-allergic subjects received peanut SLIT. Maintenance doses reached 2 mg protein. After 60 months of active treatment, subjects underwent an OFC to assess desensitization. Subjects were taken off therapy for 1 month before undergoing an OFC to assess SU. For the purpose of these experiments, a 1,750 mg peanut cut-off was used to define SU.

2.2.b Plasma samples

For each of these studies, venous blood was drawn into sodium-heparin tubes. Whole blood was centrifuged and plasma collected. Within 24 hours of the blood draw, plasma samples were stored at -20°C until analysis. Peanut-specific IgE, IgG4, and IgG values were

collected using a Phadia ImmunoCAP100 (Thermo Scientific, Portage, MI) according to manufacturer's instructions.

2.2.c IgG antibody depletion

Pierce Protein A/G Agarose Beads (Pierce Biotechnology, Rockford, IL) were washed 3 times with PBS prior to use. An equal volume of beads (suspended in PBS) and plasma, or PBS and plasma were mixed and incubated on a rotator for 90 minutes for IgG depletion or sham depletion, respectively. Plasma was separated from the beads by centrifuging at 1,000 x g for 90 seconds and the supernatant collected. Peanut-specific IgE, IgG4, and IgG values from the sham and IgG-depleted samples were quantified using a Phadia ImmunoCAP100 according to manufacturer's instructions.

2.2.d Basophil activation and inhibition assays

For basophil assays on blood from peanut-allergic donors, 200 µL whole blood was centrifuged at 300 x g for 10 minutes. Plasma from the allergic donor was removed and replaced with an equal volume of plasma from a subject on OIT, plasma from a subject on placebo (Figure 2-1), or pooled plasma in the case of IgG-depleted plasma (Figure 2-2). Cells were incubated at 37°C and 5% CO_2 for 1 hour. In the case of the plasma removal experiment to test the bound versus unbound plasma fraction's blocking ability (Figure 2-3), the plasma was removed by centrifuging the sample at 300 x g for 10 minutes and replacing the plasma with an equal volume of PBS. For all experiments, following incubation with plasma, cells were stimulated with 200 µL of peanut extract (final concentration of 0.01 µg/mL) diluted in RPMI containing 2 ng/ml human IL-3 at 37°C and 5% CO_2 for 30 minutes. For each assay, a negative control consisting of blood from the allergic donor stimulated with RPMI and IL-3 was used to confirm that cells were not being activated non-specifically. Degranulation was stopped promptly at 30 minutes by adding cold 20 mM EDTA to the sample. Cells were then stained with antibodies specific for CD63-FITC (BD Biosciences, San Jose, CA), CD203c-PE (Beckman

Coulter, Indianapolis, IN), CD123-PE-Cy5 (BD Biosciences, San Jose, CA). Following staining, red blood cells were lysed and remaining cells fixed with FACS Lysis Buffer (BD Biosciences, San Jose, CA) for 15 minutes. Samples were centrifuged at 800 x g for 15 minutes, and isolated cell pellets were resuspended in staining buffer consisting of PBS, 2mM EDTA, 0.5% BSA. Samples were analyzed on a CyAn ADP (Beckman Coulter, Indianapolis, IN) flow cytometer and gated using FlowJo V10 (FlowJo, LLC, Ashland, OR) software.

For passive sensitization of basophils from a non-allergic donor (Figures 2-4 and 2-5), whole blood from a donor with no known allergies was centrifuged at 300 x g for 10 minutes. The plasma from the non-allergic donor was removed and replaced with pooled plasma from peanut allergic subjects. These pools were created by adding equal parts of plasma from 2-3 subjects and had an average PN-sIgE of 327.98 kU/L and an average PN-sIgG4 of 0.50 µg/mL. Cells were incubated with the pooled plasma from allergic subjects for 2 hours at 37°C and 5% CO_2 and mixed every 30 minutes. Following passive sensitization, blocking plasma from subjects on OIT or SLIT were applied and cells stimulated, stained, and analyzed as described above.

2.2.e Statistical analyses

GraphPad/Prism version 7.02 was used to analyze all data. Mann-Whitney U, Wilcoxon, and paired and unpaired t-tests were performed and a p-value <0.05 was considered significant. Percent inhibition of basophil activation was calculated by subtracting the %CD63+ basophils in the presence of OIT or SLIT plasma from the %CD63+ basophils at baseline, dividing by the %CD63+ basophils at baseline, and then multiplying by 100%.

2.3 Results

2.3.a Plasma from subjects on peanut OIT, but not placebo, inhibits peanut-stimulated basophil activation

Plasma from peanut-allergic subjects was removed and replaced with plasma from subjects on either 0 months or 12 months of peanut OIT as shown in the schematic in Figure 2-1A. Following stimulation with peanut extract, activated basophils (CD123+CD203c+ Lymphocytes) were identified by upregulation of cell-surface CD63 (Figure 2-1B). Incubation with 12 month active OIT plasma resulted in decreased basophil activation compared to incubation with autologous 0 month plasma ($p<0.0001$); however, this blocking of basophil activation was not observed in basophils incubated with plasma from subjects on 12 months of placebo (Figure 2-1C). This blocking capability was accompanied by small decreases in PN-sIgE (Figure 2-1D) and larger increases in PN-sIgG4 (Figure 2-1E). Similarly, we investigated the blocking capabilities of plasma from subjects who started on 12 months of placebo before crossing over to active OIT. Basophils incubated with plasma from 12 months of active therapy inhibited basophil activation ($p<0.01$), whereas plasma from the same subjects while on placebo had no effect on basophil activation compared to baseline plasma (Figure 2-1F). Taken together, these results demonstrate that OIT-induced changes in plasma can block basophil activation.

Due to limited plasma volumes for use in further experiments, we tested the ability of pooled 12 month OIT plasma to block basophil activation. Consistent with the findings for basophils incubated with individual OIT plasma, basophils incubated with pooled OIT plasma had decreased activation ($p<0.001$, Figure 2-S1). These findings show that pooled plasma can be used to further study the inhibitory effect on basophils. As a result, pooled plasma was used for the experiments in Figures 2-2 and 2-3.

2.3.b OIT-induced IgG acts through both cellular-bound and unbound mechanisms to inhibit basophil activation

Since peanut-specific IgG subclasses such as IgG1 and IgG4 have been shown to increase throughout peanut OIT, we hypothesized that the observed basophil inhibition was at least partly due to changes in IgG. To test this hypothesis, we added IgG-depleted plasma to basophils from peanut-allergic individuals. PN-sIgG (Figure 2-2A) and PN-sIgG4 ($p<0.01$, Figure 2-2B) levels were decreased to levels <1 μg/mL following depletion with Protein A/G beads compared to sham depletion. PN-sIgE levels also decreased with depletion, although IgE was readily detectable and decreases were modest (Figure 2-2C). The ratio of PN-sIgE to PN-sIgG4 increased with depletion compared to sham depletion, though changes were not significant (Figure 2-2D). These results suggest that the depletion had a greater effect on IgG and IgG4 than IgE, as expected. Basophils incubated with either undiluted or sham depleted 12 month OIT plasma had decreased activation compared to baseline (0 mo) plasma ($p<0.01$) (Fig 2-2E). This blocking was abrogated by IgG depletion ($p<0.01$, Figure 2-2E), demonstrating that OIT-induced IgG is critical for the blocking of basophil activation, consistent with the findings of others.[164,165]

Two hypotheses have been proposed for the mechanism(s) by which IgG acts to inhibit basophils and mast cells. The first is by binding to and intercepting antigen, preventing antigen from binding to IgE on effector cells. The second involves IgG binding to FcγRIIb on effector cells and resulting in the propagation of an inhibitory signal.[165] We tested whether the IgG in OIT plasma acts through either a bound, or unbound mechanism, or a combination of both. To do this, pooled 12 month OIT plasma was incubated with cells from an allergic subject in two tubes in parallel as shown in Figure 2-3A. OIT plasma is left on one sample, testing both bound and unbound mechanisms, and removed from the other sample, leaving only the bound fraction of OIT plasma. Samples in which OIT plasma was removed had greater basophil activation than the samples still containing OIT plasma ($p<0.0001$); however basophil activation following

plasma removal was still inhibited compared to the stimulated cells at baseline ($p<0.001$, Figure 2-3B). The 12 month plasma samples used had no difference in PN-sIgE quantities, but significantly more PN-sIgG4 compared to baseline (Figures 2-3C and D). These findings indicate that OIT-induced changes in plasma, potentially IgG4, are acting through both a bound and unbound mechanism to inhibit basophil activation.

2.3.c OIT and SLIT subjects' plasma have similar basophil inhibition capacity

Due to limited availability of basophils from peanut-allergic donors, we developed an assay to passively sensitize basophils from non-allergic donors (Figure 2-4A). In this assay, the plasma from a non-allergic subject was replaced by pooled plasma from peanut-allergic subjects. Prior to this passive sensitization protocol, the donor's basophils are not activated upon stimulation with peanut extract; however, they can be activated with peanut stimulation following incubation with plasma from allergic subjects, and activation can be blocked with OIT plasma (Figure 2-4B). Using this tool, we sought to determine if the plasma from subjects on OIT and SLIT had similar effects on basophil activation. Surprisingly, plasma from a 6 month OIT regimen had a greater inhibition effect on basophils than plasma from a 48 month OIT regimen ($p<0.01$, Figure 2-4C), although both had >80% median inhibition. Both had similar levels of PN-sIgG4, but the 48 month OIT plasma samples had significantly less PN-sIgE (Figures 2-4D and E). Similar inhibition of basophil activation was observed for plasmas from 6 months of OIT and SLIT (Figure 2-4F). These samples contained similar levels of PN-sIgE, but the OIT samples had higher levels of PN-sIgG4 (Figures 2-4G and H). Plasma from the time of desensitization challenge (6 months for OIT and 60 months for SLIT) while subjects were still on therapy demonstrated no difference in blocking ability when incubated undiluted with basophils (Figure 2-4I). Diluting the plasma 1:4 in PBS resulted in decreased basophil inhibition for SLIT plasma compared to OIT plasma ($p<0.05$, Figure 2-4I). Despite these functional differences, PN-sIgG4 levels were higher and PN-sIgE samples were lower in SLIT samples compared to

OIT samples (Figures 2-4J and K), indicating that functional blocking capacity is not strictly related to PN-sIgG4 and PN-sIgE quantities.

2.3.d Extent of basophil inhibition by OIT, but not SLIT, plasma is associated with clinical outcomes following therapy

Ex vivo basophil activation has previously been shown to decrease on OIT but does not discriminate subjects who achieve SU from those that are desensitized.[167] We sought to determine whether the immunotherapy-induced plasma inhibition of donor basophils can be useful in distinguishing or predicting these clinical outcomes following either OIT or SLIT. When used undiluted, plasma from OIT subjects at the time of desensitization challenge who were later classified as SU did not induce a different percent inhibition than plasma from subjects who would later be identified as transiently desensitized (Figure 2-5A). Similarly, inhibition of basophil activation was not different between plasma from SU and desensitized subjects at the time of tolerance challenge after discontinuing OIT (Figure 2-5B). Nevertheless, when OIT plasma from the time of desensitization challenge was diluted 1:10 or 1:50, plasma from SU subjects had a greater percent inhibition than that from desensitized subjects, suggesting that this assay may be useful in predicting clinical outcomes after stopping therapy ($p<0.05$, Figure 2-5C). Plasma PN-sIgE levels were not different between groups at either challenge time point, though levels tended to be lower in the group that experienced SU (Figure 2-5D). Conversely, PN-sIgG4 levels were higher in the subjects who experienced SU than those who were desensitized (Figure 2-5E). Interestingly, these quantity differences were not significant at the time of desensitization challenge, when the functional differences were noted (Figure 2-5C). Despite these interesting findings from OIT samples, undiluted or diluted plasma samples from the time of desensitization challenge while on SLIT had no difference in percent inhibition of basophil activation for subjects who achieve SU compared to those that achieve desensitization (Figure 2-5F). PN-sIgE and PN-sIgG4 levels were not different between SLIT outcomes (Figures 2-5G and H). Together, these results suggest that inhibition of basophil activation may

be a biomarker for SU following OIT, and that PN-sIgG4 is likely not the only isotype involved in the basophil inhibition process.

2.4 Discussion

OIT and SLIT are two promising investigational therapies for peanut allergy with a substantial number of subjects demonstrating desensitization, and in some cases SU, however key knowledge gaps remain. There is currently no way to predict which subjects will have success with OIT or SLIT or how long protection persists after subjects discontinue therapy. Further, immunotherapy-induced immune change(s) that can distinguish subjects who achieve SU, from those that are transiently desensitized have yet to be identified. Finally, the mechanisms by which OIT and SLIT induce desensitization and SU have not been fully elucidated. Here, we definitively demonstrated that plasma from subjects on OIT and SLIT can inhibit basophil activation, a potentially important mechanism of desensitization and SU.

Several reports have described inhibition of *ex vivo* basophil activation following OIT for peanut, milk, and egg, however it is not clear how this effector cell desensitization occurs. We used plasma from subjects on OIT or placebo to demonstrate that OIT induces changes in plasma, that are at least partly IgG-dependent can inhibit basophil activation in response to peanut stimulation. Santos, *et al.* identified post-OIT PN-sIgG4 to be a factor in plasma responsible for blocking basophil activation.[164] However, in their study, depletion of IgG4 only partially abrogated the blocking effect, suggesting that additional plasma factors play a role. Our group has recently demonstrated that OIT causes increases in antigen-specific IgA, IgA1, and IgA2, in addition to increased IgG4 and that higher levels of these antibodies are associated with SU,[168] indicating a potential role for several isotypes. Further investigation is required to determine the potential functional role of IgA, IgG and their subclasses, which are known to increase during OIT and SLIT, in blocking basophil activation.

It has been proposed that IgG prevents basophil activation by either intercepting antigen before it can cross-link surface-bound IgE or by binding to inhibitory receptors on mast cells triggering an inhibitory rather than activating signal.[169,170] Other forms of immunotherapy including subcutaneous immunotherapy for bee venom[171] and grass pollen[172] induce an increase in IgG4 that intercepts antigen, preventing it from binding to cells. A previous study showed that peanut OIT-induced IgG acts through the IgG receptor, FcγRII on basophils to inhibit their activation.[165] Here, we demonstrated that IgG is acting through both a cellular-bound and unbound mechanism to prevent basophil activation following stimulation with peanut antigen. However, the OIT subjects' plasma used for this assay appeared to utilize each of these mechanisms to differing degrees. No obvious differences in PN-sIgE or IgG4 levels distinguish these samples. Regardless, our data indicate that the mechanisms responsible for basophil hyporesponsiveness *in vivo* following OIT may be dependent on both antigen interception and neutralization in the blood stream and by cell-bound IgG, likely by inhibitory signaling through FcγRIIb.

In addition to these mechanistic findings, this report is the first to compare the basophil inhibition ability of plasma from subjects on OIT and SLIT. When comparing plasma from subjects on either 6 months OIT or 6 months SLIT, we found that the ability to block basophil activation looked similar, suggesting that blocking antibodies are induced early on in OIT and SLIT. Similarly, incubation with plasma from peanut OIT and SLIT at the time of desensitization challenge (6 months for OIT, 60 months for SLIT), resulted in comparable levels of basophil inhibition. Diluting these plasma samples 1:4 prior to incubation with basophils was able to substantially reduce the inhibition effect for SLIT, but not OIT. Interestingly, plasma from these time points of OIT and SLIT contained equivalent levels of PN-sIgG4. These results could be explained by the hypothesis that both OIT and SLIT make enough IgG4 in excess to suppress basophil activation, but that there is differences in function. These differences could be related to epitope-specificity, avidity of the antibodies, and post-translational modifications, such as

glycosylation. Future experiments testing the ability of isolated peanut-specific IgA or IgG1 to inhibit basophil activation would prove useful in demonstrating their role or lack thereof following immunotherapy.[168]

This report is also the first to relate the degree to which plasma from OIT or SLIT inhibits basophil activation to clinical outcomes following completion of therapy. Basophil inhibition by undiluted plasma from subjects on 48 months of OIT was not predictive of which subjects would develop SU after time off therapy. In fact, even basophil inhibition by undiluted plasma at the time of tolerance challenge wasn't different between subjects who experienced SU from those who were transiently desensitized. However, the blocking effect of diluted plasma from subjects at the time of desensitization challenge was indicative of which subjects would later be classified as SU and which would be classified as desensitized after discontinuing therapy. Interestingly, PN-sIgG4 quantities were not different at this challenge time point between subjects who experienced SU from those who were desensitized, signifying that it is not just the quantity of PN-sIgG4, but perhaps functional changes that are important for the development of SU. Importantly, these findings were true for plasma samples from the time of desensitization challenge, suggesting that the percent inhibition of basophils by diluted plasma while still on OIT may eventually be useful as a predictive marker for subjects that will develop SU. On the other hand, basophil inhibition by plasma from subjects on 60 months of peanut SLIT was not associated with clinical outcome even when samples were diluted. Taken together, these results suggest that the mechanisms for the induction of SU may differ between OIT and SLIT.

The possible applications for the assays and findings presented here have valuable potential for food allergy research and more broadly, allergen immunotherapy studies. The assay using passively sensitized cells is a useful tool to analyze the functional changes in plasma while controlling for therapy-induced changes in the cells themselves. While these studies do not dispute alterations in the basophils and mast cells, they offer a means to isolate changes in the plasma alone. Future experiments are needed to determine intrinsic cellular

changes in subjects actively undergoing immunotherapy in addition to the plasma changes observed in these studies. For example, we have previously demonstrated impaired calcium flux due to actin rearrangement following desensitization in model systems.[173] The findings also assist in the understanding of the changes that distinguish subjects that develop SU. As a result, if the findings are replicated in larger experiments we may be able use these basophil blocking assays to determine if a subject needs to be on therapy for a longer period of time. More broadly, the improved understanding of immunotherapy mechanisms will allow for targeted therapies in the future.

2.5 Figures

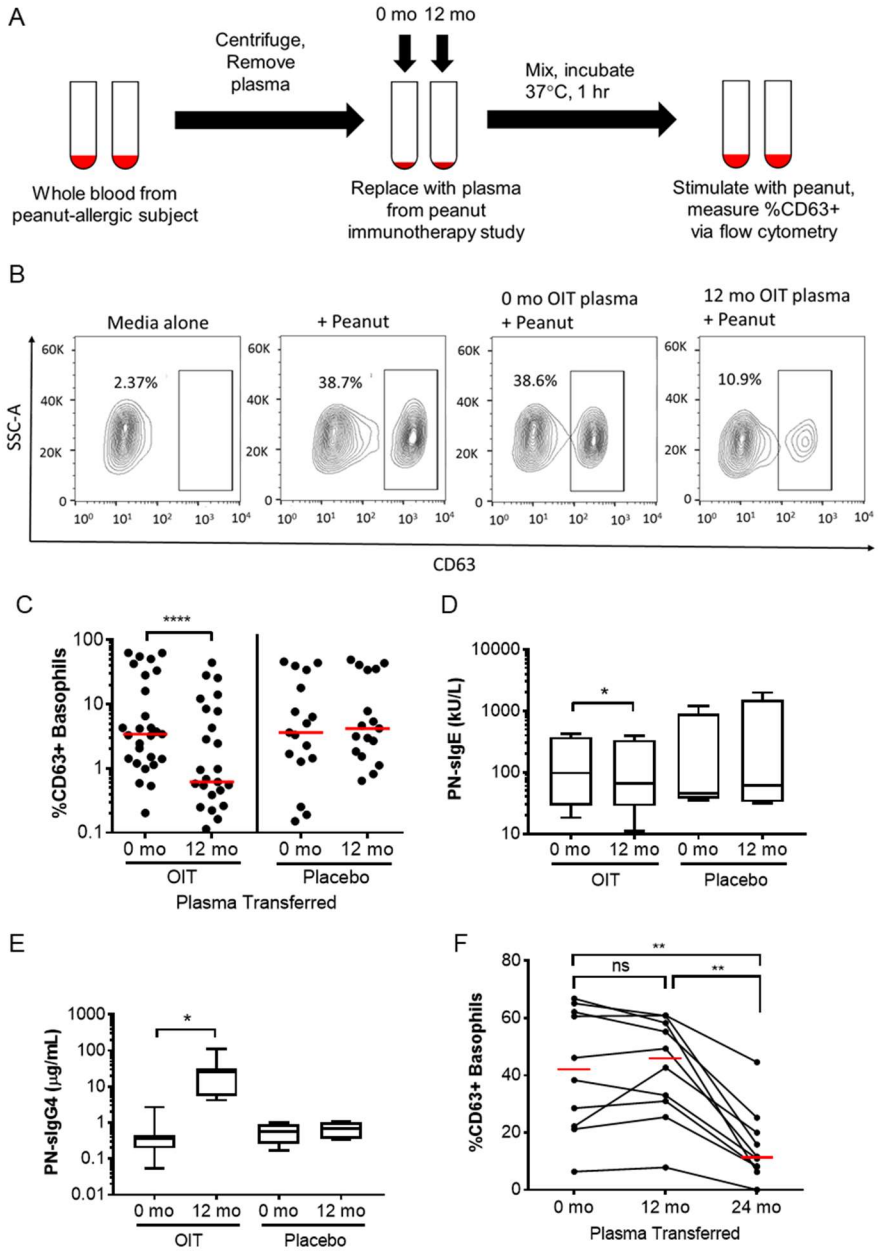

Figure 2-1. Basophil activation following pre- and post-immunotherapy plasma transfer. Assay schematic in which peanut allergic plasma is replaced with either 0 month or 12 month OIT plasma then stimulated with peanut extract (A). Representative %CD63+ basophil results are shown (B). Basophil activation for cells incubated with plasma from subjects on 0 or 12 months OIT, 0 or 12 months of placebo (C) or 12 months of placebo followed by 12 months of OIT (F). PN-sIgE (D) and PN-sIgG4 (E) shown for subjects on 0 months or 12 months of OIT or placebo. Red lines indicate medians; *p<0.05, **p<0.01, ****p<0.0001.

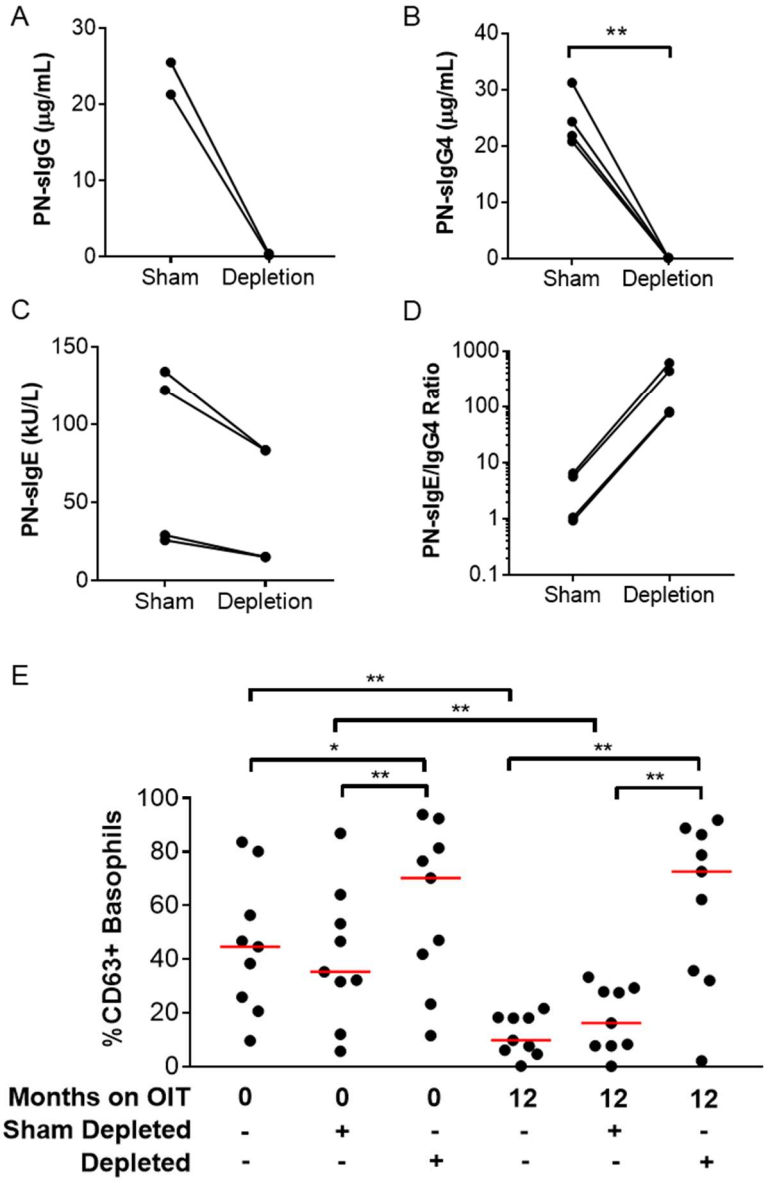

Figure 2-2. Inhibition capacity of IgG-depleted plasma. Quantities of PN-sIgG (A), PN-sIgG4 (B), PN-sIgE (C), and ratio of PN-sIgE/PN-sIgG4 (D) following sham depletion or IgG depletion of 12 month OIT plasma. Percent CD63+ basophils following incubation with undiluted, sham-depleted, or IgG-depleted 0 month or 12 month OIT plasma and stimulation with peanut extract (E). Red lines indicate medians; *p<0.05, **p<0.01.

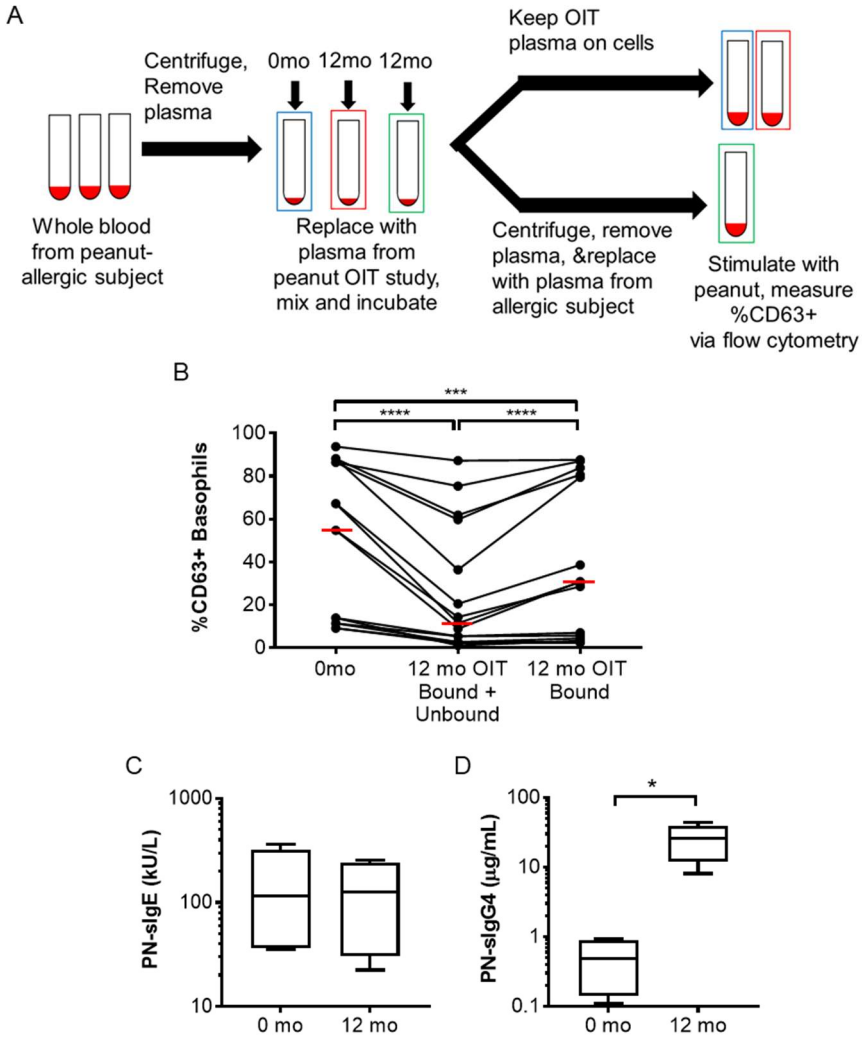

Figure 2-3. Assessment of cellular bound- and unbound-mediated plasma inhibition. Schematic for plasma transfer experiment in which basophils from a peanut-allergic donor are incubated with 0 or 12 month OIT plasma, followed by removal of 12 month OIT plasma in one tube, indicated by green outline (A) and the %CD63+ basophils for these samples following stimulation with peanut extract (B). PN-sIgE (C) and PN-sIgG4 levels for 0 and 12 month OIT plasma used in this experiment. Red lines indicate medians; *p<0.05, ***p<0.001, ****p<0.0001.

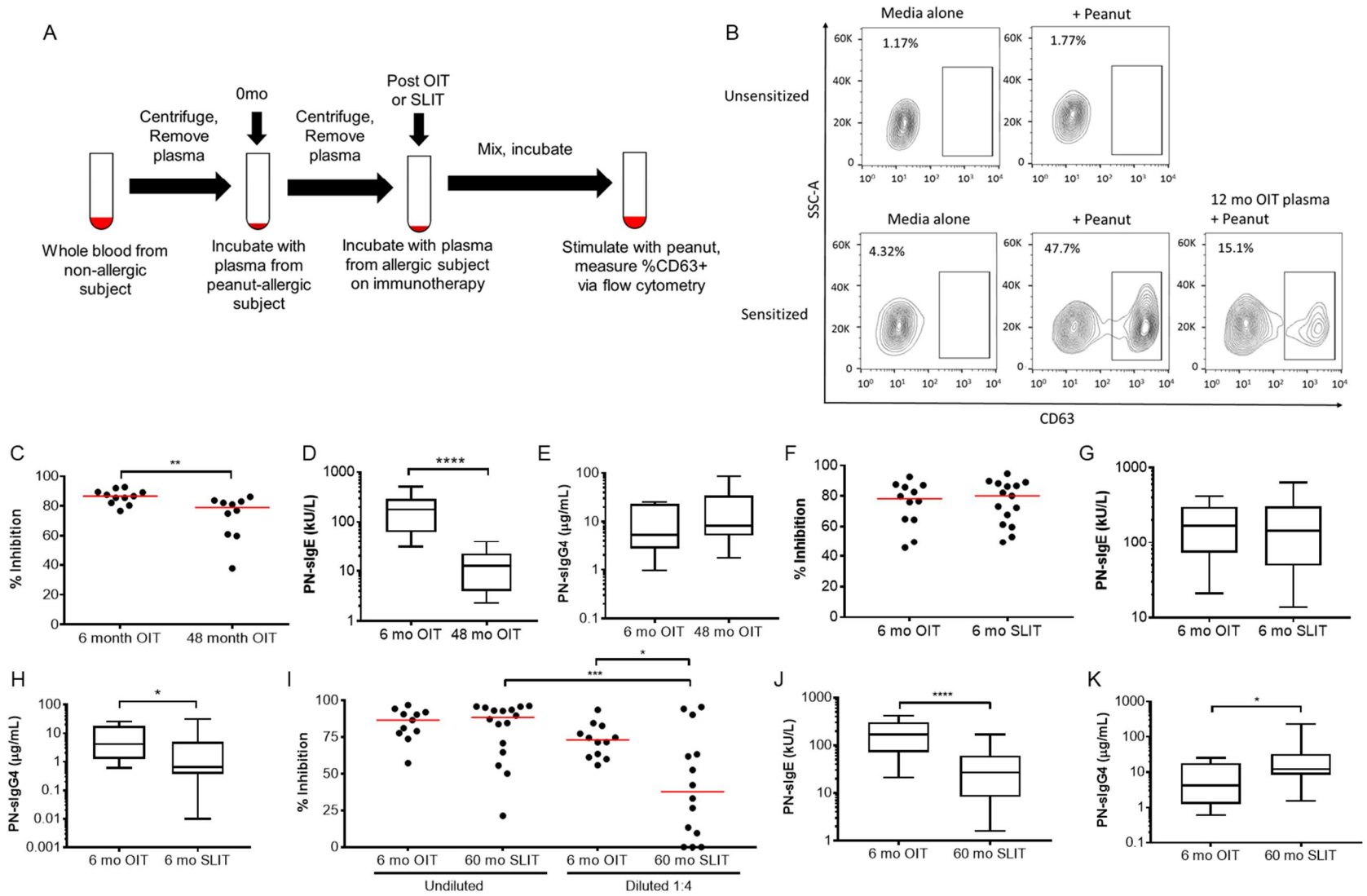
A
0mo
Post OIT or SLIT
Centrifuge, Remove plasma
Centrifuge, Remove plasma
Mix, incubate
Whole blood from non-allergic subject
Incubate with plasma from peanut-allergic subject
Incubate with plasma from allergic subject on immunotherapy
Stimulate with peanut, measure %CD63+ via flow cytometry
B
Media alone
+ Peanut
12 mo OIT plasma + Peanut
Unsensitized
Sensitized
1.17%
1.77%
4.32%
47.7%
15.1%
SSC-A
CD63
C
% Inhibition
6 month OIT
48 month OIT
D
PN-sIgE (kU/L)
6 mo OIT
48 mo OIT
E
PN-sIgG4 (µg/mL)
6 mo OIT
48 mo OIT
F
% Inhibition
6 mo OIT
6 mo SLIT
G
PN-sIgE (kU/L)
6 mo OIT
6 mo SLIT
H
PN-sIgG4 (µg/mL)
6 mo OIT
6 mo SLIT
I
% Inhibition
6 mo OIT
60 mo SLIT
6 mo OIT
60 mo SLIT
Undiluted
Diluted 1:4
J
PN-sIgE (kU/L)
6 mo OIT
60 mo SLIT
K
PN-sIgG4 (µg/mL)
6 mo OIT
60 mo SLIT

Figure 2-4. OIT and SLIT plasma blocking capabilities. Schematic for use of non-allergic donor basophils incubated with peanut-allergic plasma in the basophil activation assay (A) and representative results (B). Percent inhibition following incubation with plasma at the time of tolerance challenge for a 6 month OIT and 48 month OIT regimen (D) and corresponding PN-sIgE (D) and PN-sIgG4 (E) levels. Percent inhibition following incubation with plasma following 6 month OIT or 6 month SLIT (F) and corresponding PN-sIgE (G) and PN-sIgG4 (H) levels. Percent inhibition following incubation with undiluted and diluted 6 month OIT or 60 month SLIT plasma (I) and corresponding PN-sIgE (J) and PN-sIgG4 (K) levels. Red lines indicate medians; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

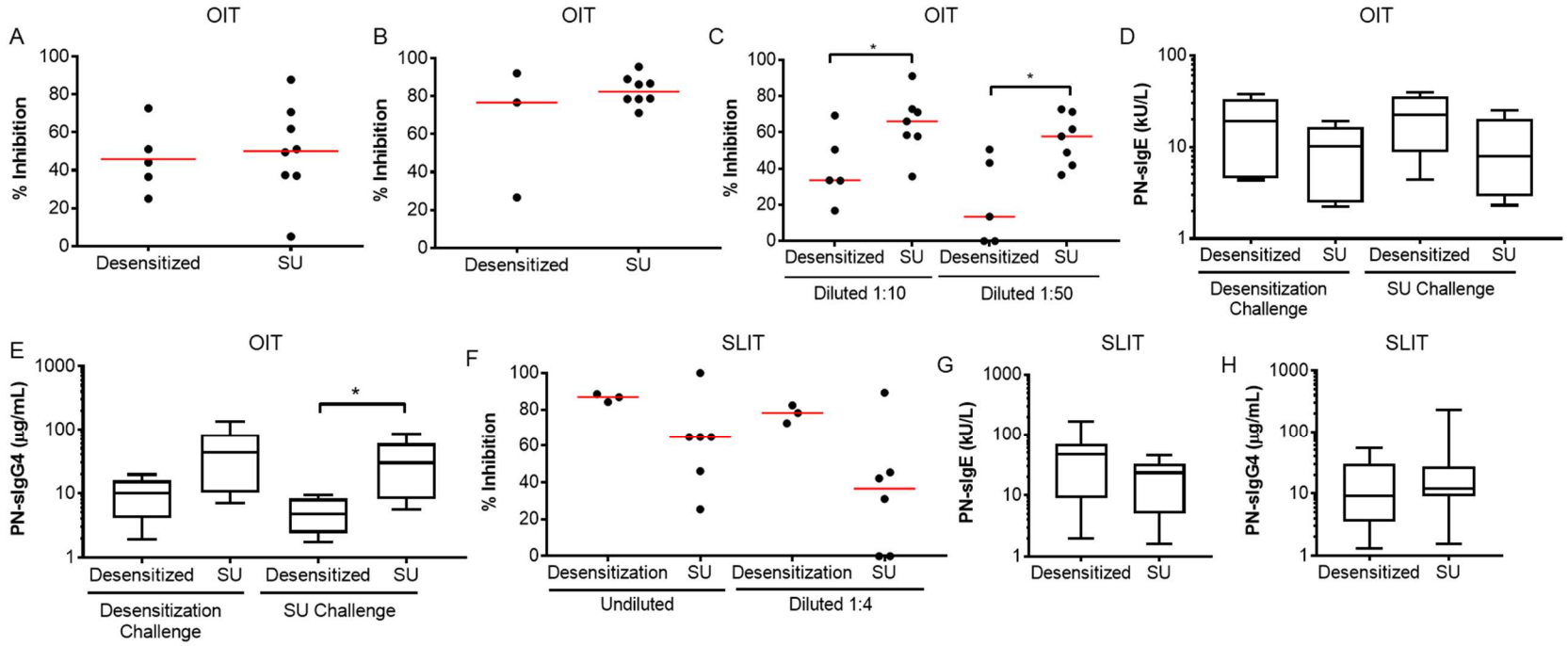

Figure 2-5. OIT and SLIT clinical outcomes in relation to basophil inhibition. Percent inhibition of basophil activation for 48 month OIT plasma (A) or tolerance challenge plasma after discontinuation of therapy (B) separated by clinical outcome. Percent inhibition of basophil activation following incubation with 48 month OIT plasma diluted either 1:10 or 1:50 (C). Corresponding PN-sIgE (D) and PN-sIgG4 (E) levels. Percent inhibition of basophil activation following incubation with 60 month SLIT plasma either undiluted or diluted 1:4 (F) and corresponding PN-sIgE (G) and PN-sIgG4 (H) levels. Red lines indicate medians; *p<0.05.

2.6 Supplementary Figures

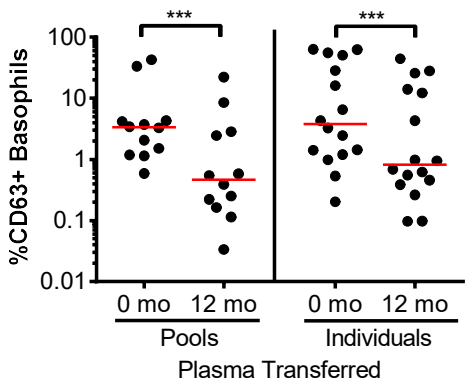

Figure 2-S1. Blocking capability of pooled plasma compared to individual plasma. %D63+ basophils following incubation with either pooled or individual plasma from 0 month or 12 month OIT. Red lines indicate medians; ***p<0.001.

CHAPTER 3: SIGLEC-ENGAGING TOLERANCE-INDUCING ANTIGENIC LIPOSOMES (STALS) IN THE PREVENTION OF PEANUT ALLERGY[1]

3.1 Introduction

As previously discussed in Sections 1.7 and 2.1, no FDA-approved therapies for food allergies currently exist. Oral, sublingual, and epicutaneous immunotherapies are under clinical study as potential food allergy treatments, yet the side effects, requirement for daily dosing, and lack of prolonged efficacy remain limitations in these human trials.[102,174] A need for targeted therapies that are not affected by these limitations persists. Peanut allergies are dominated by undesired IgE antibody responses to the 2S albumin Ara h 2,[78,175] which induce degranulation of effector cells upon exposure to the antigen. Targeting the allergen-specific B cells may limit side effects and promote long-term tolerance.

Sialic acid-binding immunoglobulin-type lectins (Siglecs) are a family of immunomodulatory receptors with cell-specific expression.[176] CD22, a Siglec expressed exclusively by B cells, is expressed early in B cell development along with CD19, but prior to the expression of CD20.[177] Important for the discrimination of "self" from "non-self" by the immune system, Siglecs bind to their glycan ligands on host cells as well as pathogens.[176] These molecules have been exploited as targets for leukemia and lymphoma cell depletion therapies.[178,179] Inhibitory Siglecs, including CD22, use immunoreceptor tyrosine-based inhibitory motifs (ITIMs) to suppress activatory receptors, such as the B cell receptor (BCR).[180]

[1] Adapted with permission from: Orgel KA, Duan S, Wright BL, et al. Exploiting CD22 on antigen-specific B cells to prevent allergy to the major peanut allergen Ara h 2. The Journal of Allergy and Clinical Immunology 2017;139:366-9.e2.

Because of its B cell-specific expression and inhibitory properties, CD22 offers a unique target for the induction of antigen-specific tolerance without causing widespread immunosuppression.

Co-localization of CD22 with the BCR, with liposomes that co-display an antigen and high-affinity CD22 ligand, not only prevents B cell activation but also causes apoptosis of the antigen-reactive B cells. As a result, robust immunological tolerance is induced due to depletion of the antigen-specific B cells from the B cell repertoire.[181] These Siglec-engaging Tolerance-inducing Antigenic Liposomes (STALs) can be formulated with any protein antigen of choice. One study found that STALs displaying Factor VIII (FVIII) inhibit antibody responses to exogenous FVIII, which interfere with FVIII replacement used to prevent bleeding in $FVIII^{-/-}$ mice.[181] Further, they found that incubation of STALs presenting either anti-IgM or anti-IgG Fab fragments and CD22 ligand with human B cells abrogated activation of both naïve and memory B cells, and resulted in decreased cell viability. In combination, these experiments demonstrate that STALs have the potential to prevent undesired B cell responses.

The experiments mentioned above motivated us to examine the potential of STALs for inducing immunological tolerance to a food allergen. We hypothesized that STALs displaying both a high-affinity and selective CD22 ligand and Ara h 2 could be an attractive strategy to prevent sensitization and subsequent anaphylaxis to Ara h 2 as well as potentially whole peanut extract.

3.2 Materials and Methods

3.2.a Peanut extract and Ara h 2 preparation

Peanut proteins were extracted by mixing peanut flour (12% fat light roast, 50% protein; Golden Peanut Co.) in a 1:5 (wt:vol) ratio of phosphate buffered saline (PBS) with 1 mol/L NaCl. The solution was mixed for 2 hours while maintaining an alkaline pH (8.5). The solution was centrifuged at 14,000 rpm for 45 minutes at 4ºC. The supernatant was collected and filter-sterilized through a 0.2 µM filter. Protein concentration was determined by bicinchoninic acid

assay (Pierce, Rockford, IL). The final preparation is referred to in the manuscript as whole peanut extract. Ara h 2 was purified according to work previously published by Sen, *et al*. *J Immunol, 2002.*

3.2.b STAL and immunogenic liposome preparation

Lipid modification of Ara h 2: A frozen stock of Ara h 2 in dH_2O was initially buffer exchanged in to PBS. The protein (2 mg/mL) was reacted with 2.5 molar equivalents of succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pierce) for 1 hr at RT, followed by desalting over Sephadex G-50 (GE Healthcare). Following deprotection with 25 mM DTT for 10 min at RT, the protein was again desalted. The thiol-modified protein was reacted with 10 equivalents of maleimide-PEG_{2000}-distearoylphosphatidylethanolamine (DSPE; NOF America) overnight under nitrogen. The following day, the reaction was passed over a Sephadex G-100 column (GE Healthcare) and the fractions containing the lipid-modified Ara h 2 in the void volume were pooled and stored at 4 ^{0}C.

Liposome formulation and extrusion: Distearoylphosphatidylcholine (DSPC; Avanti), Cholesterol (Sigma), and PEG_{2000}-DSPE (NOF America) were dissolved in chloroform and combined at an approximate ratio of 57:38:5. The solvent was evaporated under nitrogen and 100 μL of DMSO was added. To formulate the STALs, 1 mol% of PEG_{2000}-DSPE was replaced with the high affinity CD22 ligand (6'BPA-Neu5Gc-LacNAc-PEG_{2000}-DSPE) and added from a DMSO stock. The lipid solutions in DMSO were lyophilized overnight to yield a fluffy powder. To hydrate the liposomes, the dried lipids were hydrated in the appropriate amount of PBS containing the Ara h 2-PEG-DSPE such that the mol% of the protein was 0.033 mol% of the total lipids. The lipids were hydrated in 1 mL to generate liposomes with a concentration of 5 mM total lipid. The hydrated lipids were sonicated a minimum of five times at 30 second intervals and then extruded using a hand-extruder (Avanti) 20 times through a 0.8 μM filter, followed by 20 times through a 0.1 μM filter. The final liposomes passed over a CL-4B column (Sigma) and pooled fractions were

stored at 4 ^{0}C prior to being diluted in PBS to the appropriate liposome concentration and administered to the mice.

3.2.c Mouse model of peanut sensitization

All animal studies were approved by the UNC IACUC and investigated under protocol # 13-216.0. Four-week-old female Balb/cJ mice purchased from Jackson Laboratory (Bar Harbor, ME) were maintained on peanut-free food under pathogen-free conditions. Mice were housed with four animals per cage and separated into 5 groups: (1) naïve, (2) 100 μM Ara h 2 immunogenic liposomes, (3) 100 μM Ara h 2 STALs, (4)300 μM immunogenic liposomes, and (5) 300 μM Ara h 2 STALs. Naïve mice were maintained throughout the experiment but received no treatments and were not sensitized. Mice in groups 2-5 received a single tail vein injection of the designated liposome or STAL. Mice were rested for two weeks before being sensitized. After the two week rest period, mice were sensitized with 2 mg peanut extract and 10 μg CT (List Biological Laboratories, Campbell, CA) by oral gavage once a week for three weeks. On the fourth week of sensitization, mice were boosted with one gavage of 5 mg peanut extract and 10 μg CT. One week after the last sensitization dose, 200 μL blood from each mouse was collected by submandibular bleed to measure specific IgE and IgG1 by ELISA. The day following bleeding mice in groups 2-5 were challenged with 200 μg IP Ara h 2 and one week later were challenged IP with 750 μg peanut extract. Anaphylactic reactions were assessed by measuring core body temperatures with a rectal probe (Physitemp, Clifton, NJ) at 15 minute intervals. Symptoms were scored after 30 minutes using a pre-established 5 point scale: 0, no symptoms; 1, scratching around the nose and head; 2, puffiness around the eyes and mouth with reduced activity; 3, labored respiration and/or cyanosis around the mouth and tail; 4, no activity after prodding or tremor and convulsion; and 5, death.

3.2.d IgE and IgG1 measurements

Specific IgE and IgG1 were measured by ELISA using a reference curve. Plates were coated with 20 µg/mL whole peanut extract, 5 µg/mL Ara h 2, 5 µg/mL Ara h1, or 5 µg/mL CT in carbonate-bicarbonate buffer at pH 9.6 for 1 hour at 37ºC. Plates were blocked with PBS containing 0.05% Tween 20 and 2% Bovine Serum Albumin for 2 hours at 37ºC. Serum samples were added for 1 hour at 37ºC. Detection of IgE was performed using sheep antimouse IgE (0.5 µg/mL; Binding Site, Birmingham, UK), followed by biotinylated donkey antisheep IgG (0.5 µg/mL; Accurate Chemical, Westbury, NY) and neutravidin-horseradish peroxidase (HRP; 0.2 µg/mL; Pierce) for 1 hour at 37ºC. IgG1 was detected by HRP-conjugated goat antimouse IgG1 (Southern Biotech, Birmingham, Al) used at 1:40,000 for 1 hour at 37ºC. HRP activity was measured by blue color development of Sure Blue TMB Microwell Peroxidase Substrate (KPL, Gaithersburg, MD). Plates were read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT).

3.3 Results

Schematics representing an Ara h 2 STAL and the experimental design is shown in Figure 3-1A and Figure 3-1B, respectively. Four-week old female BALB/cJ mice (Jackson Laboratories, Bar Harbor, Maine) were injected intravenously with 200 µL of 100 µM Ara h 2 STALs (n=8), 300 µM Ara h 2 STALs (n=8), 100 µM immunogenic Ara h 2 liposomes (n=8), or 300 µM immunogenic Ara h 2 liposomes (n=7). All liposomes consisted of 0.03 mol % Ara h 2, which amounted to a dose of 0.12 µg of Ara h 2 in the 100 µM group. STALs additionally consisted of 1% BPA-Neu5Gc, the high affinity and selective CD22 ligand.[181] Two weeks following infusion of STALS, a timeframe previously determined to maximize tolerance induction through STALs,[181] the mice were orally-sensitized, with 2 mg peanut extract and 10 µg CT weekly for three weeks followed by a boost dose of 5 mg peanut extract and 10 µg CT. A group of naïve mice (n=8) underwent the same protocol and were injected with PBS to determine

baseline titers. Serum was collected one week later to quantify specific IgE and specific IgG1 to Ara h 2, peanut, Ara h 1, and CT by ELISA. Mice were initially challenged with 200 µg Ara h 2 via an intraperitoneal (IP) injection. One week later, mice were challenged IP with 750 µg peanut extract. To assess anaphylaxis during challenge, rectal temperatures were recorded for 30 minutes, and symptom scores were documented at 30 minutes using a 0-5 point scale where 0 represents no symptoms and 5 represents death, as described previously.[78]

On day 42, prior to the challenge, Ara h 2-specific IgE (sIgE) levels were significantly lower in animals injected with either 100 µM or 300 µM Ara h 2 STALs compared to those injected with the same dose of immunogenic controls (100 µM, p=0.0002; 300 µM, p=0.0006; Figure 3-1C). Pre-treatment with Ara h 2 STALs also inhibited production of Ara h 2-specific IgG1 (sIgG1) compared to controls (100 µM, p=0.0047; 300 µM, p=0.0006; Figure 3-1D). Upon challenge with 200 µg Ara h 2, mice pre-treated with either 100 µM or 300 µM Ara h 2 STALs were protected from hypothermia, an objective feature of anaphylaxis in mice, compared to mice pre-treated with immunogenic controls that had severe reactions (Figure 3-1E). The symptom scores, as defined previously,[78] also reflected more severe reactions in animals pre-treated with immunogenic Ara h 2 liposomes compared to those pre-treated with Ara h 2 STALs (Figure 3-1F; 100 µM, p=0.0126; 300 µM, p=0.0002). CT-sIgE was not different amongst treatment groups (Figure 3-2), indicating that tolerance induction is antigen-specific and that no intrinsic differences in an ability to mount antibody responses were present between the groups. These findings validated the results of a pilot study where 100 µM, but not 20 µM Ara h 2 STALs, led to significantly lower Ara h 2-sIgE and Ara h 2-sIgG1. Taken together, these results suggest that Ara h 2 STALs induce antigen-specific tolerance toward the major peanut allergen, severely blunting Ara h 2-sIgE and Ara h 2-sIgG1 levels and reactions upon IP challenge with Ara h 2.

Since Ara h 2 is only one of several related antigens in peanuts and sensitization was done with whole peanut extract, we also examined the impact of Ara h 2 STALs on sIgE levels

to peanut (Figure 3-3A) and to another peanut allergen, Ara h 1. Pre-treatment with 100 μM Ara h 2 STALs by intravenous (IV) injection resulted in lower PN-sIgE compared to the immunogenic control mice, though differences were not statistically significant (p=0.1296). However, mice that received 300 μM Ara h 2 STALs (IV) had significantly less PN-sIgE than their control counterparts (p=0.0037). Interestingly, mice injected with Ara h 2 STALs had lower Ara h 1-sIgE than their controls at each dose (100 μM, p=0.0146; 300 μM, p=0.0140; Figure 3-3B). Extensive cross-reactivity between Ara h 1 and Ara h 2 has previously been demonstrated and is likely to account for this effect.[82,182] Finally, these mice were challenged with 750 μg peanut and, consistent with the PN-sIgE results, the body temperatures of mice pre-treated with 300 μM Ara h 2 STALs were significantly greater than mice that received 300 μM immunogenic controls, demonstrating that the STALs attenuated anaphylaxis to peanut (Figure 3-3C). Symptom scores reflected similar results with the 300 μM immunogenic control mice reacting more severely than the Ara h 2 STALs mice (p=0.0350; Figure 3-3D). The groups treated with 100 μM Ara h 2 STALs were not significantly different in body temperatures or symptom scores from the immunogenic control group, suggesting a dose effect. These results demonstrate for the first time that antigen-specific B cells for a single component can be selectively targeted to diminish an immune response to a complex mixture of several allergens.

3.4 Discussion

Here we demonstrate that liposomes simultaneously targeting CD22 and the BCR specific for the major peanut allergen, Ara h 2, can be used to induce antigen-specific B cell tolerance. Currently, there are no FDA-approved treatment options for peanut allergy, a potentially life-threatening disease. Leading investigational treatments include oral, sublingual, and epicutaneous allergen immunotherapies. However, these therapies, which involve exposing the allergic subject to the allergen over years of treatment, can cause allergic symptoms and seem to induce a transient desensitization rather than long-lived SU. Thus, the

need for targeted therapies that have limited side effects and the potential to induce permanent tolerance remains.

In this study, we showed that pre-treatment with Ara h 2 STALs prevents sensitization to Ara h 2 in a mouse model of peanut allergy. A single injection with Ara h 2 STALs blunted the IgE and IgG1 responses to Ara h 2 as well as prevented reaction upon IP challenge with Ara h 2. We believe this effect to be antigen specific, as treatment with Ara h 2 STAL had no effect on CT-sIgE levels. Not surprisingly, treatment with Ara h 2 STALs also resulted in a reduced humoral response to an Ara h 2-cross-reactive antigen. Based on previous studies using other antigens, we hypothesize that simultaneous engagement of CD22 and the Ara h 2-specific BCR leads to deletion of the Ara h 2-specific B cells. Unfortunately, tools to detect Ara h 2-specific B cells are not readily available, making this hypothesis difficult to test.

The results of this study demonstrate that STALs specific for a single component can be used to prevent sensitization to a complex mixture of antigens. Injection with Ara h 2 STALs partially blunted the IgE response to peanut, most likely because peanut-sensitized mice mount an IgE response to other peanut allergens in addition to Ara h 2. We were encouraged that targeting Ara h 2-specific B cells alone, was sufficient to lessen reaction severity following peanut challenge. Future experiments include developing STALs specific for each of the major peanut allergens. The findings in this study suggest that combining STALs targeting several different antigen-specific B cells may lead to an additive effect in preventing sensitization to whole peanut extract.

This study utilized STALs as a preventative measure. As such, mice were injected with Ara h 2 STALs prior to sensitization. While the outcomes are promising, this methodology would be similar to a preventative vaccine in humans. Limitations to a preventative approach in humans include cost, unknown timing and route of sensitization, and lack of predictive risk factors for disease. Future work is needed to develop Ara h 2 STALs as a post-sensitization therapy. Following sensitization, memory T cells, as well as memory B cells, are activated. Previous work

has shown that STALs can deplete human memory B cells, despite low CD22 expression on these cells.[181] A combination of therapies targeting the T cell compartment and B cell compartment may provide optimal effect in a post-sensitization therapy.[176] The findings in this study provide the foundation for the development of a novel therapy for peanut allergy using a highly targeted, antigen-specific approach. This targeted approach may be beneficial in the prevention and eventual treatment of other IgE-mediated food allergies, in addition to peanut allergy.

3.5 Figures

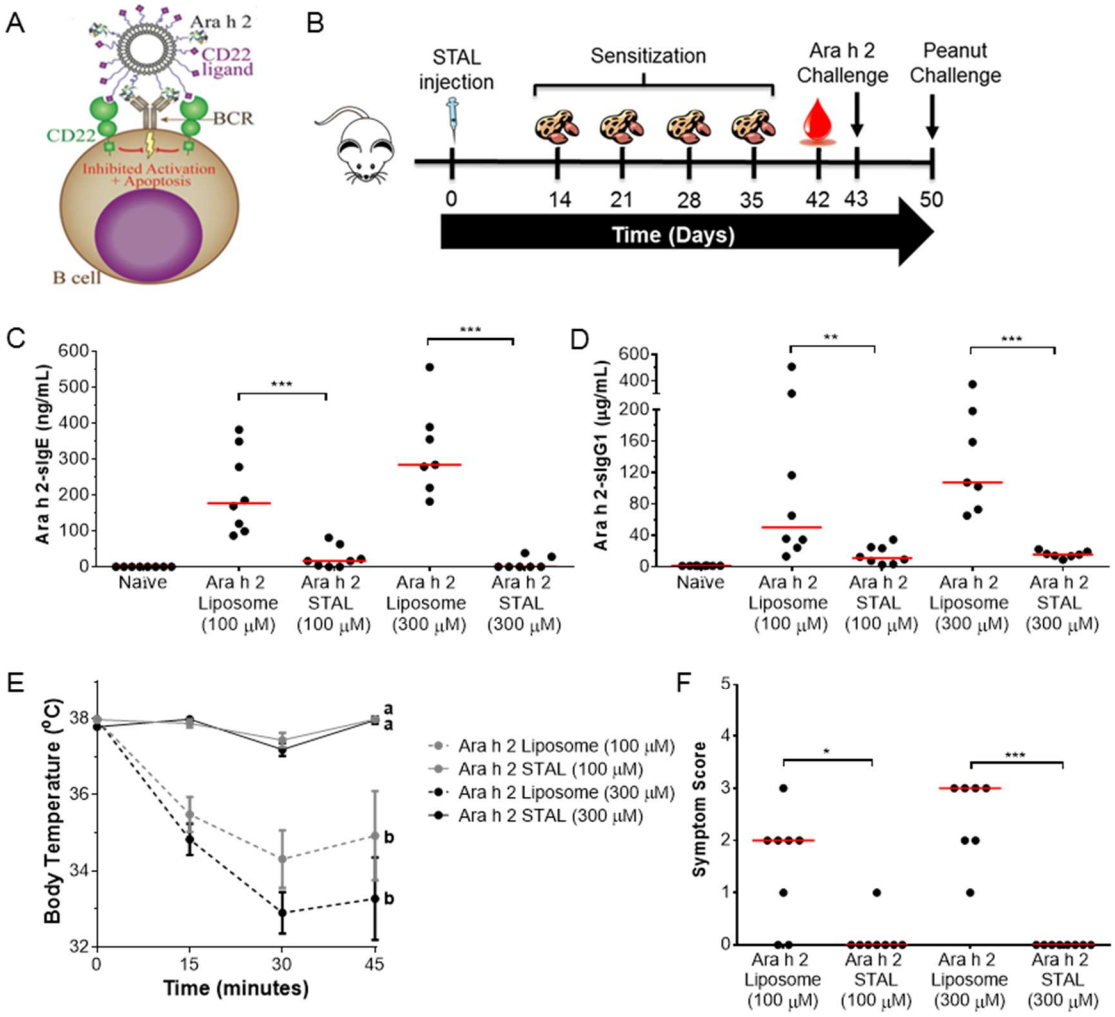

Figure 3-1. Ara h 2-specific immune responses are prevented in mice administered Ara h 2 STALs. Schematic of Ara h 2 STALs (A) and experimental protocol (B); Serum levels of Ara h 2-sIgE (C) and Ara h 2-sIgG1 (D) following oral sensitization; Body temperatures (E) and symptom scores (F) after challenge with 200 μg Ara h 2 IP Lines "a" are statistically different from lines "b" ($p<0.001$) at 15 and 30 min; Mann-Whitney U test *$p<0.05$, **$p<0.01$, ***$p<0.001$.

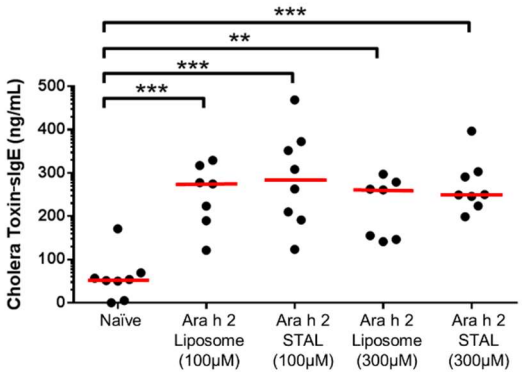

Figure 3-2. Ara h 2 STALs specificity. Cholera toxin-sIgE in mice treated with Ara h 2 STALs or immunogenic Ara h 2 liposomes. Mann-Whitney U test **p<0.01, ***p<0.001.

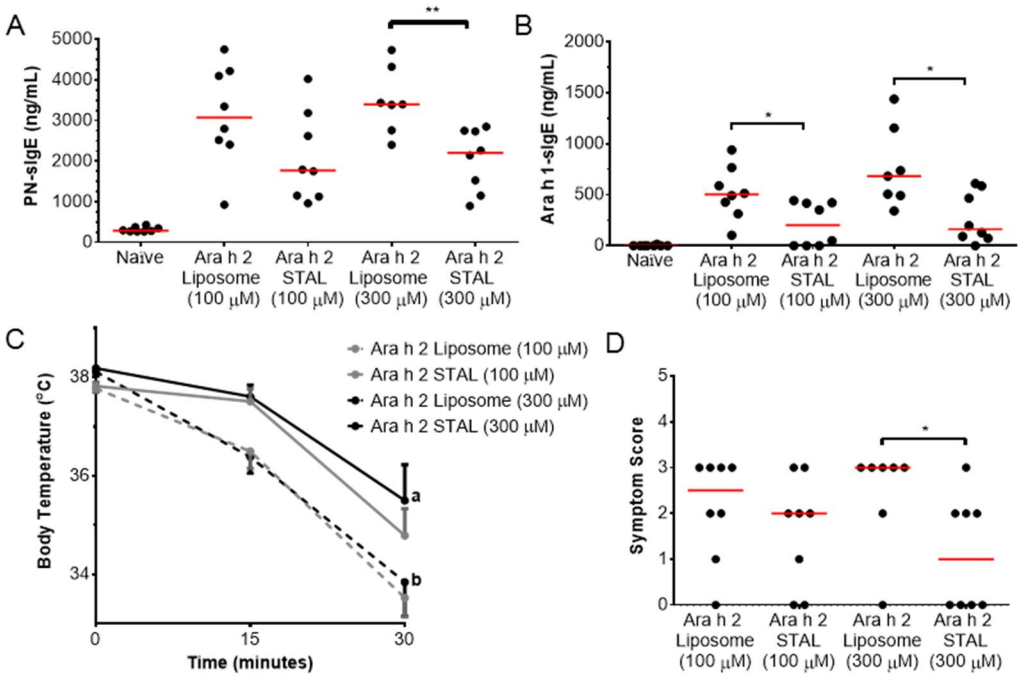

Figure 3-3. Immune responses to peanut and Ara h 1 in mice treated with Ara h 2 STALs. PN-sIgE (A) and Ara h 1-sIgE (B) following oral sensitization with peanut and cholera toxin. Body temperatures (C) and symptom scores (D) after IP challenge with 750 μg peanut. Line "a" is statistically different from line "b" ($p<0.05$) at 15 and 30 min; Mann-Whitney U test *$p<0.05$, **$p<0.01$.

CHAPTER 4: GENETIC DIVERSITY BETWEEN MOUSE STRAINS ALLOWS IDENTIFICATION OF CC027/GENIUNC AS AN ORALLY REACTIVE MODEL OF PEANUT ALLERGY[2]

4.1 Introduction

As previously discussed, food allergy is a potentially life-threatening disease characterized by IgE-mediated degranulation of mast cells and basophils upon allergen ingestion. Affecting 6% of children and 4% of the general population, food allergy is a growing public health concern, with peanut allergy present in at least 1% of the US population.[5,9,183] Although many food allergies are outgrown before adulthood, peanut and tree nut allergies persist in roughly 80-90% of the affected population.[7] Significant progress in food allergy research has occurred over the last 10 years, such as the development of potential therapies,[102,103,109-111,184] identification of improved diagnostic approaches,[5] and discovery of underlying immunologic mechanisms driving food allergies.[185,186] However, critical knowledge gaps exist about the etiology of peanut allergy, including genetic, microbial, and environmental influences.

The laboratory mouse has been the premier model organism for understanding complex human diseases, and developing therapies for a variety of diseases. Despite concerns about the translation of data from specific mouse strains to larger human health responses,[187] there has been a growing appreciation for the role that genetic diversity between inbred mouse strains

[2] Under Review: Orgel KA, Smeekens J, Ye P, Fotsch L, et al. Genetic diversity between mouse strains allows identification of CC027/GeniUnc as an orally reactive model of peanut allergy.

has in different outcomes within experimental models of human diseases.[188,189] In order to better leverage and identify the causal genetic variants driving such disease differences, a number of mouse genetic reference panels (GRPs) including the Collaborative Cross (CC),[190] the BxD panel,[191] and the Diversity Outbred (DO)[192] have been developed. These resources, panels of diverse mice with well characterized genetics have been used to (a) characterize the breadth of disease phenotypes that can be attributed to genetic variation; (b) define new models of disease phenotypes not found in the small pool of classic mouse strains used in standard studies; and (c) identify those polymorphic genes driving differential disease responses. Critically, such systems improve upon the utility and rigor of experimental models, ultimately making them more relevant for modeling diverse human disease responses.

Since peanut allergy within the human population is a heritable (i.e. genetically influenced) trait,[48,54] we sought to utilize the genetic diversity present in the CC mice to improve our understanding of peanut allergy and its contributing factors. Numerous murine models are currently in use by our group and many others to study mechanisms and treatments of peanut allergy.[137,193,194] However, these models often require powerful Th2-skewing adjuvants (e.g. CT,[78] SEB[144], or alum[146]) to sensitize animals, IP challenge to elicit a reaction,[78,134] or complex modifications such as humanization.[150,151,195] In a model commonly used by our group and others, C3H/HeJ mice are sensitized by weekly oral gavage of peanut extract and CT and challenged by IP injection with peanut extract.[78] Importantly, while some reports demonstrate reactions upon oral challenge in the C3H/HeJ model described above,[137] groups, including our own, have not been able to successfully reproduce these findings.[195,196] A model that can both be sensitized and reproducibly react orally would allow for the study of therapies that alter the immune system in the gastrointestinal tract such as OIT, as well as genetic and environmental factors driving these severe allergic reactions in a more physiologically relevant model of human disease. Here we report our screen of CC strains to identify orally-induced peanut anaphylaxis, the characterization of the peanut-specific immunologic responses, and novel insights into the

anaphylactic reactions in mice through the GI tract. In concordance with prior assessment in the human population, we identified strong genetic control of the propensity to experience anaphylaxis after sensitization. We also identified the CC strain CC027/GeniUnc as a mouse strain that develops anaphylaxis following oral sensitization and challenge.

4.2 Materials and Methods

4.2.a Mice

CC mice were purchased from the UNC Systems Genetics Core.[197] C57BL/6J and C3H/HeJ mice were obtained from colonies maintained for less than five generations by the Pardo-Manuel de Villena lab from mice purchased from The Jackson Laboratory. All mice were bred at UNC, raised on standard mouse chow, kept on a 12:12 light:dark cycle and transferred for sensitization at 4-6 weeks of age. Female mice were weaned into cages at a common cage density (between 3-5 mice/cage depending on the experiment), but with a diverse set of strains within each cage. In this way, effects of cage density and cage-specific effects were removed from these studies. Throughout these studies, where possible, experimenters were blinded to the mouse strains being studied. All mouse work was conducted in compliance with UNC IACUC protocol 16-045.

4.2.b Reagents

Peanut extract was created by mixing peanut flour (12% fat light roast, 50% protein; Golden Peanut Co.) in a 1:5 (wt:vol) ratio of phosphate buffered saline (PBS) with 1 mol/L NaCl and the soluble fraction was filter-sterilized as described previously.[134] Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). Peanut extract was run on a NuPage gel to identify and compare relative quantities of peanut allergens before using.

4.2.c Sensitization and challenge

4-6 week old female mice underwent weekly sensitization with 2 mg peanut extract and 10 µg CT (List Biological laboratories, Campbell, CA) in 200 µL volume for three weeks followed by 1 week of 5 mg peanut extract and 10 µg CT by oral gavage. One week after sensitization, mice were bled by submandibular bleed to collect serum for immunoglobulin quantification. The following day, mice undergoing an oral challenge were gavaged with 9 mg peanut extract while mice undergoing IP challenge received 200 µg peanut extract. Core body temperatures were monitored every fifteen minutes using a rectal thermometer (Physitemp, Clifton, NJ). For serum MMCP-1 and Ara h 2 measurements, blood was collected 60 min after oral challenge. Serum levels of MMCP-1 (eBioscience, San Diego, CA) and Ara h 2 (Indoor Biotechnologies, Charlottesville, VA) were measured by ELISA. Assays were run according to manufacturer's instructions.

4.2.d Immunoglobulins

For PN-sIgE, PN-sIgG1, and PN-sIgG2a/c quantification, plates were coated with 20 µg/mL peanut extract diluted in carbonate-bicarbonate buffer (Sigma Aldrich, St Louis, MO). Samples were assayed on plates at 1:100, 1:20,000, and 1:1250 respectively. Ara h 1-sIgE, Ara h 2-sIgE, and Ara h 3-sIgE plates were coated with 5 µg/mL of the appropriate purified peanut component diluted in carbonate-bicarbonate buffer. Samples were plated at a 1:20 dilution. IgE plates were all detected using the following antibodies in sequence: sheep anti-mouse IgE (0.5 µg/mL; Binding Site, Birmingham, UK), biotinylated donkey anti-sheep IgG (0.5 µg/mL; Accurate Chemical, Westbury, NY), neutravidin-horseradish peroxidase (HRP; 0.2 µg/mL; Pierce). IgG1 and IgG2a/2c ELISAs were detected with HRP-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL) or HRP-conjugated goat anti-mouse IgG2a (Southern Biotech, Birmingham, AL) and HRP-conjugated goat anti-mouse IgG2c (Southern Biotech, Birmingham, AL) respectively. Sure Blue TMB Microwell Peroxidase Substrate and

Stop Solution (KPL, Gaithersburg, MD) were applied to all plates. Plates were read on an Epoch Microplate Spectrophotometer (BioTek Instruments, Winooski, VT). Total IgE was analyzed by ELISA, (Affymetrix, Santa Clara, CA) and run according to manufacturer's instructions. All ELISA data was analyzed using Gen5 software.

4.2.e mRNA and cytokine protein quantification

Spleens were collected from both naïve as well as peanut-sensitized mice 1 week after oral challenge. mRNA abundance levels were quantified using real-time PCR and Sybr green methodology, as previously described.[198] Briefly, total RNA was extracted using RNA kits (Qiagen, Germantown, MD). Reverse transcription was performed using random decamers as primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The abundance of resultant mRNA-derived cDNA was determined by qRT-PCR analysis, using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), primers specific for genes of interest, and a StepOne Plus cycler (Applied Biosystem, Foster City, CA). Each set of primers spans at least two introns so that contaminating genomic DNA either cannot be amplified due to large product size or can be easily identified based on its size on agarose gel. Primers for 18S rRNA were obtained from Ambion (Austin, TX). The specificity of each real-time PCR target was confirmed by melt temperature analysis and agarose gel fragmentation of amplicons. To quantify mRNA abundance, a standard curve for each target mRNA, as well as for 18S rRNA, was generated from serial dilutions of cDNAs derived from a pooled intestinal cDNA library. The relative abundance of mRNA of interest in each sample was determined based on its corresponding standard curve, and normalized against the abundance of 18S rRNA. For protein analysis, splenocytes were isolated and cultured for 96 hours in the presence of 200 µg/mL peanut extract. Supernatants were collected and run on Meso Scale Discovery plates to determine levels of IL-4, IL-5, IL-13, TNF-α, IFNγ, IL-12p40, IL-10 according to manufacturer's instructions (MSD, Rockville, MD).

4.2.f Flow cytometry

For analysis of Treg cells, splenocytes were collected 1 week after oral challenge. Splenocytes were stimulated with 200 µg/mL peanut extract for 7 days. Tregs were then labeled using FITC rat anti-mouse CD4 Clone RM4-5 (BD Bioscience, San Diego, CA), PE anti-mouse/rat/human FoxP3 Clone 150D (Biolegend, San Diego, CA), and APC rat anti-mouse CD25 Clone PC61 (BD Biosciences, San Diego, CA). For determination of basophil levels, whole blood was collected by submandibular bleed 1 week after sensitization. Cells were stained with anti- mouse IgE FITC Clone 23G3 (eBioscience, San Diego, CA), PerCP/Cy5.5 anti-mouse CD49b Clone DX5 (Biolegend, San Diego, CA), PE anti-mouse CD200R Clone OX-110 (Biolegend, San Diego, CA). Flow Cytometry was performed on a Beckman Coulter CyAn ADP and analyzed using FlowJo (v10). Basophils were gated as IgE+CD49b+ and expressed as a percentage of lymphocytes. Tregs were gated as CD4+CD25+FoxP3+ and expressed as a percentage of CD4+ lymphocytes.

4.2.g Histology

Proximal jejunum was harvested from peanut-sensitized mice 1 week after challenge, and fixed with cold 4% paraformaldehyde in phosphate-buffered saline overnight and paraffin-embedded. Cross-cut sections (at a thickness of 7 µm) were subjected to immunostaining with an antibody specific for mast cell tryptase (1:180, Abcam, ab151757) or for CD117 (c-kit, 1:150, ThermoFisher, PA5-16770). Antibody-antigen complexes were detected using an ABC kit (Vector Laboratories, Burlinggame, CA) and visualized by incubation with DAB (Vector Laboratories or Sigma-Aldrich, St Louis, MO). For mast cell tryptase-positive cell quantification, immunostained sections were then subjected to counterstaining for cell nuclei with 0.1% methylene blue in acetic acid. To estimate the number of mast cell tryptase-positive cells in mucosa, 2 – 4 villi and the crypts under the villi were randomly selected, mast cell tryptase-positive cells with clear nuclei within delineated villi and crypts were counted. Total number of cells was determined by counting

methylene blue-stained cell nuclei, and the percentage of mast cell tryptase-positive cells were calculated. For each mouse, 850-1,640 cells were counted.

4.2.h Statistical analysis

GraphPad/Prism version 7.02 was used to analyze all data. Mann-Whitney U, Spearman Correlation, and unpaired-t tests were performed and a p-value <0.05 was considered significant. For cytokine protein level, values at or below the lower limit of detection were assigned half of the value of the lower limit of detection for that particular MSD assay.

4.3 Results

4.3.a CC027/GeniUnc female mice react severely to both oral and IP challenge with peanut extract

To assess the role that genetic variation plays in controlling anaphylaxis following sensitization with peanut allergen, female mice from 16 CC strains were screened using an established sensitization model (Figure 4-1).[134] These CC strains were chosen based on the fact that their well characterized genetic makeup is representative of the CC population,[199] as well as prior reports[188,200] of aberrant disease present in specific strains. All mice underwent the same four week sensitization regimen followed by half of the mice in each strain receiving a 200 µg peanut extract challenge via IP injection and the other half receiving a 9 mg peanut extract challenge via oral gavage. Following either OFC or IP challenge, CC strains were grouped into three types of reactors: Strains that do not react regardless of challenge route (Figure 4-2A: OFC, Figure 4-2D: IP); strains that reacted mildly (mean body temperature decreases between 1.5-3^{o}C; Figure 4-2B: OFC, Figure 4-2E: IP), and strains that reacted severely (mean body temperature decreases > 3^{o}C; Figure 4-2C: OFC, Figure 4-2F: IP). As expected, the screen identified many more mild and severe reactors following IP challenge than following oral challenge, however responses were highly concordant across routes of challenge (e.g. if a strain was a non-reactor in the IP cohort, it was also a non-reactor in the OFC cohort).

Two strains, CC027/GeniUnc, referred to as CC027 in figures, and CC012/GeniUnc, were classified as strong reactors following OFC, and were also classified as strong reactors to IP challenge with peanut extract, suggesting that they could be potential models for severe anaphylaxis following peanut sensitization.

In order to validate the findings of this initial screen, we conducted a second experiment with the OFC severe responders CC027/GeniUnc, CC012/GeniUnc, as well as the non-responder CC028/GeniUnc and IP-only responder CC011/Unc. While the results of this experiment were largely concordant with the initial screen (Fig 2G-J), only CC027/GeniUnc exhibited a severe reaction following OFC. We therefore concluded that CC027/GeniUnc represents a robust OFC-reaction model derived from our screen of the CC.

4.3.b CC027/GeniUnc but not C3H/HeJ or C57BL/6J react on oral challenge despite all making IgE to peanut allergens

Immune responses of sensitized CC027/GeniUnc females were compared to those of female mice from the classical inbred C3H/HeJ and C57BL/6J strains. All three strains were sensitized using the previously described 4 week sensitization schedule and then underwent an OFC with 9 mg peanut extract. Consistent with our previous experiments, CC027/GeniUnc mice experienced severe systemic reactions with body temperatures decreasing more than 3°C over the course of 60 min following OFC (Figure 4-3A), whereas C3H/HeJ and C57BL/6J mice had essentially no change in body temperature following OFC. These results confirm the utility of CC027/GeniUnc as an orally reacting allergy model. Despite only CC027/GeniUnc mice reacting upon OFC, all three strains make PN-sIgE, peanut-specific IgG1 (PN-sIgG1), and peanut-specific IgG2a/2c (PN-sIgG2a/2c) as well as IgE to the major peanut components, Ara h 1, Ara h 2, and Ara h 3 (Figure 4-3B-G). After sensitization, CC027/GeniUnc make significantly more PN-sIgE than C3H/HeJ but not C57BL/6J (Figure 4-3B; $p<0.05$). PN-sIgG1 levels are not different between the three strains (Figure 4-3C) while PN-sIgG2a/c levels are higher in CC027/GeniUnc than C57BL/6J but not different from C3H/HeJ (Figure 4-2D; $p<0.01$).

CC027/GeniUnc mice also had significantly more total IgE than C3H/HeJ ($p<0.01$) but not significantly different total IgE levels from C57BL/6J after sensitization (Figure 4-3H).

Given that PN-sIgE and PN-sIgG1 levels were different between CC027/GeniUnc and at least one of the two classical inbred strains, we assessed whether PN-sIgE or PN-sIgG1 levels within CC027/GeniUnc mice were correlated with anaphylaxis reaction severity. We found that PN-sIgE and PN-sIgG1 levels did not correlate with reaction severity in CC027/GeniUnc mice and thus do not explain the increased reactivity of these mice (Figure 4-S1A-B). Together, these data show that CC027/GeniUnc make immunoglobulins to peanut and peanut components, but that the strain-specific production of immunoglobulins alone does not distinguish CC027/GeniUnc from C3H/HeJ or C57BL/6J.

4.3.c CC027/GeniUnc mounts a Th2 cellular response with little Th1 or regulatory cytokine response to peanut

Secreted cytokines from peanut-stimulated splenocytes were quantified for CC027/GeniUnc, C3H/HeJ, and C57BL/6J mouse strains to determine T cell phenotypes. All three strains produce IL-4 levels, which are not significantly different across the strains (Figure 4-4A). IL-12-p40 was significantly elevated in C3H/HeJ relative to both C57BL/6J and CC027/GeniUnc (Figure 4-4F). For the remaining five cytokines (IL-5, IL-13, TNF-α, IFNγ, IL-10), we found that CC027/GeniUnc had significantly lower levels than either C57BL/6J or C3H/HeJ (Figure 4-4B-E, H). As a result, CC027/GeniUnc appears to have an increased Th2-skew relative to either C3H/HeJ or C57BL/6J. We illustrate this with the well-accepted[201-203] ratio of IFNγ to IL-4 (Figure 4-4G) across these strains. Concurrent with the finding that CC027/GeniUnc mice show a Th2-skew, we found that CC027/GeniUnc have higher levels of *Gata3* mRNA than C3H/HeJ ($p<0.05$), albeit similar levels to C57BL/6J (Figure 4-4I). CC027/GeniUnc also had a lower T cell regulatory response as indicated by reduced CD4+CD25+Foxp3+ Treg levels (Figure 4-4K) and decreased IL-10 protein production than the classical inbred strains ($p<0.01$; Figure 4-4H).

Sensitization-induced changes in mRNA expression levels were also analyzed for a few selected genes. Interestingly, CC027/GeniUnc have lower expression levels of *Il10* and *Il12* after sensitization than they do at baseline ($p<0.05$; Figure 4-S2A-B), suggesting that sensitizing these animals results in decreased production of regulatory and Th1-type cytokines. However, sensitization did not change *Il10* or *Il12* mRNA levels in either C3H/HeJ or C57BL/6J (Figure 4-S2A-B). Other reports have shown that *Ox40L* expression increases in dendritic cells following sensitization.[139] We found *Ox40L* mRNA levels in the small intestine to be increased in CC027/GeniUnc compared to C3H/HeJ ($p=0.0592$) and C57BL/6J ($p<0.05$) showing an additional effect of sensitization in these mice (Figure 4-4J). Overall, T cell responses appear to favor pro-allergic responses to peanut with the presence of Th2 cytokines and limited Th1 cytokine production, lower numbers of Tregs, and less regulatory cytokine IL-10.

4.3.d Effector cells are more prevalent in CC027/GeniUnc mice than classic inbred strains

Basophils and mast cells are the two main effector cells implicated in food allergy reactions.[21] We quantified basophil frequency in blood after sensitization using flow cytometry. CC027/GeniUnc had an increased percentage of IgE+CD49b+ basophils circulating after sensitization compared to C3H/HeJ ($p<0.05$) and C57BL/6J ($p<0.01$; Figure 4-5A). Furthermore, the CC027/GeniUnc basophils also had less of the inhibitory receptor, CD200RI than the other two strains ($p<0.001$; Figure 4-5B).[204] Tissue samples of the small intestine were stained for tryptase+ mast cells. CC027/GeniUnc had an increased percentage of tryptase+ cells, suggesting increased mast cell presence in the tissue (Figure 4-5C-D). Taken together, CC027/GeniUnc have an increased number of basophils in circulation that may lack negative feedback mechanisms driven by CD200R, and also an excess of mast cells in the GI tract.

4.3.e Reaction severity in CC027/GeniUnc correlates with serum levels of Ara h 2 but not MMCP-1 during oral challenge

To further characterize the severe reaction observed in CC027/GeniUnc mice, blood was collected from the mice 60 min following OFC. Serum levels of mucosal mast cell protease-1 (MMCP-1), a mediator released by degranulated mast cells in the gastrointestinal tract was measured to verify that mast cell degranulation could be detected in the reacting animals. Serum MMCP-1 was detectable in both C3H/HeJ and CC027/GeniUnc, but not C57BL/6J (Figure 4-6A), though only CC027/GeniUnc showed signs of a systemic reaction. Within CC027/GeniUnc mice, serum levels of MMCP-1 were not correlated with reaction severity (Figure 4-6B; Spearman r=0.2196, p=0.4109). Concurrently, serum levels of the major peanut allergen, Ara h 2, were measured 60 min post-challenge by ELISA to determine the amount of allergen being absorbed into the blood stream. CC027/GeniUnc had significantly higher levels of Ara h 2 in serum, compared to C57BL/6J ($p<0.05$) and C3H/HeJ ($p<0.0001$; Figure 4-6C). Interestingly, Ara h 2 quantity positively correlates with reaction severity in CC027/GeniUnc mice (Figure 4-6D; Spearman r=0.69, p=0.0028).

4.4 Discussion

An accurate translation between small animal models and human health outcomes requires that models accurately recapitulate key aspects of the human disease. Previously, we utilized a mouse model of food allergy that requires IP challenge with peanut extract to elicit an anaphylactic response after sensitization with peanut and a Th2-skewing adjuvant.[78,134] However, a mouse that reacts on oral challenge would provide a more physiologically-relevant platform to study both the etiology of the disease as well as potential treatments. Within the human population, increasing evidence has shown that host genetic variation impacts allergic responses. A twin-study[48] estimated the heritability (proportion of genetic contribution) to peanut allergy at approximately 0.8. However, identification of genetic variants contributing to peanut allergy responses and outcomes has been limited to associations with the MHC locus

and others associated with asthma and eczema.[51,54,55] Undoubtedly, there are additional genetically variable factors driving propensity for, and severity of allergic responses to peanut. Therefore, we sought to determine whether genetic variation between mouse strains could explain variation in food allergy disease severity, and whether we could develop a more relevant oral challenge model by assessing genetically diverse inbred mouse strains.

Here, we described the use of 16 strains from the CC GRP to screen for an orally reacting animal model of peanut allergy. The CC offers high genetic diversity, and has been used to both identify genetic factors driving aberrant disease outcomes,[205-207] but also has enabled the development of more relevant models of human disease responses.[188,200,208] We identified a single strain, CC027/GeniUnc as a promising model of food allergy. CC027/GeniUnc experiences a severe systemic reaction, evidenced by decreased body temperature following OFC with peanut extract, whereas the other 15 CC strains (as well as the well-studied inbred strains C3H/HeJ and C57BL/6J) did not react accordingly. CC027/GeniUnc mice produce detectable levels of IL-4 protein and produce PN-sIgE, Ara h 1-sIgE, Ara h 2-sIgE, Ara h 3-sIgE. We showed that, similar to peanut allergy in humans,[209] CC027/GeniUnc mount a Th2-skewed response to peanut and the major peanut allergens. CC027/GeniUnc have increased levels of Th2-promoting transcription factor, *Gata3* mRNA relative to C3H/HeJ mice, which do not react on oral challenge. Also, similar to human disease, PN-sIgE does not correlate with disease severity in these mice. Furthermore, CC027/Geni/Unc have a lower number of Tregs based on flow cytometry data as well as lower levels of the important regulatory cytokine IL-10 at the protein and mRNA levels. Together, these results reveal a model of peanut allergy that, like other models,[194] has Th2-skewed immune responses to the allergen, a decreased regulatory response, but also demonstrates signs of a severe, systemic reaction on oral challenge with the allergen, making it a highly relevant model recapitulating key features of peanut allergy in humans.

As in human food allergy, the exact mechanistic causes of the increased reactivity of CC027/GeniUnc need to be further studied. Our findings suggest many potential contributing factors likely driven by the underlying genetic differences in these mice. As already stated, PN-sIgE does not correlate with reaction severity, signifying that differences beyond IgE levels must be important for the severe oral reactions observed. In addition to hallmarks of acquired immune differences in CC027/GeniUnc, this strain has a greater quantity of basophils and mast cells; those effector cells responsible for the manifestations of allergic symptoms. Recent reports suggest an important interplay between activating and inhibitory signals from the surface of mast cells on allergic disease.[210] While we did not assess mast cell activation, we found that the increased numbers of basophils possess less of the inhibitory receptor CD200R1 than C3H/HeJ or C57BL/6J, similar to what has been reported for subjects with birch pollen allergy.[165] Thus, CC027/GeniUnc may have a larger number of more easily activated effector cells than the other less reactive strains.

Lastly, we demonstrated that CC027/GeniUnc absorbed higher levels of Ara h 2 protein into their blood stream during OFC than either C3H/HeJ or C57BL/6J, and these levels of serum Ara h 2 in CC027/GeniUnc correlated with reaction severity. C57BL/6J had detectable levels of serum Ara h 2 protein following OFC, but did not exhibit any signs of a systemic reaction or any detectable serum MMCP-1 following OFC. Taken together, these findings suggest mast cells in C57BL/6J are difficult to degranulate compared to CC027/GeniUnc mast cells. However, C3H/HeJ had high levels of MMCP-1 following OFC, but no serum Ara h 2 or symptoms of anaphylaxis. It is possible that only local mast cells in the mucosa degranulate in C3H/HeJ following oral challenge whereas CC027/GeniUnc experienced both local and systemic degranulation. The positive correlation observed between serum Ara h 2 levels and reaction severity in CC027/GeniUnc suggests that CC027/GeniUnc experience more severe reactions because of increased allergen absorption into their blood stream, which can trigger anaphylaxis by degranulation of mast cells, basophils, and/or neutrophils. These findings suggest that both

Ara h 2 absorption into systemic circulation along with readily-degranulating effector cells is required for anaphylaxis upon OFC. Increased Ara h 2 absorption could be due to increased gut permeability in CC027/GeniUnc. Though a role for intestinal permeability in food allergy has been suggested,[211,212] attempts by our own group and others[213] to measure Ara h 2 in human serum following ingestion has proven difficult and inconclusive. Thus, CC027/GeniUnc offer insight into a potential disease mechanism that is currently difficult to investigate in humans. Future investigation of the uptake of Ara h 2 through the gastrointestinal tract is needed.

CC027/GeniUnc represents a highly relevant model of peanut allergy to the field at-large. This small-animal model should allow for more robust evaluation of therapeutic treatments in a pre-clinical setting prior to transition into clinical trials. Leading investigational treatments in the field include various routes of peanut immunotherapy including OIT, SLIT, and EPIT. Despite promising results from OIT, SLIT, and EPIT studies,[109,110,115,161] these therapies have limitations including daily dosing, side effects, and difficulty in achieving long-term tolerance after stopping therapy. Therefore, new therapies that induce immunologic tolerance are needed. CC027/GeniUnc provides a pre-clinical model to develop these therapies and study the effects on the development of oral tolerance. Furthermore, genetic dissection of the repressive and enhancing phenotypes observed across mouse strains can lead to the identification of novel genes and pathways that may be critical in promoting peanut allergy within the human population. More broadly, our results highlight the utility of integrating the experimental robustness of inbred small animal models of disease with defined and broad genetic diversity in attempting to better understand and address human disease needs.

4.5 Figures

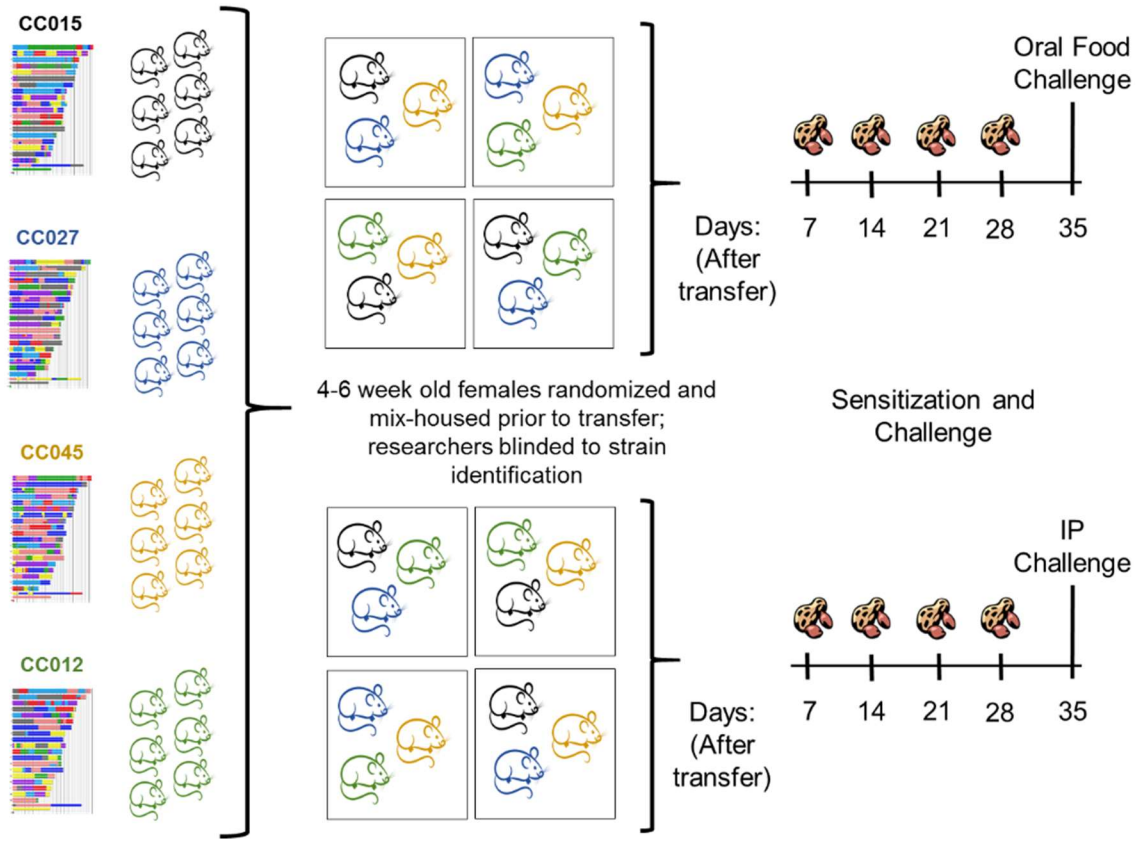

Figure 4-1. Collaborative Cross screening approach. Schematic shows 4 representative strains of the 16 strains screened. Six female mice between the ages of 4 and 6 weeks from each strain were mixed so that each cage contained 3-5 mice from different strains. Mice were then transferred from the UNC Systems Genetics Core to the UNC Food Allergy Initiative where researchers were blinded to the identification of each strain. Mice were sensitized intragastrically with peanut extract and cholera toxin for 4 weeks before undergoing either an OFC (n=3/strain) or IP challenge (n=3/strain) with peanut extract.

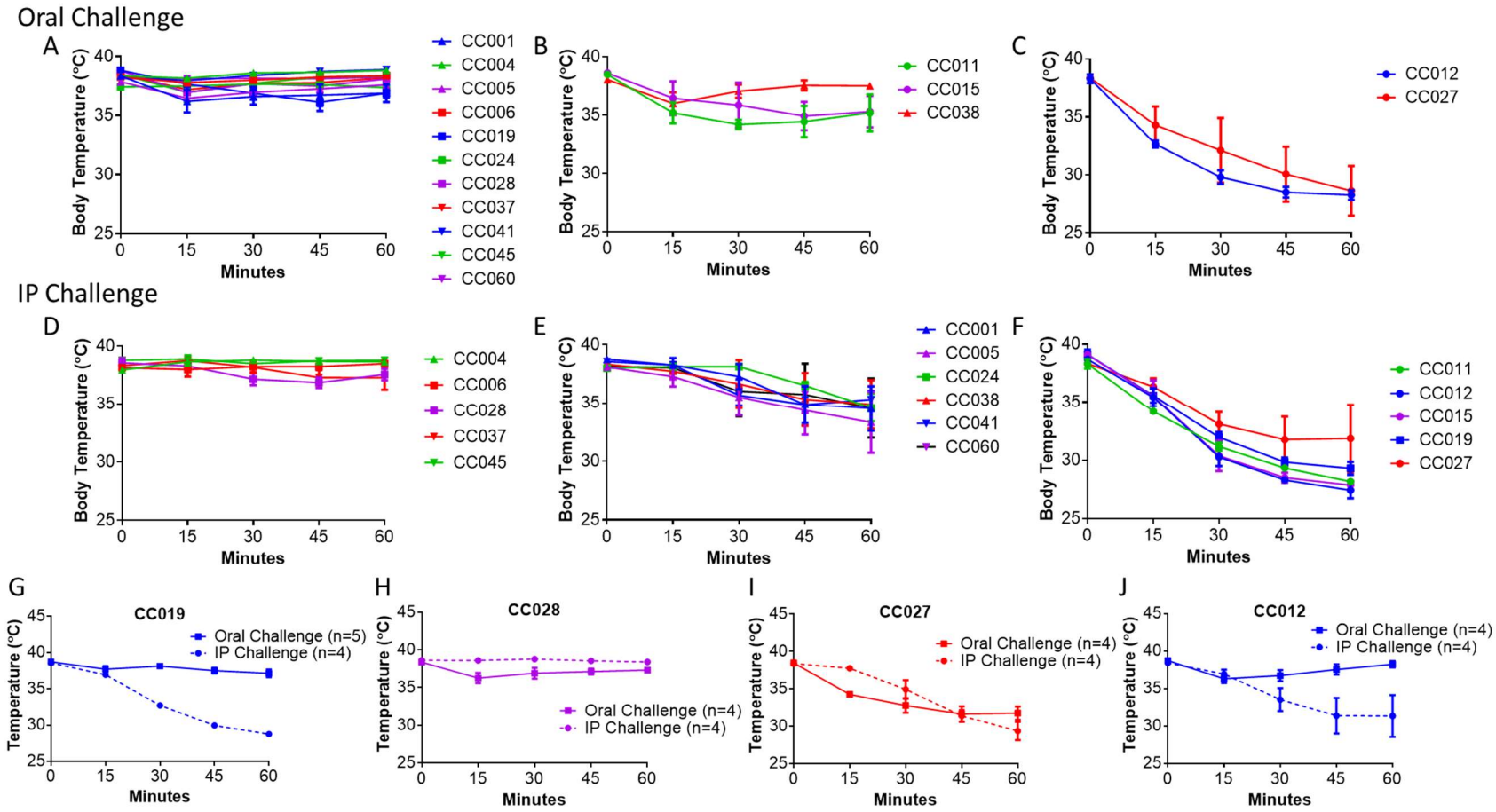

Figure 4-2. Anaphylaxis in peanut-sensitized Collaborative Cross strains following peanut challenge. Oral and IP challenges revealed Collaborative Cross strains that are non-reactors (A, D), mild reactors (B, E) and severe reactors (C, F) as measured by decreased body temperature. Challenges were repeated with a non-reactor control (G), IP reactor control (H), and oral reactors (I, J).

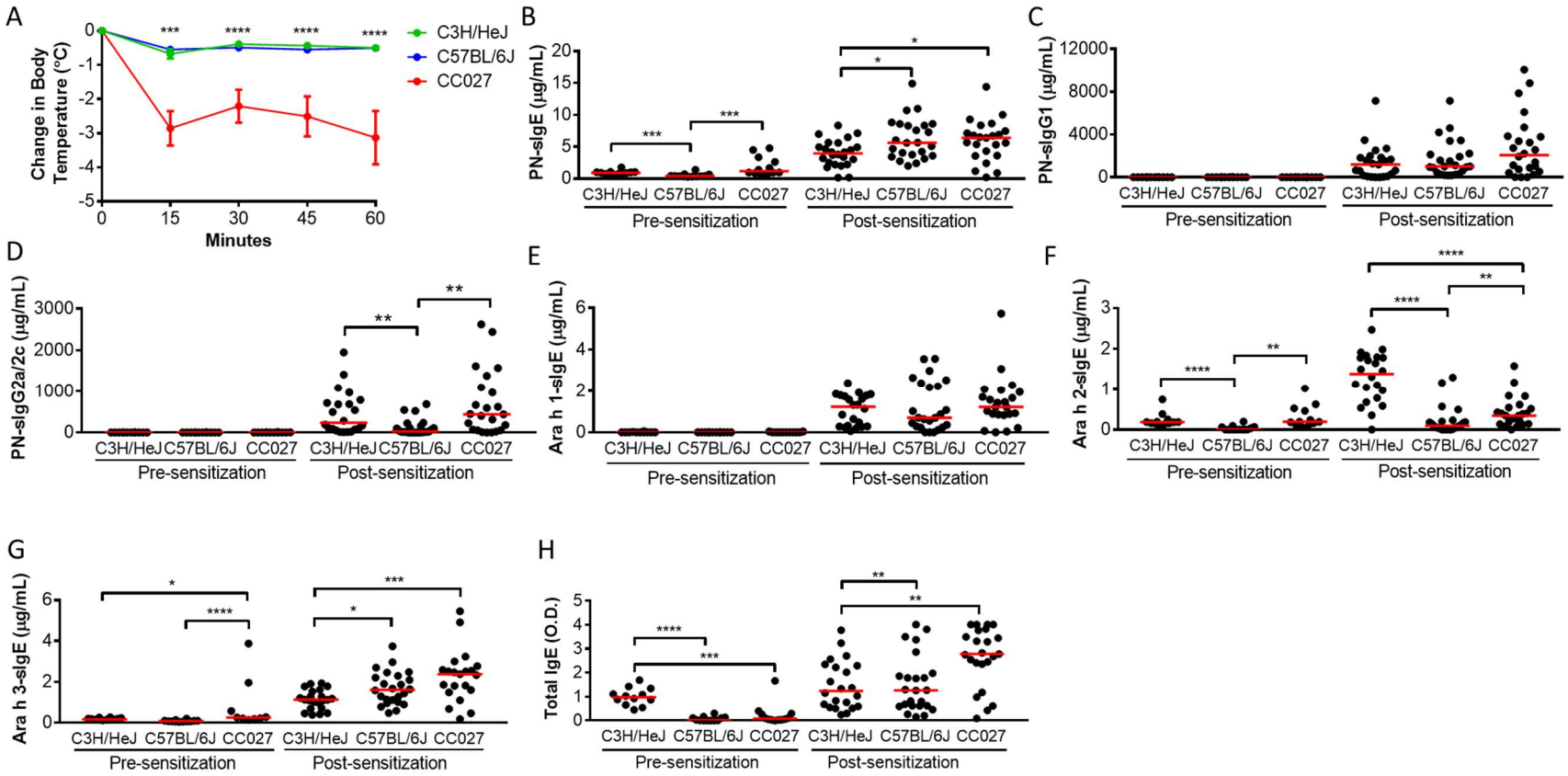

Figure 4-3. Immune response of CC027/GeniUnc to peanut extract relative to that of C3H/HeJ and C57BL/6J mice. CC027/GeniUnc is represented as CC027 in figures. Body temperatures following oral challenge with peanut extract (n=12/strain) (A). Serum levels of immunoglobulins following 4 weeks of sensitization (B-H). Mann-Whitney U Test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Statistical significance represents comparisons of both C3H/HeJ and C57BL/6J relative to CC027/GeniUnc (A).

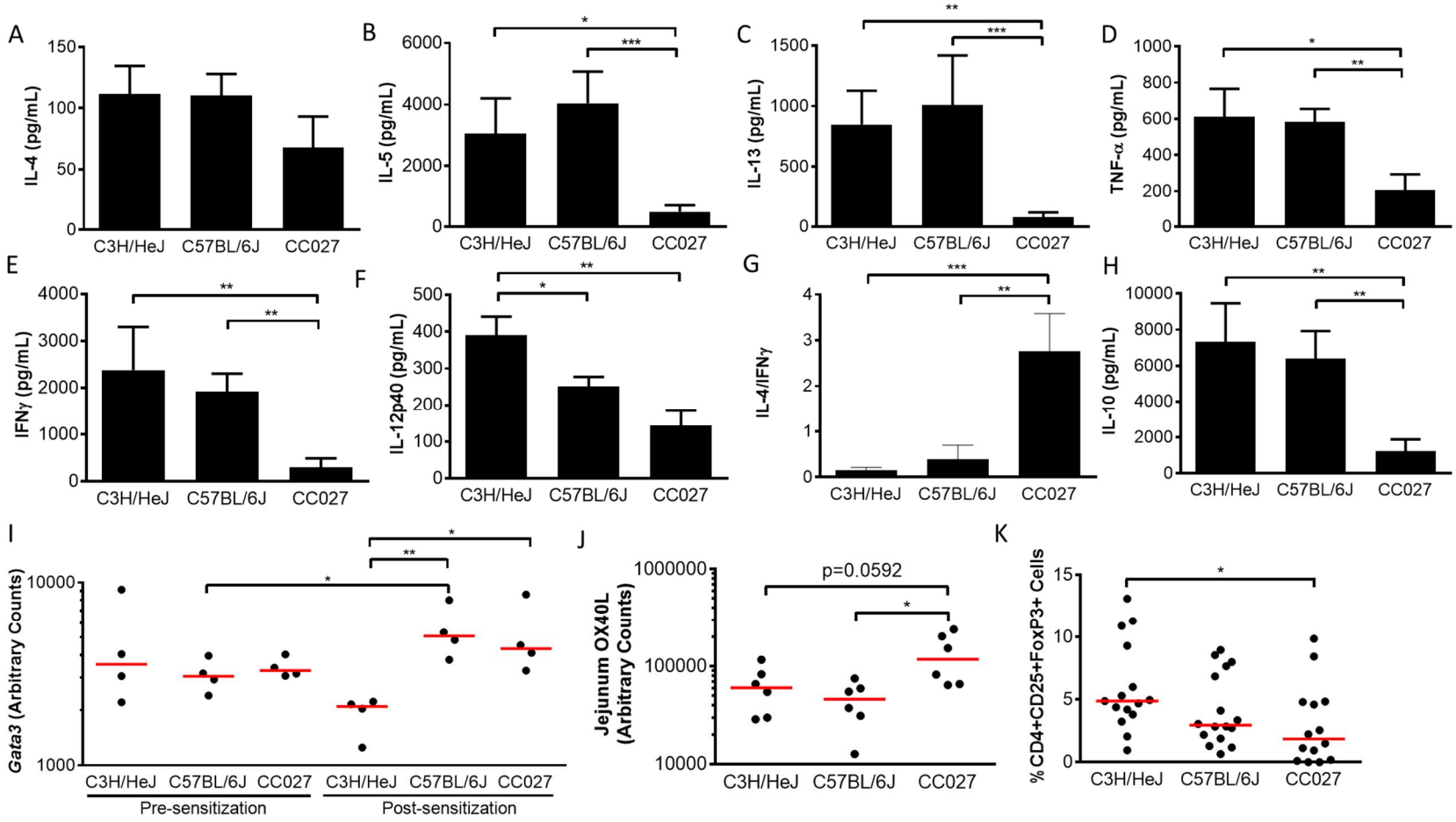

Figure 4-4. Cellular responses in CC027/GeniUnc, C3H/HeJ, and C57BL/6J. Splenic cytokines 96 hours following peanut-stimulation (n=10/strain) (A-H), mRNA expression (I-J), and CD4+CD25+FoxP3+ regulatory T cells 1 week following oral challenge (K). Mann-Whitney U Test *p<0.05, **p<0.01, ***p<0.001 (A-H; K); unpaired t-test *p<0.05, **p<0.01 (I-J).

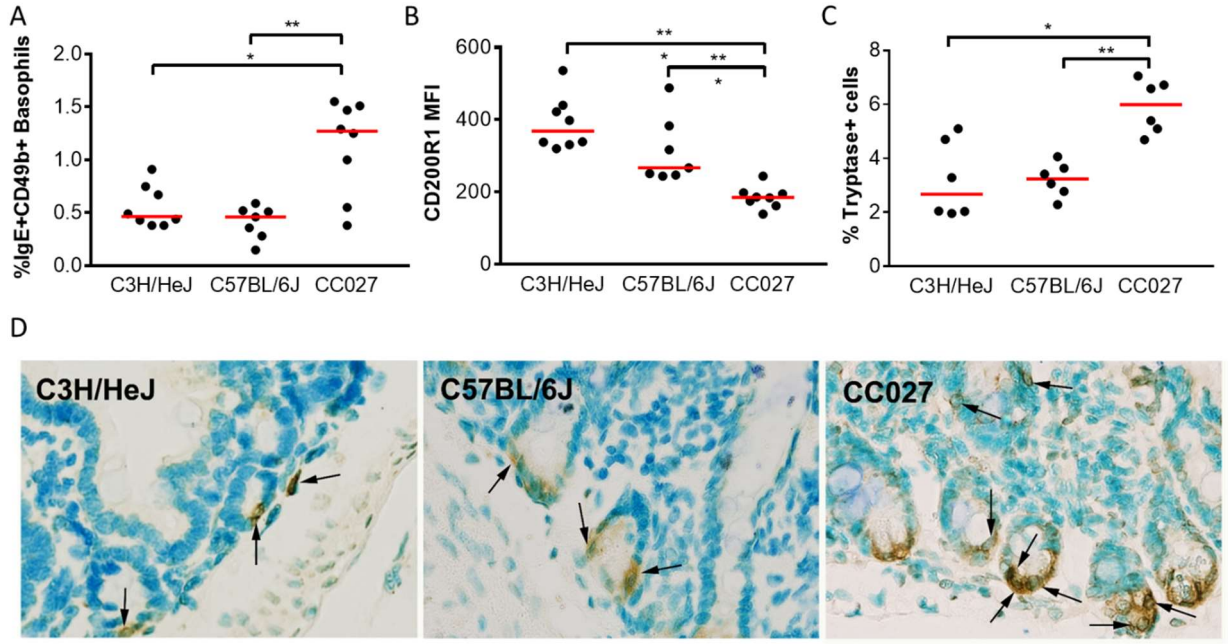

Figure 4-5. Enumeration of effector cells in CC027/GeniUnc, C3H/HeJ, and C57BL/6J. Percent IgE+CD49b+ basophils (A) and basophil inhibitory receptor, CD200R1 in whole blood (B). Jejunal tryptase+ mast cells quantified 1-3 weeks following challenge (C) and representative staining images shown with arrows indicating tryptase+ cells (D). Mann-Whitney U Test *p<0.05, **p<0.01, ***p<0.001.

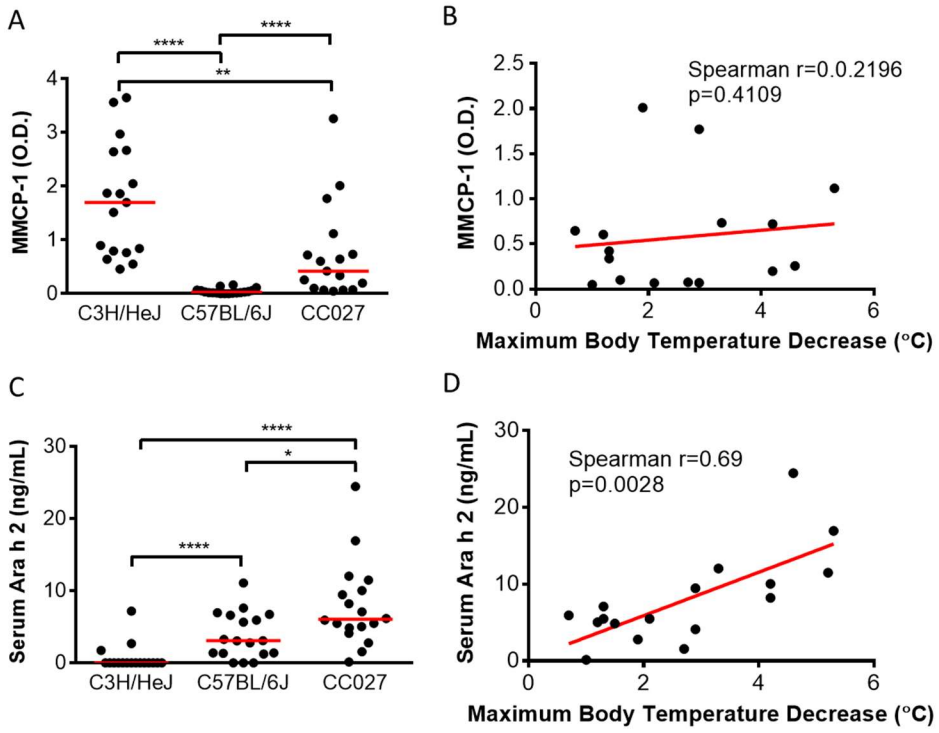

Figure 4-6. Post-OFC serum levels of mast cell degranulation marker and the major peanut allergen Ara h 2. Serum levels of MMCP-1 (A) and Ara h 2 (C) 60 min after oral challenge; Mann-Whitney U Test *p<0.05, **p<0.01, ****p<0.0001. Correlations between MMCP-1 (B) or Ara h 2 (D) and maximum body temperature decrease following oral challenge in CC027/GeniUnc.

4.6 Supplementary Figures

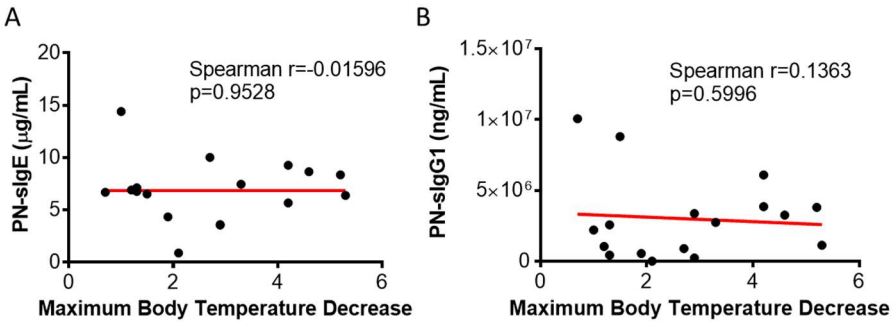

Figure 4-S1. Post-sensitization peanut-specific immunoglobulins and reaction severity correlation. Correlations between serum levels of PN-sIgE (A) and PN-sIgG1 (B) and maximum body temperature decrease following oral challenge; Spearman Correlation.

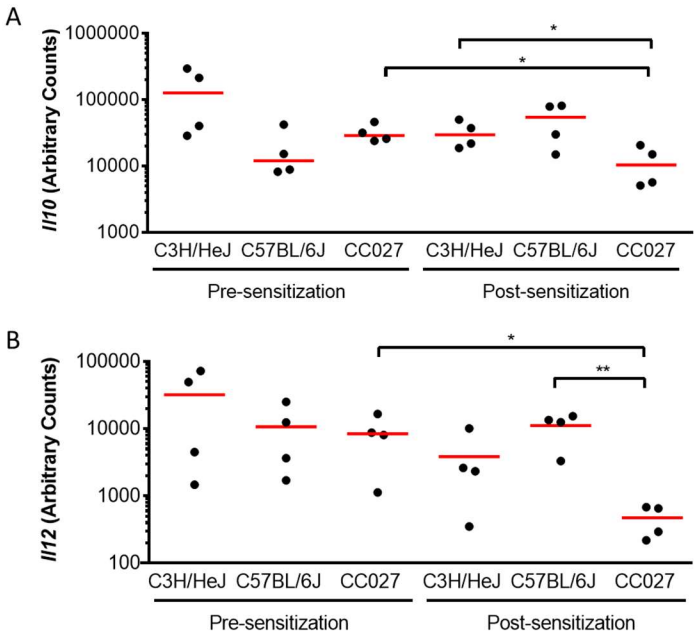

Figure 4-S2. Pre- and post-sensitization mRNA levels. *Il10* (A) and *Il12* (B) mRNA levels at baseline and post-sensitization for C3H/HeJ, C57BL/6J, and CC027 mice. Unpaired t-test *p<0.05, **p<0.01.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Over the past two decades there has been great progress in determining the underlying causes of food allergies as well as development of investigational treatments. Recently, a great deal of work has focused on potential food allergy contributing factors such as routes of sensitization,[43,194] genetics,[28,49,54,55] and microbiome.[57,65,214] While these studies have been both novel and informative, their findings have not been entirely conclusive as to a single cause of food allergies. This is most likely because food allergy is a complex disease with contributions from multiple factors including genetics as well as the environment. Further work is needed to examine the interplay of these many factors. Though food allergies to a wide variety of foods are similar in terms of the IgE-mediated reaction that occurs, it remains unclear whether the causes of allergy development are the same across all allergens.

Peanut allergies are of particular interest because they are one of the most common food allergies of childhood, are uncommonly outgrown, and are increasing in prevalence. Despite the great public health concern posed by peanut allergies, there are currently no FDA-approved treatments. Over the past 20 years, an immense amount of work has been done to develop and test investigational therapies for this disease. The leading therapies in the field include OIT, SLIT, and EPIT.[215] OIT and SLIT have both shown efficacy in the treatment of peanut allergy in clinical trials; however, these therapies each have their limitations. In their current forms, both require daily dosing, have risks of adverse events, and seem to offer limited protection once therapy is discontinued. Further, the mechanisms behind OIT and SLIT remain unclear. Undeniably, there is a need for improved, more targeted therapies, and the understanding of

OIT and SLIT mechanisms may provide insight into those targets. Finally, the development of improved, physiologically relevant animal models of peanut allergy are needed to both better understand the etiology of the disease as well as to provide a tool in which to develop these new therapies. The work in this dissertation contributes to each of these knowledge gaps: understanding the mechanisms of peanut immunotherapies, development of a targeted treatment, and the development of an improved mouse model, all with the long-term goal of improving therapy options for peanut allergies.

5.1 Mechanisms of Immunotherapy

5.1.a Summary of results

The work presented in Chapter 2 investigated the mechanism of both peanut OIT and SLIT. It was confirmed that OIT-induced plasma changes are capable of inhibiting effector cell activation, and that IgG is at least one of the necessary factors for this inhibition. Further, we demonstrated that the plasma factors act through both bound and unbound mechanisms to inhibit basophil activation. This work was the first in the field to demonstrate that this blocking capability of therapy plasma was also present in samples from subjects on SLIT. When comparing the blocking capability of OIT and SLIT plasma, we found that diluted SLIT plasma was less effective at blocking basophil activation than diluted OIT plasma, despite no difference in IgG4 quantity. These results suggest that either the IgG4 is functionally different between the two routes of therapy or that different isotypes play a role. Lastly, we showed that plasma blocking capability was different in subjects who experience long-lived SU from those who were transiently desensitized in OIT but not SLIT, suggesting that at least in OIT, the plasma blocking capability is important for clinical outcome.

5.1.b Future directions

Much work is still needed to understand the implications of these mechanistic findings. Data from others has shown that during OIT, IgG down-regulates basophil activity in an FcγRIIb-dependent manner and IgG4 inhibits basophil degranulation.[165,216] However, the depletion of IgG4 in these studies only partially reversed basophil hyporesponsiveness, suggesting other IgG subclasses may play a role. Future studies in which IgG1 and IgG4 are specifically depleted, isolated, and re-introduced to basophils in activation assays will be useful in defining a role for other IgG isotypes. Additionally, performing this experiment while blocking the FcγRIIb will identify the isotype(s) binding to this receptor. Because the IgG-depletion beads used in our previous studies are also capable of biding IgA and previous work has shown that allergen immunotherapy induces changes in IgA,[168] future studies will also investigate whether IgA plays a role in blocking effector cell activation. Importantly, performing the above experiments from samples at different time points of therapy would allow for identification of changes in humoral mechanisms throughout therapy. In other words, one isotype may be important early in therapy while a different isotype is important later in therapy. Once the important isotypes are identified, investigation into therapy-induced functional differences would be interesting. Future work will investigate differences in post-translational modifications such as glycosylation in these proteins during therapy that are not obvious when looking at quantity differences.

While the human studies above would benefit our understanding of peanut immunotherapy mechanisms, animal studies allow for further manipulation that is not possible in humans. Mouse studies in which whole plasma and separately specific antibody isotypes from mice that have undergone OIT is added to peanut-sensitized, untreated mice would also prove useful in demonstrating that these antibodies are sufficient to block anaphylaxis. Similar animal studies were performed by Burton, *et al.,*[165] but these studies were limited by the fact that they investigated the inhibitory role of the IgG fraction, rather than individual isotypes. Overall, these

studies would provide added mechanistic understanding to peanut immunotherapies with a specific focus on the humoral responses.

5.2 STALs

5.2.a Summary of results

Due to the limitations of the current investigational therapies for peanut allergy, new therapies are needed. In the work presented in Chapter 3, we showed that antigen-specific B cells can be targeted to prevent sensitization to peanut allergen in mice. In this study, we exploited an inhibitory siglec, CD22 on B cells using STALs, which are liposomes that simultaneously display a CD22 ligand and major peanut antigen, Ara h 2. A single intravenous injection with Ara h 2 STALs prevented sensitization, as measured by Ara h 2-specific IgE as well as reaction severity following Ara h 2 challenge. Mice that received Ara h 2 STALs also had decreased Ara h 2-specific IgG1 levels compared to controls. The effects were antigen specific and blunted the allergic response to whole peanut extract, despite targeting only one antigen. These animal studies were proof-of-concept that STALs could be used to target Ara h 2-specific B cells, and in doing so, could have an effect on the immune and clinical responses to peanut.

5.2.b Future directions

Future experiments will demonstrate whether STALs specifically deplete antigen-specific B cells. We can infer from our previous specificity results in which injection with Ara h 2 STALs resulted in decreased Ara h 2-specific IgE but had no effect on CT-specific IgE that this is the case; however it has not yet been definitely shown. We, along with collaborators with expertise in identification of rare antigen-specific B cells,[217] are currently developing a fluorescently labeled Ara h 2 tetramer (four biotinylated Ara h 2 proteins bound to streptavidin) that binds Ara h 2-specific B cell receptors, thus allowing for the identification of Ara h 2-specific B cells by flow

cytometry. This tool will allow us to show that the number of Ara h 2-specific B cells decreases in mice injected with Ara h 2 STALs. In early studies, Ara h 2 was chosen as the antigen to conjugate to STALs because it is one of the major peanut allergens, and has been shown to affect clinical reactivity when used in a mouse model of immunotherapy.[78] Preliminary attempts to use Ara h 1 STALs to prevent sensitization were unsuccessful. However, Ara h 1 is a trimer, making it possible that the multimer was not stable throughout the creation and injection of Ara h 1 STALs. However, developing STALs to the other peanut allergens may be useful to provide broader protection. Ideally, STALs specific for each peanut allergen would be combined into one therapy to offer greater protection. Furthermore, our work on this prevention model was all done in BALB/cJ mice. Initial studies using C3H/HeJ mice were unsuccessful. Further work is needed to determine whether other strains require a different dose of STALs, or alternatively if STALs cannot prevent sensitization in C3H/HeJ.

Further work is required to develop STALs as a therapeutic approach rather than the preventative approach discussed previously. In unpublished work, we have tested whether STALs can deplete memory B cells. In these experiments, splenocytes from sensitized animals were transferred into naïve animals. Naïve animals then received an intravenous injection with Ara h 2 STALs followed by an injection of Ara h 2 to boost antibody production. These mice produced less Ara h 2-specific IgE and exhibited less severe reactions following challenge with Ara h 2, demonstrating that STALs can deplete memory B cells in mice. These findings are consistent with previous work showing that STALs deplete human memory B cells *in vitro*.[181] In order to truly develop this technique as a therapy, other cell populations will need to be targeted simultaneously. Sensitized subjects have peanut-specific antibodies circulating, and treatment with STAL may lead to cross linking of IgE on mast cells, resulting in allergic symptoms. Alternatively, circulating antibodies may bind the STALs preventing them from reaching their B cell targets. One report showed that the use of proteasome inhibitor, Bortezomib, for 21 weeks decreased quantities of free-floating IgE, but had no effect on reaction severity after challenge,

suggesting that cellular-bound IgE persisted.[218] It is possible that prolonged treatment with Bortezomib in combination with STALs may result in treatment of peanut-allergic subjects. Overall, targeting antigen-specific B cells is a novel approach for allergy therapies that is effective at preventing sensitization in a mouse model of peanut allergy. While initial results are promising, much work still needs to be done to develop this CD22-targeted approach as a treatment.

5.3 Oral Challenge Model

5.3.a Summary of results

As discussed previously, there is a need for new treatments for peanut allergy. In order to develop treatments in pre-clinical models of food allergy, we first need improved animal models of the disease. Currently, there is not a reproducible model of peanut allergy that is both sensitized orally and reacts upon oral challenge. To identify such a model, we screened 16 strains from the genetically diverse Collaborative Cross, as described in Chapter 4. Female mice from each strain were sensitized orally to peanut with co-administration of CT. Of these 16 strains, two reacted upon oral challenge, and one, CC027, reacted reproducibly. We then characterized the immune responses in CC027 mice and compared them to well-established strains, C3H/HeJ and C57BL/6J. We found that CC027 mice mounted an IgE response to peanut, and more specifically to the major peanut allergens Ara h 1, Ara h 2, and Ara h 3. Furthermore, CC027 exhibited Th2-skewed immune responses, as indicated by an increased ratio of IL-4 to IFNγ compared to the other two strains. CC027 also had diminished regulatory responses, demonstrated by a decreased frequency of regulatory T cells and production of IL-10. Effector cell frequencies were increased in CC027, and basophils had decreased inhibitory receptor expression, suggesting that CC027 effector cells may be more easily activated. Finally, CC027 and C57BL/6J had detectable Ara h 2 circulating in the blood stream after challenge. Levels of circulating Ara h 2 correlated with reaction severity for CC027 mice,

suggesting that the increased antigen absorption is essential to the oral reactions observed. The fact that MMCP-1 was circulating during a reaction suggests that these reactions are in fact IgE-mediated, though confirmation that IgE is necessary for these reactions are still needed. Taken together, these results demonstrate that CC027 mice are an improved, relevant model of peanut allergy and further characterization may provide insight into etiology of the disease in CC027 mice as well as in humans.

5.3.b Future directions

Identification of an orally reacting model of peanut allergy can be used to better understand the etiology of the disease. We have shown that antigen absorption is correlated with reaction severity in CC027 mice. Further experiments are needed to determine the cause of increased antigen absorption in these mice. Initial Ussing chamber studies found that intestinal permeability after sensitization is higher in CC027 mice compared to C3H/HeJ and C57BL/6J, though the differences are not significant. These studies need to be repeated with fluorescently labeled Ara h 2, rather than the large FITC-labeled Dextran molecule that was used initially, as size of the molecule may be important. Currently the microbiome differences between the three strains studied both before and after sensitization are under investigation. Initial findings show that despite co-housing, the three strains have distinct microbiomes. Furthermore, the microbiome of CC027 shifts in diversity following sensitization. Work is currently being done to elucidate which microbes appear to be important for both conferring protection as well as leading to increased sensitization. We plan to use antibiotics to deplete the gut microbiome of mice and then perform fecal transplants with stool samples from other strains to see if this can result in transferred protection or risk of sensitization. In addition to microbiome, we are currently investigating the genetic underpinnings of the observed phenotype in CC027 mice. CC027 mice have been crossed with either C57BL/6J or C3H/HeJ. F1 progeny have been sensitized, and mice that react as well as those that do not react following oral

challenge have been identified. Genotypes of these mice are being analyzed for genomic regions that appear important for the oral reaction phenotype. Our long-term goal is to find differences in these mice and investigate whether they can be translated to humans with the disease.

Currently our group is expanding the use of CC027 mice outside of the original model. Our early studies found that CC027 mice had detectable levels of peanut-specific IgE even prior to sensitization with peanut and CT. We suspect that these mice mount an IgE response to seed storage proteins (homologous with Ara h 1, 2, and 3) contained in their chow with known cross-reactivity to peanut. These findings led to the hypothesis that CC027 are capable of mounting an IgE response to antigen in the absence of adjuvant. Preliminary experiments in which mice were sensitized orally once per week or three times per week for four weeks with peanut extract in the absence of adjuvant have demonstrated that these mice do in fact make peanut-specific IgE and that they react upon oral challenge with peanut extract. Interestingly, they seemed to be sensitized in a dose-dependent manner with the mice that were sensitized once per week producing less peanut-specific IgE and reacting less severely than those sensitized three times a week. This initial experiment needs to be repeated with a larger number of mice and with control groups that receive a combination of CT and peanut. These preliminary results raise the question of whether CC027 mice are capable of achieving oral tolerance. In a future experiment, we plan to challenge the mice with an extract created from their chow. CC027 mice may serve as a model of peanut allergy in which mice can be sensitized orally in the absence of adjuvant and react orally.

In addition to being sensitized without adjuvant, one pilot study on CC027 mice showed that both male and female mice can be sensitized orally to peanut with CT and both sexes react upon oral challenge. Traditional animal models of food allergies exclusively use female mice because males are typically more difficult to sensitize. This experiment needs to be repeated in a larger number of animals, but the initial findings are encouraging that CC027 could be used as

a model of peanut allergy in which both males and females react following oral challenge. A model that uses both sexes would allow for sex-based differences in treatment outcomes to be noted in future studies.

A model that is sensitized orally and reacts following oral challenge can be used for the development of an OIT model. Pilot studies in these mice have found it difficult, though possible to desensitize CC027 mice over a month long regimen of treatment. Optimization of dosing and time is still required. This model will allow for further mechanistic studies on OIT as well as improvements in OIT such as the use of adjuvants. Furthermore, the ability of other therapies including DNA vaccines and STALs, to alter oral tolerance can only be tested in an orally reacting model. Therefore, CC027 mice provide a valuable tool to the food allergy research community as a platform for drug discovery and etiology investigations.

5.4 Concluding Remarks

The past two decades have seen promising results from peanut immunotherapies in clinical trials. These are likely to become the first FDA-approved therapies for food allergies. However, they come with their limitations, requiring the discovery of new future therapies. The allergy field has seen an explosion of research into disease etiology, specifically the contributions of the microbiome and genetics to the development of food allergy. The ultimate goal of these studies is to identify targets for future therapies. The findings presented here contribute greatly to possible future developments. These results provide mechanistic insight into investigational allergen immunotherapies, suggesting that antibody functional differences are important for clinical outcome in OIT. Additionally, a novel treatment approach that targets peanut-specific B cells for deletion is described in a mouse model of peanut allergy. Perhaps the greatest contribution of all is the development of an improved food allergy model that can be used to better understand causes of the disease and allows for the identification of new therapy targets. Together, these findings provide the foundation for future allergy treatments.

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