The study of immune mechanisms underlying peanut allergy

vs. clinical tolerance

by

Larisa Lotoski

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree

MASTERS OF SCIENCE

Department of Immunology

University of Manitoba

Winnipeg

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The Study of Immune Mechanisms Underlying Peanut Allergy vs. Clinical Tolerance

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Abstract

Objective: Study peanut-driven cytokine responses, plasma cytokine biomarkers, and immunoregulatory mechanisms differentiating peanut allergic and non-allergic human populations.

Methods: Plasma IL-10 and, following primary culture of Ag-stimulated PBMC, type 1, type 2, cytokine production and its regulation was quantified using high sensitivity ELISA.

Results: Elevated plasma IL-10 is not associated with clinical tolerance to peanut. We identify a role for elevated IL-9 production in peanut allergy. Seeking immune mechanisms responsible for clinical tolerance or limiting allergy, we found inhibition of IL-10/IL-10R, TGF-β, or IDO results in enhanced cytokine production among peanut allergics, but plays a minimal role in tolerant individuals.

Conclusion: Clinical allergy is associated with quantitatively elevated IL-5, IL-9, and IL-13 production. IL-10, TGF-β and IDO act as a brake on excessive type 2 responses amongst allergics but do not show evidence of a role in maintaining clinical tolerance. Plasma IL-10 levels are not predictive of clinical tolerance towards peanut.
Acknowledgements:

I would like to express my sincere gratitude towards my supervisor, Dr. Kent HayGlass, for his continuous support and academic guidance throughout my program whilst giving me the opportunity to develop independence in my work. Dr. HayGlass' extensive knowledge, logical way of thinking, and constructive comments has been of great value during my entire program.

I am indebted to Dr. Estelle Simons, Dr. Joel Liem, and Dr. Allen Becker and the entire MICH Allergy Lab for their immense contribution to the work found in this thesis. This thesis would not be possible without the tireless work of Rishma Chooniedass, whose endless enthusiasm and excellent organizational skills were a critical component in my study population base.

I would like to thank my committee members, Dr. Abdel Soussi Gounni and Dr. Estelle Simons, for their support, contribution of expertise, and review of the present thesis.

I am grateful for being given the opportunity to be a member of the multidisciplinary National Training Program in Allergy and Asthma (NTPAA) and the AllerGen NCE program. I would like to acknowledge CIHR, AllerGen NCE, and NTPAA for their generous funding throughout my program.

I wish to thank my colleagues, Bill Stefura, Dr. Ruey Su, Isha Ostopowich, Sara Courtis, Christine Fortier and all the summer students for providing me with a supportive and always fun workplace. To Stephanie, you made two years fly
by, whether it was during a just another day in the lab or while skiing, kayaking, hostelling, etc. Thank you for being a great friend.
Dedications:

This thesis is dedicated to my parents, my sisters and my husband. To my parents, Joe and Karin Lotoski, for never defining a limit to my education and always providing an encouraging and supportive environment. To my sisters, Avila and Sabina— for providing peer pressure and understanding. To my husband, Dan, for allowing me to choose my own path versus the safe path and unequivocally supporting me the entire way.
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List of Abbreviations

\( \gamma \text{c}: \) \( \gamma \)-chain

\textbf{DC}: dendritic cells

\textbf{DC-SIGN}: DC-specific ICAM-grabbing nonintegrin

\textbf{ELISA}: enzyme-linked immunosorbent assay

\textbf{GALT}: gut associated lymphoid tissue

\textbf{Gfi-1}: growth factor-independent-1

\textbf{DBPCFC}: double-blind placebo controlled food challenge

\textbf{IDO}: indole-2,3-dioxygenase

\textbf{1-MT}: 1-methyl-D-tryptophan

\textbf{Hx+SPT+}: history positive, peanut Ag SPT positive (peanut allergic)

\textbf{Hx-SPT+}: history negative, peanut Ag SPT positive (peanut sensitized)

\textbf{Hx-SPT-}: history negative, peanut Ag SPT negative (clinically tolerant)

\textbf{Ag}: antigen

\textbf{LPS}: lipopolysacchride

\textbf{IL}: interleukin

\textbf{GI}: gastrointestinal
Ab: antibody

FAHF-2: food allergy herbal formula-2

CI: confidence interval

CTLA-4: cytotoxic T lymphocyte Ag-4

IL-9Rα: IL-9 receptor α-chain

Ig: immunoglobulin

IFNγ: interferon-γ

EDTA: Ethylene diamine tetraacetic acid

Med: complete media

mAb: monoclonal Ab

ME: 2-mercaptoethanol

MDDC: monocyte-derived DC

MHC: major histocompatibility complex

mRNA: message ribonucleic acid

NPV: negative predictive value

OIT: oral immunotherapy

OT: oral tolerance
PHA: phytohemagglutinin

PBMC: peripheral blood mononuclear cells

PMA: Phorbol 12-myristate 13-acetate

pNPP: p-nitrophenyl phosphate

PPV: positive predictive value

R: receptor

rIL-10: recombinant interleukin-10

SHP-1: Src homology 2 domain-containing protein-1

Smad: Smad ubiquitin regulatory factor

SPT: skin prick test

SOCS: suppressor of cytokine signalling

TCR: T cell receptor

TGF-β: transforming growth factor-β

Treg: regulatory T cells

WPE: whole peanut extract
Introduction

An introduction to peanut allergy

The peanut is a highly cultivated legume that has been consumed by humans for over 2000 years. Peanuts are a high source of protein, oil and a wide variety of nutrients. They are inexpensive to cultivate, making their use in our diet highly favourable. Negatively, peanuts are one of the most common foods in which allergic reactions are formed (1), and affect over 250,000 individuals in the U.S (2), and accounted for 20% of 32 hospital treated food-induced anaphylactic fatalities recorded between 1994 and 1999 (3).

Peanut allergy can be defined as the intolerance towards peanut allergens. The result is an immunological memory towards peanut antigen, which leads to negative immune response and clinical reactions upon peanut re-exposure (1). Approximately 6% of children and 4% of the total U.S. population suffers from food allergy (4). More specifically, 1.34% of children and 1-2% of North American adults suffer from peanut allergy (5-7). In an unselected Danish population peanut allergy was found to be the most common food hypersensitivity in adults (8).

Overall, there is no convincing evidence to support the outgrowth of peanut allergy. Skolnick et al. recorded over a 20% outgrowth of peanut allergy (9), but Emmet et al. and Sicherer et al. indicate that there is little difference in prevalence between child and adult population (2,10). Therefore, to understand the pathophysiology and potential subsequent outgrowth of peanut allergy may be critical in the process of developing new therapies and cures for this disease. Savage et al. have reported that
peanut allergy is diagnosed at the median age of 3 years, but additionally have noted, a less frequent but distinct second population has been identified. A second population of peanut allergic individuals are diagnosed later in life. Late onset of peanut allergy occurs at a median of 25 years and is often associated with lower peanut specific IgE and milder symptoms (11).

A report by Grundy et al. showed there was no significant increase in the prevalence of peanut allergy in children (12). Interestingly, Sicherer et al. compared U.S. peanut allergy prevalence data from 1997 to that collected in 2003 using a random phone survey. This report showed a significant increase from 0.4 to 0.8% of peanut allergy in a child population, but no significant change was recorded in the adult population (2). Therefore, there is no convincing current indication that peanut allergy prevalence has changed over the last 11 years.

It was once thought that peanut allergy was a lifelong disease, but Fleischer et al. have shown that up to 20% of peanut allergic children will outgrow their allergy. In a re-examination of the same study population, recurrence most commonly occurred in those who avoided peanut after a negative oral challenge outcome (9,13,14). Fleischer et al. reported that those who are able to outgrow their allergy have low levels of peanut specific IgE and have never suffered from peanut induced anaphylaxis (13). This was confirmed during a subsequent study that showed age and initial IgE levels are not factors that enhance the probability of resolution, but the lack of complete involvement of the skin, gastrointestinal (GI), and respiratory systems and lower IgE levels at the time of resolution improved these chances (9).
Upon ingestion of peanut, those suffering from peanut allergy experience a wide variety of symptoms affecting the skin, GI, and respiratory systems. Clinical symptoms arise at a median onset time of 3 minutes but can occur within seconds to up to 2 hours after ingestion, with 95% of patients experiencing their symptoms within 20 minutes. The average age of those experiencing their first reaction towards peanut is 24 months. The extremely low level of peanut antigen required to elicit a clinical response (as low as a few milligrams) as well as the average age of onset are two of the many factors that makes peanut allergy one of the more dangerous allergies. Skin symptoms, which are most common, often include a pruritic erythematous skin rash, acute urticaria, and/or angioedema. The GI and respiratory involvement result in acute vomiting, and/or abdominal pain, diarrhoea, and laryngeal oedema, repetitive coughing, voice changes, and wheezing of the lower respiratory tract, respectively. Reactions are often localized, but in severe cases may involve multiple organ systems, leading to anaphylaxis (4,15).

"Anaphylaxis is a serious allergic reaction that is rapid in onset and may cause death," according to the representatives of 16 organizations and government bodies present at the Second Symposium on the Definition and Management of Anaphylaxis. Symptomatically, anaphylaxis can be defined as occurring with acute onset of illness, and at least one or two of the following, depending on the circumstance: reduced blood pressure, respiratory compromise, skin-mucosal tissue involvement and persistent GI symptoms (16). Organ systems involved include the skin, respiratory tract, GI tract, cardiovascular system, and central nervous system (17). Anaphylactic induced fatalities are most commonly due to respiratory arrest, may occur in those with previous mild allergic reactions (18), and are most commonly caused by peanut (19,20). Up to 20%
of all patients who experience anaphylaxis have secondary late-phase (or biphasic) reactions within 1 to 72 hours of resolution of their initial reaction. The review of biphasic reaction prevalence by Tole et al. demonstrated that study populations are very limited and never focus on a single allergy. Biphasic reactions are more difficult to treat (4,21,22). Predisposition to anaphylaxis is multifactorial, making predictors of peanut allergy severity difficult to identify. Progress in this area has been made, as platelet-activating factor acetylhydrolase is significantly reduced in children with fatal anaphylactic reactions to peanut; platelet-activating factor is greatly elevated in these patients (23).

To date, strict avoidance of peanut ingestion is the only way to effectively prevent allergic reactions to peanut. Consequently, peanut allergic individuals must diligently avoid ingestion of peanut in their diet. Therapies for IgE-mediated peanut allergy have been explored in the past, with some more successful than others (24). Leung et al. reported that TNX-901, a humanized IgG1 monoclonal Ab (mAb) against IgE, was successful in enhancing peanut allergic individual's threshold towards peanut to levels greater than those that could occur through accidental ingestion (25). The use of recombinant engineered food proteins and bacterial adjuvant have been used in immunotherapy approaches (26). More traditional therapies have also been explored. In murine models of peanut allergy, a Chinese formula made from nine different herbs, Food Allergy Herbal Formula 2 (FAHF-2), has been shown to reduce histamine release, peanut-specific serum IgE, and peanut specific type 2 cytokines associated with peanut allergy. Interestingly, the 9 herbs act synergistically, and do not carry the same effect when used on an individual basis (27). Recently, oral immunotherapy (OIT) has shown
to successfully reduce peanut dose thresholds in at least 75% of peanut allergic individuals (28,29). OIT recipients demonstrated long term decreased levels in peanut specific IgE, increases in peanut specific IgG₄ and modulations in cytokine response profiles (29). Others have taken alternative paths by investigating the enhanced secretion of immunoregulatory cytokines to abrogate allergy. Frossard et al. have used an immunoregulatory-IL-10-secreting *Lactococcus lactis* strain to reduce anaphylactic scores in food sensitized mice (30). Studies, such as this one, emphasize that understanding regulatory mechanisms underlying peanut allergy and clinical tolerance are as important as the mechanisms resulting in disease. The initial treatment of peanut-induced anaphylaxis is epinephrine. Epinephrine is administered intramuscularly with the onset and identification of anaphylaxis. Lack of symptom resolution may result in multiple dose administration, but it is unclear if multiple doses are required for all anaphylactic reactions, as the singular study by Jarvinen et al. focused on children with more severe reactions (31).

*Diagnosis of peanut allergy*

Peanut allergy diagnosis is a life changing event. Aside from psychological stress associated with knowing the possible results of accidental exposure (32), strict avoidance of peanuts is challenging, as peanuts are a common ingredient in many manufactured foods due to their nutritional value (33). Therefore, it is imperative that accurate diagnoses occur. Food allergy diagnosis begins with a medical history and clinical examination. The main focus is to identify the causative agent, the organ system involvement, other factors that may have provoked the symptoms, the presence of other allergies, and the time between contact/ingestion and symptom presentation.
Although the double-blind placebo-controlled food challenge (DBPCFC) is considered the gold standard of food allergy diagnosis, it is often considered unnecessary in the presence of a convincing clinical history. More commonly, skin prick tests and quantification of peanut specific IgE are used in initial diagnosis of peanut allergy (34). Food challenges are potentially dangerous to the patient, however, when performed by an expert they can be safe, although time consuming (35,36).

Skin prick tests (SPT) provide a rapid means of identifying peanut sensitization in potentially allergic patients. Whole protein extracts resuspended in glycerol and saline are applied to the skin, which is punctured, and the measurement of a reaction incurred is taken. Roberts and Lack determined that a SPT diameter ≥8mm has a positive predictive value (PPV) of 94.4% (95% CI) with a specificity of 98.5% and a negative predictive value (NPV) of 57.3% with a sensitivity of 25.4%. This cut-off point is generally accepted as variables such as age and time between testing did not affect the PPV (37). As discussed later in detail, the poor negative predictive value of the SPT results in a large percentage of the peanut allergic population with negative SPT results. For this reason we have used a lower cut-off value of ≥3mm for this study. SPT determine the presence of mast cell degranulation within the dermis in the presence of antigen (Ag) (38). Unfortunately, with the exception of purified peanut antigens (39), SPT performed with standard commercially available reagents results are unable to predict clinical severity (40). More commonly, a positive SPT is defined as a wheal diameter of at least 3 mm, or 3 mm greater than the negative saline control (41). 75% of those who react to peanut during an oral food challenge have SPT ≥3mm, but only 25% elicit SPT responses ≥8mm (37).
immunological reaction within the dermis and the actual mechanism underlying peanut allergy remains unclear. That is, no study has investigated if clinical SPT reactivity is predictive of peanut specific effector function in primary cultures of peanut allergic populations.

The commercial reagents used in SPT diagnoses have little to no regulation (38). They are often purchased not with a predetermined concentration, but as a dilution from a combination of select whole peanut extracts in glycerol and saline. These extracts may contain variable amounts of undefined non-allergenic and allergenic substance (e.g. carbohydrates, histamines, other proteins, etc.) (42). The variations between commercial SPT reagent brands, which include total protein content, preservatives, glycerol levels, and the extract food matrix, can lead to differential SPT results (43-45). These factors may potentially affect the diagnosis outcome. As described by Roberts and Lack, “standardization of allergen content of (SPT) reagents are the single most important determinant of outcome (46).” For allergen extract manufacturers in the U.S. The Food and Drug Administration provides guidelines to measure the potency of allergen extracts, whereas in Europe, potency is determined using in-house reference standards. In Europe, the exact allergen content and concentrations may be measured, but are not released to those purchasing the reagents (47). The result of non-standardized methods leads to reagents differing in RAST inhibition curves, peanut allergen concentrations, and overall protein/allergen content. Commercially available SPT reagents do not always produce equivalent mean wheal diameters (48). As Rancé et al. have shown, for the use of SPT, raw peanut extract have a PPV of 100%, whereas commercially available reagents provided negative results in 18% of the
proven peanut allergic population (49). These data clearly outline the need for improved diagnostics in the field of peanut allergy.

Peanuts contain several allergens that are able to elicit an immune response in peanut allergic subjects. Eight allergens have been identified, and subsequently named, Ara h 1 to 8. Peeters et al. have shown that the in vivo reactivity pattern to these purified allergens has been shown to correlate with clinical severity. Over 80% of peanut allergics show a positive SPT and specific IgE binding to Ara h 2 and Ara h 6. Reactivity to Ara h 3 is significantly higher in those with more severe peanut allergy. These observations are limited to allergic populations, and did not investigate a viable method for differentiating between clinically tolerant and peanut allergic populations, which remains a shortcoming of food allergy diagnosis (39). Flinterman et al. has also shown that Ara h 2 and 6 as the most highly recognized allergens, which remain stable over time, but in contrast, clinical severity does not correlate with recognition of any of the allergen epitopes (40).

Most basic science studies investigating the mechanism of peanut allergy utilize purified peanut Ag extracts (containing one or more of the six major peanut allergens) or ground whole peanut prepared in the lab (6,50-52). In a mouse model of peanut allergy, van Wijk et al. examined the effect of food matrix in vivo in comparison to purified peanut allergen. They found that peanut extract (containing soluble peanut protein), but not purified peanut Ag (Ara h 1,2 or 6), was able to enhanced cell proliferation, expression of costimulatory molecules, as well as cytokine production (45). This data implies that whole peanut extract may better represent true immunologic
reactions related to peanut allergy. To date, no studies characterizing the correlation between peanut allergen extracts for in vivo skin prick testing and in vitro stimulation from both commercial and laboratory sources exist.

The quantification of peanut specific IgE in combination with a SPT and a patient’s medical history is commonly used in clinical practice for determining sensitization to peanut. Peanut specific IgE isolated from human venous blood is quantified using the ImmunoCAP Test, and is presented as a range from <0.35 to >100 kUA/L. Sampson et al. determined that a decision point of 15 kUA/L (95% CI) provided a sensitivity, specificity, PPV, and NPV of 73, 92-100, 95-100 and 36%, respectively (53,54). In a subsequent study, Perry et al. determined that 41% and 24% of those who failed DBPCFC had peanut specific IgE between 0.5 and 1.9 kUA/L (p<0.001) and <0.35 kUA/L, respectively (36). To obtain PPV of 100%, an ImmunoCAP result must exceed 57 kUA/L (49). Interestingly, the presence of IgE specific to peanut antigen, Ara h 1 and Ara h 3 are present in only 20-40% and a much higher 87% of patients with symptoms of peanut allergy, respectively (55). These data indicate that although quantification of peanut specific IgE is a useful diagnostic tool for peanut sensitization, it presents problems in the gray areas present below decision points and, like the peanut SPT, is unable to predict clinical severity (54).

The utilization of individual diagnostic methods can prove to be challenging during diagnosis of potentially allergic patients. Whereas, in combination clinical history, peanut SPT, and ImmunoCAP Test results can yield 100% PPV when SPT and ImmunoCAP results exceed 16mm and 57 kUA/L, respectively (49). As discussed
earlier, SPT and ImmunoCAP Test values exceeding these cut-off limits are unreasonable, as the majority of those with peanut allergy do not fall within these limits (37). The use of SPT can be helpful in determining if a food challenge is necessary when peanut specific IgE levels are <10kUA/L. A SPT diameter ≥7mm provides a specificity, PPV, and sensitivity of 97, 93, and 83%, respectively (56). As emphasized by Rancé et al., the gray areas presented by ImmunoCAP and SPTs result in costly oral food challenges (49). A more statistically reliable diagnostic method is warranted.

The use of serum biomarkers is a common tool in the diagnosis of several diseases. Candidate disease biomarkers have been identified in asthma (57), atopic dermatitis (58), chronic obstructive pulmonary disease (59), inflammatory bowel disease (60), and anaphylaxis (23). Attempts at finding a convincing biological marker of peanut allergy have proven to be difficult. As described in detail above, diagnostic tools in peanut allergy are most useful for the extremes of sensitization, presenting gray areas with poor PPV and NPV for a large percentage of the population. An allergic reaction to peanut is complex and multifactorial, thus, individual components associated with allergy have not provided a strong correlation with biological allergic reaction events (61). Factors, such as reduced serum angiotensin-converting enzyme, are only able to help clinicians assess an enhanced risk of anaphylaxis and do not provide a definitive answer to patients concerned of such risks upon accidental exposure to peanut (62). Alonso et al. reported elevated levels of serum interleukin-10 (IL-10) in hazelnut allergic patients who had recently outgrown their allergy in comparison to those who were hazelnut allergic. This study is unique, as it identified a potential marker of clinical tolerance, rather than a disease state (63). The role of IL-10 (see Introduction Section 10}
1.3: *Regulatory mechanisms of peanut allergy*, or its potential as a biomarker of clinical tolerance, has yet to be investigated in peanut allergy.

*The immunologic mechanisms that underlie peanut allergy*

The process of sensitization in food allergic individuals remains unclear. Upon ingestion, food proteins are degraded in the GI system. Upon entering the GI tract, soluble proteins are passed from the gut lumen into the gut associated lymphoid tissue (GALT) (64) via specialized epithelial cells, M cells. Dendritic cells (DC) within the GALT uptake, process, and present protein fragments with Class II major histocompatibility complex (MHC) molecules. Upon T cell receptor (TCR) recognition of the MCH-Ag complex by T cells, priming and activation ensue. Type 2 humoral and cellular responses are formed leading to the characteristic cytokine-associated mechanism of food allergies. Activated type 2 cells produce IL-5, and IL-13, among others. These cytokines shape the activation of B cells, causing the production of peanut specific IgE. Upon re-exposure to the food in question, Ag-bound IgE binds and cross-links the high affinity FcεRI found on basophils and mast cells. Degranulation of basophils and mast cells results in the release of proinflammatory mediators, such as histamine, prostaglandins, leukotrienes, and platelet-activating factor, leading to the symptoms of food allergy. This basic understanding of food allergy is based on studies involving animal models, allergen sensitization using cholera toxin, and food Ag other than peanut (4).

In contrast to our previous understanding of the mechanisms underlying allergy (65), Thottingal *et al.* demonstrated that peanut allergic individuals build a type 2
cytokine (interleukin (IL)-5, IL-13) and chemokine (CCL22) response, independent of type 1 cytokine (IFN-γ) and chemokine (CXCL10) production (6). In agreement with this finding, Turcanu et al. showed that there is no significant negative correlation between peanut specific Th1 cytokine and IgE production. Additionally, peanut specific type 2 cytokine production positively correlated with B cell IgE responses, but only in peanut allergic (IL-4: \( r'=0.635, p=0.02 \); IL-13: \( r'=0.641, p=0.025 \)) and not clinically tolerant populations (66). In peanut allergic individuals 27 distinct peanut specific IgE epitopes have been mapped on the three of the major allergens of peanut. Ara h 1, Ara h2, and Ara h 3 contain 17, 5, and 5 of these epitopes, respectively. In a population of 24 peanut allergic children, thirty to forty percent of peanut allergics recognized 4 dominant sequences located on Ara h 1 (2), Ara h 2 (1) and Ara h 3 (1), but an increase in epitope diversity correlates positively with greater clinical severity. The limitations of this study include a small non-atopic control population of only 6, which were used to determined the cut-off point of IgE reactivity towards the select peanut Ags (67).

The induction of peanut-induced anaphylaxis is dependent on the Ag-specific interaction of T and B cells. In a murine model of peanut-induced anaphylaxis, CD40L-/- B cell-deficient mice were unable to undergo immediate anaphylaxis. Interestingly, the type 2 cytokine profile of sensitization and the late phase of anaphylaxis, involving enhanced neutrophil, lymphocyte, and eosinophil recruitment remained unchanged from the sensitized WT mouse model (68).

Peanut Ag is able to directly activate CD14+ monocyte-derived DC, inducing upregulation of activation-associated surface molecules and direct a type 2 skewed
response. These responses are dictated by binding of Ara h 1 to DC-specific ICAM-grabbing nonintegrin (SIGN) on DC, which subsequently activate MAPK Erk 1/2 in DC (50).

Interleukin-5

IL-5 is a homodimeric glycoprotein linked by two disulfide bonds and is the most conserved member of the IL-4 cytokine family (69). IL-5 is produced primarily by Th2 lymphocytes, but has been reported to be produced by Tc2, mast cells, eosinophils, γδT cells, NK, and NK T cells (70,71). It is widely accepted that IL-5 is a type 2 cytokine. The overproduction of which is associated with allergic and asthmatic disease. Peanut Ag-driven IL-5 is produced by non-allergic populations, but to a lesser extent than allergic populations. The production of peanut specific IL-5 has been demonstrated in both peanut allergic humans (6) and peanut sensitized mice. In mice, the peanut specific IL-5 response occurs in a dose dependant manner upon peanut Ag challenge (72). In egg allergy, OVA-specific IL-5 (vs. IL-13) most closely correlates with disease severity (73), and IL-5 KO mouse models show a prevention in allergic reactions (74). Taking into account these studies discussed, it is apparent that the mechanism of allergy remains controversial, as Foster et al. suggests that abrogation of IL-5 prevents allergy, whereas Thottingal et al. emphasize that peanut specific IL-5 is produced in non-allergic populations.

IL-5 was first identified as a B cell growth factor but is best characterized for its regulation of eosinophils (71). In concert with IL-4 and IL-13, IL-5 exhibits eosinophil chemotactic properties, is able to prolong eosinophil survival, promote eosinophil
adhesion to VCAM-1 on epithelial cells and enhance eosinophil effector function (69,70). The study of the involvement of eosinophils in peanut allergy is limited. Mediators produced by eosinophils have been identified in the serum, stool, gut lavage and urine samples of IgE mediated food hypersensitivity patients (75,76). Seven duodenal biopsies from food allergic patients (1 with peanut allergy) expressed IL-5 mRNA, and 87% of the cells expressing IL-5 were eosinophils, but it is unclear what levels of IL-5 mRNA exist in healthy individuals (77). Enhanced Ag-specific IL-5 mRNA expression in gut lesions of OVA-sensitized mice has also been detected (78). Eosinophil recruitment is abrogated with anti-IL-5 mAb treatment in OVA sensitized mice (79).

Ligation of IL-5 to its primary receptor, IL-5R, results in the activation of cytoplasmic kinases and transcription factors, which include Btk, JAK2, PI3 kinase, PLCγ2, and STAT5 in B cells. Additionally, IL-5R signalling results in activation of JAK2, Lyn, Raf-1, SHP2, and the Ras-ERK pathway in eosinophils. In B cells, these events cause rapid upregulation of the blimp-2 transcription factor, and c-Myc, c-Fos, c-Jun, Cis, Cish1/Jab, and pim-1 gene expression in B cells (70). In T cells, The Ras-ERK pathway is TCR-mediated, and absolutely necessary for Th2 differentiation. Inhibition of mediators involved in this pathway lead to a Th1 shift (80).

T lymphocyte activation and differentiation leading to the Th2 phenotype is induced by the transcription factor, GATA 3. Stable expression of GATA 3 is dependent on Growth factor independant-1 (Gfi1), which is expressed in an IL-4/STAT 4 dependent manner. Differential regulation of IL-5 and other type 2 cytokines (such as IL-4 and IL-
The expression of IL-5, but not IL-4 or IL-13, is impaired at the promoter level in the absence of Gfi1 (80). The regulation of the IL-5R is controlled synergistically by IL-4, IL-5 and IL-13 (70). Although IL-5 is able to bind the unique IL-5 α-chain, the common β-chain is necessary for signal transduction to occur (81). These reports suggest that enhanced peanut Ag driven IL-5 production is an integral component of the peanut allergic reaction, but the regulatory mechanisms controlling peanut specific IL-5 have yet to be investigated.

Interleukin-9

IL-9 is a Th2 cytokine that was first identified as a T cell and mast cell growth factor (82,83). Umezu-Goto et al. stated that in atopic asthmatics the only detectable source of IL-9 is CD4+ T cells (vs. CD8+ T cells) (84). In contrast, others have demonstrated that eosinophils from dust mite allergic humans, BALB/c derived mast cells, and eosinophils and neutrophils from human atopic asthmatic bronchial biopsies are capable of producing IL-9 (83,85,86). Excessive production of IL-9 has been implicated in spontaneous proliferation of malignant T cells (87), in asthma and allergy (88), and is involved in T cell development (89). IL-9 and the IL-9R have been shown to be upregulated in the bronchial tissue of asthmatics and allergics vs non-allergic lung diseases (86), thus leading to the possibility that it may be involved in the pathogenesis of food allergy. Interestingly, mice with tolerogenic skin graft transplantations have high levels of IL-9 producing CD4+CD25+ regulatory T cells (Treg). In this same model, a delay in CD8+-mediated graft rejection was reversed with anti-IL-9 treatment, implying that IL-9 may play an important role in peripheral suppression (90).
IL-9 signalling occurs through the IL-9 receptor (IL-9R). The IL-9R has two components, the IL-9 specific α-chain (IL-9Rα) and the common γc. When bound to IL-9, the heterodimeric receptor components, IL-9Rα and γc, signal through JAK1 and JAK3, respectively. As a result of receptor signalling, phosphorylation of the IL-9R leads to docking sites for STAT1, STAT3, and STAT5 (91-93). These series of events lead to the differentiation, proliferation, and inhibition of apoptosis in T and B cells (93,94). The regulation of apoptosis by STAT1, STAT3, and STAT5 was found to be redundant in a lymphoma cell line, but this data may not translate to peanut allergy. IL-9 signalling through the JAK/STAT pathway also induces inhibitory signals via the expression of the cytokine-inducible SH2-containing protein, suppressor of cytokine signalling (SOCS)-1, and SOCS-3. It has been suggested that IL-9 may provide negative feedback inhibition on its own production via STAT-dependant SOCS-3 production (95). Furthermore, type 1 cytokine production is able to inhibit IL-9 production (96).

IL-9 biological activity has been implicated in the mechanism of allergy. IL-9 is involved in the development of OVA-induced anaphylaxis (97), but is not absolutely necessary for it to occur (98). In an IL-9-overexpressing mouse model, eosinophilia and B cell activation were induced. The overexpression of IL-9 was achieved using transgenic mice and therefore may not correctly represent Ag-specific allergic state (99). A report by Little et al. demonstrated that the HBE4-E6/E7 cell line responded to rhIL-9 treatment with the release of T cell chemotactic activity (100). IL-9 has also been shown to be a contributing factor in mastocytosis, using an IL-9 deficient mouse with a Th2 Schistosoma mansoni-dependant phenotype (101). The effects of IL-9 on mouse-
derived CD4+ T cells include the enhancement of IL-5, but although IL-9 neutralisation led to abrogation of IL-5 production, the function of IL-5 was not diminished (102). IL-9 is also able to significantly enhance FcεRI on allergic asthmatic human neutrophils (103) and promote of IgE synthesis. IL-9 is unable to induce IgE synthesis unaided, and requires the synergistic activity of IL-4 (104,105). Approximately 800 inducible genes are controlled by IL-9, which is similar to IL-5, but twice that of IL-4 (106). The IL-9 studies described above have been limited to cell lines, healthy human donors (100), or its effects have been examined using non-Ag specific methods (99). Although, the above data does provide evidence that IL-9 may be involved in the pathogenesis of peanut allergy, it remains unclear if IL-9 contributes to the peanut allergic state.

Interleukin-13

IL-13 is a significant component involved in the pathogenesis of several diseases and clinical conditions, including pulmonary fibrosis, cancer, and allergies, such as allergic asthma (107-109). Polymorphisms in and interactions between IL-13 and GATA-3 have been shown to increase the risk of allergic sensitization in children (110). Reports by Turcanu et al. and Thottingal et al. have demonstrated that both children and adult peanut allergic PBMC produced IL-13 in response to peanut Ag stimulation in vitro (6,111). Sensitization and subsequent food challenges in peanut allergic mouse models have confirmed the role of IL-13 in peanut allergy with enhanced expression following peanut stimulation (112). These data are not surprising based on our current understanding of the involvement of IL-13 in the pathogenesis of allergy.

IL-13 was originally cloned as an activator of T cells and is 30% homologous to IL-4. IL-13 is secreted primarily by antigen stimulated T cells that have undergone Th2
differentiation. As a result of IL-4-dependant Th2 differentiation, IL-13s mRNA half life dramatically increases with Phorbol 12-myristate 13-acetate (PMA) stimulation in comparison to baseline levels (113). Although IL-13 and IL-4 share structural characteristics as well as receptor components, their regulation is distinct. In a murine model, co-expression of IL-13 and other type 2 cytokines does not always occur, as nearly all IL-5 producing cells make IL-13, but not the reverse (114). Interestingly, in an atopic human population, grass pollen driven IL-13 production strongly correlate with IL-5, but not IL-4, production, but the production of IL-13 is independent of these two cytokines (115). Negative regulation of IL-13 may occur through the expression of T-bet, a transcription factor necessary for Th1 differentiation (116), but Thottingal et al. reported that peanut tolerant individuals show no detectable production of type 1 cytokines in the presence of Ag-driven type 2 cytokine production (6). Alternatively, IL-13 production by mast cells may be upregulated by IL-33, independent of Ag-stimulation (117), but whether this occurs in T cells remains to be determined. More importantly, the transcription factor found to be enhanced in the gut of peanut sensitized mice after a food challenge, GATA-3, positively regulates IL-13 production (112,114). GATA-3 is induced by IL-4/STAT6 signalling (118,119) and has been shown to be expressed in food induced intestinal inflammation (112).

IL-13 signalling occurs through a heterodimeric receptor, IL-4Rα and IL-13Rα1 (120). Signal transduction events are initiated by low affinity binding of IL-13 to the IL-13Rα1 component. Subsequent binding of the IL-4α1 chain leads to enhanced binding affinity (121) and the activation of MAP kinases, resulting in eotaxin release and airway smooth muscle cell relaxation in asthmatic models (122). Additionally, IL-13-mediated
activation of STAT-6 (107) results in expression of SOCS-1 and -3, and thus, the inhibition of type 1 cytokine production (123). It has been suggested that a second IL-13R, IL-13Rα2, has antagonistic properties (124). These antagonistic properties may be overcome by sufficient levels of IL-13 production (125). In contrast, a recent study has shown that this receptor coupled with IL-13 may have proinflammatory effects (126). It is unclear if peanut specific type 2 cytokines actively suppress type 1 cytokines production and if IL-13 is capable of producing both pro- and anti-inflammatory signalling.

The biological effect of IL-13 in the pathogenesis of peanut allergy has not been defined. Our current understanding is primarily data extrapolated from asthmatic mouse models. IL-13 is involved in B cell IgE synthesis (127-129) and contributes to exacerbation of peanut induced anaphylaxis (130). In addition to B cells, the biological effects of IL-13 have been described in monocytes and endothelial cells, but the expression of IL-13’s primary receptor on these cells is surprisingly low (107). Through IL-13Rα1 expression, IL-13 enhances FcεRI expression on human lung mast cells (125). Expression of IL-13 in mouse models leads to Th2 development, chemokine and adhesion molecule expression, eosinophil recruitment, and mucus secretion (113,131). This data provides insight into the possible effects IL-13 may be exhibiting in peanut allergy as well as how it is regulated. The regulation of IL-13 in peanut allergy has not been explored.
Clinical tolerance and regulation of peanut allergy

It was once believed that those who exhibited clinical tolerance towards peanut did so through unresponsiveness, or a failure of the immune system to recognize and respond to peanut Ag. This was shown to be inaccurate when Thottingal et al. demonstrated that clinically tolerant individuals mount a type 2 cytokine and chemokine response when stimulated with peanut (6). The mechanism of oral tolerance (OT) and the maintenance of clinical tolerance toward peanut remain unclear. OT is the state of systemic immunological hyporesponsiveness in reaction to ingestion of soluble Ag. It is thought that OT is achieved through T cell clonal anergy or deletion, and/or regulatory T cell immune suppression (132). The study of oral tolerance in peanut allergy has proven to be challenging. No known preclinical stage of peanut allergy has been identified, limiting experimental designs to mouse models, and cholera toxin-induced Th2 polarization (133,134).

The mechanism of oral tolerance is dependent on Ag availability, uptake and presentation. The availability of a food Ag may change with environmental factors and physiological conditions, such as the presence of infection, intestinal microflora and the integrity of the gut epithelial barrier (135,136). Ag concentration affects immune response outcome. High Ag dose administration effects were shown to produce Ag specific T cell anergy in OVA sensitized mice. No such effect occurred when unsensitized T cell clones were stimulated with Ag. (137). This data shows that many early studies using Ag concentrations well above physiological levels may have led us to an inappropriate understanding of events related to OT and food allergy.
As food Ag is ingested and moves into the gut it may be transferred from the lumen into the subepithelial dome by DC, M cells, and follicle associated epithelial cells. The DC of the gut are not inherently tolerizing, but use the microenvironment present to regulate T cell responses (135). OVA fed mice (non-sensitized) have shown that DC and T cell synapse formation occurs, but signalling is impaired, in comparison to sensitized mice (132). In those who are sensitized, a DC-Ag-T cell synapse formation leads to the development of the type 2 phenotype. In addition to synapse impairment, regulatory T cells and DC are capable in the production of immunoregulatory indole-2,3-dioxygenase (IDO), and secretion of immunoregulatory cytokines, such as IL-10, and transforming growth factor (TGF)-β to maintain clinical tolerance towards food Ag (137,138). In a CD4+CD25+ regulatory T cell depleted mouse model sensitized to peanut with cholera toxin, type 2 cytokine production, peanut specific IgE production, and peanut challenge responses were enhanced. Interestingly, CD4+CD25+ depleted mice showed none of these changes with peanut challenge (139), suggesting regulatory T cells are not definitive factor controlling OT induction. Conversely, Ahn et al. demonstrated that DCs are capable of reversing the inhibitory effects of regulatory T cell (140), further complicating our understanding of the induction and maintenance of OT.

*Interleukin-10*

IL-10 is an immunoregulatory cytokine that is involved in controlling both acute and chronic inflammation (141). Produced by monocytes, DC, T cells, B cells, eosinophils and mast cells (142,143), IL-10 is capable of suppressing a wide variety of inflammatory type 1 and type 2 cytokines (142). Evidence suggests that IL-10 may be associated with clinical tolerance preventing development of allergy, as several allergies
are associated with IL-10 deficiencies. In some studies children sensitized to food allergens, including peanut, and adults with penicillin allergy, have demonstrated reduced levels of IL-10 positive cells in comparison to healthy controls (144, 145). IL-10 polymorphisms have been reported to be associated with an increased susceptibility to food allergy in an atopic population of Japanese children (143). In addition, IL-10R mRNA is downregulated in acute eczema lesions of atopic individuals (146). In agreement, Tiemessen et al. showed that milk tolerant individuals produce IL-10, rather than characteristic allergic type 2 cytokines, towards milk (147). These studies show that IL-10 may potentially be associated with clinical tolerance towards peanut.

IL-10 achieves its inhibitory effects by signalling through the IL-10 receptor (IL-10R), a heterodimeric protein complex composed of IL-10R1 and IL-10R2. IL-10 signalling is initiated with the binding of the IL-10R1 component. The affinity of IL-10 to its receptor is substantially enhanced with subsequent binding of IL-10R2 (148). Downstream signalling occurs through the JAK/STAT pathway, and primarily involves STAT-1, STAT-3 and in some cells, STAT-5. One inhibitory effect of IL-10 is generated through the activation of the Src homology 2 domain-containing protein (SHP)-1. Through activation of SHP-1’s SH2 domain, co-stimulatory molecules required for T cell activation, CD28 and ICOS, are inhibited (144). Interestingly, Perez et al. illustrated that CD80 and CTLA-4 ligation induce IL-10, and consequently, IL-10-dependant production of TGF-β (149). In support of this theory, IL-10 involvement in regulatory T cell formation (150) and IL-10 inhibitory effects on proliferation and activation of T cells have been demonstrated (151, 152). MHC expression on antigen presenting cells are downregulated in the presence of exogenous IL-10 (153). IL-10 is also able to inhibit
asthma- and dust allergen-induced type 2 cytokine production (151) and when overexpressed, is able to suppress anaphylaxis (154). The effects of IL-10 inhibition of asthma associated cytokines results in direct eosinophil inhibition. IL-10 is also able to inhibit T cell activation (152) and apoptosis (155). STAT-3 is necessary for IL-10 mediated expression of FcεRI on mast cells (154) and IgE synthesis (156). IL-10 is able to negatively regulate IL-4-dependant IgE synthesis in B cells by inhibiting the accessory function of monocytes (157). Evidence of this mechanism is clear, as IL-10 KO mice with OVA-induced asthmatic reactions show a greater allergen-specific Ab response than controls (158). The possibility of IL-10 as a therapeutic agent in food allergy has also been explored. A strain of IL-10 secreting lactobacilli administered to β-lactoglobulin sensitized mice showed reduced anaphylaxis scores and disease severity (30). These results collectively demonstrate that IL-10 is involved in the negative regulation of other allergies, and provide strong evidence of its potential involvement in the clinical tolerance towards peanut Ag. The role of IL-10 in peanut allergy and the maintenance of clinical tolerance towards peanut have not been explored.

Transforming growth factor β

Transforming growth factor (TGF)-β is a pluripotent cytokine with both immunogenic and immunoregulatory properties (159). This cytokine is essential for maintenance of peripheral and oral tolerance (160,161) and may play a role in oral tolerance-associated apoptosis (159). It is produced by almost all cell types (162), including human lamina propria mononuclear cells (163). Although the exact role of TGF-β in tolerance towards food Ag is not well understood, it has been shown to be expressed on the surface of spleen-derived CD25+Foxp3+ regulatory T cells in mice.
When TGF-β is blocked in this same system, the inhibitory effects of CD25+Foxp3+ Tregs are reversed (164). In addition, the Th3 subset of Tregs has been shown to produce substantial levels of CTLA-4-dependent TGF-β (135,140).

TGF-β regulates multiple innate and adaptive cellular functions, including proliferation, differentiation, migration and cell survival (165), but its exact role in peanut allergy has not been explored. What is clear is that TGF-β plays an important role in the regulation of other allergic and immunological diseases. In the absence of TGF-β RII, mice develop lethal autoimmunity (166). TGF-β modulates precursor DC entering the intestinal tract where they promote tolerance (135), inhibiting T cell activation and differentiation (166). Other reports showing TGF-β's potential role in allergy includes evidence showing airway hyperresponsiveness reduction in the presence of TGF-β (167). Additionally, healthy individuals have been shown to produce significantly enhanced levels of TGF-β in response to ragweed, whereas atopic ragweed allergic individuals did not when compared to baseline levels (168). In agreement with the above evidence, Mucida et al. has stated that the mucosal interface of the gut requires the most critical balance in the regulation of TGF-β to maintain a functional immune system (169).

Three isoforms of TGF-β exists: TGF-β1, TGF-β2, and TGF-β3 (170). The TGF-β signalling cascade is initiated by TGF-β binding to its serine/threonine heterodimeric receptor components, TGF-β receptor types I and II. This ligation results in the transcriptional factor, receptor-regulated (R)-Smad's, activation and recruitment to the nucleus (171,172). Smad 3 is the fundamental signal transducer of TGF-β signalling.
This TGF-β signalling pathway is tightly regulated. Inhibitory signals are rapidly produced by Smad/Smad ubiquitin regulatory factor (Smurf) complex formation, resulting in the ubiquitination and consequent proteolytic cleavage of the TGF-β receptor (172). Evidence exists showing that IL-10 may control the release of TGF-β from naturally occurring CD4+CD25+ regulatory T cells, which are a critical component of the regulatory mechanisms in allergic airway disease (167, 173). The engagement of CTLA-4 also results in an upregulation of TGF-β and T cell hyporesponsiveness (174).

The negative regulation of TGF-β can occur through multiple pathways. Transgenic mice overexpressing GATA-3 showed a lack of TGF-β-dependent Treg Foxp3 induction. This inhibition is a result of GATA-3 binding directly to, and consequently inhibiting the FOXP3 promoter (175). Interestingly, a reciprocal regulation of type 2 cytokine is evident in TGF-β signalling. GATA-3 mRNA expression was significantly enhanced in the lung tissue of mice unable to participate in Smad signalling, meaning that Smad may negatively regulate type 2 immune response development (119).

TGF-β is a multifaceted cytokine. It is widely accepted that TGF-β1 inhibits thymic Treg cell expansion and peripheral T cell proliferation, activation, and differentiation. This is not to say that TGF-β is a negative regulator of Tregs, as intrinsic TGF-β1 is required for Treg cells to exist (162). With this said, it is thought that TGF-β may be involved in the conversion of CD25+Foxp3- T cells to CD25+Foxp3+ T regs, as TGF-β is critical for the maintenance of peripheral tolerance (160). Pyzik et al. provided evidence of this by showing that TGF-β1 induced expression of Foxp3 mRNA in
CD4^+CD25^-CD45RB_Low T cells. In addition, TFG-β1 can induce Foxp3 expression, but does not potentiate the inhibitory function of these regulatory T cells (173). The biological result of TGF-β inhibitory signalling is a reduction in IgE, GATA-3, type 2 cytokines, and anaphylaxis, all of which are characteristic of allergy, as shown in an OVA-sensitized mouse model (176). Due to its inhibitory properties, the investigation of using TGF-β as a potential therapeutic agent in the reduction of allergy has been explored. Orally administered TGF-β is able to maintain biological activity and promote tolerance in OVA-sensitized mice, and thus shows promise in becoming a potential therapeutic for allergy (161). The above evidence suggests that TGF-β may be involved in the limiting peanut allergy. The role of TGF-β in controlling clinical tolerance towards peanut Ag has not been explored.

*Indole-2,3-dioxygenase*

Indole-2,3-dioxygenase (IDO) is a rate-limiting enzyme involved in the breakdown and depletion of tryptophan (177,178). IDO has been reported to be produced by a wide variety of cells. Studies focusing on immune system related diseases demonstrate that human and mouse macrophages and DCs are a common producer of IDO (178,179,179,180,180-182). In the past, studies of IDOs biological properties have primarily focused on its role in prevention of foetal rejection in pregnancy, suppression of bacterial growth (183), and anterior chamber-associated immune deviation (177). More recently, IDOs role in the pathogenesis of atopy, allergy, and asthma has been explored. IDO has been reported to play a role in tolerance induction and maintenance in OVA allergic sensitization of the airways (184). Similarly, asymptomatic atopics have a significant correlation between IDO activity and IL-10.
production, whereas symptomatic atopics do not, suggesting these factors may be important in mediating T cell anergy in asymptomatic atopics but not allergen-sensitized individuals (185). In agreement, von Bubnoff et al. showed that there is differential regulation of IDO between atopics and non-atopics (186). IDO has also been shown to contribute to the beneficial effects of immunotherapy. In OVA sensitized mice, IDO metabolites are crucial for the suppression of allergen induced allergic airway type 2 cytokine production (187). Funeshima et al. also demonstrated that DC transfected with the IDO gene were highly capable of inhibiting proliferation of alloreactive T cells which is thought to be caused by the induction of regulatory T cells (188). Similarly, corticosteroids induce IDO-dependant protection in allergic airway mouse models (189), but the role of IDO in type 2 mediated hypersensitivity remains controversial (190,191).

The mechanism by which IDO exerts its inhibitory properties on the immune system is thought to be achieved through two different pathways. IDO converts tryptophan into N-formylkynurenine, which is catabolised into kynurenine, and made finally into terminal metabolites, quinolinic or piconlinic acid (188,192). This results in the depletion of the essential amino acid, tryptophan, from the affected cell, and thus impedes its proliferation (183). IDOs inhibitory property on cell proliferation has been demonstrated in T cell populations (180,181). A second mechanism by which IDO exhibits its inhibitory properties is believed to involve the downstream metabolites in the tryptophan breakdown pathway, which are thought to induce apoptosis (183). Induction of Fas-mediated apoptosis by IDO-dependent tryptophan depletion has been exhibited in T cells (193). Similarly, the tryptophan metabolites, Kynurenine, 3-Hydroxykynurenine, and 3-Hydroxyanthranilic Acid exhibit cytotoxic effects, can inhibit T
cell proliferation, and preferentially kill activated T cells (194). The generation of tryptophan metabolites is thought to affect not only the cell being targeted, but neighbouring cells in the local environment. Bone marrow DCs have a higher affinity for extracellular kynurenines in comparison to tryptophan (195) and IDO-expressing DCs can transfer their tolerogenic properties to cells lacking functional IDO via the secretion of soluble metabolites (196).

IDO induction in T cells is thought to occur through ligation of co-stimulatory molecules and IFNγ (197). Early signals in T cell activation induce IFNγ and CD40L, which in turn induce IDO (182). TGF-β has also been implicated in granting IDO competence and subsequent tolerogenic properties of DC (198). With the use of CTLA-4 lg, DC derived IDO-dependent T cell proliferation inhibition and cell death has been shown in both DC/Tcell human and mouse models (199, 199-201). It has been proposed that Treg CTLA-4 engagement upregulate IDO expression on DC and monocytes (197). In addition, the cross-linking of CTLA-4's target ligand also prevents the down-regulation of IDO. APC-T cell co-stimulatory molecules associated with T cell activation have also been shown to be involved in the induction of IDO expression. Ligation of CD80/CD86 prevents downregulation of IDO. Blocking CD80/86 and CTLA-4's target ligand, CD28, prevents induction of IDO expression (202). Fallarino et al. suggest that T cell activation may be involved in the enhanced expression of IDO and Tregs may act through multiple mechanisms, such as CTLA-4, to induce IDO expression (203). The only negative regulator of IDO that has been identified is IL-6. Through autocrine signalling, IL-6 downregulates IFNγ receptor expression, which results in an irreversible inhibition of IDO that cannot be reversed by CD40 and
CD80/CD86 ligation in DC (204). Thottingal et al. showed that peanut Ag driven cytokine production is independent of IFNγ and no studies exploring the role of IDO in peanut allergy exist (6).

It is widely accepted that T cell inhibition can occur through IDO dependent mechanisms (180,181,183). More recently, Vogel et al. showed that IDO may contribute to upstream activation of Foxp3, causing development of Tregs and consequently immunosuppression and tolerance (205). These results have confirmed tryptophan metabolism’s role in studies regarding prevention of autoimmunity (206), but no studies to date examine such possibilities in allergy. On the other hand, anti-FcεRI mAb-dependant cross-linking of FcεRI on monocytes induced IDO-dependent tryptophan degradation (207) and the generation of the subsequent immunotherapy-induced metabolites is critical for the inhibition of eosinophilia and type 2 cytokines in allergic airway mouse models (187). These recent studies suggest that IDO may play an important role in the development and maintenance of tolerance towards peanut. To date, no studies exist looking at food antigen driven T cell activation, and the effects of IDO on its regulation. Existing studies have limited our understanding of IDOs role in allergy as most involved stimulation with LPS (180,203), IFNγ stimulation (186,208), or basal IDO levels (209) and are typically limited to mouse models (187,189). Terness et al. reminds us that tryptophan pathways differ greatly between animal models used (183). With this said, it is proposed that IDO may play a role in the maintenance of clinical tolerance towards peanut.

**Experimental Hypotheses**
Experimental Design and summary of results obtained:

Throughout this investigation we sought to create an experimental design that accounted for the heterogeneity present in the human population by studying a large population of peanut allergic individuals, as well as those who are able to ingest peanut without consequence. In the past, the study of peanut allergy in humans was limited to T cell lines (95,210) or small study populations (77,210). In our study, we aimed to build on our current understanding of peanut allergy by including the necessary, detailed history in over 270 participating subjects. Mouse models have been widely used to study peanut allergy (112,134,139), but have been largely limited to cholera toxin-induced sensitization (4,45,211). A failure to include a clinical history in study population categorization has also limited the information obtained in studies involving peanut sensitized individuals (38,212).

Chapter I: Comparison of in vitro vs. in vivo responses to peanut Ag

Based on the evidence provided above, we hypothesized that commercially available reagents will produce differential SPT and cytokine results in response to commercially available and laboratory prepared peanut Ag stimulation. Extrapolating from studies examining the content and reactivity of commercially and laboratory or internally prepared reagents (38,43-46), we proposed that the reagents tested will vary in protein content, SPT diameter results, and the capacity to elicit in vitro cytokine production in human primary cultures using peripheral blood mononuclear cells (PBMC). Additionally, based on a previous study by Rancé et al. we hypothesize that laboratory prepared SPT reagents will produce greater in vitro and in vivo responses,
and have higher protein yield in comparison to commercially available content (49). We tested these hypotheses by performing protein assays on 2 commercially available, 1 externally, and 2 internally prepared peanut Ag SPT reagents. The internally prepared peanut Ag, whole peanut extract (WPE)-3 presented the largest mean SPT diameter of the SPT Ag reagents tested in peanut allergic individuals. Of all SPT reagents tested, none produced SPT diameters that significantly correlate with peanut driven primary culture cytokine responses. Using these peanut Ag preparations, human PBMC were stimulated in primary cultures using identical concentrations of peanut Ag and the resulting peanut driven cytokine production was quantified using high sensitivity ELISA. Whole peanut extract (WPE)-3 in borate buffered saline (BBS), provided the greatest Ag-specific enhancement of type 2 cytokine responses in comparison to the medium alone in primary culture. These data suggest that improved regulation of peanut Ag for diagnostic purposes is needed.

Chapter II: Are serum IL-10 levels a useful marker of clinical tolerance in peanut allergy?

SPT and ImmunoCAP diagnostic methods used in peanut allergy diagnosis have proven to present gray areas, where a large subset of the potentially peanut allergic population cannot definitively be diagnosed, forcing the use of oral challenge to eliminate uncertainty (36,49,53,54,56). Alonso et al. recently provided evidence for the potential of serum IL-10 as a biological marker of clinical tolerance towards hazelnut (63). Therefore, we hypothesized that differential levels of IL-10 would exist between peanut allergic and clinically tolerant populations, and provide useful tools for the diagnosis of peanut allergy. To test our specific hypothesis that IL-10 would be
elevated in clinically tolerant populations in comparison to those with peanut allergy, plasma IL-10 was quantified in peanut allergic vs. clinically tolerant adult and children populations. In contrast to this hypothesis, plasma IL-10 did not differ significantly between peanut allergic and clinically tolerant populations. Additionally, when populations were stratified based on gender in the adult population, or by age, no significant difference of plasma IL-10 were detectable between any of the populations. These data indicate that plasma IL-10 is not a promising candidate as a biological marker of clinical tolerance towards peanut.

Chapter III: The mechanisms of peanut allergy

There is substantial evidence that IL-9 is involved in several types of allergies, but its role in peanut allergy has not been investigated. Based on data provided by numerous studies involving IL-9 in other allergies (83,85,86,88,97,99-102,213), it is hypothesized that enhanced IL-9 production is associated with recall peanut allergic immune responses. Therefore, we hypothesized that peanut allergic populations produce significantly enhanced levels of IL-9 in response to peanut, whereas sensitized and clinically tolerant populations would not. Thottingal et al. showed that a greater frequency of peanut sensitized individuals produce type 2 cytokine compared to those who are clinically tolerant. Therefore, it was also hypothesized that peanut allergic populations would respond at a greater frequency than those who are able to ingest peanut. To test these hypotheses, primary cultures of human PBMC were stimulated with peanut Ag and the type 2 cytokine response produced was quantified using high sensitivity ELISA. In agreement with our hypotheses, peanut allergic populations produced significantly enhanced levels of peanut driven type 2 cytokines, including IL-9.
in comparison to the medium alone. These responses were both quantitatively greater and occurred at a greater frequency in the allergic population compared to those who are able to safely ingest peanut. These data indicate that IL-9, as well as IL-5 and IL-13, are involved in the physiological process of peanut allergic reactions.

Chapter IV: Regulatory mechanisms underlying peanut allergy and clinical tolerance

The mechanism(s) controlling tolerance towards food allergens, including peanut, remain unclear. A great deal of studies investigating the mechanism of oral tolerance have been carried out (132,132-138,214), but these studies have yet to clearly define the mechanism of oral tolerance in humans. Moreover, studies to date rarely focus on induction or maintenance of clinical tolerance towards peanut. Based on current understanding of the biological function of oral tolerance-associated immunoregulatory mediators, we hypothesized that elevated production of IL-10, TGF-β, and IDO would be preferentially associated with clinical tolerance towards peanut. We tested this hypothesis by quantifying the effects of IL-10R, TGF-β, and IDO inhibition on peanut driven recall cytokine production in both peanut allergic and clinically tolerant populations. In contrast to our initial hypothesis, IL-10, TGF-β, and IDO inhibition did not result in significant enhancement of peanut driven type 2 cytokine production in the clinically tolerant population. These results indicate that IL-10, TGF-β, and IDO show no detectable role in the maintenance of clinical tolerance towards peanut in populations able to ingest peanut without consequence. IL-10 may however be involved in limiting the intensity of type 2 cytokine responses associated with ongoing peanut allergy.
Materials and Methods

Study Participants

The study, "Peanut allergy vs. clinical tolerance in teenagers and young adults" was approved by the University of Manitoba and University of Windsor Ethics Boards. In collaboration with Dr. E. Simons (Dept. Paediatrics, University of Manitoba), Dr. J Liem (Windsor Allergy Asthma Education Centre, ON), and Dr. A Becker (Dept. Paediatrics, University of Manitoba), written informed or parental written informed consent was obtained from 94 adults and 153 children, respectively, between the ages of 1 and 57. The candidates were randomly recruited in response to advertisement, were in good health and not obese. Participants did not regularly take any medications and had abstained from ingesting anti-histamines 48h prior to blood sampling.

Participants were divided into study groups based on their age and clinical reactivity toward peanut (convincing history of anaphylaxis upon peanut exposure within the last 5 years, ImmunoCAP (>0.35 kUA/L) and peanut skin prick test (SPT) result (>3mm mean SPT wheal in comparison to the negative saline control). Among study participants three groups were studied: peanut allergic (history positive/SPT positive, Hx+SPT+), peanut sensitized (history negative, SPT positive, Hx-SPT+) and individuals clinically tolerant towards peanut (history/SPT negative, Hx-,SPT-) (Figure 1). In total, 129 participants were peanut antigen (Ag) skin prick test (SPT) positive, had a convincing recent history of peanut induced anaphylaxis and were ImmunoCAP
Figure 1: Clinical status characterization of study population

- **Clinically Tolerant**
  - history-NEGATIVE
  - skin prick test NEGATIVE
  - no peanut-specific IgE detectable

- **Sensitized**
  - history-NEGATIVE
  - skin prick test POSITIVE
  - peanut-specific IgE POSITIVE

- **Peanut Allergic**
  - history-POSITIVE (*recent reaction to peanut*)
  - skin prick test POSITIVE
  - peanut-specific IgE POSITIVE
positive, termed “peanut allergic”. Fifteen participants had a positive peanut Ag SPT, but no history of peanut allergy and were able to regularly ingest peanut, termed “Peanut sensitized”. One hundred and three participants were unsensitized (negative SPT and <0.35kU peanut specific IgE) and ingested peanut at least monthly without negative outcome, termed clinically tolerant.

*Isolation of PBMC*

Twenty and 50 mL of peripheral whole blood was taken by venipuncture and placed in a tube containing 1 mL 2.7% EDTA per 20 mL of blood from children and adults, respectively. The blood/EDTA mixture was mixed immediately to prevent clotting. For every 10 mL of blood, 10mL of 0.85% Saline was added and mixed thoroughly. Diluted blood was then layered on top of 3.5 mL of Ficoll (Histopaque-1077; Sigma, St. Louis, MO) and centrifuged at 460 x g for 30 min (Eppendorf Centrifuge 5804 (1850 rpm) to isolate mononuclear cells from polymorphonuclear cells, red blood cells and most platelets. Following centrifugation, 4 mL of the top plasma layer was stored at -20°C in 0.2% sodium azide for future analysis, while the remainder was discarded. The layer between the plasma and Ficoll containing mononuclear cells, or “buffy coat,” was removed using a pasture pipette and resuspended in 0.85% saline. Cells were spun at 250g (1378 rpm) for 10 min. The saline layer was discarded and a second wash was repeated in saline. Isolated PBMC were resuspended in 4 mL of complete media (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 10 mM L-glutamine, 2x10(5) M 2-mercaptoethanol (ME), and antibiotic-
antimycotic (Life Technologies, Burlington, Canada)). Cell concentrations were determined by hemocytometer count. The viability of the cells was assessed using Trypan Blue exclusion. A viability of ≥95% was obtained for all samples collected.

PBMC Culture Reagent Preparation

Crude Peanut Protein/Antigen Extract: 2g of peanut butter containing no additives (Just Peanuts Peanut Butter; President’s Choice™) was resuspended in 40 mL of Borate Buffered Saline, pH 7.4 or RPMI-1640, pH 7.4 supplemented with 10% heat-inactivated FCS. The peanut butter was dissolved for 20 min in a 37°C hot water bath. The mixture was rotated slowly for 72h at 4°C. After centrifugation at 2500 rpm for 30 min (1187 g), the top fatty layer was discarded. The center fraction containing soluble protein was saved, and the bottom solid fraction was discarded. The protein concentration of the peanut antigen isolation was determined using the BCA™ Protein Assay Kit (Pierce, Rockford, IL), and the standard curve and all samples were analyzed in triplicate.

1-Methyl-D-tryptophan (1-MT): 40 mg of 1-MT (Sigma, Oakville, ON) was reconstituted in 10 mL of 1.0 N NaOH, and stored at 4°C until further used. The 1-MT mixture was further diluted in 1/10 in complete media, pH 7.4, to a final concentration of 400 µg/mL or 1.8x10⁻³ M.
**PBMC stimulated with peanut antigen**

Cells were cultured in triplicate at 500,000 cells/well in 200 µL using 96 well U-bottom plates (Nunc, Roskilde, Denmark) in complete media (RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM L-glutamine, 2x10⁻⁵ M 2-ME, and antibiotic-antimycotic) at 37°C with 5% CO₂ for 48h and 1-5 days for phytohemagglutinin (2.5 µg/mL) (PHA-L, Sigma, Cat # 4144) and all other conditions, respectively.

For each subject, the following conditions were established:

1. medium control

2. with phytohemagglutinin-L, 2.5 µg/mL

3. with commercial crude Peanut Allergenic Extract 1:10 w/v (ALK Abelló, Port Washington, NY) (designated "ALK Peanut Antigen")

4. with commercial crude Peanut Mix Allergenic Extract (Hollister-Stier Laboratories LLC, Spokane, WA) ("Omega Peanut Antigen")

5. with crude peanut protein in complete media (RPMI Peanut Antigen) prepared as described above, designated “whole peanut extract” (WPE) 1

6. with crude peanut protein in borate buffered saline (Borate Peanut Antigen), WPE 2

7. with crude peanut protein (provided by Wesley Burks) (Burks Peanut Antigen), WPE 3
*Immunoregulatory inhibition assays*

Cells were isolated and cultured as described above.

For each subject, the following conditions were established:

1. medium control
2. with phytohemagglutinin (PHA), 2.5 ug/mL
3. with PHA at concentration of 2.5 ug/mL and LEAF™ purified anti-human CD210 (IL-10 R) (BioLegend, San Diego, CA) at concentrations of 0.16, 6.3, 2.5, and 10* ug/mL
4. with PHA at concentrations of 2.5 ug/mL and Recombinant Human IL-10 (BioLegend, San Diego, CA) at concentrations of 1.56, 6.25*, 25, and 100 ng/mL.
5. with PHA at concentrations of 2.5 ug/mL and anti-TGF-β mAb (R&D Systems, Minneapolis, MN) at concentrations of at 0.31, 1.3, 5.0, and 20* ug/mL
6. with PHA at concentrations of 2.5 ug/mL and 1-Methyl-D-tryptophan, 95% (Sigma Aldrich, St. Louis, MO) at concentrations of at 1.6, 6.3*, 25, and 100 ug/mL.
7. with Borate Peanut Antigen at concentrations of 50 and 100 ug/mL
8. with Borate Peanut Antigen (50 and 100 ug/mL) and Recombinant human IL-10 (rIL-10) (BioLegend, San Diego, CA) at concentrations of 1.56, 6.25*, 25, and 100 ng/mL.
9. with Borate Peanut Antigen (50 and 100 ug/mL) and LEAF™ purified anti-human CD210 (IL-10 R) (BioLegend, San Diego, CA) at 0.16, 6.3, 2.5, and 10* ug/mL

10. with Borate Peanut Antigen (50 and 100 ug/mL) and anti-TGF-β mAb (R&D Systems, Minneapolis, MN) at 0.31, 1.3, 5.0, and 20* ug/mL

11. with Borate Peanut Antigen (50 and 100 ug/mL), anti-IL-10R mAb (0.6 and 2.5 ug/mL) and anti-TGF-β mAb (5.0 and 20 ug/mL)* Antibody

12. with Borate Peanut Antigen (50 and 100 ug/mL) and 1-Methyl-D-tryptophan, 95% (Sigma Aldrich, St. Louis, MO) at 1.6, 6.3*, 25, and 100 ug/mL

*optimal concentration

PBMC stimulated with LPS

Cells were cultured in duplicate as described above in PBMC stimulated with peanut antigen. Cells were cultured in the absence of stimuli (medium alone) or in the presence of LPS (In vivogen, San Diego, CA) at 5.0, 0.5, 0.05, and 0.005 ng/mL.

Inhibition of TCR-mediated T cell activation

Cells were cultured in duplicate wells at 500,000 cells/well in 200 µL using a 96 well Ubottom plate (Nunc, Roskilde, Denmark) in complete media (in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mM L-glutamine, 2x10(5) M 2-ME, and antibiotic-antimycotic) at 37°C with 5% CO₂ for 24h and 5 days. Cells were cultured in the absence of stimuli (medium alone), with peanut Ag (50 ug/mL) alone or
in the presence of CTLA-4 Ig (Biosource, Nivelles, Belgium) at 0.25, 0.75, 2.5 and 7.5 ug/mL to determine if cytokine responses were peanut Ag-TCR driven.

Analysis of human cytokine responses

Human cytokine responses in supernatants from above experiments were measured using specific ELISA for IL-5, IL-9, IL-10, IL-13, and IFNγ.
Table 1: ELISA Materials

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Function</th>
<th>Company</th>
<th>Catalog No.</th>
<th>Working Concentration (ug/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5, Purified</td>
<td>Primary Antibody</td>
<td>BD PharMingen</td>
<td>554393</td>
<td>0.5</td>
</tr>
<tr>
<td>IL-5, Biotin</td>
<td>Secondary Antibody</td>
<td>BD PharMingen</td>
<td>554491</td>
<td>0.25</td>
</tr>
<tr>
<td>Purified anti-human IL-9</td>
<td>Primary Antibody</td>
<td>BioLegend</td>
<td>507602</td>
<td>1.0</td>
</tr>
<tr>
<td>Biotin anti-human IL-9</td>
<td>Secondary Antibody</td>
<td>BioLegend</td>
<td>507702</td>
<td>0.25</td>
</tr>
<tr>
<td>Purified anti-human IL-10</td>
<td>Primary Antibody</td>
<td>BioLegend</td>
<td>501402B</td>
<td>0.35</td>
</tr>
<tr>
<td>Biotin anti-human IL-10</td>
<td>Secondary Antibody</td>
<td>BioLegend</td>
<td>501502</td>
<td>0.05</td>
</tr>
<tr>
<td>Purified anti-human IL-13</td>
<td>Primary Antibody</td>
<td>BioLegend</td>
<td>501902B</td>
<td>0.4</td>
</tr>
<tr>
<td>Biotin anti-human IL-13</td>
<td>Secondary Antibody</td>
<td>BioLegend</td>
<td>555054</td>
<td>0.25</td>
</tr>
<tr>
<td>Purified anti-human IFNy</td>
<td>Primary Antibody</td>
<td>BioLegend</td>
<td>507502B</td>
<td>0.25</td>
</tr>
<tr>
<td>Biotin anti-human IFNy</td>
<td>Secondary Antibody</td>
<td>BioLegend</td>
<td>502504B</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Table 2: Standard Curve Reagents

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Company</th>
<th>Range of Detection</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-5</td>
<td>Peprotech</td>
<td>250 – 1.95 pg/mL</td>
<td>5.86 pg/mL</td>
</tr>
<tr>
<td>IL-9</td>
<td></td>
<td>300 – 2.34 pg/mL</td>
<td>9.38 pg/mL</td>
</tr>
<tr>
<td>IL-10</td>
<td>BD PharMingen</td>
<td>500 – 3.91 pg/mL</td>
<td>11.7 pg/mL</td>
</tr>
<tr>
<td>IL-13</td>
<td>Peprotech</td>
<td>250 – 1.95 pg/mL</td>
<td>5.86 pg/mL</td>
</tr>
<tr>
<td>IFNγ</td>
<td>NIH Research Ref.</td>
<td>20.0 – 0.156 U/mL</td>
<td>0.47 U/mL</td>
</tr>
</tbody>
</table>
ELISA Protocol

Coating: Ninety-six well plates (Costar 96 Well Easy Wash™ 3369 EIA/RIA Plate, Corning Incorporated, Corning, NY) were coated with 50 µL of specific coating antigen in coating buffer overnight in a sealed moisture box to prevent evaporation at 4°C. All optimal coating concentrations were determined during previous experiments.

Blocking: The coating mixture was removed and before the addition of 80 µL of blocking buffer. Plates were incubated at 37°C for 2h and washed 4 times using an automatic plate washer. Excess wash buffer was removed by hitting plates against absorbent paper three times.

Sample and Standard Curve Addition: Sample initial dilutions and standard curve starting concentrations were determined through ELISA optimization experiments. The total volume of each well was 50 µL.

The samples were added to the plate at an initial dilution of 1 in 3 and 1 in 4 for cytokines IL-5, IL-10, and IL-13 and IL-9 and IFNγ, respectively. All were diluted in two or four two-fold dilutions using dilution buffer. The standard curve was diluted in eight two-fold dilutions repeated on each plate. Plates were stored overnight at 4°C in a moisture box to prevent evaporation.

Detection: Plates containing samples and standard were washed four times using an automatic washer and hit three times against absorbent paper to remove excess wash buffer. Specific cytokines were detected with the addition of 50 µL of biotinylated detection antibody at pre-optimized concentrations (see Table 1) in dilution buffer to
each well. Optimal concentrations of detection antibody were determined during previous ELISA optimization experiments. Plates were stored overnight at 4°C in a moisture box to prevent evaporation.

**Development:** Plates containing detector antibody were washed four times using an automatic washer and hit three times against absorbent paper to remove excess wash buffer. Fifty microlitres of Streptavidin-alkaline phosphatase (Jackson ImmunoResearch, Mississauga, ON) in dilution buffer was added to each well at a 1 in 3000 dilution. Plates were incubated at 37°C for 45 min.

Plates were then washed 4 times using an automatic washer and hit three times against absorbent paper to remove excess wash buffer. At least 30 min. prior to use, p-nitrophenyl phosphate tablets (Sigma, Oakville, ON) (pNPP) were dissolved in ELISA diluents (1 tablet/5 mL). Fifty microlitres of the pNPP solution was added to all wells and incubated at 37°C for 1 to 6.5h, based on the cytokine being analyzed.

Plates were read using a microtitre plate reader (Molecular Devices, Sunnyvale, CA) at 405-690 nm. A sigmoidal standard curve was constructed using a 4-parameter curve fit and the eight standard serial dilutions; log scale of optical densities (x-axis) vs. concentrations (y-axis). Standard curves with a percent confidence interval <10% were deemed suitable for data analysis. Samples concentrations were determined using the x-axis (log scale) to determine the concentration (and corrected for dilution factors). Statistical analysis was performed using Prism 5 (GraphPAD Software, San Diego, CA).
Results

Chapter I: In vitro and in vivo characterization of peanut allergy diagnostic reagents

Chapter Introduction

Peanut allergy is diagnosed using a combination of clinical history, SPT, quantification of peanut specific IgE (ImmunoCAP Test), and in some cases, a food challenge. Several studies examining large populations of peanut allergic individuals have determined cut-off values that provide strong PPVs for the SPT and ImmunoCAP Test methods to achieve positive diagnosis of peanut allergy. Unfortunately, these cut-offs or decision points have poor NPVs and the finding that approximately half the peanut allergic population lies below these specified points (37,49,53,54). The use of peanut specific IgE as a biomarker for peanut allergy can confirm diagnosis with a value $\geq 15$ kU/mL (100% PPV, 95% C.V.) but at least 46% of peanut allergic individuals are within the range of 0.35-14 kU/mL (53), emphasizing the need for improved peanut allergy diagnostic methods. The diameter of a skin prick test can also, in some cases, predict the likelihood of having peanut allergy, but like peanut specific IgE levels, it cannot predict the clinical severity. Moreover, Peanut specific IgE and SPT results may remain positive years after clinical tolerance towards peanut have been achieved (4) Thus, a better understanding and subsequent improvement to the existing diagnostic methods are warranted.
In addition to having poor NPVs, SPT reagents are not regulated by external governing bodies over their content (38), which may result in variable lot to lot or supplier to supplier SPT outcome (45). Studies exploring the allergenicity of peanut Ag suggest that roasted peanuts exhibit enhanced IgE binding affinity and thus may be more allergenic than boiled or fried peanuts (215,216). As reviewed by Maleki, several reports also demonstrate no difference in allergenicity between raw and roasted peanut (217). As the content of commercially available SPT reagents is not regulated by law, it is unclear if their content is ideal for skin prick testing as well as in vitro examination of immune responses in individuals with allergy. To date, no published studies have examined the reactivity of commercially available and internally prepared peanut Ag both in vivo (SPT) and in vitro (primary culture).

The goal of this study was to examine if SPT results are predictive of peanut driven recall cytokine responses in vitro. We hypothesized that (i) commercially available and internally prepared peanut Ag reagents will contain varying amounts of protein and elicit differential SPT and cytokine responses and (ii) the intensity of SPT results will not positively correlate with cellular response profiles generated with peanut Ag stimulation. To test these hypotheses, protein content, SPT reactivity, and type 2 cellular response profiles, as determined by ELISA, were examined in clinically tolerant, peanut sensitized, and peanut allergic populations. Commercially available SPT reagents showed dramatic variability in protein content and cellular response profiles generated in vivo and in vitro. SPT and in vitro cytokine responses did not positively or significantly correlate for any of the Ag preparations used.
Chapter Results

Characterization of peanut diagnostic methods

It has been previously determined that SPT and peanut specific IgE diagnosis methods provide the strongest PPV at levels ≥8 mm and ≥15 kUA/L, respectively, but a consequence of a high PPV are low NPV, hence limited clinical utility when used in isolation (37,53). Commercially available SPT reagents contain varying levels of protein and glycerol among other substances, which in turn may lead to variable SPT results (43-45). Our first objective was to confirm if commercially available peanut Ag reagents vary in protein content. As Table 3 demonstrates, protein concentrations vary up to 4-fold between commercially available peanut Ag extracts and up to 35-fold between commercially available and internally prepared peanut Ag reagents. Commercial SPT reagents content is not regulated by an external governing body, creating variability amongst Peanut Ag reagent manufacturers (38).

As Omega, ALK Abélllo brands, and WPE 1-3 showed dramatic variability in protein content, we sought to make use of these reagents at standardized protein levels to better assess the capacity of these preparations under equivalent conditions in skin prick testing. Omega, ALK-Abelllo, and WPE 2 were diluted to 300 and 3000 ug/mL in saline, and SPT were performed on 24 peanut allergic individuals. The cut-off points between those used in our study (≥3 mm) and those suggested by Roberts and Lack (≥8 mm) were examined (37). Our data argues that a lower SPT cut-off value of ≥3mm is optimal as inclusion criteria in the diagnostic process, as higher cut-off values exclude one quarter of the allergic population. Thus, when Omega, the reagent with the most
Table 3: Protein concentrations vary dramatically between commercially available and internally prepared Peanut Ag reagents.

<table>
<thead>
<tr>
<th>Peanut Ag Reagent</th>
<th>Listed Concentration</th>
<th>Matrix</th>
<th>Source</th>
<th>Protein (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALK-Abelló</td>
<td>10% w/v</td>
<td>Glycerol/water</td>
<td>Commercial</td>
<td>7100</td>
</tr>
<tr>
<td>Omega</td>
<td>10% w/v</td>
<td>Glycerol/water</td>
<td>Commercial</td>
<td>29,000</td>
</tr>
<tr>
<td>WPE 1</td>
<td>-</td>
<td>RPMI Serum Free Media, pH 7.4</td>
<td>Internally prepared</td>
<td>1400</td>
</tr>
<tr>
<td>WPE 2</td>
<td>-</td>
<td>Borate Buffered Saline, pH 10.0</td>
<td>internally prepared</td>
<td>2900</td>
</tr>
<tr>
<td>WPE 3</td>
<td>-</td>
<td>RPMI Serum Free Media, pH 7.4</td>
<td>externally prepared by Dr. Wesley Burks</td>
<td>820</td>
</tr>
</tbody>
</table>

WPE – whole peanut extract
protein content, was reduced to 3000 μg/mL, it produced 4 fewer positive results than did the other two commercial reagents at ≥3 mm above the saline control. Omega was able to elicit a positive response in only 75% of those diagnosed with clinical allergy when cut-off levels were increased to ≥8 mm. With all reagents tested, only 1 of the 24 peanut allergic individuals did not produce a positive SPT at the lowest cut-off level.

The mean SPT diameter did not vary dramatically between reagents tested, as shown by the largest difference of only 1.5 mm between ALK-Abélló and WPE 2's mean diameter responses (Table 4). In almost all cases, Omega, ALK-Abélló, and WPE 2, at a total protein concentration of 300 μg/mL, elicited a SPT result of equal or lesser diameter (data not shown). Furthermore, we sought to examine the outcome of positive diagnosis using higher peanut specific IgE cut-off points of 15 kUA/L in comparison to our study's method (>0.35 kUA/L), in defining a positive diagnosis in peanut allergic individuals. As Table 5 shows, 57% of our peanut allergic population did not have peanut specific IgE above 15 kUA/L. One hundred percent of all peanut allergic individuals had detectable levels peanut specific IgE (or levels >0.35 kUA/L). In conclusion, commercial peanut Ag SPT reagents clearly lack standardization. Use of >8 mm SPT wheal diameters as the suggested cut-off levels for peanut allergy diagnosis methods exclude an unreasonable proportion of the peanut allergic population.
Table 4: Commercially available and internally prepared Peanut Ag reagents produce differential SPT results.

<table>
<thead>
<tr>
<th><em>Diameter (mm)</em></th>
<th>ALK Peanut Ag</th>
<th>Omega Peanut Ag</th>
<th>Borate Peanut Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%) Positive (%) Negative</td>
<td>No. (%) Positive (%) Negative</td>
<td>No. (%) Positive (%) Negative</td>
</tr>
<tr>
<td>≥3</td>
<td>23 96 4</td>
<td>22 92 8</td>
<td>23 96 4</td>
</tr>
<tr>
<td>≥4</td>
<td>21 88 13</td>
<td>22 92 8</td>
<td>21 88 13</td>
</tr>
<tr>
<td>≥5</td>
<td>20 83 17</td>
<td>20 83 17</td>
<td>19 79 21</td>
</tr>
<tr>
<td>≥6</td>
<td>20 83 17</td>
<td>20 83 17</td>
<td>18 75 25</td>
</tr>
<tr>
<td>≥7</td>
<td>18 75 25</td>
<td>20 83 17</td>
<td>18 75 25</td>
</tr>
<tr>
<td>≥8</td>
<td>17 71 29</td>
<td>18 75 25</td>
<td>18 75 25</td>
</tr>
<tr>
<td>Total Pop:</td>
<td>24</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>Mean SPT Diameter (mm):</td>
<td>8.42</td>
<td>8.56</td>
<td>9.96</td>
</tr>
</tbody>
</table>

All SPT reagents – 3000 ug/mL
Table 5: The IgE ImmunoCAP positive diagnosis decision cut-off value of ≥15 kU/L excludes the majority of peanut allergics.

<table>
<thead>
<tr>
<th>Category</th>
<th>kUA/L</th>
<th>Peanut allergics (n)</th>
<th>Population Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>absent/undetectable</td>
<td>&gt;0.35</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>low positive</td>
<td>0.35 - 0.70</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>moderate positive</td>
<td>0.70 - 3.5</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td>high positive</td>
<td>3.5 - 17</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>very high positive</td>
<td>17 - &gt;100</td>
<td>15</td>
<td>43</td>
</tr>
<tr>
<td>Total No.</td>
<td></td>
<td></td>
<td>35</td>
</tr>
</tbody>
</table>
Internally prepared peanut Ag produces stronger type 2 cytokine response in primary culture

We have demonstrated that commercially available peanut Ag reagents show dramatic concentration differences and variable SPT results, but it remains unclear if these reagents elicit similar Ag-driven type 2 cytokine responses in vitro. We asked if our selection of peanut Ag reagents had an equivalent capacity to induce type 2 cytokine production in a peanut allergic population when used at a standardized equivalent concentration. As shown in Figure 2, internally prepared peanut Ag reagents elicited more intense IL-13 responses vs. commercially available reagents. Internally prepared peanut Ag reagents showed a significant increase in Ag-driven IL-13 (WPE1, 69.1; WPE2, 67.8; and WPE3, 85.2 pg/mL) but not IL-9 (WPE1, 17.3; WPE2, 45.7; and WPE3, 14.75 pg/mL) production in comparison to commercially available peanut Ag driven IL-13 (ALK-Abélo 42.7; Omega 72.5 pg/mL) and IL-9 (ALK-Abélo 9.38; Omega 31.1 pg/mL) production. Therefore, even at standardized concentrations, peanut Ag reagents elicit differential in vitro Ag-driven cytokine responses.

*In vivo SPT diameter does not significantly correlate with peanut specific in vitro cytokine responses*

Peanut Ag SPT is a measure of mast cell degranulation within the skin tissue (38). It is unclear if the intensity of the peanut specific immune reaction that occurs in the skin is representative of other peanut specific cellular responses. To determine if SPT diameter significantly correlates with the intensity of Ag specific cytokine responses in primary culture, 13 peanut allergic individuals were skin
Figure 2: Internally prepared peanut Ag produces stronger type 2 cytokine responses in primary culture in comparison to commercially available peanut Ag. PBMC from peanut allergic individuals were stimulated with commercially available (ALK and Omega) and internally prepared (WPE1-3) peanut Ag (n=40). Type 2 cytokine responses were quantified using ELISA. Each point represents one individual. Significance between the commercially available and internally prepared Peanut Ag was determined using a paired t test (*p<0.05, **p<0.005, ***p<0.0001).
prick tested using standardized concentrations of ALK-Abélo, Omega, and WPE 2 peanut Ag (3000 ug/mL). The mean SPT diameters were compared to peanut specific IL-5, IL-9, IL-10, and IL-13 responses. As shown in Table 6, SPT diameter and peanut driven in vitro cytokine responses did not significantly correlate. In conclusion, SPT values are not representative of peripheral cellular responses produced in reaction to peanut Ag stimulation.

These data collectively emphasize that although our current diagnostic methods may be able to provide us with strong PPVs above high cut-off points, a large percentage of the allergic population exists within the gray areas presented. The understanding of the mechanisms underlying peanut allergy may elucidate potential markers, leading to improved diagnostic methods.
Table 6: SPT diameter and peanut specific *in vitro* cytokine responses do not significantly correlate. The mean SPT diameter was determined using commercially available or internally prepared peanut Ag reagents (3000 ug/mL) in a peanut allergic population (n=22). In the same population, the peanut Ag (50 ug/mL) were used to stimulate PBMC in culture. Cytokine responses were quantified using ELISA. Correlation was determined using a Spearman test.

<table>
<thead>
<tr>
<th>Peanut Ag</th>
<th>IL-5</th>
<th>IL-9</th>
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<tr>
<td>WPE 2</td>
<td>0.09</td>
<td>0.8</td>
<td>-0.04</td>
<td>0.8</td>
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</table>
Chapter Discussion

Peanut allergy is a life threatening allergy. Anaphylactic fatalities are most commonly caused by peanut (19,20), yet the correct diagnosis of peanut allergy remains difficult and individuals who incorrectly believe that they have a true clinical allergy to peanut are not uncommon. The peanut Ag SPT is a method commonly used in combination with clinical history and peanut specific IgE quantification for the diagnostic of peanut allergy (4). Aside from a concerning lack of regulation over the content of peanut Ag for diagnosis purposes, several studies suggest that uncontrolled content may lead to variable SPT results (44,45).

In this study we examined multiple aspects of peanut Ag reagents used for diagnosis. We found that protein content varied greatly amongst commercially available and internally prepared peanut Ag reagents. Akkerdass et al. illustrated a similar trend with hazelnut Ag SPT reagents. Hazelnut Ag for SPT was found to vary by up to 65-fold between commercially available brands. Brands with lower total protein content also produced false negative results, which were not found with higher concentration brands (218). In all cases, it is unclear why such a wide range of protein content exists. Guo et al. showed that certain peanut cultivars have higher levels of allergenic proteins (219). Manufacturers of peanut Ag often give an option of a mix of peanut cultivars or specific varieties, but the proportion of each peanut cultivar or the total amount of peanut Ag or protein extracted are not provided. The method of drying the peanut may also affect the protein content. Pomes et al. demonstrated that protein content can be reduced up to 78% when roasting is used as a drying method (220). These results are controversial, as Beyer et al. showed that frying, boiling and roasting did not result in differential total
protein levels in peanuts (215). Until there are regulations implemented requiring the manufacturing of peanut Ag to publish the allergen and total protein content in their SPT products, as well as post-harvesting drying methods, concentrations and other important information it will remain unavailable to the public (47).

In order to combat the drastic differences noted in protein content between preparations, peanut Ag were adjusted to equivalent concentrations for skin prick testing and use in primary culture. SPT results using the highest concentration of 3000 ug/mL showed little to no difference in SPT diameter amongst Omega, ALK-AbéIlo, and WPE 2 peanut Ag. Although WPE 2 peanut Ag gave the largest mean SPT diameter at the cut-off of ≥8mm, ALK AbéIlo demonstrated the highest number of positive responses above this lower limit. Only one third to one quarter of the entire peanut allergic population responded with SPT diameters above ≥8mm. Similarly, less than half the allergic population had peanut specific IgE levels ≥15kUA/L. These data demonstrate that a diagnostic tool with improved NPV would be indispensable to clinicians dealing with diagnosis of peanut allergy.

The capacity of commercially available vs. internally prepared peanut Ag reagents to drive type 2 cytokine production in vitro was assessed. At standardized, EQUIVALENT concentrations, commercially available reagents elicited a less or non-significant type 2 cytokine response in comparison to those internally prepared. The processes involved in manufacturing the commercially available reagents may be a contributor to this lack of activity in vitro, as Pomes et al. reported that there is no significant difference in Ara h 1 levels between different peanut varieties (220). These
results emphasize the importance of needed implementation of regulations by an external governing body on the preparation of commercially available food Ag.

To date, no studies have examined if SPT diameter is representative of the cellular response profiles generated with peanut Ag stimulation \textit{in vitro}. We have determined that SPT diameter does not correlate with peanut driven type 2 cytokine production (IL-5, IL-9 and IL-13) in primary culture. The lack of correlation between SPT diameters and recall cytokine responses emphasize our minimal understanding of how events leading to a positive SPT relate to cellular mechanisms underlying peanut allergic reactions. Determining the relationship between either of these biomarkers and the intensity of clinical anaphylaxis would be most valuable but is not achievable experimentally. Improving our understanding of cytokine and regulatory responses associated with peanut allergy and clinical tolerance towards peanut may lead to the identification of a marker for improved diagnosis of peanut allergy.

For study populations discussed in this and subsequent chapters DBPCFC were not performed during diagnosis for both practical and ethical reasons, creating limitations in this study design. Diagnosis was made by an experienced allergist based on a recent convincing history of peanut induced anaphylaxis, detection of peanut specific IgE, and peanut skin prick test.

\textit{Chapter Conclusion}

Commerially available peanut Ag reagents vary dramatically in total protein content. The current methods for diagnosing peanut allergy have high NPV and are unable to predict the PBMC response generated in primary culture, suggesting that the
immune mechanism that underlies peanut allergy differ between the skin and peripheral blood.
Chapter II – Are serum IL-10 levels a useful marker of clinical tolerance in peanut allergy?

Chapter Introduction

Current methods used in diagnosis include oral food challenges, detailed medical history, skin prick test, quantification of peanut specific IgE in serum and self-report (i.e. in telephone surveys). These methods are commonly used in combination as no single method is faultless. While time consuming and potentially risky, double blind placebo controlled food challenges (DBPCFC) are the gold standard of food allergy diagnosis for an individual, but it is well established that the severity of food reactivity in the community does not correlate with severity diagnosed during DBPCFC (221). Skin prick tests and peanut specific IgE levels cannot reliably differentiate between sensitization and allergy in all cases (53). Access to subspecialty trained allergists is limited in many regions, leading to incorrect diagnoses. Given that incorrect positive diagnoses regularly lead to negative psychological effects and a reduced quality of life (32) and that the prevalence of individuals with clearly positive SPT is several fold higher than individuals with verifiable peanut allergy (222), there is great interest in development of novel, potentially more useful quantitative diagnostic tools to complement current methods of food allergy diagnosis. Therefore, to improve diagnosis of potentially peanut allergic individuals we propose that a cytokine biomarker may be a novel method to effectively diagnose peanut allergy.

Biomarkers for differentiation of clinical status and disease severity are a major focus in disease diagnosis research. Markers with sufficient sensitivity and specificity to
offer clinical utility have been identified for conditions ranging from food-induced anaphylaxis (23), cancers (223) and heart disease (224).

No single biomarker of sufficient sensitivity and specificity for diagnostic use is currently available for peanut allergy. In a small clinical study involving 12 DBPCFC-negative individuals and 29 hazelnut allergic adults, Alonso et al. recently reported the potential use of IL-10 serum levels as a marker of clinical tolerance to hazelnut. They reported that serum levels of IL-10 >2.28 pg/mL were diagnostic of clinical tolerance. The significant difference in serum IL-10 was limited to subjects with a previous diagnosis of hazelnut allergy but a recent negative DBPCFC, implying a newly acquired tolerance to hazelnut. Here, in paediatric and adult populations, we sought to determine the potential utility of serum IL-10 levels as a marker of clinical tolerance towards peanut. To date, research has predominantly concentrated on markers associated with disease, but rarely on markers of clinical tolerance.

In this study, using >200 individuals, we quantified IL-10 protein levels in plasma from clinically tolerant and peanut allergic adults and children to determine if elevated IL-10 levels in plasma are a good biological marker of clinical tolerance towards peanut. Specifically, we hypothesized that plasma IL-10 levels differ significantly between peanut allergic (Hx+SPT+) and clinically tolerant (Hx-SPT-) populations.
Chapter Results

Elevated plasma IL-10 levels are not a good marker of clinical tolerance towards peanut

To determine if plasma IL-10 differs significantly between individuals with clear clinical history and positive skin tests to peanut Ag vs. clinically tolerant individuals who are regularly exposed to dietary peanut, plasma from each population was analyzed to quantify IL-10 protein using a high sensitivity ELISA. Plasma IL-10 protein levels were detectable in all individuals examined, ranging from 11.87 to 9862 pg/mL (our detection limit 2.0 pg/mL). Despite use of substantially sized populations, no statistically significant difference was evident between the two populations (median response: Hx-SPT-, 197.4; Hx+SPT+, 127.7 pg/mL, p=0.19) (Figure 3).

It is well established that allergy prevalence is affected by age and gender. During early life peanut allergy, as most allergies, is more frequently diagnosed in males. Throughout adolescence and into adulthood females predominate, having higher and more severe incidences of asthma and allergy (225) The prevalence of sensitization as determined by skin prick test, as well as the mean SPT diameter, is also increased as children age (226). The effect of gender on adult plasma IL-10 was analyzed, as shown in Figure 4. On average, plasma from male peanut allergic adults contained 157 pg/mL IL-10, a greater, but statistically insignificant amount (p>0.05) in comparison to adult peanut allergic females (35.7 pg/mL). Plasma IL-10 did not differ significantly between adult, clinically tolerant, female (96.4 pg/mL) and male populations (103 pg/mL) (p=0.81) (Figure 4). Similarly, within a given gender there is no significant difference in plasma IL-10 levels within the
Figure 3: Clinically tolerant individuals do not have significantly higher levels of IL-10 in their plasma in comparison to peanut allergics. Clinically tolerant (CT) (circles, n = 107) and Peanut Allergic (PN) (squares, n = 94). Red bars indicate medians. Significance was determined using a Mann-Whitney unpaired T test.
Figure 4: Gender differences do not reveal significantly different plasma IL-10 levels in adult populations. Clinically tolerant (CT) (female – solid squares, n= 14; males – circles, n= 8) and Peanut Allergic (PN) (females –triangles, n = 21; males – diamonds, n = 10). Red bars indicate medians. Significance was determined using a Mann-Whitney unpaired T test.
adult population (female p=0.07, male p=0.6). Thus, plasma IL-10 levels do not differ significantly between genders.

We next asked if constitutive plasma IL-10 levels in healthy individuals show age related increases or decreases. Therefore, we asked if plasma IL-10 differed significantly in children and/or adults and if it did between clinically tolerant and peanut allergic population at the two age cohorts. Neither clinically tolerant children (291 pg/mL) nor adults (90.0 pg/mL) showed significantly higher levels of plasma IL-10 in comparison to children (138 pg/mL) and adult (57.7 pg/mL) peanut allergic populations, respectively (p = 0.48 and 0.27, respectively) (Figure 5).
Figure 5: Clinical tolerance is not associated with increased IL-10 in plasma. Clinically tolerant (CT) (children, n= 77; adults, n= 23) and Peanut Allergic (PN) (children, n= 61; adults, n= 31) represent clinically tolerant and peanut allergic individuals. Red bars indicate medians. Significance was determined using a Mann-Whitney unpaired T test.
Chapter Discussion

Unlike most food allergies, peanut allergy is usually life-long. Of all food allergens it poses the greatest risk of anaphylaxis (4). Improved methods for diagnosing food allergies, here peanut allergy, or for identifying clinical tolerance are urgently needed, as current methods create “probable” and “possible” food allergic populations, which are problematic. Oral challenges are costly, time consuming and inherently of higher risk, all factors that severely limit their usage, whereas SPT and ImmunoCAP peanut specific IgE result in large numbers of individuals in the “gray” areas characterized by poor PPV and NPVs. A 2007 report by Alonso et al. suggested that serum IL-10 could act as a marker for clinical tolerance towards hazelnut (63).

IL-10 is involved in the suppression of type 1 and 2 cytokines (4), but here, using a population of ~200 individuals, we found that plasma IL-10 is not an effective marker of clinical tolerance to peanut. Our finding that plasma IL-10 levels are not useful markers of clinical tolerance to foods is supported by Qiao, H.L. et al. who found that there was no significant difference between IL-10 plasma levels in clinically tolerant and penicillin-allergic individuals (145). In the past, attempts at using markers that are not specific to the disease in question have also shown a lack of success. Total IgE was once measured for the diagnosis of allergy. It was later shown that total IgE may vary depending on the season, by gender, and race, making decision point cut-offs difficult to establish (227). These results emphasize that Ag non-specific marker discovery is likely to be difficult in allergy diagnosis.
Alonso et al. presented a possible successful use of serum IL-10 in the detection of clinical tolerance in those who once were diagnosed with hazelnut allergy (63). Although allergies share common characteristics, mechanisms that underlie each disease are unique. For instance, mouse models of experimental allergic conjunctivitis and asthma showed that IL-10 KO mice may have reduced rather than elevated allergic symptoms relative to WT mice (228). Higher levels of IL-10 producing monocytes are found in atopic asthmatics in comparison to controls, which may perpetuate Th2 responses (229), respectively. Similarly, while Akdis et al. reported that both healthy and dust mite and birch pollen allergic individuals produce IL-10 secreting Tr1 cells in response to allergen, allergen-specific IL-10-secreting T cells are more predominant in healthy individuals (230).

Chapter Conclusion

Here we have demonstrated that constitutive plasma IL-10 levels do not serve as a useful marker of clinical tolerance to peanut. The mechanisms which are responsible for continued clinical tolerance towards peanut in the ~97% of the population which is not allergic to peanut in the industrialized world remains unclear. Further research is required to identify clinically useful biomarkers by distinguishing factors between those who are peanut allergic and those who ingest peanut without the development of inappropriate reactions. With greater understanding of how tolerance is achieved, identification of such biomarkers may be made possible, thereby improving the diagnostic methods used to diagnose peanut allergy.
Chapter III – Characterization of Ag-Dependant Cytokine Responses in Peanut Allergy

Chapter Introduction

Peanut allergy is characterized by expression of inappropriate immune responses to peanut Ag. Upon ingestion of peanut, elevated levels of type 2 cytokines and peanut specific IgE is formed leading to the development of allergy. A current, widely accepted model of peanut allergy suggests that peanut allergic populations mount Ag-driven type 2 cytokine responses and clinically tolerant populations form protective type 1 cytokine response that underlie clinical tolerance. One such model demonstrating this theory uses a mouse model sensitized to peanut using cholera toxin (72). In opposition to this hypothesis, Thottingal et al. found that peanut sensitized and clinically tolerant PBMC showed no detectable “protective” type 1 cytokine production with peanut Ag stimulation in primary cultures (6).

IL-9 is a type 2 cytokine whose Ag-driven elevated production has been associated with allergy (231), such as house dust mite allergy (84) and asthma in a mouse model (88). In house dust mite allergy, IL-9 is produced by CD4$^+$ T cells in response to allergen (84). Both studies by Devos et al. and Umezono-Goto et al. used small human populations (n=2-5) and did not explore the Ag-driven responses of clinically tolerant populations (84,88,231).

IL-9 acts synergistically with IL-4 to induce IgE expression via the IL-9R on human B cells (104). Alphonse et al. also demonstrated that IL-9 stimulated neutrophils express higher levels of FcεRI-α chain (103). Studies of food allergy induced
anaphylaxis show that, although not necessary for, IL-9 is a contributing factor to
anaphylaxis (97,98). Forbes et al. reported that no more than 20% of IL-9−/− mice
experienced experimental intestinal anaphylaxis upon OVA-challenge. Overexpression
of IL-9 in OVA-sensitized mice also induced the experimental intestinal anaphylactic
phenotype and was enough to predispose these animal models to anaphylaxis (97). In
agreement, Knoops et al. reported that IL-9 transgenic mice had increased susceptibility
to OVA-induced active systemic anaphylaxis, but neither IL-9R deficient mice nor anti-
IL-9 blocking Ab in OVA-challenged mice showed a significant difference in anaphylaxis
as compared to controls (98). These data suggest that elevated Ag-driven IL-9
production may be characteristic of peanut allergy.

Despite the compelling evidence of an important role for IL9 in murine models of
food allergy, the role of IL-9 in peanut allergy has not been explored in peanut allergic or
peanut clinically tolerant human populations. The goal of our study was to determine if
elevated IL-9 synthesis is associated with peanut allergy. We hypothesized that
enhanced peanut specific IL-9 production would be associated with peanut allergy.
Human PBMC from 88 clinically tolerant, peanut sensitized and peanut allergic
populations were stimulated with peanut Ag. In agreement with our hypothesis, IL-9
production was significantly enhanced in peanut allergic populations. Additionally,
peanut non-allergic populations demonstrated the capacity to produce peanut specific
IL-9, but less frequently and in quantitatively lesser amounts. In conclusion, enhanced
peanut driven IL-9 is associated with peanut allergy.
Chapter Results

Peanut allergy is characterized by quantitative, not qualitative, differences in peanut specific type 2 cytokine production

Peanut allergy was defined as a food hypersensitivity characterized by type 2 cytokine production by de Jong et al. and Higging et al. (210,232). These early studies were carried out using T cell clones from peanut allergic populations, thus limiting the size of the population of T cells (several clones vs. the diversity of Ag-reactive T cells present in PBMC), the number of individuals examined (5-7 individuals used to develop T cell clones vs. ~200 individuals studied here), and were unable to assess the inherent variability present in the human population. Subsequent studies involving peanut allergic human PBMC responses were restricted to proliferation assays and were typically carried out using very high concentrations (1 mg/mL) of peanut Ag (233). We sought to characterize representative peanut specific cytokine responses in a peanut allergic population using low concentrations of peanut Ag that may better represent physiological levels (137). As shown in Figure 6, the optimal concentration for peanut driven type 2 cytokine production in a peanut allergic population is 50 μg/mL. At this concentration, peanut Ag stimulation of PBMC from peanut allergics produced significantly greater levels of peanut driven IL-5 (325 pg/mL, p<0.0001) and IL-13 (63.2 pg/mL, p<0.0001) in comparison to the medium alone (IL-5, 37.4; IL-13, 10.2 pg/mL) (Figure 6).
Figure 6: Peanut allergic populations produce type 2 cytokines in response to peanut. PBMC from peanut allergic individuals were stimulated with peanut Ag in primary culture. Supernatant cytokine response profiles were quantified using high sensitivity IL-5 (n=33) and IL-13 (n=37) ELISAs. Each point represents one individual. Significance between the medium control and peanut Ag was determined using a paired t test (*p<0.05, **p<0.005, ***p<0.0001). PHA – 2.5 ug/mL.
Peanut non-allergic individuals exhibit type 2 cytokine production in response to peanut Ag stimulation ex vivo

Thottingal et al. demonstrated that peanut sensitized and clinically tolerant populations both regularly exhibit the capacity to produce type 2 cytokines in the presence of peanut Ag (6). While we wished to examine the peanut sensitized (n=9) and clinically tolerant (n=39) populations' response to peanut Ag stimulation separately, an imbalance in population strength did not allow for a critical statistical evaluation of peanut driven type 2 cytokine responses in primary culture. As shown in Figure 7, peanut non-allergic (sensitized and clinically tolerant) populations demonstrated the capability to produce peanut driven type 2 cytokines. Peanut driven IL-5 (15.7 pg/mL, p=0.032) and IL-13 (9.17 pg/mL, p=0.0024) production were significantly enhanced in comparison to the medium alone to the greatest extent at peanut concentrations of 50 ug/mL (Figure 7). This data supports the conclusion that peanut non-allergic populations demonstrate the capacity to produce peanut driven type 2 cytokines thought to be only associated with allergy, while maintaining the ability to ingest peanut without consequence. Additionally, lower concentrations of peanut Ag are capable of inducing statistically significant changes in cytokine production in both peanut allergic and non-allergic populations. These lower concentrations may better represent the physiological process that occurs during a peanut allergic reaction.
Figure 7: Peanut non-allergic populations produce peanut driven type 2 cytokines. Peanut non-allergic (peanut sensitized and clinically tolerant) individuals PBMC were stimulated with peanut Ag in primary culture (n=48). PHA (2.5 ug/mL). Supernatant cytokine response profiles were quantified by ELISA. Each point represents one individual. Significance between the medium control and peanut Ag was determined using a paired t test (*p<0.05, **p<0.005, ***p<0.001).
Peanut driven IL-9 is significantly enhanced in peanut allergic populations

IL-9 is a well established marker type 2 cytokine that has been shown to promote IgE synthesis and has been implicated in allergy (103,104). Umezu-Goto et al. demonstrated that CD4+ T cells are a detectable source of IL-9 in atopic asthmatics (84). The role of IL-9 in peanut allergy has not been explored. In order to determine if IL-9 is involved in the mechanism underlying peanut allergy, supernatant IL-9 from PBMC stimulated with medium alone or in the presence of peanut Ag was quantified using ELISA. For the detection of peanut driven recall IL-9 responses optimal peanut Ag concentrations of 50 and 100 ug/mL were determined, as peanut driven recall IL-5 and IL-13 responses were also the strongest and most significantly enhanced in comparison to the medium alone at these concentrations. Peanut Ag stimulation at 50 and 100 ug/mL resulted in a significant increase in IL-9 production in comparison to the medium alone (50.6, p=0.038; 73.9, p=0.039, medium alone: 10.2 pg/mL) (Figure 8A). In 40 peanut allergic individuals we then demonstrated that peanut driven IL-9 (45.7 pg/mL, p=0.021) production is significantly enhanced in comparison to the medium (9.38 pg/mL) alone (Figure 8B). As with IL-5 and IL-13, peanut non-allergic individuals demonstrated the capacity to produce significantly enhanced levels of peanut driven IL-9 in comparison to the medium (Med, 50, and 100 ug/mL Peanut Ag: 9.38; 9.38, p=0.015; and 9.38 pg/mL, p=0.033). In all cases, peanut allergic (vs. non-allergic) individuals exhibited quantitatively greater levels of IL-9 production, which were more significantly enhanced in comparison to the medium alone (Figure 9). These data provide insight that IL-9 may be associated with peanut allergy, based on the
Figure 8: Peanut allergic individuals produce significantly enhanced levels of peanut driven IL-9. A. Determination of optimal Ag concentration for elicitation of peanut driven IL-9 responses (n=15). B. Peanut recall IL-9 responses of peanut allergic individuals (n=40). Peanut allergic PBMC were stimulated with peanut Ag and PHA (2.5 ug/mL) in primary culture and IL-9 levels quantified using ELISA. Significance between the medium control and peanut Ag and PHA was determined using a paired t test (*p<0.05, **p<0.005, ***p<0.0001).
Figure 9: Non-allergic populations do not produce significant levels of IL-9 in response to peanut. Peanut non-allergic (clinically tolerant and peanut sensitized) PBMC were stimulated with peanut Ag in primary culture (n=49). Supernatant cytokine response profiles were quantified using high sensitivity IL-9 ELISAs. Each point represents one individual. Significance between the medium control and peanut Ag was determined using a paired t test (*p<0.05, **p<0.005, ***p<0.0001).
significant median differences between peanut allergic and non-allergic populations. The data provided in Figures 6-9 also demonstrates that 50 ug/mL is an optimal concentration for evaluating peanut driven type 2 cytokine responses in peanut allergic, sensitized, and clinically tolerant populations.

*Peanut allergics produce significantly greater levels of peanut driven type 2 cytokines more frequently than non-allergics*

In a previous study by Thottingal et al. peanut sensitized individuals demonstrated a higher IL-5 and IL-13 response frequency than those who were clinically tolerant (6). To date, no one has examined if peanut allergic and non-allergic populations exhibit different peanut driven IL-9 responder frequencies. To evaluate if a differential IL-5, IL-9 and IL-13 responder frequency existed between peanut allergic and non-allergic populations, their response frequencies were recorded. As Figure 10A demonstrates, peanut allergic individuals (IL-5, 80.6; IL-9, 64.1; and IL-13, 89.2%) respond to peanut Ag at a greater frequency than peanut non-allergic individuals (IL-5, 21.7; IL-9, 14.3; and IL-13, 31.3%). Peanut allergic populations also produce significantly greater levels of peanut driven IL-5, IL-9, and IL-13 in comparison to clinically tolerant recall responses (Figure 10B). Of the entire peanut allergic population, all demonstrated the capacity to produce peanut driven recall type 2 cytokine responses, whereas only 36.7% of the peanut non-allergic population produce a ≥2-fold increase in one or more type 2 cytokines in comparison to the medium alone. Interestingly, only 48.4 and 8.9% of all peanut allergic and non-allergic individuals...
Figure 10: Peanut allergics produce elevated levels of type 2 cytokines more frequently than non-allergic populations. (A) Peanut driven responder frequency. A positive response to peanut Ag (50 ug/mL) is defined as ≥2 fold increase in comparison to the medium alone (peanut non-allergic, gray; peanut allergic, black). (B) Median type 2 cytokine production in peanut non-allergic (O, n=49) and peanut allergic (△, n=39) populations. Significance between the populations was determined using an unpaired Mann-Whitney t test (*p<0.05, **p<0.005, ***p<0.0001).
demonstrate the capacity to produce IL-5, IL-9, and IL-13 in response to peanut. Therefore, while there is clearly not a qualitative difference in the capacity of peanut allergic and peanut non-allergic individuals to mount food-specific type 2 cytokine responses, peanut allergic populations produce type 2 cytokines both more frequently and at higher levels than those who are non-allergic.

In agreement with Thottingal et al. clinically tolerant and peanut sensitized populations are capable of producing peanut driven type 2 cytokine responses, but those who response are substantially fewer in comparison to the peanut allergic population. It is unclear if these type 2 cytokine responses differ significantly between peanut allergic and non-allergic populations. In order to determine if peanut allergic populations produce significantly enhanced type 2 cytokine production in comparison to non-allergic populations, median peanut Ag (50 ug/mL) responses were evaluated. As Figure 10B shows, median peanut allergic peanut driven type 2 cytokine responses were significantly higher than non-allergic responses (all median responses are listed in Figure 6-9 results). Thus, peanut allergic populations produce a significantly higher response at a greater frequency than non-allergic populations.
Chapter Discussion

We have demonstrated that peanut allergy is associated with a significant increase in IL-5 and IL-13 production when stimulated with peanut Ag. Type 2 cytokine polarization in peanut allergy has been shown in several studies (72,111,210,234). Peanut allergy studies involving humans are often restricted to limited population size and the use of T cell clones (111,210,234). In this study 40 peanut allergic individuals were recruited for analysis of peanut driven cytokine profiles generated in primary cultures. These results demonstrate that IL-9 may play an important role in the pathophysiology of peanut allergy.

Thottingal et al. reported that non-allergic populations demonstrate the capacity to generate a peanut driven type 2 cytokine response, but maintain clinical tolerance towards peanut with ingestion. Here we not only confirmed that peanut non-allergic individuals demonstrate the capacity to produce peanut driven IL-5 and IL-13, but 1 in 10 individuals also produce IL-9. These data are intriguing as orally tolerized T cells are able to form stable conjugates with APC (132). Furthermore, the ligation of the costimulatory molecule, CD80, is necessary for oral tolerance induction (133). Why signalling events continue that allow the production of peanut driven type 2 cytokines is not understood and unexpected as Ise et al. demonstrated that immunologic synapse formation and lipid raft translocation is impaired in OVA tolerized mice (132). Why peanut non-allergic populations produce type 2 cytokines in response to peanut, and how these individuals maintain the capability of ingesting peanut without a negative clinical outcome remains unclear.
We have demonstrated IL-9 is associated with peanut allergy. These results are supported by several studies that implicate IL-9 in allergy. In a transgenic mouse model IL-9 is able to induce eosinophilia and B cell activation (99). IL-9 is also able to enhance IL-5 production (102) and plays a role in FcεRI (103) and IL-4-depandan IgE synthesis (104). The role of IL-9 in Ag-induced anaphylaxis has also been explored. Knoops et al. showed that IL-9 is able to promote OVA-induced anaphylaxis in mice, but is not absolutely necessary for it to occur (98). Intestinal IL-9 expression has been shown to be a predictor of intestinal anaphylaxis and oral sensitization is able to occur in an IL-9 deficient mouse model (97). The production of Ag-driven IL-9 has also been shown to be stable over time, and thus may be able to categorize allergic status (231). These data collectively suggest that IL-9 plays an important role in peanut allergy and may be a candidate in predicting peanut induced anaphylaxis.

In this study we show that peanut allergic populations produce quantitatively greater levels of type 2 cytokines in response to peanut in comparison to non-allergics. Peanut allergic populations also produce peanut driven type 2 cytokines at a greater frequency than non-allergics. This observation was also noted by Thottingal et al. but within a smaller population (6). Peanut driven IL-13 was most commonly produced by allergic, sensitized, and clinically tolerant populations but peanut driven IL-5 presented the highest median response of the type 2 cytokines analyzed. Similar response frequency patterns have been observed in atopic vs. non atopic populations (231). Although overexpression of IL-5, IL-9 and IL-13 are accepted as cytokines associated with allergy, the magnitude of their peanut driven production varies substantially in comparison to one another and between individuals. These results extend our
understanding of how these cytokines are regulated differentially and play important but distinct roles in peanut allergy.

Chapter Conclusion

Our results in this study expose the association of IL-9 in allergy. Peanut allergy is associated with the Ag-driven enhancement of IL-9 and this understanding may help further elucidate the mechanism of the immune response underlying peanut allergy. Clinically tolerant and peanut sensitized populations demonstrate the capacity to produce type 2 cytokines in response to peanut Ag stimulation, but they produce quantitatively less and at a lesser frequency than peanut allergic populations.
Chapter IV - Clinical tolerance and the regulation of peanut-specific cytokine production

Chapter Introduction

Little is understood concerning the immune mechanisms that underlie peanut allergy, delaying advances in prophylaxis, effective treatments and a possible cure. Up to Twenty percent of peanut allergic patients will outgrow their allergy (9), but the severity of the disease (3) and the commonality of peanut as a food source in the North American diet (1) pose a large risks for those who retain this allergy for life. Despite often extremely intensive efforts, it is virtually impossible to totally avoid exposure to environmental peanut Ag exposure. Thus, there is a critical need for a greater understanding of regulatory mechanisms that differentiate peanut allergic and clinically tolerant populations.

Regulatory (i.e. immunosuppressive) mediators, such as IL-10, TGF-β, and IDO, have been reported to be involved in tolerance towards food Ag (208,235,236). Evidence exists suggesting that these three regulators may contribute to the maintenance of clinical tolerance towards food Ag. However, the causal mechanisms that underlie the decision of the immune system to develop an “allergic” vs. a “clinically tolerant” clinical phenotype are very poorly understood. IL-10, an inhibitor of type 1(IFNγ) and more recently type 2 cytokine production (145,237), has also been shown to inhibit T cell activation and inhibit antigen presentation (238) in a wide variety of murine, and several human experimental systems. Elevated plasma IL-10 levels have been suggested to be associated with the outgrowth of food allergy (63,73). Some
studies report that food allergic children have significantly fewer IL-10 secreting cells (144) and IL-10 is necessary for natural immune responses to allergens in clinically tolerant individuals towards bee venom (239). Li et al. demonstrated that inhibition of TGF-β function results in enhanced anti-CD3 and anti-CD28-dependant IL-4 expression (162). In agreement, TGF-β signalling-impaired mice demonstrate increased levels of IL-4 in BAL fluid (119). Furthermore, simultaneous OVA feeding and TGF-β treatment in OVA-sensitized mice produced decreased Ag driven IgE, but Ag doses given were at high concentrations (161). The tolerogenic role of IDO has also been explored using OVA sensitized mouse models. One such model indicated that IDO is essential for tolerogenic responses, but allergy was only measured using histology and the measurement of a single biomarker of airway hyperresponsiveness (184). The above evidence suggests that IL-10, TGF-β, and IDO may play important roles in maintaining clinical tolerance towards peanut.

Here, we have chosen to examine the roles IL-10, TGF-β, and IDO play in peanut allergy in humans, as all peanut allergy studies involving these immunoregulatory cytokines have been restricted to mouse models and/or have not directly examined these cytokines' role in peanut allergy (51,52,240).

We hypothesized that clinical tolerance towards peanut would be associated with enhanced IL-10 production in response to peanut. Functional responses by PBMC from clinically tolerant and peanut allergic individuals were studied to determine their type 1, type 2 and regulatory cytokine production in response to peanut. We also evaluated the ability of exogenous IL-10 to functionally inhibit the production of peanut driven type 2 cytokines in both allergic and tolerant populations. To determine if endogenous IL-10,
as well as TGF-β and IDO, are important in inhibiting peanut driven type 1, type 2, and regulatory cytokine production, IL-10 Receptor (IL-10R), TGF-β, and IDO function were blocked. We have demonstrated that IL-10, TGF-β, and IDO play only a limited role in controlling the intensity of type 2 immune responses seen in peanut non-allergic individuals. In contrast to our initial hypothesis, we found that IL-10, TGF-β, and IDO play their most substantive role in limiting peanut driven cytokine responses in peanut allergic (rather than clinically tolerant) populations.

Further investigation revealed that, IL-10 and TGF-β act synergistically in negatively regulating select peanut driven type 1 and type 2 cytokine production in peanut allergic and clinically tolerant populations, with the most substantive impact again evident in the allergic rather than in the tolerant population. In conclusion, the data available best supports a model where IL-10, TGF-β, and IDO play a minimal role in preventing overexpression of type 2 immunity (and by implication, clinical allergy) among peanut non-allergic individuals whereas it plays a substantial role in inhibiting the intensity of ongoing, undesirable overexpression of type 2 cytokine production as neutralization of any of these three inhibitor mechanisms leads to significant increases in the already elevated peanut-driven type 2 cytokine production.
Chapter Results

Peanut allergic populations produce significant levels of IL-10 in response to peanut Ag

Our work in Chapter II established that both peanut allergic and clinically tolerant populations produce type 2 cytokines in response to peanut, but those who are clinically tolerant produce quantitatively less. The mechanisms regulating such responses have yet to be determined. IL-10 has been widely described as an immunoregulatory cytokine, enhanced expression of which is often associated with inhibition of immune responsiveness and both immunologic and clinical tolerance (30). To determine if elevated IL-10 levels are associated with clinical tolerance towards peanut in peanut non-allergic individuals, cytokine protein levels from primary Ag-stimulated cultures were measured using ELISA in both peanut allergic and clinically tolerant populations. We hypothesized that elevated peanut driven IL-10 production is associated with clinical tolerance. However, in opposition to our hypothesis, peanut allergic populations also produced significant levels of IL-10 and the intensity of IL-10 production was highest not in the clinically tolerant primary culture supernatants following peanut Ag stimulation but in cultures derived from allergic individuals (Figure 11A). Furthermore, peanut allergic populations produced significantly greater levels of IL-10 in comparison to clinically tolerant populations (Figure 11B). These data suggest that an Ag-driven elevation in IL-10 production does not play a major role in the maintenance of immune tolerance towards peanut.
Figure 11: Peanut allergic populations produce significant levels of IL-10 in response to peanut Ag. (A) Differential prevalence of IL-10 production in response to peanut Ag stimulation. Peanut driven cytokine production by human PBMC were measured by ELISA (peanut allergic, n = 41; clinically tolerant, n = 18). Significance between the medium control and peanut Ag stimulation was determined using a Paired t test. (B) Differential intensity of IL-10 production in the two main groups. Peanut driven IL-10 production was compared between clinically tolerant (blue circles) and peanut allergic (yellow triangles) populations using a Mann Whitney test. *p < 0.5, **p<0.005, ***p<0.0001.
Peanut allergic populations exhibit a greater peanut driven IL-10 responder frequency

Peanut allergic and clinically tolerant populations both produce type 2 cytokines in response to peanut, albeit at different intensities. Allergic populations respond (i.e. express Ag driven type 2 cytokine production) at a higher frequency than those who are clinically tolerant. To elucidate if a similar difference in response pattern existed in terms of peanut driven IL-10 production between the two clinically distinct populations, response frequencies were calculated for each population studied. Positive responses were defined as cytokine responses (shown in Figure 11) that were ≥2 fold than the medium response alone. In Table 7, it is shown that of the peanut allergic population, 31-38% produce IL-10 in response to peanut restimulation (11-14/27) in primary culture, whereas from 0-11% (0 to 2 of 17 individuals) produce a detectable peanut driven IL-10 response. These differences in peanut driven IL-10 response frequency are significantly different between peanut allergics and those who are clinically tolerant. Therefore, peanut allergic populations not only produce enhanced peanut driven type 2 cytokines at a greater frequency than those who are non-allergic (Chapter III), but regulatory cytokines also exhibit this pattern within the distinct populations. This suggests that a relationship between peanut driven type 2 and regulatory cytokine production exists.

Peanut driven IL-10 production significantly correlates with type 2 cytokine production

To determine if peanut driven IL-10 positively correlated with expression of type 2 cytokines produced in response to peanut, a correlation analysis was performed for all individuals studied (Table 8, Figure 12). Peanut driven IL-10 levels significantly correlated with peanut driven IL-5, IL-9, and IL-13 cytokine production in the collective
Table 7: Peanut allergics produce peanut driven IL-10 at a greater frequency than those who are clinically tolerant. Values are expressed as a fraction of the population exhibiting a positive response to peanut Ag (defined as ≥2 fold increase in comparison to the medium alone).

<table>
<thead>
<tr>
<th></th>
<th>Peanut Ag (ug/mL)</th>
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<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Clinically Tolerant</td>
<td>0/17</td>
</tr>
<tr>
<td>Peanut Allergic</td>
<td>11/36</td>
</tr>
</tbody>
</table>
Table 8: Peanut driven IL-10 production significantly correlates with type 2 cytokine production. Peanut driven human PBMC IL-5, IL-9, and IL-13 cytokine production significantly correlates with IL-10 production in both peanut allergic and clinically tolerant populations, n = 37 and 18, respectively. Cytokine production was measured using ELISA. Correlation was determined using the Spearman test.

<table>
<thead>
<tr>
<th>Clinical Status</th>
<th>IL-5</th>
<th>IL-9</th>
<th>IL-13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>All individuals</td>
<td>0.6</td>
<td>&lt;0.0001</td>
<td>0.7</td>
</tr>
<tr>
<td>Peanut Allergic</td>
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<td>0.003</td>
<td>0.6</td>
</tr>
<tr>
<td>Clinically Tolerant</td>
<td>0.6</td>
<td>0.008</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Figure 12: Peanut driven human PBMC IL-5, IL-9, and IL-13 cytokine production significantly correlates with IL-10 production. Peanut driven cytokine production was measured using ELISA. Correlation was determined using the Spearman test (n = 57).
population. Similarly, stratified populations based on clinical status showed that peanut driven type 2 cytokines significantly correlate with peanut driven IL-10 production in both allergic and clinically tolerant populations (Figure 13). As demonstrated in Chapter III and Figure 11, peanut allergic individuals produce significantly quantitatively greater levels of type 2 and regulatory cytokines in response to peanut in comparison to those who are clinically tolerant. To examine if a different relationship exists between peanut driven type 2 cytokine and IL-10 production in peanut allergic vs. non-allergic individuals, correlation analyses were performed within the stratified populations based on clinical status. The data show similar relationships in both populations, this elevated type 2 production may be causing elevated IL-10 production as a response.

Exogenous rIL-10 inhibits Type 2 cytokine production in peanut allergic populations

Recombinant IL-10 is able to inhibit both peanut driven Type 1 and Type 2 cytokine production in primary PBMC cultures (237). We sought to confirm that IL-10 and the IL-10R signalling pathways were functionally able to decrease peanut driven cytokine production with the addition of rIL-10 to peanut stimulated culture (Figure 14). For clarity, Figure 14A represents a typical experiment conducted with the full population studied in Figure 14. It shows the result of medium alone, peanut Ag stimulation, and the effects of different concentrations of rIL-10 added during peanut Ag stimulation on IL-13 cytokine production as determined by ELISA.

In a dose dependant manner, rIL-10 was able to decrease peanut driven Type 2 cytokine production to the ELISA lowest limit of quantitation (LLOQ). Peanut driven IL-9 production was reduced from 370 pg/mL to the to the ELISA lowest limit of quantitation.
Figure 13: The intensity of peanut driven IL-10 production significantly correlates with type 2 cytokine production. Peanut driven human PBMC IL-5, IL-9, and IL-13 cytokine production significantly correlates with IL-10 production in both peanut allergic and clinically tolerant populations, n = 37 and 18, respectively. Cytokine production was measured using ELISA. Correlation was determined using the Spearman test. Each point represents one individual.
Figure 14: Exogenous rIL-10 inhibits Type 2 cytokine production in peanut allergic populations. (A) Each line represents 5 individuals (B) Each point represents one individual and is expressed in IL-9 (n=13) and IL-13 (n=11) % Inhibition from peanut Ag (Pn Ag) stimulation alone. 0% change representing no change. Significance was determined using One sample and Wilcoxon Signed Rank t test (*p<0.05, **p<0.005, ***p<0.0001).
(9.38 pg/mL). Similarly, peanut driven IL-13 production was reduced from 89.8 pg/mL to the ELISA LLOQ (5.86 pg/mL). Therefore, rIL-10 was able to functionally inhibit peanut driven Type 2 cytokine production in peanut allergic populations.

*Endogenous IL-10 negatively regulates peanut driven Type 1 and Type 2 cytokine production in peanut allergic populations*

Although subtle, the sensitivity of exogenous and endogenous IL-10 have been shown to vary on mature DC (241). Here, we studied the effects of neutralising the IL-10R to verify if endogenous IL-10 is being produced by peanut allergic individuals. Neutralisation of the IL-10R would also help establish if inhibitory signalling is occurring through the IL-10R and thus functionally inhibiting peanut driven cytokine production. Type 2 cytokines were measured in response to this inhibition in the presence of peanut Ag stimulation using ELISA. As shown in Figure 15, neutralisation of the IL-10R resulted in a 46.8 (p = 0.003), 62.1 (p<0.001), 66.2 (p<0.0001), and 0% (p <0.0001), increase in peanut driven IL-5, IL-9, IL-13 and IFNγ from 259.7, 137.0, 71.16 pg/mL, and 4.241 IU/mL, respectively. When quantifying peanut driven cytokine production with the neutralisation of the IL-10R two distinct populations formed, those who show enhanced cytokine production in comparison to peanut Ag stimulation alone (>0% Change in cytokines quantified), and those who do not (0% Change in cytokines quantified). The median IL-5, IL-9, IL-13 and IFNγ response of those who produced a >0% change increase in the presence of anti-IL-10R mAb was 100, 251, 74, and 266 %
Figure 15: Endogenous IL-10 negatively regulates peanut driven type 1 and type 2 cytokine production in peanut allergic populations. PBMC from peanut allergic individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of anti-IL-10R mAb (n=47). Each point represents one individual and is expressed in % change from peanut Ag stimulation alone. 0% change represents no change. Significance between peanut Ag stimulation alone and Peanut Ag in the presence of anti-IL-10R mAb was determined using One sample and Wilcoxon Signed Rank t test.
Change in the peanut allergic population. These results confirm that the IL-10/IL-10R pathway is functioning and endogenous IL-10 is able to inhibit peanut driven cytokine production. Furthermore, peanut allergic populations retain the capacity to negatively regulate their harmful immune response formed against a normally innocuous food Ag.

*Endogenous IL-10 demonstrates only a limited role in negatively regulating peanut driven Type 2 cytokines responses in clinically tolerant populations*

Sebastiani et al. have shown that although a cytokine may not be detectable in supernatants of cultures, they are still expressed, and may be internalized too rapidly to be detected by ELISA (242). As clinically tolerant individuals had provided evidence in Figure 11 that IL-10 does not play a detectable role in negative regulation of peanut driven Type 2 cytokine production, we sought to reduce the possibility that endogenous IL-10 was undetectable in supernatants due to rapid internalization. PBMC from clinically tolerant individuals were stimulated with peanut Ag alone and peanut Ag in the presence of anti-IL-10R mAb. As shown in Figure 16, neutralisation of the IL-10R resulted in a 96.9 (p<0.0001), 0 (p = 0.06), 0 (p = 0.004), and 0% (p = 0.001) increase of peanut driven IL-5, IL-9, IL-13 and IFNγ from peanut Ag stimulation cytokine levels of 22.50, 23.08, 29.47 pg/mL and 1.790 IU/mL, respectively. Unexpectedly, clinically tolerant IL-5 production was enhanced significantly with anti-IL-10R mAb treatment, and to a greater extent than peanut allergic populations. PBMC stimulated with anti-IL-10R mAb in the absence of peanut Ag resulted in no significant change of any of the cytokines analyzed in both clinically tolerant and peanut allergic populations (data not shown). IL-5, IL-9, IL-13 and IFNγ median responses of the subset clinically tolerant population demonstrating >0% Change in response to anti-IL-10R mAb treatment during
Figure 16: Endogenous IL-10 also has only a selective impact in limiting peanut allergic cytokine production in clinically tolerant populations. PBMC from clinically tolerant individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of anti-IL-10R mAb (n=57). Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
peanut Ag stimulation were 300, 308, 155 and 96.0% Change, respectively. This data suggested that differential regulation of IL-5 and other Th2 cytokines, such as IL-9 and IL-13, exists. In addition, IL-10 is not associated with the negative regulation of peanut driven IL-9 and IL-13, and thus shows only a limited role in the maintenance of clinical tolerance towards peanut.

*TGF-β negatively regulates peanut driven cytokine production in peanut allergic populations*

Shevach *et al.* provided evidence that TGF-β is a critical component in the maintenance of peripheral tolerance (160). To evaluate if TGF-β is involved in the maintenance of clinical tolerance towards peanut, PBMC from peanut allergic and clinically tolerant populations were stimulated with peanut Ag alone or in the presence of anti-TGF-β mAb. As demonstrated in Figure 17, inhibition of TGF-β led to a significant enhancement of peanut driven cytokine production in the peanut allergic population. Peanut allergy-associated Type 2 cytokine production of IL-9 and IL-13 were significantly enhanced by 0% (p<0.0001) and 57.9% (p<0.0001), respectively. The regulatory cytokine, IL-10, was also significantly enhanced by 106% (<0.0001) with simultaneous peanut Ag stimulation and TGF-β inhibition (Figure 17). All of the peanut driven cytokines analysed revealed a significant enhancement in production with the neutralisation of endogenous TGF-β. These results suggest that TGF-β is involved in the negative regulation of not only peanut driven Type 2, but Type 1 cytokine production and regulatory cytokines shown to control peanut allergy. The formation of populations that exhibit TGF-β-dependant negative regulation of peanut driven cytokine production.
Figure 17: TGF-β negatively regulates peanut driven type 1, type 2, and regulatory cytokine production in peanut allergic populations. PBMC from peanut allergic individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of anti-TGF-β mAb (n=49). Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
and those who do not suggest that differential regulation of the allergic cytokines may exist in a seemingly singular peanut allergic population.

*TGF-β selectively negatively regulates peanut driven cytokine production in peanut allergic populations*

The role of TGF-β in clinical tolerance towards peanut was examined. In contrast to our initial hypothesis, TGF-β appeared to play a lesser effect in controlling Type 2 cytokine production in clinically tolerant populations than in peanut allergic populations. A smaller proportion of clinically tolerant individuals exhibited increased Type 1 or type 2 cytokine production (compared to allergic individuals, (Figures 17 vs. 18). Moreover, the strong effects of anti-TGF-β mAb on peanut driven IFNγ and IL-10 production may not be the result of the presence of peanut Ag. When PBMC were stimulated with anti-TGF-β mAb in the absence of peanut Ag, IFNγ and IL-10 were significantly enhanced to levels similar to simultaneous peanut Ag stimulation and TGF-β inhibition (*data not shown*). TGF-β neutralisation did not significantly enhance peanut driven IL-13 production (0%, p=0.06). Clinically tolerant subset populations demonstrating a >0% Change in peanut driven cytokine production in the presence of anti-TGF-β mAb showed a 342, 340, 281, and 222% increase in IL-9, IL-13, IFNγ and IL-10 production, respectively (*Figure 18*). These results demonstrate that two sub populations of clinical tolerance exists, those control their peanut driven cytokine response with TGF-β and those who maintain clinical tolerance through differential mechanisms.
Figure 18: TGF-β negatively regulates peanut driven type 1, type 2, and regulatory cytokine production in clinically tolerant populations. PBMC from clinically tolerant individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of anti-TGF-β mAb (n=49). Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
IL-10 and TGF-β synergistically negatively regulate peanut driven Type 1 cytokine production

The immune system is infamous for having redundant characteristics. The immunoregulatory mediators, IL-10 and TGF-β, have shown act synergistically, in IL-2-mediated Treg suppressive activity (243). To examine if IL-10 and TGF-β act synergistically in the negative regulation of peanut driven cytokine production, PBMC were stimulated with peanut Ag in the presence of anti-IL-10R and anti-TGF-β mAb. Peanut allergic and clinically tolerant populations showed a significant increase in peanut type 2 cytokines production with inhibition of IL-10R and TGF-β. As shown in Figure 19, peanut driven type 2 cytokine production was significantly enhanced with simultaneous inhibition of IL-10R and TGF-β in both peanut allergic and clinically tolerant populations. As with single inhibition, two distinct populations formed—responders to simultaneous inhibition and non-responders (Figure 19). Peanut driven Type 1 cytokine production was significantly enhanced with simultaneous inhibition of IL-10R and TGF-β in peanut allergic and clinically tolerant population by 1725 (p<0.0001) and 178% (p<0.0001), respectively (Figure 20). Whether this IL-10R- and TGF-β-dependant inhibition occurs in a synergistic manner remained unclear. Therefore, to examine if IL-10 and TGF-β act synergistically in negatively regulating peanut driven cytokine production the cumulative and simultaneous effects of anti-IL-10R and anti-TGF-β mAb on peanut driven cytokine production were examined. As shown in Table 9, when independent IL-10R or TGF-β inhibition-dependant % Change of peanut driven IL-13 was compared to simultaneous neutralization, neither an additive nor synergistic effect was detectable in either population. In contrast, simultaneous
Figure 19: IL-10 and TGF-β negatively regulate peanut driven Type 2 cytokine production. PBMC from (A) Peanut allergic (n=46) and (B) clinically tolerant (n=49) individuals were stimulated with Peanut Ag alone or simultaneously with Peanut Ag, anti-IL-10R and anti-TGF-β mAb. Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
Figure 20: IL-10 and TGF-β negatively regulate peanut driven Type 1 cytokine production. PBMC from (A) peanut allergic (n=43) and (B) clinically tolerant (n= 45) individuals were stimulated with Peanut Ag alone or simultaneously with Peanut Ag, anti-IL-10R mAb and anti-TGF-β mAb. Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
Table 9: Peanut driven IL-13 and IFNγ production are synergistically negatively regulated by IL-10 and TGF-β in peanut allergic and clinically tolerant.

<table>
<thead>
<tr>
<th></th>
<th>IL-9</th>
<th>IL-13</th>
<th>IFNγ</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Hx-SPT-</td>
<td>Hx+SPT+</td>
<td>Hx-SPT-</td>
</tr>
<tr>
<td>anti-IL-10R mAb + PN Ag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% Change)</td>
<td>0</td>
<td>62.1</td>
<td>0</td>
</tr>
<tr>
<td>n (responders)</td>
<td>4</td>
<td>27</td>
<td>9</td>
</tr>
<tr>
<td>anti-TGF-β mAb + PN Ag</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% Change)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>n (responders)</td>
<td>6</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>cumulative enhancement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(% Change)</td>
<td>0</td>
<td>62.1</td>
<td>0</td>
</tr>
<tr>
<td>anti-IL-10R mAb +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-TGF-beta mAb + PN Ag</td>
<td>0</td>
<td>159</td>
<td>0</td>
</tr>
<tr>
<td>(% Change)</td>
<td></td>
<td></td>
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</tr>
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<td>15</td>
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</tr>
<tr>
<td>synergistic enhancement</td>
<td>0</td>
<td>96.5</td>
<td>0</td>
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</tbody>
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inhibition of IL-10R and TGF-β lead to a dramatic and significant increase in peanut driven IL-9 and IFNγ production in peanut allergic and both populations, respectively. These results demonstrate that a subset of both peanut allergic and clinically tolerant populations have the capacity to control peanut driven cytokine production synergistically through the IL-10R pathway and TGF-β.

As presented in Chapter II, both peanut allergic and clinically tolerant populations demonstrate the capacity to produce peanut driven Type 2 cytokine production. Whether this IL-10R- and TGF-β-dependant inhibition is dependent on the production of peanut driven cytokine production remains unclear. As shown in Table 10, IL-10R and TGF-β negatively regulate peanut driven Type 1, Type 2 and regulatory cytokine production even though only a fraction of these individuals demonstrate the capacity to produce these cytokines with peanut Ag stimulation. With the exception of IL-5, a larger percentage of the peanut allergic population’s peanut driven cytokine response is negatively regulated by IL-10R and TGF-β in comparison to those who are clinically tolerant. These results suggest detectable levels of peanut driven cytokine production is not necessary for IL-10R- and TGF-beta-dependant Type 1, Type 2 and regulatory cytokine inhibition in both peanut allergic and clinically tolerant populations.

**IDO negatively regulates peanut driven Type 2 cytokine production in peanut allergic populations**

In addition to IL-10 and TGF-β, recent data suggests that IDO may play a role in maintenance of tolerance (184) and has been shown to significantly correlate with
Table 10: Pre-existing peanut driven cytokine production is not necessary for IL-10- and TGF-β-dependant negative cytokine regulation. Of the subpopulation demonstrating >0% Change response, Peanut Ag Responders are defined as those individuals who also show a 2-fold or greater response to peanut in comparison to the medium alone (Peanut Ag responders/Responders to inhibitory treatment). Pn Ag – Peanut Ag.

<table>
<thead>
<tr>
<th></th>
<th>anti-IL-10 R mAb + Peanut Ag</th>
<th>anti-TGF-β mAb + Peanut Ag</th>
<th>anti-IL-10 R mAb + anti-TGF-β mAb + Peanut Ag</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Responses &gt;0% Change (%)</td>
<td>Peanut Ag responders</td>
<td>Responses &gt;0% Change (%) Peanut Ag responders</td>
</tr>
<tr>
<td>Hx-SPT-</td>
<td>Hx+SPT+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 40)</td>
<td>(n = 57)</td>
<td>60.0</td>
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<td>10/17</td>
</tr>
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<td>Hx+SPT+</td>
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<td>1/4</td>
</tr>
<tr>
<td>(n = 46)</td>
<td>(n = 44)</td>
<td>60.0</td>
<td>17/27</td>
</tr>
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<td>4/9</td>
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<td>(n = 44)</td>
<td>(n = 45)</td>
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<tr>
<td>Hx-SPT-</td>
<td>Hx+SPT+</td>
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<td>-</td>
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<tr>
<td>(n = 49)</td>
<td>(n = 49)</td>
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asymptomatic atopy vs. symptomatic atopy (185). These studies suggest that IDO may play a contributing role in the maintenance of clinical tolerance towards peanut. To examine this possibility, PBMC were stimulated with Peanut Ag alone or in the presence of an IDO-inhibitor, 1-methyl-D-tryptophan (1-MT). As shown in Figure 21, all peanut-driven cytokines analysed showed a significant increase when IDO was inhibited. These results suggest that peanut-driven cytokine production is in part, negatively regulated by IDO. In contrast, in clinically tolerant populations, peanut-driven Type 2 (IL-9 and IL-13) cytokine production did not show a significant increase with this inhibition of IDO, whereas Type 1 (IFNγ) and regulatory (IL-10) cytokines did. In the clinically tolerant population, median peanut-driven increases in IL-9, IL-13, IFNγ and IL-10 were 0%. The median response of peanut allergics was 1050% (IL-9), 170% (IL-13), 147% (IFNγ) and 154% (IL-10 production), respectively (Figure 22). PBMC stimulated with 1-MT in the absence of peanut Ag resulted in no significant change in any of the cytokines analyzed in both clinically tolerant and peanut allergic populations (data not shown). In addition, Table 11 demonstrates that IDO-dependant negative regulation occurs in individuals who do not necessarily normally demonstrate the capacity to produce peanut-driven cytokine production. Peanut allergic populations show a greater frequency of individuals within the population that have enhanced peanut-driven IL-9, IL-13, IFNγ and IL-10 production with the inhibition of IDO. These data allow us to conclude that IDO may play an important role in limiting peanut-driven Type 2 cytokine production in peanut allergic populations but that IDO is minimally involved in the maintenance of clinical tolerance towards peanut among non-allergic individuals.
Figure 21: IDO negatively regulates peanut driven cytokine production in peanut allergic populations. PBMC from peanut allergic individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of 1-MT (n=49). Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
Figure 22: IDO play no detectable role in negatively regulating peanut driven Type 2 cytokine production. PBMC from clinically tolerant individuals were stimulated with Peanut Ag alone or with Peanut Ag in the presence of 1-MT (n=49). Each point represents one individual and is expressed in % change from peanut Ag antigen stimulation alone. 0% change represents no change. Significance was determined using One sample and Wilcoxon Signed Rank t test.
Table 11: Pre-existing peanut driven cytokine production is not necessary for 1-MT-dependant negative cytokine regulation. Of the subpopulation demonstrating >0% Change response, Peanut Ag Responders are defined as those individuals who also show a 2-fold or greater response to peanut in comparison to the medium alone (Peanut Ag responders/Responders to inhibitory treatment). Pn Ag – Peanut Ag.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td></td>
<td>Responses &gt;0% Change (%)</td>
</tr>
<tr>
<td><strong>IL-9</strong></td>
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<tr>
<td>Hx-SPT- (n = 49)</td>
<td>34.7</td>
</tr>
<tr>
<td>Hx+SPT+ (n = 48)</td>
<td>42.9</td>
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</tbody>
</table>
Chapter Discussion

IL-10 is an immunoregulatory cytokine associated with the control of acute inflammation and tolerance (141,145,244). Altered expression of IL-10 has been implicated in several diseases (229,237,245), but its contribution to peanut allergy remained unstudied. In the present study we investigated the role of IL-10 in peanut allergy.

In opposition to our initial hypothesis, that elevated IL-10 production might underlie the clinical tolerance to peanut, peanut driven IL-10 protein production was not significantly increased in clinically tolerant individuals. As IL-10 was only enhanced in peanut allergic populations this may lead one to conclude that IL-10 is a Type 2 cytokine, and is associated with the manifestation of allergy, as this has been reported in several incidences (229). Our results show that this is not the case, and in fact, IL-10 is acting as a negative regulator of peanut driven cytokine production. These results are supported by evidence that ligation of B7 co-stimulatory (inhibitory) molecules increase IL-10 production (246), implying antigen driven, TCR-mediated T cell activation results in enhanced IL-10 production. As well, an abundance of evidence exists stating that IL-10 is able to negatively regulate cytokine production and is critical for oral tolerance (238,247), but these finding are not without disagreement (248). Reports have demonstrated an increase in peanut driven IL-10 in murine peanut allergy models during sensitization (72), and higher levels of IL-10 in the gut mucosa of food allergic children as compared to those who were healthy (249). Enhanced allergen driven IL-10 has also been demonstrated with nickel allergic individuals in comparison to healthy controls and (237). In addition, IL-10 mRNA expression has been found after allergen
challenge in patients with contact dermatitis and in severe asthmatics (229). It has also been suggested by Barnes et al. that IL-10 is a late breaking mechanism that occurs approximately 12-18 hrs. after antigen driven cytokine production (142). The significant correlation between peanut induced IL-10 and Type 2 cytokines suggests that IL-10 is consequently induced to counter the cytokines known to be important in the pathology of allergy. IL-10 production at early time points (ie <5d) is very low to undetectable (data not shown).

IL-10 is able to inhibit accessory cell function. In the presence of IL-10, T lymphocyte proliferation and cytokine production in response to allergen are inhibited. In some cases these effects render T cells irreversibly nonresponsive. Reports studying the effects of IL-10 on B-lymphocytes are controversial. IL-10 has also been shown to skew antibody production from immunoglobulin E (IgE) towards the non-inflammatory isotypes IgG4 and IgA (250). In contrast, IL-10’s effect on B cells have resulted in proliferation and Ig secretion (145) and IL-10 gene polymorphisms may be involved in the pathogenesis of food allergy (143). The reduction of Type 2 cytokines with rIL-10 treatment in peanut allergic individuals maintains the hypothesis that IL-10 is an immunoregulatory cytokine capable of inhibiting allergen driven Type 2 cytokines. In agreement, Minang et al. has shown that addition of rIL-10 resulted in a pleiotropic down-regulation of Type 2 cytokines in response to nickel (237).

To assess the relevance of the IL-10 and IL-10R signalling pathway, neutralisation of the IL-10R was performed concomitant with peanut allergen re-stimulation. This neutralisation resulted in enhanced peanut driven Type 2 cytokine production in peanut allergic populations. These results suggest that IL-10 actively
inhibits cytokines associated with peanut allergy. Interestingly, IL-10 showed a greater inhibition of IL-9 and IL-13 production than IL-5 and IFNγ. Thottingal et al. has shown that both peanut allergic and clinically tolerant populations produce Type 2 cytokines in responses to peanut, independent of Type 1 (IFNγ) production (6), and our results (Figure 14) suggest that, although more subtle than IL-9 and IL-13, active suppression of peanut driven Type 1 cytokine production is also occurring in peanut allergic individuals.

Throughout this study, clinically tolerant populations have consistently shown no association with elevated levels of peanut driven IL-10. Intriguingly, neutralisation of the IL-10R in clinically tolerant people resulted in enhanced IL-5 and IL-13 production, indicating differential regulation of Type 2 cytokines. Differential IL-13 enhancement between peanut allergic and clinically tolerant populations may also suggest that IL-13 is more strongly upregulated in peanut allergics and/or they are less sensitive to the immunosuppressive behaviour of IL-10 in terms of IL-13 production. Transcription of IL-5, but not IL-13, is controlled in part by a transcription factor, Gfi1 (80), which may partly explain differential effects of IL-10 inhibition.

Administration of TGF-β has been shown to enhance oral tolerance towards food Ag (161), but its direct role in regulating peanut driven cytokine production in peanut allergy has not been explored. In contrast to our initial hypothesis, we have shown that TGF-β plays a significant role in negatively regulating peanut driven cytokine production. This control of Ag driven cytokine production is also apparent in clinically tolerant populations, but to a lesser extent. This may be a result of TGF-β acting as a
braking mechanism rather than an early stage control of Type 2 cytokine production. As discussed in Chapter II, peanut allergic populations produce quantitatively greater levels of peanut driven Type 2 cytokines than those who are clinically tolerant. In human cultured bronchial epithelial cells Type 2 cytokines have been shown to induce TGF-β production in a dose dependant manner (251). Interestingly, we have shown that individuals that respond to anti-TGF-β mAb treatment with significant enhancement of peanut driven Type 2 cytokine production do not necessarily (normally) produce detectable levels of peanut driven cytokines. We have also provided evidence that TGF-β is not involved in limiting all peanut driven Type 2 cytokine production. Our suggestion of TGF-β’s limited role in the involvement in the maintenance of clinical tolerance is not without warrant. Chung et al. demonstrated that neutralisation of TGF-β does not prevent oral tolerance induction (252) and T cell derived TGF-β can inhibit both Treg and peripheral T cell proliferation, activation, and differentiation (162).

To date, no evidence exists demonstrating TGF-β's role in negatively regulating IL-10, but in reverse, IL-10 has been shown to negatively regulate TGF-β signalling molecules in human bronchial epithelial cells (253). In addition to our work, this study suggests that immunoregulatory cytokines, such as TGF-β, play an important role not only in controlling peanut allergy associated cytokine production, but in inhibiting other inhibitors of the peanut driven response.

The inhibition of IDO resulted in enhanced peanut driven Type 2 cytokine responses in peanut allergic populations but had little impact in the tolerant individuals. Although unexpected, these results are not entirely surprising. Mellor et al. demonstrated that enhanced IDO expression occurs with CTLA-4 Ig ligation in splenic
APC, suggesting that IDO expression is induced with T cell activation and subsequent regulatory events (199). Ligation of CD80/CD86 has also been shown to suspend downregulation of IDO (202). These data may help explain why peanut driven Type 2 cytokine production in the peanut allergic population was more greatly affected in comparison to the clinically tolerant population. As shown in Chapter II, both allergic and non-allergic populations produce Type 2 cytokines in response to peanut. Peanut allergic populations produce peanut driven Type 2 cytokines at a greater frequency, therefore the responding portion of this population is greater, and may show a greater response to IDO-driven inhibition than the clinically tolerant population. Similarly, with experiments examining asymptomatic atotics and symptomatic atotics, von Bubnoff et al. concluded that increased IDO activity during allergen is an attempt to control an unwanted response (185)

**Chapter Conclusion**

In conclusion, the activity of endogenous TGF-β or IDO appears to not be associated with the maintenance of clinical tolerance. The activity of endogenous interleukin-10/interleukin-10 receptor pathway and TGF-β are able to functionally inhibit the intensity of peanut driven Type 1 and Type 2 cytokine production and may play a role in controlling the extent of undesirable type 2 cytokine production that underlies peanut allergy. TGF-β and IL-10 synergistically negatively regulate peanut driven Type 1 and Type 2 cytokine responses. Thus, all three mechanisms are likely to contribute to the allergic individuals’ efforts to contain the intensity of undesirable peanut-specific cytokine responses. However, the key factors which are responsible for preventing
development of cytokine responses in clinically tolerant individuals remain to be determined.
General Thesis Discussion

What are peanut allergy diagnostic methods really telling us?

Peanut allergy is the leading cause of anaphylactic reactions in children (19). Peanuts are a common component of our diets because they are inexpensive to cultivate, yet are high in nutrients, protein and oil (1). When taking into consideration the risks of exposure in allergic individuals and the potentially hazardous outcomes of an allergic or anaphylactic reaction, the diagnosis of peanut allergy calls for methods that provide strong PPVs and NPVs. The current laboratory methods of diagnosis are able to provide such answers above high cut-off values, but this leaves a large percentage of the population in a questionable diagnostic state unless a clear history is present or an oral food challenge is performed. Our results showed that these high diagnostic cut-off values exclude 25-30 and 57% of the population for peanut Ag SPT and peanut specific IgE ImmunoCAP tests, respectively. When SPT results were compared to in vitro primary culture peanut driven cytokine production, no significant correlation was observed. SPT reagents are comprised of whole raw peanut (42), but as several studies have revealed, the methods used to prepare peanuts before they are consumed result in differential allergenicity. Roasting, a common practice in North America, results in higher levels of the major peanut allergen, Ara h 1, in comparison to levels found in boiled peanuts (220). In agreement, Beyer et al. using SDS PAGE demonstrated that frying peanuts reduced both the monomeric and trimeric forms of Ara h 1, whereas boiling peanut resulted in reduced IgE binding to Ara h 1, Ara h 2 and Ara h 3 in comparison to roasting (215). Roasting not only alters protein content but function as well. Ara h 2 functions as a trypsin inhibitor, preventing breakdown of itself and other
peanut allergens during digestion. This inhibitor function is enhanced 3-fold during the roasting process (216). These data suggest that roasting alters the allergenicity of peanuts through enhancement of protein content, IgE binding affinity, and protein function. Thus, the most common form of peanut consumed in North America has enhanced allergenicity properties. It remains unclear what drying methods are employed prior to the manufacturing of commercial peanut Ag SPT reagents. Although speculative, this may be a cause of a larger SPT diameter observed with internally prepared peanut Ag reagents (derived from peanut butter) in comparison to commercially available SPT reagents. Quantitatively greater type 2 cytokine response profiles were also observed using the internally prepared peanut Ag reagent. Collectively, these data suggest that utilizing a peanut Ag reagent for diagnosis of peanut sensitization and primary cultures that best represent the peanut as it is found in our diet and most likely to cause allergic reactions may provide results that are more representative of a peanut induced allergic reaction. By examining the relationship between SPT results and in vitro reactivity of PBMC to peanut, we may begin to clarify why our current methods of diagnosis present gray areas making definite diagnosis difficult.

The importance of biomarkers in disease diagnosis

Biomarker discovery for use in disease diagnosis has proven to be challenging. To identify a single molecule only associated with one disease state remains highly improbable, thus leading to the use of several diagnostic methods to confirm disease. In the case of hazelnut allergy, Alonso et al. identified a possible marker for clinical tolerance towards hazelnut, but elevated levels of IL-10 were identified only in a discrete
population of individuals who had outgrown an existing allergy towards hazelnut. Our attempt to identify IL-10 as a plasma biomarker of clinical tolerance towards peanut was unsuccessful. Plasma biomarker discovery has proven to be difficult for several reasons. Plasma contains proteins that span 10 orders of magnitude in concentration (254), with cytokines being some of the least abundant components (255). Cytokines located in plasma or sera are often found in their inactive form and can be considered "leakage markers." Furthermore, the relationship between plasma and the gut lymphatic tissue is not well understood (255). As identifying a single marker that is specific for only one disease may be difficult, methods employing an array of biomarkers have been researched. Li et al. demonstrated that five discriminatory biomarkers of irritable bowel disease were able to identify a disease state, subtype and severity (60). The possibility of using multiple cytokine or cellular excretion markers specific to peanut allergens may be a successful means of developing new diagnostic methods. With this said, a critical component of this development would be to expand our current understanding of the mechanism of peanut allergy, which we sought to do throughout Chapters III and IV.

Peanut driven cytokine production in peanut allergy

The realization that our current diagnostic methods do not correlate with peanut driven cytokine response profiles also addresses that there is much to be learned in our understanding of peanut allergy. It is therefore important to understand not only why peanut allergic populations mount a strong type 2 cytokine response in reaction to peanut, but why sensitized and clinically tolerant populations also exhibit the same capacity (although at quantitatively lower levels as a population) yet lack the symptoms
of allergy. In this series of studies we have examined the two distinct populations: peanut allergic and those who were non-allergic. We have found that both peanut allergic and non-allergic populations produce type 2 cytokines in response to peanut Ag. Upon peanut Ag stimulation approximately 40% of peanut non-allergic individuals, and 100% of allergic individuals, produce peanut driven type 2 cytokines. Evidence shows that CD28/CTLA-4-B7 signalling occurs in both populations (133). As peanut allergic populations produce quantitatively greater levels of peanut driven type 2 cytokines and at a high frequency than those able to ingest peanut, this may indicate that a certain threshold of type 2 cytokine production must be achieved to elicit an immune response characteristic of allergy. The caveat to this hypothesis is that while the median population response is clearly different, there is substantial overlap between the individuals within the groups. In several cases, highly allergic individuals exhibit weaker recall IL-5, IL-9, and IL-13 responses than do individuals who readily tolerate peanut. Another possibility that exists may be beyond our understanding of the downstream signalling events that occur through the release of type 2 cytokines. It is unknown if differential type 2 cytokine signalling occurs in clinically tolerant and peanut allergic populations. Early experiments suggest that soluble type 2 cytokine receptors may have antagonistic or even inhibitory properties. sIL-13RA2, when bound to IL-13, has a 4- to 5-fold increase in half life, and results in altered gene expression (126). Similarly, IL-5 is unable to carry out downstream signal transduction via the IL-5R unless the common β-chain is present (81). Thus, while the picture is clearer at the population level, at the individual (or diagnostic level) much remains to be learned.
As demonstrated in **Chapter II**, enhanced peanut driven IL-9 production is associated with peanut allergy. Although Umezu-Goto *et al.* has stated CD4+ T cells are the only detectable source of IL-9 in atopic asthmatics (84), others have demonstrated that eosinophils and neutrophils are detectable sources of IL-9 in allergy mouse models (83,85,86). Our experimental design excluded multinucleated cells, so it is uncertain if eosinophils or neutrophils may contribute to IL-9 production in peanut allergy. When considering the target cells of IL-9, cell types yet to be defined as contributors to peanut allergy may be implicated. B cells and eosinophils are both activated by IL-9 overproduction in transgenic mice (99), suggesting that eosinophils, as identified in one peanut allergic patient’s duodenum section, may play an important role in peanut allergy (77). IL-9 is capable of inducing IL-5 production (102) and is involved in OVA-induced anaphylaxis (97). IL-9 may be responsible for, or contribute to, these same biological activities in peanut allergy.

*The regulatory mechanisms underlying peanut allergy and clinical tolerance*

When exploring differential regulatory mechanisms underlying clinical tolerance and peanut allergy we are often faced with two major roadblocks: we have yet to identify the exact turning point at which a person develops an allergic response or oral tolerance and in those who are able to ingest: what maintains this tolerance? In an effort to determine if and how immunoregulatory mediators, such as IL-10, TGF-β, and IDO, are involved in the maintenance of clinical tolerance, we have surprisingly identified their involvement in limiting peanut-driven type 2 cytokine production, and perhaps allergy, rather than identifying a role for them in the maintenance of clinical tolerance among those able to ingest peanut. These results show that the body is still attempting to form
a means of regulation, even in a disease state. Here we have shown that expression of one of these immunoregulatory mediators, IL-10, is proportional to peanut driven type 2 cytokine production, suggesting that the production of IL-10 is a reaction to this type 2 event. Barnes et al. showed that IL-10 is a late braking mechanism that occurs approximately 12-18 hrs after LPS-dependant cytokine production (142). Our data suggests that endogenous IL-10 is functioning in a similar manner, limiting the intensity of peanut driven type 2 cytokine responses. It is also possible that IL-10 is acting to suppress anaphylaxis, as Kennedy Norton et al. showed that IL-10 overexpression in mice is capable of this (154). Whether humans with higher levels of peanut driven IL-10 production exhibit reduced risk of anaphylaxis is unclear, but given the non-significant differences in serum levels and the elevated levels of IL-10 seen in those most at risk of anaphylaxis (i.e. peanut allergic), this is difficult to support.

TGF-β is produced by almost all cell types (162) and can induce oral tolerance towards food Ag (161,162). Therefore, we hypothesized that enhanced peanut driven TGF-β production would be associated with clinical tolerance towards peanut. We were again surprised to discover that TGF-β appears to play only a limited role in maintaining clinical tolerance (as its functional neutralization led to minimal impact on the intensity of type 2 cytokine production. Rather, its neutralization led to substantive increase in type 2 cytokine production among allergic individuals, arguing that it predominantly acts to limit the intensity of (established) allergy.

The type 2 cytokine transcription factor, GATA-3, is significantly enhanced in mice incapable of signalling through the Smad-TGF-β inhibitory pathway (119). As reviewed by van Wijk et al. blockade of IL-10 nor TGF-β does not prevent oral tolerance
induction (135). Therefore, as with IL-10, TGF-β may be produced as a result of type 2 cytokine production in an effort to control the allergic response, but our evidence showed that not all individuals showing TGF-β-inhibition-dependant enhancement of cytokine production had detectable levels of these cytokines with the stimulation of peanut.

Thottingal et al. demonstrated that peanut non-allergic populations produce type 2 cytokines, independent of "protective" type 1 cytokine production, in response to peanut (6). Here we found that IL-10 and TGF-β synergistically negatively regulate peanut driven IFNγ production in clinically tolerant, and even more so in peanut allergic populations. These results suggest that not only does GATA-3 inhibit the IFNγ expression via STAT-4-dependant inhibition of T-bet, negative regulators of allergy also participate in this downregulation (256).

Fallerino et al. showed that T cell activation results in enhanced expression of IDO (203). Our results showed that IDO significantly controls the intensity of peanut driven type 2 cytokine production in peanut allergic populations. However, in contrast to our hypothesis and current thinking, the role of IDO in maintaining clinical tolerance was less convincing. As with IL-10 and TGF-β, the biological inhibitory activity of IDO is more strongly associated with controlling the intensity of responses in peanut allergic individuals. Collectively, the results regarding IL-10, TGF-β and IDO show us that the majority of individuals unable detectable type 2 responses exhibit that capacity when we neutralise these immunoregulatory molecules. While experimental systems of oral tolerance and allergy suggest there is an active suppression of Ag-driven cytokine production preventing Th2-like overexpressing in non-allergic individuals, these
particular immunoregulatory mediators show a limited role in maintaining clinical
tolerance towards peanut.

Several studies have focused on key subsets of regulatory cells in understanding
oral tolerance, but as van Wijk et al. describes, DC of the gut are not inherently tolerant,
but use their surrounding microenvironment to aide in a decision of how they will guide
T cell responses (135). Therefore, our newfound understanding of IL-9, IL-10, TGF-β,
and IDO in peanut allergy has contributed to our understanding of this
microenvironment.

Global Conclusion

In an effort to improve our understanding of peanut allergy we have presented
work involving 247 human participants. This study population provides a new
understanding that SPT responses do not represent in vitro cytokine profiles generated
in response to peanut, the association of enhanced peanut driven IL-9 in peanut allergy,
and regulatory mechanisms underlying peanut allergy. By using internally prepared
peanut Ag produced from a common source of peanut in our diets we have
demonstrated cellular responses that may better represent peanut allergic reactions
outside of the laboratory.
Global Summary

Chapter I: In vitro and in vivo characterization of peanut allergy diagnostic reagents: Commercially available SPT reagents show dramatic protein concentration differences. Peanut Ag SPT results do not correlate with peanut driven cytokine response profiles generated in primary cultures.

Chapter II: Are serum IL-10 levels a useful marker of clinical tolerance in peanut allergy?: IL-10 is not a useful marker of clinical tolerance.

Chapter III - Cytokine Responses in Peanut Allergy: Enhanced peanut driven IL-9 is associated with peanut allergy. Both clinically tolerant and peanut sensitized populations demonstrate the capacity to produce elevated peanut specific type 2 cytokines.

Chapter IV - Clinical tolerance and the regulation of peanut allergy

Introduction: IL-10, TGF-β and IDO do not show a convincing role in maintaining clinical tolerance towards peanut. Instead, they may play an important role in limiting peanut allergy.
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