CHARACTERIZATION AND IMMUNE REGULATION OF PEANUT-SPECIFIC CYTOKINE AND CHEMOKINE RESPONSES IN PEANUT NON-ALLERGIC HUMANS

BY

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In Partial Fulfillment of the Requirements for the Degree of:

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3 Dedication

First and foremost, I would like to thank my supervisor, Kent HayGlass for helping me realize and develop my potential in research. For his encouragement in the best and worst of times and continued support. The chicken curry recipe, I swear, is coming.

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The candidate, Tina B. Thottingal, performed all of the experiments contained within this thesis (other than those mentioned above).

5 Abstract

Peanut allergy is an increasingly important concern that may lead to severe or potentially fatal symptoms. It most often develops in childhood and usually persists into adulthood with ~15% of individuals developing tolerance to peanut.

We developed a novel system using short-term primary culture of peripheral blood mononuclear cells, to define immunoregulatory mechanisms of the peanut-specific immune response of peanut *non-allergic* individuals. Participants included: A) Skin Prick Test Negative (SPT-) to peanut and B) Skin Prick Test Positive (SPT+): sensitized to peanut, yet asymptomatic to peanut ingestion. Cells were stimulated with whole peanut extract (WPE) or *Ara h1*/*Ara h2* and neutralizing antibodies against essential co-stimulatory molecules and endogenously produced cytokines. Th1/Th2 cytokine were subsequently measured responses by ELISA.

The data shows that the response of non-allergic humans to WPE is defined predominantly by production of type 2 cytokines IL-5, IL-13 and CCL22, with type 1 cytokine production (IFNγ, CXCL10) measured at levels similar to background. When comparing SPT- versus SPT+ populations we found that IL-5, IL-13 and CCL22 responses were substantially more intense ($p<0.04$ to $p<0.0008$) and frequent ($p=0.04$ to 0.002) in the latter. These non-allergic individuals demonstrate a clear *Ara h1* response (IL-13 and CCL22) but no detectable *Ara*

h2-specific response. No allergen-specific type 1 response (IFNγ) was observed in either group.

Type 2 peanut-specific cytokine recall responses are dependent upon T cell recognition of antigen in the context of MHC class II. We subsequently demonstrated co-stimulatory requirements of peanut-specific responses by in SPT- and SPT+ groups. SPT+ individuals depend solely on CD86 for type 2 peanut-specific cytokine production, and are CD80 independent. Furthermore, endogenously produced immunosuppressive cytokines IL-10 and TGFβ have the ability to regulate a type 1 (IFNγ) but not a type 2 (IL-5 and IL-13) response following WPE stimulation in SPT- individuals.

Collectively, these data clearly demonstrate that peanut-specific type 2 cytokine and chemokine responses are frequent among non-allergic individuals and that differential regulation of this response through CD80 and CD86 may be fundamental in the determination of clinical "sensitivity" vs clinical tolerance to peanut.

6 Abbreviations

Alpha	α
Antigen Presenting Cell	APC
Arachis Hypogaea (1-3)	*Ara h* (1-3)
B Lymphocyte	B cell
Beta	β
Bovine serum albumin	BSA
Cytotoxic T lymphocyte associated antigen-4	CTLA-4
Delta	δ
Deoxyribonucleic acid	DNA
Dermatophagoides pteronyssinus 1	*Der p1*
Double-blind placebo-controlled food challenge	DBPCFC
Enzyme Linked Immunosorbant Assay	ELISA
Fluorescein isothiocyanate	FITC
Food allergy herbal formula 1	FAHF-1
Gamma	γ
Gata binding protein-3	GATA-3
Human Lymphocyte Antigen	HLA
Immunoglubulin (D,M,G,E,A)	Ig (D,M,G,E,A)
Inducibile Co-stimulator	ICOS
Interferon	IFN
Interleukin (1-13)	IL-(1-13)
Kilodalton	kDa

Lipopolysaccharide	LPS
Ligand of ICOS	LICOS
Major Histocompatibility	MHC
Messenger ribonucleic acid	mRNA
Microgram	μg
Nanogram	ng
Natural Killer Cells	NK Cells
Natural Killer T Cells	NKT Cells
Oral allergy syndrome	OAS
Peripheral blood mononuclear cell	PBMC
Phosphate buffered saline	PBS
Picogram	pg
R- phycoerythrin	R-PE
Signal Transducing Activator of Transcription (1-6)	STAT-(1-6)
Single Nucleotide Polymorphisms	SNPs
Skin Prick Test (+/ -)	SPT (+/-)
Sodium azide	NaN_3
Sodium cholride	NaCl
Standard Error of the Mean	SEM
Streptokinase	SK
T Cell Receptor	TcR
T Helper Cell (0-3)	Th
T Lymphocyte	T Cell

T Regulatory 1 cells	T_r1
T-box Expressed in T cells	T-bet
Transforming Growth Factor	TGF
Tumor Necrosis Factor	TNF
Units	U
Whole Peanut Extract	WPE
Zeta	ζ

7 Introduction

7.1 Allergy: History and Prevalence

ALLERGY: ***An undesirable immunological reaction to common and normally innocuous environmental allergens***.

The term "allergy", introduced almost one hundred years ago by von Pirquet, is defined as a "changed reactivity" that can either be protective (immunity) or harmful (allergic response) (1). For almost 200 years, efforts have been made to further clarify the processes of these extremely complex responses.

Allergic disease is on the rise, affecting up to 40% of people all over the world (2). Those suffering from allergic disease often experience a decreased quality of life as a result of physical or emotional impairment during everyday life activities such as working, going to school, and attending social activities. In addition, billions of dollars are spent every year on the treatment of allergic disease (3, 4).

Key mediators of the immune system, which contribute to the development and maintenance of allergic disease will be reviewed including a discussion of current hypotheses and the genetic and environmental factors postulated to explain mechanisms of allergy.

7.2 Processes of Allergy

Communication between cells of the immune system is essential in protecting the body against harmful agents and maintaining a healthy immune system. This section will review the basic immunological processes leading to allergic reactions.

7.2.1. Antigen Presenting Cells and B Cells

Professional antigen presenting cells (APCs) include dendritic cells, macrophages and B cells. They share the function of processing and presenting antigen in the context of major histocompatibility (MHC) molecules (human leukocyte antigen (HLA) in humans) to T cells. In contrast to macrophages and dendritic cells, which phagocytose antigen non-specifically, B cell uptake of antigen occurs through an antigen-specific membrane IgM receptor. MHC class I molecules, displayed on all nucleated cells, are complexed with peptides from processed endogenous antigens such as proteins from intracellular pathogens including bacteria and viruses. MHC class II molecules are complexed with processed exogenous peptides from foreign antigens such as allergens, proteins from extracellular pathogens or released viral products, and are displayed on the surface of professional APCs.

7.2.2 $CD4^+$ and $CD8^+$ T Cells

T cells play an integral role in primary immunological responses to foreign and pathogenic antigens, as well as in the maintenance of memory responses should the immune system re-encounter antigen. T cells are characterized as either $\alpha\beta$ or $\gamma\delta$ based on the combination of Ig-like polypeptide chains which make-up their T cell receptor (TcR). Each $\alpha\beta$ TcR has an antigen specificity thus, demonstrating its' diversity in comparison to the $\gamma\delta$ TcR which is less variable and more restricted recognizing conserved motifs identifying pathogenic infection. The α and β chains of the TcR are associated with the invariant accessory chains in the CD3 complex: CD3γ, CD3δ, CD3ε and CD3ζ chains. Specific subsets of $\alpha\beta$ T cells are further distinguished based on cell surface expression of co-receptors CD4 and CD8 that bind the different classes of MHC. Specifically, $CD4^+$ T cells recognize exogenous peptide-MHC class II complexes and $CD8^+$ T cells recognize endogenous peptide-MHC class I complexes (5, 6).

7.2.3 Co-Stimulatory Requirements for T Cell and APC Activation

Activation of antigen-specific T cells requires co-stimulation provided by interaction with molecules on APCs. B cells, dendritic cells, and macrophages require co-stimulation signals provided in turn by T helper cells or other sources (pathogen associated molecular patterns such as lipopolysaccharide (LPS)) to become activated. Several interactions are essential. As previously mentioned, the initial activation signal occurs when the TcR/CD3 complex and CD4 or CD8

co-receptor bind the antigen-peptide MHC complex (5, 6). The best characterized co-stimulatory pathway is that of CD28 or cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) on T cells ligating B7 family members, CD80 and CD86, on APCs. Other potential T cell/APC co-stimulatory pairs are 4-IBB/4IBBL and inducible co-stimulator (ICOS)/ligand of ICOS (LICOS) (7, 8). Various cell surface proteins become up regulated and contribute to signals that further clonal expansion and differentiation. One such protein is CD40 on APCs, which binds CD40-ligand on T cells. CD40 ligation signals the beginning of B cell differentiation, antibody isotype switching, and is important in triggering IL-12 production from dendritic cells and macrophages (9) (6).

7.2.5 Type 1 Immediate Hypersensitivity

When a normally innocuous antigen is presented through MHC class II on APC and is recognized by a T cell, an allergic reaction can ensue. Allergic reactions are characterized by the production of specific IgE to the offending protein. IL-4 cytokine production by antigen-specific T helper cells causes B cells to divide and become plasma cells that release antigen-specific IgE into the circulation. IgE binds $F_c\varepsilon RI$ receptors on mast cells and basophils. Cross-linking of $F_c\varepsilon RI$ bound IgE by circulating antigen, during subsequent exposures, results in the degranulation of mast cells and basophils. These cells release preformed mediators such as histamine, prostaglandins and leukotrienes resulting in increased itching, vascular permeability, swelling, smooth muscle contraction and loss in blood pressure (as reviewed by 10).

7.3 T Helper Cells: The Type 1/Type 2 Hypothesis

Upon activation, naive T cells undergo differentiation into effector cells that direct the immune response. When naive T cells recognize antigen peptide complexed with MHC, they begin to secrete IL-2, driving clonal proliferation and differentiation into effector T cells. It was found that naive $CD4^+$ T cells can develop into one of two subsets based upon the cytokine profile produced following stimulation. These subsets are termed Th1 or Th2 cells and give rise to the Th1/Th2 hypothesis (11, 12). Thereafter, it was found that $CD8^+$ T cells, natural killer cells (NK cells), dendritic cells and B cells could also be divided into two subsets based on cytokine production (13-16). The Th1/Th2 hypothesis was subsequently referred to as the Type 1/Type 2 hypothesis to acknowledge that a variety of cells contribute to the development and expression of the immune response. Cytokines produced by each subset also inhibits both the development and function of the reciprocal subset. For example, IFNγ production by Th1 cells inhibits the development and production of cytokines from Th2 cells and IL-4 exerts the same effects on responses from type 1 cells (17).

It was originally postulated that Th1 and Th2 cells were derived from two distinct cellular lineages, but studies have since demonstrated that they are derived from the same T helper cell precursor under the influence of genetic and

environmental factors (17, 18). The strength of the TcR and MHC/peptide complex interaction has been postulated to affect lineage commitment of naive T cells (19, 20). Additionally, signaling through APC co-stimulatory molecules, such as B7 members CD80 and CD86, have been shown to differentially induce Th1 or Th2 responses through CD28 (21, 22). At present, the most clearly defined factors contributing to the development of Th1 or Th2 cells include the cytokine milieu and the transcription factors, induced in the early stages of T cell differentiation. The following section will discuss these factors that contribute to the development of each Th subset.

7.3.1 Th1 Differentiation

As the major responders to infection by pathogens, macrophages and dendritic cells are the primary sources of IL-12, the chief cytokine influencing the development of the Th1 cell phenotype (23, 24). IL-12 acting through the signal transducing activator of transcription-4 (STAT-4) signaling pathway, results in increased levels of RNA and protein levels of T box expressed in T cells (T-bet), a T box transcription factor which controls the expression of the Th1 cytokine IFNγ (25). Furthermore, IFNγ acting through STAT-1 signaling also up-regulates T-bet expression (26). IFNγ, produced by T cells and NK cells, acts in a positive feedback loop regulating the production of IL-12 by macrophages (24).

Type 1 lymphocytes also demonstrate distinct chemokine receptor expression including CXCR3 and CCR5 (27). CXCR3 ligands, including type 1 chemokines

CXCL9 and CXCL10 produced by a variety of cell types in response to IFNγ, are important in the recruitment of Th1 cells to the inflammatory site (28-31).

The Th1 subset is most often associated with the term cellular immunity activating macrophages and cytotoxic cells that participate in the clearance of invading pathogens. IFNγ, in concert with other cytokines such as IL-2 and TNFα, induce B cells to produce opsinizing and complement fixing antibodies directed against pathogenic proteins and infected cells (32). Although, antigen that is not removed quickly results in dangerously chronic type 1 responses as demonstrated in some autoimmune diseases (33). IFNγ can regulate type 2 responses by indirectly inhibiting antibody switching to IgE responses (34). Additionally, type 1 responses to environmental allergens have also been characterized as non-allergic or clinically tolerant responses (35).

7.3.2 Th2 Differentiation

Naïve T cells activated through their TcR, in the presence of IL-4, results in the development of Th2 type cells (36, 37). T cell binding of IL-4 initiates signaling through STAT-6, mediating the induction of the transcription factor GATA binding protein-3 (GATA-3). GATA-3 expression is required for the production of all Th2 cytokines (38, 39). The origin of initial IL-4 production is still unclear, although possible sources are subsets of T cells such as NKT cells, MHC class II restricted $CD4^+$ T cells or cells such as eosinophils, mast cells and basophils

(17). Interestingly, IL-4 can dominate the development of effector cells if present at threshold levels despite the presence of type 1 cytokines (23, 37).

The type 2 response has been associated with humoral immunity, the production of antigen-specific antibody to environmental antigens, and protection from helminth infections. T cells that differentiate in the presence of IL-4 are capable of producing cytokines such as IL-5 and IL-13, which are important in type 2 responses. IL-4 is important in the development of B cells and the induction of antibody isotype switching to IgE production characteristic of allergic disorders. IL-13, initially thought to be functionally redundant with IL-4, also induces IgE production, plays a role in the expulsion of worms in parasitic infections and is a key mediator in allergic inflammation (40). IL-5 has been shown to affect the growth and activities of eosinophils having roles in both allergic inflammation and protection against parasitic infections (41).

Th2 cells found in allergic disorders, such as airway inflammation, also express distinct chemokine receptors, which include CCR3, CCR4 and CCR8. Cells involved in allergic inflammation expressing receptors for chemokines such as CCL11, CCL17, CCL22 suggest their role in the pathogenesis of allergic disorders (31).

7.3.3 Limitations of the Type 1/Type 2 Hypothesis

The Type 1/Type 2 hypothesis in both mouse and human has provided a basis for the definition of the factors that contribute to the development of an inflammatory or humoral response. Since then, Th1 and Th2 profiles have been used to characterize a number of atopic and autoimmune disorders but no causal relationship between type of response and disorder type can be absolutely defined based on this system. Each disorder must be investigated individually and may be a combination of both Th1 and Th2 responses. Roles played by other cell types, such as Th0 and T regulatory cells and immunoregulatory cytokines IL-10 and TGFβ add to the complexity of the regulation of potential immune responses.

7.3.4 Th0 Cells

Th0 cells are $CD4^+$ T cells or clones, which are capable of producing both Th1 and Th2 cytokine responses (42, 43). Conceivably, having a balance of both cell-mediated and humoral immunity may contribute to the efficient elimination of harmful pathogens without damage to the host. Whether Th0 cells are precursors to Th1, Th2 cells and T regulatory cells remains to be elucidated (18, 44, 45).

7.3.5 T Regulatory Cells

Several subsets of regulatory T cells have been studied that demonstrate suppressive effects on immune responses. Th3, T_r1 and $CD25^+$ $CD4^+$ T cells

have been studied extensively in the processes of oral tolerance and in the context of suppressing autoimmune disorders. Factors including the dose and frequency of antigen feeding, presence of adequate co-stimulation, and the role of IL-10 and TGFβ in the development of T regulatory cells will be further discussed under the section of *Mechanisms of Oral Tolerance.*

Th3 cells, defined by their capacity to secrete only TGFβ, have been implicated as key players in the maintenance of oral tolerance to antigens by contributing to IgA production (45-47). **T_r1 cells** do not proliferate or secrete IL-2 but display a cytokine profile consisting of both IL-10 and TGFβ. It has been found that IL-10 and IFNα, rather than TGFβ, are required for the development of T_r1 cells (48). T_r1 cells have been shown to play a role in the down regulation of immune responses in allergic disease and autoimmune disorders. Alternatively, T_r1 cells induced by bacteria and viruses can contribute to the pathogenesis of infectious disease by suppressing the natural protective immune response (49, 50). Naturally occurring **$CD25^+CD4^+$ T cells**, similar to T_r1 cells, produce IL-10 and TGFβ. These cells do not produce IL-2 and thus, do not proliferate (51). The role of IL-10 and TGFβ in mediating suppressive effects by these cells remains to be clarified. *In vivo* studies indicate that both cytokines are required for suppression whereas *in vitro* studies demonstrate IL-10/TGFβ independent suppression (52). Mechanisms of suppression are potentially mediated by cell to cell contact and TGFβ production through ligation of CTLA-4 (53, 54). The role of any of these cell types in human immune regulation is controversial.

7.4.3 Role of IL-10 and TGFβ in Immunoregulation

The cytokines IL-10 and TGFβ are pleiotropic in function. They have the ability to either enhance or inhibit the growth, activation and differentiation of various types of immune cells and thus play a potentially important role in controlling the immune response to foreign, self, and pathogenic antigens.

IL-10 is produced by a variety of cells and can down-regulate production of pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, CCL4, IL-12 and CXCL8 by macrophages and monocytes (34, 55). IL-10 down-regulation of co-stimulatory molecules CD80 and CD86, as well as MHC class II expression, results in decreased efficiency of antigen presentation by APC and their ability to activate T cells (50, 55). IL-10 -/- mice demonstrate chronic colitis characterized by a strong Th1 response (56). In addition to inhibiting Th1 responses, IL-10 down-regulates Th2 responses associated with the allergic response. It promotes B cell differentiation and acts as a switch factor for IgG isotypes (IgG_4 in humans), inhibiting IL-4 induced IgE synthesis. IL-10 can decrease mast cell and eosinophil numbers and the release of mediators (57-59).

TGFβ is shown to have both immunostimulatory and inhibitory activities. It limits T cell proliferation through the inhibition of IL-2 synthesis, affects cell cycle progression and prevents Th1/Th2 cell differentiation (60-62). Like IL-10, TGFβ can down-regulate MHC class II on APCs (50) indirectly inhibiting T cell

responses. The production of TGFβ triggered by the ligation of co-stimulatory molecule CTLA-4 may potentially explain the suppression of immune responses associated with CTLA-4 (63). This cytokine also serves a protective effect by helping maintain the integrity and function of the epithelial barrier (64). In combination with IL-10, TGFβ serves as a switch factor for IgA isotypes thus preventing the production of IgE (55). IgA secreted from B cells plays a role in blocking transport of microbes and antigens into the body returning them to the intestinal lumen (65). Other immunostimulatory effects of TGFβ include promoting cell growth of fibroblasts, enhancing naive T cell growth and differentiation of dendritic cells (63).

7.4 Potential Factors Involved in the Development of Allergic Disease

7.4.1 Genetic Factors

Studies on monozygotic and dizygotic twins clearly demonstrate the contribution of genetics to the development of allergic disease. Monozygotic twins show higher concordance rates of developing an allergic disorder than dizygotic twins (66, 67). Genetic predisposition for atopy, based on parental allergic status, increases the risk of development of allergic disease. Notwithstanding this, it has been demonstrated that children who exhibit atopic symptoms in early life do not necessarily belong to groups deemed to be at high risk of developing allergic disease. This indicates the involvement of other non-genetic risk factors (68).

Studies to date have determined that genetic factors contributing to the development of allergic disease are numerous and most likely characterized by multi-gene profiles. Several candidate genes are linked with the development of allergic disease including a large section of chromosome 5, which encodes several mediators associated with allergic disease such as IL-4, IL-5, IL-13, IL-12 and IL-9 (69, 70). Additionally, gene variation in the form of single nucleotide polymorphisms (SNPs), have been linked with the development of asthma and allergic disease (71, 72). The tremendous variability of the human genome highlights the importance of the study of gene polymorphism associated with the development of allergy. Now that a draft of the human genome has been mapped, further intensive study will be beneficial in determining possible markers of allergic disease. It is important to remember that genetic influences cannot be considered the sole determinant in the pathogenesis of allergic disease and that environmental factors are considered to play a significant, if not major role.

7.4.2 Environmental Factors

The Hygiene Hypothesis and Related Proposals

Strachan proposed that westernized lifestyle characterized by declining family size and higher standards of household and personal cleanliness has contributed to the rise in allergic disease (73). The hygiene hypothesis suggests that infections in early childhood reduce the risk of developing allergic disease. Bacterial and viral infections induce type 1 cytokine and antibody response

patterns, which can potentially suppress type 2 responses involved in IgE-mediated responses associated with allergic reactions. Studies focusing on differences in gut flora colonization and urban versus rural living have provided information in accordance with the hypothesis that may account for the increase in allergy (74, 75). Several studies show an inverse relationship between exposure to endotoxin or hepatitis A virus through the oral fecal route and allergic disease (75, 76). Reports indicating the opposing relationship have been similarly documented when examining pertussis infection and other childhood infections (77-79).

Other disease states are not fully explained by the Th1/Th2 dichotomy and the effects of infections on the development of allergy. Parasitic helminth infection results in a dominant Th2 immune response sharing similar characteristics with allergic disease (80). Studies of populations with helminth infections in developing countries show that despite exposure to high concentrations of common allergens, skin test positivity was likely to be lower in comparison to those with light or no helminth infection (81). The "IgE blocking hypothesis" postulates that high levels of polyclonal IgE produced in response to helminth infection may bind IgE receptors on mast cells resulting in decreased bound antigen-specific IgE. The chances of IgE receptor cross-linking decreases, inhibiting mast cell degranulation and thus immediate hypersensitivity results (82, 83). Studies show that allergen sensitization of patients with high polyclonal IgE was not possible (84) although other studies find no relationship between

helminth infection and atopy (85). Those with chronic helminth disease demonstrate high levels of specific IgG_4. A possible explanation for the observed decrease in allergic sensitivity in those with chronic helminth infections has been attributed to the role that IgG_4 plays in competing with allergen binding to IgE. However, IgG_4 is functionally limited, being monovalent, unable to fix complement and has a weak affinity for their receptor Fcγ. Therefore, although filarial-specific, the resulting immunological consequences are not harmful (86). Alternatively, IL-13 responses contribute to susceptibility and infection to *Leishmaniasis mexicana* (87).

The hygiene hypothesis also fails to describe the association of the increased prevalence of autoimmune disease with an increase in allergic disease (88). The presence of immunosuppressive cytokine IL-10, whether due to helminth/bacterial infection or produced by T regulatory cells, down-regulates proliferative and cytokine responses. Suppression of these responses may prevent the development of allergic disease and autoimmune disease (50, 85, 89). Although the hypothesis has been useful in defining trends of allergic disease study of other potential factors contributing to this immunological framework are required for future research and therapy.

Allergen Exposure

Several studies examining cord blood cell immune responses to mitogens and allergens, including inhalant and food allergens, suggest that allergen

sensitization may occur *in utero* (90, 91). Although mechanisms underlying *in utero* sensitization have not been confirmed and this field remains highly controversial, allergen has been measured in amniotic fluid and cord blood (92). The development of allergy is most likely to occur within the first years of life, during the maturation of the immune system. Allergens measured in amniotic fluid and the passage of allergen through the placental wall may allow intrauterine sensitization (93). Breastfeeding is used as a preventative strategy, it has been suggested that allergen in breast milk contributes to the development of allergies (94) and thus, variation in maternal diet during pregnancy has been recommended. Studies on sensitization to indoor allergens, such as house dust mite and animal dander, and the subsequent development of asthma remains to be clarified (95-97). Another source of sensitizing food protein, and a major concern of already food-allergic individuals, is accidental ingestion of improperly labeled food products.

While these studies indicate that many aspects contribute to the development and maintenance of allergic disease, further study of both genetic and environmental factors is required to better our understanding of this extremely complex disorder.

7.5 Food Allergy

7.5.1 Prevalence and Demographic

Food allergy is most commonly experienced within the first few years of life. It affects between 2-8% of infants and young children approximately 1-2% of adults of developed countries (98). The most common foods children are allergic to are cow's milk, eggs, peanut, soybean, fish, wheat and tree nuts while adults are most often allergic to peanuts, tree nuts, fish and shellfish (99). Allergies to foods developed early in life are most often outgrown by the age of five. Longitudinal studies show that up to 80% of egg allergic children become tolerant to egg by the age of five and 85% of milk allergic children can have cow's milk by the age of three (100). In contrast, children experiencing severe allergy to peanut rarely become tolerant although, studies examining those experiencing mild to moderate peanut allergy report that between 10 and 20% may outgrow their (100, 101)

Variations in food preparation or consumption, due to different cultural and environmental influences, may account for the differences in common food allergies frequently seen in different areas of the world (102-104).

7.5.2 Food Allergens

Various characteristics of food proteins make them potentially immunogenic allergens with the ability to cause severe systemic allergic reactions. Food allergens are low molecular weight glycoproteins (<70kDa) with acidic isoelectric

points. Heating of fruits and vegetables can result in reduced protein allergenicity through protein/peptide degradation. Alternatively, increased allergenicity by heating can result from the formation of new bonds which contribute to the protein's stability as is the case with the roasting process of peanuts (105). Food allergens are resistant to proteolysis and the processes of denaturation that occur in the digestive and intestinal tract. Thus, intact immunogenic peptides are allowed to pass through the intestinal mucosa into circulation causing local or systemic allergic reactions (106, 107).

Not surprisingly, the possibility exists of being allergic to a genetically similar food or environmental antigens. For example, those allergic to shrimp may also become allergic upon the ingestion of other crustaceans and those allergic to cow's milk may have allergic reactions to goat's milk (99). Similarity between plant and pollen allergenic epitopes can result in clinical allergy, known as the oral allergy syndrome (OAS) due to cross-reactive IgE antibodies (108).

7.5.3 Pathogenesis of Food Allergy

Protein Breakdown in the Digestive System

As previously mentioned, many food allergens are stable at low pH and resist cleavage by enzymes in the stomach and pancreas. Protein breakdown begins in the stomach with pepsin cleaving long peptide chains into shorter fragments. Precursor enzymes, trypsinogen and chymotrypsinogen, are released from the

pancreas into the duodenum of the small intestine and converted to trypsin and chymotrypsin at low pH. These proteases further cleave digested protein into smaller fragments for absorption. Typically, food allergens are resistant to these three main proteases resulting in peptide fragments that retain immunogenic sequences (106, 107).

Antigen Absorption in the Small Intestine

Consisting of one layer of epithelial cells, the intestine must be able to prevent the passage of possible antigens and pathogens, as well as absorb dietary nutrients during the digestive process. Dietary nutrients are absorbed through the microvilli on enterocytes facing the intestinal lumen. Soluble proteins and microbes cross the epithelial barrier by vesicular transport from the lumen via M cells which deliver antigen to APC's in the Peyer's Patches (aggregations of lymphoid follicles) located in the gut-associated lymphoid tissues (GALT) (109). A specialized dendritic cell has also been implicated in transport of antigen across the intestinal epithelial layer by extending dendrite-like processes through tight junctions directly into the lumen (110).

Although the majority of food antigens ingested are broken down, about 2% are absorbed through the mature adult gut and retain the ability to cause systemic immune responses. It is thought that the immaturity of the infant gut reduces the efficiency of the intestinal barrier resulting in increased gastrointestinal infections and food allergy that is observed in the first years of life (111).

7.5.4 Mechanisms of Oral Tolerance

Although the majority of dietary protein becomes degraded after passage through the digestive tract, intact food antigen can still cross the intestinal barrier into the GALT and the circulation. The GALT consists of Peyer's patches which are lymphoid nodules containing villus epithelial cells, intraepithelial lymphocytes, B cells, macrophages, dendritic cells and T cells. These components work together to produce an immunosuppressive environment limiting cellular and humoral hypersensitivity and inducing tolerance to oral allergens and bacterial products from mucosal flora (45). Three potential mechanisms of oral tolerance include anergy, deletion and the development of T regulatory cells.

7.5.5 Factors Determining the Development of Oral Tolerance

It has been shown in various experimental systems that T_r1, Th3 and $CD25^+CD4^+$ cells can be induced after oral administration of antigen. Several factors have been implicated in defining the type of tolerance that may develop, although the mechanisms underlying the development of tolerance are still unclear, despite decades of study.

One of the main factors determining the development of T regulatory cells is dose and frequency of feeding. Studies show that multiple low-dose or a single high dose feeding can result in the deletion of cells or the development of anergic

T cells. On the other hand, a single low dose feeding results in the development of suppressive or regulatory responses by T cells (45, 112).

Antigen presentation under certain co-stimulatory conditions can also determine the form of tolerance induced to ingested antigens. Resting APC express low levels of surface co-stimulatory molecules. In the absence of inflammation or adequate co-stimulation, antigen presentation alone fails to activate effector or helper T cell development which results in non-responsive T cells (46, 113). APCs expressing high levels of co-stimulatory molecules can efficiently activate cytokine production thus influencing the development of resting T cells into either Th0, Th1, Th2 or T regulatory cells (46, 114).

7.5.6 Role of IL-10 and TGFβ in Food Allergy

Much attention has focused on the role of immunosuppressive cytokines IL-10 and TGFβ in the development of regulatory T cells. Up-regulation of basal levels of IL-4, IL-10 and TGFβ have been measured in the domes of Peyer's patches after oral administration of antigen (115). This modified Th2 profile is thought to facilitate the development of regulatory T cells (109, 116). Antigen presentation in the presence of IL-10 results in anergic T cells that retain the ability to secrete immunosuppressive cytokines IL-10 and TGFβ after repeated stimulation with αCD3 (117, 118). Much of the research focusing on the suppressive effects of IL-10 and TGFβ have been in autoimmunity and allergy although, there are a few studies concerning food allergy.

As previously mentioned, genetic predisposition based on parental history of atopy, particularly maternal status, can increase the risk of developing allergic disease (119). Several studies have been carried out examining the levels of IL-10 and TGFβ in breast milk in addition to other potential mediators acquired from the mother such as IL-4, TNFα and IFNγ that may contribute to the initiation or suppression of allergic responses. By comparing the allergic status of the mother with the production of these cytokines, Laiho *et al* conclude that TGFβ levels were lower in mothers with a history of allergic disease. It is postulated that this deficiency in maternal breast milk TGFβ levels may interfere with the development of protective mucosal tolerance in the breast-fed child (120). In contrast, IL-10 and TGFβ responses specific to cow's milk allergens of memory T cells from cord blood of infants of atopic mothers show higher IL-4 and TGFβ responses in comparison to infants of non-atopic mothers with no difference in IL-10 response (121). Hauer *et al* suggests that an increase in these cytokines provide a Th2 skewing environment to dietary antigens.

Studies comparing young children with IgE mediated egg allergy at different ages in comparison to those who had successfully outgrown their allergy (based on clinical history and skin prick testing) and non-allergic children show different PBMC cytokine profiles after *in vitro* stimulation with egg allergen. Egg-allergic children less than 18 months old demonstrate a Th0 profile characterized by IL-5 and IL-13 PBMC responses with detectable IFNγ and IL-10 responses after egg

ovalbumin (OVA) stimulation. When examining children between the ages of 18 months and 3 years, more intense OVA-specific IL-5 and IL-13 responses in response to OVA were observed in comparison to levels from children between the ages of 3 and 5. No change in detectable IL-10 or IFNγ was observed. Children who had outgrown their egg allergy showed undetectable OVA stimulated IL-5 responses and low IL-13 responses but show strong IL-10 and IFNγ responses to OVA. The non-allergic group showed strong IL-10 responses and very low IFNγ and Th2 responses to OVA (122). These data suggest that as egg allergy is outgrown, the Th0 cytokine profile change to a dominant Th1 or immunosuppressive cytokine profile plays a potentially protective role in the regulation of responses to egg.

$CD25^+CD4^+$ T cells are naturally occurring in the human immune system. Tiemessen *et al* report that suppression of *in vitro* cultures of $CD25^-CD4^+$ T cell milk-specific proliferation responses by $CD25^+CD4^+$ T cells from milk allergic individuals is comparable to the suppressive ability of $CD25^+CD4^+$ T cells isolated from non-allergic individuals (123). Despite identical function of $CD25^+CD4^+$ T cells in allergic and non-allergic individuals *in vitro,* these data indicate that the mechanisms determining the particular response of these cells *in vivo* is still unclear.

Probiotic therapy studies using *Lactobacillus rhamnousus* in milk allergy (inducing IL-10) and administration of heat killed *Escherchia coli* with peanut

allergen (induction of TGFβ) demonstrate the potential contribution of the appropriate bacteria to suppression of allergic responses to common food allergens (124, 125).

Although the mechanisms by which IL-10 and TGFβ may contribute to immunosuppression are complex, their contribution in the development of oral tolerance, in addition to their role in down regulating responses in food allergic animal models, demonstrate their importance in regulating immune responses to food allergens.

7.5.7 Clinical Manifestation of Food Allergy

Food allergy results when normally innocuous dietary antigens elicit a harmful immune response. Although a Th2 dominant profile exists early in development, it is thought that Th1 skewing mechanisms, such as normal bacterial gut flora and exposure to microbes through food or the oral fecal route, balance this response to environmental antigens (126, 127). When this response to dietary allergens becomes unbalanced, food allergy can develop.

IgE mediated reactions are most often the mechanism by which food allergy occurs. Mixed IgE/non-IgE and strictly non-IgE mediated mechanisms have also been implicated in several food hypersensitivity reactions. Food allergic reactions can have various clinical manifestations. Cutaneous symptoms can include hives, swelling and other rashes. Gastrointestinal hypersensitivity can present as

nausea, abdominal pain or cramping, vomiting and/or diarrhea. Respiratory and systemic symptoms such as asthma exacerbation and anaphylaxis are usually secondary to IgE mediated food allergic symptoms (111).

IgE-Mediated Food Allergy

Initial exposure in a predisposed individual to a food can result in the production of food-specific IgE antibodies, which are subsequently bound to FcεRI present on mast cells and basophils. Upon re-exposure to the food allergen, this food-specific surface IgE can become cross-linked by the circulating multiple-epitope food allergen. Several mediators are released initiating an immediate hypersensitivity reaction occurring within minutes up to two of hours following ingestion of the culprit food. IgE mediated food allergic reactions are usually a combination of more than one organ system. Reactions can vary from itching and swelling of the skin and gastrointestinal anaphylaxis (abdominal pain/cramping, nausea, vomiting or diarrhea) to severe systemic anaphylaxis.

In contrast, OAS presents as itching and swelling restricted to the mouth and throat. Patients exhibiting allergic rhinitis to airborne pollens such as birch or ragweed frequently experience the OAS after ingestion of fresh fruits and vegetables elicited by cross-reacting IgE-binding proteins (128).

Mixed IgE and Non-IgE Mediated Food Allergy

Cell-mediated food-allergic disorders, such as atopic dermatitis, can demonstrate food-specific IgE. Allergic eosinophilic gastroenteritis or esophagitis may not demonstrate food-specific IgE but most often present with elevated total IgE. Pathology is characterized by increased lymphocyte and eosinophilic infiltration of target organs which can include the skin, esophagus, stomach or intestinal wall (as reviewed by 98, 111, 129). Interestingly, approximately a third of children with moderate to severe cases of atopic dermatitis suffer from IgE mediated hypersensitivities to food proteins (130, 131).

Non-IgE Mediated Food Allergy

Non-IgE mediated food hypersensitivities are generally delayed reactions occurring many hours to 3 days after ingestion of food. It is typically seen within the first year of life and most often provoked by proteins in cow's milk or soy based formulas. Food-induced enterocolitis and proctocolitis demonstrate eosinophilia of the small intestine. In contrast, food protein-induced enteropathy demonstrates a cellular infiltrate that is low in eosinophils. Food protein-induced enteropathy and celiac disease (associated with sensitivity to gliadin found in wheat, rye and barley) present with atrophied villi, the latter demonstrating complete atrophy (132). The end results of celiac disease are thought to be due to the prominent Th1 profile of IFNγ and IL-12 found in the intestinal mucosa and blood stream (133). A great majority of patients with celiac disease have an

association with HLA-DQ2, HLA-B8 or HLA-DR17 suggesting a genetic predisposition (111, 132).

7.5.8 Diagnosis and Treatment

Physicians employ many diagnostic tools to diagnose food allergy in their patients. Primary evaluation of a patient involves taking a detailed clinical history including food allergic events. Skin prick testing is useful in diagnosing food allergy. Negative skin prick test results are more useful in predicting the absence of food allergy with >95% predictive value. The existence of a high positive rate (~50%) of skin prick test positive results in the population as a whole indicates sensitization, which may or may not be associated with symptoms (134). Interestingly, although food-specific IgE can be found in those who are not clinically allergic, the concentration of food-specific IgE, as measured by CAP System FEIA (fluorescent enzyme immunoassay), can be correlated with the risk of clinical reaction (135, 136). Elimination diets can be used to identify, in the absence of signs and symptoms, the suspected food causing reactions and noting any improvement after a set amount of time. The gold standard for diagnosing food allergy is the double-blind placebo-controlled food challenge (DBPCFC). This oral challenge is usually carried out based on convincing history and either skin prick tests or CAP System FEIA results to the particular food. Depending on the suspected food disorder and delayed reactions, endoscopy, biopsy or studies of blood or stool samples can also be useful in confirming disease identity (137).

Currently there are no curative treatments for food allergy. Antihistamines can be used to reduce itching and rash. During systemic reactions, such as anaphylaxis, administration of epinephrine is the only definitive treatment should such a severe reaction occur. The best treatment and the only way to prevent subsequent reactions is food avoidance.

7.6 Peanut Allergy

Of the major food allergies that individuals experience, peanut allergy deserves particular attention for various reasons. It is the most common cause of near fatal and fatal food-induced anaphylaxis (138). Like many other food allergies, ingestion of peanut elicits an immediate type 1 hypersensitivity reaction occurring within minutes and is most often associated with peanut-specific IgE. In comparison to other food allergies, peanut allergy is far more likely to persist into adulthood. With the increased use of peanut, due to its low cost as a source of protein and an increasing movement towards vegetarianism, children are becoming increasingly exposed to peanut at an earlier age. Avoiding accidental exposure becomes a priority to peanut allergic patients and their families, directly influencing everyday activities and having an impact on the overall quality of life of peanut allergic individuals and their families.

7.6.1 Prevalence and Demographics

Many studies have been attempted to determine the true prevalence of peanut allergy. DBPCFC in a suspected peanut allergic individual proves to be risky, as anaphylaxis may result. The high percentage of false positive skin prick test results to peanut (45%) must be acknowledged when estimating the prevalence of peanut allergy (134). Therefore, the assessment of the natural history and prevalence of peanut allergy has been limited to surveys, DBPCFC in low risk peanut allergic, skin prick testing, RAST levels of peanut specific IgE, and a convincing history of peanut allergy.

Peanut allergy is most prevalent in developed countries. Studies carried out in the USA, UK and France find the prevalence of peanut allergy to be between 0.5% and 1.0% (134, 138-140). Studies carried out by Grundy *et al* show a trend towards an increase in the prevalence of peanut allergy from 0.5% to 1.5 % on the Isle of Wright over a period of 6 years (134). Although peanut consumption is high in China and other Asian countries, the rate of peanut sensitization and allergy is low in comparison to the USA and even in the UK. The difference probably relates to the consumption of boiled or fried peanuts, which have reduced antigenicity compared to dry-roasted peanuts (102-104).

Studies carried out by Bock show that peanut allergy does not resolve within 2-14 years based on DBPCFC in children of mean age 7 years (141). The idea that peanut allergy does not resolve has been questioned in recent studies. It has

been found that approximately 10-20% of children diagnosed with peanut allergy in infancy or within the first two years of life can become tolerant to peanut (100, 101, 142). Many of these studies did not use DBPCFC but rather open oral challenges or tested a subset of individuals who had histories of milder reactions to peanut. From these studies, we can conclude that resolution is unlikely for those with severe peanut allergy diagnosed in early childhood.

7.6.2 Peanut Allergens

Peanuts belong to the legume family, which is the third largest source of dietary protein (107). Protein comprises 22-30% of the peanut, which can be divided into albumins as well as the storage proteins arachin and conarachin. Many studies indicate that the major allergens are seed storage proteins. Peanut specific IgE antibody cross-reactivity has been found to other legume family members such as soybean and pea (143, 144). Amino acid sequence homology between seed storage proteins in pea and peanut and to glycinin in peanut, soybean and pea has been described. Originally, it was thought that although cross reactivity existed *in vitro*, legume-allergic individuals did not demonstrate adverse reactions to other legumes. Recent studies show that some peanut-allergic individuals have experienced adverse reactions to lentils, chickpea, soybean, pea and lupine flour (143).

Three major peanut allergens, *Ara h1*, *Ara h2* and *Ara h3,* have been identified based on IgE-antibody binding from peanut allergic sera to these proteins

isolated from peanut extract. Linear epitopes of these allergens were determined by synthesizing peptide fragments representing the entire primary amino acid sequence as determined by cDNA and incubating with serum IgE from peanut allergic individuals.

Ara h1

Ara h1 is a 64.5kDa protein (isoelectric point 4.55) belonging to the vicilin family of storage proteins. European studies indicate that the percentage of patients possessing *Ara h1*- specific IgE varies from 35-70% (145, 146) and greater than 90% in American studies (147). Through binding of serum IgE from peanut allergic patients to overlapping peptide fragments, it was found that 23 epitopes exist on the linear form of the protein. Four immunodominant epitopes showing the greatest amount of serum IgE binding by >80% of the peanut allergic patients in comparison to other epitopes were identified (148). Amino acids critical for IgE binding are hydrophobic residues located in the center of the linear epitopes. Structurally, *Ara h1* forms oligomeric proteins which consist of homotrimers and concentrate the 23 linear epitopes in two main areas (149). Experiments carried out using digestive proteases trypsin, chymotrypsin and pepsin results in several protease resistant peptide fragments that retain the ability to bind IgE from peanut allergic sera (107). In addition to protease resistance, the stability of *Ara h1* conformation is demonstrated by its ability to withstand high temperatures while retaining structure and IgE binding properties. Although purified *Ara h1*

denatures easily, it still has the ability to bind IgE indicating that conformational epitopes are not a factor in its allergenicity (150).

Ara h2

Ara h2, weighing 17.5 kDa (isolelectric point 5.2), belongs to the conglutin family of seed storage proteins. Similar to *Ara h1*, greater than 70-90% of peanut allergic patients have serum IgE which binds *Ara h2* (145, 146, 151). As in work done with *Ara h1*, three immunodominant peptides of ten possible IgE binding epitopes were identified along the length of the *Ara h2* protein (152). Ara h2 does not form an oligomeric structure like *Ara h1*, but contains eight cystiene residues that form disulfide bonds, which contributes to its stability and allergenicity. Upon incubation with digestive tract proteases, a 10kDa peptide results that contains 6 of the 10 epitopes (i.e. retains IgE binding from peanut allergic patients), 6 of 8 cystienes and 11 potential chymotrypsin sites (153) demonstrating its' structural stability.

Ara h3

In a study carried out by Rabjohn *et al*, a 14kDa protein bound by IgE from peanut allergic sera was found to have high sequence homology to a family of storage proteins called glycinins. Glycinin protein is initially synthesized as a 60kDa protein, which is then cleaved by proteases into two proteins that are 40kDa and 20kDa. The process by which the 14kDa protein is produced and that has ultimately been recognized by IgE from peanut allergics, has not been determined. Linear B cell epitopes were mapped using synthetic peptides of the

primary protein sequence and it was found that all four *Ara h3* epitopes were found within the 40kDa subunit (154). Recombinant *Ara h3* is recognized by serum IgE from approximately 44% of patients (154), whereas native *Ara h3* appears to be recognized by a sub-population of peanut- allergic patients (144).

7.6.3 Pathophysiology of Food Allergy

Antibody Responses to the Major Peanut Allergens

The antibody profile to peanut has been characterized by antibody specificity experiments testing pooled sera from peanut allergic and non-allergic individuals. As previously mentioned, several studies have been carried out demonstrating peanut-specific IgE in the sera of a substantial percentage of peanut allergic individuals binding various proteins in peanut extract as well as binding the major allergens *Ara h1, Ara h2* and *Ara h3.* Although the humoral antibody response in allergic individuals to peanut is characterized by high amounts of peanut-specific IgE , the response can also be polyisotypic. Studies by de Jong *et al* and Kolopp-Sarda *et al* demonstrate peanut-specific IgM, IgG, IgG1, IgG2, IgG3, IgG4, and IgA in both peanut allergic and non-allergic individuals (145, 155). Results from Kolopp-Sarda *et al* state observed differences in sera IgG1, IgG2 and IgG4 where de Jong *et al* only finds higher plasma IgG4 in peanut allergic versus non-allergic individuals. In addition, no peanut-specific IgE was detected by their assays in peanut non-allergic individuals, whereas high levels were found in

allergic individuals. Differences in peanut-specific antibody isotopes between peanut allergic and non-allergic individuals, other than IgE, remain unclear.

T Cell Responses to Peanut Allergens

Early studies examining T cell responses to peanut allergens focus on proliferation of PBMC *ex vivo* and measurement of mRNA or endogenous production of cytokines IFNγ(Th1) or IL-4 (Th2) after short-term primary culture with peanut allergens. The relationship between peanut-specific proliferation, cytokine production, or IgE responses and predicting clinical severity was also examined. Whether peanut-stimulated proliferation is increased in peanut-allergic individuals (156) or non-allergic individuals actually demonstrate a proliferative response still remains unclear (156-159).

Initial studies by Dorion *et al* used *Ara h2* to study T cell cytokine responses (160). They found that peanut allergic individuals express mRNA for and secrete IFNγ, although a weaker response is observed in comparison to non-allergic individuals. IL-4 production in cell supernatants could not be measured in response to *Ara h2* stimulation, but mRNA levels could be measured in both peanut-allergic and non-allergic. Further, they correlate increased IFNγ production with decreased proliferation. PBMC from peanut allergics also demonstrate IFNγ production in the presence of pepsin digested and intact peanut extract (161). Similarly, other studies show that T cell clones derived from severely peanut allergic individuals demonstrate both secreted Th1 (IFNγ) and

Th2 (IL-4 and/or IL-5) cytokines in response to peanut extract (158, 162). In contrast, studies by Laan *et al* show that IFNγ induced by peanut extract stimulation of PBMC could not be readily measured from peanut allergic individuals. Secreted IL-4 could not be measured, but IL-4 mRNA production was significantly correlated with proliferation in peanut allergic individuals (156). Collectively, these findings suggest that the peanut allergic response is more Th2-like than Th1-like based on the relationship between proliferation of PBMC and differential IL-4/IFNγ mRNA levels, and further supported by weaker peanut-specific IFNγ responses in peanut allergic patients than non-allergic controls.

A study by Turcanu *et al* is the first to describe differences in cytokine mRNA levels from peanut-specific T cells (cells detected by carboxyfluorescein succinimydyl ester by flow cytometry) between peanut allergic, peanut non-allergics and those who have outgrown peanut allergy. They report that peanut-allergic donors' PBMC are Th2 polarized defined by high levels of IL-4, IL-5 and IL-13 mRNA levels whereas children that have outgrown their allergy and those are non-allergic demonstrate low levels of the type 2 cytokines and high levels of IFNγ and TNFα (163).

Collectively, these studies suggest that T cell responses by peanut allergic individuals are characterized by the production mRNA of Th2 cytokines including IL-4, IL-5 and IL-13 in comparison to non-allergics and demonstrate low levels of IFNγ.

Restricted Use of TcR and HLA Class II: Potentiating Susceptibility to Peanut Allergy

Studies have been carried out examining possible differences in expanding a specific T cell receptor upon recognition of peanut allergens. The expansion of T cells based on one dominant TcR-Vβ family paired with varying D and J regions are thought to play a role in superantigenic polyclonal activation (164). Antigen-driven T cell expansion is usually the result of specific antigen-recognition by a TcR created as a combination of common V, D and J segments. One early study demonstrates selective expansion of the TcR-Vβ2 family by PBMC from peanut allergic individuals in response to peanut extract (164) suggesting restricted T cell receptor usage for peanut recognition. Subsequent studies on TcR use by Bakakos *et al* state no difference in the use of TcR-Vβ2 between peanut allergic and non-allergics. The restricted use of TcR-Vβ11 by peanut allergic individuals in a clonal or oligoclonal manner is proposed, as demonstrated by characteristic plots by molecular genetic analysis of PCR products (165). Several studies demonstrate the use of specific polymorphisms of HLA class II molecules in peanut allergen recognition by peanut allergic T cell clones (166, 167).

In determining differences in TcR usage and specific HLA molecule polymorphisms between peanut allergic and non-allergic, factors contributing to the susceptibility to peanut allergy can be further defined.

7.6.4 Investigation of Treatment for Peanut Allergy

Animal Models

Considering the potentially severe reactions of patients to peanut and the limited access to human tissues and cells for the study of food allergy, animal models have been developed to investigate immunological mechanisms and therapeutic options for peanut allergy. Early studies employing intraperitoneal sensitization and challenge show that sensitization can vary between strains of mice (168). Pig and dog models of IgE-mediated peanut allergy are more similar to the physiological state of allergic disease in humans than are murine models (169, 170). One drawback of using the aforementioned models in examining peanut allergy in humans, is the route of sensitization. Sensitization by intraperitoneal injection does not mimic the route of sensitization in humans and may not be suitably comparable to the induction of peanut allergic responses in humans.

Li *et al* have described a peanut-specific IgE mediated murine model of peanut allergy presenting with immediate hypersensitivity reactions following intragastric sensitization. These mice demonstrate B and T cell responses through specific IgE and T cell proliferation to the major allergens *Ara h1* and *Ara h2*. In addition, epitope mapping reveals that similar sequences are recognized in comparison to those found in peanut allergic patients (171). This model has been used to investigate immunological responses and potential therapeutic treatments for peanut allergy.

7.6.5 Experimental Models of Potential Therapies

Use of Modified Peanut Allergens

The production and characterization of recombinant forms of the major peanut allergens has made the investigation of therapies possible. These recombinant forms of allergens can be modified resulting in low affinity IgE binding epitopes (148, 152, 154) yet still retain the ability to stimulate T cell proliferation in peanut-allergic individuals (172).

In unpublished data, Li *et al* use a modified *Ara h2* or overlapping peptide sequences of *Ara h2* containing T cell epitopes (unable to cross-link IgE) to reduce amounts of specific IgE and lower anaphylaxis in a murine model of peanut anaphylaxis (as reviewed in 173). Desensitization using overlapping peptides also results in the production of IFNγ from splenic cells.

Administration of modified *Ara h1, Ara h2* and *Ara h3* in conjunction with adjuvants such as heat-killed *Listeria monocytogenes* or *Escherichia coli* is quite effective in desensitizing established peanut allergy in a murine model of peanut anaphylaxis (125, 174). Treated mice show decreased peanut-specific IgE, increased specific IgG2a, decreased symptoms scores and abrogation of anaphylaxis reactions. In addition, decreased splenocyte peanut-specific type 2

cytokine IL-4, IL-5, IL-13, and increased IFNγ responses were observed after heat killed bacteria and modified allergen treatment.

Several studies have made use of these modified allergens to desensitize peanut-allergic reactions in animal models by down-regulating type 2 responses underlying established food allergy. Immunotherapy using modified peanut allergens with heat-killed bacteria has proven useful in treating peanut allergy in this model. In the event that this application is used in treating peanut allergy in humans, the responses to bacterial components contributing to potentially serious side effects must be considered. The determination of the exact component of the organism that helps to induce the protective or down regulatory response would be beneficial in creating a more efficient therapy.

DNA-Based Therapies

Advances in cloning the peanut allergen *Ara h2* led to studies employing plasmid DNA vectors to investigate the development of a protective response upon subsequent peanut allergen challenge. The response to peanut challenge after immunization with plasmid DNA encoding *Ara h2* is strain dependent in mice (168) and does not result in reduction of peanut-specific IgE (as reviewed in 173).

The use of CpG motif-*Ara h2* conjugates to prevent the development of peanut allergy has been explored to improve DNA based immunotherapies. Mice

previously immunized with immunostimulatory sequence (ISS) and *Ara h2* conjugates and then sensitized and subsequently challenged with peanut did not demonstrate any symptoms, whereas control mice sensitized with ISS-ragweed allergen did (as reviewed in 173). Although promising, the ability of plasmid DNA constructs, along with immunostimulatory oligonucleotide immunization, to desensitize or redirect the allergic response to peanut in humans has not been explored and therefore requires further study.

Suppression or Neutralization of Type 2 Peanut-Allergic Responses

The immunological backbone of peanut-allergy is defined by a Th2 response. Suppression of Th2 responses by administering type 1 cytokines has been explored though oral administration of type 1 cytokine IL-12 after peanut sensitization in a murine peanut allergy model (175). This study demonstrates reduction in symptoms and peanut-specific IgE, in addition to increasing IFNγ responses without a decrease in type 2 cytokine production. Although beneficial in the murine model of allergy, studies by Bryan *et al* show that the use of IL-12 in human allergic disease results in severe side effects and even death (176, 177).

Since the engagement of allergen-specific IgE on mast cells and basophils contributes to the severity of an IgE-mediated reaction, neutralization of circulating IgE could be beneficial in preventing this process. TNX-901 is an IgG1 monoclonal antibody that binds and masks the epitope on IgE responsible for

binding the Fcε receptor on mast cells and basophils. Neutralization of IgE using TNX-901 treatment has been found to be effective in increasing the threshold of sensitivity to peanut upon oral challenge, which can protect against most incidents of accidental ingestion of peanut (178). At present, TNX-901 is being investigated in human clinical trials in patients with peanut allergy with promising regimen results. Treatment may be impractical, as the regimen will involve monthly injections of the short-term acting anti-IgE antibody over a lifetime and costs of treatment will be high.

Alternative Medicine

Traditional Chinese medicine has been used as alternative to conventional therapies for a variety of diseases. The use of the traditional Chinese herbal medicine, food allergy herbal formula-1 (FAHF-1), has been shown to have anti-allergic properties that substantially reduce mast cell degranulation, histamine release and can completely abrogate peanut-induced anaphylaxis in a mouse model of peanut-induced anaphylaxis (179).

More investigation is required before a truly effective treatment or therapy can be adapted to the human system. Thus at best, avoidance of peanuts is the only solution.

Rationale

Why are we not allergic? Allergic individuals develop undesirable immune responses to normally innocuous proteins resulting in the clinical manifestation of allergy. In contrast, healthy individuals are regularly exposed to these proteins yet do not develop any apparent response. Although healthy individuals do not present with clinical symptoms in response to these proteins, it does not exclude the possibility that they respond at the immunological level. The type of response an individual has may be a determining factor in the development of versus the protection against allergy.

Peanut allergy is becoming an increasingly important area of study. Unlike other allergies, peanut allergy most often persists into adulthood with a small percentage becoming tolerant to peanut. With the relatively recent development of peanut extracts and recombinant peanut allergens, the body of literature is slowly increasing. Type 1 and type 2 cytokines and chemokines are important immunological mediators in the development of allergy and tolerance. The non-allergic immune response to this common food allergen has not been extensively characterized. Defining immunological responses based on cytokine and chemokine production by non-allergic individuals to peanut allergen can help us understand the regulatory factors and mechanisms that underlie the development of tolerance versus allergy to peanut.

7.7 Project Summary

We hypothesize that the production of type 1 and type 2 cytokines and chemokines may serve as potential markers of the non-allergic response to peanut allergen and that the initiation of these responses are dependent on key molecules involved in the classical T cell activation pathway. Additionally, we hypothesize a role for immunosuppressive cytokines in the regulation of peanut stimulated responses by PBMC from peanut non-allergic individuals.

We developed a novel *in vitro* primary culture system using freshly isolated PBMC culture from peanut non-allergic individuals with peanut extract or the major peanut allergens *Ara h1* and *Ara h2*. This enabled us to characterize the immune response of peanut non-allergic individuals by measuring type 1 (IFNγ and CXCL10) and type 2 (IL-5, IL-13, CCL17 and CCL22) cytokine and chemokine production. Specifically, we were able to A) determine the prevalence of cytokine and chemokine responses in the general peanut non-allergic population B) characterize and compare the peanut-specific cytokine and chemokine production profile of peanut non-allergic skin prick test negative and skin prick test positive individuals C) identify T cell and APC co-stimulatory requirements for the initiation of peanut-specific cytokine and chemokine production and D) determine the role of endogenously produced cytokines IL-10 and TGFβ in the suppression of peanut-specific cytokine and chemokine production from PBMC.

A strength of this study lies in the utilization of short-term primary culture of PBMC from peanut non-allergic individuals to measure actively produced cytokine and chemokine in response to peanut-stimulation as opposed to examining peanut-induced mRNA cytokine and chemokine levels or responses from peanut-specific T cell clones. The aim of this study is to characterize the peanut-specific cytokine and chemokine profile of non-allergic individuals and identify potential factors in the regulation of these responses through the examination of the involvement of co-stimulatory molecules and immunosuppressive cytokines.

8 Methods

8.1 Participants

Blood was obtained from approximately 60 healthy adults between the ages of 18 and 35 years old, who were randomly recruited in response to an advertisement. They were divided into the following three groups based on peanut skin prick testing (50% peanut extract in glycerine, Bencard, Mississauga, Ontario) and clinical history of peanut allergy for primary cell culture:

A) **Skin Prick Test Negative (SPT-) Non-Allergic:** Defined by a negative skin test to peanut and no clinical history of allergy despite regular exposure to peanut. Many of these patients are skin test positive to other allergens and have a clinical history of other allergies (68%). n~50

B) **Skin Prick Test Positive (SPT+) Non-Allergic:** Defined by a positive skin test to peanut and a negative clinical history of allergy to ingested peanut (sensitized but asymptomatic). All of these individuals are skin test positive to other allergens and have a clinical history of other allergies. n~10.

C) **Peanut Allergic:** While our focus was on peanut non-allergic individuals, we included a small number of clinically peanut allergic individuals for purposes of comparison. The peanut allergic individuals were defined by a positive skin test to peanut and a positive clinical history of allergy to peanut. n~3

The University of Manitoba Faculty Committee on the Use of Human Subjects in Research approved this study and written informed consent was obtained from each individual.

8.2 Peripheral Blood Mononuclear Cell (PBMC) Isolation

50ml blood was collected into 2ml of 2.7% ethylene diamine tetra-acetic acid (Sigma, St. Louis, Missouri). PBMC were obtained from whole blood diluted with 0.85% sodium chloride (NaCl) (Fisher Scientific, New Jersey) by density centrifugation on Histopaque-1077 Ficoll (Sigma, St. Louis, Missouri) for 30 minutes at 1600 rpm (Eppendorf Centrifuge 5810, Brinkmann Instruments Inc., Westbury, New York). Cells collected from the plasma-ficoll interface were washed and centrifuged twice at 250g for 10 minutes with 0.85% NaCl. Cells were re-suspended in 4 ml of RPMI 1640 supplemented with 10% fetal bovine serum, 2mM L-glutamine, penicillin/streptomycin/fungizone and 2 x 10^{-5} mM 2-mercaptoethanol. Cells were counted using a haemocytometer (>95% viability by trypan blue exclusion) and stored on ice until cultured.

8.3 Short Term Primary Culture of PBMC

We developed and optimized a short-term *in vitro* primary culture system using freshly isolated PBMC from blood acquired from peanut non-allergic and allergic

individuals. The culture system was optimized using a range of concentrations of whole peanut extract (WPE) and peanut allergens *Ara h1* and *Ara h2* (between 2ug/ml and 200ug/ml) and culturing at 37°C for 5 and/or 7 days in 96 well roundbottom microwell plates (NUNC Brand products)(duplicate wells at minimum). Subsequent experiments were performed using either 10 or 100ug/ml peanut extract (optimal concentration inducing cytokine and chemokine production) cultured for 5 days (peak time of cytokine and chemokine production).

Table 1: List of Allergens and Antibodies used in Short Term Primary PBMC Culture

Allergen	Final Concentration	Source
Whole Peanut Extract (WPE)	Between 2 μg/ml –200 μg /ml	All peanut allergens kindly provided by Dr.Gary A . Bannon and Dr. Steve Stanley, University of Arkansas
Ara h1	Between 15 ug/ml and 150ug/ml	
Ara h2	Between 15 ug/ml and 150ug/ml	
Streptokinase	5000 U/ml	Aventis
T Cell Activation Molecules		
αCD4	2 ug/ml	BD Pharmingen
αHLA-DR	2 ug/ml	BD Pharmingen
Co-Stimulatory Molecules		
αCD80	5 ug/ml	BD Pharmingen
αCD86	1 ug/ml	BD Pharmingen
CTLA-4 Ig	5 ug/ml	Kindly provided by Dr. Peter Nickerson , University of Manitoba
Endogenous Cytokines		
αIL-10	25 ug/ml	Grown from JES 3-19F1.1.1. HB 10487, rat IgG)
αTGFβ	10 ug/ml	R & D Systems

αIL-10 and αTGFβ added as a cocktail

A concentration of 10ug/ml of WPE was used when PBMCs were co-cultured with antibodies to T cell activation molecules, co-stimulatory molecules or endogenous cytokines (refer to Table 1). Supernatants were then collected and stored at –20°C until analyzed by enzyme linked immunosorbant assay (ELISA).

8.4 Cytokine/Chemokine Enzyme Linked Immunosorbant Assays (ELISA)

Sandwich ELISA was used to determine IL-5, IL-13, CCL17, CCL22, IFNγ and CXCL10 production in cell supernatants after *in vitro* stimulation of PBMC with peanut allergens and the addition of antibodies to co-receptors and co-stimulatory molecules. After ELISA plates (Costar, Corning Inc., Corning, New York) were coated with primary antibody overnight at 4°C in a moist box with carbonate buffer (0.075M, 0.02% NaN_3, pH 9.6), blocking buffer (0.17% bovine serum albumin (BSA), phosphate buffered saline (PBS), 0.02% NaN_3, pH 7.4) was added to all wells and incubated for 2 hours at 37°C. Plates were then washed four times (Skatron 300 Version B, Skatron Instruments, Sterling, Virginia) with wash buffer (PBS, 0.05% Tween 20, 0.02% NaN_3, pH 7.4). Recombinant cytokine was applied to plate and titrated 2 fold in dilution buffer (0.085% BSA, 0.05% Tween 20, PBS, 0.02% NaN_3, pH 7.4) for 8 wells. Samples were appropriately diluted in dilution buffer and then titrated 2 fold for four wells. Dilution buffer was applied to all remaining wells. Samples were incubated in a moist box overnight at 4°C. Plates were then washed and biotinylated secondary

antibody diluted in dilution buffer was added to all wells of plate. After incubation overnight at 4°C, streptavidin alkaline phosphatase (Jackson Immunoresearch Laboratories Inc., Pennsylvania) in dilution buffer was added to all wells and incubated for 45 minutes at 37°C. p-nitrophenol phosphate tablets (Sigma, St. Louis, Missouri) dissolved in substrate buffer (0.5mM $MgCl_2$-$6H_2O$, 9.7% diethanolamine (Fisher Scientific, New Jersey, pH 9.8)) was then added to plates. Plates were read at 405-690nm (SOFTmax Pro 3.2.1program, SpectraMax 190, both from Molecular Devices Corp., San Diego, California) after the appropriate time of development. Recombinant cytokine, capture and biotinylated secondary antibodies for cytokine were obtained from BD Pharmingen (Canada), Peprotech (Ottawa, Ontario) or The National Institutes of Health (Maryland, USA). Recombinant proteins and antibodies for chemokine assays were obtained from R & D Systems (Minneapolis, Minnesota) or Peprotech. See Table 2 for standard concentrations, sample dilutions, and development times.

Table 2: List of Cytokines and Chemokines Analyzed by ELISA

Cytokine/Chemokine	*Range of Detection*	*Sample Dilutions*	*Final Sensitivity*
Type 1			
IFNγ	20- 0.16 U/ml	1/4	0.600 U/ml
CXCL10	1500- 11.4 pg/ml	1/20	235 pg/ml
Type 2			
IL-5	250- 1.95 pg/ml	1/3	5.90 pg/ml
IL-13	200- 1.6 pg/ml	1/3	4.70 pg/ml
CCL17	600- 4.7 pg/ml	1/10	47.0 pg/ml
CCL22	2200- 17 pg/ml	1/10	170 pg/ml

8.5 Flow Cytometry

Flow cytometry was used to determine levels of cell surface expression of co-stimulatory molecules on T cells and APCs, specifically monocytes in response to WPE stimulation. PBMCs isolated from freshly drawn blood from peanut allergic and non-allergic humans were cultured with LPS, Concanavalin A (Con A) or WPE for up to 4 days at 37°C. Supernatants from cells were removed and then re-suspended in 150ul of wash buffer (PBS, 1% BSA, 0.02% NaN_3) with 10% homologous plasma. After incubation for 15 minutes on ice, cells were spun down for 4 minutes at 200g. After removing wash buffer, the appropriate amount of ice cold wash buffer was added such that the final volume equaled 50ul after fluorochrome-conjugated antibodies were added. Contents of each well were gently mixed and then incubated on ice for 30 minutes in the dark. After incubation, 100ul of cold wash buffer was added to all wells and then spun for 4 minutes at 200g. Cells were washed two more times with 150ul cold wash buffer each time and pelleted at 200g after each wash for 4 minutes. After removing wash buffer, cells were re-suspended in 150ul of 2% paraformaldehyde in plate then transferred to flow tubes (Falcon, Becton Dickinson Labware, New Jersey) and topped to 500ul. Cells were stored in the dark at 4°C until analyzed. Appropriate isotype and compensation controls were used. Fluorescein isothiocyanate (FITC), R- phycoerythrin (R-PE) or Cy-Chrome conjugated antibodies were used in combination for staining monocytes and lymphocytes. CD14 stained monocytes were also stained for either CD80 or CD86. CD3

stained lymphocytes were stained for CTLA-4. All antibodies were obtained from BD Pharmingen. Acquisition and analysis was determined by EXPO 2 Software using the Beckman-Coulter Flow Cytometer-EPICS ULTRA CELL SORTER.

Table 3: List of Fluorochrome-Conjugated Antibodies for Cell Surface Molecules Analyzed by Flow Cytometry

Isotype Antibody	*Fluorochrome*	*Matched With*	*Analyzed Population of Cells*
Mouse IgG_1	R-PE	**CD80-R-PE**	Monocytes
Mouse IgG_{2b}	R-PE	**CD86-R-PE**	Monocytes
Mouse IgG_{2a}	FITC	**CD14- FITC**	Monocytes
Mouse IgG_{2a}	Cy-Chrome	**CTLA-4 or CD3 Cy-Chrome**	Lymphocytes

8.6 Statistical Analysis

All samples for ELISA were cultured and analyzed at minimum in duplicate. Data was obtained from at least four dilutions against a standard curve for each assay plate. All statistical analysis was carried out using Graph Pad Prism 3 software. To account for the non-Gaussian distribution of data obtained from our study population of humans, non-parametric tests were used in the analysis of differences in cytokine production. The Wilcoxon matched pairs or the Mann-Whitney U tests (both non-parametric statistical tests) were used to compare cytokine and chemokine median responses between conditions and sub-population groups. The Fisher's T test was used to compare the frequency of cytokine and chemokine responses between the skin test negative and skin test positive groups. Statistical significance: $p < 0.05$.

9 Results

9.1 Introduction

The focus of this thesis is to characterize the nature of the peanut-specific immune response of peanut non-allergic humans. In particular, we examine the production of cytokines, chemokines and expression of co-stimulatory molecules known to be important immunological mediators. By investigating the role of important co-stimulatory molecules CD80, CD86 and CTLA-4 and the immunosuppressive cytokines IL-10 and TGFβ, we aim to identify possible mechanisms that underlie regulation of allergen-specific responses to this common food in non-allergic people.

9.2 Optimization of Primary Culture of PBMC

At the initiation of this project, there were limited publications examining *in vitro* peanut-specific cytokine and chemokine responses PBMC from peanut non-allergic humans. Indeed, it was unclear if these responses existed. Therefore, it was important to establish a working experimental system. We developed a short-term, *in vitro* primary culture system using PBMC isolated directly *ex vivo* from healthy peanut non-allergic individuals. Optimization studies were carried out to determine i) if such responses existed, ii) the concentration of WPE capable of eliciting peanut-specific cytokine and chemokine responses, as well as iii) their peak time of production. PBMC from 7 peanut non-allergic individuals

were incubated for 5 or 7 days at various concentrations of WPE between 2 and 200 μg/ml. After collecting culture supernatants, analysis by ELISA showed that production of type 2 cytokines (IL-5, IL-13) and chemokines, (CCL17, CCL22) was evident in a significant proportion of peanut non-allergic individuals and that maximal responses were evident at 10 or 50μg/ml of WPE on day 5 of culture (representative data shown in Figure 1). The type 1 response, as measured by IFNγ and CXCL10 production, was not affected by the presence of peanut extract (representative data shown in Figure 2).

PBMC from one peanut allergic individual was incubated with concentrations of 10, 50 or 100μg/ml of WPE for 5 days. In contrast to peanut non-allergic individuals, this peanut-allergic individual responded to WPE in a dose-dependent manner. Measurement of type 2 cytokines and chemokines by ELISA showed detectable IL-5, IL-13, CCL17, and CCL22 responses to WPE (Figure 3A). Type 1 IFNγ cytokine production was slightly increased (Figure 3B).

Through the establishment of this system, we have shown that non-allergic individuals can demonstrate a peanut-specific cytokine and chemokine response. Based on these results, concentrations of 10 or 100μg/ml of WPE were used for stimulation of cell cultures in subsequent experiments.

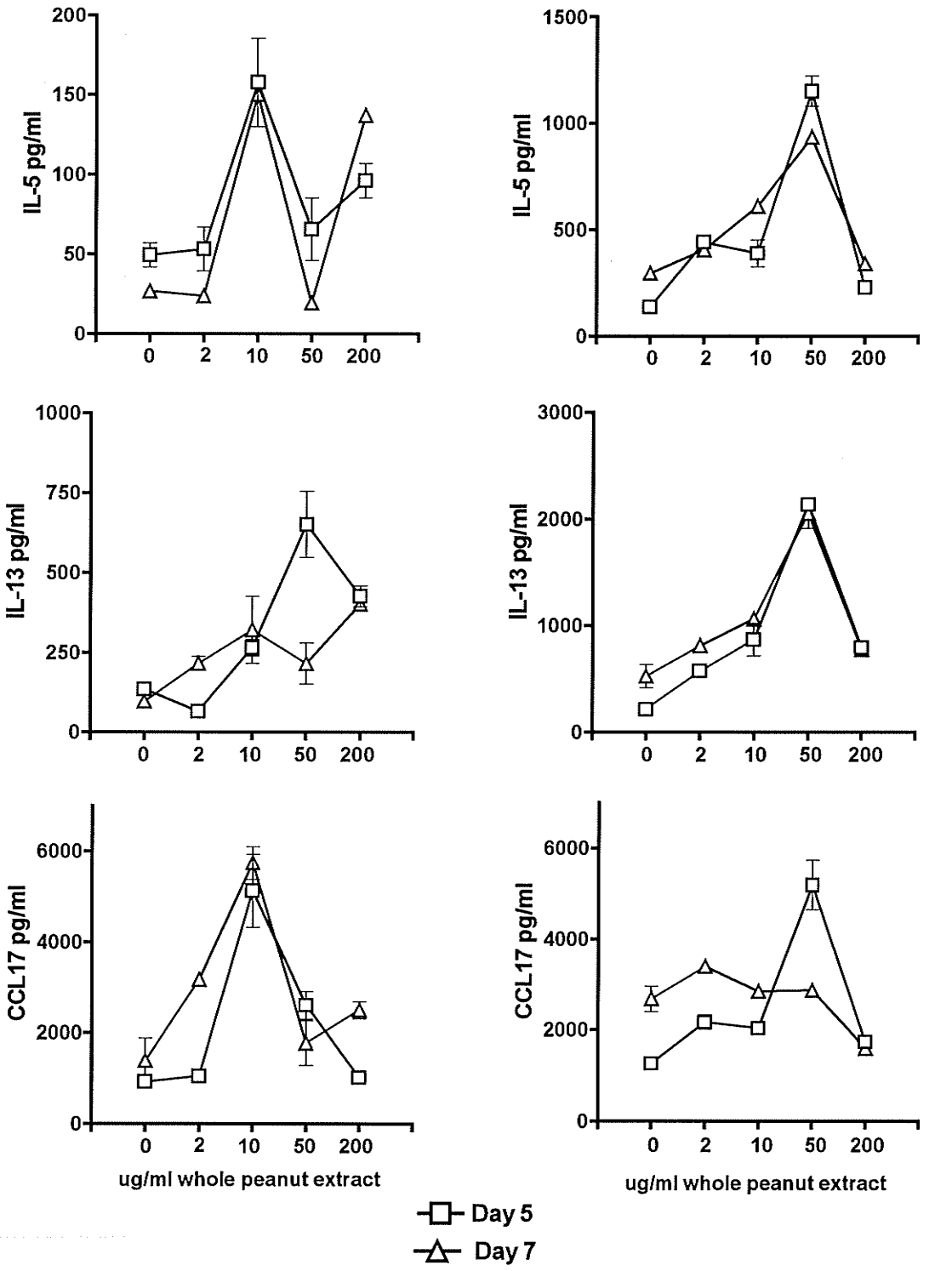

Figure 1: Optimal Time and Dose Response Curves to Whole Peanut Extract for Type 2 Cytokines and Chemokines (IL-5, IL-13 and CCL17). PBMC from 7 peanut non-allergic individuals were cultured directly *ex vivo* in triplicate with 2, 10, 50 or 200μg/ml of whole peanut extract. Supernatants were then collected at 5 and 7 days and analyzed using cytokine-specific sandwich ELISA. Mean cytokine production (+/- SEM) from 2individuals representative of 7 examined in detail.

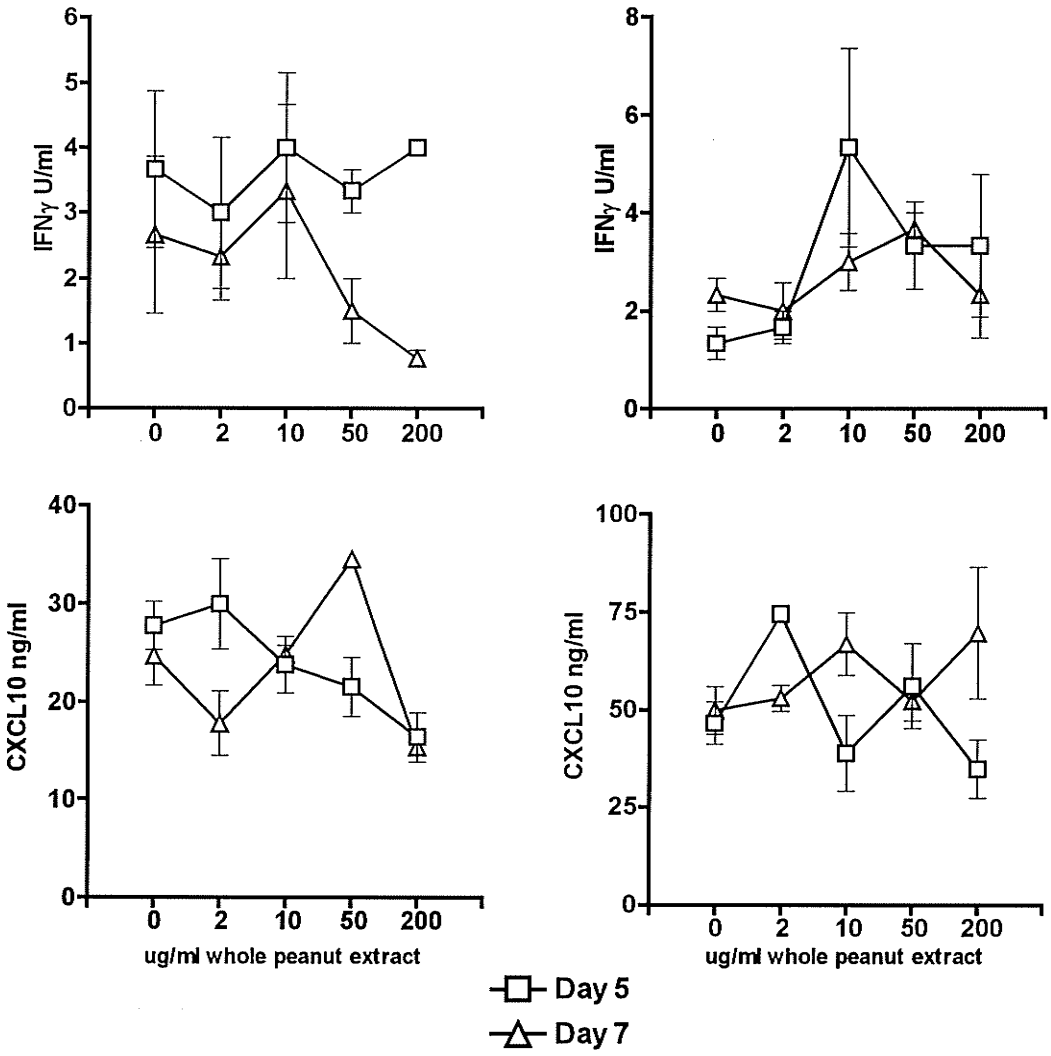

Figure 2: Optimal Time and Dose Response Curves to Whole Peanut Extract for Type 1 Cytokines and Chemokines (IFNγ and CXCL10). PBMC from 7 peanut non-allergic individuals were cultured directly *ex vivo* with 2, 10, 50 or 200μg/ml of whole peanut extract for 5 or 7 days. Supernatants were then collected for cytokine and chemokine analysis. Mean cytokine production (+/- SEM) from 2 individuals representative of 7 examined in detail.

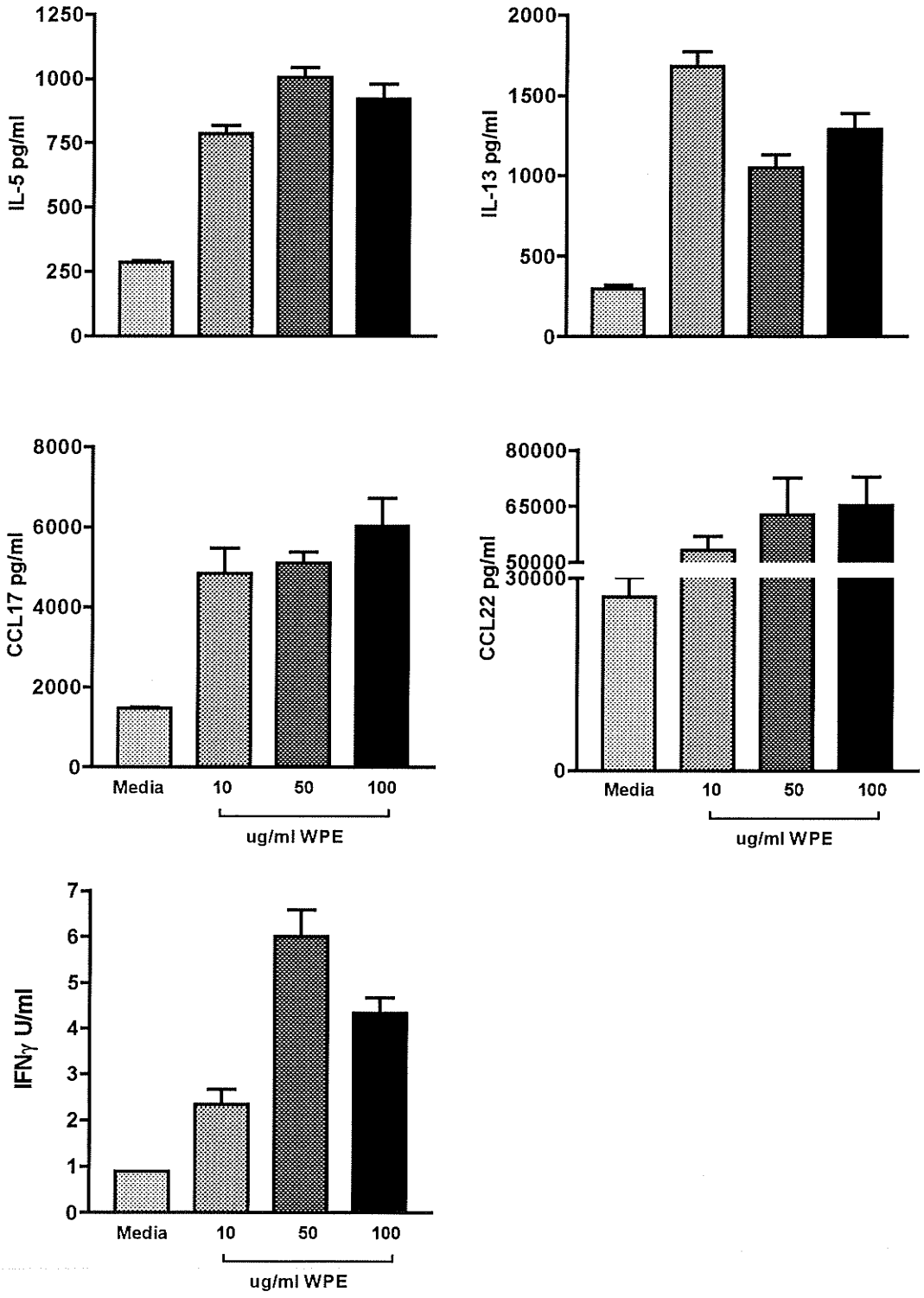

Figure 3: Type 2 Cytokine and Chemokine Dose Response to Whole Peanut Extract (WPE) of a Peanut Allergic Subject. After culturing PBMC from one peanut allergic subject +/- 10, 50 and 100μg/ml whole peanut extract (WPE) for 5 days A) type 2 cytokines (IL-5, IL-13) and chemokines (CCL17, CCL22) and B) type 1 cytokine (IFNγ) were measured by ELISA (each condition measured in triplicate). Mean cytokine production (+/- SEM) from 1 individual.

9.3 Peanut-Specific Cytokine and Chemokine Responses in Non-Allergic Humans

9.3.1 WPE-Specific Cytokine and Chemokine Responses in Non-Allergic Humans

We propose that peanut non-allergic individuals mount a measurable immunological response to peanut allergen and that the nature of this response is what likely determines the manifestation or absence of clinical allergic disease to peanut. Specifically, ***we hypothesized*** that peanut non-allergic individuals would demonstrate cytokine and chemokine recall responses to peanut allergen. To explore this, we examined cytokine and chemokine responses normally associated with the manifestation (type 2) or absence (type 1) of clinical allergic disease. By examining *in vitro* PBMC responses to 10 and 100μg/ml of WPE from a large population **of peanut-exposed non-allergic humans** (n~ 55 individuals) we showed that markedly increased levels of type 2 cytokines, IL-5 and IL-13, were observed following peanut extract stimulation (Figure 4).

When characterizing type 2 chemokine responses in this population, a difference in production of CCR4 binding family members CCL17 and CCL22 was found. CCL17 responses to either concentration of WPE were not seen among the non-allergic population (Figure 5). In contrast, a substantial increase in peanut-specific CCL22 was observed in peanut non-allergic individuals (Figure 5). When type 1 cytokine and chemokine responses (IFNγ and CXCL10 respectively) were measured, WPE did not stimulate production above that seen in media control

cultures (Figure 6). These cells are capable of both type 1 (IFNγ and CXCL10) and type 2 (IL-5, IL-13, CCL17, and CCL22) cytokine and chemokine production as demonstrated by their response to recall antigen streptokinase.

These data indicate that although non-allergic individuals do not demonstrate clinical allergic manifestations despite regular exposure to peanut, they mount detectable immune responses characterized by the production of type 2 dominated peanut-specific cytokines (IL-5 and IL-13). Interestingly, differential production of peanut-specific type 2 chemokines was found when analyzed. We showed that peanut non-allergic individuals produced detectable CCL22 responses and that no change in CCL17 production was measured after peanut extract stimulation. In contrast to the type 1 dominated immune response of PBMC from non-allergic individuals when stimulated with common aeroallergens (180), the responses of PBMC following WPE simulation from peanut non-allergic humans demonstrated no peanut-specific type 1 cytokine and chemokine responses.

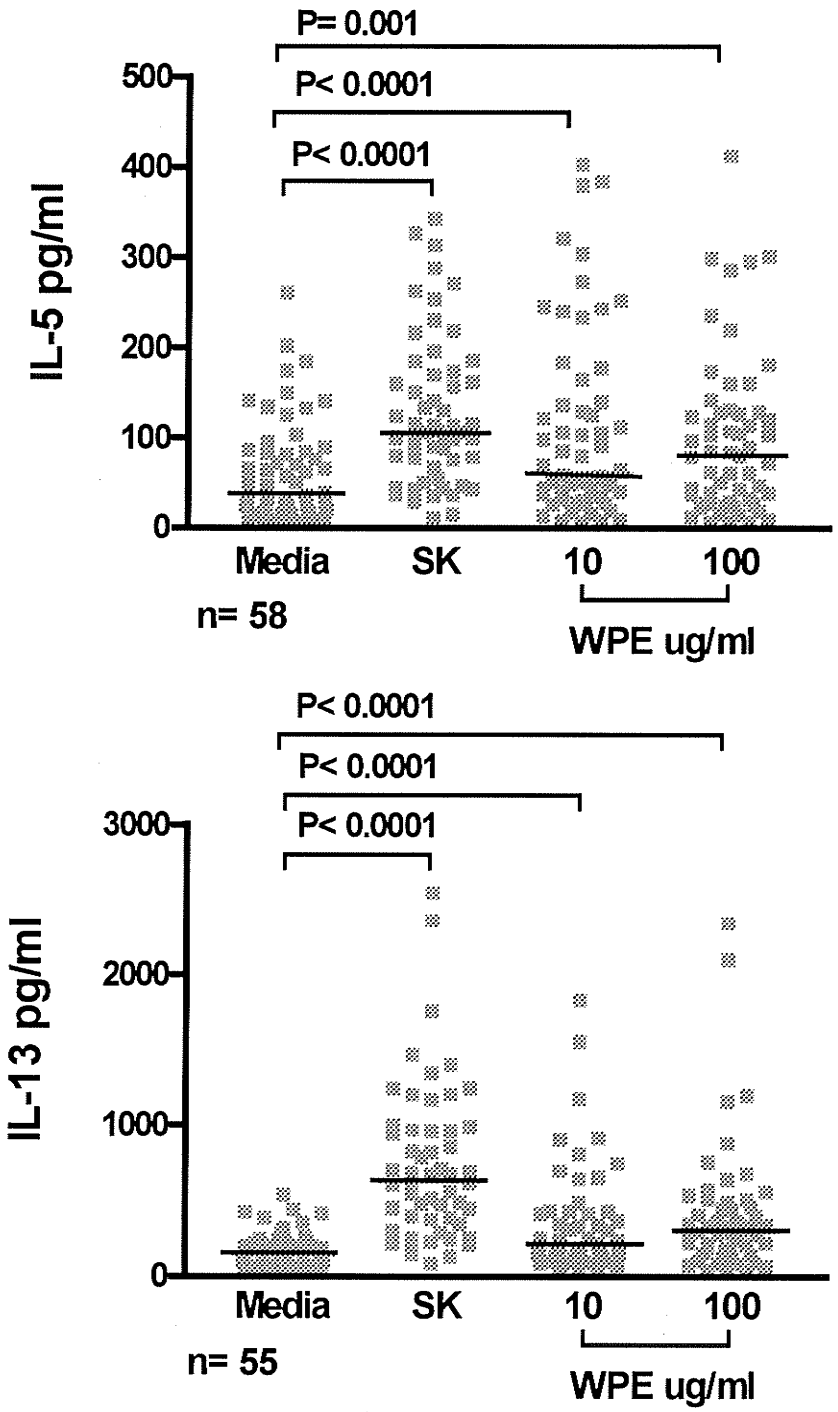

Figure 4: PBMC from Peanut Non-Allergic Individuals Demonstrate Type 2 Peanut-Specific Cytokines in Response to WPE. PBMC freshly isolated from blood collected from peanut non-allergic individuals were cultured in the presence of 5000U/ml streptokinase (SK), 10 or 100μg/ml of WPE for 5 days. Type 2 cytokines, IL-5 and IL-13, were measured by cytokine-specific ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

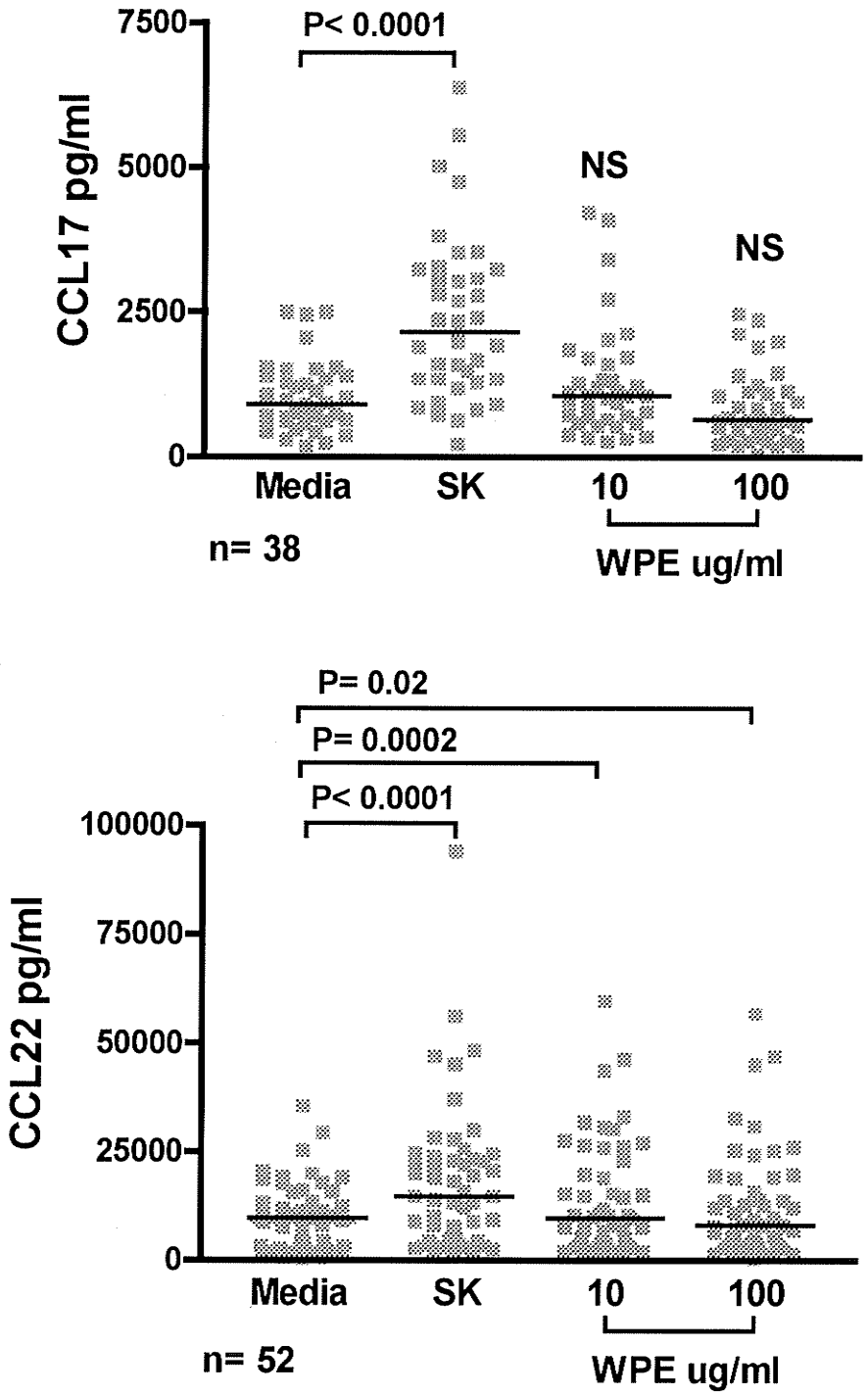

Figure 5: PBMC from Peanut Non-Allergic Individuals Demonstrate Differential Type 2 Peanut-Specific Chemokines in Response to WPE. PBMC freshly isolated from blood collected from peanut non-allergic individuals were cultured in the presence of 5000U/ml SK, 10 or 100μg/ml of WPE for 5 days. Type 2 chemokines, CCL17 and CCL22, were measured by chemokine-specific ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test. NS= Not Significant when comparing media versus WPE stimulated condition. P> 0.05

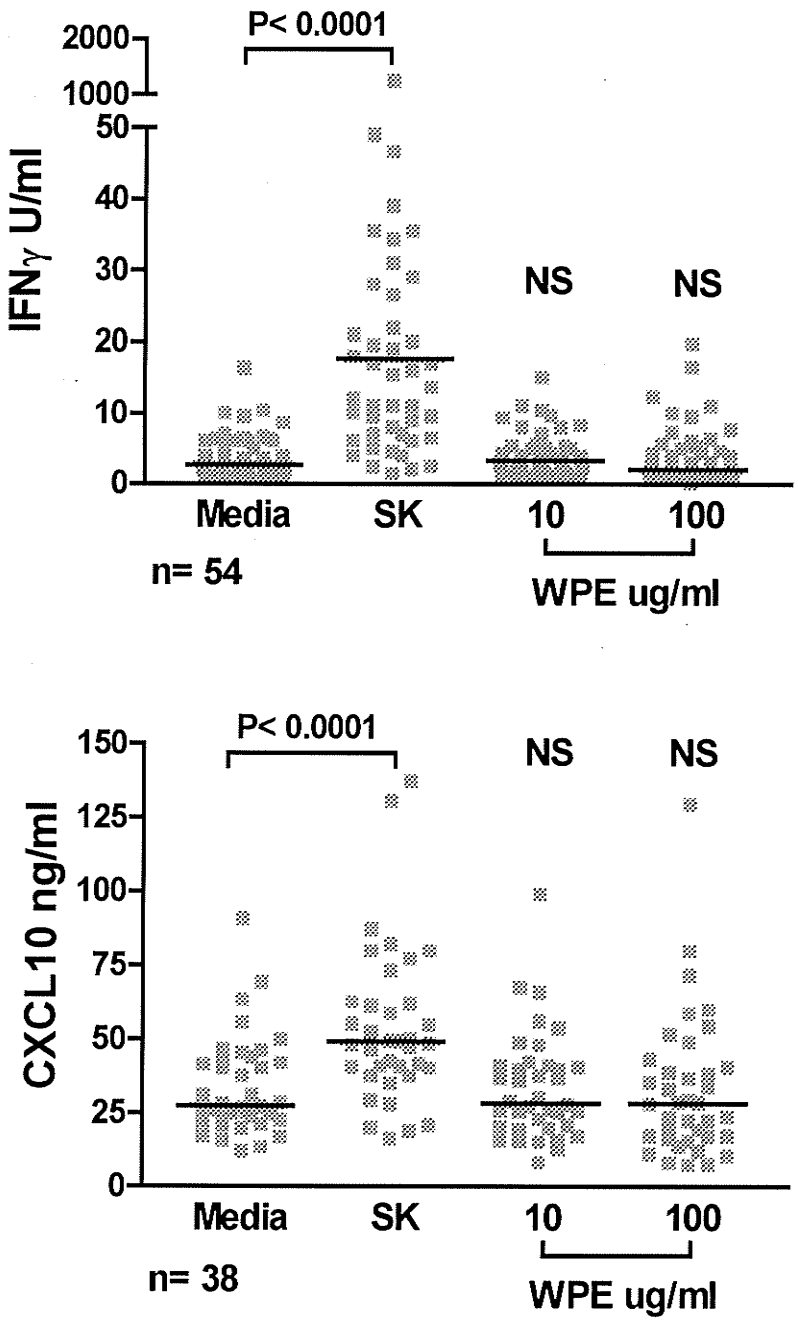

Figure 6: PBMC from Peanut Non-Allergic Individuals do not Demonstrate a Type 1 Peanut-Specific Cytokine or Chemokine Response to Whole Peanut Extract. PBMC freshly isolated from blood collected from peanut non-allergic individuals were cultured in the presence of 5000U/ml SK, 10 or 100μg/ml of WPE for 5 days. Type 1 cytokine, IFNγ, and chemokine, CXCL10, were measured by specific sandwich ELISA. NS= Not Significant when comparing media versus WPE stimulated condition. P>0.05

9.3.2 Ara h1 and Ara h2 Cytokine and Chemokine Responses in Non-Allergic Humans

Of the major peanut allergens, *Ara h1* and *Ara h2* are immunodominant, binding sera IgE from >90% of peanut allergic individuals (146, 147, 151). No studies describe a detailed analysis of cytokine and chemokine production by PBMC from peanut non-allergic individuals to these allergens. To address this issue, PBMC were cultured from peanut non-allergic individuals with concentrations of purified *Ara h1* or *Ara h2* between 15 and 150μg/ml for 5 days. Measuring type 1 and type 2 cytokine levels revealed that *Ara h1*-specific responses are type 2 dominated as demonstrated by increased IL-13 (at 150μg/ml) and CCL22 (at all concentrations tested) responses (Figure 7A). IL-5 production following *Ara h1* stimulation was no different than the characteristically low responses seen in unstimulated conditions (Figure 7A). Type 1 responses to *Ara h1* were only significantly different from media controls when cells were stimulated with 150μg/ml (Figure 7B). In contrast, peanut non-allergic individuals rarely showed an *Ara h2*-specific cytokine or chemokine in response (Figure 8).

Similar experiments using PBMC from peanut allergic individuals show that a type 2 response occurs at 150μg/ml of *Ara h1* (Figure 9A). Overall, a slight increase in IL-5 and no changes in IL-13, CCL22 production were observed in response to *Ara h2* at the 150μg/ml dose (Figure 9B). Similarly, no type 1 response was detected after stimulation with either *Ara h1* or *Ara h2*.

Collectively, these data indicate the presence and involvement of *Ara h1*-specific B and T cells in the production of *Ara h1*-specific type 1 and type 2 cytokine and chemokine production from peanut non-allergic individuals. The dominance of the *Ara h1* response, over the *Ara h2* response, demonstrates the importance of *Ara h1* in initiating peanut allergen responses.

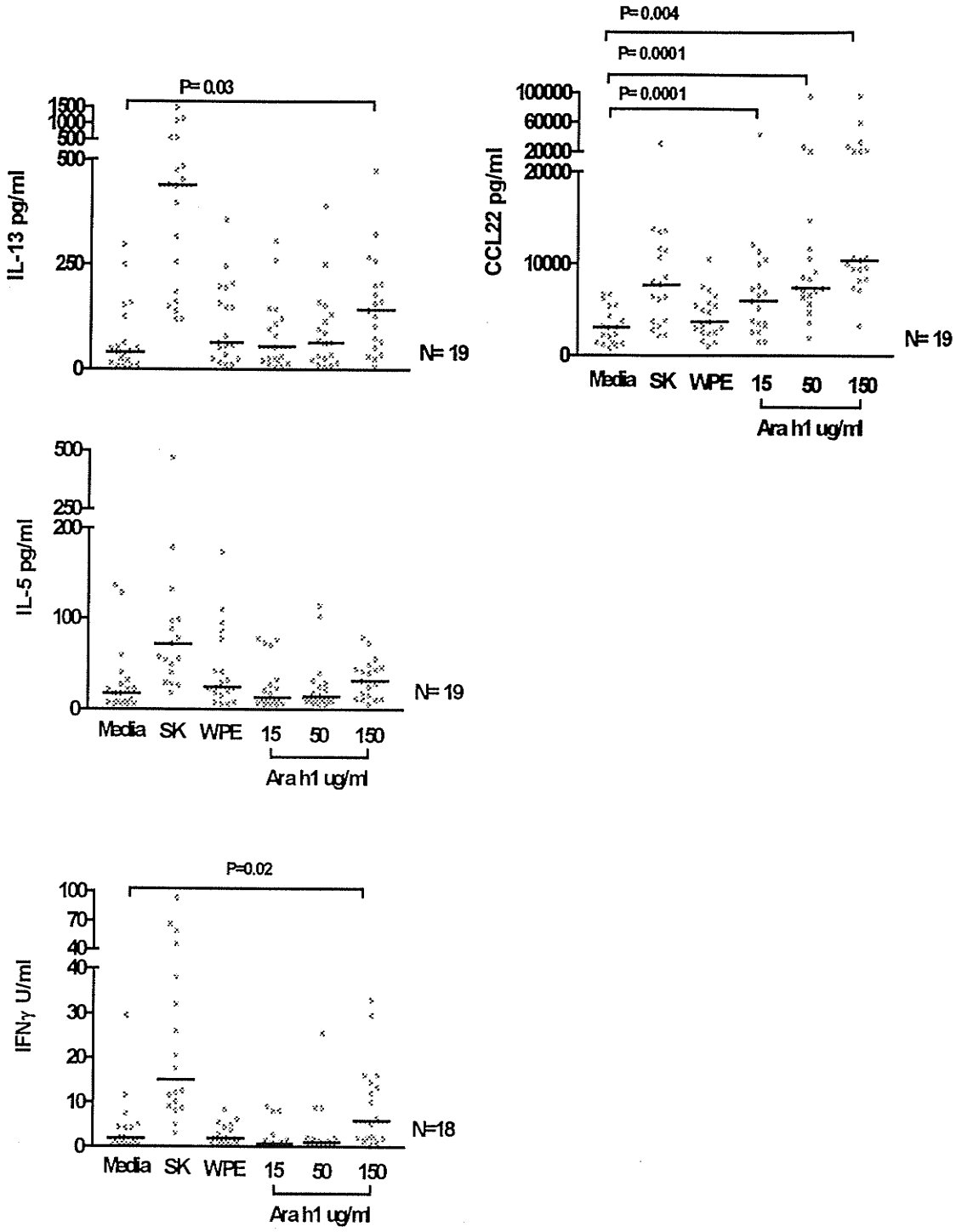

Figure 7: PBMC from Peanut Non- Allergic Humans Demonstrate an Antigen-Specific and Dose Dependant IL-13 and CCL22 response to the Major Peanut Allergen *Ara h1.* Freshly isolated PBMC from peanut non-allergic humans were cultured with SK (5000U/ml), WPE (10μg/ml) or *Ara h1* (15, 50 or 150μg/ml) for 5 days for the measurement of A) type 2 and B) type 1 cytokine and chemokine production. Cell supernatants were then analyzed by cytokine and chemokine specific ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

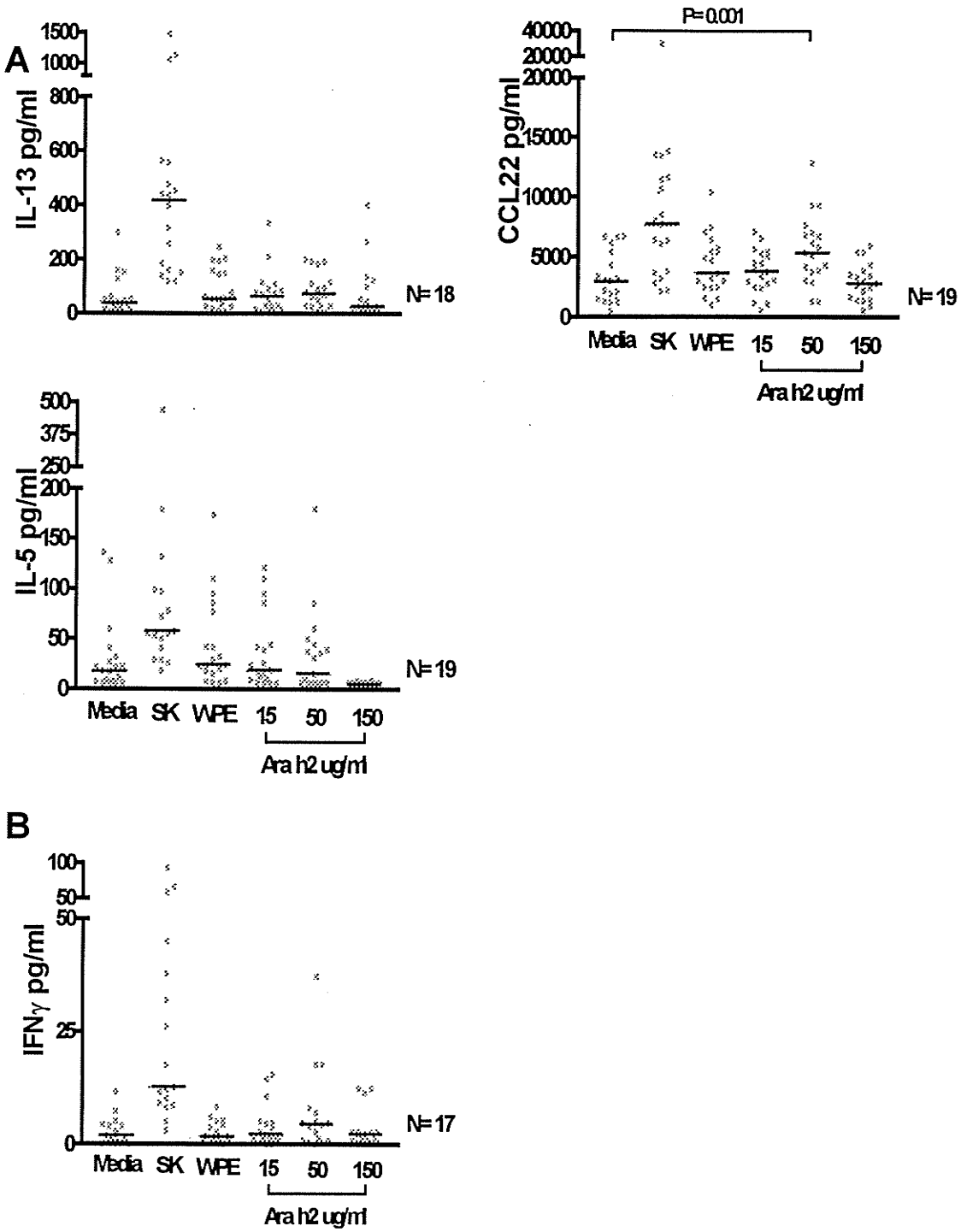

Figure 8: PBMC from Peanut Non-Allergic do not Demonstrate an Allergen-Specific Response to the Major Peanut Allergen *Ara h2.* PBMC isolated directly *ex vivo* were stimulated with SK (5000U/ml), WPE (10μg/ml) or *Ara h2* (15, 50 or 150μg/ml) for 5 days at which time supernatants were collected for cytokine and chemokine analysis of A) type 2 and B) type 1 cytokines and chemokines by ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

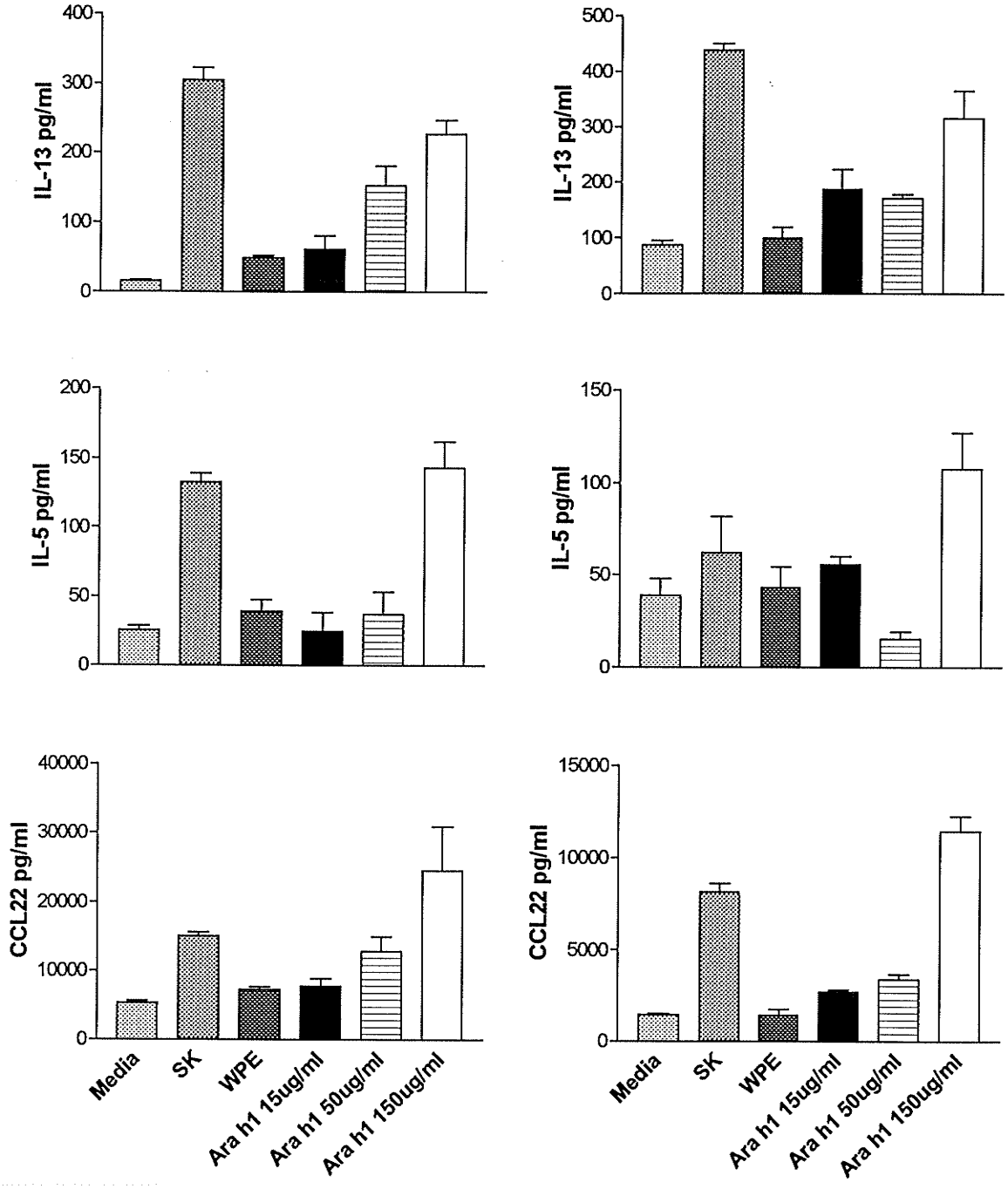

Figure 9A: *Ara h1* Specific Production of Type 2 Cytokines and Chemokines by Peanut Allergic Individuals. After stimulating PBMC isolated from blood of two peanut allergic humans with major peanut allergens *Ara h1* for 5 days, type 2 cytokines, IL-5 and IL-13, and chemokine CCL22 were measured by ELISA. Mean cytokine production (+/- SEM).

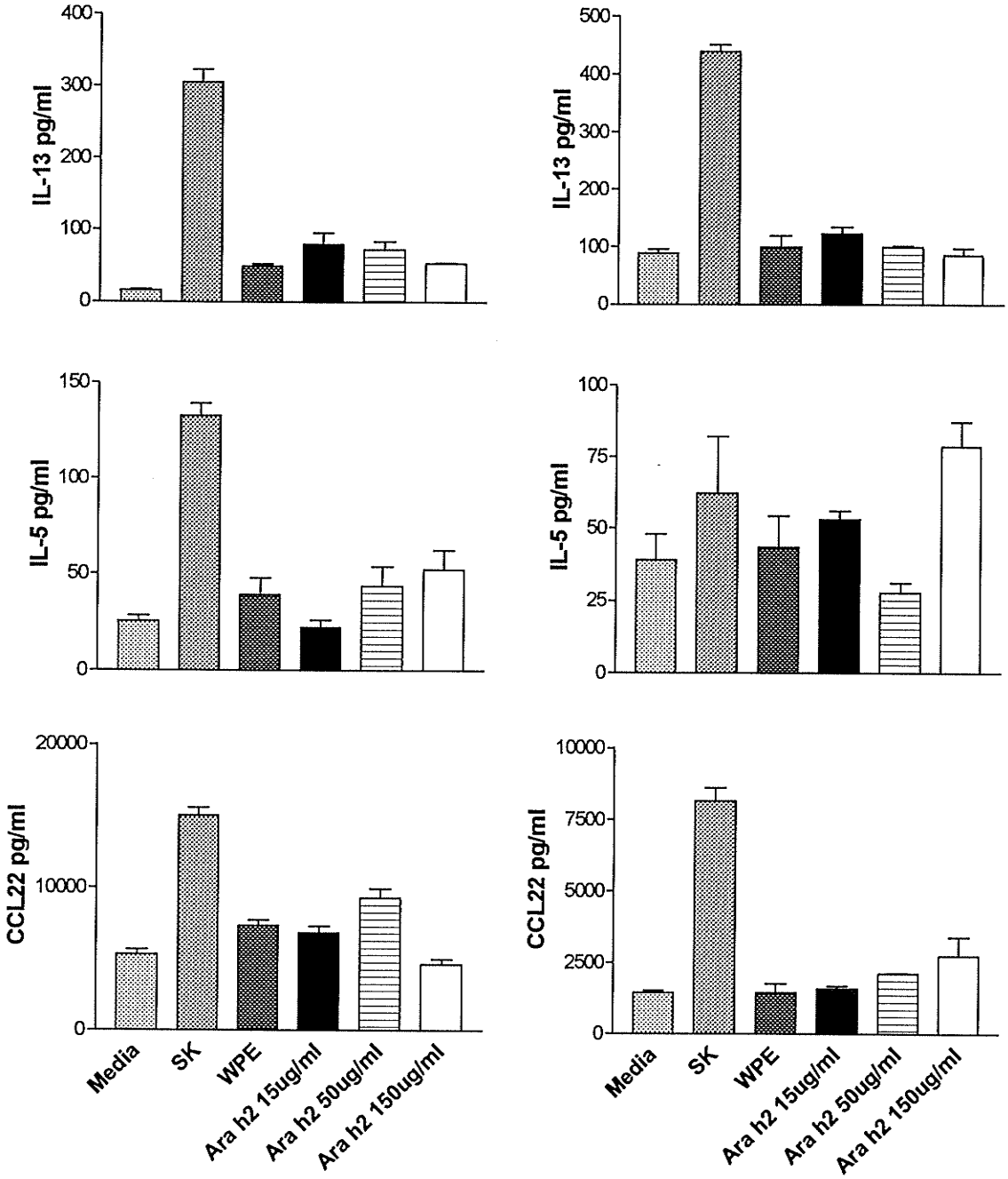

Figure 9B: Type 2 Cytokine and Chemokine Response of PBMC from Peanut Allergic Individuals in Response to *Ara h2*. After stimulating PBMC isolated from blood of two peanut allergic humans with major peanut allergens *Ara h2* for 5 days, type 2 cytokines, IL-5 and IL-13, and chemokine CCL22 were measured by ELISA. Mean cytokine production (+/- SEM)

9.4 Comparison of Peanut-Specific Cytokine and Chemokine Responses between Peanut SPT- and SPT+

As shown previously, the response to peanut allergen in this population of non-allergic individuals is characterized by a type 2 dominated cytokine response. Our peanut non-allergic population does not suffer from clinical symptoms although exposed to peanut, yet approximately 17% (10 of 58 peanut non-allergic individuals) exhibit positive skin prick test results to peanut. On the basis of skin prick test results, we classified our peanut non-allergic individuals into one of two groups: A) **Skin Prick Test Negative (SPT-)**: those who are skin prick test negative to peanut and experience no clinical symptoms upon the ingestion of peanut and B) **Skin Prick Test Positive (SPT+)**: those who demonstrate skin prick test positivity to peanut, yet experience no clinical symptoms despite regular exposure to peanut (negative clinical history). When comparing cytokine responses of the SPT- group (n~ 50 individuals) versus those of the SPT+ group (n~ 10 individuals), IL-5 and IL-13 responses were substantially more intense in the SPT+ group (Figure 10). When comparing peanut-specific chemokine responses, similar differences were also found based on sensitization. SPT+ individuals demonstrated significant increases in the intensity of the CCL22 response in comparison to SPT- individuals (Figure 11). Neither the SPT- nor the SPT+ group demonstrated statistically significant CCL17 response after stimulation with peanut extract (Figure 11). Upon examining IFNγ and CXCL10

responses, peanut-specific responses were not detected in either group or when comparing the SPT- to the SPT+ group (Figure 12).

When examining individual responses from SPT- individuals, up to 48% (23 of 48) exhibited either peanut-specific IL-5, IL-13 or CCL22 responses whereas 90% of SPT+ individuals (9 of 10) had a response as characterized by the production of one of these type 2 cytokines or chemokines after stimulation with peanut extract (data not shown). When examining each cytokine separately, the SPT+ group had responded with IL-5, IL-13 and CCL22 responses more frequently than the SPT- group ($p= 0.04$ to 0.002, Table 4). In addition to finding that the SPT+ group demonstrated a more intense type 2 response, up to 75% of SPT+ individuals had a concomitant IL-5, IL-13 and CCL22 response in comparison to approximately 25% of SPT- individuals (data not shown). Furthermore, when examining type 1 responses from both SPT- and SPT+ groups 0% of peanut non-allergic individuals showed an IFNγ or CXCL10 response (Table 4).

Taken together, data thus far indicates that a difference in the production of and frequency of type 2 responses can be distinguished based on skin prick test results of our peanut non-allergic group. The SPT+ group showed a more intense peanut-specific IL-5, IL-13 and CCL22 response. Furthermore, the SPT+ group responded with a type 2 cytokine or chemokine response more frequently than the SPT- group.

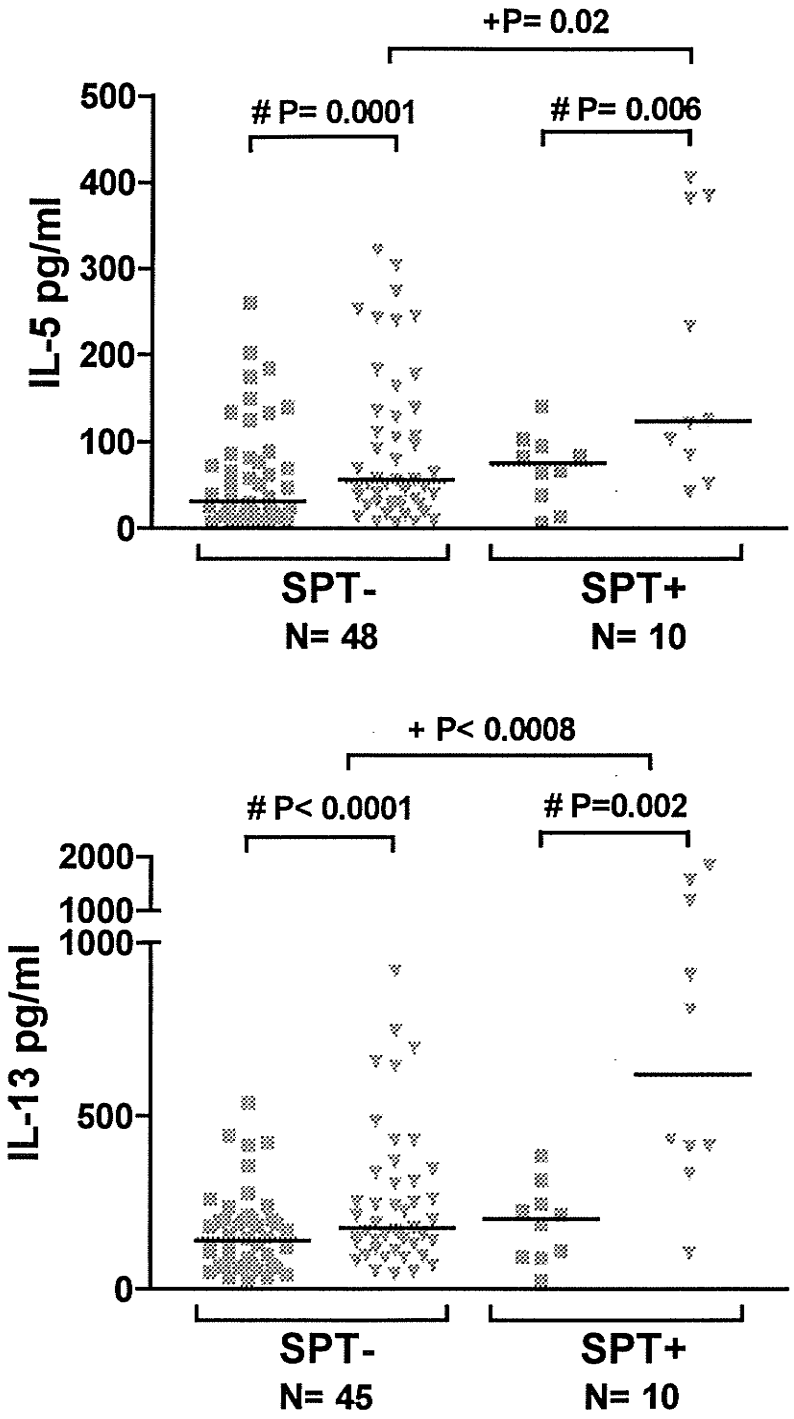

Figure 10: Skin Prick Test Negative (SPT-) and Skin Prick Test Positive (SPT+) Individuals Demonstrate Differential Intensities of Type 2 Cytokines IL-5 and IL-13 Responses. PBMC from blood collected from SPT- and SPT+ peanut non-allergic individuals were cultured in the presence of 5000U/ml SK, 10ug/ml or 100ug/ml of WPE for 5 days. Type 2 cytokines IL-5 and IL-13 were measured by specific sandwich ELISA. Statistics: Medians were compared using # Wilcoxon matched pairs test, + Mann-Whitney U test.

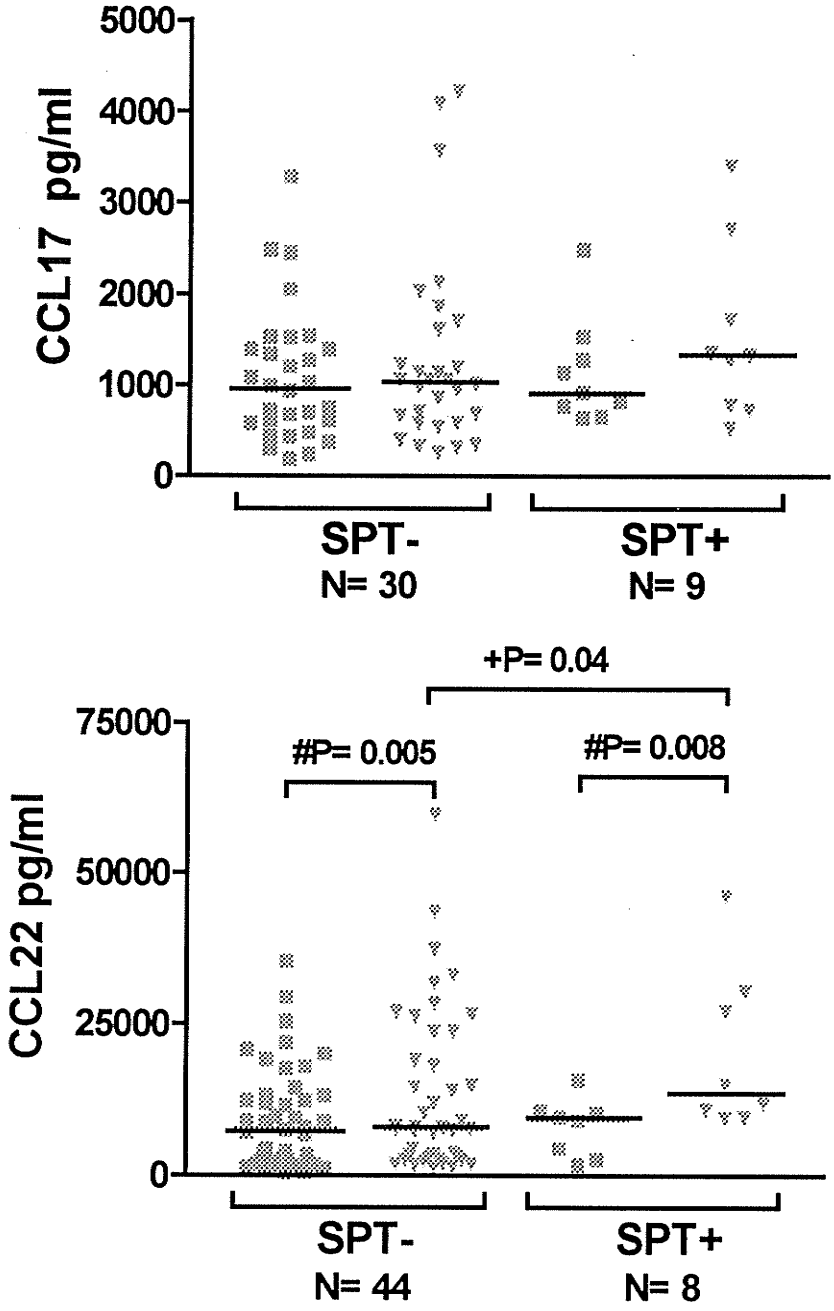

Figure 11: SPT- and SPT+ Individuals Demonstrate Differential Type 2 CCL17 and CCL22 Chemokine Responses. PBMC from blood collected from SPT- and SPT+ peanut non-allergic individuals were cultured in the presence of 5000U/ml SK, 10ug/ml or 100ug/ml of WPE for 5 days. Type 2 chemokines CCL17 and CCL22 were measured by specific sandwich ELISA. Statistics: Medians were compared using # Wilcoxon matched pairs test, + Mann-Whitney U test.

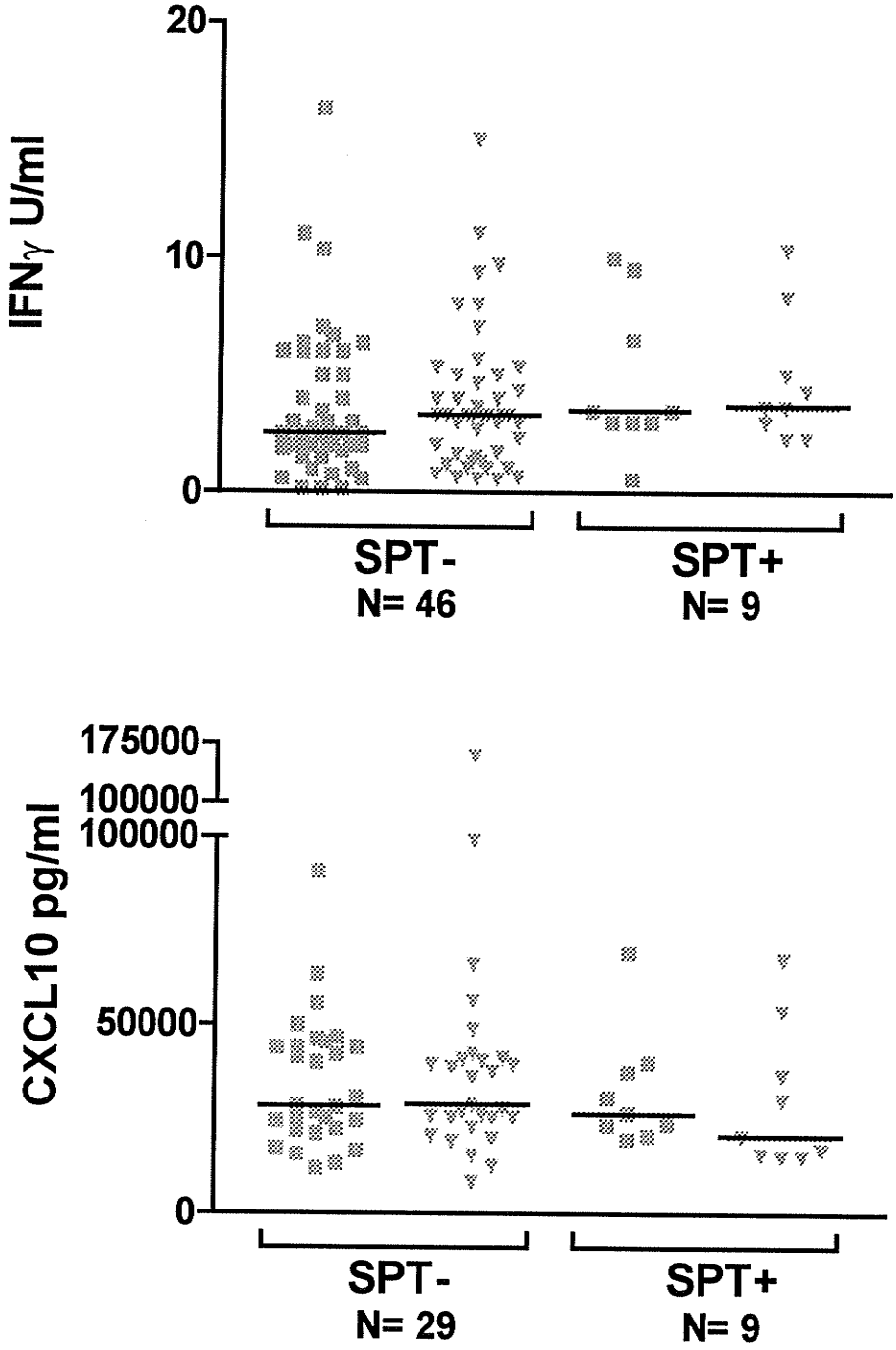

Figure 12: No difference in Type 1 Cytokine Production Between SPT- and SPT+ Peanut Non-allergic Individuals. PBMC from blood collected from SPT- and SPT+ peanut non-allergic individuals were cultured in the presence of 5000U/ml streptokinase, 10ug/ml or 100ug/ml of WPE for 5 days. Type 1 cytokines IFNγ and CXCL10 were measured by specific sandwich ELISA.

Table 4: Skin Prick Test Positive Individuals Demonstrate a Greater Frequency of Responders of Type 2 cytokines IL-5, IL-13 and CCL22 in Comparison to the Skin Prick Test Negative Group

Cytokine Test	Skin Test-	Skin Test +	Fisher's
IL-5	13/48	8/10*	*p= 0.002
IL-13	13/45	8/10*	*p= 0.004
CCL22	12/44	6/8*	*p= 0.02
IFNγ	0/46	0/9	
CXCL10	0/29	0/9	

9.5 T Cell and Antigen Presenting Cell Co-Stimulatory Molecule Requirements for Peanut-Specific Responses

Immune responses to exogenous antigen depend on optimal signaling through the TcR complex. The complex includes the binding of the TcR to antigen in the context of MHC class II and CD4 co-receptor-MHC class II molecule interactions. A second co-stimulatory signal from the APC is required for complete T cell activation. The ligation of APC co-stimulatory molecules, CD80 or CD86, to CD28 on T cells plays an important role in T cell activation. An alternative, and competitive ligand, for CD80 and CD86, CTLA-4, is commonly up regulated on T cells after activation resulting in down-regulation of potentially uncontrolled responses.

To investigate the immunological mechanisms that may control peanut-specific recall cytokine and chemokine responses in non-allergic humans and exclude the possibility of mitogenic or bacterial component stimulation being responsible for the cytokine production described above, peanut-stimulated PBMC were cultured with blocking antibodies against key co-receptors and co-stimulatory molecules required in the classical T cell/APC activation pathway (CD4, HLA-DR, CD80, CD86).

In the presence of antibody to CD4 or MHC class II molecule HLA-DR, peanut-specific IL-5, IL-13 and CCL22 responses by PBMC from peanut non-allergic humans are identical to or lower than responses from media control cultures

(Figure 13). These data indicate that the previously observed type 2 cytokine production is in response to peanut-specific stimulation and not an unspecific trigger such as mitogenic or bacterial proteins. In addition, the second co-stimulatory molecule signal involving CD80 and CD86 was required for IL-5, IL-13 and CCL22 production as demonstrated by significant decreases in cytokine production in the presence of αCD80, αCD86 or the fusion protein CTLA-4 Ig that blocks the active binding site for both CD80 and CD86 (Figure 13). Similarly, decreased peanut-specific IL-5 and IL-13 production was observed in cultures of PBMC from 2 peanut allergic individuals in the presence of antibodies to CD4, HLA-DR or co-stimulatory molecules CD80 and CD86 (Figure 14).

These data indicate that type 2 cytokine and chemokine production is elicited by peanut antigen ruling out potential contamination of the extract preparation. The dependence on the formation of the TcR complex involving CD4 and the recognition of peanut antigen-MHC class II, in addition to signaling through co-stimulatory molecules CD80 and CD86, is important in regulating peanut-specific cytokine and chemokine responses.

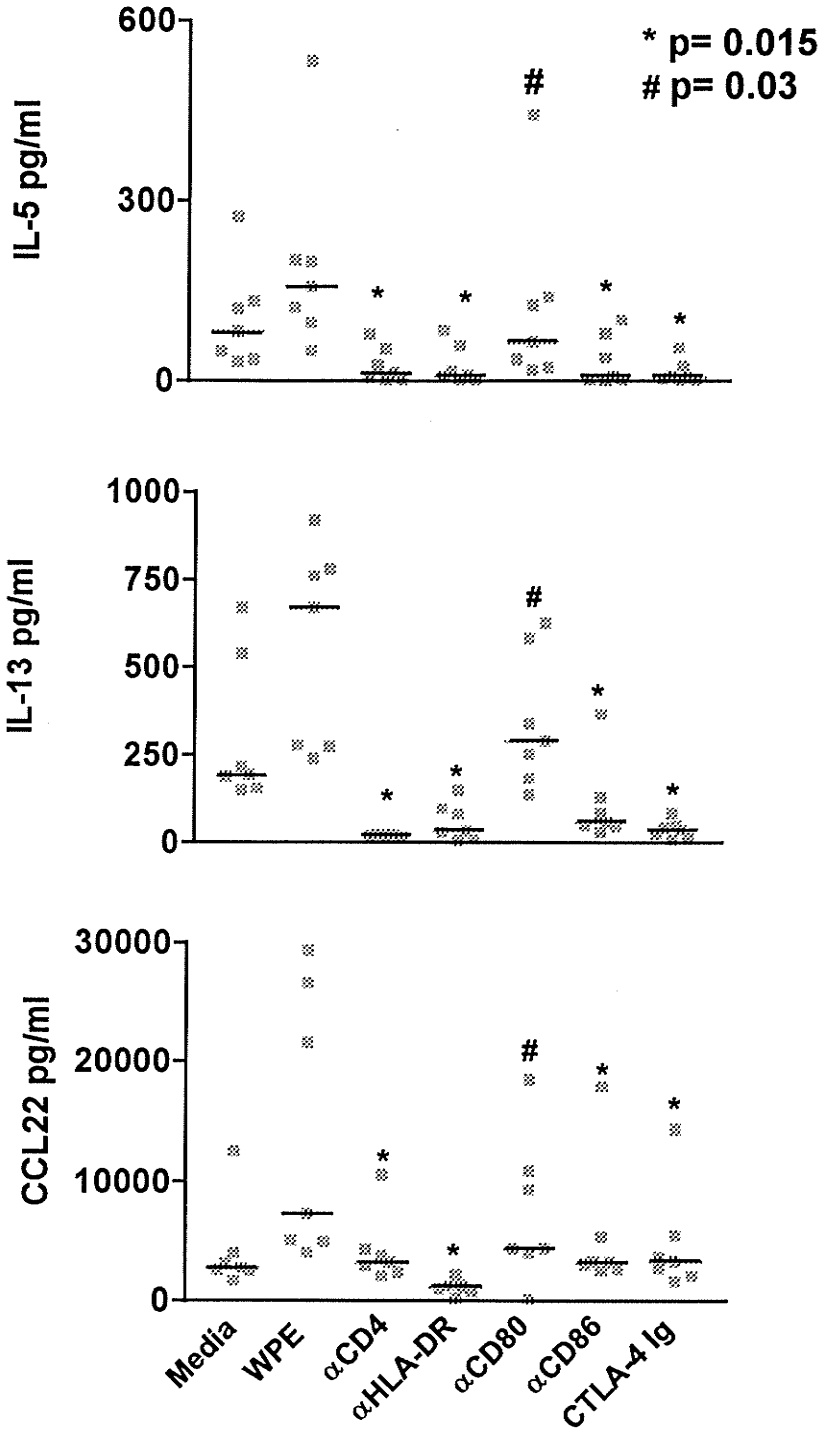

Figure 13: Peanut-Driven IL-5, IL-13 and CCL22 Production of PBMC from Peanut Non-Allergic Humans is Dependent on the Recognition of Peanut Allergen in the Context of MHC class II and CD4 and Co-Stimulatory Molecules CD80 and CD86. PBMC from 7 peanut non-allergic humans were stimulated *in vitro* with10ug/ml of WPE +/- antibodies to CD4, HLA-DR, CD80, CD86 or the fusion protein CTLA-4Ig for 5 days. IL-5, IL-13 and CCL22 were measured by ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

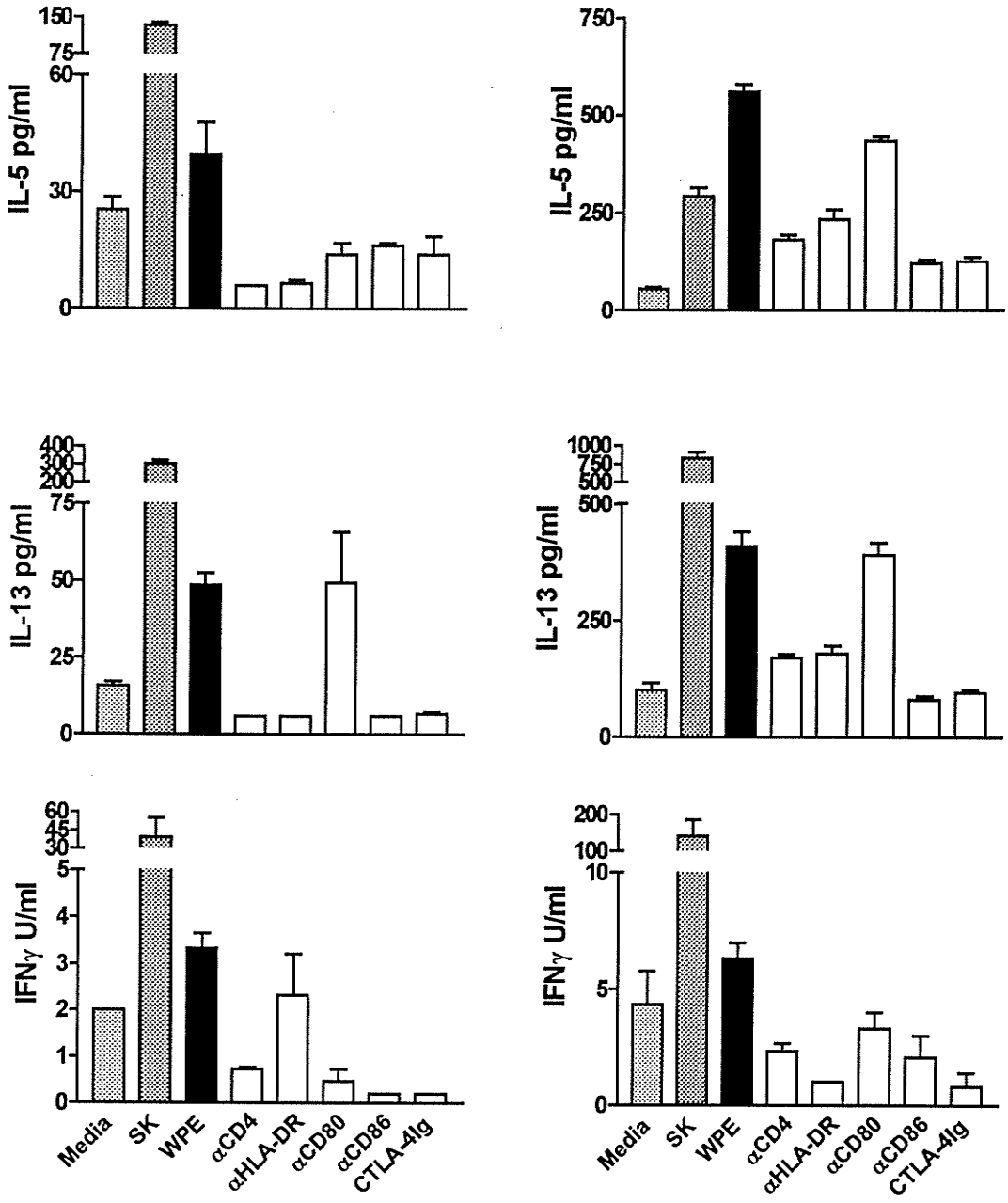

Figure 14: Peripheral Blood Mononuclear Cells from Peanut Allergic Humans Demonstrate Peanut-Specific T Cell Dependant IL-5 and IL-13 responses. PBMC isolated from fresh blood from peanut allergic individuals were cultured with SK (5000U/ml) or 10ug/ml of WPE +/- blocking antibodies to CD4 (2ug/ml), HLA-DR (2ug/ml), CD80 (5ug/ml), CD86 (1ug/ml) and CTLA-4 (5ug/ml). Cells were cultured for 5 days after which supernatants were collected and stored at -20°C until further analyzed by cytokine specific ELISA. Mean cytokine production (+/- SEM)

9.6 Differential Use of CD80 and CD86 in Peanut-Specific Cytokine and Chemokine Response

The role that CD80 versus CD86 play in directing Th1 and Th2 responses remains controversial. Some studies report that co-stimulation through CD86, but not CD80, results in the activation of Th2 immune responses (22, 181, 182). Although allergen stimulated responses are dependant on T cell activation through CD86, Th2 responses of recently activated T cells and Th clones are unaffected by blocking CD86 (22, 181, 183).

Preliminary data from this non-allergic population concerning co-stimulatory molecules suggests the differential dependence on CD86 versus CD80 ($p=0.015$ vs $p=0.03$, Wilcoxon matched pairs test, Figure 13) in the stimulation of IL-5, IL-13 and CCL22 peanut-specific cytokine production. To determine the requirement for CD80 versus CD86 for type 2 peanut-specific cytokine and chemokine production in peanut non-allergic individuals, PBMC from peanut non-allergic individuals demonstrated a type 2 peanut-specific response were cultured with peanut extract in the presence of antibody to CD80 and CD86. We found that type 2 peanut-specific cytokine production by PBMC from peanut non-allergic individuals was dependent on signaling through both CD80 and CD86. However, use of blocking antibodies to these co-stimulatory molecules showed αCD86 to consistently have a greater effect on cytokine production than αCD80 (Figure 15). Having previously determined differences in cytokine and chemokine intensity in response to peanut stimulation, we then decided to investigate

differences in the use of CD80 or CD86 for peanut-specific cytokine production by SPT- and SPT+ individuals. Comparing the differential dependence of cytokine responses on CD80 versus CD86 in the SPT- and SPT+ group yielded interesting results. Both αCD80 and αCD86 (to a greater extent) decreased peanut-driven IL-5 and IL-13 responses in SPT- individuals (Figure 16). In contrast, production of peanut-specific IL-5 and IL-13 recall responses by PBMC from SPT+ individuals was only inhibited by αCD86 (Figure 17). Blocking CD86 signaling results in the inhibition of peanut-antigen driven CCL22 production by both SPT- and SPT+ groups, while antibody to CD80 has no effect (Figure 16 and 17).

These data indicate that differential regulation of type 2 responses occurs between SPT- and SPT+ individuals. Only CD86 participates in stimulating IL-5, IL-13 and CCL22 production in SPT+ individuals. In contrast, IL-5 and IL-13 responses from SPT- individuals are dependent on both CD80 and CD86.

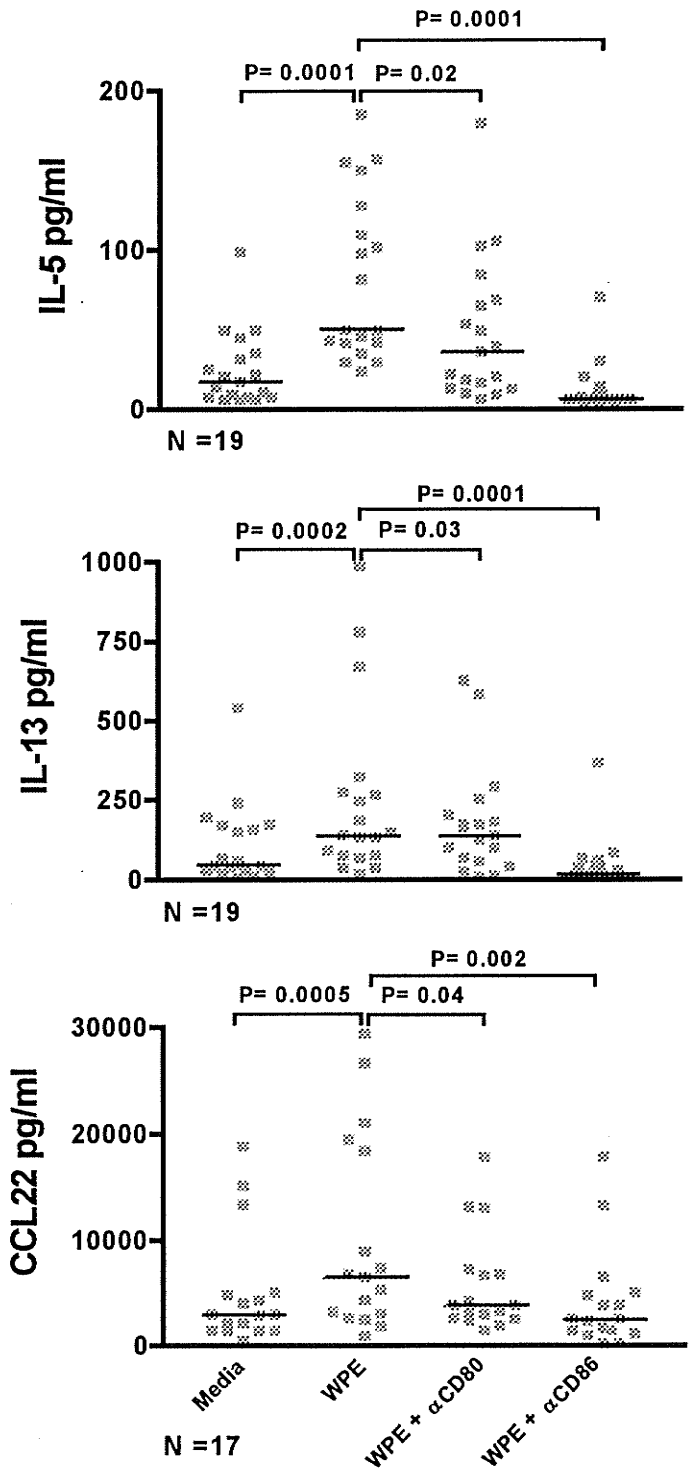

Figure 15: Peanut-Specific IL-5, IL-13 and MDC Production is Decreased in the Presence of αCD80 and to a Greater Extent by αCD86 in Peanut Non-Allergic Humans. PBMC from peanut non-allergic humans were cultured with whole peanut extract in the presence of antibody to either CD80 or CD86 and incubated for 5 days at 37°C. Supernatants were then analyzed for IL-5, IL-13 and CCL22 by ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

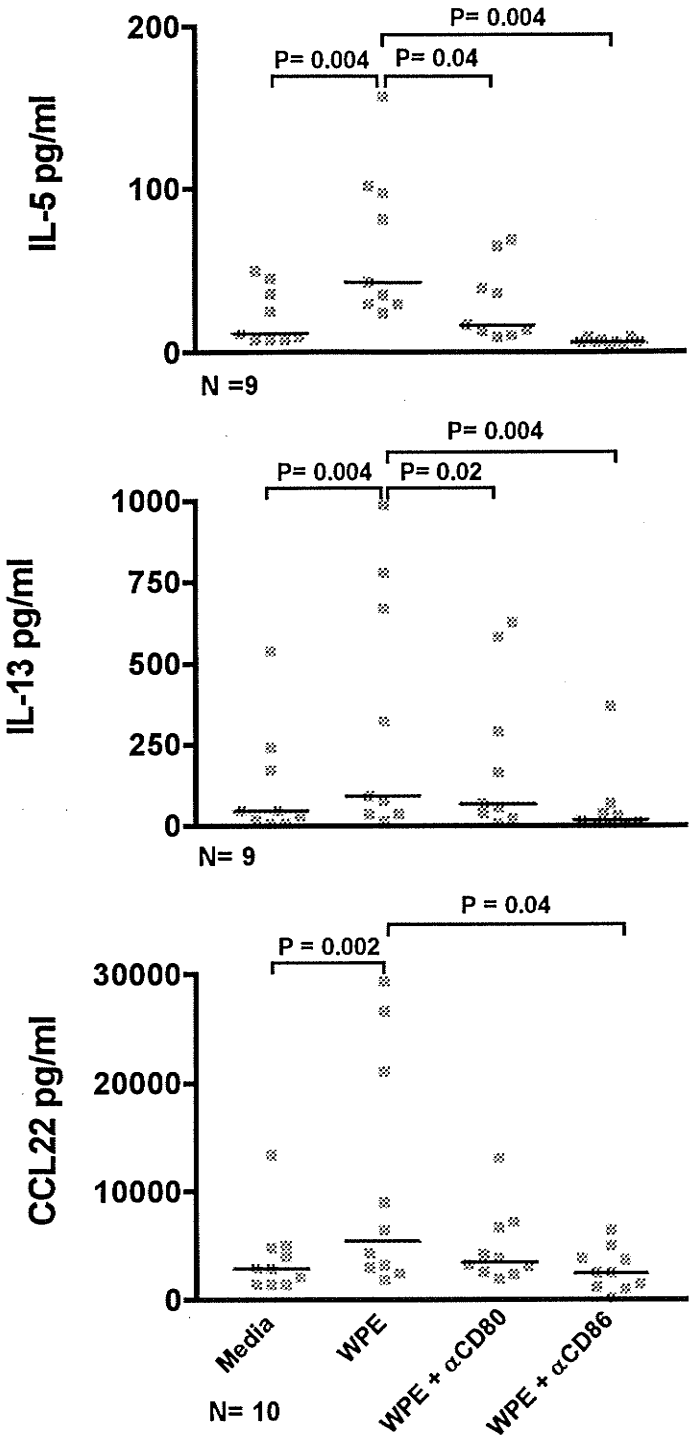

Figure 16: Peanut-Specific IL-5, IL-13 and CCL22 Production by Peanut SPT- Humans is Dependent on Co-Stimulatory Molecules CD80 and CD86. PBMCs isolated from the blood of peanut non-allergic humans were cultured with 10ug/ml WPE +/- blocking antibodies to CD80 or CD86. After 5 days, cell supernatants were collected for IL-5, IL-13, CCL22 and IFNγ analysis by ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

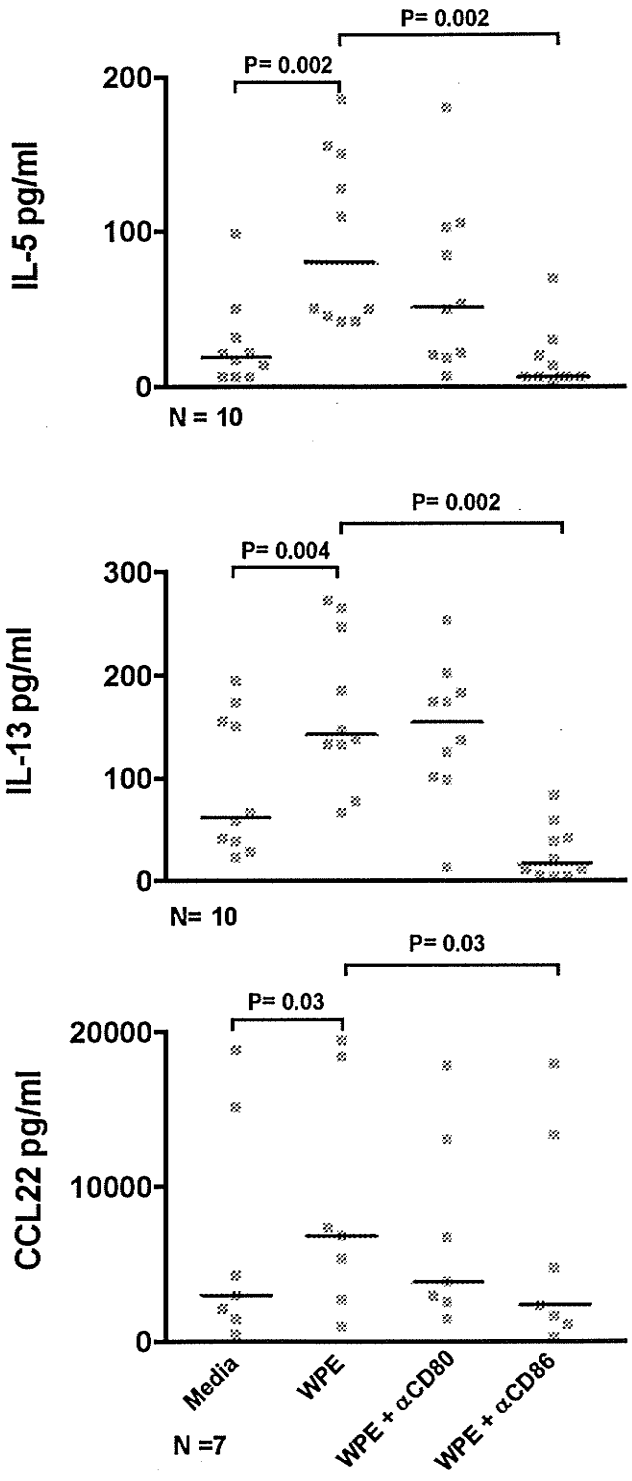

Figure 17: Peanut-Specific IL-5, IL-13 and CCL22 Production by Peanut SPT+ Humans is Dependent Only On Co-Stimulatory Molecule CD86. PBMCs isolated from the blood of peanut non-allergic humans were cultured with 10μg/ml WPE +/- blocking antibodies to CD80 or CD86. After 5 days cell supernatants were collected for analysis IL-5, IL-13, CCL22 by ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

9.7 Endogenous IL-10 and TGF-β Regulation of Type 1 and Type 2 Peanut-Specific Cytokine and Chemokine Production

High levels of IL-10 and TGFβ in Peyer's patches are hypothesized to play a role in the development of T regulatory cells in response to oral antigens and peripheral tolerance, thus contributing to the induction of tolerance to foods (48, 109, 115, 116). Immunosuppressive cytokines IL-10 and TGFβ have been shown to down-regulate production of both type 1 and type 2 cytokines. It remains unclear if cellular interactions, contributing to the development of T regulatory cells thought to contribute to oral tolerance, occur locally or systemically (184-186). No studies have been conducted on the effects of IL-10 and TGFβ on systemic responses to peanut allergens. We hypothesized that endogenous production of these immunosuppressive cytokines plays a role in the down regulation of peanut-specific cytokine and chemokine responses in peanut non-allergic people.

To test this hypothesis, PBMC were cultured from 24 peanut non-allergic individuals with peanut extract in the presence of neutralizing antibody to IL-10 and TGF-β. A significant increase in the production of peanut-specific IFNγ (Figure 18A) but no striking alterations in IL-5 and IL-13 production were found; however, peanut non-allergic individuals maintained a peanut-specific type 2 response (Figure 18B). In the presence of antibodies to IL-10 and TGFβ, the IFNγ response to streptokinase was markedly increased, the response being more intense than the peanut specific response. Neutralizing IL-10 and TGFβ in

peanut extract stimulated culture of PBMC from skin test negative individuals resulted in a significant increase in IFNγ production. In contrast, there was a global increase in cultures of PBMC from skin test positive individuals, including media alone controls and peanut stimulated conditions (Figure19A). The addition of αIL-10 and αTGFβ to culture had no demonstrable effect on any peanut-driven type 2 cytokine production in either skin test negative or skin test positive groups (Figure 19B).

Collectively, these data indicate that endogenously produced immunosuppressive cytokines IL-10 and TGFβ do not play a role in regulating the intensity of type 2 cytokine IL-5 and IL-13, but rather suppress a potentially undesired peanut-specific IFNγ response.

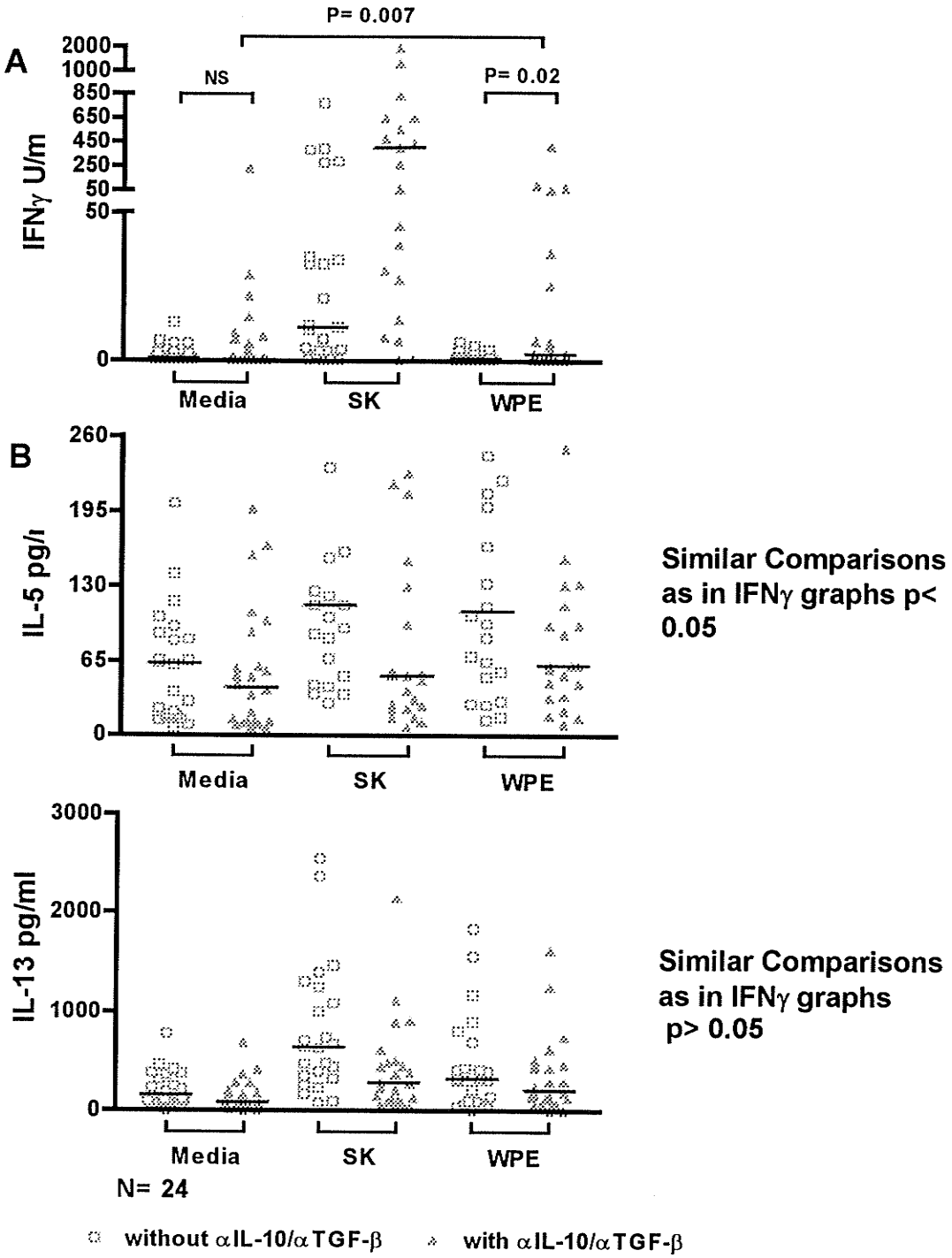

Figure 18: Peanut-Specific IFNγ Production is Markedly Increased in Peanut Non-Allergic Humans in the Presence of Antibody to Immunosuppressive Cytokines IL-10 and TGFβ. PBMCs, isolated directly *ex vivo* from peanut non-allergic humans, were stimulated with either SK (5000 U/ml) or WPE (10ug/ml) in the presence or absence of antibodies to IL-10 and TGF-β. Sample supernatants were examined for A) IFNγ B) IL-5 and IL-13

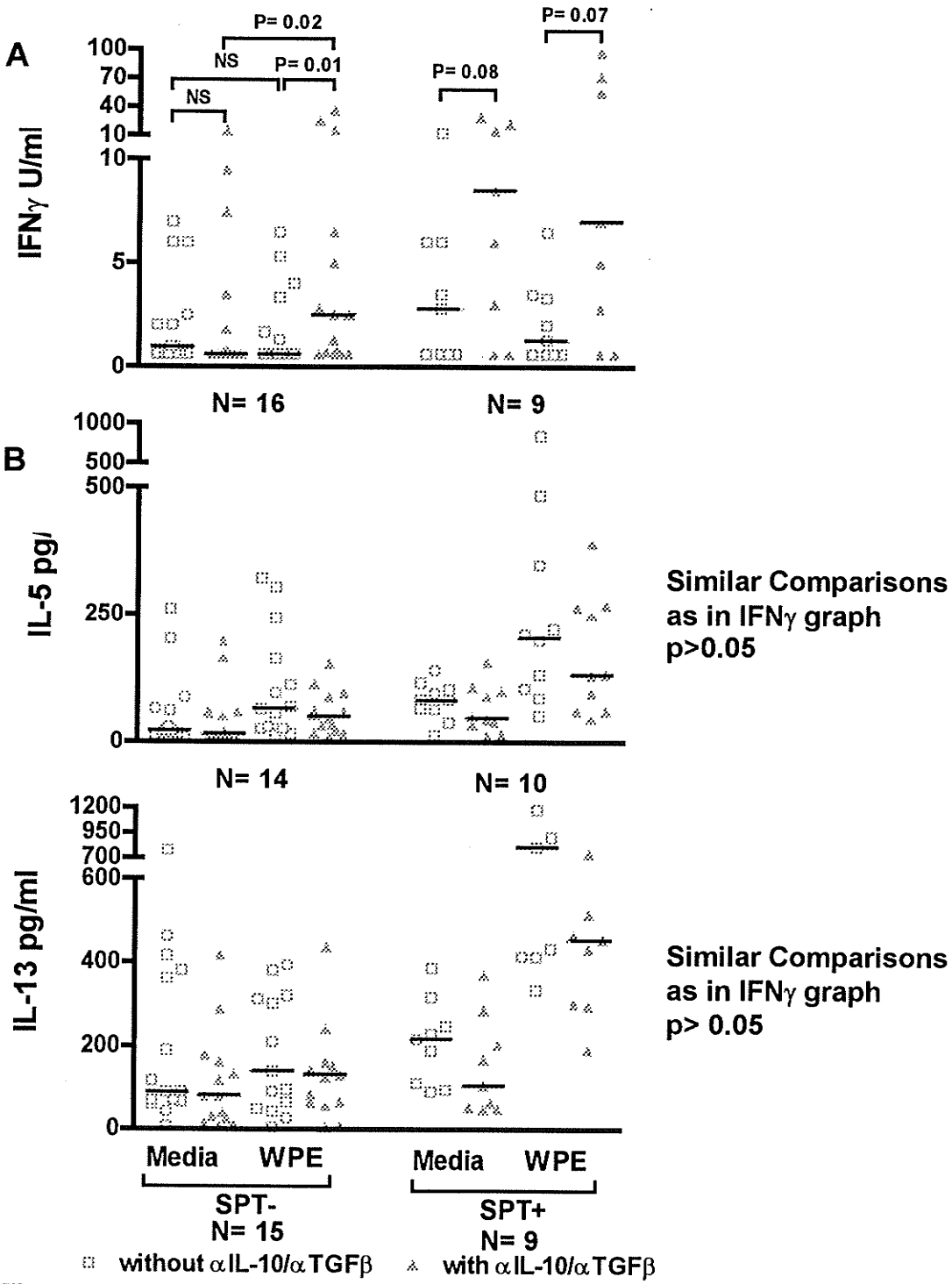

Figure 19: PBMC from Peanut SPT- Humans Demonstrate Markedly Increased Peanut-Specific IFNγ Production in the Presence of Antibody to IL-10 and TGFβ in Comparison to Peanut SPT+ Humans. PBMCs isolated directly *ex vivo* from peanut non-allergic humans were stimulated with either SK (5000 U/ml) or WPE (10μg/ml) in the presence or absence of antibodies to IL-10 and TGF-β. Sample supernatants were examined for A) IFNγ B) IL-5 and IL-13 production by cytokine-specific ELISA. Statistics: Medians were compared using the Wilcoxon matched pairs test.

8.8 Expression of Co-Stimulatory Molecules CD80, CD86 and CTLA-4 on PBMC from Non-Allergic Humans in Response to Peanut Allergen

Several reports indicate differences in peripheral blood APC surface expression of co-stimulatory molecules CD80 and CD86 depending on allergic status. Studies show that the percentage of $CD86^+$ B cells from allergic individuals can become up regulated using anti-CD40 and IL-4 (187). In addition, stimulation with cedar pollen extract results in the increased intensity of CD86 expression on B cells of allergic individuals (188). Although the up regulation of CD80 expression occurs on B cells from both allergic and non-allergic individuals, studies show that no differences in expression are seen after stimulation (187, 188).

Few studies have been carried out examining the differences in CD80 and CD86 surface expression on APCs other than on B cells after allergen stimulation. We decided to investigate differences in monocyte cell surface expression of CD80 and CD86 in response to peanut allergen stimulation as an explanation for peanut specific type 2 cytokine and chemokine responses exhibited by our peanut non-allergic population. We hypothesized that both the percentage of $CD14^+$ monocytes expressing CD86 and the intensity of CD86 on these cells would vary from high (peanut allergic), medium (non-allergic SPT+) to low (non-allergic SPT-) on PBMC following stimulation with WPE. To test this hypothesis, we cultured PMBC in media alone, LPS, or peanut extract for up to four days. APC cell surface expression of CD80 and CD86 was analyzed using two-color staining with flourochrome-conjugated antibodies by flow cytometry.

In preliminary experiments, CD80, CD86 and CD14 expression was determined following LPS stimulation. The intensity of CD14 expression on monocytes increased after 48 hours of stimulation with LPS. Although the intensity of CD80 expression did not change, the percentage of $CD14^+CD80^+$ monocytes increased (data not shown). In contrast, the intensity of CD86 and the percentage of $CD14^+CD86^+$ decreased in response to LPS stimulation (Figure 20).

Kinetic studies of CD80 expression in response to peanut extract show that the induction of CD80 occurred optimally on day 2. The percentage of $CD14^+$ peripheral blood monocytes expressing CD80 was slightly increased in skin test negative, skin test positive and peanut allergic individuals after peanut extract stimulation ($p= 0.04$ Table 5). On the contrary, constitutive expression of CD86 was seen on a high percentage of $CD14^+$ monocytes at a similar intensity on day 0 (data not shown) with no change seen after two days of peanut extract stimulation as compared to media controls (Table 5). Even after four days of culture with peanut extract, no detectable change in either the percentage of cells expressing CD86 or its' intensity was noted on CD14+ monocytes from either SPT-, SPT+ or peanut allergic individuals (Figure 20).

The alternative ligand for CD80 and CD86, CTLA-4, has been shown to down-regulate responses after T cell activation. We hypothesized that differential cell surface expression of the negative regulatory molecule CTLA-4 may play a role

in directing peanut-specific type 2 responses. Specifically, that CTLA-4 expression would vary in intensity with lymphocytes from non-allergic SPT- individuals displaying a high intensity, non-allergic SPT+ demonstrating a moderate intensity and peanut allergic exhibiting low to no cell surface expression of CTLA-4. Kinetic studies of PBMC stimulated showed that although Con A stimulated expression of CTLA-4 was measurable after 2 days, peanut induced CTLA-4 expression was practically undetectable even after 4 days of peanut extract stimulation. A small increase of CTLA-4 on $CD4^+$ T cells from two of three peanut non-allergic individuals was found whereas no increase was observed on $CD4^+$ lymphocytes from PBMC from peanut allergic individuals (Table 5).

Contrary to our hypothesis, we have shown that CD86 expression on $CD14^+$ monocytes did not change in response to peanut extract when we compared peanut non-allergic SPT-, peanut non-allergic SPT+ of peanut non-allergic and allergic individuals. Although, the majority of individuals (regardless of grouping) showed an increase in CD80 expression on monocytes suggests that CD80 may play a role in the regulation of responses to exogenous antigens.

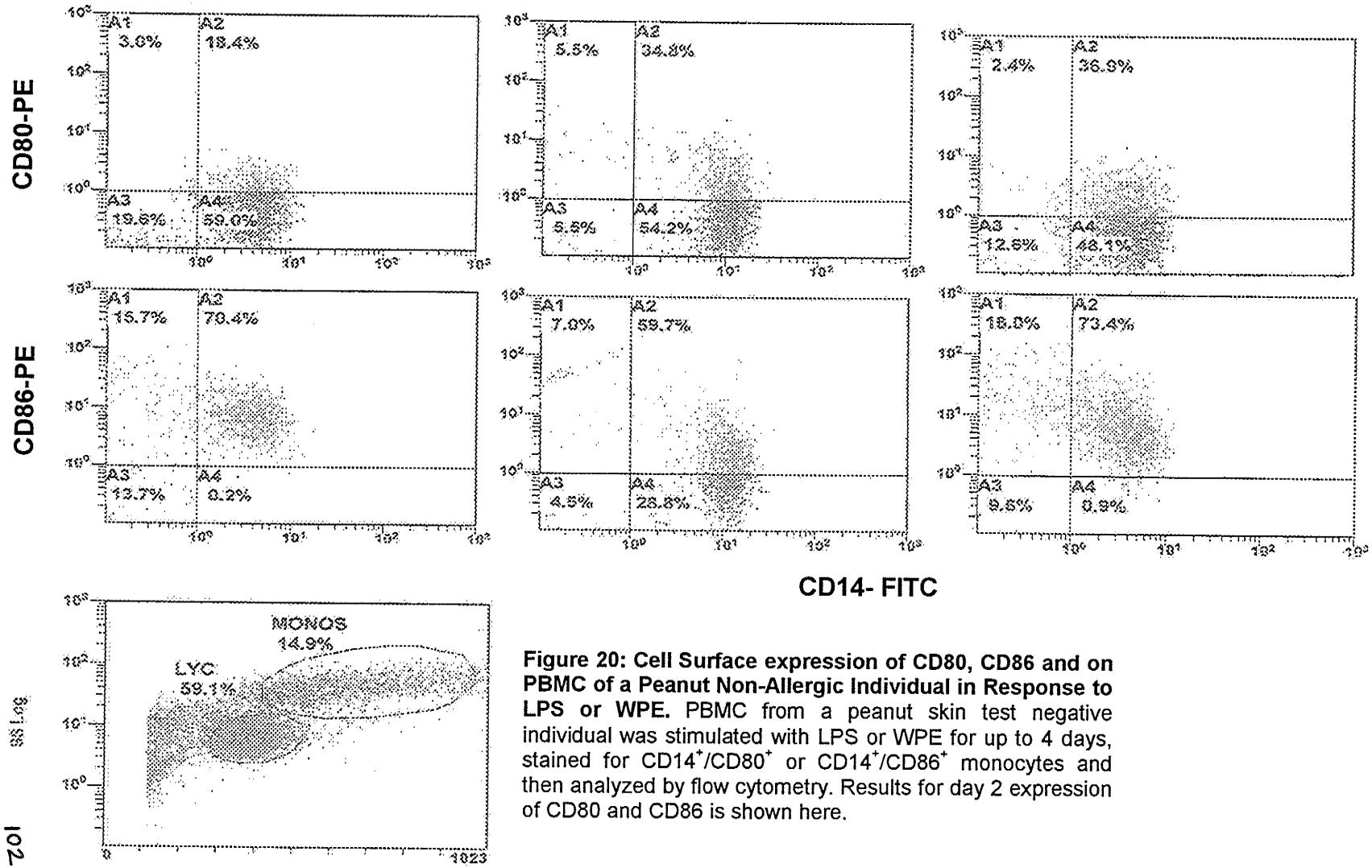

Figure 20: Cell Surface expression of CD80, CD86 and on PBMC of a Peanut Non-Allergic Individual in Response to LPS or WPE. PBMC from a peanut skin test negative individual was stimulated with LPS or WPE for up to 4 days, stained for $CD14^{+}/CD80^{+}$ or $CD14^{+}/CD86^{+}$ monocytes and then analyzed by flow cytometry. Results for day 2 expression of CD80 and CD86 is shown here.

Table 5: Cell Surface expression of CD80, CD86 or CTLA-4 in PBMC from SPT-, SPT+ and Peanut Allergic Individuals in Response to WPE. Cells were cultured with 10μg/ml of WPE for 2 days. Monocytes ($CD14^+$) and lymphocytes ($CD4^+$ or $CD3^+$) were then stained for the expression of CD80, CD86 and CTLA-4, as described in Materials and Methods, and then analyzed by flow cytometry.

Subject Group	N	% of CD80 Expressing $CD14^+$ Monocytes		% of CD86 Expressing $CD14^+$ Monocytes		% of CTLA-4 Expressing $CD4^+$ Lymphocytes	
		Media	*WPE*	*Media*	*WPE*	*Media*	*WPE*
Peanut Allergic	3	17.3	23.6	69.6	71.6	0.4	0.2
		7.7	10.3	67.0	62.1	0.6	0.6
		14.1	13.0	75.1	75.7	0.3	0.4
SPT+	2	24.9	23.6	81.2	74.9	38.2	38.4
		5.3	12.2	77.4	79.6	8.50	12.7
SPT-	3	34.5	35.9	69.3	68.2	0.2+	2.0+
		32.5	36.6	75.3	74.3	1.7+	2.0+
		18.4	36.9	70.4	73.4	7.90	16.3
Total Average	8	19.3	24.0 *	73.3	72.5	9.30	11.4

* p= 0.04 Wilcoxon matched pairs

+ measurement on $CD3^+$ lymphocyes, values not included in average

10 Discussion

10.1 Introduction

Peanut allergy is an immediate hypersensitivity reaction involving the production of peanut-specific IgE. Allergy to peanut may be associated ranging from mild hives to life threatening situations soon after ingestion, including laryngeal edema and systemic anaphylactic shock. Most often persisting into adulthood, only a small percentage of individuals may outgrow peanut allergy (10-20%). Although clinical manifestations of this allergy are well known, the immunological mechanisms determining the development of allergy versus clinical resistance to peanut require elucidation.

The opportunity to intensively research the pathophysiology of peanut allergy has been made possible through the characterization and cloning of peanut allergens. Although limited, studies thus far have focused on the characterization of *Ara h1, Ara h2* and *Ara h3* protein structure, PBMC proliferative responses, the identification of immunodominant IgE binding epitopes, and the characterization of the peanut-specific antibody response to peanut allergens from both peanut allergic and non-allergic individuals.

Cytokines and chemokines are important mediators of allergic disease. Their role in the pathogenesis of peanut allergy has not been thoroughly studied. Equally important, yet understudied, is the role of cytokines and chemokines in

mechanisms of tolerance and the protective response to peanut allergens. ***How do peanut non-allergic individuals respond to peanut allergen?***

The aim of this study was to characterize the immune response of ***peanut non-allergic humans*** to peanut allergen as defined by the production of peanut-specific type 1 and type 2 cytokines and chemokines. Focusing on PBMC responses from ***peanut non-allergic humans***, we sought to A) determine the prevalence of peanut-specific T cell responses in the general peanut non-allergic population B) characterize the type 1 (IFNγ and CXCL10) and type 2 (IL-5, IL-13, CCL17 and CCL22) immunoregulatory response profile to WPE and major allergens *Ara h1* and *Ara h2* C) determine the T cell and APC co-stimulatory requirements for the activation of peanut-specific cytokine production and D) determine the role of endogenously produced immunosuppressive cytokines, IL-10 and TGFβ, hypothesized to inhibit peanut-specific cytokine/chemokine production. We aimed to identify specific mediators that may contribute to the nature of the peanut-specific immune response in peanut non-allergic individuals and thus the subsequent development of, or absence thereof, of clinical sensitivity to peanut.

10.2 System Optimization for the Measurement of Peanut-Specific Cytokine and Chemokine Responses

Early studies show that PBMC from both peanut allergic and non-allergic humans proliferate in response to peanut extract in a dose dependant manner (157, 158). Studies examining T cell cytokine responses to peanut antigen characterize

cytokine profiles of T cell clones derived from peanut allergic and/or non-allergic individuals by measuring endogenous production or mRNA levels of IL-4 and IFNγ mRNA (156, 158, 161-163).

To date, studies of cytokine production in response to peanut-specific stimulation are limited. No studies have been carried out investigating peanut-specific chemokine responses in peanut allergic or non-allergic humans. Therefore, we developed and optimized a system in which we could measure these responses. Our preliminary studies, using *in vitro* short-term primary culture of PBMC isolated from blood of peanut-allergic and non-allergic individuals, showed that both groups demonstrated a detectable cytokine and chemokine response on day 5 to peanut extract in a dose dependant manner. Measurement of peanut stimulated (10 and 100μg/ml) type 1 (IFNγ and CXCL10) and type 2 (IL-5, IL-13, CCL17 and CCL22) responses showed that type 2 cytokines and chemokines are produced by PBMC from both peanut allergic and non-allergic individuals (Figures 1-3). Studies that demonstrate a dose dependent response to peanut allergen measure optimal proliferation over a seven day period at much higher concentrations of peanut extract between 200-300μg/ml (157, 158).

Having established a novel *in vitro* primary culture system and the utilization of sensitive ELISA assays to measure type 1 and type 2 cytokines we proceeded to examine the response to peanut antigen from a larger population of peanut non-allergic individuals.

10.3 Peanut-Specific Cytokine and Chemokine Responses by Peanut Non-Allergic Humans

10.3.1 Peanut Non-Allergic Humans Demonstrate a Type 2 Dominated Cytokine and Chemokine Response to WPE

Allergic individuals develop adaptive immune responses and display clinical symptoms to specific food allergens to which they have been previously exposed. Often, initial exposure to the food cannot be recalled but must have occurred at an early age. In contrast, current theories suggest that normal individuals mount an immune response against these allergens and in the end, become clinically tolerant to them. Several studies show that the response of non-allergic individuals can be characterized by a type 1 or a mixed Th0 mediator profile (189-192). Some studies indicate that the non-allergic profile can be described as type 1 dominated, while others show that non-allergic individuals can produce type 2 cytokine IL-4 in the absence of a substantial difference in IFNγ production as compared to allergic individuals (193). The demonstration of type 2 chemokine production (CCL17 and CCL22) by both allergic asthmatics and normal controls after house dust mite stimulation also provides evidence that non-allergic individuals can exhibit type 2 responses to allergen stimulation (194). Studies by Li *et al* show that non-atopic individuals demonstrate detectable IL-5 and IL-13 in response to grass allergen (195). In addition, both of these studies respectively suggest that although non-allergic individuals exhibit type 2 responses, these responses are not as strong as those of allergic individuals.

Few studies have focused on B or T cell responses of peanut non-allergic individuals to peanut. To begin our study, we examined the production of type 1 and type 2 cytokine and chemokine PBMC responses from a large population of peanut non-allergic individuals. We found that this response was defined predominantly by a type 2 response as characterized by the production of substantial amounts of peanut-specific type 2 cytokines including IL-5 and IL-13 (Figure 4). The production of peanut specific CCL22 with an absence of a peanut-specific CCL17 indicates the differential production of CCR4 chemokine family members (Figure 5). Using our assays to examine type 1 cytokine production, we were unable to detect differences in peanut-specific IFNγ and CXCL10 responses when comparing peanut stimulated and media control cultures (Figure 6). When examining peanut-driven IL-13 responses from peanut non-allergic individuals we found that peanut-specific IL-5 and IL-13 production by peanut non-allergic individuals were equal to or greater than grass pollen driven responses from both grass pollen allergic and non-allergic individuals as studied by Li *et al* (195). This indicates that peanut allergens could be considered more potent stimulators of cytokine production than aeroallergens such as grass pollen in both allergic and non-allergic individuals.

Our findings differ from those reported by Turcanu *et al* with regard to comparison of results from peanut non-allergic individuals. They report the isolation of peanut-specific lymphocytes through carboxyfluorescein succinimidyl

ester by flow cytometry. They show that cloned peanut-specific lymphocytes from non-allergic children and children who have outgrown their peanut allergy, display a Th1 phenotype after peanut extract stimulation characterized by high IFNγ and TNFα and low IL-4, IL-5, and IL-13 mRNA production (163). Although interesting, this study does not address the non-allergic response in terms of actively released cytokine from the cell in response to peanut extract. The strength of our study lies in our approach of measuring secreted cytokines and chemokines from freshly isolated PBMC in primary culture in response to peanut extract stimulation. Using our assays, we did not detect released type 1 cytokine after peanut extract stimulation.

10.3.2 The Importance of the Major Allergen Ara h1 in the Initiation of Peanut Non-Allergic PBMC Responses

Studies show that *Ara h2* is the predominant allergen in populations located in the UK, as demonstrated by 70% of peanut allergics having *Ara h2*-specific IgE compared to 35% with *Ara h1*-specific IgE (145). In contrast, studies in North America and the Netherlands report that greater than 70-90% of peanut allergic patients have *Ara h1* and *Ara h2*-specific IgE (146, 147, 151). One study examines *Ara h2* stimulated human PBMC cytokine responses. This study shows that *Ara h2* induces significantly higher levels of IFNγ in normal and peanut non-allergic asthmatics in comparison to peanut allergic individuals (160). No other studies have been carried examining human T cell cytokine or chemokine responses to these allergens. Thus, we addressed this issue by investigating the

peanut non-allergic cytokine and chemokine PBMC response to these major allergens.

When examining responses to individual purified peanut allergens in non-allergic individuals, we found *Ara h1*-specific (Figure 7) and very rarely, a detectable *Ara h2* specific response (CCL22 response at 50ug/ml of *Ara h2* (Figure 8). Similar to the WPE response, the peanut non-allergic *Ara h1* response (n=20) was Th2 dominated (IL-13, CCL22, Figure 7A). No detectable allergen-specific type 1 response (IFNγ) was observed (Figure 7B).

Upon examining allergen-specific responses from 2 peanut allergic individuals, we observed that *Ara h1*-specific IL-5, IL-13 and CCL22 responses were more intense than those found in non-allergic individuals (at 150ug/ml of *Ara h1*, Figure 9A) and *Ara h2*-specific responses were very low or undetectable (Figure 9B). Allergen-specific IFNγ responses were undetectable or similar to media alone conditions (data not shown).

Our observation of PBMC responses from peanut non-allergic and peanut allergic individuals to the major peanut allergens suggests the immunodominance of *Ara h1* in comparison to the *Ara h2* response. This argues for the importance of *Ara h1* in initiating peanut allergen responses through interactions between allergen-specific B cells and T cells in peanut non-allergic and allergic individuals.

Our results thus far argue that non-allergic individuals can display an immunological response to peanut extract and its major allergen *Ara h1* and are not necessarily allergen non-responsive or ignorant. The presence of a peanut-specific type 2 response in peanut non-allergic individuals, normally associated with the allergic response, suggested to us that a continuum of responses might potentially exist in determining the development of allergic disease to peanut.

10.4 Differential Cytokine and Chemokine Responses Comparing Skin Test Positive Group *vs.* the Skin Test Negative Group

Although the number of peanut allergic individuals we tested was small, we noted that their type 2 responses could be more intense than those observed from the general peanut non-allergic population (Figure 3 and 14). We divided our population of non-allergic individuals into two groups based on skin prick test results to peanut. **Skin prick test negative (SPT-)**; those who are skin test negative and asymptomatic to peanut and **skin prick test positive (SPT+)**; individuals who are characterized by a positive skin test to peanut and have a negative clinical history, despite regular exposure, to peanut. We hypothesized that a difference in type 2 cytokine and chemokine production might also exist between these two groups of peanut non-allergic individuals.

When comparing the SPT- group (n~ 50 individuals) to the SPT+ group (n~10 individuals) we found that IL-5, IL-13 and CCL22 responses by SPT+ individuals

were substantially more intense than responses of SPT- individuals after WPE stimulation compared to media controls ($p< 0.04$ to $p< 0.0008$, Figures 10 & 11). Comparison of CCL17 responses between SPT- and SPT+ individuals showed no difference in chemokine production between media and peanut-stimulated conditions (Figure 11).

As an alternative approach, we chose to examine the frequency of type 2 responses among SPT- and SPT+ individuals. When examining each cytokine separately, we found that the SPT+ group had responded with IL-5, IL-13 and CCL22 responses more frequently than the SPT- group ($p= 0.04$ to 0.002, Table 4). In data not shown, we determined that up to 48% of SPT- individuals (23 of 48) versus 90% of SPT+ individuals (9 of 10) showed a type 2 response, as characterized by the production of either IL-5, IL-13 or CCL22, suggesting that a substantial proportion of SPT+ peanut non-allergic individuals demonstrate a commitment to type 2 response. Furthermore, up to 75% of SPT+ individuals demonstrate a simultaneous IL-5, IL-13 and CCL22 response in comparison to only 25% of SPT- individuals. Additionally, when examining type 1 responses, 0% of peanut non-allergic individuals showed a response as measured by IFNγ or CXCL10 (Table 4).

When we compare the three different groups in terms of the intensity of cytokine and chemokine production, we see that SPT- individuals have a demonstrable but milder type 2 cytokine response compared to SPT+ individuals (Figures 10 &

11). The responses from peanut allergic individuals were more intense than responses from either clinically non-allergic group. Differences in cytokine and chemokine production may serve as one of many other factors in the determination of the absence of manifestation of clinical allergy to peanut. These data are important as evidence testing our initial hypothesis that a continuum of immune responses to peanut exists between peanut SPT- and SPT+ individuals.

10.5 Peanut-Specific Cytokine and Chemokine Responses are CD4 and MHC Class II Dependent in Peanut Non-Allergic Humans

To investigate the immunological mechanisms controlling peanut-specific cytokine and chemokine recall responses observed in non-allergic humans, we considered the importance of interactions between T cells and APCs in this process. Non-specific activation by mitogens or by bacterial protein through contamination can occur. Thus to exclude these possibilities, we sought to confirm peanut-specific T cell activation by allergenic proteins in our peanut extract by examining the involvement of T cell co-receptor CD4 and the APC MHC class II molecule, HLA-DR, in the production of peanut-specific cytokine by PBMC from non-allergic humans.

Although no other study has directly examined the role of CD4 or HLA-DR in peanut-specific cytokine responses in either peanut allergic or non-allergic humans, studies have shown differential frequencies of HLA-DR haplotypes in human peanut allergic groups (166, 167). This suggests the involvement of

different HLA-DR haplotypes in the presentation of peanut allergen immunodominant epitopes and thus, in the absence or development of peanut allergy. In our study, the addition of antibody to CD4 and MHC class II molecule HLA-DR to peanut-stimulated cultures, we observe marked decreases of IL-5, IL-13 and CCL22 recall responses to levels that are identical to or lower than those measured from media alone cultures (Figure 13). Similarly, examination of two peanut allergic individuals showed decreased peanut-specific IL-5, IL-13 and CCL22 responses (Figure 14). We show for the first time that TcR/CD4 recognition of peanut allergen peptide in the context of MHC class II molecules is essential for peanut-specific T cell cytokine and chemokine production in non-allergic individuals.

10.6 CD86 Regulation of Peanut-Specific Cytokine and Chemokine Responses in Non-Allergic Humans

As previously discussed in the *Introduction* and *Results* section, the association of CD80 with type 1 and CD86 with type 2 responses remains controversial. The production of type 2 cytokines has been linked to the preferential involvement of CD86 versus CD80 (22, 181, 182). Alternatively, studies by Kuchroo *et al* investigate the role of co-stimulatory molecules in diabetes and show that CD80 is a major player in the regulation of type 2 cytokine production, IL-4 (196). The role of CD80 and CD86 has not been examined in T cell responses to any food allergens.

In preliminary experiments, we sought to examine co-stimulatory molecule requirements for peanut-specific cytokine and chemokine production in peanut non-allergic humans by blocking these molecules with specific antibody and the fusion protein CTLA-4Ig. Initial studies showed that when we blocked both CD80 and CD86, through the addition of CTLA-4Ig or directly with antibody, peanut-specific IL-5, IL-13 and CCL22 responses were inhibited to background (media) levels (Figure 13). Although we demonstrated the dependence of peanut-specific cytokine and chemokine production on both CD80 and CD86, we also found that antibody to CD86 had a greater effect than antibody to CD80 in blocking this production after peanut extract stimulation (Figure 13).

A similar observation was made when a larger population was examined, confirming the involvement of both CD80 and CD86 but that blocking CD86 had a greater effect on inhibiting peanut-specific IL-5, IL-13 and CCL22 production than blocking CD80 (Figure 15). We hypothesized that differences in co-stimulatory requirements existed between SPT- and SPT+ individuals. Differential co-stimulatory requirements were identified between SPT- and SPT+ individuals, the latter depending solely on CD86 for IL-5, IL-13 and CCL22 production (Figures 16 and 17). Our findings are important because they indicate that, in contrast to the literature, both CD80 and CD86 participate in regulating peanut-specific Th2 cytokine production from PBMC of non-allergic individuals. Additionally, finding that CD86 regulates Th2 cytokine production by peanut SPT+ individuals independently of CD80 indicates differential co-stimulatory

requirements for cytokine production between SPT+ and SPT- and thus, may be an underlying mechanism determining clinical sensitivity versus clinical unresponsiveness.

10.7 IL-10 and TGFβ Regulates Type 1 Peanut-Specific Responses In Peanut Non-Allergic Humans

The cytokines IL-10 and TGFβ are multifunctional in nature and are produced by a large variety of cells. They have the ability to promote or inhibit cell development and immunological processes depending on their environment. They play a significant role in the development of tolerance to oral antigens and peripheral tolerance, in the regulation of autoimmune diseases and the elimination of parasitic infections through down regulation of either type 1 or type 2 responses.

We have demonstrated that non-allergic humans display a detectable systemic T cell dependent response to peanut that is dominated by type 2 cytokine and chemokine production. We postulated that systemic production of IL-10 and TGFβ regulates peanut non-allergic human type 1 and type 2 responses to peanut antigen. Specifically, we hypothesized IL-10 and TGFβ played a role in suppressing an unregulated type 2 response to peanut allergen in non-allergic humans. To test this, we cultured PBMC with WPE in the presence or absence of antibody to IL-10 and TGFβ and then measured indicators of type 1 and type 2

responses. We found that peanut-specific IL-5 and IL-13 production was not significantly affected by the addition of αIL-10/TGFβ to culture, maintaining a detectable peanut-specific response of unchanged intensity (Figure 18B). Instead, we found a detectable peanut-specific IFNγ response after neutralizing IL-10 and TGFβ in vitro (Figure 18A). When comparing responses within the SPT- group, we found that IFNγ responses were modestly increased after treatment with αIL-10/TGFβ. Responses from the SPT+ group showed increased, although not peanut-specific, levels of IFNγ (Figure 19).

In data unpublished by Ulczak *et al*, PBMC responses of non-allergic individuals to β-lactoglobulin showed that detectable IFNγ and IL-13 responses were similarly affected in the presence of antibody to IL-10 and TGFβ. In the presence of antibody to IL-10 there was up to an 80% increase in β-lactoglobulin specific IFNγ production whereas in the presence of antibody to TGFβ alone the increase was detected but to a lesser degree. Culturing cells with antibody to both IL-10 and TGFβ resulted in up to 20% further enhancement of the β-lactoglobulin IFNγ response. In contrast, the β-lactoglobulin IL-13 response was unaffected in the presence of antibody to either IL-10 or TGFβ.

We have demonstrated that peanut non-allergic individuals exhibit no peanut-specific IFNγ response. Collectively, these data suggest that IL-10 and TGFβ may be suppressing a potentially undesired systemic type 1 response to peanut in this group. More importantly, the production of endogenously produced IL-10

and TGFβ does not regulate peanut-specific type 2 (IL-5 and IL-13) responses in peanut non-allergic humans.

10.8 Differential Expression of Co-Stimulatory Molecules in Response to Peanut Extract

Cell surface molecules can become up regulated or down regulated facilitating T cell and APC activation. Differential expression of co-stimulatory molecules CD80, CD86 and CTLA-4 has been associated with the pathogenesis of both autoimmune and allergic disease (197, 198). Several studies examine B cell expression of CD80 and CD86 in response to allergen stimulation but few studies have documented the expression of these molecules on monocytes (188, 199). CD86 is constitutively expressed at low levels on APC whereas CD80 expression is induced after cell activation (200). Other approaches include examining allergen stimulated cell surface expression of these co-stimulatory molecules on monocyte-derived dendritic cells. Studies by Hammad *et al* found that cell surface CD86 was increased on house dust mite allergen, *Der p1*, pulsed dendritic cells (201).

In order to determine the kinetics of cell surface expression of these co-stimulatory molecules on monocytes, LPS was used as a stimulant based on its effect of up regulating CD80 and CD86 expression on APCs (202, 203). We found that the percentage of $CD14^+$ monocytes expressing CD80 increased and the percentage and intensity of CD86 decreased (Figure 20). LPS is known to

induce strong type 1 responses (204) thus, our findings of increased CD80 and decreased CD86 surface expression supports the hypothesis of the association of preferential use of CD80 in type 1 responses and CD86 in type 2 responses.

We have shown that differential intensities of type 2 peanut-specific cytokine exist between peanut non-allergic SPT- and SPT+ individuals. We hypothesized that this, in part, could be explained by differential monocyte surface expression of CD80 and CD86 after peanut extract stimulation. Contrary to our initial belief, no change occurred in the intensity of CD86 or the percentage of $CD14^{+}CD86^{+}$ monocytes over two days in response to WPE (Table 5). To our surprise, PBMC stimulated with WPE for 2 days resulted in an increase in the percentage of $CD14^{+}CD80^{+}$ cells from peanut allergic, non-allergic SPT+ and non-allergic SPT- individuals (Table 5). Studies on monocyte surface expression of CD80 and CD86 from healthy humans in response to mistletoe extract demonstrate similar results, although this group does not explain the differential expression pattern of co-stimulatory molecules (199).

As an alternative hypothesis, we examined the role of CTLA-4 as a negative regulator through the binding of CD80 and CD86 in the regulation of peanut-specific PBMC responses. Substantial amounts of CTLA-4 are expressed intracellularly, yet only a fraction of it becomes rapidly expressed at the cell surface upon T cell activation and is quickly internalized (205, 206). The kinetics of CTLA-4 expression occurs rapidly, thus as an alternative, many studies

analyze intracellular expression. Although stimulation with Con A resulted in measurable surface expression of CTLA-4, measurement of levels in response to WPE over the course of four days proved to be difficult. Two of three peanut non-allergic individuals presented with two-fold increases in CTLA-4 expression on $CD4^+$ lymphocytes after 2 days of stimulation with peanut extract whereas no change in surface expression was detected on $CD4^+$ T cells from peanut allergic individuals (Table 5).

One potential explanation for our findings is that peanut extract does not alter CD86 surface expression but rather maintains its' expression, thus allowing the development of a Th2 profile as suggested by our data. Although CD80 expression increases, potential signaling of a type 1 response through CD80 does not outweigh signaling through CD86 but instead contributes to the Th2 response in skin test negative individuals (as shown previously). By increasing the numbers of individuals per group, potential differences may become apparent when examining the expression of these important co-stimulatory molecules.

10.9 Global Summary

In order to characterize the type 1 and type 2 cytokine and chemokine response profile of peanut non-allergic individuals, we developed an *in vitro* short-term primary culture system in which we examined PBMC directly *ex vivo* stimulated with peanut extract and the major peanut allergens *Ara h1* and *Ara h2.*

Additionally, we examined differences in peanut-driven cytokine production peanut in our non-allergic population as two sub-populations based on skin prick test sensitivity: A) Skin Prick Test Negative (SPT-) and B) Skin Prick Test Positive (SPT+). Using cytokine and chemokine specific ELISAs we were able to determine the following:

1) The general, peanut non-allergic, population mounts an immune response characterized by substantial production of type 2 (IL-5, IL-13 and CCL22) peanut-specific cytokine and chemokines. Non-allergic individuals demonstrated no type 1 cytokine (IFNγ) or chemokine (CXCL10) production in response to peanut.
2) Upon examining differences between non-allergic sub-populations, SPT+ individuals demonstrated a more intense peanut-specific IL-5, IL-13 and CCL22 response than SPT- individuals. 75% of SPT+ individuals demonstrated a type 2 response in comparison to 25% of SPT- individuals.
3) Peanut non-allergic individuals exhibit peanut allergen-specific type 2 responses to *Ara h1* and very rarely to *Ara h2*. This is important as it indicates the dominance of *Ara h1,* in comparison to *Ara h2*, in the initiation of peanut-driven cytokine and chemokine responses.
4) The use of CD4 and MHC class II is critical for peanut-driven type 2 cytokine and chemokine production. Recognition of peanut allergen in the context of MHC class II illustrates the requirement of antigen specific T cell and APC

involvement in the initiation of peanut-specific cytokine and chemokine production.

5) Differential co-stimulatory requirements for peanut-specific cytokine and chemokine production exist between SPT- and SPT+ individuals. SPT- individuals require both CD80 and CD86 for type 2 cytokine production whereas SPT+ individuals depend solely on the use of CD86 for type 2 cytokine production.
6) Immunosuppressive cytokines IL-10 and TGFβ do not play a role in the regulation of type 2 peanut-specific cytokines (IL-5 and IL-13) produced by PBMC peanut non-allergic individuals but suppress the expression of existing peanut-specific type 1 cytokine (IFNγ) responses.

In summary, these data show that non-allergic individuals are capable of mounting an immune response to peanut, regardless of clinical sensitivity to peanut. We demonstrate that PBMC from peanut non-allergic individuals respond with type 2 cytokine and chemokine production following peanut extract and Ara h1 stimulation. Differences in the intensity of peanut specific cytokine production between non-allergic SPT- and SPT+ indicates that a spectrum of type 2 responses may underlie the development of or absence of clinical peanut allergy. The specific recognition of peanut antigen through MHC class II molecules indicates the specificity of the immune response to this common food allergen. Immunosuppressive cytokines IL-10 and TGFβ play a role in the regulation of peanut-specific IFNγ production which may result in potentially undesired

responses to peanut. Differential requirements of co-stimulatory molecules CD80 and CD86 indicates their role in the regulation of peanut-specific cytokine and chemokine production in the continuum of responses that may be fundamental in the determination of clinical hypersensitivity to peanut.

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