Assessment of allergen specific response in humans

Luis Diego Archila Diaz

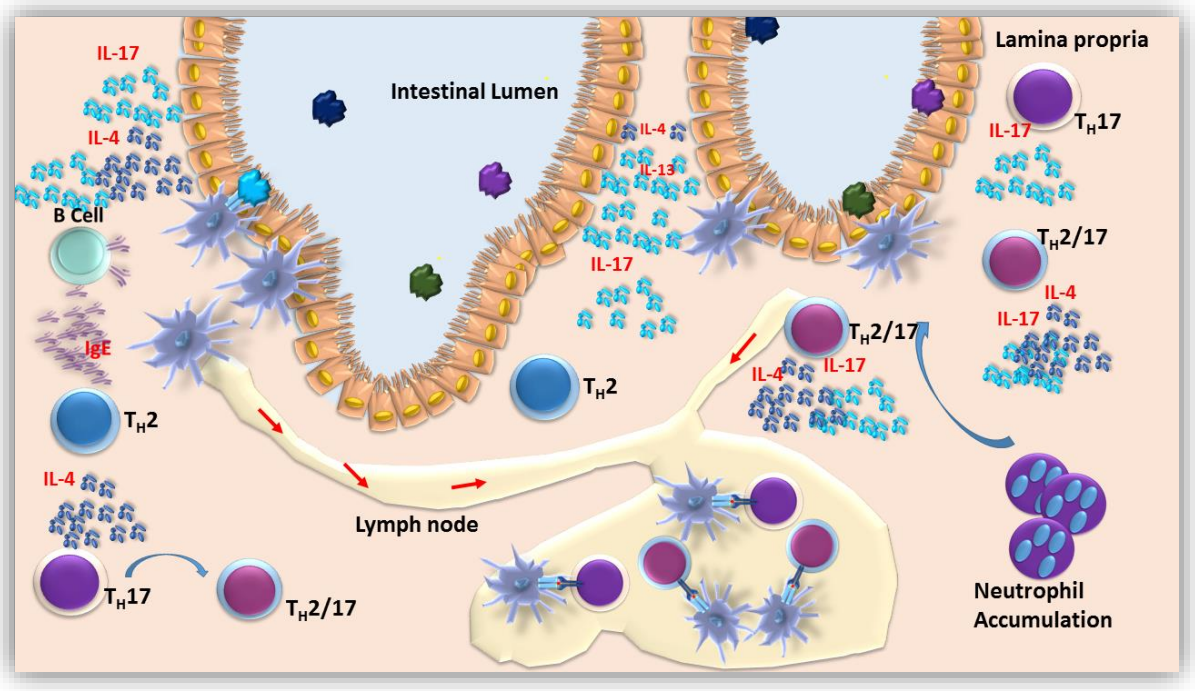
ASSESSMENT OF ALLERGEN SPECIFIC RESPONSE IN HUMANS

Thesis memoir presented to obtain the degree of

Doctor of Philosophy in Biomedicine by the University of Barcelona,

July 2015

By **Luis Diego Archila Diaz**



Supervision: **William W Kwok**
(Benaroya Research Institute and University of Washington, Seattle).
Tutorship: **Manel Juan i Otero** (Hospital Clínic, Barcelona)

Universitat de Barcelona

Department of Medicine

Doctoral program in Biomedicine

Immunology

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Certifies:

That the doctorate thesis "Assessment of allergen specific response in humans" carried out by Luis Diego Archila Diaz was under my direction and that he considers that is acceptable to be presented to obtain the degree of Doctor in Biomedicine through the University of Barcelona.

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Certifies:

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Stating, I sign the following certification in Barcelona, May 2015.

Manuel Juan i Otero, PhD

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To my parents who have always encouraged me to follow my dreams,

To my family, who has made me the person I am today,

And to Gabi, my lifetime inspiration.

It is not to see something first, but to establish solid connection between previously known and hitherto unknown that constitutes the essence of scientific discovery. To discover does not mean to see, but to uncover sufficiently that many can see and continue to see forever.

Hans Selye

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ABBREVIATIONS

Ana o	*Anacardium occidentale*
APC	Antigen Presenting Cell
BAT	Basophil Activation Test
Bcl-6	B-cell CLL/lymphoma 6
CCA	Cytokine capture assay
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
Cor a	*Corylus americana*
CRTH2	Chemoattractant receptor-homologous molecule expressed on T_H2 cells
CSA	Cytokine Secretion Assay
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) Ligand
CXCR	Chemokine (C-X-C motif) receptor
Dac g	*Dactylis glomerata*
DC	Dendritic Cell
ELISA	Enzyme-linked immuno assay
ELISPOT	Enzyme-Linked ImmunoSpot
FcεRI	High-Affinity Receptor for the Fc Region of Immunoglobulin E (IgE)
FcγRIIB	High-Affinity Receptor for the Fc-Gamma Receptor-IIB
GATA3	Gata binding protein 3

GITR	glucocorticoid-induced TNF receptor
HLA	Human histocompatibility leukocyte antigen
Hol l	*Holcus lanatus*
Hor v	*Hordeum vulgare*
ICAM	Intercellular Adhesion Molecule 1
ICOS	Inducible T cell costimulatory
ICS	Intracellular cytokine staining
IFN-ϒ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILC2	Innate Lymphoid Cell 2
ILIT	Intralymphatic Immunotherapy
ISAC	ImmunoCAP® Solid-Phase Allergen Chip
Jug r	*Juglans regia*
Lol p	*Lolium perenne*
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
LTP	Lipid Transfer Protein
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
Phl p	*Phleum pretense*
Pis v	*Pitacia vera*
Pha a	*Phalaris aquatica*
pMHCII	Peptide/MHC class II

PNAd	Peripheral node addressin
Poa p	*Poa pratensis*
RAST	Radioallergosorbent Test
RORC	RAR-Related Orphan Receptor C
RORγ	RAR-related orphan receptor gamma
SCIT	Subcutaneous Immunotherapy
SIT	Specific Immunotherapy
SLC	Solute carrier family
SLIT	Sublingual Immunotherapy
SPT	Skin Prick Test
STAT	Signal Transducer and Activator of Transcription
T-bet	T-box transcription factor
TCC	T-cell clone
TCL	T-cell line
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{eff}	Effector T cell
T_{FH}	Follicular helper T cell
TGEM	Tetramer Guided Epitope Mapping
TGF	Transforming Growth Factor
T_H	T helper
TNF	Tumor Necrosis Factor
T_R1	Regulatory T_H1-like T cell
T_{reg}	Regulatory T cell
TSLP	Thymic stromal lymphopoietin

Part I.

Introduction

A. Allergies are complex ailments:

I. Definition and prevalence

According to the American Academy of Allergy, Asthma and Immunology (A.A.A.I), an allergy is defined as an adverse or hypersensitivity reaction involving responses from the human immune system against a foreign harmless substance ("allergen") that is touched, injected, breathed into the lungs or eaten (**Figure I**). The World Allergy Organization (W.A.O) states that allergies are increasing worldwide and it is certainly the most pervasive disorder globally, affecting between 20 – 30% of the world's population in both developed and developing countries **[Pawankar, et al., 2013]**. Allergies involving polysensitization and multiple organ involvement are also increasing, thus, resulting in decreased quality of life, increased morbidity and mortality **[Pawankar, et al., 2013] [Holgate, 1999] [DeKruyff, 2002]**. In many countries, attempts to tackle these problems on a national basis are widely fragmented and the lack of such care leads to substantial increased costs in health care and national budgets **[Cookson, 1999]**.

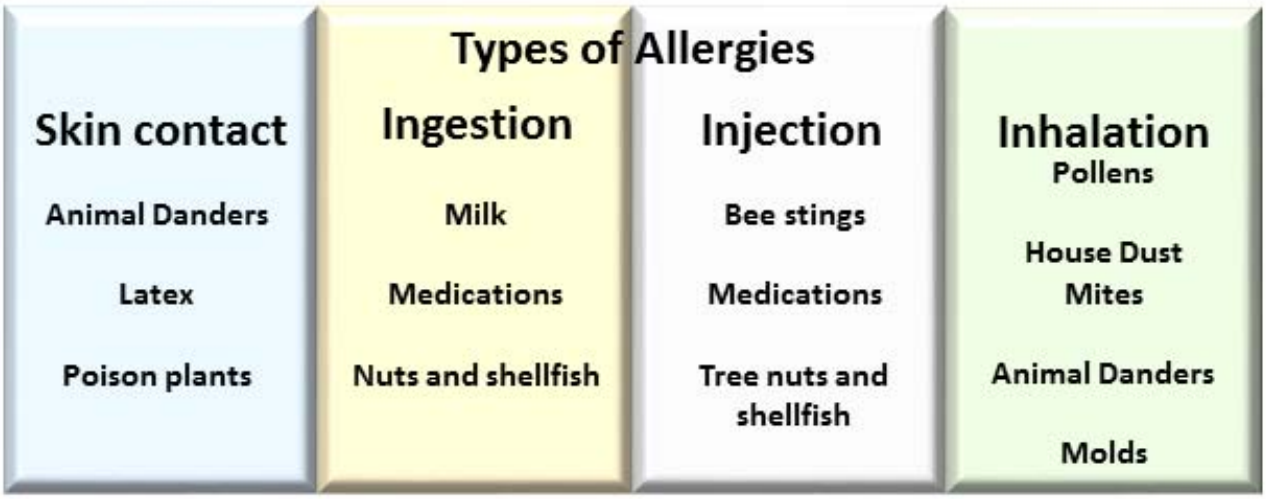

Figure I. Example of allergens and their route of exposure.

Although significant effort has been put to clarify the molecular mechanisms of allergic disease and susceptible (candidate) genes have been identified **[Vercelli, 2008]**, the precise effects for which allergic ailments occur are not yet fully understood. Allergies are triggered by crosstalk amongst multiple

susceptible genes and environmental factors, such as allergen exposure and infection **[Vercelli, 2008] [Umetsu, et al., 2002]**. Development of allergic disorders involves various factors including genetic components, environmental exposure, maternal diet, reproductive physiology and birth outcomes, breast feeding, child nutrition and Vitamin D level, obesity, physical activity and psychological stress **[Ninabahen, et al., 2011]**.

It is often questioned whether the changes in our lifestyles have a profound effect on the prevalence of this disease as the consequences of modernity (including excessive cleanliness) in developing countries alter and diminish exposure to infectious pathogens **[Strachan, 1989]**. The so-called "hygiene hypothesis" is one of the main propositions to explain this phenomenon. Epidemiological and clinical evidence support the hygiene hypothesis as cause of both autoimmune and allergic diseases **[Akdis, 2006]**. It has been suggested that limited exposure to microbial, viral and macro parasites in early childhood results in an insufficient stimulation of T-helper 1 (T_H1) cells, which in turn cannot maintain homeostasis of T_H2 cells resulting in predisposition to allergy. This lack of counterbalance promotes exaggerated T_H2 responses towards allergens and antagonizes tolerance through a protective T_H1 or T regulatory response **[Maizels, et al., 2014] [Umetsu, et al., 2006]**. Additionally, in the case of food allergies, it is suggested that delayed introduction of foods in the diet can be associated with higher incidence of disease and yet again, the modernity of our lifestyles changes the appearance in which these food products are eaten **[Fiocchi, 2006]**.

II. Clinical Manifestations

The most common allergic symptoms are eczema, asthma, allergic rhinitis, atopic dermatitis and anaphylaxis **[Holgate, 2000]**, the symptoms are broad, and however, symptom manifestation highly depends on the route of exposure and the targeted organ (**Table I**).

Target Organ	Symptoms
Cutaneous	Erythema Pruritus Urticaria Morbilliform eruption Angioedema
Ocular	Pruritus Conjunctival erythema Tearing Periorbital edema
Upper respiratory	Nasal congestion Pruritus Rhinorrhea Sneezing Laryngeal edema Hoarseness Dry staccato cough
Lower respiratory	Cough Chest tightness Dyspnea Wheezing Intercostal retractions Accessory muscle use
GI (oral)	Angioedema of the lips, tongue, or palate Oral pruritus Tongue swelling
GI (lower)	Nausea Colicky abdominal pain Reflux Vomiting Diarrhea
Cardiovascular	Tachycardia (occasionally bradycardia in anaphylaxis) Hypotension Dizziness Fainting Loss of consciousness
Miscellaneous	Uterine contractions

Table I. Symptoms for Allergic Reactions **(Adapted from [NIAD, 2010])**

For example, airborne allergic symptoms are observed in the upper and lower airway respiratory organs while food allergic symptoms may target several organ systems and may lead to anaphylaxis.

III. The allergic march

As previously mentioned, allergic disease may manifest clinically as a combination of different allergic disorders. It has been illustrious that both food allergies and atopic dermatitis are frequently the earliest manifestation of atopic predisposition in young children. Nearly 50% with atopic dermatitis will develop asthma and about 75% develop allergic rhinitis **[Saarinen, et al., 2005]**. The so-called "allergic march" suggests that allergic disorders may progress in allergic individuals in a sequential manner from infancy to adolescence and adulthood (**Figure II**).

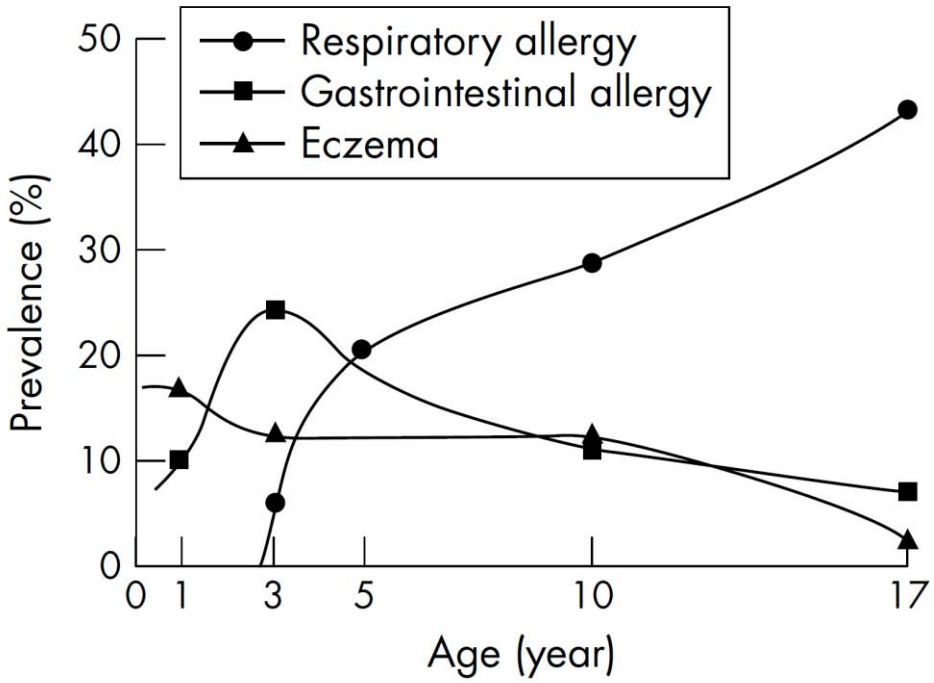

Figure II. The "allergic march"; allergic disease progression. (Reproduced from **[Saarinen, et al., 2005]**).

IV. The Immunological Mechanisms of Allergy

Hypersensitivity is defined, as an inappropriate immune response that can be damaging and sometimes fatal when it is excessive; these types of reactions require a pre-sensitization stage in the host. Hypersensitivities, as classified by Philip Gell and Robin Coombs in 1963, can be categorized into 4 different groups depending on the physiological mechanisms of their response. These are briefly mentioned in **Table II**.

Type I	Type II	Type III	Type IV
IgE-Mediated Hypersensitivity	IgG-Mediated Cytotoxic Hypersensitivity	Immune Complex-Mediated Hypersensitivity	Cell-Mediated Hypersensitivity
Antigen induces crosslinking of IgE bound to mast cells and basophils with release of vasoactive mediators	Antibodies directed against cell surface antigens mediates cell destruction via complement activation or ADCC	Antigen-Antibody complexes deposited in various tissues induce complement activation and an ensuring inflammatory response mediated by massive infiltration of neutrophils	Sensitized TDTH cells release cytokines that activate macrophages or TC cells which mediate direct cellular damage
Typical manifestations include systemic anaphylaxis and localized hay fever, asthma, hives, food allergies and eczema	Typical manifestations include blood transfusion reactions, erythroblastosis fetalis and autoimmune hemolytic anemia	Typical manifestations include localized Arthus reaction and generalized reactions such as serum sickness, necrotizing vasculitis, glomerulonephritis, rheumatoid arthritis and systemic lupus erythematosus	Typical manifestations include contact dermatitis, tubercular lesions and graft rejection

Table II. Classification of Hypersensitivities. (Adapted from **[Gell and Coombs, 1963]**).

The hallmark of Type I allergic immune response is production of allergen-specific IgE and activation of innate lymphoid, mast cells, basophils, eosinophils and T_H2 memory induction. This type of hypersensitivity occurs in two different stages:

1. *Sensitization and memory induction phase*. In this phase the subject becomes sensitized after first exposure to the allergen.
2. *Allergic inflammation phase.* In this phase the subject reencounters the allergen and clinical manifestations can be visible.

IV.1 The cycle that perpetuates a Type I allergic immune response

The sensitization stage corresponds to the generation of an adaptive memory response that leads to the production of specific-IgE to an allergen. However, recent literature suggests that both innate and adaptive immunity are instrumental in establishing Type I allergic immune responses **[Saenz, et al., 2008]**. The sensitization and memory induction phase is characterized by a cascade of events that initiates when an allergen, mechanical stress or viral infection leads to the secretion of IL-25, IL-33 and TSLP by epithelial cells **[Saenz, et al., 2008] [Liu, et al., 2007]**. The secretion of these cytokines generates a T_H2-promoting milieu as both TSLP and IL-33 act on dendritic cells (DC) and promote T_H2 differentiation bias **[Besnard, et al., 2011] [Liu, et al., 2007]**. Additionally, both IL-25 and IL-33 play an important role in the promotion of an adaptive T_H2 milieu, as they activate lineage negative lymphoid-like populations such as type 2 innate lymphoid cells (ILC2), nuocytes or natural helper cells **[Mjosberg, et al., 2012] [Wilhelm, et al., 2011]**. These cell populations maintain significant levels of IL-5, IL-9 and IL-13, thus promote differentiation, expansion and commitment of the T_H2 repertoire **[Wilhelm, et al., 2011]**.

Simultaneously, upon capturing and processing the allergen, DC migrate into secondary lymphoid organs where they differentiate naïve $CD4^+$ T-cells into T_H2 cells. This activation leads to the production of cytokines (IL-4 and IL-13) which induce immunoglobulin class switching to IgE, clonal expansion of naïve and IgE^+ memory B-cell populations and production of allergen-specific IgE antibodies **[Larche, et al., 2006]**. Mast cells and basophils encounter secreted allergen-specific IgE that circulates in the blood and this interaction leads to the binding of allergen-specific IgE into FcεRI (IgE-specific receptor) found on the surface of these cell types **[Mudde, et al., 1990]**.

The immediate phase of the allergic reaction occurs when cross-linking of mast cell- and basophil -surface FcεRI bound IgE by allergens leads to the initial release of vasoactive amines (such as histamines), lipid mediators (prostaglandin D, platelet-activating factor, leukotriene C4 (LTC4), LTD4 and LTE4), expression of chemokine receptors (such as CXC-chemokine ligand 8 (CXCL8), CXCL10, CC-chemokine ligand 2 (CCL2), CCL4 and CCL5), and cytokines (IL-4, IL-13 and IL-5) responsible for the acute allergic response **[Larche, et al., 2006] [Kay, et al., 2001]**. This process transits into a late-phase or allergic inflammation stage where chronic responses associated with leukocyte recruitment (such as eosinophils) and activation of tissue remodeling occur. A second encounter with the allergen would lead to an immediate phase of type I reaction and a perpetuated cycle of allergic immune responses **[Mudde, et al., 1990]**. A schematic representation of the pathogenic processes in allergic disease are presented in (**Figure III**).

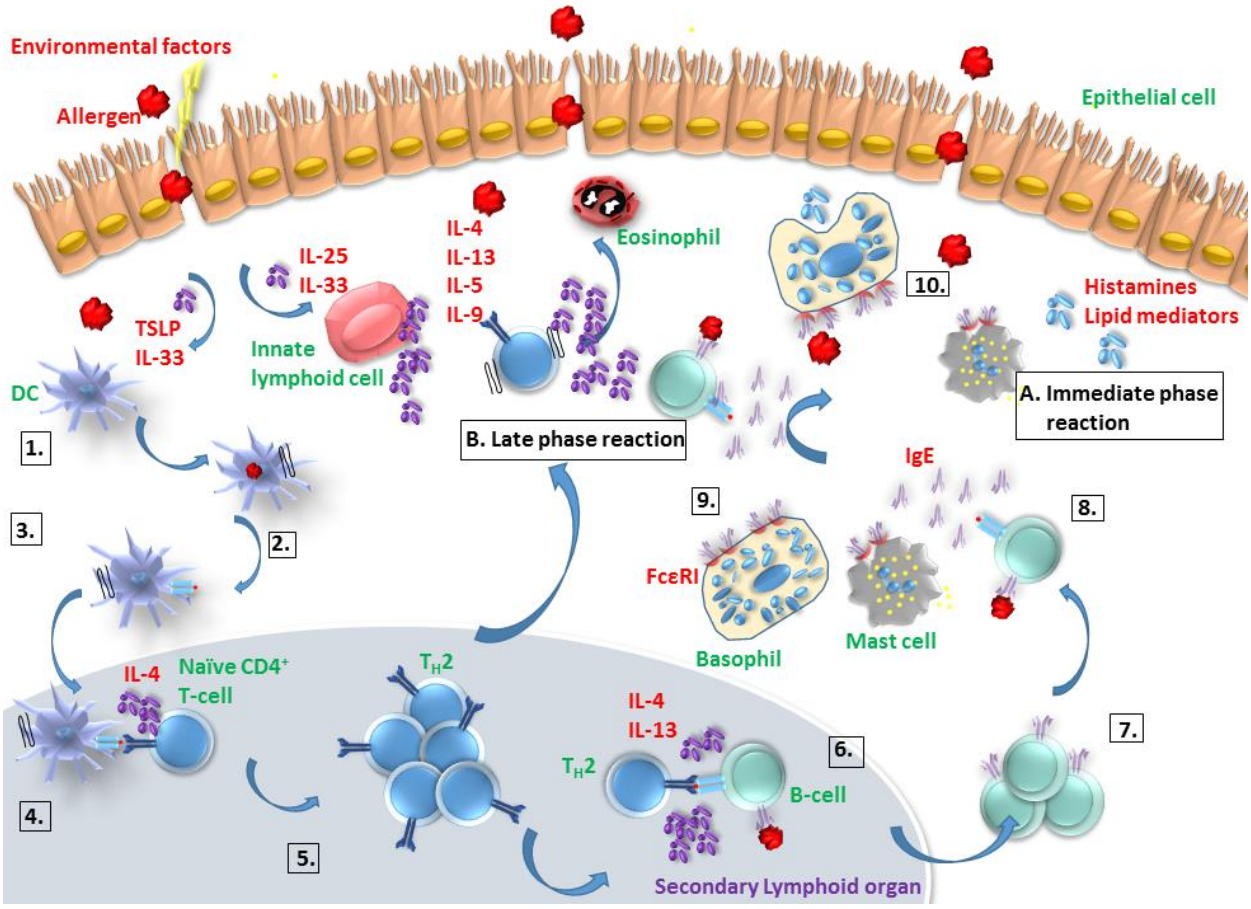

Figure III. Mechanisms of allergic reaction (Adapted from **[Wambre, et al., 2012] [Larche, et al., 2006]**).

A cascade of events initiates upon allergen exposure in a suitable environmental milieu that leads to production of IL-25, IL-33 and TSLP by epithelium cells and simultaneously activate both adaptive and innate responses. 1. DC capture the allergen in a T_H2 promoting environment. 2. DC mature and express chemokine receptors and initiate the migration towards the secondary lymphoid organs. 3. DC migrate and express MHC-II loaded complexes. 4. An immunological synapse occurs between DC and Naïve $CD4^+$ T cells. 5. Differentiation of T_H2 cells and clonal expansion. 6. Activated T_H2 cells induce class switching to IgE on B cells. 7. Clonal expansion of IgE^+ memory B cells. 8. Allergen-specific IgE antibody production by B cells. 9. Allergen-specific IgE binds FcεRI receptors on mast cells and basophils. 10. Cross-linking of basophil and mast cell FcεRI receptors-bound IgE by allergens leads to degranulation and type I reactions (A. Immediate phase reaction). B. the late phase reaction is characterized by a cycle that perpetuates a type I allergic immune response leading to recruitment and activation of pro-inflammatory cells such as mast cells, basophils and eosinophils.

B. The Allergic patient

Although allergies are increasing worldwide and reasons for its prevalence are not yet fully understood, several diagnostic tools have been implemented and are key components for the improvement of personalized therapies for allergic subjects. Additionally, it is now possible to establish what allergen is the sensitizing agent in a particular subject. However, due to phylogenetically relatedness of several species, allergenicity can be widespread in closely related groups, thus, making it more complex to determine the original source of sensitization. This section will focus on the different diagnostic methodologies used in clinical practice; we will describe allergens and how their relatedness affects allergy. Finally, we will also discuss how evolution can explain why several groups of allergens are allergenic and how this can be advantageous for disease treatment.

I. Diagnostic tools in clinical practice

An accurate diagnosis in allergic disease is pivotal to both clinical practice and epidemiological research. Recent advances in proteomics have improved our knowledge about protein structures and have allowed us to characterize protein molecules, purify natural compounds and produce recombinant proteins, which have prompted the development of new tools for better diagnosis of allergic diseases. Below, we will discuss some of the most utilized tools in practice.

I.1 Skin prick test (SPT)

A skin prick test consists of puncturing the skin with a needle containing a small amount of allergen extract dissolved in phosphate buffer saline (PBS). The basis of this procedure is to provoke a small and controlled allergic reaction to measure the presence of IgE antibodies to certain allergens. If a wheal appears about 30 minutes after in the skin, it is very likely that the subject is allergic to that allergen **[Mari,**

et al., 2010]. This method, although simple, still remains as one of the preferred methods for allergy diagnosis as it is not time-consuming and tends to be accurate **[Bernstein, I.L., et al,. 2008]**. Currently, other tools that are based with the same principle have evolved. An example of this is the patch skin test, frequently utilized for diagnosing contact dermatitis **[Fall, et al., 2015]**, where a patch that contains an allergen is placed upon the subject's skin, generating a wheal the size of a patch. Other provocation tests that can be useful to confirm allergies are food challenges and nasal/bronchial mucosa exposure to allergens.

I.2 Serum-Specific IgE Testing

A combination of skin tests and in vitro quantification of specific IgE constitute the cornerstone of allergic disease diagnosis **[Sicherer, S.H., et al., 2012]**. Total and specific IgE can be detected *in vitro* using a variety of commercially available tools. The basis of this method (applied to different systems) is similar to a enzyme-linked immunosorbent assay (ELISA); in allergy it is defined as a radioallergosorbent test (RAST). First, serum specific IgE binds to an allergen that can be in fluid or a solid phase (depending on the assay). Second, an anti-IgE that is conjugated to an enzyme detects IgE, and next catalyzes the transformation of a specific substrate with a quantifiable property (fluorescence or color) **[Cox, L., 2011]**.

The ImmunoCAP technology from Phadia, is an innovative version of the RAST test. In this procedure the allergen is covalently bound to a solid phase consisting of an activated hydrophilic polymer. The anti-IgE antibodies are bound to the enzyme β-galactosidase which can convert added methylumbelliferyl-β-D-galactoside into a fluorescent product (4-methylumbelliferone) **[Goikoetxea, M.J., et al., 2013]**. The fluorescence intensity depends on the concentration of the product and correlates with the IgE bound to the allergen (**Figure IV**).

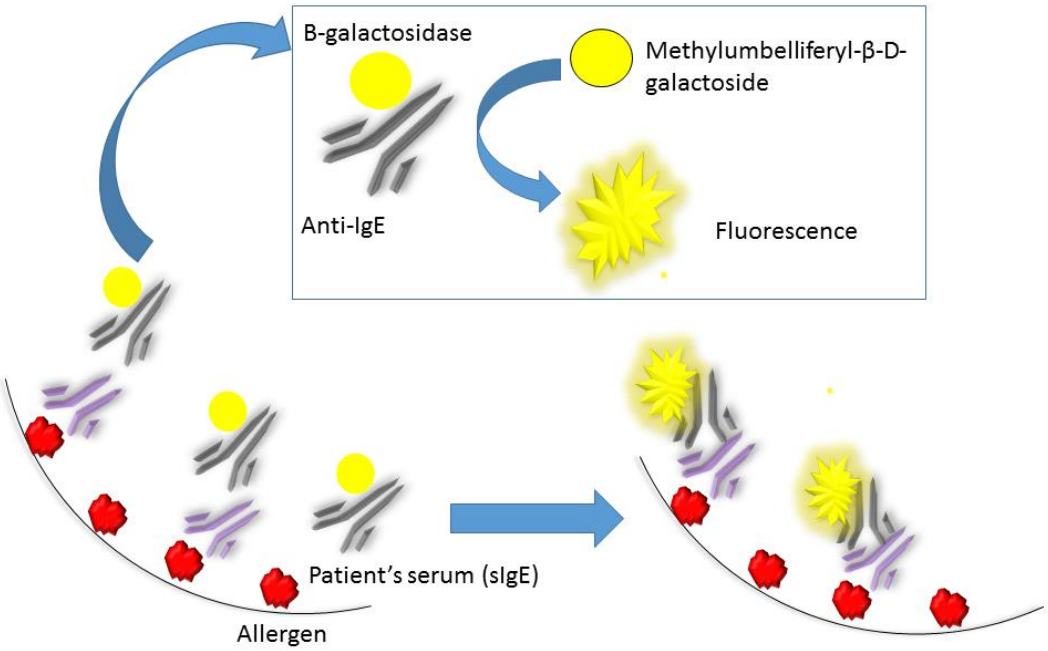

Figure IV. Methodology of specific IgE detection systems: ImmunoCAP and the ISAC microarray. (Adapted from **[Goikoetxea, M.J., et al., 2013]**).

The fact that allergic subjects are generally polysensitized requires better diagnostic tools that can determine the precise proteins responsible for the allergic disease, thus determining specific IgE to a monocomponent **[Melioli, G. et al., 2012]**. For this matter, the diagnosis is to be made by replacing the complete extract of a biological source by a recombinant or purified allergenic component. These technologies are usually arrays that contain a vast number of allergens and can provide a wealth of information about the allergic subject. Such examples of these technologies are ImmunoCAP with proteins or allergenic components and ImmunoCAP ISAC microarray, the latter contains about 112 single components derived from 51 allergens **[Phadia, 2010]**.

Other technologies such as Western blotting can also be utilized to detect specific IgE to a particular allergen or component that is not available in ImmunoCAP ISAC microarray. This approach is time consuming and requires the allergen or component source.

I.3 Addition in vitro diagnostic tests

Other than measurement of specific-IgE levels, *in vitro* diagnostic tests are rarely used in clinical practice. An example is the basophil activation test (BAT). The basis of this test is to stimulate whole blood with allergen extracts and to detect expression of certain markers such as CD203c or CD63 that are suggestive of allergy inflammation **[Burtin, D. et al., 2009] [Santos, A.F., et al., 2015]**. This tool can be ancillary to both SPT and specific IgE measurements when tests are negative **[de Weck, A.L., et al., 2008]**. Although this is not a time consuming approach it requires standardization and flow cytometry expertise.

Other cell types that can be useful for diagnosis of a particular component are T-cells. Numerous *in vitro* assays such as proliferation assays, cytokine synthesis, CD69 and CD154 up-regulation on cell surface, can be used to detect hypersensitivities **[Porebski, G. et al., 2001]**. The basis of these methods is to stimulate PBMC from patients with whole extract or peptides and to observe the nature of their responses. Although these methods are very specific and can provide useful information they are time consuming, expensive and require expertise in the field, thus are only commonly used in research, and not in clinical practice. The advantages and disadvantages of these methods are presented in **Table III**.

Test (functional/ phenotypic marker)	Method (readout systems)	Duration (day)	Advantages	Disadvantages
Proliferation of allergen-specific T-cells	3H thymidine incorporation, radioactivity measurement (CFSE or non-reactive methods also possible, but less sensitive)	6-7	Highly specific, Many allergen concentrations testable and many replicates	Long-lasting radioactivity and expensive equipment
CD69 and CD154 up-regulation on cell surface	Flow cytometry	1	Rapid; identification of reactive cell subset	Flow cytometry is difficult to standardize
Cytokine synthesis and secretion (e.g. IL-4, IL-13 and Il-5)	ELISA, ELISPOT, Flow cytometry	1-3	Rapid; sensitive; informative regarding pathological mechanism	Expensive, minimally 2 cytokines needed

Table III Comparison of *in vitro* tests with T-cells to detect hypersensitivities **(Adapted from [Porebski, et al., 2011]**).

II. Categories of allergic disorders

II.2. Allergens

As briefly mentioned in the first chapter, an allergen is a "harmless" protein source that can generate vigorous immune responses in humans. The current opinions in the field suggest that a protein source is considered an allergen if it is able to provoke specific IgE synthesis on first contact and, later, inflammatory reactions **[Marth, et al., 2014]**. Allergens vary in size (10 to 50 KD), most are biochemically activated, have acidic isoelectric points, and have enzymatic properties that enables their ability to enter mucosal tissues in events where epithelial injury occurs, thus, focalizing an allergic response **[Musu, et al., 1997]**. Due to tremendous advances in proteomics, the identification and characterization of allergens from a variety of substances is now possible. Allergens from three different kingdoms (Animalia, Plantae and Fungi) have been identified.

Allergens can be categorized as major and minor allergens. Major allergens represent components to which the majority of patients (by definition >50%) reacting to a given allergen source are sensitized, while minor allergens are recognized by a limited number of subjects **[Andersson, et al., 2003]**. In many circumstances major allergens serve as marker allergens for sensitization to certain kinds of organisms e.g. Phl p 1 for Timothy grass, Bet v 1 for birch, Alt a1 for *Alternaria alternata,* Asp f 1 *Aspergillus* fumigatus, etc **[Gieras, et al., 2011] [Chruszcz, et al 2012] [Madan, et al., 2004]**. Depending on their nature and route of exposure allergens can be divided into three main groups:

a. Airborne allergens (e.g. grass-pollen, house dust mite, cat dander, fungal spores).
b. Food allergens (e.g. peanut, tree nuts, crustaceans)
c. Injected allergens (e.g. bee venom)

II.2.1. Airborne allergy

The environmental agents responsible for the burden of allergic disorders worldwide are those derived from house dust mite, cockroach and animal dander (Indoor allergens) and grass and tree pollens and fungal spores (Outdoor allergens) **[Holt, et al., 2005].** As mentioned in the first section (A. Allergies are complex ailments), changes in our lifestyles, housing and/or extrinsic factors such as pollution and global warming can also affect both exposure to allergens and susceptibility to sensitization. Global warming has a direct effect upon the immediate accumulation of carbon dioxide in our environment, which leads to an augmentation in the stimulatory effect on pollinosis; thus, higher pollen counts in polluted cities where demographic populations are denser and were allergies are common **[Ziska, et al., 2003]**.

Airborne allergens can be seasonal or perennial, depending on the nature of their presence. Seasonal allergens include grass and tree pollens. These members from the Plant Kingdom require several extrinsic factors such as, temperature, amount of water and amount of daylight to enter a period of pollination **[Krämer, et al., 2005] [Holt, et al., 2005]**. Pollen count concentrations in outdoor air increase in spring and summer, when all these factors are favorable, which at the same time increase the severity of the symptoms in allergic patients (**Figure V**).

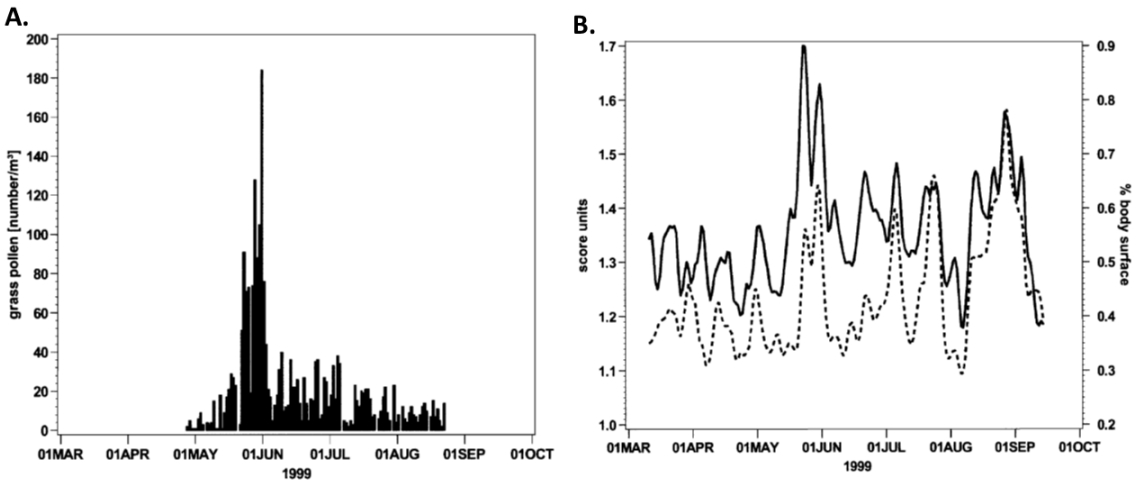

Figure V. Seasonality influences symptom severity. The following figure represents data from Germany in 1999. A. Daily mean grass pollen concentration in outdoor air throughout the year. B. Mean symptom scores itch (eczema) in 18 nine year old children throughout the year. (Adapted from **[Krämer, et al., 2005]**).

On the other hand, perennial allergens are present around the year and are related to non-seasonality. The most common indoor allergens are house dust mites, animal danders and molds. Therefore, allergic subjects are constantly exposed to these allergens **[Mandhane, et al., 2011]**.

Airborne allergies are the most frequent type of pathogenesis, affecting around 65% of allergic subjects **[Jones, et al., 2010]** and affect the respiratory system. Rhinitis is the most common type of inflammation. It is characterized by IgE mediated reactions that affect the membranes lining of the nasal cavity, and is characterized by combination of sneezing, itching, nasal congestion, rhinorrhea, conjunctivitis, rhinitis, and wheezing. Airborne allergies may also lead to asthma, a common disorder characterized by recurrent airway obstruction and wheezing that affects 8.2% of the total population in the United States **[Knutsen, et al., 2012]**. Patients with perennial allergic rhinitis are at more risk of developing bronchial symptom and airway inflammation, the likely risk factors for asthma **[Downie, et al., 2004]**.

II.2.1.1 Grass pollen allergens

Grass pollens are one of the most important airborne allergen sources world-wide, they affect approximately 40% of allergic individuals **[Andersson, et al., 2003]**. Pollen from the grass family Poaceae is one of the key causes of allergies in North America and Europe **[White, et al., 2003] [Jaeger, 2008]**. Members of the Pooideae subfamily are responsible for pollen exposure and sensitization in allergic subjects. The major allergens for the Pooideae subfamily can be classified into two groups: Group 1 (beta-expansins) and Group 5 (ribonucleases). The various species have variable importance in different geographical areas and prevalence is non-uniform amongst this subfamily. Pooideae grasses exhibit similar pollen morphology **[Heijl, et al., 2009]** thus neither pollen counts nor microscopic analyses can distinguish pollens from a distinct grass species **[Moingeon, et al., 2008]**. Therefore, it has been an important topic of debate that immunotherapy with a mixture of grass pollen from different Pooideae species would be able to tackle all of these issues and is suited for therapy instead of therapy with one species only (Generally *Phleum pratense*).

II.2.2. Food allergy

In contrast to airborne allergy, less than 30% of allergic subjects have food allergy; affecting greater than 2% and less than 10% of the US population **[Chafen, et al., 2010]**. The prevalence of food allergy peaks in the first few years of life and the most common sources of allergens are milk, egg, peanut, tree nuts, seafood and fruit **[Jones, et al., 2010] [Berin, et al, 2013] (Figure VI)**. Most children outgrow milk, egg during their first 10 years of life, however, peanut, tree nut and sea food allergies are rarely outgrown.

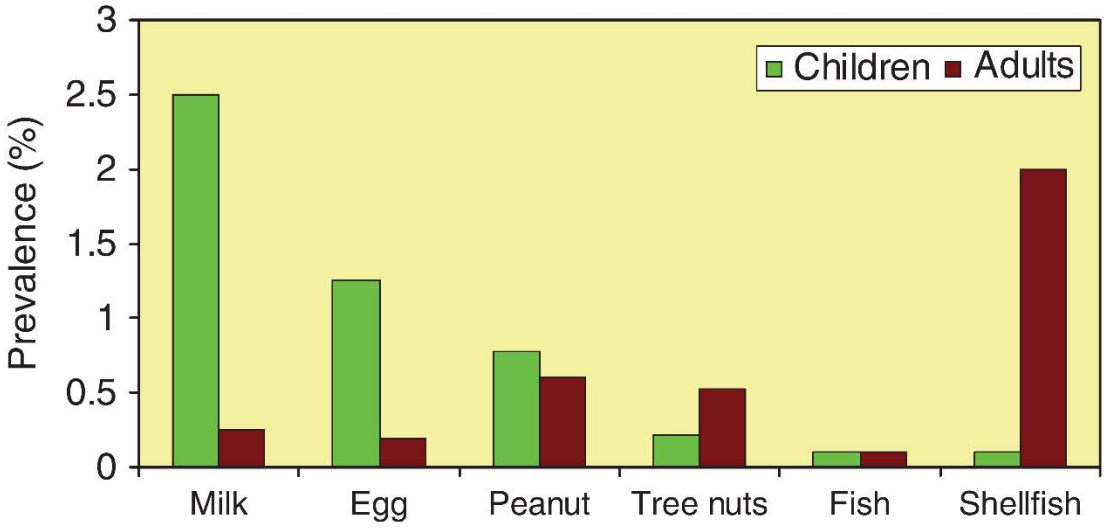

Figure VI. Prevalence profile of food allergy among children and adults in the United States. (Taken from **[Lopata, et al., 2010]**).

These ailments are increasing in industrialized countries worldwide for reasons that are not fully understood **[Osborne, et al., 2011]**. Food allergies can be either IgE mediated or non-IgE mediated; usually with delayed onset, gastrointestinal or non-specific symptoms, where the mechanism is unclear and harder to diagnose **[Fox, et al., 2009]**. Although food allergies are less common compared to airborne allergy, these ailments are able to cause severe allergic reactions as itchy flushing of the skin and/or urticarial, nausea, abdominal pain and/or vomiting, mild to severe bronchospasm and respiratory distress, hypotension, cardiovascular collapse **[Berin, et al., 2013]**. A combination of systemic reactions involving two or more organ systems leads to anaphylaxis; responsible for the death of approximately 1 to 3 individuals per million population **[Fox, et al., 2009] [Moneret-Vautrin, et al., 2005]**. Food avoidance is the only therapeutic option; however, the ubiquity of these foods in the diet makes avoidance difficult, and accidental ingestion is a common occurrence **[Sicherer, et al., 2001]**.

II.2.2.2 Tree nut allergies

Allergic reactions to tree nuts, including walnut, cashew and almond, are common affecting approximately 1.1% of children younger than 18 years and 0.5% of adults in the United States **[Sicherer, et al., 2010]**. Similar to peanut allergy, tree nut allergy generally has an onset in early childhood and persists throughout life **[Fleischer, et al 2007]**. It is estimated that only 9% of patients outgrow tree nut allergy. In the United States the most commonly reported tree nut allergies are: walnut (34%), cashew (20%), pecan (9%), pistachio (7%), hazel, Brazil, pine, macadamia and hickory less than 5% each. Allergic reactions to tree nuts can be serious and life threatening **[Kulis, et al., 2011]**. It has been shown that severe clinical reactions, including higher incidence of anaphylaxis, occurs more frequently in cashew than peanut allergy **[Grigg, et al., 2009] [Clark, et al., 2007]**. Anaphylactic shocks to tree nut allergens are extremely severe and sometimes fatal in sensitized individuals **[Sampson, et al., 1992]**.

Currently, 5 groups of major allergens have been reported. 7s vicilin-like proteins, 2s albumin, 11S legumin-like, and profilin are allergenic in all tree nuts and have been described as important major allergens in the United States **[Roux, et al., 2003]**. On the other hand, lipid transfer protein (LTP) has been proposed as a major allergen in the Mediterranean area **[Pastorello, et al., 2004]**.

II.3. Evolution of species and its effect on cross-allergenicity in allergic disease

Natural selection, Darwin's grand idea of evolution, is very simple, yet some of its principles can even be applied to explain cross-allergenicity in allergic disease. This theory suggests that the more advantageous traits that enable the survival of the species will become more common in the population. Because these traits have a genetic basis, point mutations may occur spontaneously, generating genetic variability in the populations **[Cockerham, et al., 1987]**.

Cross-allergenicity inferred to systematics relies on 2 principles **[Weber, et al., 1985]**. The first is that closely taxonomically related species will have a higher degree of shared characteristics (synapomorphies), thus greater possibility of shared antigens **[Weber, 2003] [Weber, et al., 1985]**. The second principle relies on the fact that it is revealed by the phylogeny; meaning that two species in the same genus truly evolved from a common progenitor, two in the same family evolved from a distant ancestor and so on **[Judd, et al., 1999]**. In systematics, this is considered as monophyly, a group of species that descended from a common ancestor **[Cockerham, et al., 1987]**. Thus it can be implied that species from the same family or genus may share similar characteristics due to this consequence.

In allergy, this approach has been validated **[Weber, 2003]**. Closely related allergen sources frequently induce similar symptoms. As an example, cross-allergenicity has been observed in grass pollen allergy from Pooideae grass pollen (closely related species), where the ailment can be triggered by any of the distinct members from the subfamily **[Martin et al., 1985]**. Nonetheless, distant related species can also have a similar effect **[Zuidmeer, et al., 2006]**. It has been validated that allergens not only can induce similar symptoms but they can also sensitize patients in such manner that sensitization generated by a specific species can also generate poly-sensitization to different allergens from distant related species

[Diaz-Perales, et al., 2000]. This type of cross-allergenicity has been observed in allergies to distinct foods, such as: fruits, nuts, vegetables and pollen (distant related species).

II.3.1 Cross-allergenicity in closely related species (Grass-pollen)

As briefly mentioned in (II.2.2.1 Grass pollen allergens), most of the grass species belonging to the Pooideae subfamily frequently cause grass pollen allergy **[Andersson, et al., 2003]**. The allergenic relationship between these species correlates closely with their phylogenetic relatedness (**Figure VII**).

Grasses are anemophilous plants, meaning that the male reproductive cells (pollen) are light and easily dispersed by wind, over large areas so that they can pollinate the ovule of another plant **[Jaeger, 2008]**. The structure of grass pollen is common to all Pooideae species since it has been an advantageous form of pollination, thus, a conserved trait (**Figure VIII**). Pooideae pollens are perfect vectors for allergenic molecules as they are small in diameter (15-55 μm) and have a spheroid to ovoid shape making it easy to disperse and are found in high concentrations throughout the air during the pollen season, making it almost impossible for allergic individuals to avoid exposure **[Jaeger, 2008] [Moingeon, et al., 2008]**. Grasses of the Pooideae subfamily are widespread throughout temperate zones and their pollination periods overlap, therefore, allergic subjects are concomitantly exposed and polysensitized to pollen from different grass species **[Moingeon, et al., 2008]**.

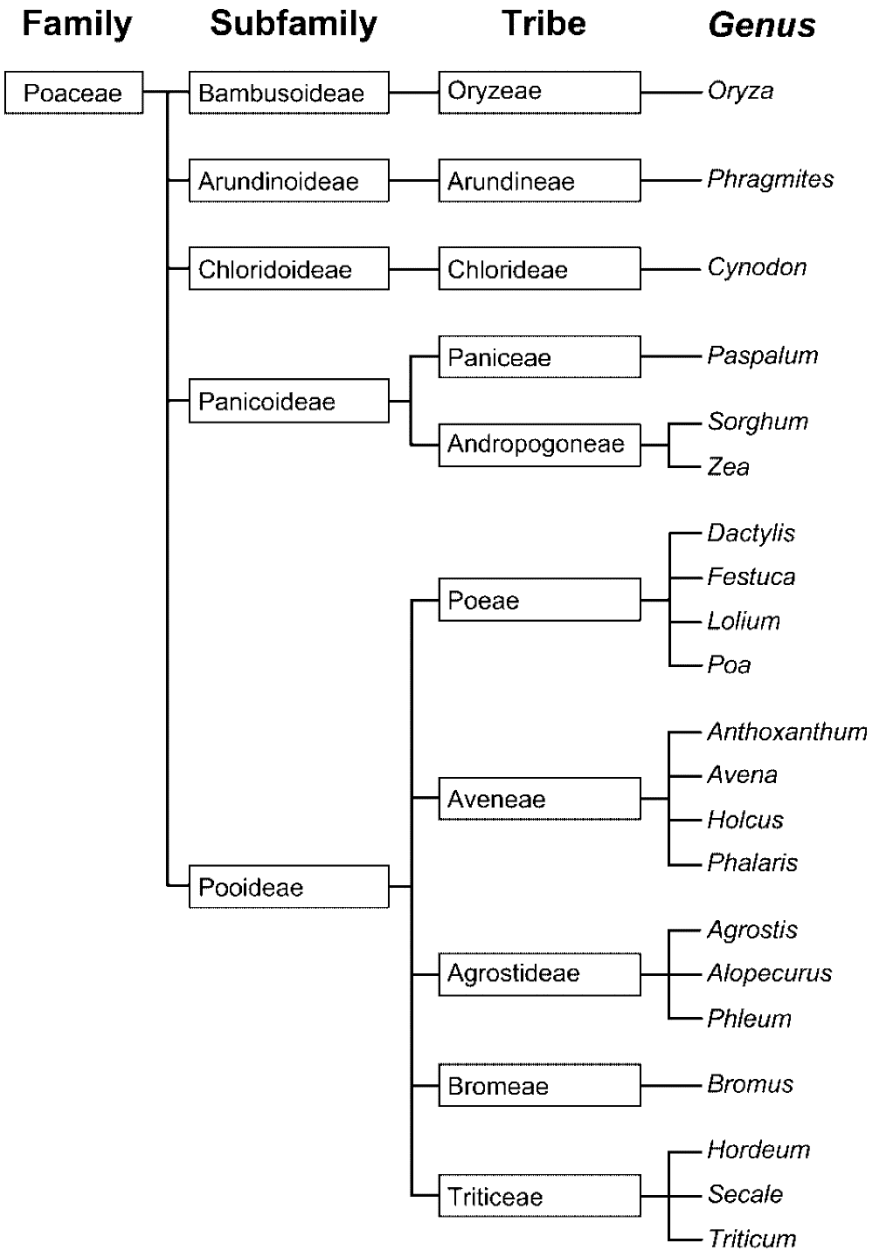

Figure VII. Phylogenetic relationships between grass genera representing important allergenic species, (taken from **[Lewis, et al. 1983]**).

Due to cross-allerginicity it is difficult to identify which grass species was the original source of sensitization **[Jaeger, 2008] [Moingeon, et al., 2008]**. This has important implications in terms of vaccine design, because a vaccine that combines different grass species would be more advantageous as it reflects natural exposure **[Moingeon, et al., 2008]**.

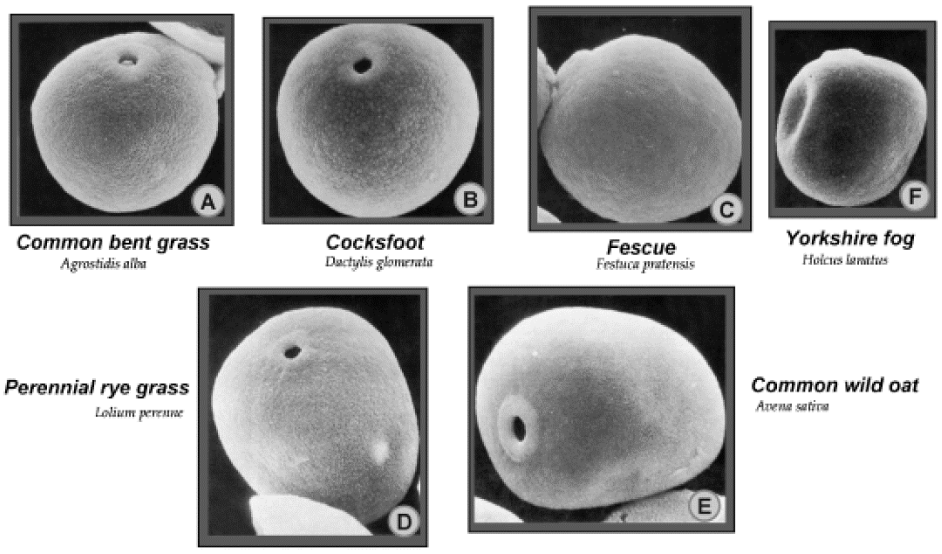

Figure VIII. The structures of grass pollen grains from the Pooideae sub-family have common features, (taken from **[Jaeger, 2008]**).

II.3.1.1 Group 1 and Group 5 grass pollen allergens

Although major allergens Group 1 (beta-expansins) and Group 5 (ribonucleases) dominate the allergenicity of grasses, Group 5 allergens have been identified exclusively in the Pooideae subfamily but not outside this taxon **[Niederberger, et al., 1998]**.

Group 1 allergens (expansins) are glycoproteins in a reported size range of 31-35 kD **[Schramm, et al., 1996]**. Isoform variation among this group of allergens has been demonstrated in several species with respect to the isoelectric point, molecular weight and amino acid sequence **[Smith et al., 1994]**. The family of expansins has been divided into α- and β-expansins; the latter including group 1 grass pollen allergens **[Cosgrove, et al., 1997]**. The overall amino acid sequence identity among known group 1 allergens from different species ranges in the 90% and >90% **[Weber, 2003]**.

Notwithstanding their phylogenetic confinement, more diversity has been found within Group 5 allergens than within Group 1 allergens **[Niederberger, et al., 1998]**. Group 5 allergens are similar in size compared to Group 1 allergens, with a size range of 27- to 33-kD **[Schramm, et al., 1996]**. For this group, two main isoforms have been identified, denoted as "a" and "b" with amino acid sequence variation **[Petersen, et al., 1992]**. The overall amino acid sequence identity found in group 5 allergens from different grass species ranges between 55% and 85% **[Andersson, et al., 2003]**. Thus, because the Pooideae sub-family forms a consistent clade of species that are highly homologous, cross-allergenicity for both group 1 and 5 allergens can be expected.

II.3.2 Cross-allergenicity in closely related species (Tree nuts)

In the case of tree nut allergens, most of the patients are allergic to widely consumed tree nuts **[Roux, et al., 2003]**. It has been estimated that at least 86% of subjects who are allergic to at least one tree nut are allergic to several foods in the tree nut group **[Clark, et al., 2005]**. Although, no propagation system has naturally evolved like grass pollens, enabling them to be present in high concentrations in the air, foods are another type of a perfect allergenic vector due to their ubiquity in food diets. However, we must also acknowledge that food processing conditions, not only may play a role in increasing allergenicity but also act as blind sensitization in allergic subjects, as subjects that eat one tree nut and develop reactions may in fact remain unaware of traces of other nuts present in foods **[Masthoff, et al., 2013]**. Cross-contamination occurs when a safe food comes in contact with a food allergen such as peanut, tree nut, seafood or milk. The slightest trace of an allergic food can cause a potentially threating reaction **[http://www.foodallergy.org]**.

Most of tree nut allergens belong to the seed storage protein family, this proteins are resistant or susceptible to enzymatic digestion and extreme pH upon consumption **[Schulten, et al., 2012]**; this factors should also be taken into consideration as they may play a crucial role in the allergenicity of these allergens.

II.3.2.1 Seed storage proteins, definition and evolution

In botany, a nut is defined as a fruit part of a flowering plant that contains the plant or trees' seeds. These are generally edible, and are defined as dried seeds, because the fleshy tissue is reduced when compared to a fruit **[Fuleki, 2009]**. Not only are seeds an organ of propagation and dispersal, but also a part of the embryonic plant. Seeds contain a high number of protein groups that provide storage for nutrients and amino acids that are useful during germination and seedling growth **[Fuleki, 2009]**. Despite wide variation in their structures, all seed storage proteins have common properties due to their significance in

species survival. First, they are synthetized at high levels in specific tissues at certain stages of development. Second, these structures are also present in mature seeds **[Shewry, et al., 1995]**. Interestingly, it has been suggested that all seed storage protein fractions are mixtures of components that exhibit polymorphisms both between single genotypes and among genotypes of the same species **[Argos, et al., 1985] [Shewry, et al., 1995]**. This polymorphism is characterized by the presence of multigene families. As previously mentioned, seed storage proteins are common in tree nuts, legumes (e.g. including peanuts and soy) and plants. These proteins coexist in tree nuts, although the relative levels of expression may vary among species **[Roux, et al., 2003]**. The major seed storage proteins in for tree nuts groups are:

a. The legumin or 11s family
b. The vicilin or 7s types
c. 2s albumins

The evolution of the legumin and vicilin families has been previously studied **[Gibbs, et al., 1989]**. Gibbs, et al. showed that an ancestral gene encoding one copy of the repeat domain first underwent duplication, by homologous recombination, or, unequal crossover. This two gene domains underwent a subsequent duplication that gave rise to ancestors of the vicilin and legumin families. These duplicated domains have persisted in vicilins, while a different sequence has replaced, in part, the N-terminal domain in the legumins (**Figure IX**). Gene conservation within vicilins and legumins explains why structural similarities are observed at the protein level amongst this group of proteins **[Shutov, et al., 1995]** and probably why both are allergenic.

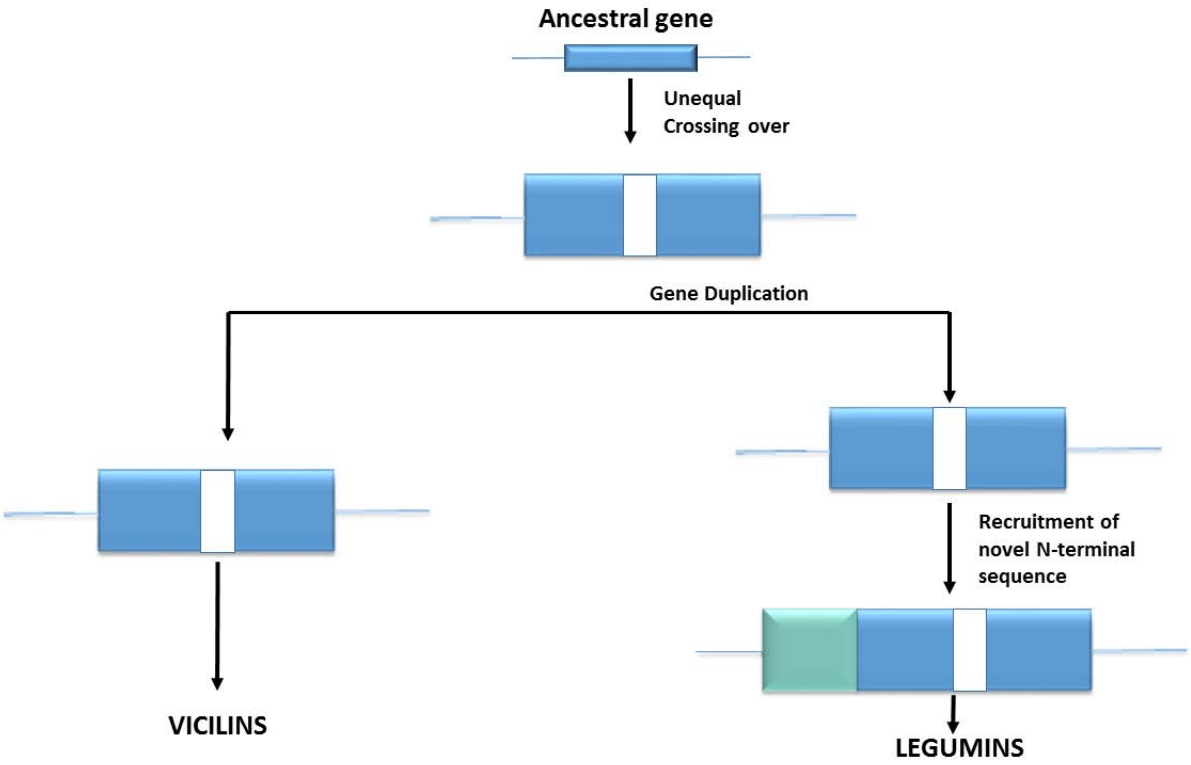

Figure IX. Evolution of vicilins and legumins from a common precursor. (Adapted from **[Gibbs, et al., 1989]**).

In food allergy, cross allergenicity is found among the major allergens of the tree nut group **[Roux, et al., 2003]**. As explained earlier, the fact that protein structures from the seed storage protein group evolved from a common ancestor gene might explain why these proteins are allergenic. Second, the tree nut clade forms a comprehensive group of nuts that have similar characteristics due to its nature of germination capacity. Therefore, it might be expected that because of their phylogenetically relatedness sensitization to one component may be triggered by another homologous component that was not the original source of sensitization. Indeed, common clinical practice suggests tree nut avoidance when food allergy has been initially diagnosed for one tree nut, as cross-recognitions with reactions are common **[Roux, et al., 2003]**.

II.3.3 Cross-allergenicity in distantly related species

Additionally to major allergens, minor allergens have also been shown to be accountable for cross-recognition of unrelated species. Many minor allergens are structures that are involved in vital functions and can therefore be found in plants to humans **[Judd, et al., 1999]**. Sequence conservation is reflected by highly similar structures that retain equivalent biological purposes **[Cockerham, et al., 1987]**. Examples of these types of allergens are Panallergens and have been identified in the plant kingdom **[Moreno-Aguilar, 2008]**. Profilins and lipid transfer proteins (LTP) are the main examples of panallergens **[Borges, et al., 2008]**. As an example, Lipid transfer proteins seem to be the major allergen found in Rosaceae fruits **[Diaz-Perales, et al., 2000]**. Recent reports have identified that lipid transfer proteins (LTPs) have a unique importance because they frequently cross-react with non-Rosaceae food (nuts, vegetables, legumes and cereals) and pollen (Birch) homologues **[Schulten, et al., 2011] [Tordesillas, et al., 2009]**. Hence, the majority of patients sensitized to LTP seem to display adverse reactions upon contact to multiple allergen sources **[Flinterman, et al., 2008]**.

C. $CD4^+$ T cells in allergy

It is now firmly established that $CD4^+$ T-cells play a central role in allergic inflammation in airway diseases and food allergy **[Akdis, et al., 2004]**. The aim of this section is: 1) to give an overview of the current knowledge of the various co-stimulatory molecules; 2) to summarize the different T cell sub-populations that play a role in allergy; 3) to explain the differentiation stages and plasticity of T cells; 4) to discuss pMHC class II tetramers and related methods for detection of antigen-specific T cells; 5) to explain how epitopes contribute to cross-reactivity at the T cell level and 6) to explain the types of immunotherapies commonly used in practice.

I. T cell recognition

Human beings are exposed to a variety of pathogens, which induce customized immune responses and thus, generate highly diverse populations of pathogen-specific T cells with different roles of protection. $CD4^+$ T cells have a central role in adaptive immunity and provide crucial help for both cytotoxic T cell and antibody-mediated responses **[Geginat, et al., 2013]**.

The adaptive immune response initiates with an early interaction between the antigen (allergen) and immune cells. As previously mentioned in the previous section (*IV.1 The cycle that perpetuates a Type I allergic immune response*), Antigen Presenting Cells (APC), such as Dendritic cells, Langerhans cells, monocytes, macrophages and B cells, are chemo-attracted into the site of exposure and depending on other factors produced by epithelial cells can promote the induction of particular phenotypes. After APC capture the allergen, these cells go through a maturation stage that enables them to migrate to secondary lymphoid organs. Subsequently, APC process the allergen into a small set of peptides (T cell epitopes) and express major histocompatibility complexes bound to peptides on their cell surface. Antigen-mediated immune

responses are controlled and driven by the specific activation of T cells. The recognition of allergen-derived epitopes by $CD4^+$ naive T cells requires the integration of multiple positive and negative signals to properly induce cellular activation, proliferation and differentiation into an effector status **[Banchereau, et al., 1998]**. These three different signals required for T cell activation are (**Figure X**):

a. Signal 1, derived from engagement of the T cell's TCR with peptide bound MHC-II molecules on the surface of APC.

b. Signal 2, delivered by co-stimulating receptor interactions between T cells and APC.

c. Signal 3, derived from cytokines that are produced in response to inflammation.

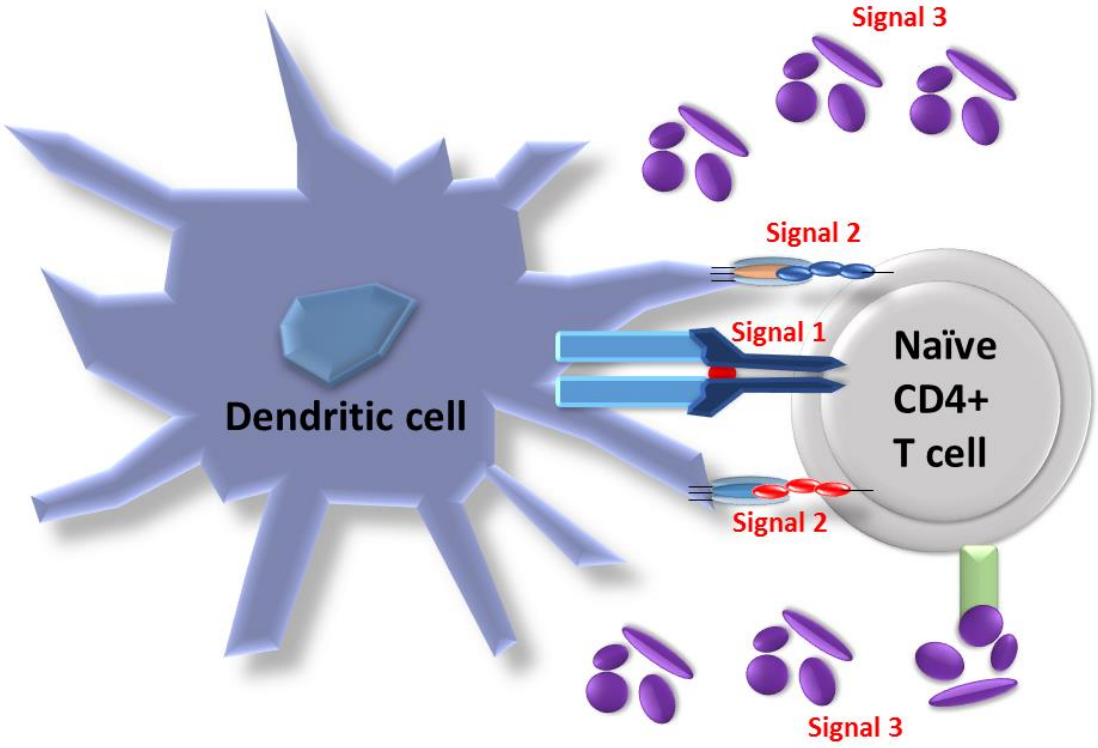

Figure X. T cell activation requires three signals. (Adapted from [**Yin, et al., 2014]**).

I.1 Signal 1, TCR recognition of specific epitopes

The so-called T cell receptors (TCR) for foreign antigens are composed of two chains, alpha and beta **[Dai, et al., 2008]**. The genes coding for these chains are created by gene rearrangements and thus, several combinations of different Vα and Jα chains can code about 5000 different TCRα chains **[Dai, et al., 2008]**. Depending on the gene rearrangements, different nucleotides can be added or removed, resulting in considerable variability in DNA sequence, and therefore amino acid sequence, at this junction **[Yin, et al., 2013]**. This very variable region lies within what is known as the CDR3 loop of the TCRα chain (**Figure XI**), and random DNA sequences in this junction can occur in an organism by several orders of magnitude, up to 10^7 **[Garcia, et al., 2009]**. A similar phenomena applies to rearrangements for the TCR beta chain, leading again to a very large number of possible TCRβ chain sequences **[Garcia, et al., 2009]**. These gene rearrangements account to the most hyper variable gene sources found in nature **[Dai, et al., 2008]**. The capacity to generate multiple gene rearrangements explains why TCR are so variable and capable of targeting a particular pathogen.

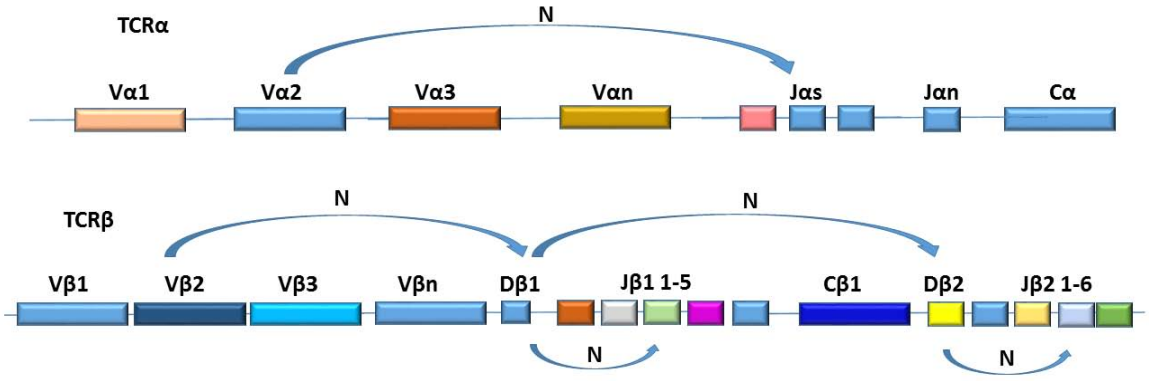

Figure XI. Diagram of the rearrangements that give rise to genes coding for T cell receptor alpha and beta polypeptides (Adapted from **[Yin, et al., 2014]).**

The TCR usually reacts with foreign antigens in the form of small peptides generated from the allergen material bound to MHC-protein complexes **[Steinman, et al., 1985]**. The allergenic peptide fills

the groove of the MHC molecule and the TCR lies above the two in an approximately diagonal orientation **[Garcia, et al., 1996] [Garcia et al., 1998]**. Class II MHC/peptide structures have been thoroughly characterized, and it is established that peptide side chains at positions p1, p4, p6 and p9 project into pockets along the MHC peptide-binding groove acting as anchor motifs **[Southwood, et al., 1998] [Yassai, et al., 2002]**. Conversely, peptide side chains at position p-1, p2, p3, p5, p8, p10 project directly upward from the MHC-molecule surface and impact T-cell recognition through TCR **[Southwood, et al., 1998] [Yassai, et al., 2002] (Figure XII)**. T cell activation by this receptor interaction alone fails to induce cytokine production and sustained proliferation, but rather results in T cell apoptosis, or the induction of specific non-responsiveness (anergy) to subsequent stimulation with the same antigen **[Mueller, et al., 1989]**.

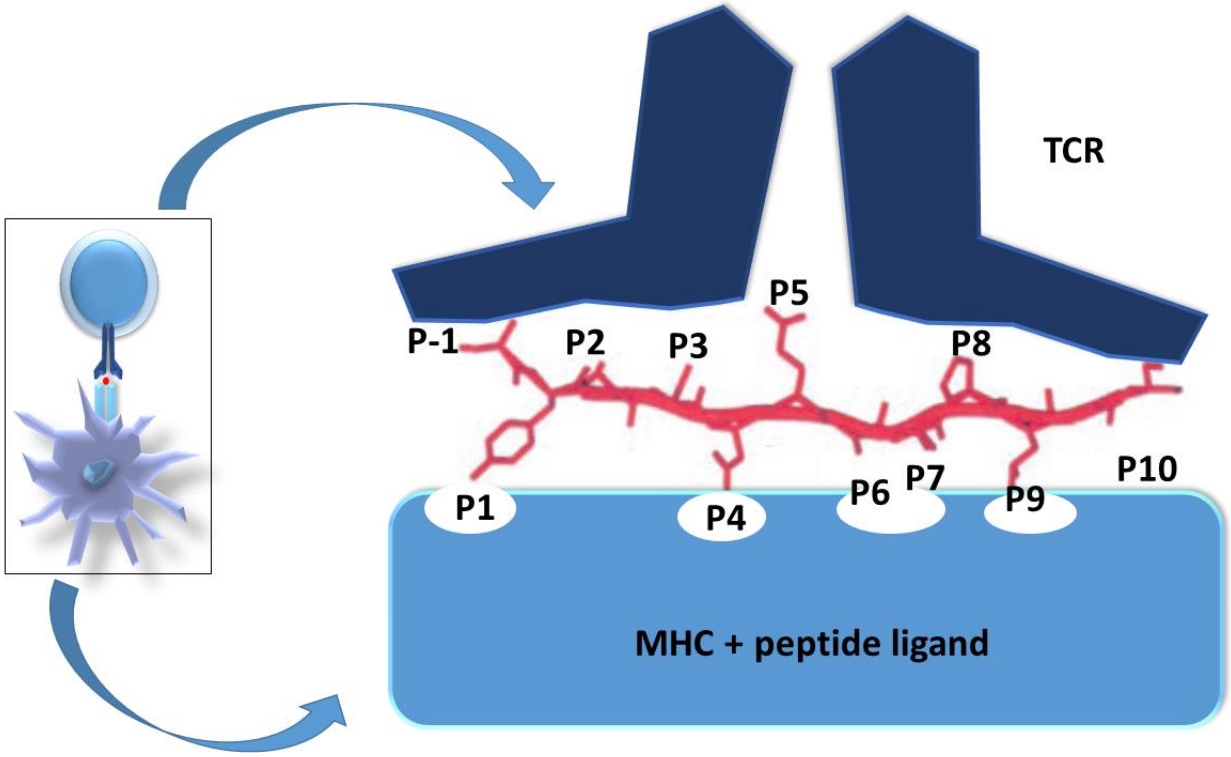

Figure XII. Illustration of the position of T cell receptor on a MHC + peptide ligand (Adapted from **[Yassai, et al., 2002]**).

I.2 Signal 2, co-stimulation molecules

Signal 1 alone is not usually sufficient to trigger the T cell into full activity; however, T cells usually need to receive additional, so-called co-stimulatory signals **[Jenkins, et al., 1987]**. Signal 2 is delivered by interaction between proteins on both T cells and APC. These molecules are transmembrane proteins that induce an intracellular signaling cascade via their cytoplasmic tail that modifies the TCR-mediated signal **[Jenkins, et al., 1987]**. Without concomitant TCR crosslinking, co-stimulatory molecules cannot activate T cells **[Jenkins, et al., 1987]**. On the other hand adhesion molecules are not considered to be stimulatory molecules, as they only enhance the immunological synapse, contact of T cell and APC **[Beier, et al., 2007]**. Furthermore, co-stimulation refers to a signal that is delivered to the T cell exclusively. An example is the CD40/CD40L interaction, important receptor/ligand interaction T and B cell cooperation **[Beier, et al., 2007]**.

Co-stimulatory molecules can be constitutively expressed or inducible, can generate positive (enhancement) or negative (inhibition) signals, have an effect on T cell function and can provide information about the location of action. Some of the most important co-stimulatory molecules in allergy are summarized in **Table IV**.

FUNCTIONAL ASPECT	CHARACTERIZATION	CO-STIMULATORY MOLECULE	LIGAND
EXPRESSION PATTERN	Constitutively	CD28	B7-1, B7-2 (CD80, CD86)
		CD27	CD70
	Inducible	ICOS	ICOS-L
		OX40(CD134)	OX40L
		CTLA-4(CD152)	B7-1, B7-2 (CD80, CD86)
T CELL MODULATION	Positive/enhancement	CD28	B7-1, B7-2 (CD80, CD86)
		ICOS	CD70
		OX40	ICOS-L
		CD27	OX40L
	Negative/inhibition	CTLA-4(CD152)	B7-1, B7-2 (CD80, CD86)
		PD-1	PD-L1, PD-L2
T CELL DIFFERENTIATION	TH2	ICOS	ICOS-L
		OX40	OX40L
		CD30	CD30L (CD153)
T CELL FUNCTION	T effector/helper	ICOS	ICOS-L
		CTLA-4	B7-1, B7-2 (CD80, CD86)
		OX40	OX40L
	Central	CD28	B7-1, B7-2 (CD80, CD86)
		CTLA-4	B7-1, B7-2 (CD80, CD86)
	Central and Peripheral	ICOS	ICOS-L
		OX40	OX40L

Table IV. Currently known co-stimulatory molecules, their ligands, and functional classification. (Adapted from **[Beier, et al., 2007]**).

After the extent of TCR ligation, other contact-dependent signals important in allergy are OX40L-OX40 and B7-CD28 interactions. As previously mentioned TSLP is a cytokine that induces differentiation of T_H2 cells from naïve T cells through a DC-mediated pathway involving OX40L/OX40. OX40 co-stimulation enhances differentiation of naïve $CD4^+$ T cells and IL-4 expression after priming. OX40L/OX40 interaction also increases the expression of B7-1 and B7-2, promoting an efficient activation of T cells **[Ohshima, et.al., 1997]**.

The role of B7/CD28 interactions are more complex. Production of IL-4 by naïve T cells is also dependent on B7 molecules **[Hathcock, et al., 1994]**. Naïve CD4 T cells seem to be receptive to CD28-

dependent IL-4 production only if they receive weak TCR signals **[Harding, et al., 1992]**. The CD27/CD70 interaction promotes T cell survival **[Hintzen, et al., 1995]**. CD27 is constitutively expressed on T cells, and is subsequently down regulated on T effector cells after several rounds of cell division, in a similar fashion as CD28 **[Tesselaar, et al., 2003]**. Effector cells lacking CD27 display a high antigen recall response, whereas $CD27^+$ memory T cells require additional co-stimulation for T cell receptor triggering **[Tesselaar, et al., 2003]**. Conversely, CTLA-4/B7 interactions inhibit the activation of $CD4^+$ T cells, through the blockade of IL-2 production. It has been previously shown that injection of anti-CTLA-4 antibodies in mice during the sensitization phase generates an exacerbation of the T_H2 response and down regulation of T regulatory cells that produce IL-10 **[Nuriya, et al., 2001] [Botturi, et al., 2008]**. THE PD-1/PD-L1 interaction also down modulates T cell responses **[Bennet, et al., 2003]**.

Some co-stimulatory molecules are down regulated upon repetitious stimulation. Therefore, naïve T cells, recently activated, or memory T cells express a unique combination of co-stimulatory receptors depending on their history of activation and state of differentiation **[Beier, et al., 2007]**.

I.3 Signal 3, cytokines

Signal 3 is thought to be often derived from the soluble cytokines that are produced in response to inflammation (e.g. IL-4, TNF-α, IL-6, IFN-γ, etc) **[Dinarello, 2002] [Croft, et al., 2010] [Hintzen, et al., 1994]**.

Thus, depending on the integration of multiple positive and negative signals generated upon T cell priming, T cells can differentiate into virtually all different types of effector, memory or regulatory cells **[Gabrysova, et al., 2010]**. This highly depends on the characteristics of the allergens and the epitopes as TCR signaling can affect the outcome of the cascade of events that lead to a particular phenotype or tolerance **[Gabrysova, et al., 2010]**.

II. T cell sub-populations

Human $CD4^+$ T cells are critical regulators of the immune system. Different subsets have evolved with specialized functions permitting them to counterattack a high number of pathogens **[Geginat, et al., 2013**]. Helper T cell subsets are defined by the production of cytokines and/or the expression of characteristic lineage-defining transcription factors **[Mucida, et al., 2010]**. Seven main subsets of $CD4^+$ T cells have been identified so far: T_H1, T_H2, T_H9, T_H17 and T_H22 cells that target specific classes of pathogens, regulatory T_{reg} cells that maintain self-tolerance and follicular helper T cells (T_{FH}) that provide help to B cells for antibody production (**Figure XIII**) **[Mucida, et al., 2010] [Jabeen, et al., 2012] [Souwer, et al., 2010] [Allan, et al., 2008] [Crotty, 2011]**. Depending on the cytokine environment naïve T cells can differentiate into various types of effector cells **[Sallusto, et al., 2004]**. T_H1 and T_H2 central memory cells are arrested at an early stage of differentiation, are highly plastic and some can still switch lineage **[Sallusto, et al., 2004]**. Conversely, antigen experienced cells are less flexible, but many subsets retain some plasticity and rather become polyfunctional after antigenic re-stimulation **[Sallusto, et al., 2004]**. Although allergic diseases are primarily driven by T_H2 cells, other subsets like T_H9, T_H2/T_H17, T_H17, T_H22, T_{reg} (T_R1-like) and T_{FH} subsets have different functional roles in allergy **[Romagnani, 2000]**.

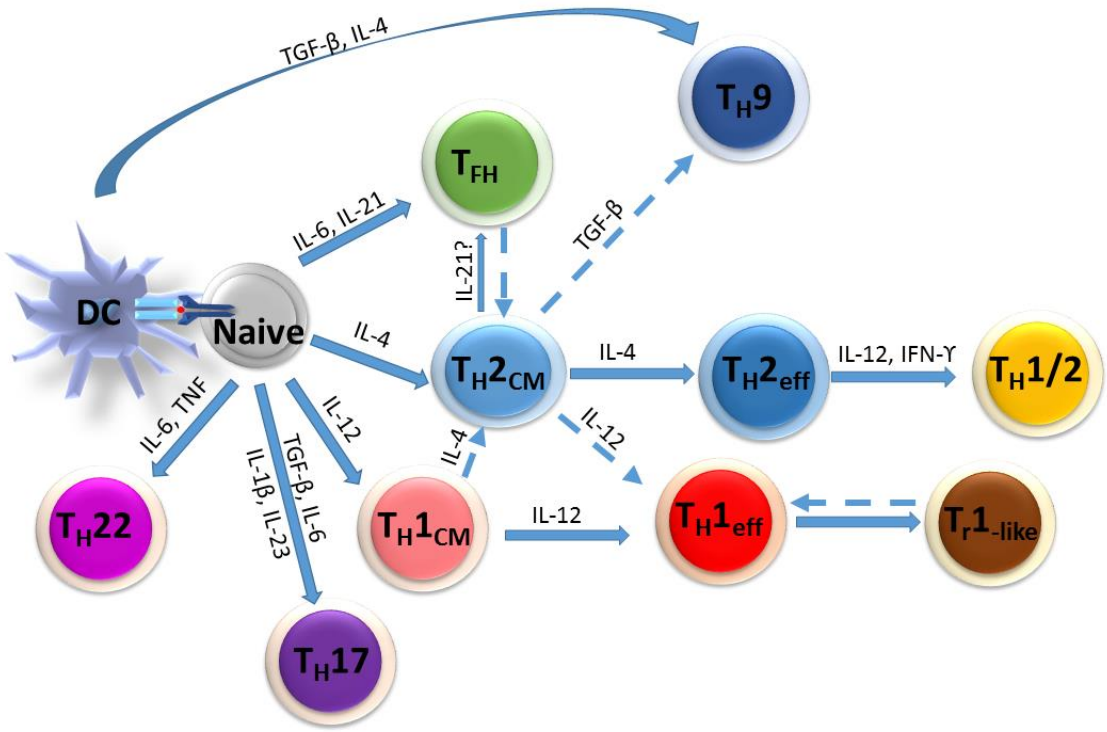

Figure XIII. Plasticity of human T cells. Naïve CD4+ T cells are "stem-cell-like' that under the influence of different cytokines can differentiate to various types of effector cells. (Adapted from [**Geginat, et al., 2014]**).

II.1. T helper 1 cells

T_H1 cells are promoted through IL-12 rich environments that induce the expression of their characteristic transcription factor T-box 21(*tbx21*, T-bet) and phosphorylation of STAT4. They primarily produce IFN-γ, and T_H1 cells were considered essential for antiviral immunity and providing help to $CD8^+$ cytotoxic T cells **[Kanno, et al., 2012]**. Recent literature suggests that T_H1 cells can also play a role in allergic disease, such as acute lesional skin, where IFN-γ induces keratinocyte and epithelium apoptosis, a common trait of the pathology found in atopic dermatitis **[Akkoc, et al., 2008]**. Recent studies suggest that, T_H1 cells are the only subsets found in non-allergic subjects, implying that these cells play a protective role **[Wambre, et al., 2012] [Wambre, et al., 2014]**.

In addition, IL-10 producing T_H1 cells have also been described **[Akdis, et al., 1998] [Akdis, et al., 2004] [Verhoef, et al., 2005]**. It has been shown that IL-12 rich environments promoted the production of IL-10 in T_H1 cell clones expressing T-bet as its master regulator **[Gerosa, et al., 1996] [Maynard, et al., 2008**]. IL-10 has potent anti-inflammatory functions and inhibits maturation and T cell stimulatory capacities on APC, thus this type of T_H1 cells that are capable of producing IL-10 have been defined as Type 1 regulatory-like T cells (T_R1) **[Akdis, et al., 1998]**, because IL-10 plays an important role in both induction and maintenance of specific T cell tolerance and might be implicated in the mechanism of desensitization **[Akdis, et al., 1998] [Nouri-Aria, et al., 2004]**. Interestingly allergen-specific T_R1 cell populations have been detected in non-allergic subjects as allergic subjects ongoing immunotherapy, suggesting a tolerance/protective role **[Wambre, et al., 2014]**.

II.2. T helper 2 cells

T_H2 cells require IL-4 rich environments to promote the phosphorylation of STAT6 signaling, which induce the expression of the master transcription factor GATA3 (*GATA-3*) **[Rivino, et al, 2004]**. IL-4, IL-5 and IL-13 are the signature cytokines of type 2 responses, but other cytokines such as IL-9 and IL-10 can also be produced **[Veldhoen, et al., 2008]**. This subset plays major roles in orchestrating humoral immune responses against extracellular parasites **[Coffman, et al., 1991]**. Allergic diseases are primarily driven by T_H2 cells, the effector portion of this response includes IgE production, infiltration of eosinophils and mast cells, leading to the propagation of inflammation **[Romagnani, et al., 2000].** Moreover, T_H2 cells have additional plasticity. Under rich TGF-β cytokine environment T_H2 cells can switch from IL-4 to IL-9 production **[Veldhoen, et al., 2008]**.

II.3. T helper 9 cells

Recent reports have described a new subset of the T helper population separate from T_H2 populations that produces IL-9 in large quantities and contributes uniquely to immune responses **[Jones, et al., 2012]**. This subset requires TGF-β and IL-4 cytokine environments to express the PU.1 (*PU.1*) transcription factor **[Jones, et al., 2012]**. T_H9 cells have pro-inflammatory properties and respond to helminth and allergenic antigens **[Jones, et al., 2012]**.

II.4. T follicular helper cells

T follicular helper cells were first identified as a subset of $CD4^+$ T cells isolated from human tonsils **[Crotty, 2011]**. Early differentiation toward the T_{FH} lineage requires ICOS expression and signaling to induce expression of the transcriptional repressor Bcl-6 **[Johnston, et al., 2009] [Crotty, 2011] [Hale, et al., 2015]**. In addition, cytokines such IL-6, IL-21 and other molecules such as SAP are important for T_{FH} differentiation and function **[Choi, et al., 2011]**. T_{FH} professional helper T cells secrete IL-21 in B cell follicles, where they promote antibody production, germinal center formation and survival of memory B cells **[Crotty, 2011]**. T_{FH} cells can express low to intermediate levels of T-bet, GATA3, or RORγt, resulting in a variety of subsets that can influence antibody class switching **[Hale, et al., 2015]**.

II.5. T helper 17 cells

The differentiation from naïve $CD4^+$ T cells into the T_H17 lineage requires IL-1, Il-6, IL-21, IL-23 and TGF-β cytokine rich environments that promote the phosphorylation of STAT3, thus, inducing the expression of the lineage-defining transcription factor RORC2 **[Acosta-Rodriguez, et al., 2007] [Annunziato, et al., 2007] [Unutmaz, 2009]**. T_H17 cells have important immune roles in fighting extracellular bacteria and fungi **[Unutmaz, 2009]**. T_H17 cells are highly heterogeneous and can produce

great amounts of other cytokines besides IL-17, like IL-21, IL-22, IL-25 and TNF-α. T_H17 are partially unstable (**Figure XIV**) and can switch to co-produce T_H1 cytokines (in rich IL-12 cytokine environments) **[Annunziato, et al., 2007]** and co-produce IL-17 and IL-4 (in rich IL-4 cytokine environments) **[Cosmi, et al., 2010]**. These T_H2/T_H17 cells were proposed to be highly pro-inflammatory in allergic asthma, but their role in immune responses against pathogens remains to be understood.

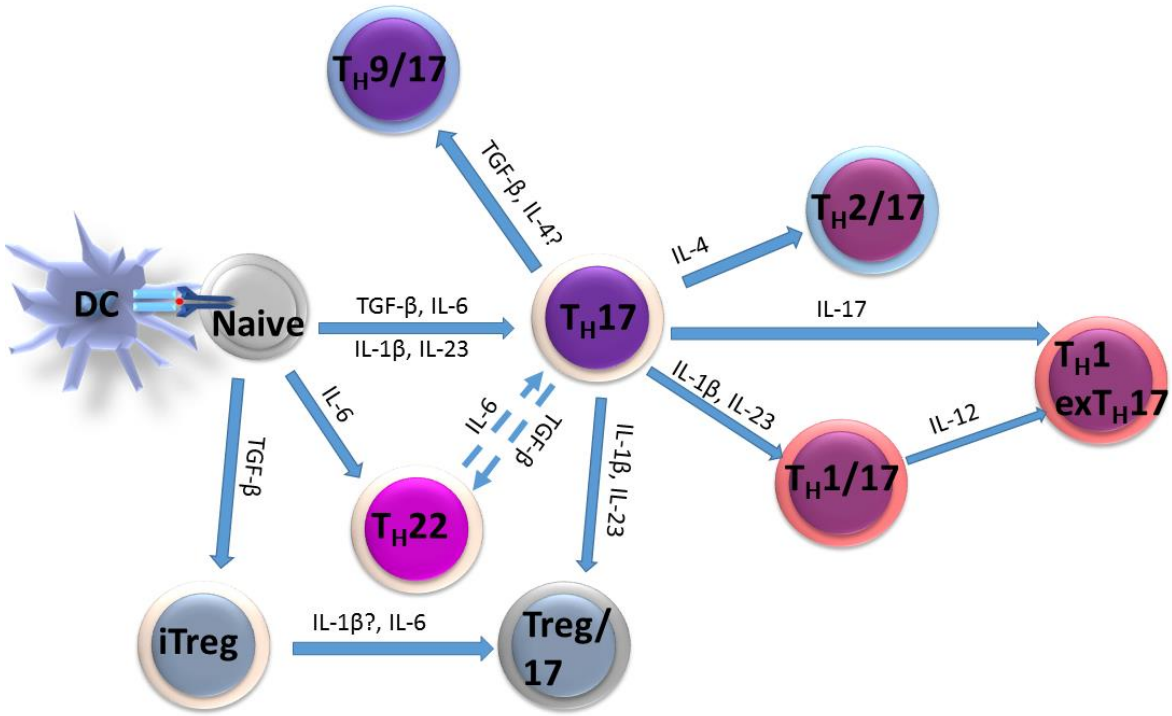

Figure XIV. Heterogeneity and plasticity of human T_H17 cells. T_H17 CD4+ T cells are highly heterogeneous and produce various types of cytokines under the influence of different cytokine environments. (Adapted from [**Geginat, et al., 2014]**).

II.6. T helper 22 cells

T_H22 cells require IL-6 and TNF rich cytokine environments for their commitment into this lineage. T_H22 cells are characterized by IL-22 production and play an important role against microbial pathogens **[Liang, et al., 2006]**. IL-22 has both pro-inflammatory and protective properties, but the dual effects of IL-22 are not yet fully understood **[Souwer, et al., 2010]**.

III. T cell migration and maturation

III.1 T cell migration

Passage of T cells through the endothelial venule barrier follows 4 subsequent steps: rolling, chemokine-mediated activation and subsequent firm adhesion, followed by diapedesis and migration (**Figure XV**). This process requires both extravasation and chemotaxis.

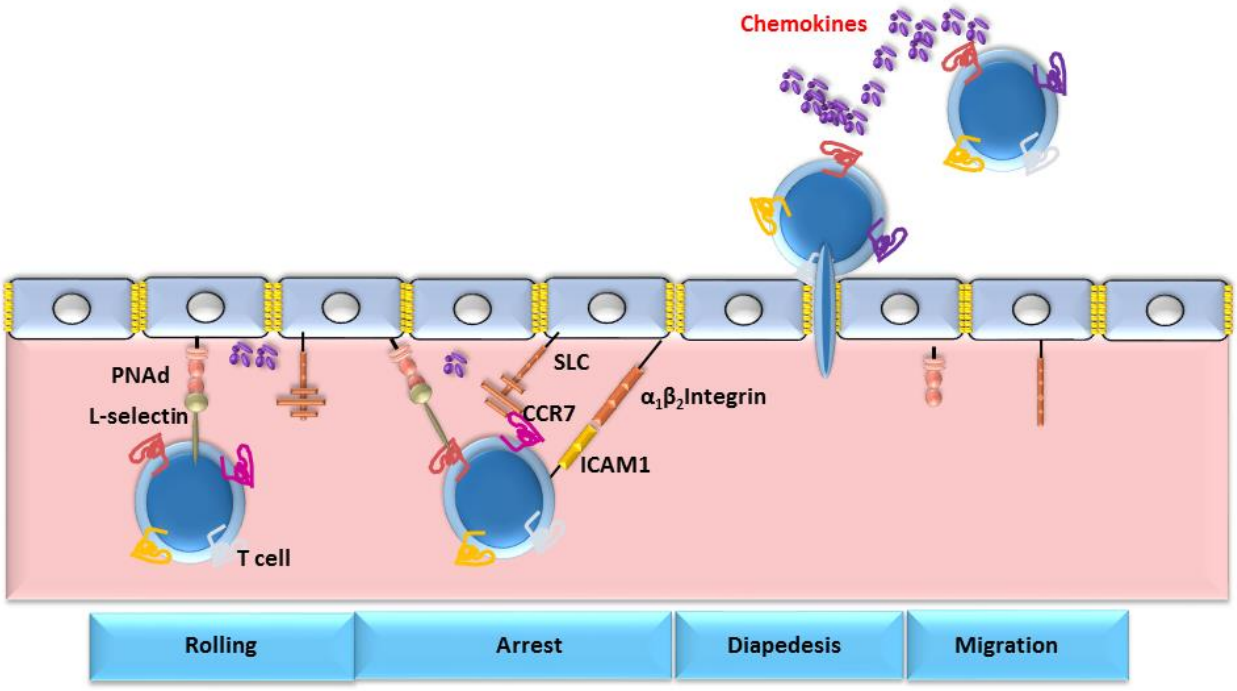

Figure XV. The process of T cell migration is divided into 4 steps. (Adapted from [**Pease, et al., 2006]**).

Naïve T cells are able to home to lymph nodes through a process of "rolling", an interaction between L-selectin CD62L (T cell) and peripheral node-addressins (PNAds) or mucosal cell adhesion molecule-1 (MAdCAM-1) (endothelial venules) **[Butcher, et al., 1999]**. This contact permits the chemokine receptor CCR7 to engage its ligand SLC (CCL19/ELC and CCL21), which is displayed by endothelial cells **[von Andrian, et al., 2003]**. Next, the CCR7/SLC interaction triggers a gradual, global increase in integrin affinity and avidity that promotes firm adhesion **[Cambpell, et al., 1998]**. After firmly arresting on endothelial cells, T cells must overcome physical barriers to enter the lymph node parenchyma through a mechanism known as transmigration **[Cambpell, et al., 1998]**. The increasing concentrations of chemokines or other chemoattractants enable T cells to find their way through inter-endothelial junctions through diapedesis and migrate into the focal site **[Carman, et al., 2008]**.

III.2. T cell maturation

The ability to mount adaptive immune responses requires antigen induced activation and expansion of naïve T cells into memory T cells (**Figure XVI**) **[Sallusto, et al., 2004]**. Naïve lymphocytes express CCR7, CD62L, CD27 and CD45RA and circulate mainly through secondary lymphoid organs **[Sallusto, et al., 1999] [Fritsch, et al., 2005]**. On the other hand, antigen experienced lymphocytes express CD45RO, and are able to upregulate the expression of various combinations of adhesion, chemokine and lipid chemoattractant receptors as a part of their differentiation into memory cells **[Sallusto, et al., 1999] [Sallusto, et al., 2004]**. These combinations of several receptors enable $CD45RO^+$ T cells to migrate into different peripheral tissues for both immunosurveillance and inflammatory responses **[Pease, et al., 2006]**.

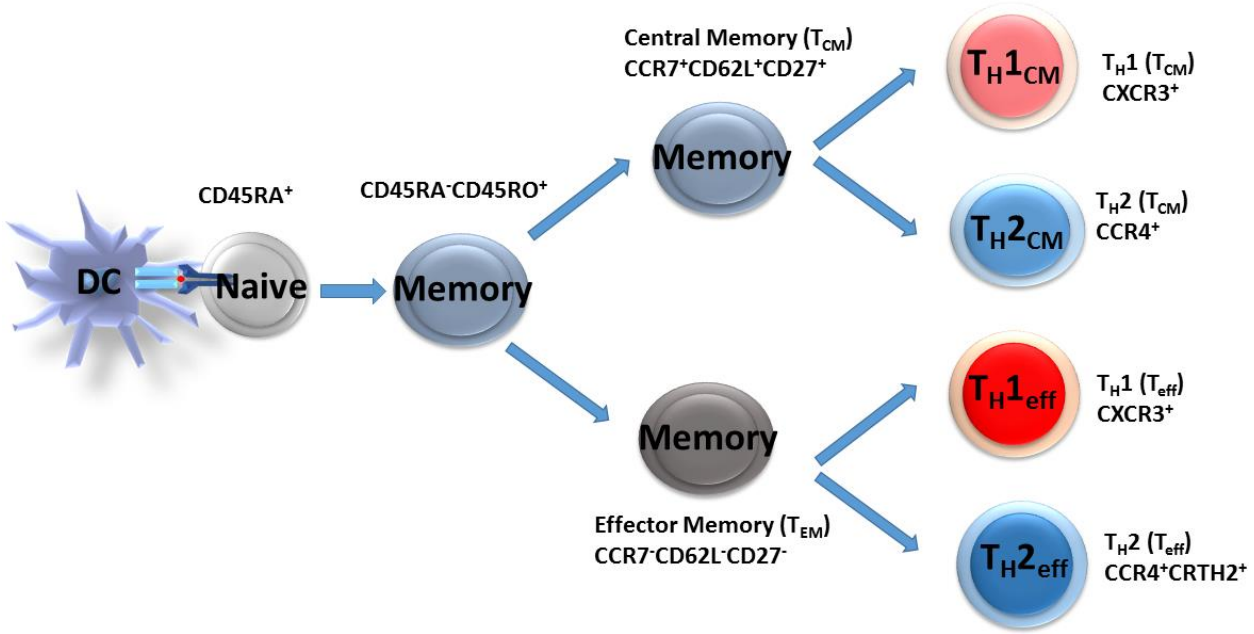

Figure XVI. Phenotypic properties of human $CD4^+$ T cell subsets. (Adapted from [**Woodfolk, et al., 2007]**).

Memory $CD4^+$ T cells have been classified into 2 distinct types on the basis of expression of CCR7, CD62L and CD27 **[Lanzavecchia, et al., 2002] [Fritsch, et al., 2005]**. Central memory T cells (T_{CM}) that express these three markers preferentially home to secondary lymphoid tissues and can readily proliferate into effector status after antigen encounter **[Sallusto, et al., 1999]**. By contrast, effector memory T cells (T_{EM}), which lack CCR7, CD27 and CD62L, traffic to sites of inflammation and can exert their effector functions rapidly **[Sallusto, et al., 1999] [Sallusto, et al., 2004]**. In the allergic scenario, T cells that loose CD27 have increased capacity of secreting IL-4 **[Fritsch, et al., 2005]**. Depending on the cytokine environments and the characteristics of the epitopes, DCs imprint T cells to express specific homing markers committing them into a particular lineage **[Sallusto, et al., 2004]**.

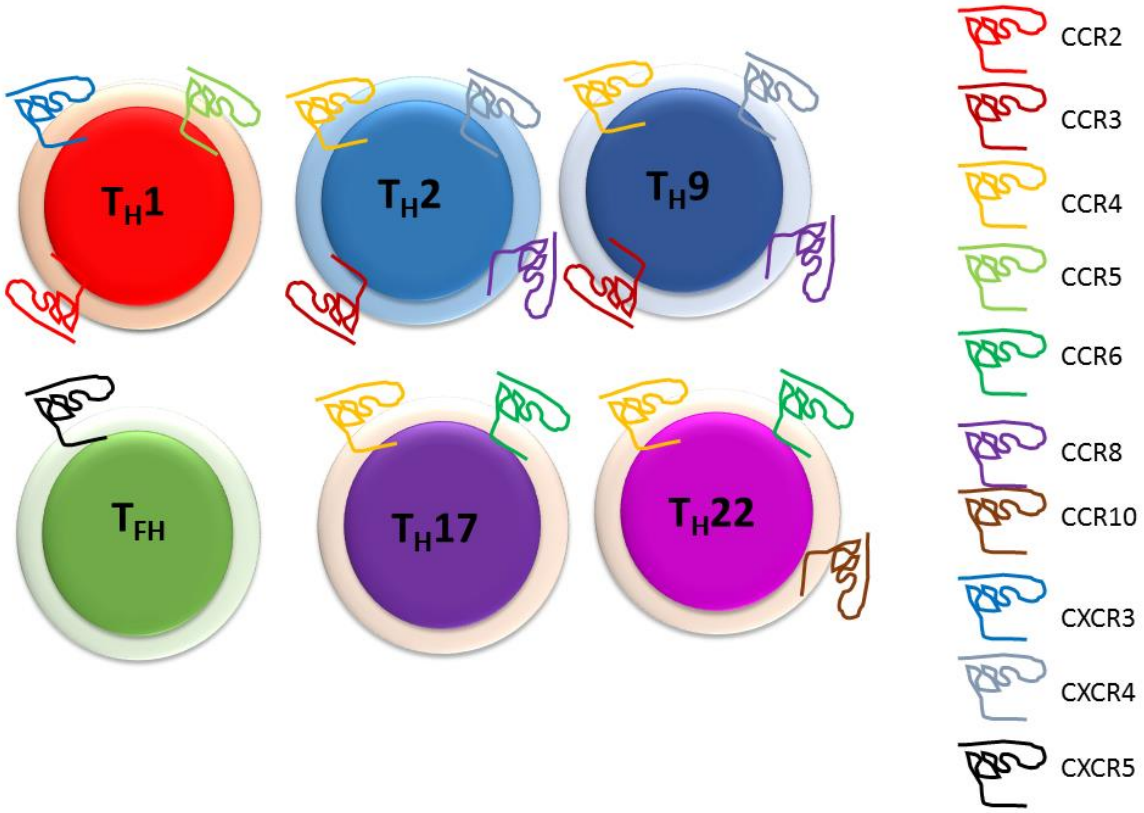

Figure XVII. The chemokine receptor repertoires of lymphocytes implicated in allergic disease. (Adapted from [**Pease, et al., 2006] [Duhen, et al., 2009] [Acosta-Rodriguez, et al., 2007] [Annunziato, et al., 2007**]).

After acquiring a memory status, T_H1 cells express CXCR3, CCR5 and CCR2, conversely, CCR4, CCR3, CXCR4 and CCR8 defines the T_H2 cells lineage. (**Figure XVII**) The T_H17 and T_H22 cell populations co-express CCR4 and CCR6 **[Acosta-Rodriguez, et al., 2007] [Annunziato, et al., 2007]**, however, CCR10 expression in the latter distinguishes both lineages **[Duhen, et al., 2009]**. T_H9 cells have a similar phenotype as T_H2, though the identification of a surrogate marker for this lineage remains to be identified. Finally, T_{FH} express CXCR5 **[Crotty, 2011]**. In the allergic context, the expression of particular chemokine receptors play different roles in the recruitment of allergen specific $CD4^+$ T cells into peripheral tissue where receptor-ligand interaction takes place **[Bromley, et al., 2008]**. Depending on the co-expression of different chemokine receptors, T cells have different capacities of migration (**Table V**).

RECEPTOR	LIGAND(S)	EXPRESSION PATTERN	FUNCTION(S)
CCR4	CCL17, CCL22	Skin-tropic $CD4^+$ T cells T_H2 cells T_H17 cells T_{reg} cells	Migration to inflamed tissues Migration to asthmatic airways Migration to sites of inflammation
CCR5	CCL11, CCL14, CCL16	T_H1 cells	Migration to inflamed tissues
CCR6	CCL20	T_H17 cells	Migration to inflamed tissues
CCR8	CCL1	T_H2 cells T_{reg} cells	Migration to normal skin Migration to sites of allergic inflammation
CCR9	CCL25	Gut-tropic $CD4^+$ cells	Migration to lamina propria and GALT
CCR10	CCL27	Skin-tropic $CD4^+$ cells	Migration to normal and inflamed skin
CXCR3	CCL9-CCL11	T_H1 cells	Migration to inflamed tissues
CXCR5	CXCL13	Follicular helper $CD4^+$ cells	Migration to T-B cell border and GC in lymph nodes

Table V. The chemokine receptors on T cell subsets and its ligands. (Adapted from **[Bromley, et al., 2008])**.

IV. Detection of allergen-specific $CD4^+$ T cells with pMHC-II tetramers and related methods

As a result of the high diversity of the T cell repertoires that mediate specific immune responses in allergy, it becomes important to understand the mechanisms of immunopathology as well as to correlate the frequencies, phenotypes and functional capacities to the particular allergen-specific T cell response and the immune status of an individual. This information as a whole is essential to develop new immunotherapies and understand the nature of disease. In this sub-chapter we will discuss the methods that are currently utilized to identify T cell epitopes and characterize allergen-specific $CD4^+$ T cells.

IV.1. T cell epitope identification methods

An epitope is part of a protein that is recognized by the immune system **[Li Pira, et al., 2010]**. In the allergy context, antigens are considered to have allergenic properties if they bind specific IgE antibodies **[Andersson, et al., 2003] [Basketter, et al., 2011]** and induce IgE mediated reactions **[Gieras, et al., 2007]**. On the other hand, epitopes within the allergen that trigger T cell reactivity can provide crucial knowledge for the design of peptide vaccines for immunotherapy and in many cases are not well studied. Several approaches have been utilized to identify T-cell epitopes, such as: ELISPOT, CD154 activation assay, Class II Tetramer guided epitope mapping (TGEM) and Mass spectrometry. The advantages and disadvantages of these methods are shown in **Table VI**.

METHOD	ADVANTAGES	LIMITATIONS	TIME
ELISPOT	Sensitive, provides both qualitative and quantitative information.	Limited to a few cytokines. Dependent on specific T cell function. T cell phenotype cannot be defined. Number of secreting cells can be underestimated	*Ex vivo*: 2-3 days. *In vitro*: 5-14 days.
CD154 ACTIVATION ASSAY	Provides quantitative information. T cell phenotypes can be identified. T cell assay compatibility (ICS, CFSE, CCA). Cells are not sacrificed and can be further utilized.	Dependent on specific T cell activation. Responses may not be antigen-specific due to by-stander activation	*Ex vivo*: 1 day. *In vitro*: 7 days.
MHC-II PEPTIDE TETRAMER	Sensitive, epitope-specific. Cells are viable. T cell phenotypes can be identified. T cell assay compatibility (ICS, CFSE, CCA, CD154).	Availability of recombinant MHC-II molecule. Knowledge of HLA information is required.	*Ex vivo*: 6 hours. *In vitro*: 14 days.
MASS SPECTROMETRY	Identification of large library of epitopes. Identification of unconventional binders. Peptide binding register simultaneously	Responses may not be antigen-specific due to By-stander. Cells are not viable. Although epitopes bind they may not activate T cells.	1 day

Table VI. Methods for the identification of T cell epitopes. (Adapted from **[Wambre, et al., 2012] [Li Pira, et al., 2010])**.

IV.1.1. HLA Class II Tetramers

Soluble class II molecules with leucine zipper regions attached to the carboxyl terminals of the molecules are produced in the *Drosophila* system **[Reijonen, et al., 2002]**. HLA class-II monomers assemble into "tetramer" (tetravalent) complexes through fluorochrome labeled streptavidin and are associated with a specific peptide. Class II HLA-peptide ligands can engage αβTCR and permit the analysis of antigen-specific $CD4^+$ T cells (**Figure XVIII**).

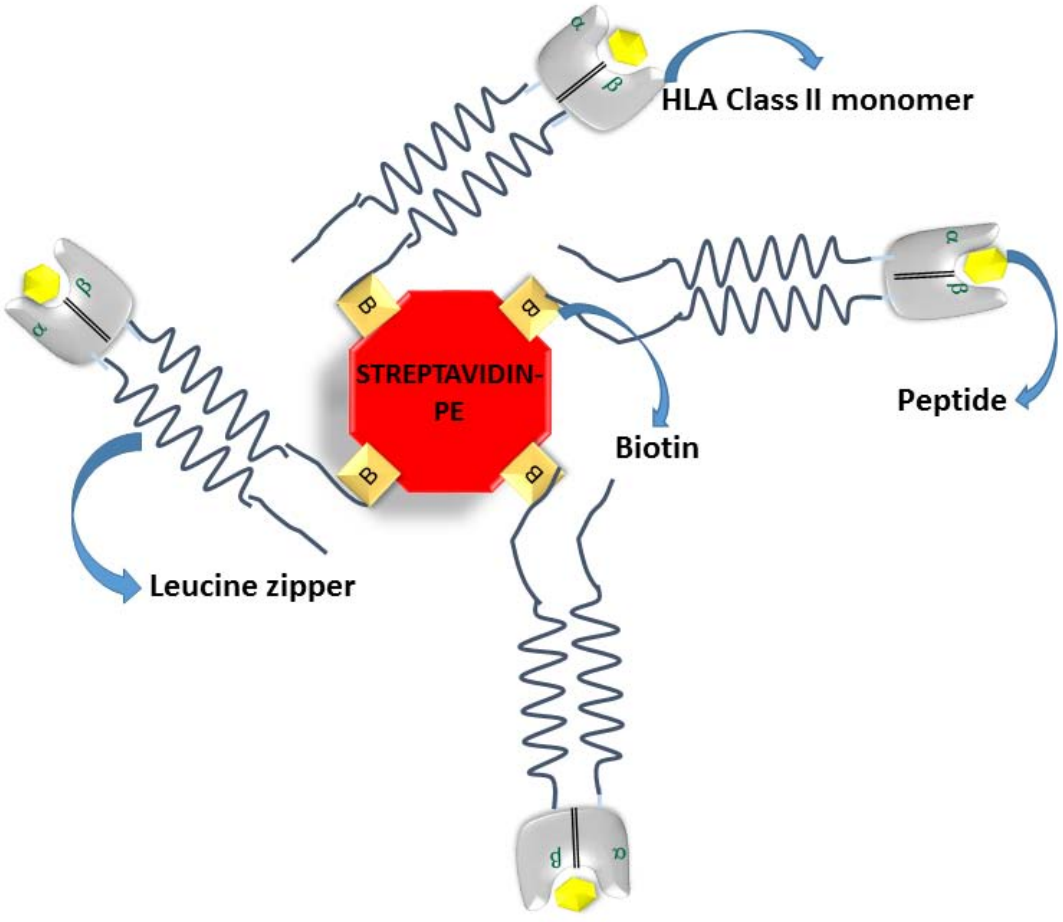

Figure XVIII. HLA Class II Tetramers. Biotinylated class II monomers oligomerize with streptavidin.

The concept of using soluble HLA-peptide labeled ligands to analyze and detect antigen-specific T cells has been a general strategy successfully applied in numerous translational and clinical contexts **[Nepom, 2012]**. HLA tetramer staining was developed for class I recognition in the context of $CD8^+$ T cells **[Altman, et al., 1996]**, staining $CD4^+$ T cells with class II tetramers appeared no longer after **[Novak, et al., 1999] [Lebowitz, et al., 1999]**. The number of publications reporting class II HLA tetramer staining experiments have substantially increased in the last 15 years, as class II tetramers are now widely used in studies of pathogen immunity and vaccine development, in evaluation of antitumor responses, in allergy monitoring and desensitization studies and in autoimmunity (**Figure XIX**) **[Nepom, 2012].**

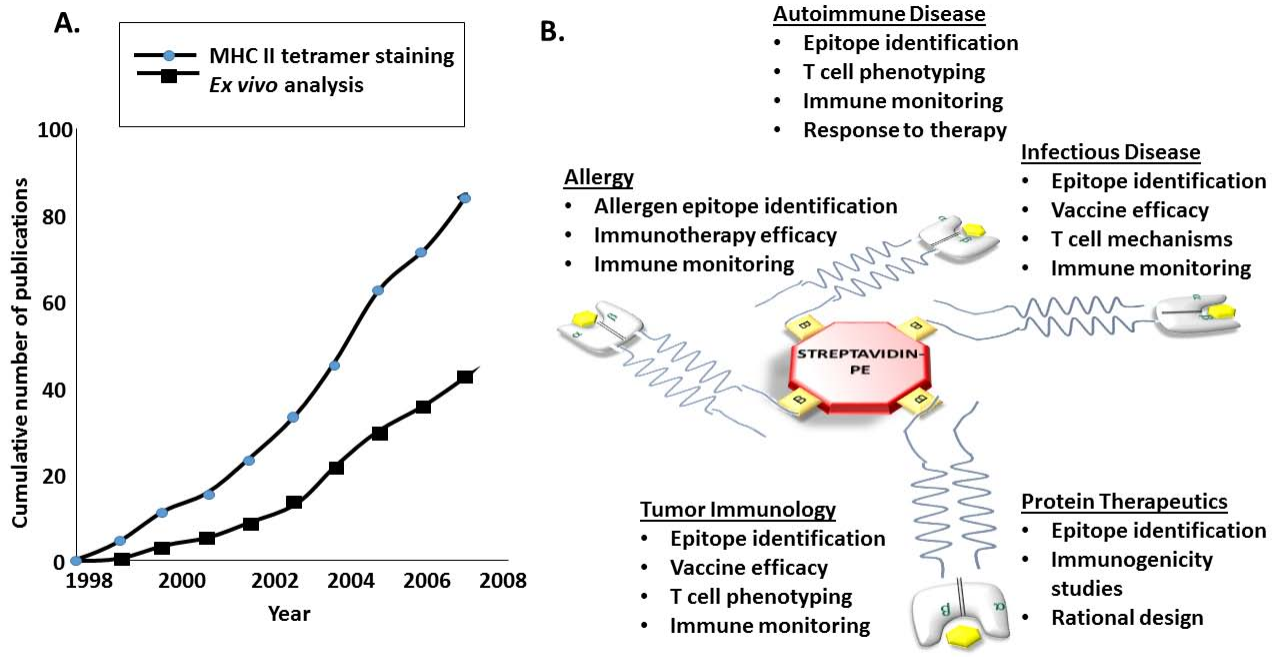

Figure XIX. A. Publications reporting HLA (MHC)-II tetramer staining experiments. **B**. Some major areas of tetramer analysis in human disease applications are listed. (Adapted from [**Nepom, et al., 2011] [Vollers, et al., 2008]**).

Tetramer assays offer important advantages over other methods, by enabling the recovery and further study of sorted cells in flow cytometry, which also permits the study of epitope specific $CD4^+$ T

cells, and can be compatible with a different number of assays. The major limitation of class II tetramers is the consideration that humans have very diverse HLA class II molecules, so that studies may require the use of many different tetramers to include a broad representation of the population **[Nepom, 2012]**.

IV.1.2. Tetramer Guided Epitope Mapping (TGEM)

As mentioned earlier, tetramers can be used to identify novel T cell epitopes. The Tetramer Guided Epitope Mapping (TGEM) was first described by Kwok's Laboratory **[Novak, et al., 2001]** to identify Herpes virus simplex virus VP16 and has been utilized to describe a variety of DR-restricted T cell epitopes derived from multiple allergens, including Fel d 1, Ara h 1, Aln g 1, Phl p 1, Phl p 5 and Pen m 2 **[Kwok, et al., 2010] [DeLong, et al., 2011] [Wambre, et al., 2012] [Wambre, et al., 2014] [Renand, et al., 2014]**.

The information regarding class II HLA type of the subject and a library of over-lapping peptides are a pre-requisite to apply TGEM **[Nepom, 2012] [Novak, et al., 1999] [Reijonen, et al., 2002]**. The basis of this approach is to stimulate PBMC from the subject with peptide mixtures. These mixtures are composed of multiple pools of peptides were each pool consists of 5 different overlapping peptides. Soluble class II molecules that matched the HLA of the subject are loaded with each mixture of peptides, and matching tetramers are used to screen individual wells of stimulated PBMC. The particular pooled peptide tetramer that gives positive staining is identified. Individual peptides from the pool are then used to generate single peptide tetramers. Positive staining in a second round of FACS analysis with a particular tetramer will identify the peptide containing the antigenic epitope (**Figure XX**).

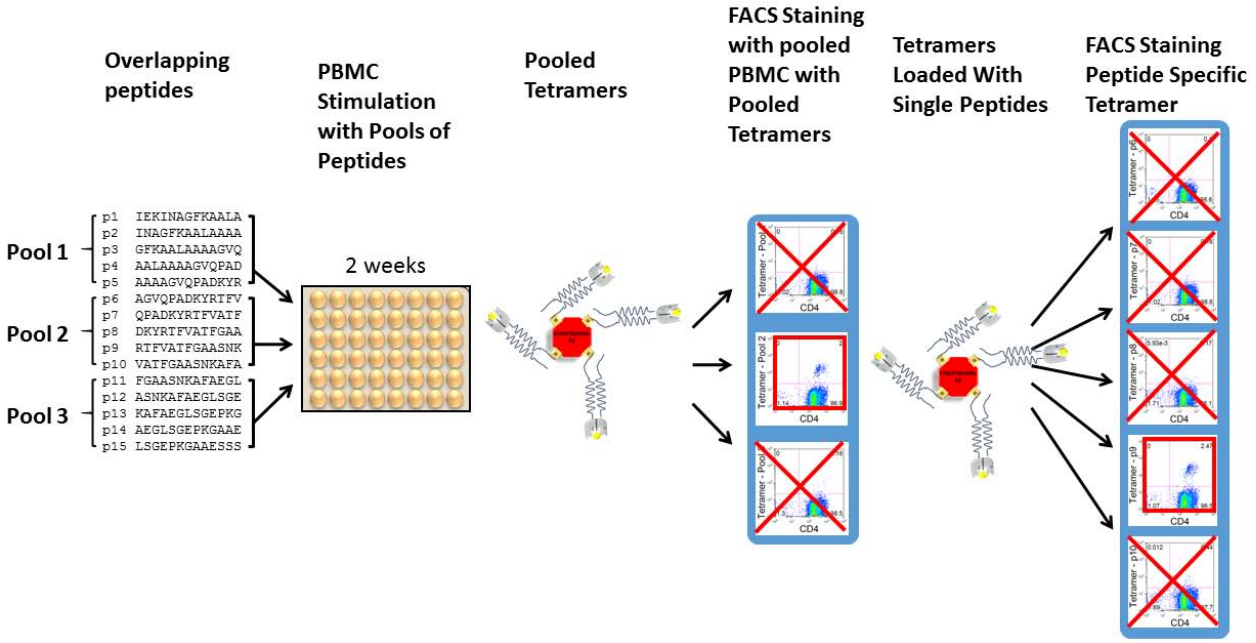

Figure XX. Tetramer Guided Epitope Mapping (TGEM). (Adapted from [**Reijonen, et al., 2003]**).

Besides the fast identification of novel epitopes, tetramer assays offer important advantages over other methods, by enabling the recovery of sorted cells in flow cytometry, epitope specific T cell clones can be generated and permits further analysis of transcription factors. Additionally the tetramer technology can be compatible with a different number of assays like Intracellular Cytokine Secretion (ICS), Cytokine Secretion Assay (CSA), and CFSE making it a robust technology for the study of the phenotype and functionality of $CD4^+$ T cells.

IV.1.3. Direct ex- vivo analysis of allergen-specific $CD4^+$ T cells with pMHC-II tetramers and other methods

IV.1.3.1. Direct ex vivo analysis of allergen-specific $CD4^+$ T cells with pMHC-II tetramers

While a variety of T cell assays require *in vitro* expansion, current second-generation tetramer approaches enable the analysis of allergen-specific $CD4^+$ T cells directly *ex vivo*. In the context of allergy. direct *ex vivo* tetramer analysis of allergen specific T cells has been reported in cat, peanut, timothy grass, shrimp and alder subjects **[Kwok, et al., 2010] [DeLong, et al., 2011] [Wambre, et al., 2012] [Wambre, et al., 2014] [Renand, et al., 2014]**.

This method is based on stringent 'dumping' criteria and enrichment with magnetic bead capturing procedures to remove cells that lack tetramer specificity, enriching the antigen-specific populations, allowing for the detection of rare events (e.g. 10 tetramer-binding T cells from 20 ml of peripheral blood) and detailed analysis of magnitude of T cell responses **[Nepom, 2012] (Figure XXI)**. In addition, multiparameter flow cytometry analysis using a variety of monoclonal antibodies simultaneously with tetramer assays permits for direct characterization of epitope specific T cells, allowing accurate confident detection of surface phenotypes **[Nepom, 2012] [Wambre, et al., 2012]**.

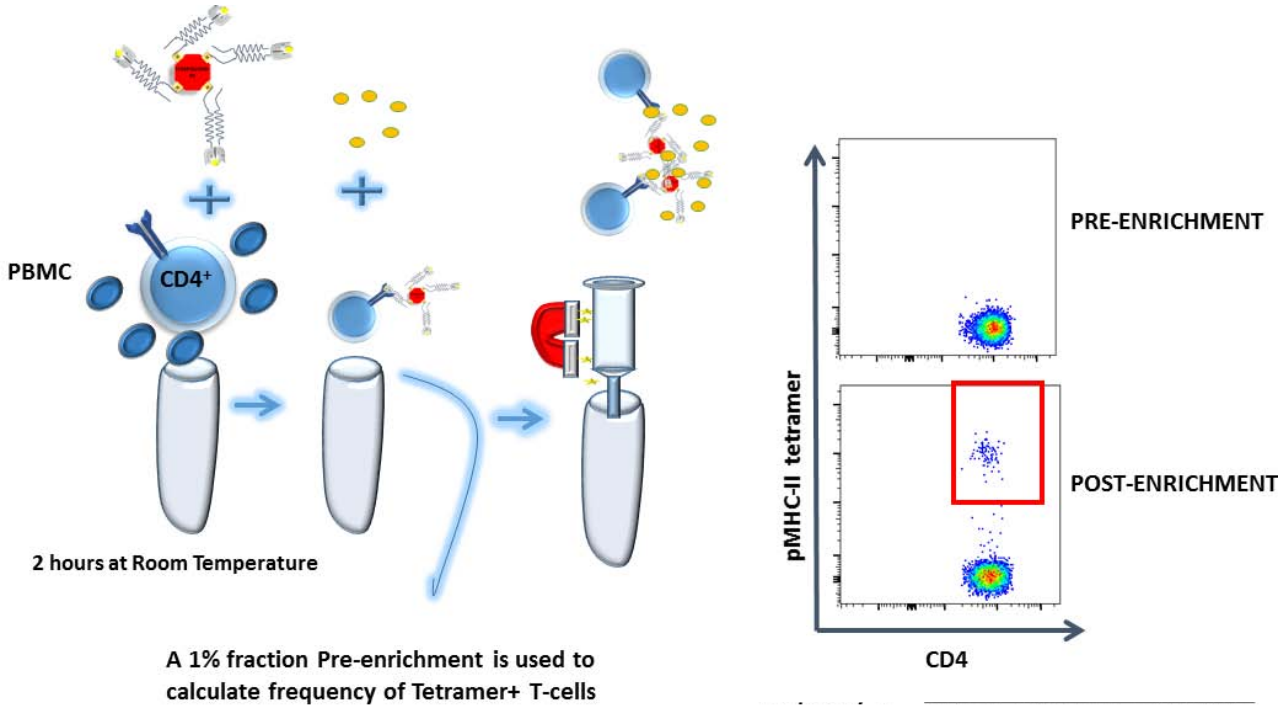

Figure XXI. *Ex vivo* tetramer staining and enrichment with magnetic beads. 30 x 10^6 PBMC are stained with 6µl of the desired PE-labeled tetramer. After 2 hours of incubation at room temperature, PBMC are incubated with anti-PE magnetic beads and subsequently enriched through magnetic columns. A 1% fraction from the pre-enrichment is used to calculate the frequency of $Tetramer^+$ T cells. Frequency=n/N where n designates the number of tetramer positive cells in the bound fraction and N designates the total number of $CD4^+$ T cells in the sample.

IV.1.3.2. Direct ex- vivo analysis of allergen-specific CD4$^+$ T cells with other methods

Although HLA class II tetramers can identify epitope specific T cells and are compatible with a number of assays; this technology is limited to the paucity of available tetramers, requires information of HLA-restriction and does not necessarily identify functionally responsive cells *ex vivo*. Chattopadhyay, et al., in 2005 described a technique that permitted the functional identification of antigen specific CD4$^+$ T cells through *de novo* CD154 expression. An extension of this technique was implemented in our laboratory utilizing the same principle of the direct *ex vivo* tetramer staining method. We also extended the CD154 technique **[Chattopadhyay, et al., 2005]** to direct *ex vivo* assessment of cytokine production for allergen-specific T-cells in a similar manner to the frequency and phenotype measurements. This method is also based on stringent 'dumping' criteria and enrichment with magnetic bead capturing procedures to remove cells that express CD154 *de novo*, enriching the antigen-specific populations, allowing for the detection of global T cell responses, and detailed analysis of frequencies of T cell populations **(Figure XXII)**.

In addition, polychromatic cytokine staining in our CD154 detection method is compatible with measurement of several cytokines by ICS. However, cytokine non-producers can also be detected with this method which can be explained by stochastic events during T cell signaling, variable expression of transcription factors or stage of cell cycle and differentiation may be responsible for heterogenous cytokine production capacity **[Gett, et al., 1998] [Zhu, et al., 2010]**. In our hands, cytokine responses are consistent between class II tetramers (*in vitro*) and CD154 methods (*ex vivo*). In conclusion, CD154 enrichment may be used to rapidly characterize allergen-reactive T-cells *ex vivo* as an alternate method to class II tetramers.

Additionally, this technique can also be used to identify T cell epitopes, however, due to the low number of T cells, a cloning step is required for the detection of these cells.

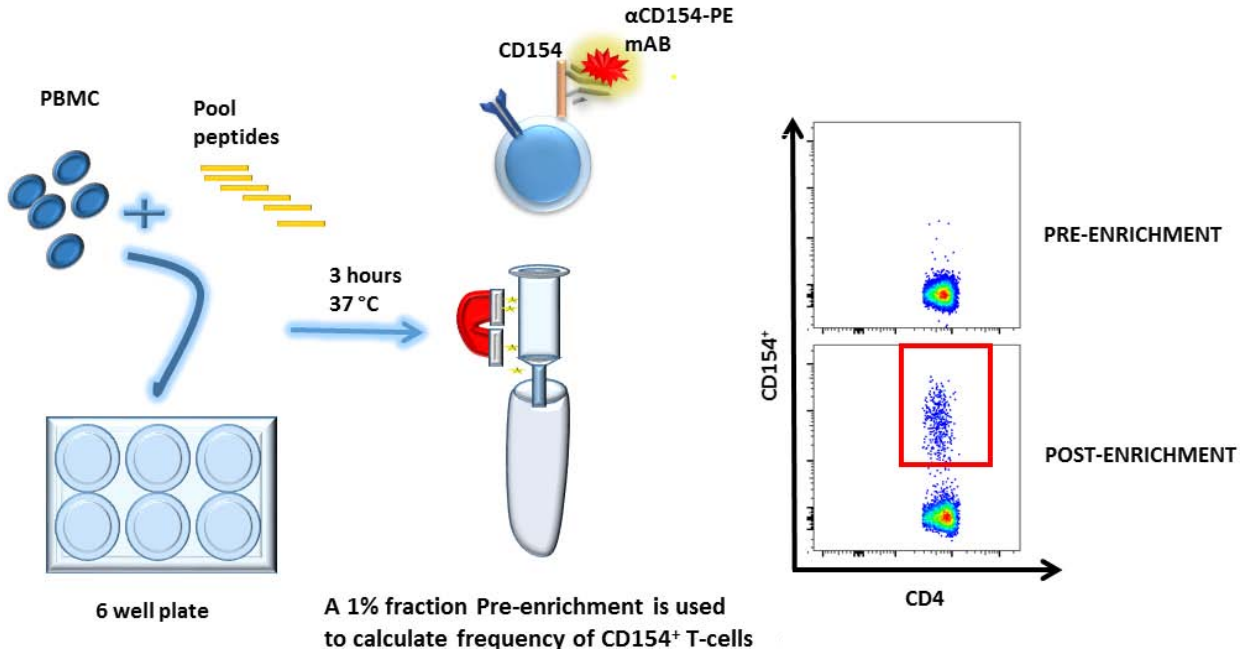

Figure XXII. *De novo* CD154 expression after stimulation identifies antigen-specific $CD4^+$ T cells after bead enrichment. 30 x 106 PBMC are stimulated with peptide libraries from the desired allergen for 3(Surface phenotypes) or 6 hours (ICS in addition to Monensin) at 37 ºC. After incubation step, cells are stained with PE-labeled anti-CD154, incubated with anti-PE magnetic beads and subsequently enriched through magnetic columns. A 1% fraction from the pre-enrichment is used to calculate the frequency of $CD154^+$ T cells. Frequency=n/N where n designates the number of CD154 positive cells in the bound fraction and N designates the total number of $CD4^+$ T cells in the sample.

IV.1.4. Allergen-specific tetramers as a specific biomarker for monitoring allergen-specific CD4$^+$ T cells in various settings

Several studies have highlighted the use of tetramer reagents to detect and characterize allergen-specific T cells **[Kwok, et al., 2010] [Delong, et al., 2011] [Wambre, et al., 2012] [Wambre, et al., 2014] [Renand, et al., 2014].** These studies confirmed that a high percentage of allergen specific T cells express CCR4 in allergic subjects. Conversely, in non-allergic subjects allergen specific T cells with a memory phenotype were lower and expression of CXCR3 was observed in epitope specific T cell populations. Wambre et al., showed that in allergic individuals, the frequency of tetramer-binding CD4$^+$ T cells is suitably high particularly within pollen season (allergen exposure) **[Wambre, et al., 2012]**. Multiparameter flow analysis documented the analysis of a variety of cell surface markers, leading to important new insights. For example, from examining PBMC from alder allergic subjects, Wambre, et al., observed that a large population of Alg n 1-specific T cells lacked expression of CD27 **[Wambre, et al., 2012]**. The loss of CD27 correlated with CRTH2 expression and IL-4 secretion. The loss of CD27 expression indicates that these T cells undergo extensive proliferation during constant exposure to the allergen.

On the other hand, for food allergy, DeLong, et al., observed that most of the Ara h 1-specific T cells in peanut allergic adults did not express CRTH2, but expressed CCR4 and produced IL-4, suggesting that antigen stimulation is essential for the expression of CRTH2 **[DeLong, et al., 2011]**. However, CD27 expression was not analyzed in this study. Recent, shrimp allergen studies observed that most of Pen m 2-specific CD4$^+$ T cells did not express CRTH2, and variable expressions of CD27 and CCR6 were detected **[Renand, et al., 2014]**. Most importantly, tetramers are currently applied in different studies to monitor progressive shifts in T cell frequencies and cell surface marker profiles during allergen immunotherapy.

V. $CD4^+$ T cells contribute to allergic disease

In the previous section (I. T cell recognition) we have mentioned how T cells are primed by APCs, and how this engagement and other factors can promote T cell maturation into a particular lineage. We also mentioned several of the chemokine receptors that play a role in migration to specific organs and sites of inflammation. In this section we will discuss how T cells contribute to allergic disease, and show how different lineages have been associated to particular ailments with supporting literature.

V.1. The role of T_H2 cells in initiation and maintenance of allergic inflammation

The role of T_H2 cells in allergic inflammation is not limited to the induction of allergen-specific IgE antibodies by B cells, but also eosinophilic infiltration in target tissues **[Romagnani, et al., 2000]**. Recent studies have shown that allergic reactions may occur in the absence of IgE. Indeed, B cell **[Maclean, et al., 1999]**, IgE **[van de Rijn, et al., 1998]**, CD40 **[Hogan, et al., 1997]** and mast cell **[Takeda, et al., 1997]** gene deficient mice develop asthma, whereas $CD4^+$ T cell IL-4 gene deficient mice do not, due to lack of IgE production **[Corry, et al., 1998]**. Thus, production of IL-4 by T cells is essential for the production of IgE antibodies.

Production of IL-4, IL-13, IL-5 and IL-9 by T_H2 cells have direct or indirect roles in the pathophysiologic manifestations in allergic patients **[Romagnani, et al., 2000]** (**Figure XXIII**). Not only is IL-4 responsible for the IgE isotype switching but also for rolling on and adhesion to, endothelial cells of circulating eosinophils, which can then be attracted by IL-5 and eotaxins **[Bochner, et al., 1994]**. Moreover, eotaxins produced by epithelial cells are under the control of IL-4 or IL-13 **[Devouassoux, et al., 1999]**. IL-4 and IL-13 also stimulate fibroblast growth and synthesis of extracellular matrix proteins **[Doucet, et al., 1998]**. IL-13 is suggested to play a critical role in, eosinophilic infiltration, goblet cell metaplasia and lung fibrosis **[Kuperman, et al., 2002]**. IL-9 is a mast cell growth and differentiation factor that can cause mast cells to release various substances including histamine **[Patkai, et al., 2001]**. Finally, IL-4, IL-9 and IL-13 induce metaplasia and hypersecretion of mucus cells **[Sempowski, et al., 1994] [Doucet, et al., 1998]**. Although IL-9 has been generally attributed to T_H2 cells, a separate population that produces IL-9 in large quantities has also been described. It has been shown that activation of naïve $CD4^+$ T cells in the presence of TGF-β and IL-4 enhances IL-9 production but no other T_H2 cytokines **[Veldhoen, et al., 2008] [Dardalhon, et al., 2008]**.

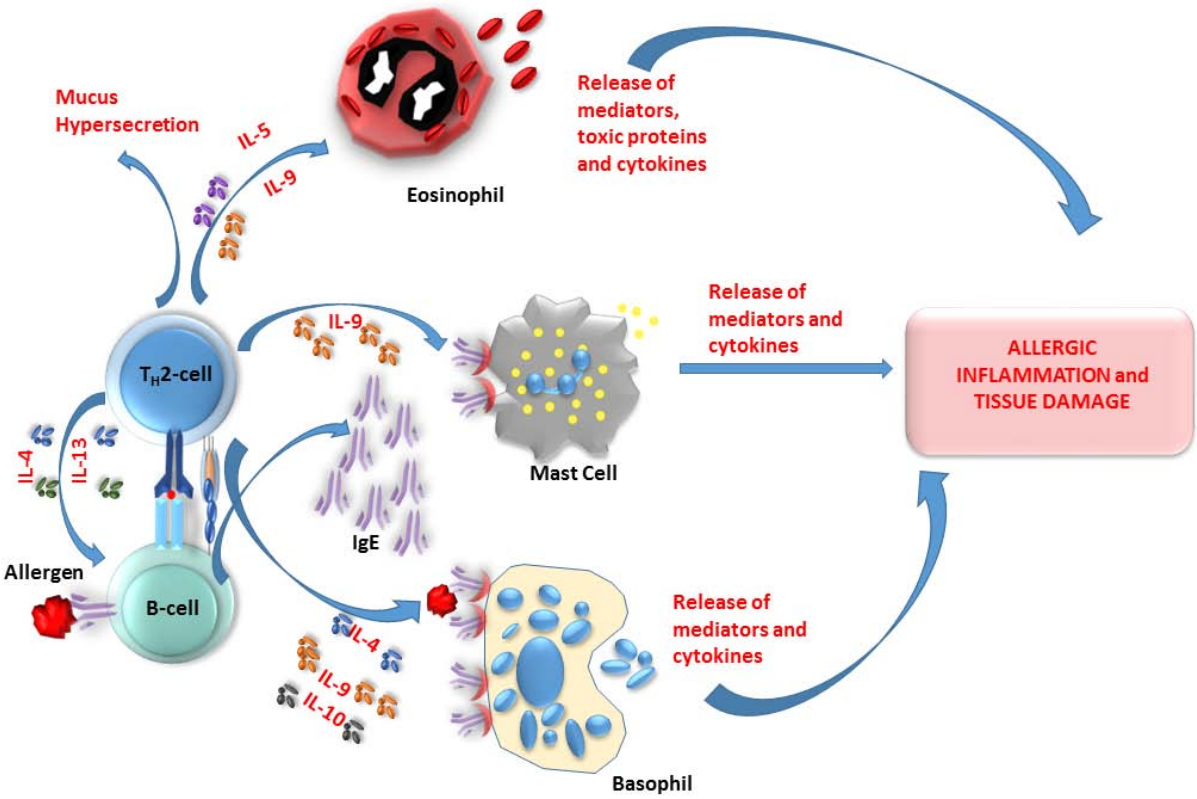

Figure XXIII. The roles of T_H2 cells in allergic inflammation (Adapted from **[Romagnani, et al., 2000]**).

During repetitive exposure to allergens or other stimuli, epithelial cells begin a repair process of damaged epithelium through the release of several growth factors and cytokines such as TGF-β, which may switch T_H2 cells into T_H9 cells **[Vedhoen, et al., 2008]** (**Figure XXIV**). IL-9 supports proliferation of T cells, enhances IgE production by B cells, promotes release of proinflammatory cytokines by mast cells and potentiates the survival of eosinophils **[Soroosh, et al., 2009]**. IL-9 in combination with IL-17 can promote the accumulation of neutrophils in the airways **[Soroosh, et al., 2009]**. Thus, IL-9 secretion contributes to the development or maintenance of allergic disease. In allergic asthma, IL-9 was proposed as a candidate gene for atopy and was associated with elevated IgE levels **[Doull, et al., 1996]**. It has also been shown that IL-9 is enhanced during asthmatic inflammation, upper airway disease and peanut allergy **[Nouri-Akia, et al., 2005] [Erpenbeck, et al., 2003] [Xie, et al., 2012]**.

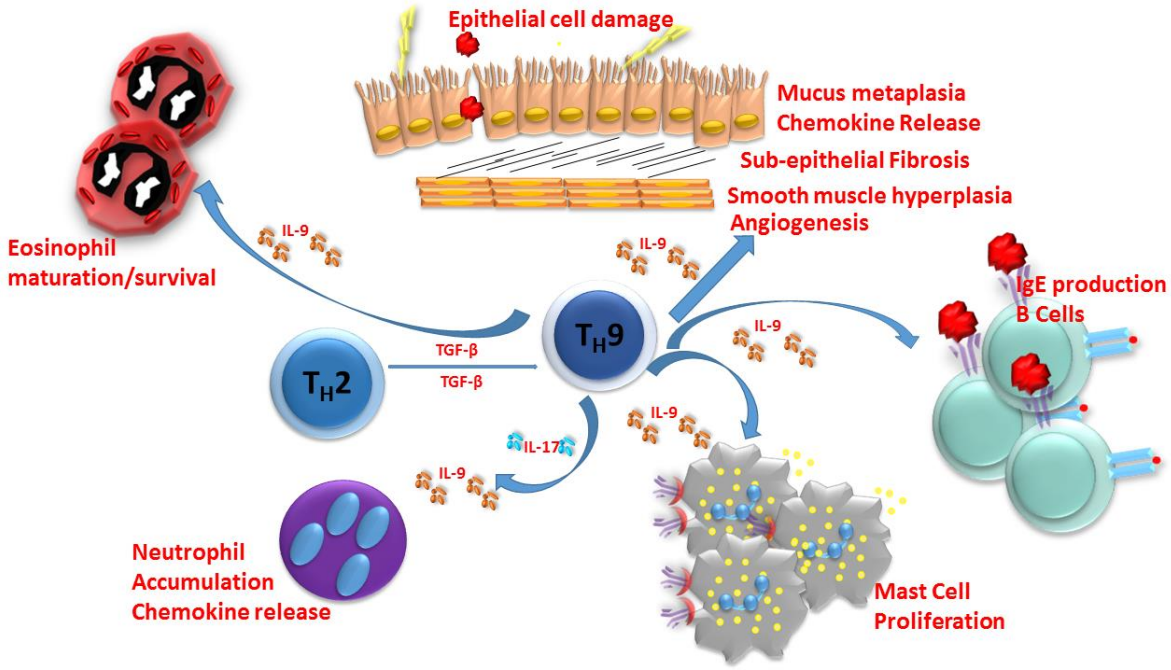

Figure XXIV. The roles of T_H9 responses during chronic lung inflammation (Adapted from **[Soroosh, et al., 2009]**)

V.2. The role of T_H1 cells for the development of allergic inflammation

The hypothesis of T_H1/T_H2 disequilibrium originally suggested that changes in the infectious environment during childhood favored a disequilibrium between T_H1 and T_H2 responses to innocuous antigens in favor of T_H2 responses. Varney et al. in 1993 observed that T_H1-type cytokines were enhanced in half of the patients' ongoing immunotherapy, thus, supporting the view that immunotherapy may possibly render a "protective" T_H1 phenotype **[Varney, et al., 1993]**. Additionally, T_H1 cells had been regarded to inhibit bronchial asthma by production of IFN-ϒ **[Cohn, et al., 1999] [Huang, et al., 2001]**. However, it has been observed that the combination of both T_H1 and T_H2 cell cytokines induce airway inflammation **[Ford, et al., 2001] [Hansen, et al., 1999]**. It has also been shown that IL-18 rich

environments promote the secretion of IL-13 and IFN-ϒ by T_H1 cells **[Hata, et al., 2004]** and T_H1 cells that produce these cytokines induce intrinsic atopic dermatitis **[Terada, et al., 2006]**.

IL-18R expression levels determine the intensity of responsiveness to IL-18 and it is highly expressed on T_H1. Accumulating evidence suggests positive relationship between IL-18 levels in allergic diseases, such as asthma, allergic rhinitis and atopic dermatitis **[Wong, et al., 2001] [Tanaka, et al., 2001] [Krakowiak, et al., 2008]**. IL-18 promotes the production of high levels of IFN-ϒ and TNF-α when stimulated through IL-18R. However, it has been also shown that IL-18 has potential to render T_H1 cells to produce T_H2 cytokines like IL-13 and IL-9, but no IL-4 **[Sugimoto, et al., 2004]** (**Figure XXV**). Recent literature has shown that T_H9 cells can modulate allergen-specific IFN-ϒ production by T_H1 cells in allergic contact dermatitis **[Liu, et al., 2014]**. In a recent study, newly described Timothy grass antigens where associated with IFN-ϒ but no IL-5 production [**Schulten, et al., 2014**]. In conclusion, T_H1 cells seem to play a role in allergic disease, it seems that T_H2 environments promote adverse effects by T_H1 cells.

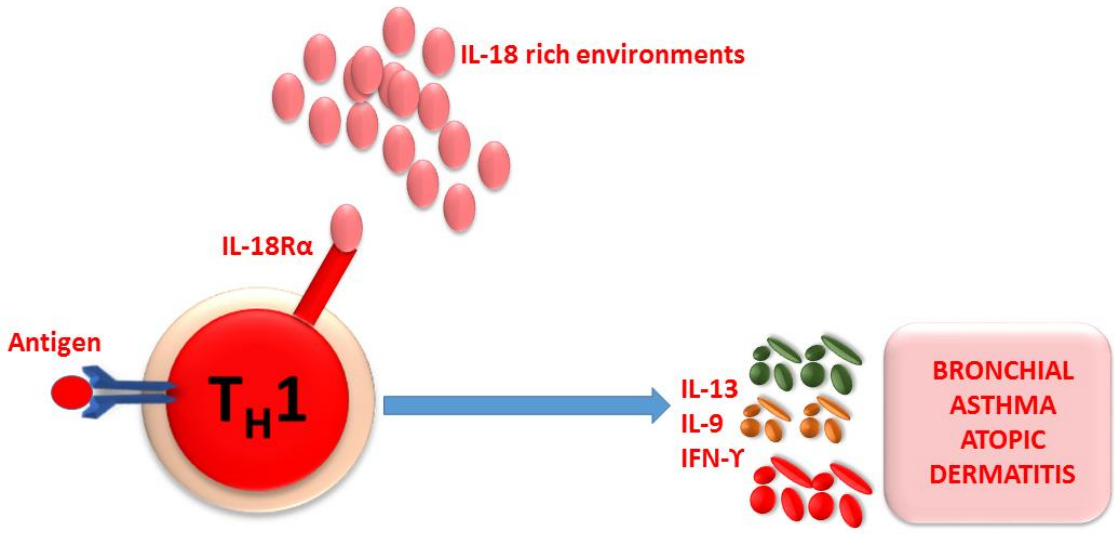

Figure XXV. T_H1 cells can produce IL-13 and IL-9 when they are activated with antigen in IL-18 rich environments. IFN-ϒ and IL-13 are critical for the development of AHR and airway fibrosis (Adapted from **[Nakanishi, et al., 2010]**).

V.3. The role of T_H17 cells in atopic allergic disease

Allergic diseases are commonly associated with chronic inflammation characterized by infiltration and accumulation of eosinophils. On the other hand, increased disease severity often coincides by influx of neutrophils and inflammation (**Figure XXVI**). Recent studies suggest that IL-17 plays a role in sustained inflammation in allergic diseases through recruitment of neutrophils. Patients with severe asthma demonstrate high numbers of neutrophil infiltration in bronchoalveolar lavage fluid and bronchial biopsy specimens when compared to control subjects **[Wenzel, et al., 1997]**. Further studies have also shown elevated numbers of T_H17 cells in allergic asthmatics compared to healthy controls **[Zhao, et al., 2010]**. T_H17 cells also contribute to allergic airway disease by inducing airway smooth muscle cell migration **[Chang, et al., 2011]** and it has also been demonstrated that IL-17, under IL-4 rich environments can induce B cell class-switching to IgE in human subjects **[Milovanovic, et al., 2010]**. In atopic dermatitis (AD), IL-17 expression is enhanced in acute lesions in the skin compared with uninvolved skin **[Toda, et al., 2003]** and a correlation between the number of T_H17 cells in peripheral blood and acute AD severity has been reported **[Koga, et al., 2008]**.

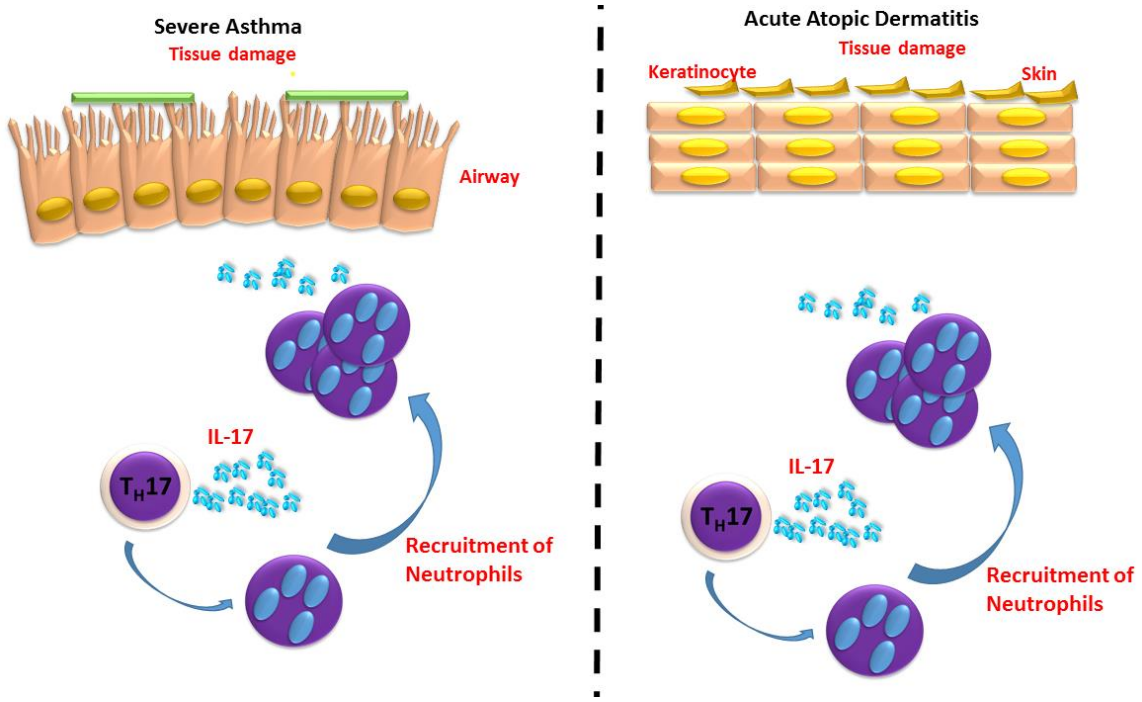

Figure XXVI. T_H17 cells in allergic asthma and atopic dermatitis, IL-17 promotes neutrophil recruitment with increasing severity of disease, though IL-17 is present in acute responses in AD, it is absent in chronic tissue inflammation (Adapted from **[Souwer, et al., 2010]**).

T_H17 cells possess an intrinsic plasticity that allows them to shift to T_H2-like cells, in response to specific cytokine environments **[Cosmi, et al., 2010]**. Human circulating memory $CD4^+$ T cells that produce both IL-4 and IL-17a (T_H2/T_H17 cells), have been suggested to play a role in the exacerbation of chronic allergic asthma **[Wang, et al., 2010] [Chiarella, et al., 2015]**. Although rare, these T_H2/T_H17 cell populations, have been identified in allergic asthmatics but not in non-allergic controls **[Irvin, et al., 2014] [Cosmi, et al., 2010]**. Der p 1-derived T cell lines with a T_H2/T_H17 phenotype has been detected in house dust mite allergic subjects **[Cosmi, et al., 2010]**. Furthermore, this population has also been identified in food allergy, specifically for peanut and shrimp allergens **[DeLong, et al., 2012] [Renand, et al., 2014]**.

V.4. The role of T_H22 cells in atopic allergic disease

The role of T_H22 cells in allergic disease has been less documented. Infiltrating T_H22 cells have been detected in inflamed skin of nickel-challenged allergic subjects **[Larsen, et al., 2009]**. T_H22 cells

promote migration of airway smooth muscle mass in asthmatic patients in a dose dependent manner **[Chang, et al., 2011]**. These structural changes reversibly influence airway obstruction and correlate with asthma severity **[Chang, et al., 2011]**. In atopic dermatitis, T_H22 and T_H17 cells are enriched in patients. These cell populations initiate inflammation through various cytokines and chemokines, leading to dermatitis and eczema. On the other hand, the role of IL-22 in airway hypersensitivity remains obscure, as it has been shown in a mouse model that IL-22 negatively regulates allergic responses because neutralization of IL-22 augmented eosinophil recruitment to the lung **[Schnyder, et al., 2010]** (**Figure XXVII**). However, neutrophil recruitment was not affected by IL-22 neutralization **[Schnyder, et al., 2010]**.

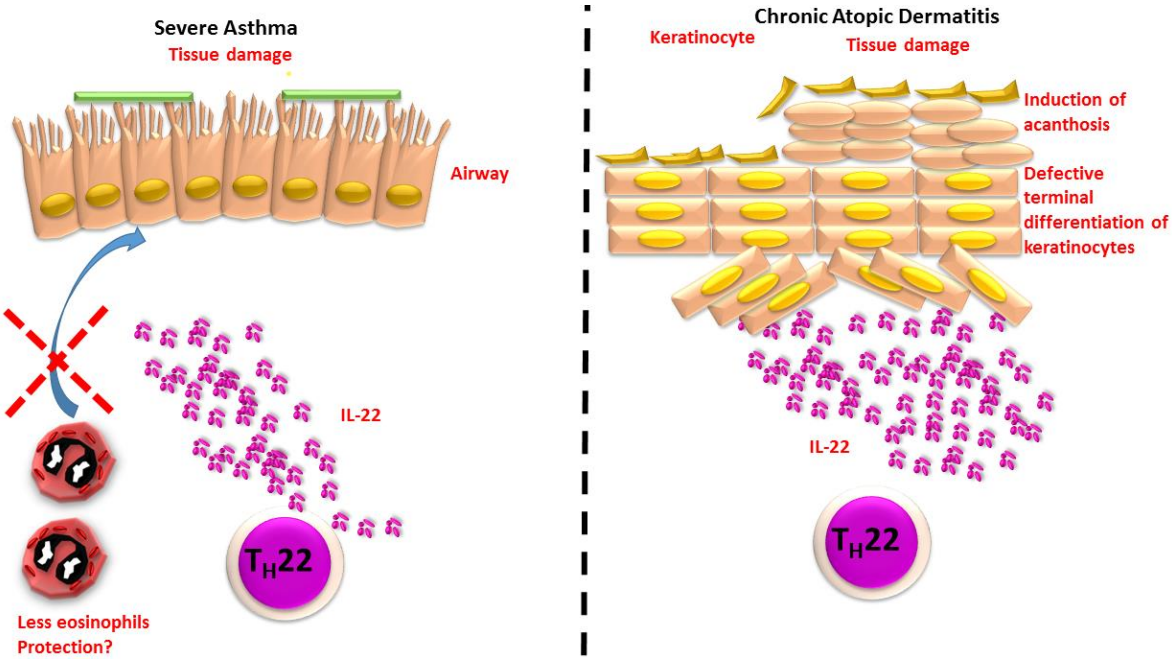

Figure XXVII. T_H22 cells in allergic asthma and atopic dermatitis, IL-22 promotes the induction of acanthosis and terminal differentiation of keratinocytes in AD. On the other hand, IL-22 reduces the accumulation of eosinophils in severe Asthma, suggesting a possible protective role (Adapted from **[Souwer, et al., 2010]**).

VI. Cross-reactivity at the T cell level in allergic disease

VI.1. Cross-reactivity in allergic disease

Allergic symptoms may be due to cross-reactivity with antibodies (IgE or IgG) or T-cells **[Weber, 2001]**. In the allergy context, cross-reactivity is the ability of an IgE antibody that was raised to a given allergen, can also bind to homologous molecules from a different sources **[Weber, 2001] [Sirvent, et al., 2001],** as IgE can bind to similar conformational epitopes with the same binding affinity **[Sirvent, et al., 2001] [Pfiffner, et al., 2010]**. Identification of IgE-binding epitopes is the first step to better our understanding of the molecular features of an allergen **[Pfiffner, et al., 2010]**. However, linear epitopes also play a role in the manifestation of allergic disease, thus, the same principle can be applied to cross-reactivity at the T cell level.

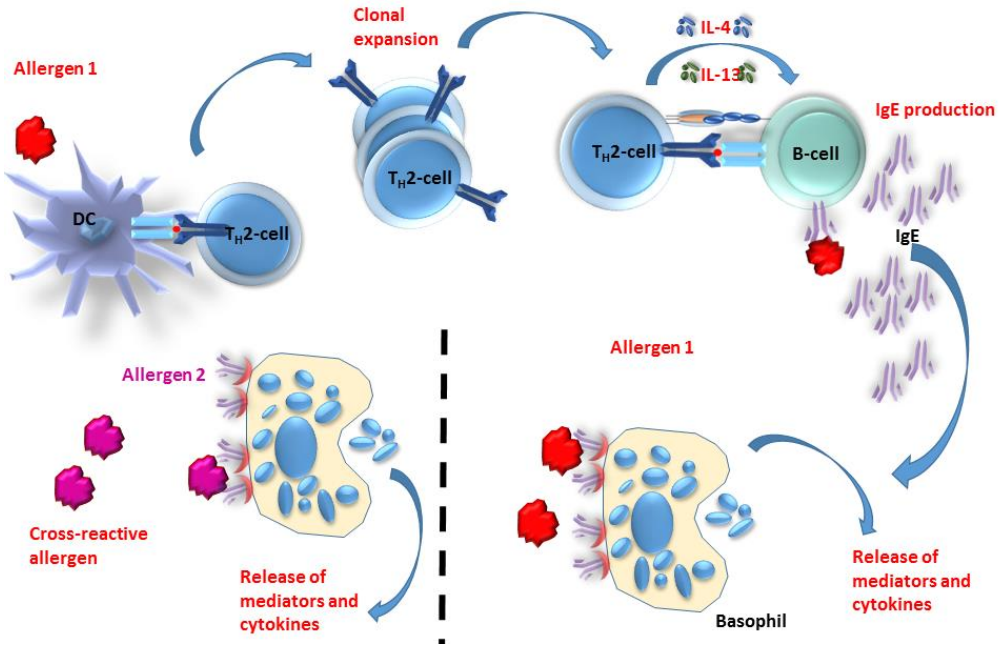

Figure XXVIII. Exposure to environmental allergens leads to a cascade of events ending in the production of allergen-specific IgE. Re-exposure either to the same allergen or homologous molecule induces cross-linking of the IgE antibodies on surface of effector cells (Adapted from **[Crameri, et al., 2008]**).

VI.2. Cross-reactivity at the T cell level in allergic disease

The recognition by T cells of antigenic peptides bound to class I or class II HLA on professional APC is a central event in the adaptive immune response **[Petrova, et al., 2012] [Davis, et al., 1998]**. The expression of a variety of HLA molecules ensures the presentation of several antigenic peptides, thus, ensuring the survival of an individual after an emerging infection **[Robinson, et al., 2003] [McAdam, et al., 1994]**. In humans, MHC molecules are encoded within the HLA locus **[Robinson, et al., 2003]**. The HLA locus is the most polymorphic region of the human genome **[Robinson, et al., 2003] [McAdam, et al., 1994]**. Each individual expresses six different HLA class I (two HLA-A, two HLA-B and two HLA-C) and at least six to eight HLA class II molecules (two to four HLA-DR, two HLA-DQ and two HLA-DP) **[Sewell, 2012]**. In addition, as both DQA1 and DQB1 genes are polymorphic, four different DQ molecules can be formed. This same phenomenon is also applied to DP. On the other hand, in terms of adaptive immune protection, the TCR repertoire must also be broad enough to respond to all potential foreign peptides that are processed and bind to an HLA molecule with a "face that fits" **[Mason, 1998] [Nicholson, et al., 2004]**.

Originally, the clonal selection theory proposed that individual T cells were specific for a single peptide-MHC complex (pMHC) molecule and that the recognition of multiple ligands is unlikely **[Jerne, 1951] [Jerne, 1971]**. However, progress in the ability to synthesize peptides facilitated the exploration of the specificity of T cell recognition of antigens and use of single amino acid substitutions in epitopes enabled different approaches to demonstrate that T cells can recognize several pMHC molecules **[Kersh, et al., 1996]**. T cell cross-reactivity is a phenomenon of the immune system related to the observation that a given TCR can respond to epitopes that show strong sequence homology or are completely unrelated **[Petrova, et al., 2012]** (**Figure XXIX**). The recognition is based on the ability of a TCR to bind sufficiently well to initiate a cellular response **[Petrova, et al., 2012]**. This effect plays an important role in shaping T-cell repertoire and effector functionality **[Wooldridge, et al., 2012].** This has been described as TCR

degeneracy. Although TCRs have the potential to discriminate between single conservative amino acid substitutions, not every substitution will be recognized so precisely **[Nicholson, et al., 2004]**. The approach of making substitutions of such sequences at multiple residues is a powerful technique to study T cell cross-reactivity **[Hemmer, et al., 1998]**.

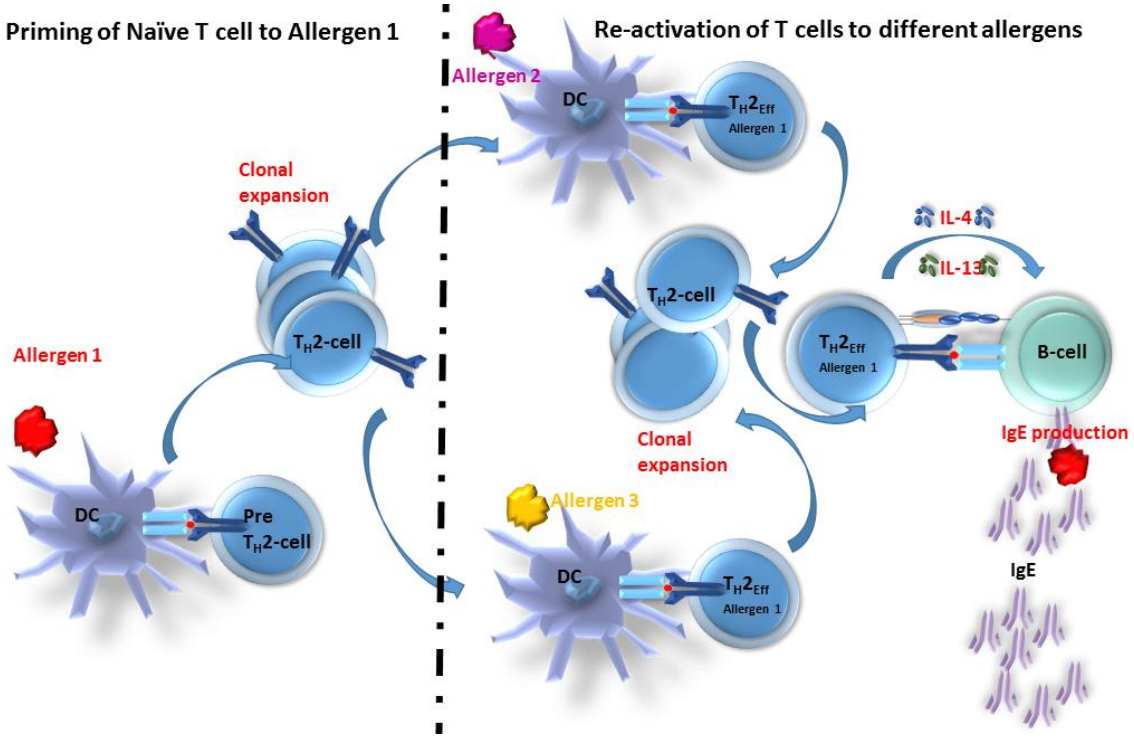

Figure XXIX. Exposure to environmental allergens leads to a cascade of events ending in the production of allergen-specific IgE. Re-exposure either to the same allergen or homologous molecule induces re-activation of T cells that leads to the production of IgE on B cells.

Therefore, the understanding of T-cell epitope recognition and its patterns of cross-reactivity become essential for practical applications such as immunotherapy and immune modulation **[Markovic-Plese, et al., 2005] [Ge, et al., 2010]**. While epitope identification and cross-reactivity at the IgE level has been discussed in a wealth of studies, cross-reactivity at the allergen-specific T-cell level has been less documented. Identification of allergen-specific T-cell epitopes that can be HLA-restricted may better our understanding on the patterns of cross-reactivity and functionality of the effect of this process in a particular type of allergy.

VI.2.1. Cross-reactivity in grass pollen allergy and implications for immunotherapy

In Pooideae grass pollen allergy, allergenic pollen is a complex mixture of several molecules including major and minor allergens. The allergenicity of these grass pollens is shared amongst these taxonomically related species and since pollination periods from distinct members of this subfamily overlap, patients are constantly exposed to a mixture of grass pollens **[White, et al., 2003]**. Nonetheless, it has been suggested that *Phleum pratense* (Phl p) has the most representative allergen grass profile **[Hrabina, et al., 2008]**(91% sequence identity for Group 1 and 55% - 85% for group 5 allergens in comparison to other members of the Pooideae subfamily). This high similarity proposes that the use of several species might be redundant for immunotherapy and the selection of one species of the subfamily may be sufficient **[Moingeon, et al., 2008]**. A wealth of studies regarding comparisons of immunotherapy using a 5-grass mix extract versus *P. pratense* extract alone have been studied extensively **[Schenk, et al., 1995] [Müller, et al., 1998] [Würtzen, et al., 1999] [Rimaniol, et al., 2003] [Moingeon, et al., 2008] [Heijl, et al., 2009] [Martinez-Cocera, et al., 2010] [Marcucci, et al., 2010]**. High patterns of cross-reactivity have been observed at the T and B cell level **[Würtzen, et al., 1999] [Moingeon, et al., 2008],** and at the IgE level **[Rimaniol, et al., 2003] [Würtzen, et al., 1999]**, suggesting that *P. pretense* alone may be sufficient for therapy. However, it has also been advised that pollen allergens moreover contain species-specific T or B cell epitopes that can be HLA-restricted **[Rimaniol, et al., 2003]** and substantial quantitative difference in pollen composition amongst species and variation in geographical prevalence. The use of tetramer reagents can help address this question by identifying and characterizing cross-reactivity at the allergen specific T cell level and thus gives the possibility for the identification of new epitopes that can be HLA-restricted and used for allergen-specific immunotherapy.

VI.2.2. Cross-reactivity in tree nut allergy

Tree nut-allergic subjects often react to other tree nuts **[Roux, et al., 2003]**. Major allergens such as legumins, vicilins and 2s albumins represent major storage protein constituents of the nuts **[Roux, et al., 2003]**. Cashew allergens Ana o 1, Ana o 2 and Ana o 3 and their corresponding allergens Pis v 1, Pis v 2 and Pis v 3 of the closely-related pistachio nut are highly homologous (78%, 80%, and 70%, respectively). Between tree nut Vicilins, Ana o 1 and Cor a 11 are only 44% homologous while Ana o 1 and Jug r 2 have low sequence homology 36%. Sequence homology for 11-s legumin like allergens is found in similar ranges between Ana o 2 and their corresponding allergens Jug r 4 (60%) in walnut and Cor a 9 in hazelnut (60%) **[www.allergen.org]**. Various IgE-reactive epitopes have been described and both native and recombinant nut allergens have been identified and characterized **[Roux, et al., 2003] [Barre, et al., 2008] [Hasegawa, et al., 2009] [Maleki, et al., 2011] [Noorbackhsh, et al., 2011]**. *In vivo* T-cell cross reactivity has been recently studied in mice sensitized with cashew alone and cashew in addition to walnut; cashew and walnut cross-reactivity was demonstrated at the T cell level **[Kulis, et al., 2009]**. However, HLA-restricted epitopes still remain unknown for the walnut and cashew allergens, the most common tree nut allergens. Characterization of these cross-reactive epitopes and epitope specific T cells may better our understanding in disease manifestation and improve the selection of better diagnostic and therapeutic tools for the treatment of this ailment.

VII. Allergen-specific immunotherapy

The prevalence of allergic disease has increased over the past decades, representing a serious health problem that decreases the quality of life and causes considerable morbidity worldwide **[Eiwegger, et al., 2012]**. To date, allergen-specific immunotherapy represents the only causal treatment for allergic disease **[Jutel, et al., 2011]**. The concept of allergen-specific immunotherapy (SIT) was first reported at the beginning of the last century **[Noon, 1911]**.

Allergen specific immunotherapy involves repeated administration of the sensitizing allergen by subcutaneous (SCIT) or sublingual application (SLIT) **[Eiwegger, et al., 2012]**. Oral immunotherapy (OIT) is being used to treat food allergy in clinical trials **[Vickery, et al., 2010]**. New routes of application have been recently proposed such as the intralymphatic route (ILIT) or epicutaneous route **[Eiwegger, et al., 2012] [Larché, et al., 2006]**. SIT is a disease modifying approach, which establishes long term tolerance, restores immune balance, and prevents the onset of new sensitization to different allergens **[Pajno, et al., 2001]**. This section will review the immunological mechanisms of action of SIT and a novel approach that is in progress to improve both efficacy and safety.

VII.1 Mechanisms of SIT

Multiple studies have shown that SIT modulates the functions of APC, T cells and B cells (**Figure XXX**). Various common traits have been observed in SIT, such as reduction in the number of eosinophils, T_H2 cells **[Durham. et al., 1996] [Varney, et al., 1993]**, basophils and mast cells after challenge **[Wilson,**

et al., 2001] [Durham, et al., 1999]. The following sections will focus on the modulation of APC, T cells and B cells after SIT.

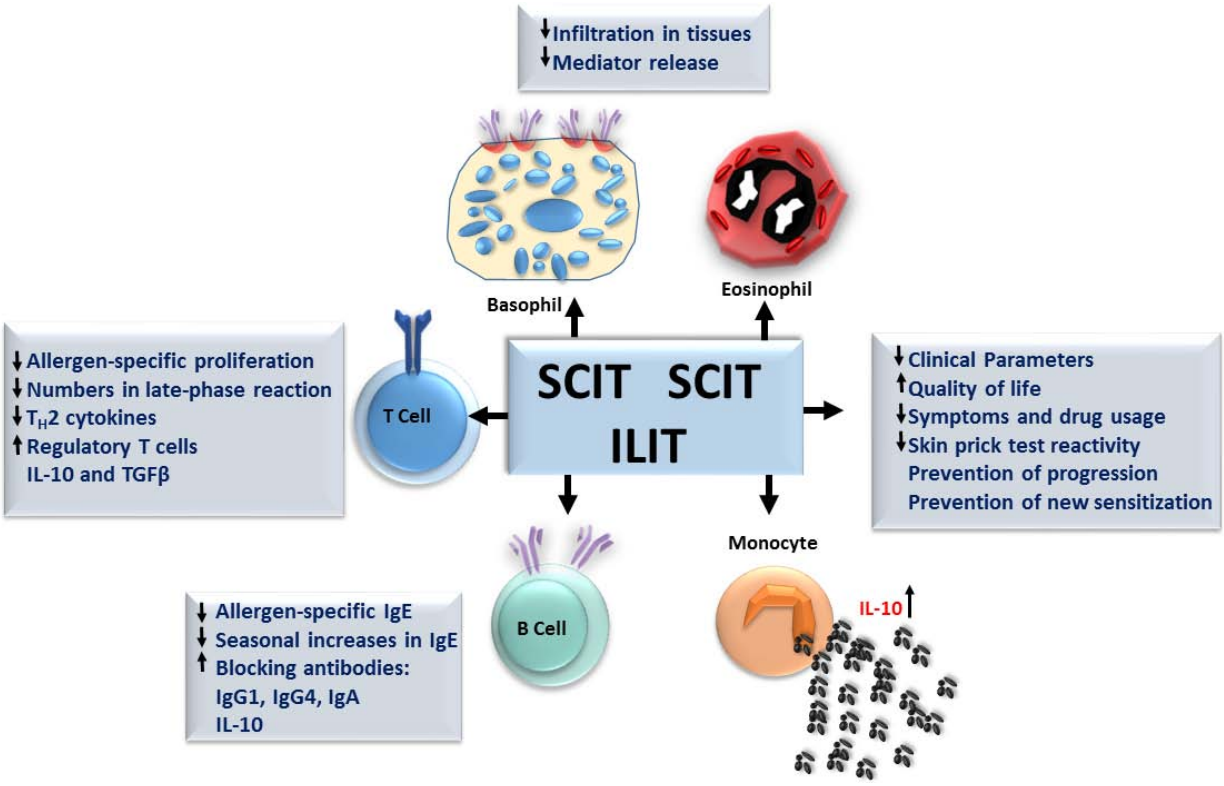

Figure XXX. Effects of allergen-specific immunotherapy on clinical and experimental immune parameters. (Adapted from **[Larché, et al., 2006]**).

VII.1.1. Modulation of Antigen-Presenting Cells

DCs play a key role in defining the type of immune response that is subjected to the interpretation of environmental signals associated with antigen encounter **[Akbari, et al., 2001]**. Depending on their maturation stage, DCs can control both peripheral tolerance and immune triggering **[Eiwegger, et al., 2012] [Larché, et al., 2006]**. Both myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) are known to induce

regulatory T cells (T_{regs}) **[Lambrecht, et al., 2000]**. This capacity highly depends on their stage of activation, co-expression of different surface receptors and absence of pro-inflammatory signals) **[Lambrecht, et al., 2000]**.

It has been shown that, partially mature DCs from the lung can produce IL-10 and promote the generation of type 1 T-regulatory (T_R1) cells **[Akbari, et al., 2001]**. Additionally, repeated stimulation of T cells with immature DCs results in the generation of non-proliferating T_R1-like cells, which inhibit subsequent inflammatory responses **[Jonuleit, et al., 2000]**. Moreover, pDCs from the palatine and lingual tonsils have the ability to generate regulatory T cells from naïve populations, which could explain the immunological mechanism of SLIT **[Palomares, et al., 2012]**.

VII.1.2. Modulation of Allergen-specific T cell responses

Recent studies suggest that active regulation might be a crucial mechanism for the induction and maintenance of peripheral tolerance to allergens **[Jutel, et al., 2011]**. IL-10 is the key player in the suppression of allergic reactions and can be produced by T_R1-like **cells [Akdis, et al., 1998]**, DCs, monocytes and B cells **[Aslam, et al., 2010]**. Mounting evidence suggests that both $CD4^+CD25^+$ regulatory T cells (T_{regs}) and T_R1-like cells and can be boosted by SIT cells **[Akdis, et al., 1998] [Akdis, et al., 2004] [Verhoef, et al., 2005]**. It has been demonstrated that T_{regs} play a key role in the prevention of allergic disease **[Ling, et al., 2004]** (**Figure XXXI**).

For example, children who outgrew milk allergy have a higher frequency of circulating T_{regs} in peripheral blood and a decreased allergen-induced proliferative T cell capacity compared with children that

remain allergic **[Karlsson, et al., 2004]**. On the other hand, T_R1-like cells bestow suppressive actions through the production of IL-10 and TGF-β, and expression of cytotoxic T lymphocyte antigen 4 (CTLA-4), glucocorticoid-induced TNF receptor (GITR) and programmed cell death 1 receptor (PD-1) **[Sakaguchi, et al., 2008] [Zimmermann, et al., 2011]**.

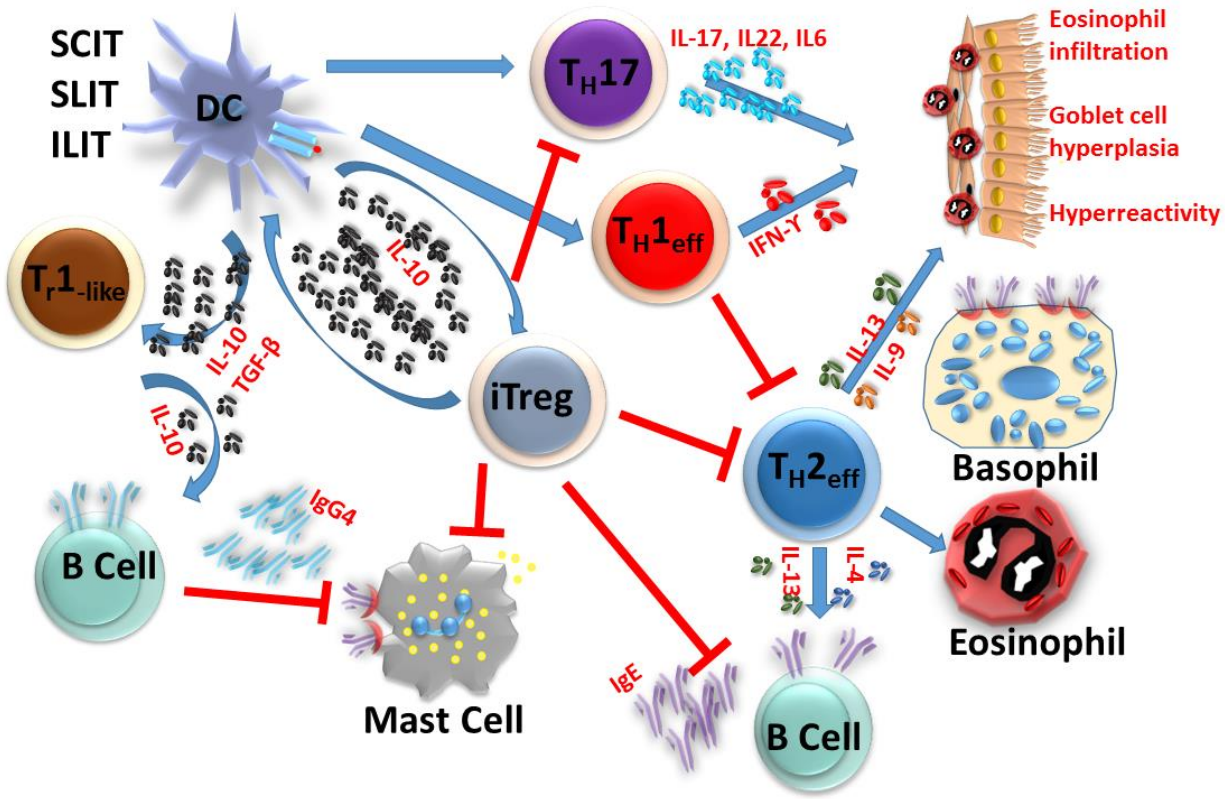

Figure XXXI. SIT initially affects APC in the draining lymph nodes, this leads to the induction of Tregs which exert potent inhibitory effects by using multiple mechanisms. (Adapted from **[Eiwegger, et al., 2012]**).

Several studies have suggested that following SIT, there is a shift toward a T_H1 phenotype **[Ebner, et al., 1997]**, as well as an increased T_H1/T_H2 ratio **[Wachholz, et al., 2002]**. However, these are controversial studies, as in vitro cultures may manipulate phenotypes of T cells. Nonetheless, an increased T_H1/T_H2 ratio, with mostly unchanged T_H2 but increased T_H1 cytokines, like IFN-ϒ and IL-12, has been demonstrated following SIT **[Wachholz, et al., 2002] [Varney, et al., 1993] [Durham, et al., 1996]**.

Recent longitudinal studies from our laboratory observed that allergen specific T_H1-like T_R1 cells that produce IL-10 become increasingly dominant during the course of SIT **[Wambre, et al., 2011] [Wambre, et al., 2014]**. Moreover, it has been shown that IL-4 negatively regulates the generation of T_R1 cells **[Hadjur, et al., 2009] [Trinchieri, 2001]**. Wambre et al, suggest that T_H2 cell depletion is a critical step for the induction of a protective response, as T_H1-like T_R1 cells that are found in low frequencies can then outcompete pathogenic responses **[Wambre, et al., 2011]**.

VII.1.3. Modulation of antibody responses; regulation of allergen-specific IgE and IgG4

IL-10 can also suppress total and allergen specific-IgE while increasing IgG4 production by B cells **[Akdis, et al., 1998]**. Moreover, it has been shown that IL-10 can induce direct switching of immunoglobulin classes expressed by naïve B cells to IgG **[Briere, et al., 1994]**, a common trait that has been reported following SIT. The dose dependent increase of allergen-specific IgG4 during successful SIT has been suggested as a surrogate marker for T_{reg} and T_R1 activity **[Meiler, et al., 2008]**. Allergen-specific IgG4 is thought to have a "blocking" effect, as it can outcompete the binding of an allergen to cell-bound IgE, thus, reducing clinical symptoms **[Gleich, et al.,1982]**. A recent study, demonstrated that Feld-1 specific IgG antibodies interact with Fc_γRIIB on mast cells, promoting IgE internalization and inhibits effector function **[Uermösi, et al., 2013]**. These results gave insight on how allergen-specific desensitization may occur and provided a mechanism for long-term desensitization of mast cells by selective removal of long-lived IgE antibodies on mast cells **[Uermösi, et al., 2013]** (**Figure XXXII**). In addition to IgG, evidence for the increase of allergen specific IgA following SIT has also been observed, suggesting a possible in clinical efficacy **[Jutel, et al., 2003]**.

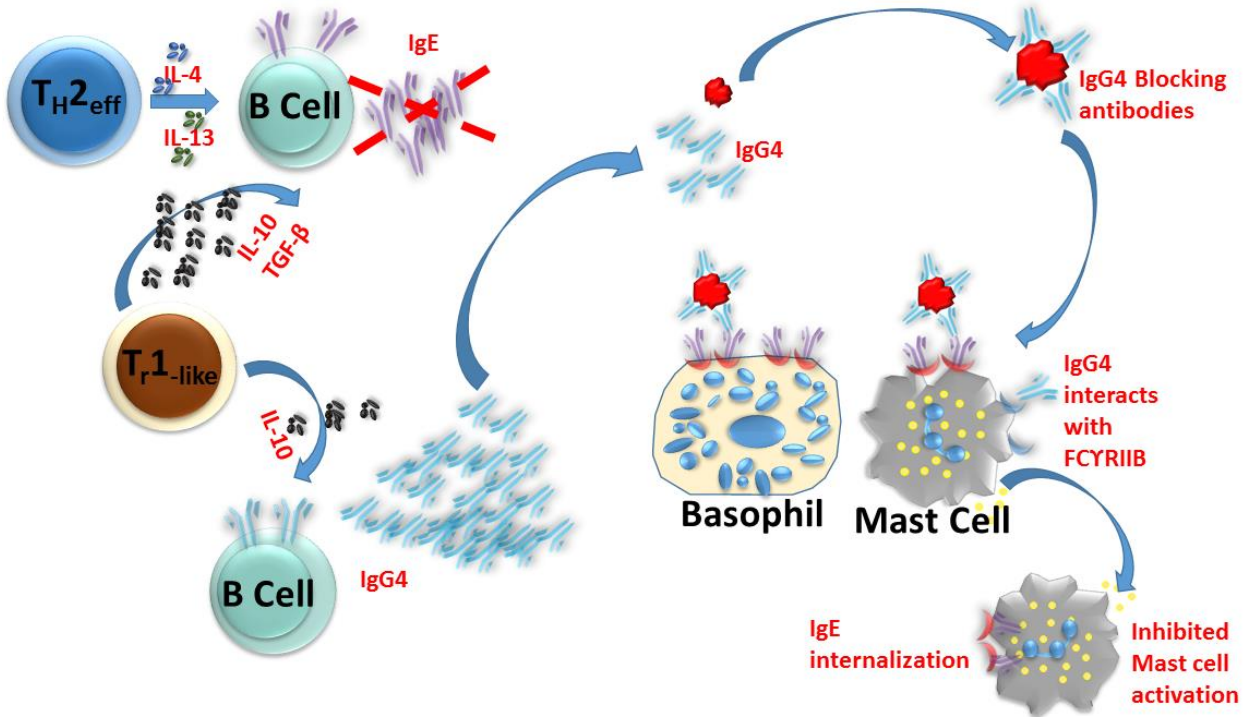

Figure XXXII. SIT leads to production IL-10 which suppresses IgE production and at the same time induces the production of blocking antibodies. IgG antibodies then interact with FcϒRIIB on mast cells and promote IgE internalization and inhibition of mast cell activation (Adapted from **[Jutel, et al., 2011] [Uermösi, et al., 2013]**).

VII.2. Peptide-Based Allergen Specific Immunotherapy for the Treatment of Allergic Disorders

A novel approach that reduces the allergenicity of immunotherapy vaccines while retaining the immunogenicity of the administered allergen molecules has been proposed **[Norman, et al., 1996] [Verhoef, et al., 2005] [Larché, et al., 2006]**. The use of synthetic peptides for allergen specific immunotherapy is a promising field in translational research and has clinical efficacy because short allergen-derived T cell epitopes lack IgE binding capacity, thus, risk of adverse reactions is low compared to conventional SIT **[Larché, et al., 2006]**. Synthetic peptide-based vaccines derived from the major cat allergen, Fel d 1 **[Norman, et al., 1996] [Oldfield, et al., 2002]** and from bee-venom phospholipase A_2 (Api m 1) **[Muller, et al., 1998]** have been developed and clinically evaluated. Mixtures of peptides have also been utilized and have been effective in the down regulation of systemic T_H1 and T_H2 cell responses to allergen **[Oldfield, et al., 2002]**, together with concomitant induction of IL-10 production **[Verhoef, et**

al., 2005] [Oldfield, et al., 2002], and reduction of immediate and late phase reactions **[Oldfield, et al., 2002]**.

D. Concepts to be improved in allergy

The rational design of T cell based therapies for allergic disease is dependent on well-designed studies that underlie the T cell mechanisms that accompany allergic inflammation in humans **[Woodfolk, et al., 2007]**. The identification of novel T cell epitopes for various allergens is required for the selection of peptide candidates for vaccine design and development. Preferably, T cell epitopes should be promiscuous, and induce T cell reactivity in a HLA-II diverse population of allergic subjects **[Woodfolk, et al., 2007]**. Further investigations of allergen-specific T cell phenotypes and responses at the epitope level should be evaluated, as understanding T cell responses to different allergens is a prerequisite for rational design of a future immunotherapy vaccine. Although IgE epitopes of most of tree nut allergens have been studied **[Roux, et al., 2003]**, $CD4^+$ T cell specific epitopes for tree nut allergens, walnut and cashew, remain uncharacterized. In particular the relationship of phenotypes of food allergen-specific $CD4^+$ to disease has not been examined for any tree nut.

The use of single vs. multiple components in SIT is another topic of debate. Subsequent studies should also focus on the capacity of T cell epitopes to cross-react with phylogenetically related species as they can generate important information that can benefit immunotherapy design. An important question is whether one species alone is sufficient to modulate $CD4^+$ T cell responses, or several species will be required to fully cover the complete set of epitopes. To better our understanding on the patterns of cross-reactivity, our work focused on two distinct types of allergens: 1. Grass pollen from the Pooideae grass subfamily and 2. Tree nut allergens.

Part II.
Hypothesis
And
Objectives

E. Hypothesis

It has been demonstrated that $CD4^+$ T cells play a crucial role in the development of allergy and that there are unique HLA-restricted epitopes within an allergen that trigger this ailment in allergic subjects. In our hypothesis, **we postulate that these epitopes of the sensitized allergen that trigger initial responses share homologous sequences with additional allergens from other members of the same family, genera and/or even class of that of the sensitizing species**. Thus, it can be inferred that an equivalent response can be generated equally or greater by another epitope from a different species that did not act as the source of the original sensitization. It is also important to note that not all homologous sequences can be cross-reactive and that this information as a whole will better our understanding for immunotherapy design. Additionally, we also hypothesize that allergen specific T cells can have **different phenotypes, and these different subpopulations will lead to different clinical manifestations of the disease.**

F. Objectives

1. Identification of allergen-specific $CD4^+$ T-cell HLA-restricted immunodominant epitopes with the use of MHC class II tetramers.
2. Characterization of $tetramer^+$ allergen specific $CD4^+$ T-cells.
3. Evaluation of $CD4^+$ T-cell cross-reactivity for identified allergen-specific epitopes using co-staining with MHC class II tetramers loaded with different homologous peptides.
4. Determination of frequency and comparison of phenotype of allergen-specific $CD4^+$ T cells in allergic subjects and non-allergic subjects.

Part III.

Results

A. Grass-specific $CD4^+$ T-cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy

Archila, L.D. J.H. Delong, E. Wambre, E.A. James, D. M. Robinson, W.W. Kwok. Grass-specific $CD4^+$ T-cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy. 2014.

Clinical and Experimental allergy. 44:986-998. **Editorial choice by P. Moingeon. 44:898-900**. **Impact factor: 4.324**

Grass-specific CD4$^+$ T-cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy

In this study, we determined the patterns of cross-reactivity of CD4$^+$ T-cells specific for homologous Pooideae-grass-pollen epitopes derived from Timothy grass against Kentucky, Orchard, Rye, Velvet, Barley and Canary grass utilizing dual pMHC-II tetramer staining. We determined whether DRB1*04:01, DRB1*07:01 and DRB5*01:01 grass-pollen allergic subjects that were diagnosed based upon IgE reactivity to Timothy grass pollen (TGP) extract were also sensitized to other related grass species at the T-cell level. The implications of our findings and the choices of using a single extract verses multiple extracts in immunotherapy were discussed. T-cells with various degree of cross reactive profiles could be detected. Poa p 1 $_{97\text{-}116}$, Lol p 1 $_{221\text{-}240}$, Lol p 5a $_{199\text{-}218}$, and Poa p 5a $_{199\text{-}218}$ were identified as minimally-cross-reactive T-cell epitopes that do not show cross reactivity to Phl p 1 and Phl p 5a epitopes. *Ex vivo* tetramer staining assays demonstrated T-cells that recognized these minimally-cross reactive T-cell epitopes are present in Grass-pollen allergic subjects. Our results suggest that not all Pooideae grass are cross-reactive. Non-cross reactive T-cells with comparable frequency, phenotype and functionality to Phl p-specific T-cells, suggest that a multiple allergen system should be considered for immunotherapy instead of a mono allergen system.

B. Ana o 1 and Ana o 2 cashew allergens share cross-reactive $CD4^+$ T-cell epitopes with other tree nuts

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Ana o 1 and Ana o 2 cashew allergens share cross-reactive CD4$^+$ T-cell epitopes with other tree nuts

In this study, we used cashew as a model to study tree-nut cross-reactivity against hazelnut, pistachio and walnut at the T-cell level. We initially investigated Ana o 1, Ana o2 and Ana o 3-specific T-cell responses using CD154 activation assay. Both Ana o 1 and Ana o 2 were identified as the predominant allergens that elicit CD4$^+$ T-cell responses and the magnitude of these were compared in allergic and non-allergic subjects. Several Ana o 1 and Ana o 2 derived epitopes were identified by using tetramer-guided epitope mapping (TGEM). Phenotypes for allergen-specific T-cells were analyzed by *ex vivo* tetramer staining and results show that allergic subjects have a predominant T_H2 phenotype, however, T_H2/T_H17 responses in some individuals were also detected. T-cell-clones specific to 7 out of 12 were generated to assess cross-reactivity by tetramer co-staining and proliferation experiments. TCC specific to cashew derived epitopes could readily proliferate with hazelnut and pistachio but not walnut peptides. This study leads to the conclusion that exposure to other tree nut allergens can trigger cashew-specific T-cell responses in these cashew allergic subjects. Strict avoidance of other tree nuts should be recommended in cashew allergic subjects.

C. Jug r 2-reactive $CD4^+$ T-cells have a dominant immune role in walnut allergy

Luis Diego Archila, MSc, David Jeong, MD, Mariona Pascal, PhD, Joan Bartra, MD, Manel Juan, MD PhD, David Robinson, MD, Mary L. Farrington, MD, William W. Kwok, PhD. 2015.

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Jug r 2-reactive CD4+ T-cells have a dominant immune role in walnut allergy

In this study, we examined T-cell reactivity towards Jug r 1 and Jug r 2, as their corresponding allergens in peanut, 2S albumin (Ara h 2) and 7S vicilin-like seed storage protein (Ara h 1) respectively, are highly immunogenic in peanut allergic subjects. Jug r 3 was also studied since we have a small cohort of samples from Spain, where LTP is the major plant food allergen. We initially investigated Jug r 1 Jug r 2 and Jug r 3-specific T-cell responses using CD154 activation assay. Jug r 2, but neither Jug r 1 nor Jug r 3, elicited dominant T-cell responses in allergic subjects. Several Jug r 2 derived epitopes were then identified by using tetramer-guided epitope mapping (TGEM). The magnitude and phenotype of the response of Jug r 2-specific $CD4^+$ T-cells in allergic and non-allergic subjects were determined directly *ex-vivo.* The predominant phenotype for Jug r 2 reactive T-cells is central memory phenotype. Results show that allergic subjects have a predominant T_H2 phenotype, however, T_H17 responses in some individuals were also observed. T-cells with $CCR4^+CD27^+$, $CCR4^+CD27^-$, $CCR4^+CCR6^+$ and $CCR4^+CCR6^-$ surface phenotypes were detected in allergic subjects. T-cells from non-allergic subjects have a T_H1 and T_H1/T_R1 phenotypes characterized by surface expression of CXCR3. Walnut-specific T-cells with T_H2, T_H17 and T_H2/T_H17 phenotypes could be detected in non-asthmatic and asthmatic walnut allergic subjects. Understanding this T-cell heterogeneity may improve our understanding of disease manifestation.

Part IV.

Discussion

A. General Discussion

In recent years, researchers have focused on the study of $CD4^+$ T cells, as they play a central role in orchestrating specific immune responses in host defense and pathogenesis. Differences between phenotypes, frequencies and functional capacities amongst healthy and allergic individuals have been reported and have provided new insightful information with intrinsic value on how different T cell lineages are responsible for several clinical features in these ailments, thus the original central dogma of allergy where it was thought that only T_H2 cells and IgE mediate allergic responses has been challenged.

Although several conceptual advances have begun to shed light on the pathways that initiate type 2 responses, there are several caveats that need to be acknowledged in allergy research. For example, a thorough comparison between the genetic profiles of several cell populations in parasitic infection and in an allergic context has not been documented. This would yield important information on whether the same elements are significant in the generation of type 2 responses in both settings, as it could lead to the identification of novel gene targets important for therapy, but also discrepancies in the regulation and generation of molecular responses. This is an important question that remains unanswered, specifically for allergen-specific $CD4^+$ T cells, as it remains a possibility that T_H2 cells specific for different antigens activate distinct genetic pathways depending on the nature of the antigen, route and frequency of exposure. For example, allergic subjects that are exposed concomitantly to perennial allergens might have different genetic profiles compared to chronic parasitic infection. Food allergen-specific T cells might have different phenotypes compared to airborne allergen-specific T cells, as the route of exposure to the allergen is distinct. Furthermore, the use of pMHC-II tetramers can provide a thorough characterization of these cells with *ex vivo* enrichment and may lead to the identification of the immunological signatures (surface receptors) of T_H2 cell responses in different allergic settings.

Recent work from our laboratory, focused on airborne allergens (alder and grass pollen), has observed that lack of CD27, correlated with CRTH2 expression on allergen-specific $CD4^+$ T cells and that this phenotype is exclusively found in allergic individuals **[Wambre et al., 2011] [Wambre, et al., 2014]**. In contrast, protective responses in non-atopic individuals are associated with $CD27^+CRTH2^-$ allergen specific T cells. Thus, Wambre et al., suggest that CD27 expression distinguishes protective ($CD27^+$) from pathogenic ($CD27^-$) responses in the allergic context. However, the examination of the pathophysiological meaning of different T cell signatures, particularly the etiology of surface markers, in allergy should also be evaluated. The discovery of disease biomarkers would also be beneficial during immunotherapy, as the direct detection of specific pathogenic subsets with pMHC-II tetramers may be important to track modulations of responses and/or depletion of T cells. Sequencing TCRs from $CD27^-$ prior to immunotherapy and $CD27^+$ allergen specific T cells after successful immunotherapy could address the previously mentioned statement. Sequencing would clarify whether IL-10 secreting T_R1 cells are generated from naïve T cells **[Roncarolo, et al., 2006] [Groux et al., 1997]** that are enhanced after depletion of T_H2 cells **[Wambre et al., 2011]**, or if a modulation of responses generates *de novo* expression of T_H1 and/or T_R1 markers on existing allergen specific T cells.

Despite it is widely believed that T_H2 cells play an important role in the generation of allergic disease, recent literature implicate that other T cell subsets with distinct homing receptors and effector functions are related to disease. The use of pMHC-II tetramers or CD154 up-regulation assay may provide new insights on what type of populations are involved in perennial, seasonal airborne and food allergy, but also further characterization of their surface receptors and their genetic profiles. The cooperation of different lineages of allergen-specific T cells with B cells should also be looked further, as different subsets can orchestrate immunoglobulin class switch and promote production of antibodies. In addition, the role of other immunoglobulin isotypes in allergy has not been investigated in detail. For example, the role of IgA in allergy and its possible effects remains controversial. Mucosal IgA antibodies are hypothesized to inhibit food allergen-induced anaphylaxis by binding antigen prior to systemic absorption. Indeed, low levels of

milk- and house dust mite (HDM)-specific IgA were associated with persistence of milk allergy **[Savilahti, et al., 2010]** and occurrence of HDM allergy in children **[Miranda, et al., 2011]**. Nonetheless, milk and egg allergic children did not show decreased allergen-specific IgA levels compared with tolerant children **[Savilahti, et al., 2012] [Vazquez-Ortiz, et al., 2013]**. Conversely, Guhsl et al. recently observed that allergic subjects had higher levels of specific IgA against apple, soy and celeriac than tolerant patients, which do not support a protective role **[Guhsl, et al., 2014]**. The role of specific-IgA in food allergy should be further evaluated.

Furthermore, the advances in genomics and proteomics, not only have permitted the synthesis of known allergens but also the identification of new allergens that play an important role in eliciting T cell responses related to allergic disease. For example, Schulten et al. compared T cell responses between Timothy grass pollen extract versus Timothy grass known allergen derived peptides **[Schulten et al., 2013]**. They found that no T cell responses could be detected by stimulation with peptides derived from known Timothy grass allergens despite detection of robust responses to Timothy grass pollen extract. This observation lead to the development of newer strategies that permitted the identification of novel Timothy grass allergens that play a role in allergic disease. The identification of novel T cell epitopes for a wide range of novel allergens is clearly warranted to hasten the bench-to-bedside transition.

Moreover, allergic responses may involve cross-reactivity by antibodies or T-cells. While IgE cross-reactivity amongst a wide range of allergens (e.g. grass pollen and tree nut allergens) has been documented, cross-reactivity at the allergen-specific T-cell level has been less studied. Identification of the patterns of cross-reactivity may improve our understanding, allowing optimization of better immunotherapy strategies as it is now firmly established that allergen-specific T-cells play an important role in allergic inflammation **[Woodfolk, et al., 2007]** and that induction of antigen specific Treg or elimination of allergen-specific T_H2 cells might be a prerequisite for the induction of specific tolerance **[Kwok, et al.,2010] [Wambre et al., 2011] [Wambre et al., 2014]**.

The rarity of allergen-specific T cells in peripheral blood requires highly specific labeling methods that enable their detection and accurate calculation of their frequencies. Regarding these considerations, the main objective of the work presented in this thesis focuses on the use of pMHC-II tetramers and other related methods to study allergen-specific $CD4^+$ T cells in different allergic settings; airborne and food allergy. We focused on Pooideae grass pollen allergy and tree nut allergy (particularly cashew and walnut allergy). Both allergic models contain species that share synapomorphies and belong to comprehensive taxonomical groups. Due to their taxonomical relatedness, high amino acid sequence homology is found within clinically relevant major allergens in different Pooideae grass species: 90% for Group 1 (Beta-expansins) and about 55-85% for Group 5 (Ribonucleases) allergens **[Weber, 2004] [Andersson, et al., 2003]**. On the other hand, amino acid sequence homology among major tree nut allergens 7s vicilin-like; Ana o 1 with Cor a 11, Pis v 3 and Jug r 2 (44%, 78% and 36.6% respectively) and 11s globulin like; Ana o 2 with Cor a 9, Pis v 5 and Jug r 4 (60%, 80.5%, and 63.2% respectively) **[http://fermi.utmb.edu/SDAP/]**, is also observed. As we mentioned earlier, cross reactivity at the IgE level has been well studied for both allergic models. However, cross-reactivity at the $CD4^+$ T cell level has been less documented. We first utilized pMHC-II tetramers to identify novel Pooideae grass pollen-, cashew- and walnut-specific $CD4^+$ T cell epitopes. Next, pMHC-II were used to evaluate the patterns of cross-reactivity of $CD4^+$ T cells specific for homologous grass-pollen epitopes derived from Timothy grass pollen and 7s vicilin- and 11s legumin-like tree nut allergen epitopes derived from cashew. The functional responses of cross-reactive and non-cross-reactive T cells was also assessed.

HLA class II tetramers have also been used to identify food allergen specific T cells **[DeLong, et al., 2012] [Renand, et al., 2014]**. The discovery of new biomarkers should better our understanding of basic immunological mechanisms involved in the development of food allergic responses so that more effective forms of therapy can be developed. For this objective, the use of pMHC-II tetramers was also advantageous to accurately characterize $CD4^+$ T cell responses towards novel tree nut epitopes directly *ex vivo* without the need for re-stimulation of cells. The implications of our findings will be discussed.

B. Identification of novel epitopes

Class II tetramers can be utilized to map antigenic epitopes of known allergens through a peptide screening procedure known as TGEM. In the current work, this method was used to identify $CD4^+$ T cell epitopes within walnut allergen Jug r 2 and cashew allergens Ana o 1 and Ana o 2. For Jug r 2, a total of 11 immunogenic epitopes restricted to *DRB1*01:01, DRB1*01:03, DRB1*04:01, DRB1*04:02, DRB1*04:04, DRB1*07:01, DRB1*09:01, DRB1*11:01, DRB1*14:01* and *DRB1*15:01* were identified. Hot spots with promiscuous Jug r 2 $CD4^+$ T cell peptides presented by multiple *DRB1* alleles were identified. Six of the Jug r 2 peptides (Jug r $2_{152\text{-}171}$, Jug r $2_{184\text{-}203}$, Jug r $2_{224\text{-}243}$, Jug r $2_{392\text{-}411}$, Jug r $2_{456\text{-}475}$ and Jug r $2_{520\text{-}539}$; 20 amino acids each) can be presented by at least 3 different *DRB1* alleles, including 3 peptides (Jug r $2_{152\text{-}171}$, Jug r $2_{184\text{-}203}$ and Jug r $2_{456\text{-}475}$) that can be presented by at least 5 different *DRB1* alleles. For peptide immunotherapy, T cell epitopes ideally should bind to multiple HLA class II molecules **[Woodfolk, et al., 2007]**. Indeed, these 6 promiscuous epitope regions should be good candidates for peptide vaccine to desensitize patients with walnut allergy. On the other hand, for cashew allergens, a total of 4 immunogenic epitopes restricted to *DRB1*01:01, DRB1*07:01, DRB1*09:01* and *DRB5*01:01* for Ana o 1, and for Ana o 2, a total of 8 immunogenic epitopes restricted to *DRB1*04:04, DRB1*07:01, DRB1*09:01, DRB1*15:01* and *DRB4*01:01* were identified. Interestingly, only one of the Ana o 1 peptides (Ana o $1_{281\text{-}300}$) could be presented by at least 3 different *DRB1* alleles. Consistent with previous studies **[Carballido, et al., 1992] [Ebner, et al., 1995] [DeLong, et al., 2011] [Wambre, et al., 2012] [Wambre, et al., 2014]** allergen-tolerant subjects and allergic patients recognize identical allergen-derived epitopes.

The identification of novel epitopes through TGEM can also be useful to identify homologous epitopes from phylogenically related species using *in silico* methods. Indeed, potential epitopes for other Pooideae grass and tree nut species were identified by selecting sequences homologous to previously reported Timothy grass pollen- **[Wambre, et al., 2014]** and to novel cashew-derived epitopes, using Blast

alignments **[Altschul, et al., 1990]**. The main advantage of TGEM is that we were able to identify the HLA-Class II restriction for each peptide, thus, we can predict if homologous peptides can be presented by a specific *DRB1* restriction as plausible T cell epitopes utilizing peptide binding assays (peptide + MHC-II).

C. Cross reactivity in grass pollen and tree nut allergens: implications in immunotherapy

I. Grass-specific CD4$^+$ T cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy

Although the efficacy and safety of single grass pollen allergen (Phl p) immunotherapy has been documented **[Dahl, et al., 2006] [Durham, et al., 2006]**, the use of single or multiple allergen products for Pooideae grass-pollen immunotherapy is still debated **[Didier, et al., 2007] [Moingeon, et al., 2008] [Chabre, et al., 2010]**. Thus, the main objective of this part of the study was to use Phl p derived epitopes and assess the patterns of cross-reactivity with homologous grass epitopes. We utilized a multiplex pMHCII tetramer staining approach to determine the patterns of cross-reactivity between TGP-derived epitopes and epitopes of pollens from multiple grass-species within the Pooideae family.

Prior studies showed that Pooideae pollen extracts demonstrate significant IgE cross-reactivity **[Leiferman, et al., 1976] [White, et al., 2003] [Hrabina, et al., 2008] [Marcucci, et al., 2010]**. In contrast, cross-reactivity at the T-cell level has not been thoroughly documented. Heijl et al showed both Phl p 1- and Phl p 5-specific T-cell lines (TCL) responded robustly to extract from other Pooideae species, suggesting cross-reactivity at the T cell level **[Heijl, et al., 2009]**. However, TCL being used were generated by pollen extract, and T cells specific for minimally cross-reactive T cell epitopes could be overlooked. In this present study, Phl p-specific CD4$^+$ T cells in allergic individuals had a range of patterns of cross-reactivity against allergens from other Pooideae species. The cross-reactivity profile depended on the HLA-

restriction and epitope involved. Consistent with previous studies **[Schenk, et al., 1995] [Muller, et al., 1998]**, our approach identified full-scale, partially and minimally-cross-reactive T cell epitopes for both Group 1 and 5 major allergens. Interestingly, minimally-cross-reactive $CD4^+$ T cells were identified utilizing a two way stimulation and detection system. The majority of T-cells specific for minimally-cross-reactive epitopes did not cross-recognize the Phl p 1 derived epitopes in the corresponding regions. However, a minor population of T cells, capable of cross-recognizing these epitopes was detected in some subjects, indicating that TCR diversity can be present in Pooideae grass-pollen specific T cells. This suggests that although high sequence homology is found amongst grass-pollen allergens from the Pooideae subfamily, a single species (Timothy grass) does not cover the broad epitope repertoire for multiple species at the T cell level. However, a caveat is noteworthy in our study, as the result of the cross-reactivity patterns might be influenced by the size of the cohort and the variety of HLA-alleles. It is possible that minimally cross-reactive epitopes may be absent in other HLA types. Interestingly, there is discordance in known cross-reactivity at the IgE level as compared to the T cell level. The findings in our study are ancillary to what is known for IgE cross-reactivity **[Leiferman, et al., 1976] [White, et al., 2003] [Hrabina, et al., 2008] [Marcucci, et al., 2010]**. IgE epitopes are conformational and Pooideae grass-pollen allergens contain IgE-binding sites that are capable of generating IgE responses to allergens from other Pooideae species **[Weber, et al., 2003] [Weber, et al., 2004]**. On the other hand, T cell epitopes are linear and selectively target allergen-specific T cells **[Focke-Tejkl, et al., 2014]**. A recent study by Focke-Tejkl et al showed discrepancy among the IgE- and T cell-reactive domains in Phl p 5 major allergens, showing the importance of this ancillary study of T cell epitopes.

Class II MHC/peptide structures have been thoroughly characterized **[Dessen et al., 1997] [Southwood, et al., 1998] [Yassai, et al., 2002]**, and it is established that peptide side chains at position p1, p4, p6, and p9 project into pockets along the MHC peptide-binding groove acting as anchor motifs **[Sette, et al., 1993]**. Conversely, peptide side-chains at position p-1, p2, p3, p5, p8, p10 project directly upward from the MHC-molecule surface and impact T cell recognition **[Sette, et al., 1993] [Dessen et al.,**

1997] [Southwood, et al., 1998] [Yassai, et al., 2002]. Interestingly, anchor positions for most of the allergenic epitopes are well conserved among Pooideae species. For example, anchor motifs in TGP derived epitopes from region $_{97\text{-}116}$ correspond to Y, D, S and G. In contrast, most of the minimally cross-reactive T cell epitopes feature variability at p-1, p2, p3 and/or p8. As shown in (**Figure XXXIII**), Poa p 1 $_{97\text{-}116}$ variant residues are present in p-1 (A) and p8 (K). For this reason, homologous peptides from diverse grass species can bind to MHC class II but have varying degrees of recognition by different TCRs. In addition, highly homologous epitopes tend to have higher binding affinities to MHC molecule compared to those with variability at T cell contact sites, suggesting that differences in affinity to MHC II may also play an important role in cross-recognition of minimally-cross-reactive epitopes.

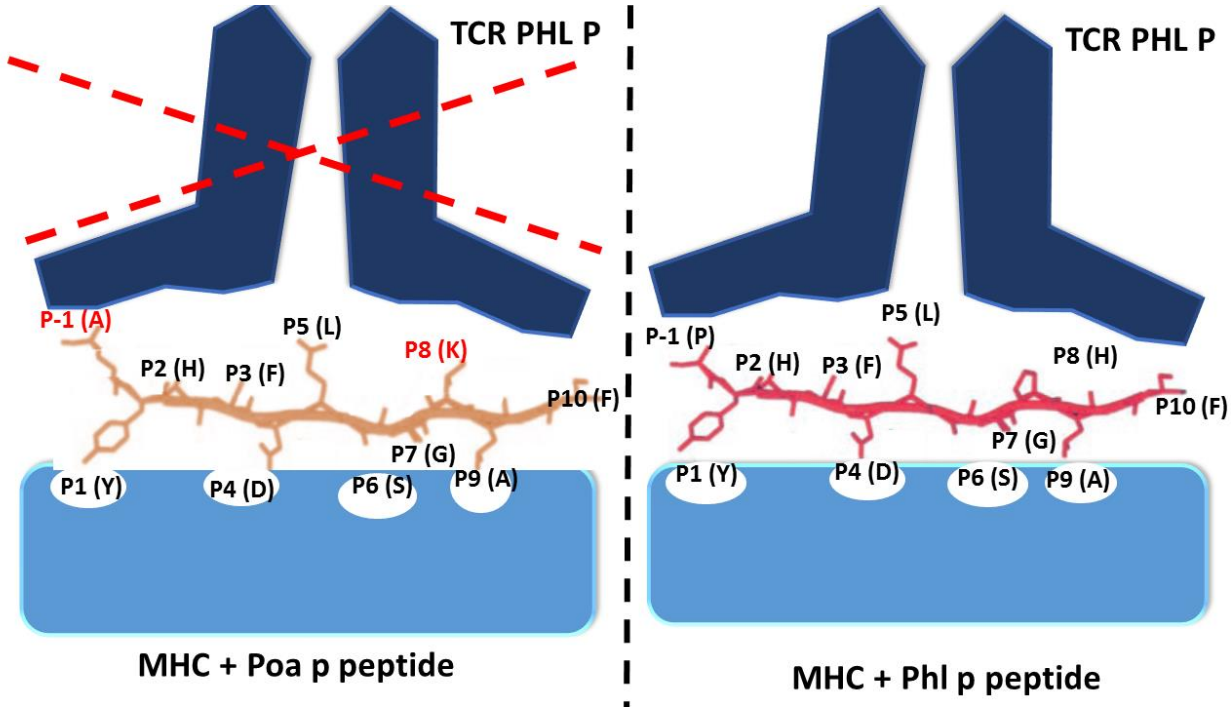

Figure XXXIII. Variability at T cell contact sites play an important role in TCR recognition. The first panel shows the amino acid sequence for Poa p $_{97\text{-}116}$; variant residues at p-1(A) and p8(K) disable the interaction with Phl p specific T cell. The second panel shows the amino acid sequence for Phl p $_{97\text{-}116}$ and interaction with Phl p specific T cell is possible.

The current study demonstrated that different grass-species contain species-specific T cell epitopes. Considering the overlap of pollination calendars and geographical distribution for Pooideae grass species,

allergic subjects can be exposed to multiple pollens in the same geographical region **[White, et al., 2003] [Moingeon et al., 2008] [Chabre, et al., 2010]**. As minimally cross-reactive T cell epitopes can also bind to MHC-II, exposure to pollens from different grass species may generate T cell population's specific to those minimally cross-reactive T cell epitopes in allergic subjects. Indeed, homologous grass-pollen epitope-specific $CD4^+$ T cells specific for minimally cross-reactive T cell epitopes of other Pooideae species could be readily detected in PBMC from grass-pollen allergic subjects. For example, frequency of Poa p $1_{97\text{-}116}$ in PBMC was found in similar range as the immunodominant Phl p epitope in DR04:01 subjects, suggesting that poly-sensitization occurs at the T cell level. Side-by-side comparison of surface phenotype and functional profiles reveals that these minimally cross-reactive $CD4^+$ T cells have a T_H2 dominated response and equal allergenic properties as Phl p-specific $CD4^+$ T cells. In addition, these homologous grass-pollen epitope-specific $CD4^+$ T cells are also activated during pollen season. The presence of the minimally cross-reactive T cells for different Pooideae species in a group recruited on the basis of IgE reactivity to TGP implies that GP allergic subjects are usually poly-sensitized to other Pooideae species at the T cell level (**Figure XXXIV**). A recent study suggested that depletion of allergen specific T cells would be essential for successful antigen specific immunotherapy **[Wambre et al., 2012]**. In addition, it has also been established that the strength of the TCR signal induced by an epitope can affect the outcome in inducing tolerance **[Gabrysova, et al., 2010]**. Conversely, lack of cross-staining and the featured variability at sites of T cell contact suggest that minimally cross-reactive CD4+ T cells specific for other Pooideae species will not be targeted by TGP specific mono immunotherapy as most of GP allergic subjects are sensitized to various Pooideae species.

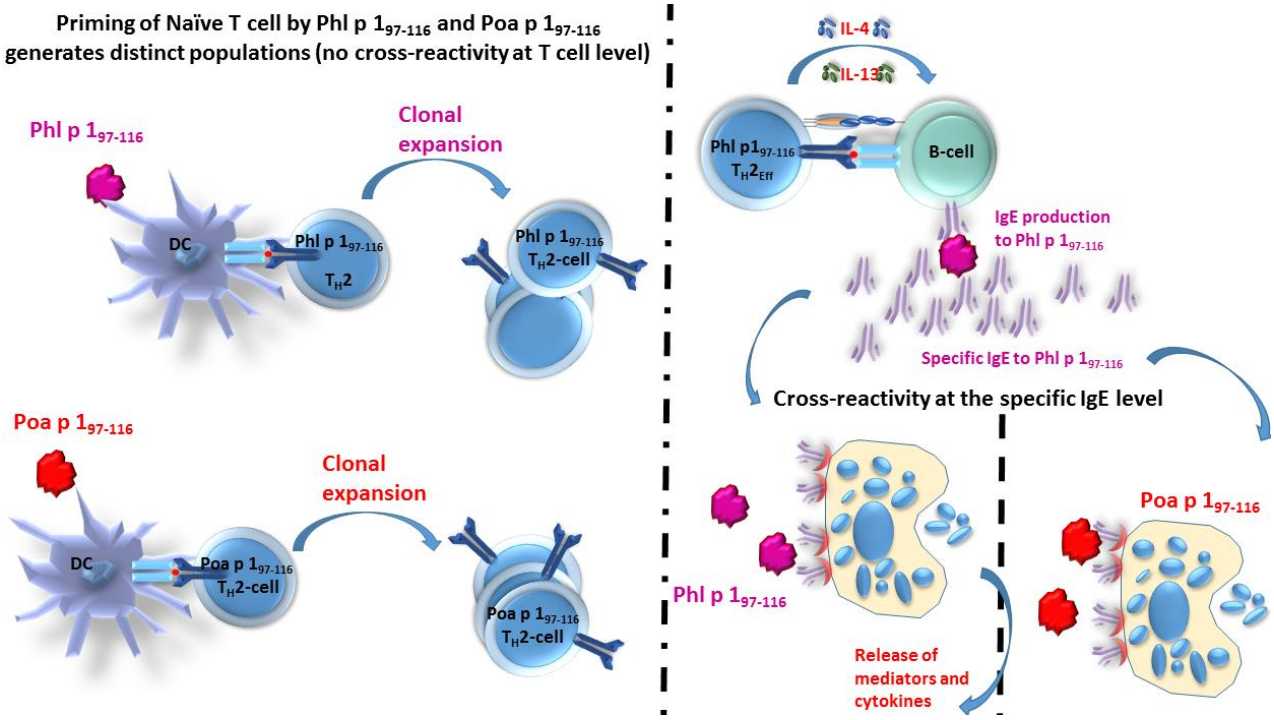

Figure XXXIV. Exposure to multiple pollens from the Pooideae grass species generates separate Poa p 1 $_{97\text{-}116}$- and Phl p 1 $_{97\text{-}116}$- specific T cells that have similar functional profiles in DRB1*04:01 allergic subjects. While no cross-reactivity is observed at the T cell level, specific IgE can recognize both Phl p 1 and/or Poa p 1 grass pollen allergens.

On the other hand, the presence of full-scale and partially cross-reactive T cell populations raises the possibility that single species immunotherapy might be effective for these cross-reactive populations with similar T_H2 phenotype. Campbell et al previously showed that peptide immunotherapy with selected epitopes from a single allergen could render T cell tolerance to one epitope **[Campbell, et al., 2009]**, enabling the suppression of the function of T cells specific for other epitopes within the same allergen **[Holan, et al., 1983]**. However, this linked epitope suppression is confined to a single allergen. Nonetheless, further investigations are required to confirm that Phl p-specific T cells can mediate suppression of immune responses elicited by Phl p-nonspecific homologous T cell populations.

In summary, class II tetramer staining experiments show that the patterns of cross-reactivity vary for 4 different DR04:01-, 1 DR07:01- and 1 DRB5*01:01-restricted TGP derived epitope regions tested.

Indeed, T cells with various degrees of cross reactivity profiles could be detected with tetramers both *in vitro* and *ex-vivo* settings. On the basis of these findings, we confirmed that both Phl p-specific as well as their Phl p- homologous $CD4^+$ T_H2 populations exist *in vivo*, suggesting GP allergic patients diagnosed upon IgE reactivity to *Phleum pratense* pollen extract were sensitized to various grass species at the T cell level. This finding implies that mono-allergen immunotherapy with Phl p allergen would fail to elicit Treg or delete those species-specific T cells that show minimum cross reactivity to Phl p. Although a direct clinical comparison between immunotherapy with single grass-extract verses multiple species-extracts utilizing cohorts with a variety of HLAs would be obligatory, the current study suggests multiple-grass-pollen-species immunotherapy should be more beneficial than single species immunotherapy.

II. Ana o 1 and Ana o 2 cashew allergens share cross-reactive $CD4^+$ T-cell epitopes with other tree nuts

It has been estimated that at least 86% of subjects who are allergic to tree nuts are allergic to multiple tree nuts **[Clark, et. al., 2005]**. Three major allergens have been reported to be allergenic in the tree nut group; 7s vicilin-like protein, 11s legumin-like protein and 2s albumin **[Wang, et al., 2002] [Robotham, et al., 2005] [Robotham, et al., 2010]**. All three are classified as seed storage proteins **[Roux, et al., 2003]**. Furthermore, amino acid sequence alignments of vicilins, legumins and albumins indicate that a high degree of homology is found within cashew allergens and allergens from other tree nuts including hazelnut, pistachio and walnut **[Radauer, et al., 2003]**. Cross-sensitization for pistachio, hazelnut and walnut are common within cashew allergic subjects **[Rance, et al., 2003]**. Studies have shown high degree of cross-reactivity between pistachio and cashew at the sIgE level, owing to their botanic relatedness **[Goetz, et al., 2005] [Noorbakhsh, et al., 2011] [Willison, et al., 2008]**. This phenomenon has also been shown between walnut, hazelnut and cashew allergens at moderate levels **[Goetz, et al., 2005] [Barre, et al., 2008]**. On the other hand T-cell cross-reactivity between tree nuts in humans has not been well documented.

In the present study, we detected basophil responses towards different tree nuts in subjects recruited on the basis of cashew allergy, confirming a poly-sensitization status. We also identified cashew derived epitopes and looked for cross-reactivity to homologous epitopes from other species of tree nuts. We found that the majority of cashew-specific TCC from Ana o 1 and Ana o 2 reacted to homologous peptides from hazelnut and pistachio via proliferation assays. Importantly, these homologous peptides are able to elicit identical T_H2 cytokine in cashew reactive T cells. Our observations infer that phylogenetic relatedness among tree nuts reflects cross-reactivity as we observed that most of cashew derived T-cell epitopes cross-reacted with pistachio epitopes. This is in agreement with the fact that cashew allergens Ana o 1, Ana o 2 and Ana o 3 and their corresponding allergens Pis v 1, Pis v 2 and Pis v 3 of the closely-related pistachio nut are highly homologous (78%, 80%, and 70%, respectively) and have a high degree of IgE-binding cross-reactivity **[Noorbakhsh, et al., 2011] [Hasegawa, et al., 2009]**. Although Ana o 1 and the hazelnut allergen Cor a 11 are only 44% homologous, we were still able to detect cross-reactive epitopes between cashew and hazelnut. In contrast, though sequence homology is found in similar ranges (60%) between Ana o 2 and their corresponding allergens Jug r 4 in walnut, neither T-cell proliferation nor class II tetramer co-staining was observed for all of the walnut homologous peptides. Additionally, all of the identified Jug r 2 epitopes did not fall into the regions identified for Ana o 1 cashew derived epitopes. This is expected as Jug r 2 and Ana o 1 have low sequence homology 36%. Nonetheless, walnut T-cell responses were still detected in these subjects, suggesting that walnut allergens contains species-specific T-cell epitopes, and this places walnut in a group of allergens within the tree nut allergens family that is distinct from cashew (**Figure XXXV**). Both walnut and pecan belong to the family of Juglandaceae **[Chen, et al., 1998]** and walnut and pecan allergens are highly homologous. Similar profiles of IgE cross-reactivity have been reported by Goetz et al., they reported that cashew, pistachio and hazelnut form a group of tree nuts which were distinct from walnut and pecan **[Goetz, et al., 2005]**. However, a low degree of cross-reactivity between cashew and walnut was still observed. In addition to the cross-reactive T cell epitopes as discussed above, TCC derived

from Ana o $1_{433-452}$ did not co-stain with class II tetramers nor proliferate to homologous peptides from other tree nut species suggesting this as a species-specific epitope for cashew. It is also possible that Ana o $1_{433-452}$ is conserved in other tree nut species not tested in our study.

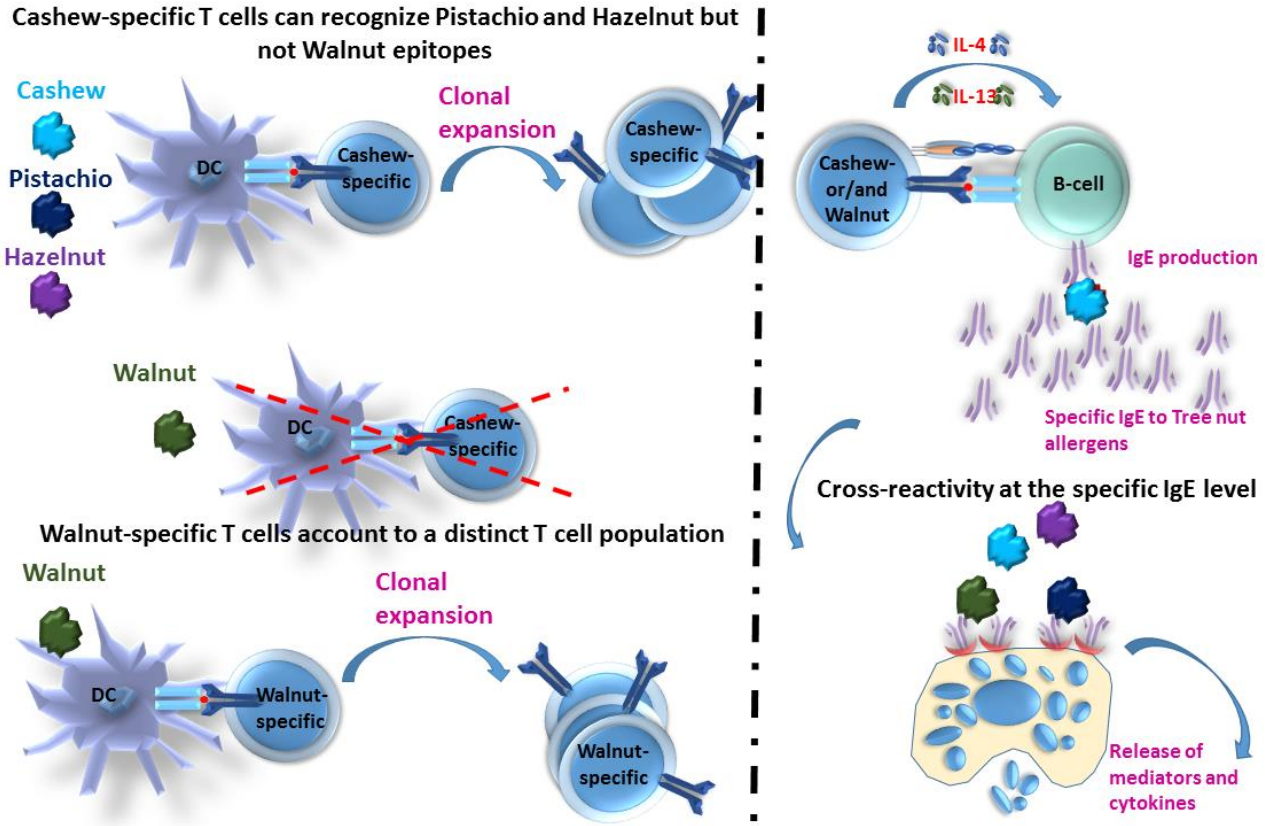

Figure XXXV. Cashew-specific T cells can recognize Pistachio- and Hazelnut- but not Walnut- derived epitopes. Distinct Walnut epitopes generate different T cell populations. While no cross-reactivity is observed between walnut and cashew at the T cell level, specific IgE can recognize both walnut and cashew allergens.

From an immunotherapeutic standpoint, the presence of cross-reactive T-cell epitopes raises the possibility that single species peptide immunotherapy might be effective in modulating or depleting **[Wambre, et al., 2012]** these cross-reactive populations with both pathological phenotype. Indeed, linked epitope suppression has been previously shown were peptide immunotherapy with selected epitopes from a single allergen resulted in suppression of responses to other epitopes **[Campbell, et al., 2009]**. Additionally, Kulis et al demonstrated that cashew immunotherapy prevented allergic responses to both cashew and pistachio in a cashew sensitized murine model and to cashew and walnut in a multisensitized

murine model **[Kulis, et al., 2011]**. Kulis et al also reported T cell cross-reactivity between walnut and cashew in a cashew sensitized mouse model **[Kulis, et al., 2011]**. It is likely that these cross-reactive epitopes are epitopes with low affinity to MHC-molecules. Low affinity cross-reactive epitope may also be presence in humans which are overlooked in our tetramer approach. However, the presence of walnut specific T-cell populations in walnut sensitized subjects of our cashew allergic cohort and their lack of cross-reactivity with cashew suggest that cashew peptide immunotherapy approach may not be most effective for walnut.

Overall, the T cell cross-reactivity between tree nuts as reported here together with the extent of IgE cross-reactivity amongst different tree nuts as reported earlier are direct evidence that exposure to one tree nut can lead to allergic immune response related to other tree nuts. Thus subjects whom have clinical reaction to one tree nut should avoid all tree nuts as a precaution.

D. Characterization of tree nut allergen-specific $CD4^+$ T cells

Although allergic reactions to walnut and cashew nut are increasing in prevalence and account for the vast majority of severe reactions in patients with tree nut allergy in the United States **[Sicherer, et al., 2010]**, (adverse reactions to tree nuts (cashew) may be more severe than peanut allergy **[Davoren, et al., 2005] [Grigg, et al., 2009] [Rance, et al., 2003]**), the role of $CD4^+$ T-cell responses in walnut and cashew allergy has not yet been studied. In this study, we identified the predominant walnut (Jug r 2) and cashew (Ana o 1 and Ana o 2) allergens that elicit $CD4^+$ T cell responses. In non-allergic subjects, Jug r 2-, Ana o 1- and Ana o 2-reactive T cells were detected at substantially lower frequencies than allergic subjects. In addition, surface phenotypes and functional properties of these T cells are distinct in non-allergic and allergic subjects. For non-allergic subjects with detectable frequency through *ex vivo* analysis of PBMC, the phenotype was $CXCR3^+CCR6^+CD27^+$, while IFN-ϒ and low IL-10 production was observed on Jug r 2-specific TCCs derived from this cohort. On the other hand, $CCR4^+$ T cells that produced T_H2 and T_H17

cytokines were exclusively observed in allergic subjects. ICS showed that the majority TCC clones derived from cashew and walnut epitopes mainly produce IL-4, IL-13 and IL-5, while a minority of TCC co-produced IL-4 and IL-17A implicating that these $CCR4^{+}CCR6^{-}$ and $CCR4^{+}CCR6^{+}$ cells were *bona fide* T_H2, T_H2/T_H17 cells, respectively. Additionally, we were also able to detect Jug r 2 TCCs that exclusively produced IL-17A alone. It remains a possibility that allergen-specific T_H2 cells are also present in non-allergic subjects at a very low frequency, which is less than the threshold of the detection method.

Our laboratory previously demonstrated that only terminally differentiated ($CD27^{-}$) allergen specific T-cells from pollen allergic subjects displayed a T_H2 phenotype **[Wambre et al., 2012] [Wambre et al., 2014]** and that lack of CD27 expression coincides with CRTH2 expression **[Wambre, et al., 2012]**. In the present study, both cashew- and walnut-specific terminally differentiated T_H2 T cells were present in allergic subjects and were essentially absent in non-allergic subjects. In addition, both $CD27^{+}CRTH2^{-}CCR4^{+}$ T_{CM} and $CD27^{-}CRTH2^{+}CCR4^{+}$ T_{eff} cells were present in allergic subjects with T_{CM} as the most prevalent phenotype. We also demonstrated that both T_{CM} and T_{eff} were capable of producing T_H2 cytokines. A previous report showed $CCR4^{+}T_{CM}$ in humans are capable of producing IL-4 even though they are not fully differentiated **[Rivino, et al., 2004]**. Variable expressions of CRTH2 have been previously observed for Ara h 1-**[DeLong, et al., 2011]** and Pen m 2-**[Renand, et al., 2014]** reactive T cells, and these cells were capable to produce IL-4. Accumulating evidence proposes that CD27 is lost after repetitive antigenic stimulation **[Hintzen, et al., 1993] [Hamman, et al., 1997]** and loss of CD27 after TCC generation suggest that occasional antigen stimulation is essential for the expression of CRTH2 in food allergen specific T cells **[DeLong, et al., 2011]**. Food avoidance in walnut allergic subjects may have resulted in the accumulation of $CRTH2^{-}$ T_{CM} in peripheral blood of allergic subjects (**Figure XXXVI**). The results from this study do not contradict to our laboratories previous pollen studies **[Wambre, et al., 2012] [Wambre et al., 2014]**, as subjects with pollen allergy are subjected to annual challenges of high doses of pollens during the pollen season. The presence of allergen specific T_{CM} as consequence of food avoidance in food

allergic subjects may complicate the treatment of food allergy, as T_{CM} are less susceptible to deletion by allergen specific immunotherapy, as shown in a murine model **[Mackenzie, et al., 2014]**.

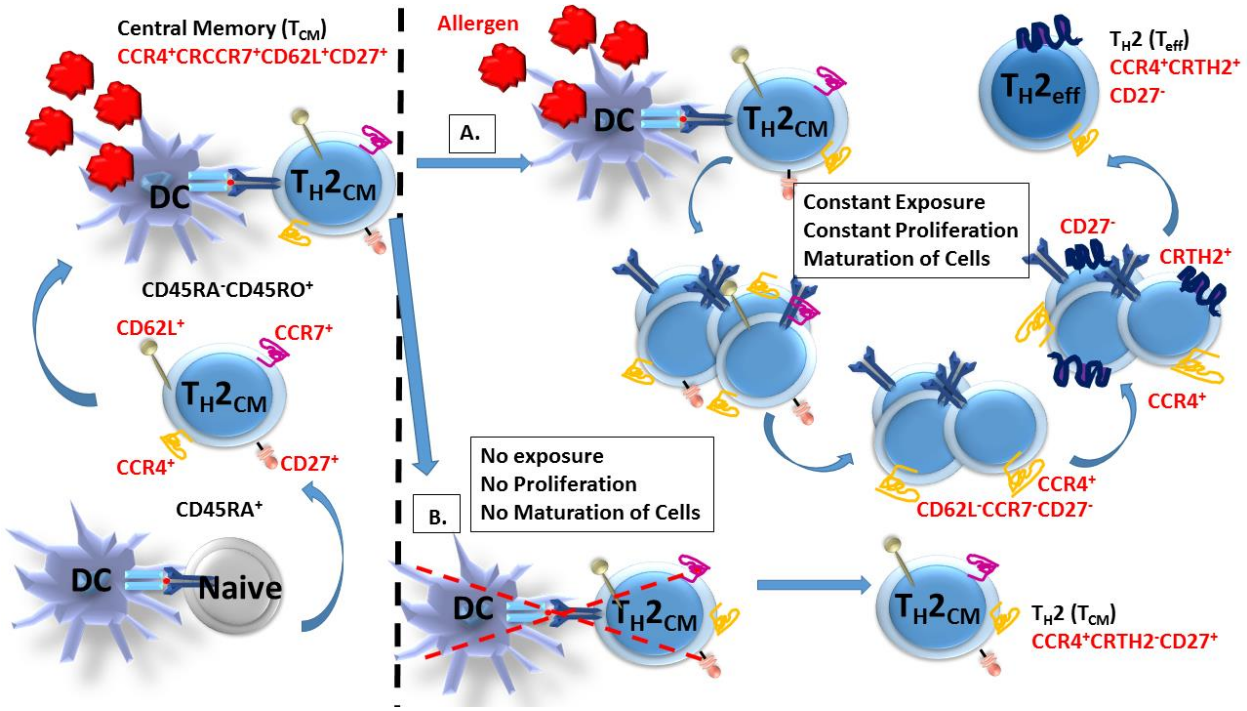

Figure XXXVI. Maturation of T_H2 cells. In a T_H2 milieu the immunological synapse commits Naïve T cells into Pre T_H2 lineage, characterized by the expression of CCR4 and Central Memory surface receptors ($CCR7^+CD62L^+CD27^+$). Two different scenarios are then possible. **A.** Constant allergen exposure (Airborne or Perennial allergens), promotes the proliferation and initiation of maturation on T_H2 cells ($CCR4^+$). It is probable that CCR7 and CD62L expression are lost in the first differentiation stages, as homing to the secondary lymphoid organs is no longer required. Constant allergen exposure commits T cells into a T_{eff} phenotype ($CRTH2^+CD27^-$). This process is essential for the evolution of an effective immune response because memory cells acquire altered capacity to produce cytokines, different migratory capability and, diminish activation requirements on rechallenge with allergen **[Fritsch, et al., 2005]**. **B.** Allergen avoidance results in the accumulation of $CRTH2^-$ T cells in blood of allergic subjects.

It has been demonstrated that IL-17A can promote class switch to IgE **[Milovanovic, et al., 2010]** and IL-17A producing $CD4^+$ T cells are more frequent in allergic subjects **[Milovanovic, et al., 2010] [Zhao, et al., 2010] [Ciprandi, et al., 2010]**. T_H2/T_H17 cells are also observed in subjects with allergic asthma **[Cosmi, et al., 2010] [Wang, et al., 2010] [Irvin, et al., 2014]**. However, the involvement of T_H17 cells in food allergy remains obscure. In the current study a sub-population of $CCR4^+CCR6^+$ cashew and walnut- T-cells which produced IL-17A alone or IL-4 and IL-17A were detected in both asthmatic and non-asthmatic cashew and walnut allergic individuals. These results are consistent with previous studies with food allergens **[DeLong, et al., 2011] [Renand, et al., 2014]**, where T_H2/T_H17 allergen-specific T-cells have been previously described. This data implicates a direct association of $CCR4^+CCR6^+$ antigen specific $CD4^+$ T_H2/T_H17 cells with food allergy disregarding the asthmatic status (**Figure XXXVII**). Compared with peanut allergy, tree nut allergy, especially cashew, causes more gastro-intestinal symptoms **[Grigg, et al., 2009]**. Indeed these symptoms may be attributed to the presence of $CCR6^+\beta7^+CCR7^+$ T-cells as they have the capacity to home to the GALT **[Ito, et al., 2011] [Kunkel, et al., 2003] [Campbell, et al., 2002] [Forster, et al., 1999]** and $CCR6^+$ T-cells play a role in gastrointestinal allergic disease, as shown in a murine model **[Blazquez, et al., 2010]**. On the other hand, Dhuban et al recently suggested that T_H17 responses are impaired in food allergic children and that lack of T_H17 responses may play a potential role in food tolerance **[Dhuban, et al., 2013]**. The presence of this population in allergic subjects raises important questions of the pathophysiological role of these $CCR4^+CCR6^+$ food allergen specific $CD4^+$ T-cells in food allergy in general. Future effort should commit to examine whether $CCR4^+CCR6^+$ allergen specific cells are more prevalent in food allergy compared to airborne allergy.

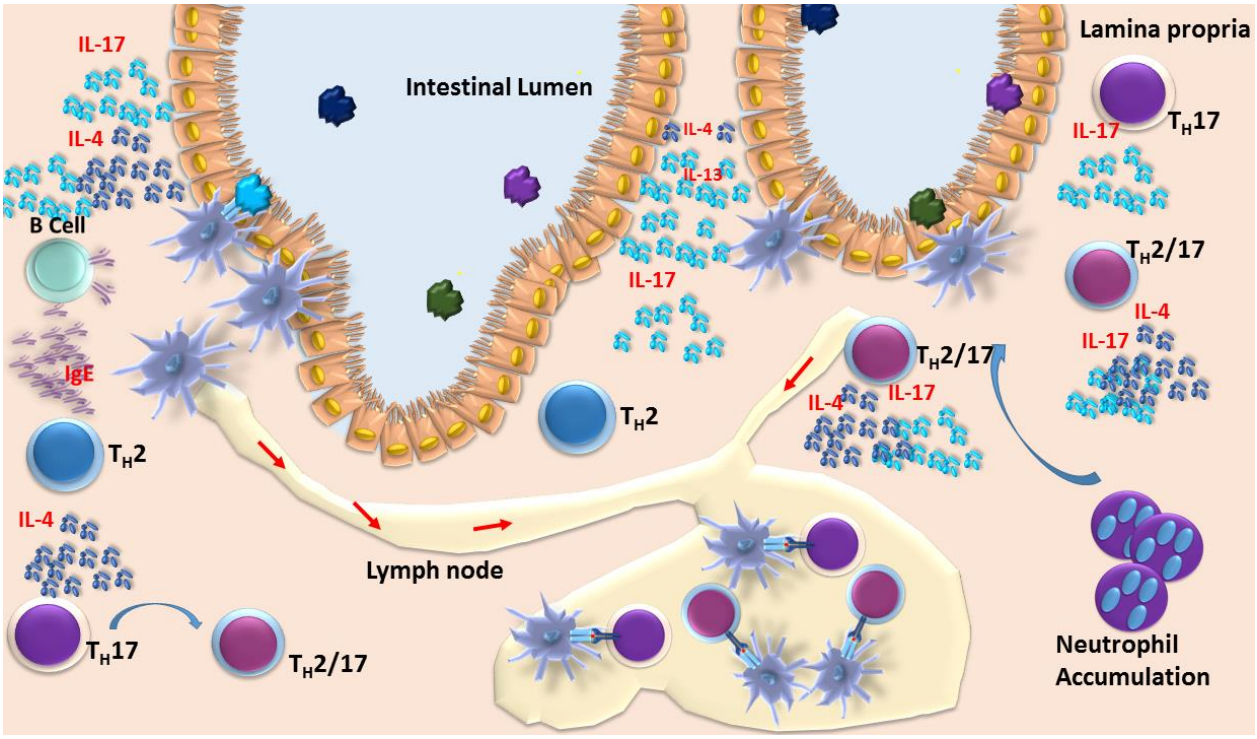

Figure XXXVII. Proposed biological mechanisms in food allergen mediated responses in the gut system. Presence of T_H2/T_H17 and T_H17 subsets raises important questions of their role in food allergy. A combination of different subsets may play a role in disease. T_H2_{CM} cells can migrate to secondary lymphoid organs, produce T_H2 cytokines and promote specific IgE production by B cells. T_H17 that recognize food derived epitopes can generate gastrointestinal symptoms. Rich IL-4 cytokine environments generate plasticity of T_H17 to co-produce IL-4 and IL-17. Finally, IL-17 can promote robust production of IgE in IL-4 rich environments **[Milovanovic, et al., 2010]**.

E. Ending remarks and future perspectives

Current outbreak in allergic disease over recent years has coincided with progressive changes in our lifestyles (including hygiene, excessive antibiotic use and dietary change). The hygiene hypothesis, suggested that microbial exposure in early childhood is crucial for normal development of the immune system **[Strachan, 1989]**. This hypothesis was revised by Wold in 1998. He proposed that lack of early colonization of microbiota in the gut has the potential to influence the risk of allergic disease **[Wold, 1998]**. A mutualistic relationship between microbes and the immune system has played a critical role in normal immune development and regulation **[West, et al., 2014]**. Lack of regulatory responses may promote chronic inflammatory disease. Immune dysregulation has been clearly related to the failure in the colonization of microbes in the GALT of mouse models **[Sudo, et al., 1997]**. Recent reports showed that infant microbiomes have lower species richness than adults. Indeed, recent studies have shown lower abundance of *Bacteroides* and *Clostridium* in infants with food allergy **[Tsuji, et al., 2012] [Penders, et al., 2013] [Azad, et al., 2013] [Jakobsson, et al., 2014] [Yang, et al., 2014]**. Interestingly, it has been reported that sensitization to a food allergen is increased in mice that have been treated with antibiotics or are devoid of commensal microbiota **[Stefka, et al., 2014]**. Furthermore, IL-17 production was abolished in mice that were treated with large-spectrum antibiotics, suggesting that their differentiation depends on foreign antigens provided by the gut microflora **[Lemaire, et al., 2011]**. T_H17 cells have critical roles in mucosal defense and are most abundant in the small intestine lamina propria **[Atarashi, et al., 2013]**. Moreover, a recent study showed that intestinal antigen-specific $CD4^+$ T cells can differentiate to become T_H17 cells, depending on cognate bacterial antigen delivery **[Yang, et al., 2014]**.

In the current work presented in this thesis, we were able to detect food allergen specific T_H17 cells that expressed GALT and secondary lymphoid organ homing markers ($CCR6^+\beta7^+CCR7^+$) **[Ito, et al., 2011] [Kunkel, et al., 2003] [Campbell, et al., 2002] [Forster, et al., 1999]**. In addition, we were also able to detect T_H2/T_H17 responses in food allergic individuals. Both T_H17 and T_H2/T_H17 have been related to

asthma severity and autoimmunity **[West, et al., 2014]**. Celiac disease is a disorder characterized by a deregulated immune response to ingested wheat gluten (Gliadin) and related cereal proteins in susceptible individuals **[Fernández, et al., 2011]**. Interestingly, it has been shown that Gliadin-specific T_H17 cells are present in the mucosa of celiac disease patients and play a role in the pathogenesis of the disease **[Fernández, et al., 2011]**. In regard to this statement, our current observations acknowledges the fact that this T cell subset may play a role in food allergy.

Moreover, it is possible that due to the lack of microbiota stimulation in early childhood and/or at precise moments in growth, T_H17 cell responses do not benefit from this mutualistic interaction that generates regulation in the gut. Naturally, the immune system of the allergic host requires to maintain homeostasis of different T cell subsets. Thus, it is possible that T_H17 cells target food allergens in the absence of particular microbiota antigens to maintain immune balance within the gut. Furthermore, it is possible that regulation can be restored with the introduction of microbiota in different stages of growth in children. Indeed, milk and egg allergy can be outgrown **[Berin, et al., 2013]**. In vivo oral milk challenges increased the number of regulatory T cells in milk-tolerant but not milk reactive children, suggesting a role for regulatory T cells in the natural outgrowth of milk allergy **[Karlsson, et al., 2004]**.

In the introduction section of this work, we talked about the plasticity of T_H17 responses. T cells can differentiate into virtually all different types of effector, memory or regulatory cells depending on the cytokine environment and nature of the epitopes, as TCR signaling can affect the outcome of the cascade of events that lead to a particular phenotype **[Gabrysova, et al., 2010]**. Indeed, Cosmi et al. first described T_H2/T_H17 cells that can co-produce IL-17 and IL-4 in IL-4 rich cytokine environments **[Cosmi, et al., 2010]**. We also discussed how different symptoms often appear in a particular order and often progress during childhood. Interestingly, it has been suggested that the first symptom in "the allergic march" may be the appearance of eczema, which may then develop to food allergy, rhinitis and finally asthma **[Saarinen, et al., 2005]**. Although this pattern of progression does not apply to every child it provides

insights on how the evolution of disease may occur. It has been suggested that skin lesions of patients with atopic dermatitis promote the expression of IL-25, IL-33 and TSLP **[Saenz, et al., 2008]**. TSLP is considered as the master switch for allergic inflammation as it triggers DC-mediated T_H2 inflammatory responses while IL-25 and IL-33 promotes a T_H2 milieu **[Saenz, et al., 2008]**. T_H2 rich environments in the absence of microbiota in the gut may play a role in the plasticity of T_H17 cells. It is possible that due to the nature of the allergen, cytokine environment, and co-stimulation signals in the absence of regulation promote the generation of a first stage of plasticity (T_H2/T_H17). The lack of regulation of T_H2/T_H17 responses and the fact that these cells have TCRs that can recognize foreign allergens may promote another plasticity event that may lead to the generation of a pathogenic T_H2 phenotype in food allergy (**Figure XXXVIII**). Indeed, it has been shown that effector T_H2 cells, those that express CRTH2, can produce IL-17 and promote the exacerbation of chronic allergic asthma **[Wang, et al., 2010]**. Further effort should commit to answer this question utilizing mouse models. Another important question to address is whether commensal microbiota share cross-reactive epitopes with tree nut allergens, it would be interesting to assess the proliferation capacities of $CCR4^+CCR6^+$ T cells against *Clostridium* and *Bacteroides*.

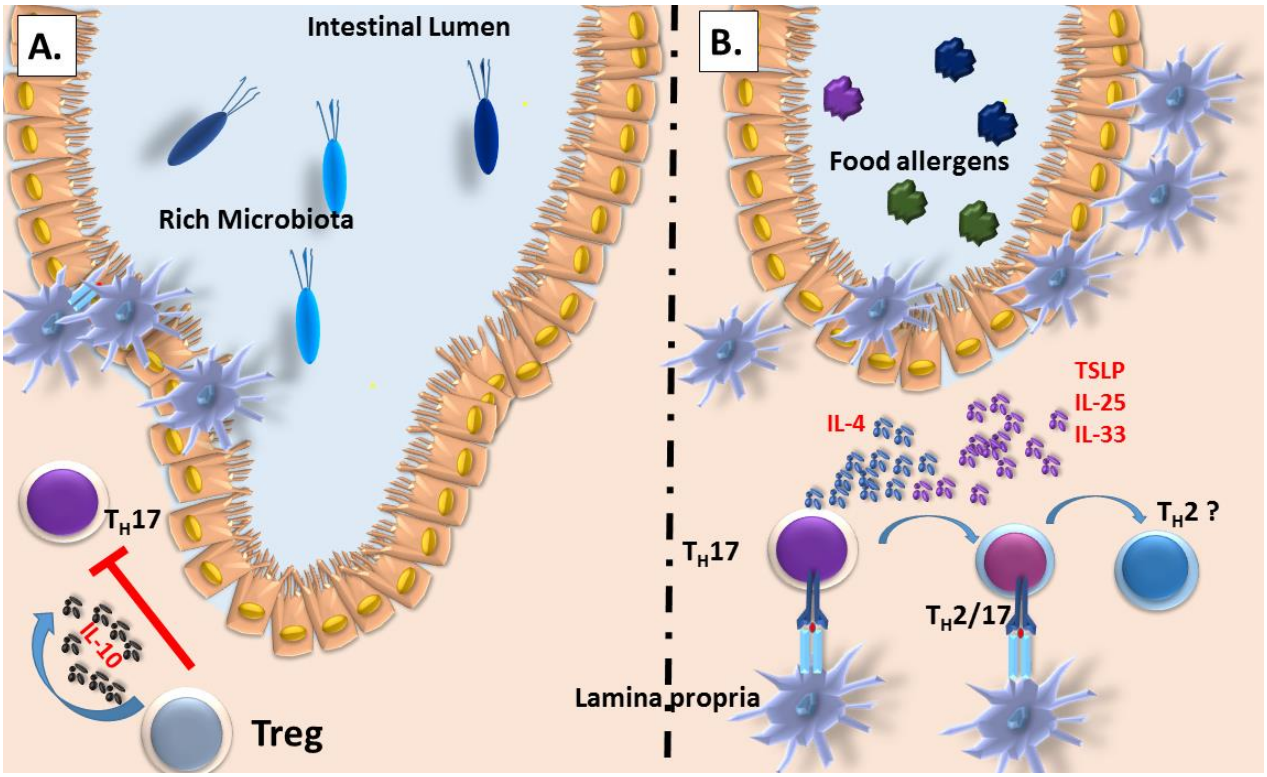

Figure XXXVIII. Hypothesis of the generation of pathological T_H17 responses in food allergy. **A.** A mutualistic relationship between microbes and the immune system plays a critical role in immune regulation in the gut. **B.** Lack of microbiota stimulation in early childhood and/or at precise moments in growth does not promote this mutualistic interaction that generates regulation in the gut. Furthermore, T_H2 cytokine milieu promotes the plasticity of T_H17 subset that can recognize food allergens into T_H2/T_H17 and possibly into T_H2 cells.

Part V.

Conclusions

A. Identification of novel epitopes

- We identify novel Jug r 2-, Ana o 1- and Ana o 2-specific $CD4^+$ T cell epitopes. Hot spots with promiscuous Jug r 2 $CD4^+$ T cell peptides presented by multiple *DRB1* alleles were also defined.
- Potential cross-reactive epitopes for other species can be identified by selecting sequences homologous to the particular allergen-derived epitopes using Blast alignments. (e.g. Phl p derived epitopes and homologous epitopes for other Pooideae grasses).

B. Cross reactivity in grass pollen and tree nut allergens: implications in immunotherapy

- Subjects that were recruited on the basis of Timothy grass pollen (for grass pollen allergy) and Cashew (tree nut allergy) allergy had basophil responses for other phylogenetically related species components.
- Cross-reactive and non-cross reactive $CD4^+$ T cell epitopes were identified in both models, grass pollen allergens from the Pooideae subfamily and tree nut allergens.
- High sequence homology does not imply cross-reactivity. Variability at sites of T cell contact can disrupt pMHC-II-TCR interaction.

I. Grass-specific $CD4^+$ T cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy

- Poa p 1 $_{97\text{-}116}$, Lol p 1 $_{221\text{-}240}$, Lol p 5a $_{167\text{-}186,}$ Hor v 5a $_{167\text{-}186}$, Hol l 5a $_{167\text{-}186}$, Poa p 5a $_{199\text{-}218}$, Lol p 5a $_{199\text{-}218}$ for DRB1*04:01, and Dac g 5b $_{89\text{-}108}$ for DRB1*07:01 were identified as minimally cross reactive T cell epitopes as they did not cross-recognize the Phl p1 and Phl p 5 epitopes in the corresponding regions.
- The presence of minimally cross reactive T cells with comparable frequency, phenotype and functionality to Phl p specific T cells in subjects recruited on the basis of Timothy grass pollen allergy, suggest that a multiple allergen system should be considered for immunotherapy instead of a mono-allergen system.

II. Ana o 1 and Ana o 2 cashew allergens share cross-reactive $CD4^+$ T-cell epitopes with other tree nuts

- Our observations infer that phylogenetic relatedness among tree nuts reflects cross-reactivity as we observed that most of cashew derived T-cell epitopes cross-reacted with pistachio epitopes.
- Although cashew and other tree nut allergens do not share high sequence homology, cross-reactive epitopes were identified in distantly related species (e.g. hazelnut: Cor a 11 $_{281\text{-}300}$, Cor a 9 $_{233\text{-}252}$, Cor a 9 $_{297\text{-}316}$, Cor a 9 $_{329\text{-}348}$ cross-recognize the Ana o 1 and Ana o 2 epitopes in the corresponding regions), and these cross-reactive epitopes also elicit identical cytokine profiles.
- Although no T cell cross-reactivity was observed between walnut and cashew, we were still able to detect walnut reactive $CD4^+$ T cells in cashew allergic subjects. These results suggested that these subjects are sensitized to both cashew and walnut and their respective allergen-reactive T cells account to different populations of T cells.

C. Characterization of tree nut allergen-specific $CD4^+$ T cells

- Jug r 2, Ana o 1 and Ana o 2 specific responses dominate walnut and cashew T-cell responses in subjects with walnut and cashew allergy, respectively.
- In non-allergic subjects, Jug r 2-, Ana o 1- and Ana o 2-reactive T cells were detected at substantially lower frequencies than allergic subjects. In addition, surface phenotypes and functional properties of these T cells are distinct in non-allergic and allergic subjects.
- Cashew- and Walnut-specific T-cells with T_H2, and sub-populations with T_H17 and T_H2/T_H17 phenotypes could be detected in allergic subjects, irrespective of their asthmatic status.
- Cashew- and Walnut- specific central memory $CD4^+$ cells and terminal effector T-cells were detected in peripheral blood with the central memory phenotype as the most prevalent phenotype.
- Gastrointestinal symptoms may be attributed to the presence of $CCR6^+\beta7^+CCR7^+$ T-cells as they have the capacity to home to the GALT.

D. Ending remarks and future perspectives

- Lack of microbiota stimulation in early childhood and/or at precise moments in growth may disrupt the regulation in the gut and promote T cell responses to food allergens. Moreover, T_H2 cytokine milieu may promote the plasticity of T_H17 subset that can recognize food allergens into T_H2/T_H17 and possibly into T_H2 cells.

Part VI.

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Part VII.

Appendix

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ORIGINAL ARTICLE **Allergens**

Grass-specific CD4$^+$ T-cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy

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Clinical & Experimental Allergy

Summary

Background Conceptually, allergic responses may involve cross-reactivity by antibodies or T-cells. While IgE cross-reactivity among grass-pollen allergens has been observed, cross-reactivity at the allergen-specific T-cell level has been less documented. Identification of the patterns of cross-reactivity may improve our understanding, allowing optimization of better immunotherapy strategies.

Objectives We use *Phleum pratense* as model for the studying of cross-reactivity at the allergen-specific CD4$^+$ T cell level among DR04:01 restricted Pooideae grass-pollen T-cell epitopes.

Methods After *in vitro* culture of blood mono-nucleated cells from grass-pollen-allergic subjects with specific Pooideae antigenic epitopes, dual tetramer staining with APC-labelled DR04:01/*Phleum pratense* tetramers and PE-labelled DR04:01/Pooideae grass homolog tetramers was assessed to identify cross-reactivity among allergen-specific DR04:01-restricted T-cells in six subjects. Direct *ex vivo* staining enabled the comparison of frequency and phenotype of different Pooideae grass-pollen reactive T-cells. Intracellular cytokine staining (ICS) assays were also used to examine phenotypes of these T-cells.

Results T-cells with various degrees of cross-reactive profiles could be detected. Poa p 1 $_{97–116}$, Lol p 1 $_{221–240}$, Lol p 5a $_{199–218}$, and Poa p 5a $_{199–218}$ were identified as minimally cross-reactive T-cell epitopes that do not show cross-reactivity to Phl p 1 and Phl p 5a epitopes. *Ex vivo* tetramer staining assays demonstrated T-cells that recognized these minimally cross-reactive T-cell epitopes are present in Grass-pollen-allergic subjects.

Conclusions Our results suggest that not all Pooideae grass epitopes with sequence homology are cross-reactive. Non-cross-reactive T-cells with comparable frequency, phenotype and functionality to Phl p-specific T-cells suggest that a multiple allergen system should be considered for immunotherapy instead of a mono-allergen system.

Keywords allergy, CD4$^+$, cross-reactivity, epitopes, grass-pollen, MHC class II tetramers, Pooideae, T-cells

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Introduction

In atopic individuals, exposure to allergens from taxonomically related species plays an important role in eliciting and maintaining clinical symptoms [1, 2]. Structural similarities among proteins derived from these species allow humoral and cell-mediated immunity to target homologous regions that originally did not serve as the source of sensitization [1, 2]. An example is found among grass-pollens from the Pooideae subfamily. Pooideae grasses coexist geographically and share pollination periods; therefore, allergic subjects in temperate zones are poly-exposed and poly-sensitized to multiple pollens from this subfamily [3–6]. Due to their taxonomical relatedness, high amino acid sequence homology is found within clinically relevant major allergens in different Pooideae species: 90% for Group 1 (beta-expansions) and about 55–85% for Group 5 (Ribonucleases) allergens [2, 7–10]. Thus, cross-reactivity for both humoral and T-cell responses can be expected among allergens of grass-pollens [2, 7–10]. *Phleum pratense* (Timothy grass) has been

accounted as an index species in this group because it exhibits the most dominant epitope profile [3, 9, 11]. Several investigators have suggested that immunotherapy with this species alone is sufficient to cover other species due to observed cross-reactivity at the IgE level [3, 9, 11]. On the other hand, it is now firmly established that allergen-specific T-cells play an important role in allergic inflammation [12] and that induction of antigen-specific Treg or elimination of allergen-specific T_H2 cells might be a prerequisite for the induction of specific tolerance [13]. Yet, evaluation of cross-reactivity at the T-cell level has been less documented. Some studies advocate that there are cross-reacting and non-cross-reacting T-cell epitopes for both major allergens [14, 15]. In this study, we determined the patterns of cross-reactivity of $CD4^+$ T-cells specific for homologous Pooideae grass-pollen epitopes derived from Timothy grass against Kentucky, Orchard, Rye, Velvet, Barley, and Canary grass. We determined whether grass-pollen-allergic subjects that were diagnosed based upon IgE reactivity to Timothy grass-pollen (TGP) extract were also sensitized to other related grass species at the T-cell level. The implications of our findings and the choices of using a single extract verses multiple extracts in immunotherapy will be discussed.

Materials and methods

Human subjects

Subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute. All subjects were recruited with informed consent and institutional review board approval (IRB title 'Allergen and T-cell reagent resources for the study of allergic diseases', Approval number IRB7109). A total of 6 DR04:01, 2 DR07:01, and 2 DRB5*01:01 grass-pollen (GP) allergic patients, diagnosed upon an ImmunoCAP score for TGP extract of ≥ 3 (Phadia AB, Uppsala, Sweden), were recruited. DNA samples were HLA-typed using Dynal Unitray™ SSP Kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The attributes of these human subjects are summarized in Table S1.

Basophil stimulation tests

Basophil activation was measured as previously described [16]. Briefly, heparinized whole blood from TGP allergic subjects was incubated with pollen extract from different grass species (2 μg/mL): Timothy grass (*Phleum pratense,* Phl p), Velvet grass (*Holcus lanatus*, Hol l), Canary grass (*Phalaris aquatica,* Pha a), Barley grass (*Hordeum vulgare*, Hor v), Rye grass (*Lolium perenne*, Lol p), Orchard grass (*Dactylis glomerata*, Dac g), and Kentucky grass (*Poa pratensis*, Poa p) (Greer Laboratories, Lenoir, NC, USA). Pollen extracts are sterile solutions containing the extractables of pollens (20 000 BAU/mL), 0.5% Sodium Chloride, 0.275% Sodium Bicarbonate, and 50% Glycerine by volume as preservative. The content of group 1 and group 5 of the extracts can be found in [17], the extract contains isoforms for both groups), and simultaneously stained with anti-CD3 (eBioscience, San Diego, CA, USA), anti-CD203 (Beckman Coulter, Pasadena, CA, USA), anti-CRTH2 (BD Biosciences, Franklin Lakes, NJ, USA) for 25 min at 37°C. Basophils were identified as $CD3^-CRTH2^+$, and activation status was assessed following the detection of CD203c in the presence of the allergens tested [16]. Whole blood stimulated with buffer without allergen and a mixture of grass-pollen extract were used as negative and positive controls, respectively.

Peptide-binding assays

Peptide-binding assays were measured as previously described [18]. Briefly, non-biotinylated target peptides were incubated with DR04:01 protein at final concentrations ranging from 0.01 to 10 μM for 1 h at 37°C, followed by additional 16-h incubation in the presence of 0.01 μM biotinylated reference peptide (Influenza $HA_{306–318}$, PKYVKQNTLKLAT). The binding reaction was stopped by adding an equal volume of 50 mM Tris-Cl buffer (pH 8.0). The DR04:01 molecules were then immobilized on 96-well plates coated with anti-HLA-DR monoclonal antibody (L243). The amount of biotinylated reference peptide-bound to DR04:01 was quantified using a europium streptavidin detection system on a Victor 2 microtiter plate reader (Perkin-Elmer, Waltham, MA, USA). The concentrations of target peptides required to inhibit 50% of maximal biotinylated reference peptide binding were retrieved from regression curves fitted by a sigmoidal dose–response equation provided by Prism software (GraphPad, San Diego, CA, USA). Relative binding was calculated using Phl p peptides as reference.

Dual tetramer staining

$CD4^+$ T-cells (5×10^6) were stimulated for 2 weeks *in vitro* with homologous grass-pollen antigenic epitopes (20-mer for Group 1 or 13-mer for Group 5a); cultures were then co-stained with allophycocyanin (APC)-conjugated pMHC II tetramers loaded with TGP-derived peptides (Phl p 1 or Phl p 5a peptides), and phycoerythrin (PE) labelled tetramer with homologous grass-pollen peptides at 37°C for 1 h. FITC-conjugated anti-CD4 (eBioscience) was then added to the cell suspension for a 20-min incubation at 4°C. Cells were analysed by flow cytometry. Data were analysed uti-

lizing FlowJo (Tree Star, Ashland, OR, USA); cells were gated on $CD4^+$ and PE-$tetramer^+$ subsets. The average of cross-reactive T-cells was calculated utilizing the percentage of co-stained T-cell populations divided by the total of $tetramer^+$-stained T-cells. $Tetramer^+$ T-cells showed three different cross-staining patterns: co-staining of greater than 85% was arbitrarily defined as full-scale cross-reactivity; T-cells that have 25–85% co-staining were designated as partially cross-reactive T-cells; and T-cells that showed co-staining of lower than 25% were designated as minimally cross-reactive T-cells. We defined epitope regions that elicit full-scale cross-reactivity as full-scale cross-reactive T-cell epitopes, partially cross-reactive T-cells as partially cross-reactive T-cell epitopes, and minimally cross-reactive T-cells as minimally cross-reactive T-cell epitopes.

Ex vivo tetramer staining to determine the frequency of Phl p- and grass homolog-specific $CD4^+$ T-cells

The frequency of Phl p 1- and Phl p 5a-specific T-cells was measured as previously described [12]. Briefly, 30 million peripheral blood mononuclear cell (PBMC) in 200 μL T-cell culture medium were stained with 20 μg/mL PE-labelled tetramers and/or APC-labelled tetramers (for *ex vivo* dual tetramer staining) for 100 min. Cells were then washed and incubated with anti-PE or anti-APC magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min at 4°C, and a 1/100 fraction was saved for analysis; the other fraction was passed through a magnetic column (Miltenyi Biotec). Bound PE- and/or APC-labelled cells were flushed and collected. Cells in the bound and pre-column fractions were stained with a panel of antibodies of interest for 20 min at room temperature. After staining, cells were stained with Via-$probe^+$ (BD Biosciences) for 10 min at 4°C before flow cytometry. Data were analysed utilizing FlowJo (Tree Star) gating on forward scatter/side scatter and excluding $CD14^+$, $CD19^+$, and Viaprobe populations. Frequency was calculated as previously described [12]. For phenotyping studies, antibodies were used against markers of interest; CCR4 (R&D systems, Minneapolis, MN, USA), CD45RA (eBioscience), and CD38 (eBioscience).

Intracellular cytokine staining

For intracellular staining of IFN-γ and IL-4, PBMC were stimulated for 2 weeks with specific peptides and stained with the corresponding PE-labelled tetramers for 30 min at 37°C. Cells were restimulated with 50 ng/mL phorbol 12-myristate-13-acetate and 1 mg/mL ionomycin in the presence of 1× brefeldin-A (eBioscience) for 5 h at 37°C, 5% CO_2. After restimulation, cells were stained with anti-CD4 (BD Biosciences) and anti-CD3 (eBioscience). After 10 min at room temperature, cells were then fixed with fixation buffer (eBioscience) and washed twice with a permeabilization buffer (eBioscience). Cells were then stained with a panel of antibodies directed against cytokines of interest, IFN-γ (Biolegend), IL-4 (eBioscience) for 20 min at room temperature; cells were washed and immediately analysed by flow cytometry. Cells were gated on CD4 and PE-tetramer subsets.

Results

GP allergic subjects have IgE sensitivity to different grass species

A total of 6 DR04:01 GP allergic subjects with an ImmunoCAP score of 3 or greater for TGP extract were recruited for this study (Table S1). DR04:01 was selected since it is the most frequent MHC class II allele found in our cohort of GP allergic subjects. Subjects were subsequently evaluated for IgE reactivity to five additional Pooideae grass-pollen extracts by using a basophil activation assay. All GP allergic individuals tested exhibited reactivity to all five additional grass species (Fig. 1). This confirms that allergic subjects sensitized to Phl p are also sensitized to other Pooideae species. However, due to significant homology at the conformational level among Pooideae allergens [2], the involvement of a specific grass species in symptom manifestation cannot be established.

Pooideae grass-pollen-specific $CD4^+$ T-cells exhibit varying degrees of cross-reactivity: High-sequence homology does not imply cross-reactivity

Previous studies in our laboratory have identified Phl p $1_{97–226}$ and Phl p $1_{221–240}$ (Phl p 1 is a Group 1 allergen), and Phl p $5a_{167–186}$ and Phl p $5a_{199–218}$ (Phl p 5a is a Group 5a allergen) as dominant DR04:01-restricted TGP T_H2 T-cell epitopes [19]. Since major allergens from different Pooideae species share high amino acid sequence homology (90% homology for Group 1 and 55–85% for Group 5 allergens) [2], T-cell cross-reactivity among these different allergens may be present. Potential epitopes for other Pooideae species were identified by selecting sequences homologous to the previously reported TGP-derived epitopes using Blast alignments [20] (Table 1). As shown in Table 1, the majority of Group 1 and Group 5a homologs from the different Pooideae species bound DR04:01 with binding affinities similar to the previously verified TGP-derived epitopes. However, we observed that, Group 1 homolog Dac g $1_{221–240}$ along with Group 5 homologs, Hol l $5a_{199–218}$, Dac g $5a_{199–218}$, and Poa p $5a_{199–218}$ bound with much higher affinity than their Phl p counterpart (10 to 15-fold higher). These data suggest that all homologous peptides could be presented by DR04:01 as plausible DR04:01 restricted T-cell epitopes.

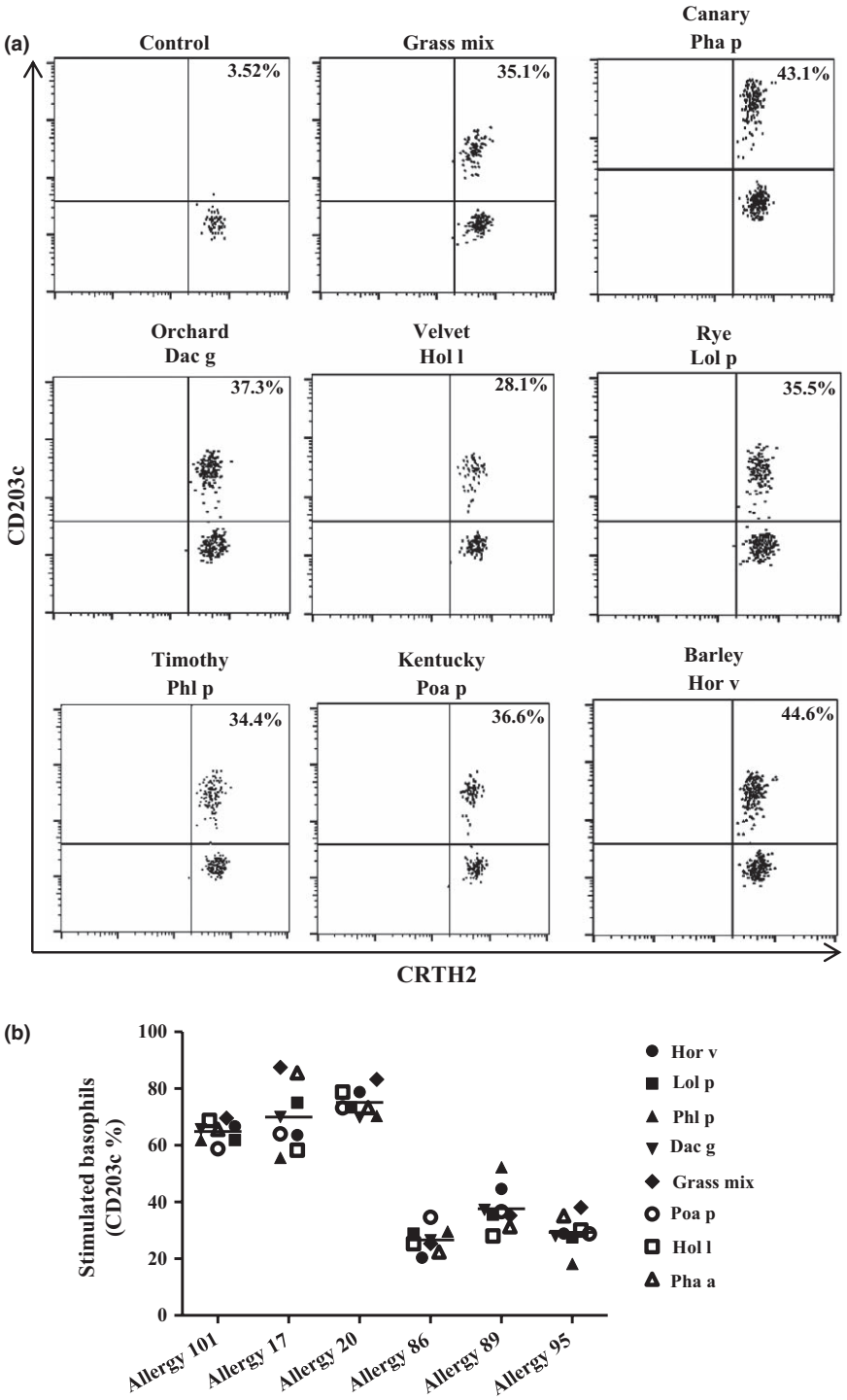

Fig. 1. Subjects allergic to TGP have IgE sensitivity to different grass species. Basophils were stimulated with pollen extracts from six different Pooideae species or a mix containing grass-pollen from all species (Greer Laboratories) and were stained simultaneously with a panel of antibodies of interest. Upregulation of CD203c indicated activation of basophils. (a) Representative result for Allergy #89. (b) Percentages of CD203c on stimulated basophils are summarized for each DR04:01 allergic subject.

PBMC from GP allergic subjects were then stimulated with the Pooideae homologs (other than Phl p peptides). T-cell cross-reactivity was evaluated by co-staining assays utilizing pMHC II tetramers loaded with TGP-derived peptides (Phl p 1 or Phl p 5a peptides) and tetramers with homologous grass-pollen peptides corresponding to the stimulating epitope (Figs 2a, b and c top panels). Three different cross-staining patterns were observed: (i)

Table 1. Phl p and grass homolog $CD4^+$ T-cell epitopes

Group allergen*	Grass species†	Amino acid sequence‡	IC_{50} (μM)§	RBA¶
Group 1 0102 $_{97–116}$	Phl p 1	EEPIAPYHFDLSGHAFGAMA	0.274590811	1
	Lol p 1	EEPIAPYHFDLSGHAFGSMA	0.435355464	0.63072784
	Pha a 1	EEPIAPYHFDLSGHAFGSMA	0.435355464	0.63072784
	Poa p 1	EEPIAAYHFDLSGKAFGAMA	0.826658242	0.33216969
Group 1 0102 $_{221–240}$	Phl p 1	TEAEDVIPEGWKADTSYESK	2.921366199	1
	Lol p 1	SEFEDVIPEGWKADTSYSAK	2.921366199	1
	Poa p 1	GEAEDVIPEGWKADTAYASK	0.453158364	6.44667832
	Dac g 1	SEVEDVIPEGWKADTSYEAK	0.176697335	16.533165
Group 5 0101 $_{167–186}$	Phl p 5a	AAFKVAATAANAA	0.215901172	1
	Hor v 5a	AAFRTAATAADAA	0.970389302	0.22248923
	Hol l 5a	AAFRIAATAANAA	0.116004811	1.86113982
	Lol p 5a	AAYRTAATAANAA	0.531949004	0.40586818
	Pha a 5a	AAFKIAATAANSA	0.38603706	0.55927576
	Poa p 5a	AAFKVAATAANAA	0.05	4.31802348
	Dac g 5a	AAYKIAATAANAA	0.05	4.31802348
Group 5 0101 $_{199–218}$	Phl p 5a	ESYKFIPALEAAV	0.970389302	1
	Hor v 5a	ESYKFIPALEAAV	0.990031471	0.98016006
	Hol l 5a	EAYKFIPSLETAV	0.104945543	9.24659849
	Lol p 5a	DSYKFIPTLVAAV	0.409955767	2.36705855
	Pha a 5a	ETYKFIPSLEAAV	0.051012076	19.0227368
	Poa p 5a	DTYKSIPSLEAAV	0.061093499	15.8836753
	Dac g 5a	ESYKFIPTLEAAV	0.074648485	12.9994507

*Two distinct regions were used for Group 1 (20-mer) and Group 5a (13-mer) major allergens; Major allergen Phl p 5 consists of two isoforms, Phl p 5a and 5b.

†Peptides for different grass species were used.

‡Homologous sequences of amino acids for each epitope region. Bold letters variant residues. Underlined amino acids depict DRB1*0401 motifs P1, P4, P6, P7, and P9.

§IC_{50}, concentration required by a target peptide to inhibit 50% binding of the reference peptide.

¶RBA, relative binding affinity (relative to that of the Phl p peptide).

Full-scale cross-reactivity (Fig. 2a); (ii) Partial cross-reactivity (Fig. 2b); and (iii) Minimal cross-reactivity (Fig. 2c). Even though Group 1 allergens share high-sequence similarity, homologous antigenic epitopes (Fig. 2d), Poa p 1 $_{97–116}$, and Lol p 1 $_{221–240}$ were identified as minimally cross-reactive T-cell epitopes. The majority of T-cells elicited by these epitopes did not cross-recognize the Phl p 1 epitopes in the corresponding region. On the other hand, along Group 5 epitopes the patterns of cross-reactivity were more diversified (Fig. 2e). Lol p 5a $_{167–186}$, Hor v 5a $_{167–186}$, Hol l 5a $_{167–186}$, Poa a 5a $_{199–218}$, and Lol p 5a $_{199–218}$-reactive T-cell populations co-stained less than 25%. The patterns of cross-reactivity were also evaluated by stimulating PBMC with Phl p epitopes, and subsequently staining with both Phl p tetramers and tetramers loaded with other homologous GP peptides (Fig. 2a, b and c bottom panels). The results were similar to those obtained by stimulating PBMC with homologous grass peptides (Fig. 2a, b and c top panels). The cross-reactive patterns for each epitope region are summarized in Table 2. These data suggest that the patterns of cross-reactivity among Pooideae GP homolog epitopes are diversified and that minimally cross-reactive T-cell epitopes are present among Group 1 and Group 5 allergens, meaning that high-sequence homology does not imply cross-reactivity.

Minimally cross-reactive $CD4^+$ T-cells can be detected in GP allergic subjects

On the basis of these findings, we asked whether T-cells that recognize the minimally cross-reactive T-cell epitopes of the different Pooideae species can be detected directly *ex vivo* in PBMC of DR04:01 GP allergic subjects without an amplification step. *Ex vivo* frequencies of minimally cross-reactive T-cell epitopes of Poa p 1 and Lol p 1 were compared with its counterpart Phl p 1. Interestingly, for TGP-derived epitope region $_{97–116}$ in Group 1 (Fig. 3), both Phl p 1 and Poa p 1 were identified as the immunodominant epitopes (frequency ranged from 40 to 65 T-cells per million), while for TGP-derived region $_{221–240}$, low frequency was observed for both Phl p 1 and Lol p 1 (frequency ranged from 2 to 6 T-cells per million). For Group 5a allergens (Fig. 3), T-cell frequencies for TGP-derived region $_{199–218}$ for Phl p 5a, Lol

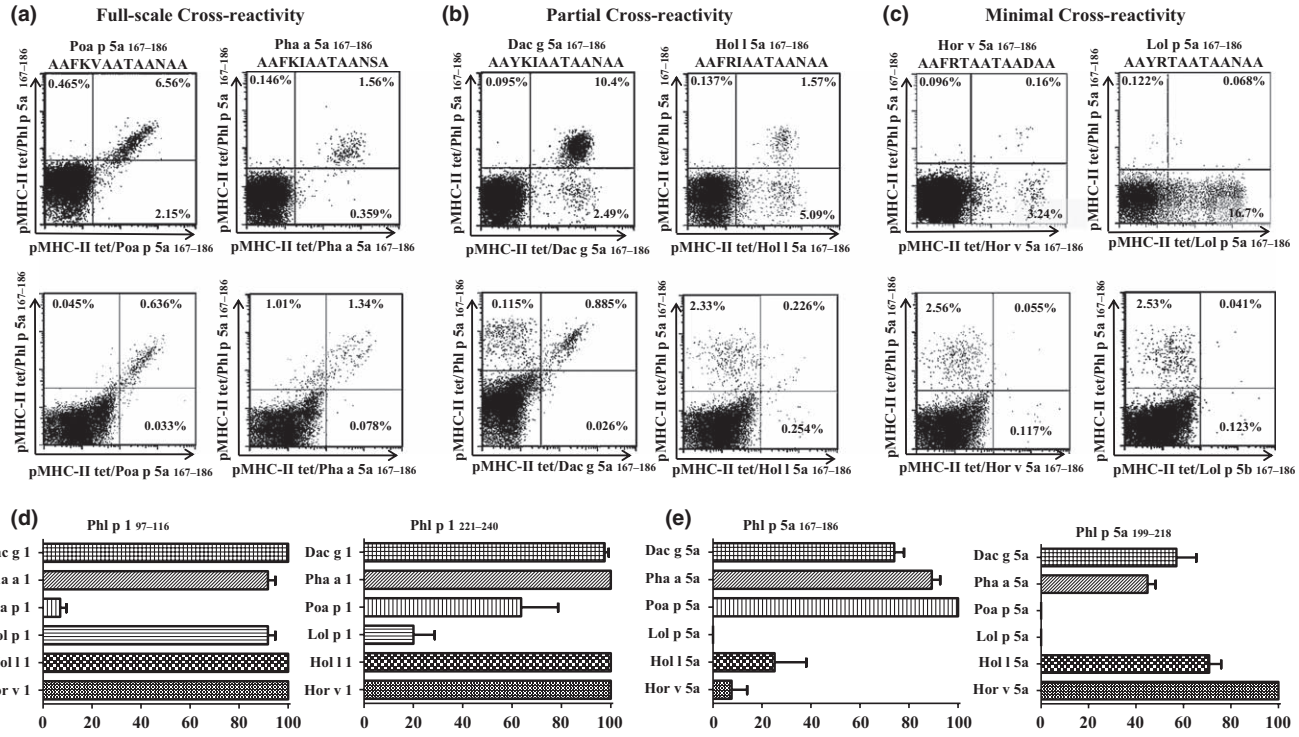

Fig. 2. Grass-specific CD4$^+$ T-cells exhibit varying degrees of cross-reactivity. After *in vitro* stimulation with Phl p homologous grass peptides [top panels of (a), (b) and (c), amino acid sequence of the stimulating peptide is specified on top of each panel] or Phl p $_{167–186}$ AAFKVAATAANAA, [bottom panels of (a), (b) and (c)], cells were co-stained with APC-labelled DR04:01/Phl p grass peptide-loaded tetramers (*y* axis) and with PE-labelled DR04:01/homologous grass peptide-loaded tetramers corresponding to the stimulating epitopes (*x* axis). The plots in (a), (b) and (c) show representative results for different cross-reactivity profiles for the TGP-derived epitopes in the Group 5a $_{167–186}$ region. (a) Full-scale cross-reactivity was observed for Poa p 5a $_{167–186}$, and Pha a 5a $_{167–186}$. (b) Partial cross-reactivity was observed for Dac g 5a $_{167–186}$ and Hol 5a $_{167–186}$. (c) Minimal cross-reactivity was observed for Lol p 5a $_{167–186}$ and Hor v 5a $_{167–186}$. (d) Comparison of percentages of cross-reactive T-cells for Group 1 homologs. (e) Comparison of percentages of cross-reactive T-cells for Group 5a homologs. Summarized results from (n = 6) allergic subjects are presented for distinct antigenic epitope regions. Each bar depicts the average of dual tetramer-stained T-cells (cross-reactive) observed per epitope.

p 5a, and Poa p 5a were similar and ranged from 5 to 14 cells per million). Thus, T-cells from TGP-derived region $_{97–116}$ in Group 1 are found in higher frequencies ($P < 0.05$) than epitope-specific T-cells from TGP-derived regions $_{221–240}$ in Group 1 and TGP-derived region $_{199–218}$ in Group 5a allergens. These experiments show that subjects with GP allergy, diagnosed upon IgE reactivity to TGP extract are also sensitized to pollen from other Pooideae species at the T-cell level.

Minimally cross-reactive CD4$^+$ T-cells have a T_H2 phenotype and are functionally active during pollen season

To further characterize the phenotype of minimally cross-reactive T-cells, antibodies directed against CCR4 (TH2 marker), CD45RA (a naïve T-cell marker) and CD38 (an activation marker) were utilized with *ex vivo* tetramer staining. The majority of epitope-specific T-cells from Group 1 $_{97–116}$ region and Group 5a $_{199–218}$ region displayed a CCR4$^+$ and CD45RA$^-$ phenotype (Fig. 4), indicating these are memory T-cells with a T_H2 phenotype. However, a higher percentage of epitope-specific T-cells from Group 1 $_{221–240}$ region displayed a mixed CCR4$^+$ and CD45RA$^+$ phenotype (Fig. 4b). The activation status of these cells was also examined during pollen season by analysing CD38 expression on CD454RA-T-cell populations. These stainings show that substantial activation could be seen for all these T-cells (Figs 4b and c) and that there is no difference in CD38 expression among the different immunodominant TGP-derived epitopes.

Functional assays were performed to confirm that minimally cross-reactive epitope-specific T-cells belong to the T_H2-lineage (CCR4$^+$); cells that had been stimulated *in vitro* with peptides and cultured for 2 weeks were stained with tetramer and co-stained for cytokine expression. A T_H2-cytokine (IL-4) and a T_H1 cytokine (IFN-γ) were used to discern among both lineages. As presented in Fig. 5, tetramer-stained T-cells predominantly produced IL-4 with minimal production of IFN-γ. Overall, these data suggest that minimal cross-reactive T-cells are memory T_H2 cells and functionally, are equally potent as its Phl p

Table 2. Cross-reactivity between Phl p and grass homologous epitopes

Group allergen*	Cross-staining†	Full-scale cross-reactivity‡	Partial cross-reactivity‡	Minimal cross-reactivity‡
Group 1 $_{97–116}$	Pha a 1 and Phl p 1	X		X
	Poa p 1 and Phl p 1			
	Lol p 1 and Phl p 1	X		
Group 1 $_{221–240}$	Dac g 1 and Phl p 1	X		
	Poa p 1 and Phl p 1		X	
	Lol p 1 and Phl p 1			X
Group 5 $_{167–186}$	Dac g 5a and Phl p 5a		X	
	Pha a 5a and Phl p 5a	X		X
	Poa p 5a and Phl p 5a	X		
	Lol p 5a and Phl p 5a			X
	Hol l 5a and Phl p 5a		X	
	Hor v 5a and Phl p 5a			X
Group 5 $_{199–218}$	Dac g 5a and Phl p 5a		X	
	Pha a 5a and Phl p 5a		X	
	Poa p 5a and Phl p 5a			X
	Lol p 5a and Phl p 5a			X
	Hol l 5a and Phl p 5a		X	
	Hor v 5a and Phl p 5a	X		

*Group 1 refers to isoform 0102; Group 5a refers to isoform 0101; and 5b to isoform 0102.

†The first epitope listed is the stimulatory epitope; the second epitope listed is the cross-stained epitope.

‡Cross-reactivity assessed on the basis of tetramer co-staining experiments.

counterparts. These data suggest that allergic subjects may experience symptoms through exposure to Phl p or other Pooideae species during the pollen season.

Pooideae grass-specific CD4$^+$ T-cells have varying degrees of cross-reactivity that depend on the HLA-allele and epitope they recognize

To generalize our findings, T-cell cross-reactivity in other HLA-DR alleles was also investigated. Homologous epitopes for Dac g 5b were identified by selecting sequences homologous to previously immunodominant DR07:01 and DRB5*01:01 Phl p-specific epitopes identified in our laboratory [19]. For DR07:01, minimal cross-reactivity was observed between Dac g 5b $_{89–108}$ or Phl p 5b $_{89–108}$ T-cells (Fig. 6a). However, Dac g 5b-specific T-cell frequency was at least twofold higher in DR07:01 subjects in comparison with Phl p-specific T-cell frequency (Fig. 6b). In DRB5 subjects, full-scale cross-reactivity was observed between both homologous TGP-derived region $_{152–160}$ in Group 5b epitopes, and the T-cell frequency was approximately 10 cells per million (Figs 6c and d).

Discussion

Although the efficacy and safety of single grass-allergen (Phl p) immunotherapy has been documented [21, 22], the use of single or multiple allergen products for Pooideae grass-pollen immunotherapy is still debated [11, 23–25]. In the present study, we utilized a multiplex pMHCII tetramer staining approach to determine the patterns of cross-reactivity between TGP-derived epitopes and epitopes of pollens from multiple grass species within the Pooideae family.

Prior studies showed that Pooideae pollen extracts demonstrate significant IgE cross-reactivity [3–6, 9, 10, 26, 27]. In contrast, cross-reactivity at the T-cell level has not been thoroughly documented. Heijl et al. [11] showed both Phl p 1- and Phl p 5-specific T-cell lines (TCL) responded robustly to extract from other Pooideae species, suggesting cross-reactivity at the T-cell level. However, TCL being used were generated by pollen extract, and T-cells specific for minimally cross-reactive T-cell epitopes could be overlooked. In this present study, Phl p-specific CD4$^+$ T-cells in allergic individuals had a range of patterns of cross-reactivity against allergens from other Pooideae species. The cross-reactivity profile depended on the HLA-restriction and epitope involved. Consistent with previous studies [14, 15], our approach identified full-scale, partially and minimally cross-reactive T-cell epitopes for both Group 1 and 5 major allergens. The majority of T-cells specific for minimally cross-reactive epitopes did not cross-recognize the Phl p 1-derived epitopes in the corresponding regions. However, a minor population of T-cells, capable of cross-recognizing these epitopes was detected in some subjects, indicating that TCR diversity can be present in Pooideae grass-pollen-specific T-cells. This suggests that although high-sequence homology is

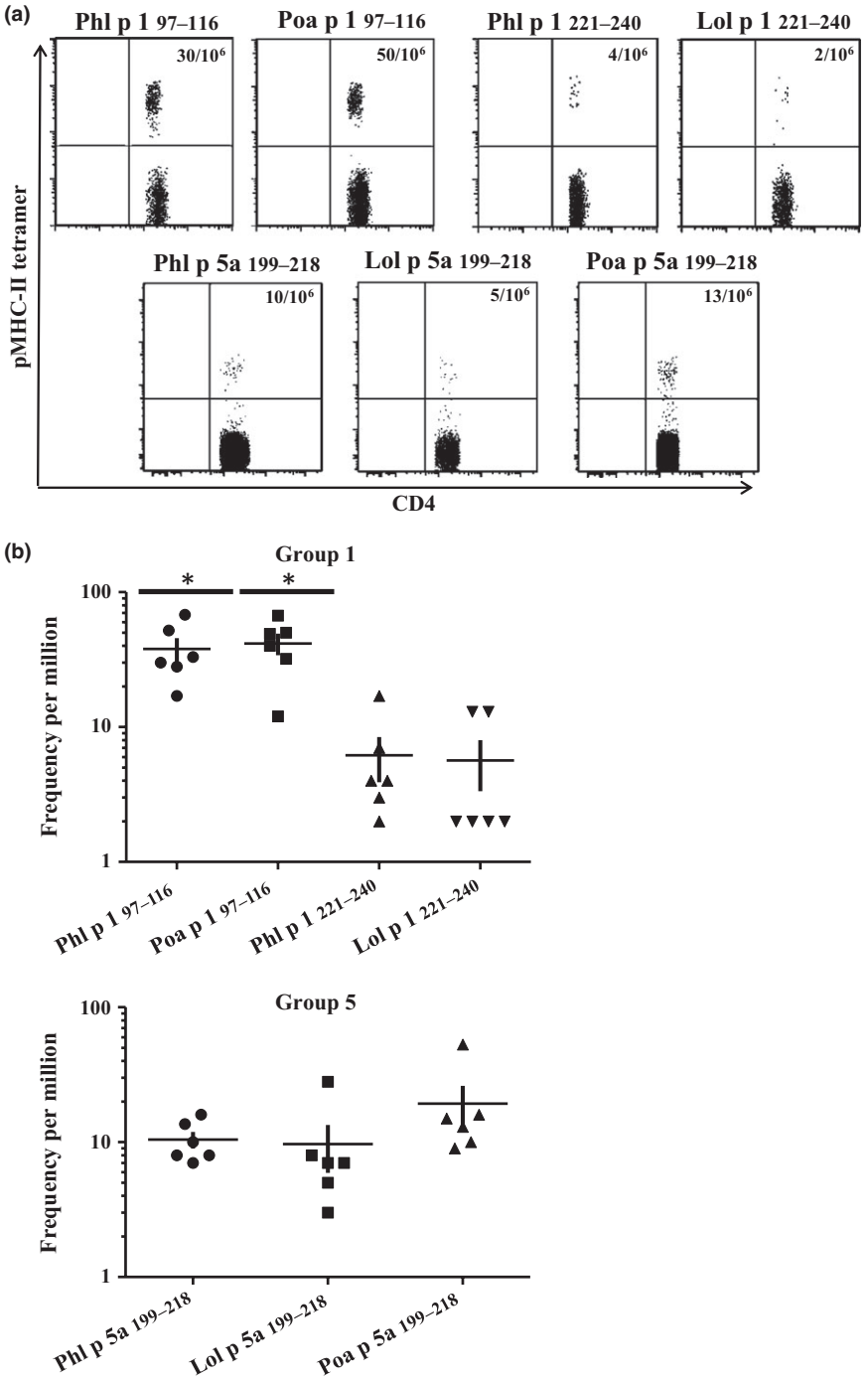

Fig. 3. Minimally cross-reactive $CD4^+$ T-cells can be detected *ex vivo* in TGP allergic subjects. Determination of frequencies of Phl p- and grass homolog-reacting $CD4^+$ T-cells for different regions of Group 1 and 5a allergens. (a) Example of *ex vivo* tetramer staining for TGP-derived Group $1_{97–116}$, Group $1_{221–240}$, and Group $5a_{97–116}$- Phl p-specific and homologous minimally cross-reactive $CD4^+$ T-cells from a grass-pollen-allergic subject with an ImmunoCAP score of 5 for Phl p-specific-IgE. (b) Frequencies for TGP-derived Group $1_{97–116}$ and Group $1_{221–240}$, and Group $5a_{199–218}$ grass homolog-reactive $CD4^+$ T-cells. Each data point denotes the frequency of epitope-specific $CD4^+$ T-cells for a different individual. A t student test was used in the statistical analysis.*$P < 0.05$ to compare populations from different epitopes.

found among grass-pollen allergens from the Pooideae subfamily, a single species (Timothy grass) does not cover the broad epitopic repertoire at the T-cell level. Interestingly, there is discordance in known cross-reactivity at the IgE level as compared to the T-cell level [3–6, 9, 10, 26, 27]. IgE epitopes are conformational, and Pooideae grass-pollen allergens contain IgE-binding sites that are capable of generating IgE responses to

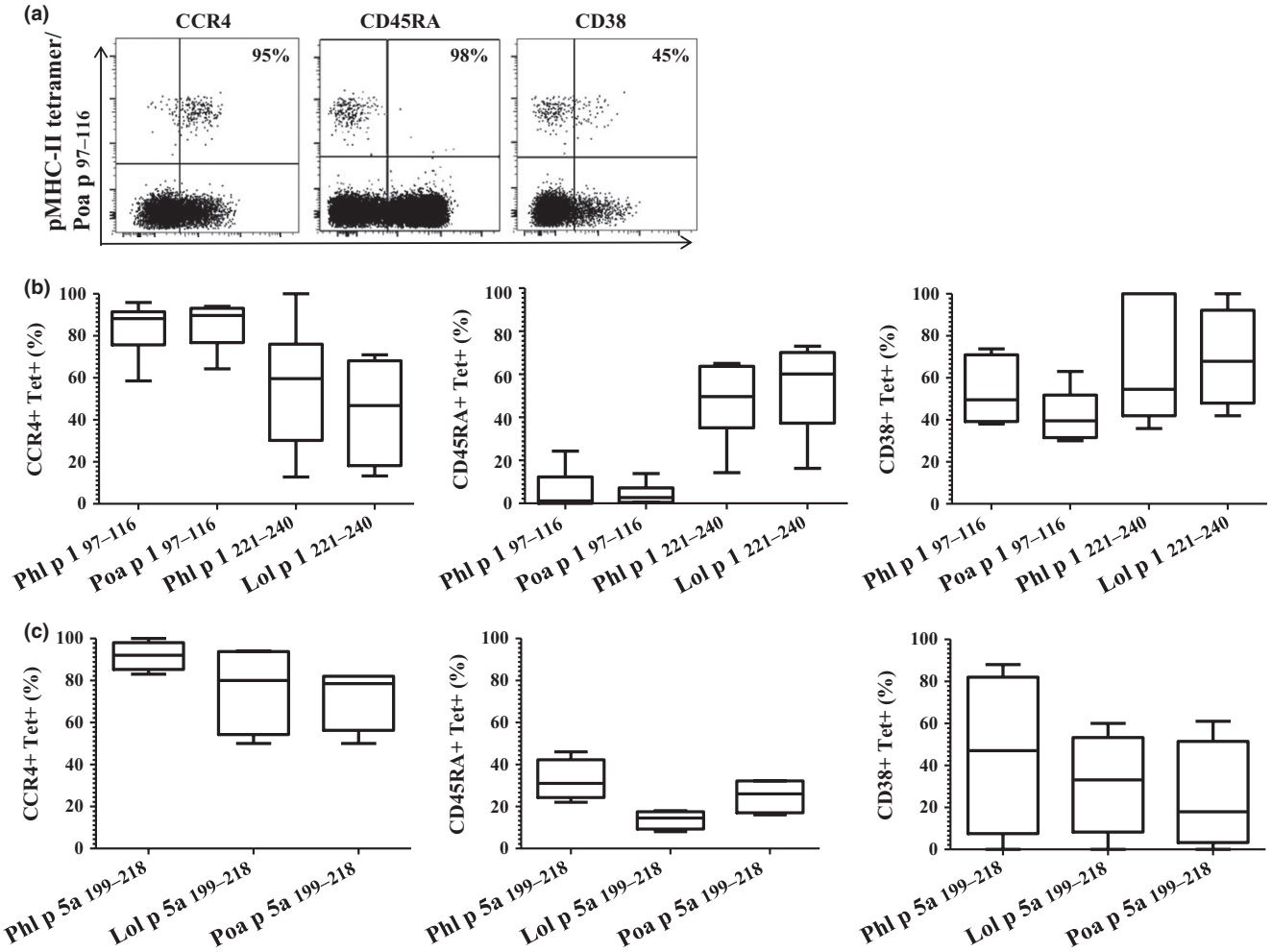

Fig. 4. Minimally cross-reactive $CD4^+$ T-cells are memory T-cells with a T_H2 phenotype and are activated during the pollen season. (a) Anti-PE enrichment permitted the analysis of surface marker expression; a naïve marker (CD45RA), a T_H2 marker (CCR4) and an activation marker (CD38) were analysed to compare Phl p grass-specific $CD4^+$ T-cells with homologous grass-specific $CD4^+$ T-cells. PBMC from a TPG allergic subject were stained with Poa p corresponding for TGP-derived region Group 1 $_{97–116}$ and are presented as an example. (b) Each bar represents surface expression of different markers for Group 1 reactive T-cells for six different allergic subjects. (c) Each bar represents surface expression of different markers for Group 5 reactive T-cells for six different subjects.

allergens from other Pooideae species [1, 2]. On the other hand, T-cell epitopes are linear and selectively target allergen-specific T-cells. A recent study by Focke-Tejkl et al. [28] showed discrepancy among the IgE- and T-cell-reactive domains in Phl p 5 major allergens, showing the importance of studying B-cell and T-cell epitopes. However, a caveat is noteworthy in our study, as the result of the cross-reactivity patterns might be influenced by the size of the cohort and the variety of HLA-alleles. It is possible that minimally cross-reactive epitopes may be absent in other HLA types.

Class II MHC/peptide structures have been thoroughly characterized [29–31], and it is established that peptide side chains at position p1, p4, p6, and p9 project into pockets along the MHC peptide-binding groove acting as anchor motifs [32]. Conversely, peptide side chains at position p-1, p2, p3, p5, p8, p10 project directly upward from the MHC-molecule surface and impact T-cell recognition [30–32]. Interestingly, anchor positions for most of the allergenic epitopes are well conserved among Pooideae species. For example, anchor motifs in TGP-derived epitopes from region $_{97–116}$ correspond to Y, D, S and G, and these residues are well conserved among Phl p, Lol p, Pha a, and Poa p (Table 1). In contrast, most of the minimally cross-reactive T-cell epitopes feature variability at p-1, p2, p3, and/or p8. As shown in Table 1, Poa p 1 $_{97–116}$ variant residues are present in p-1 (A) and p8 (K). For this reason, homologous peptides from diverse grass species can bind to MHC class II, but have varying degrees of recognition by different TCRs.

The current study demonstrated that different grass species contain species-specific T-cell epitopes. Considering the overlap of pollination calendars and

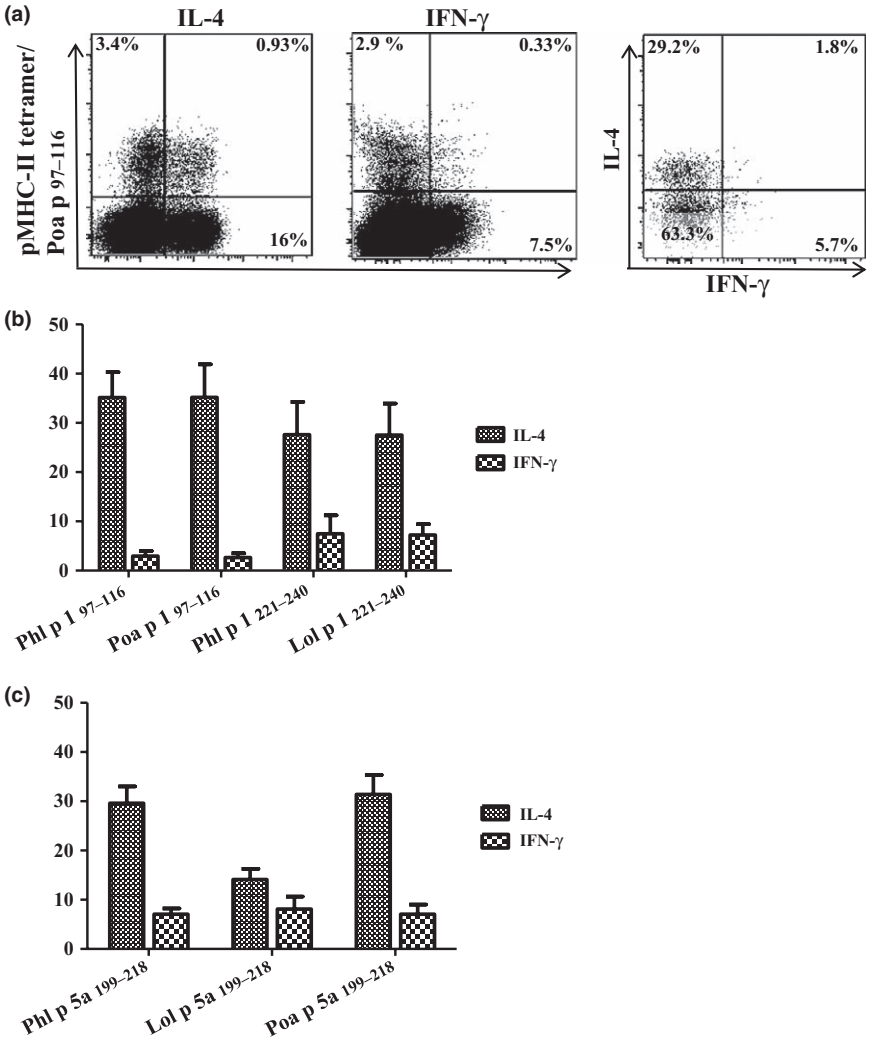

Fig. 5. Minimally cross-reactive $CD4^+$ T-cells functional profiles correlate with a T_H2 dominated response. An example of intracellular cytokine staining for Phl p- and grass homolog-reactive T-cells is provided. (a) Representative result for ICS. PBMC from a TGP allergic subject were stimulated with Poa p peptide corresponding to region Group 1 $_{97–116}$ and cultured for 2 weeks; cells were then stained with the corresponding PE-labelled DR04:01/Poa p grass peptide-loaded tetramers. $Tetramer^+$ cells were gated, and dual cytokine analysis is presented. (b) Summarized results of ICS for TGP-derived Group 1 $_{97-116}$ and Group 1 $_{221-240}$ epitopes, each bar depicts percentage of tetramer-positive cytokine producing cells in each individual. (c) Summarized results of ICS for TGP-derived Group 5 $_{199-218}$ epitopes, each bar depicts percentage of tetramer-positive cytokine producing cells in each individual.

geographical distribution for Pooideae grass species, allergic subjects can be exposed to multiple pollens in the same geographical region [3, 6, 23, 24]. As minimally cross-reactive T-cell epitopes can also bind to MHC-II, exposure to pollens from different grass species may generate T-cell populations specific to those minimally cross-reactive T-cell epitopes in allergic subjects. Indeed, homologous grass-pollen epitope-specific $CD4^+$ T-cells specific for minimally cross-reactive T-cell epitopes of other Pooideae species could be readily detected in PBMC from grass-pollen-allergic subjects. For example, frequency of Poa p 1 $_{97–116}$ in PBMC was found in similar range as the immunodominant Phl p epitope in DR04:01 subjects, suggesting that poly-sensitization occurs at the T-cell level. Side-by-side comparison of surface phenotype and functional profiles reveals that these minimally cross-reactive $CD4^+$ T-cells have a T_H2 dominated response and equal allergenic properties as Phl p-specific $CD4^+$ T-cells. In addition, these homologous grass-pollen epitope-specific $CD4^+$ T-cells are also activated during pollen season. The presence of the minimally cross-reactive T-cells for different Pooideae species in a group recruited on the basis of IgE reactivity to TGP implies that GP allergic subjects are usually poly-sensitized to other Pooideae species at the T-cell level. A recent study suggested that depletion of allergen-specific T-cells would be essential for successful antigen-specific immunotherapy [13]. In addition, it has also been

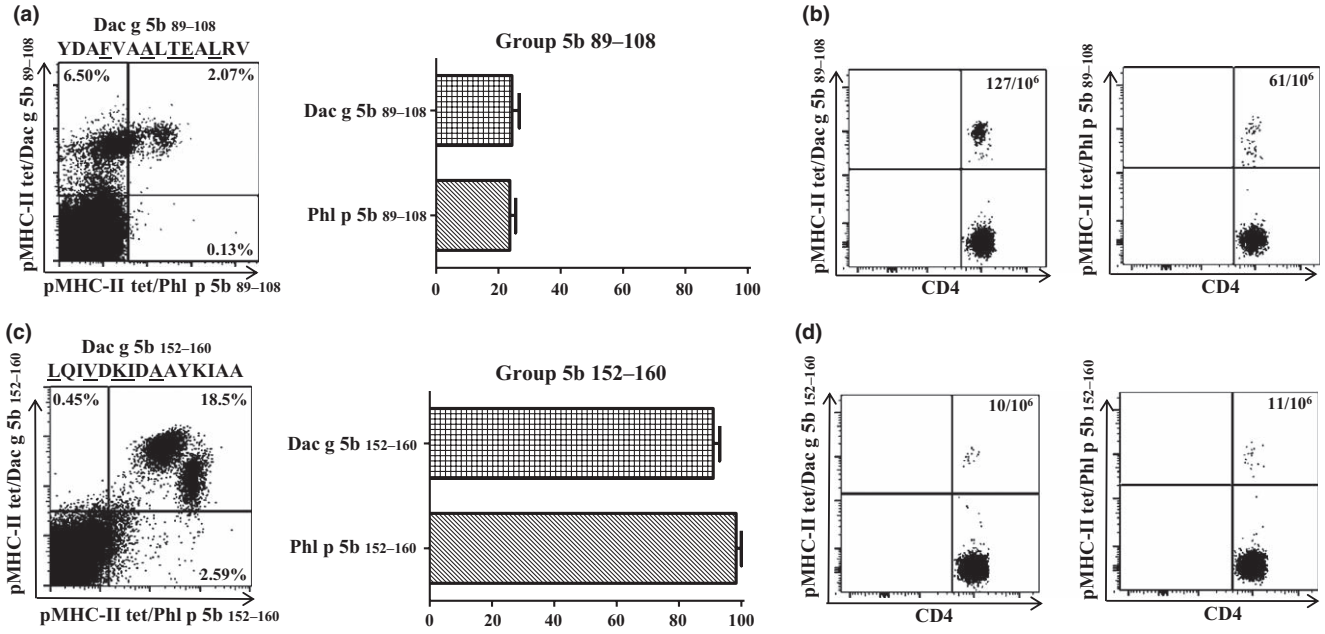

Fig. 6. Grass-pollen-specific $CD4^+$ T-cells have varying degrees of cross-reactivity that vary on the HLA and epitope they recognize. (a) PBMC ($n = 2$) from DR07:01 allergic subjects were stimulated *in vitro* with Dac g peptide corresponding to region Group 5b $_{89–108}$. Cells were then co-stained with APC-labelled DR07:01/Phl p grass peptide-loaded tetramers (*y* axis) as well as PE-labelled DR0701/Dac g 5b peptide-loaded tetramers (*x* axis). The plot shows a representative result for Group 5 $_{89–108}$ grass homologs; sequence of stimulating peptide is shown on top of plot. T-cells were gated on sidescatter and $CD4^+$. The graph represents percentage of T-cells that were able to double stain; for this particular HLA-restriction, minimal cross-reactivity was detected stimulating with either Dac g 5b or Phl p 5b peptides (about 20% of T-cells double stained) (b) *Ex vivo* frequency of allergen-specific $CD4^+$ T-cells for Group 5b $_{89–108}$ homologs. (c) PBMC ($n = 2$) from DRB5 allergic subjects were stimulated *in vitro* with either Dac g peptide corresponding to region Group 5b $_{152–160}$. Cells where then co-stained with APC-labelled DRB5/Phl p grass peptide-loaded tetramers (*y* axis) as well as PE-labelled DRB5/Dac g 5b peptide-loaded tetramers (*x* axis). The plot shows a representative result for Group 5b $_{152–160}$ grass homologs; sequence of stimulating peptide is shown on top of plot. T-cells were gated on side scatter and $CD4^+$. The graph represents percentage of double-stained T-cells; for this particular HLA-restriction, full-scale cross-reactivity was detected stimulating with either Dac g 5b or Phl p 5b peptides (≥ 90% of T-cells double stained). (d) *Ex vivo* frequency of allergen-specific $CD4^+$ T-cells for Group 5 $_{152–160}$ grass homologs.

established that the strength of the TCR signal induced by an epitope can affect the outcome in inducing tolerance [33]. Conversely, lack of cross-staining and the featured variability at sites of T-cell contact suggest that minimally cross-reactive $CD4^+$ T-cells specific for other Pooideae species will not be targeted by TGP-specific mono immunotherapy as most of GP allergic subjects are sensitized to various Pooideae species.

On the other hand, the presence of full-scale and partially cross-reactive T-cell populations raises the possibility that single species immunotherapy might be effective for these cross-reactive populations with similar T_H2 phenotype (data not shown). Campbell et al. previously showed that peptide immunotherapy with selected epitopes from a single allergen could render T-cell tolerance to one epitope, enabling the suppression of the function of T-cells specific for other epitopes within the same allergen [34, 35]. However, this linked epitope suppression is confined to a single allergen. Nonetheless, further investigations are required to confirm that Phl p-specific T-cells can mediate suppression of immune responses elicited by Phl p-nonspecific homologous T-cell populations.

In summary, class II tetramer staining experiments show that the patterns of cross-reactivity vary for 4 different DR04:01-, 1 DR07:01-, and 1 DRB5*01:01-restricted TGP-derived epitope regions tested. Indeed, T-cells with various degrees of cross-reactivity profiles could be detected with tetramers both *in vitro* and *ex vivo* settings. On the basis of these findings, we confirmed that both Phl p-specific as well as their Phl p- homologous $CD4^+$ T_H2 populations exist *in vivo*, suggesting GP allergic patients diagnosed upon IgE reactivity to *Phleum pratense* pollen extract were sensitized to various grass species at the T-cell level. This finding implies that mono-allergen immunotherapy with Phl p allergen would fail to elicit Treg or delete those species-specific T-cells that show minimum cross-reactivity to Phl p. Although a direct clinical comparison between immunotherapy with single grass extract verses multiple species-extracts utilizing a variety of HLAs would be obligatory, the current study suggests

multiple grass-pollen-species immunotherapy should be more beneficial than single species immunotherapy.

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Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Characterization of grass-pollen-allergic patients.

Ana o 1 and 11s Ana o 2 cashew allergens share cross-reactive CD4$^+$ T-cell epitopes with other tree nuts

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ABSTRACT

Allergies to cashew are increasing in prevalence with clinical symptoms ranging from oral pruritus to fatal anaphylactic reaction. Cashew-specific T-cell epitopes remain uncharacterized. Additionally, cross-reactivity amongst cashew and other tree nuts has not been examined in humans at the T-cell level. We sought to characterize cashew-specific T-cell responses associated with allergy and to identify cross-reactive epitopes among tree nut allergens. Cashew allergy was used as a model for studying cross-reactivity at the allergen-specific $CD4^+$ T-cell level. CD154 up-regulation assay was used to determine immunodominance hierarchy among cashew major allergens. The phenotype, magnitude and functionality of cashew-specific T-cells was determined by utilizing *ex vivo* staining with MHC class II tetramers. Dual tetramer staining and proliferation experiments were used to determine cross-reactivity to other tree nuts.$CD4^+$ T-cell responses are directed towards cashew allergens Ana o 1 and Ana o 2. Multiple Ana o 1 and Ana o 2 T-cell epitopes were identified. These epitopes elicited either T_H2 or T_H2/T_H17 responses in allergic subjects. These are either cashew unique epitope or cross-reactive epitopes. For clones that recognized the cross-reactive epitope, T-cell clones responded robustly to cashew, hazelnut and/or pistachio but not to walnut. Allergic responses to cashew could be triggered by cross-reactive tree nut epitopes, therefore, restricted consumption of tree nuts should be advised for cashew allergic subjects.

Key messages:

- Ana o 1 and Ana o 2 are the predominant cashew allergens recognized by T-cells.
- Cashew-specific T-cells with T_H2 and T_H2/T_H17 phenotypes could be detected in allergic subjects.
- Cashew-specific T-cell clones could respond to hazelnut and pistachio but not to walnut.

Capsule Summary:

Ana o 1 and Ana o 2 elicit T_H2 and/or T_H2/T_H17 responses in allergic individuals. Promiscuous cashew specific epitopes that elicit responses to other tree nuts should be good candidates for peptide vaccine to desensitize subjects with cashew allergy.

Key words: *Food allergy, cashew, tree nuts, Ana o 1, Ana o 2, cross-reactivity, T-cells, MHC class II tetramers, epitopes.*

List of nonstandard abbreviations used:

HLA Human histocompatibility leukocyte antigen

MHC Major histocompatibility complex

PBMC Peripheral blood mononuclear cell

Ana o *Anacardium occidentale*

Pis v *Pistacia vera*

Cor a *Corylus americana*

Car i *Carya illinoinensis*

Jug r *Juglans regia*

PE Phycoerythrin

pMHCII Peptide/MHC class II

T_H T helper

CRTH2 Chemoattractant receptor-homologous molecule expressed on T_H2 cells

TGEM Tetramer-guided epitope mapping

ICS Intracellular cytokine staining

TCL T-cell line

TCC T-cell clone

INTRODUCTION

Similar to peanut allergies, tree nut allergies are increasing in prevalence and affect 1.1% of children younger than 18 years and 0.5% of adults in the United States(1). After walnuts, cashew allergy is the next most commonly reported tree nut allergy (affecting 20% of tree nut allergic subjects)(2). It has been shown that severe clinical reactions, including higher incidence of anaphylaxis, occurs more frequently in cashew than peanut allergy(3-6). The lack of a specific treatment for cashew allergy makes food avoidance the mainstay of therapy in cashew allergic subjects(7,8).

Three major cashew allergens have been reported; Ana o 1 (7s vicilin-like protein), Ana o 2 (11s legumin-like protein) and Ana o 3 (2s albumin)(9-11) . All three are classified as seed storage proteins. Interestingly, this family of seed storage proteins are known to be allergenic in other tree nuts(12). Furthermore, amino acid sequence alignments of vicilins, legumins and albumins indicate that a high degree of homology between cashew allergens and allergens from other tree nuts including hazelnut, pistachio and walnut(13). It has been estimated that at least 86% of subjects who are tree nut allergic are allergic to multiple tree nuts(14). Cross-sensitization to pistachio, hazelnut and walnut are common within cashew allergic subjects(5). Studies have shown high degree of cross-reactivity between pistachio and cashew at the sIgE level, owing to their botanic relatedness(15-17). This phenomenon has also been shown between walnut, hazelnut and cashew allergens at moderate levels(15,18). On the other hand T-cell cross-reactivity between tree nuts in humans has not been well documented.

In this study, we used cashew as a model to study tree-nut cross-reactivity against hazelnut, pistachio and walnut at the T-cell level in humans. We initially investigated Ana o 1, Ana o2 and Ana o 3-specific T-cell responses using CD154 activation assay. Both Ana o 1 and Ana o 2 were identified as the predominant allergens that elicit $CD4^+$ T-cell responses in allergic subjects. Several Ana o 1 and Ana o 2 derived epitopes were identified by using tetramer-guided epitope mapping (TGEM). Phenotypes for allergen-specific T-cells were analyzed by *ex vivo* tetramer staining and results show that allergic subjects have a predominant

T_H2 phenotype, however, T_H2/T_H17 responses were also detected. T-cell-clones specific to these epitopes were generated to assess cross-reactivity by tetramer co-staining and proliferation experiments. T-cell clones (TCC) specific to cashew allergen derived epitopes could readily proliferate with hazelnut and pistachio but not walnut allergen derived peptides. This study leads to the conclusion that exposure to other tree nut allergens can trigger cashew-specific T-cell responses and provide help for cashew specific IgE production in cashew allergic subjects. Strict avoidance of other tree nuts should be recommended in cashew allergic subjects.

METHODS

Subjects

Subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute with informed consent and institutional review board approval (IRB title "Allergen and T-cell reagent resources for the study of allergic diseases," Approval number IRB7109.) A total of 14 subjects with an initial diagnosis that was made on the basis of history of an acute reaction to cashew and a positive ImmunoCAP score (>0.35 kU/L) for cashew extract (Phadia AB, Uppsala, Sweden) were recruited for this study. 12 non-atopic and 6 atopic subjects with no clinical symptoms to cashew, a negative ImmunoCAP score and HLA-matched were also recruited as controls for this study. The features of these subjects are shown in (**Table 1**). DNA samples were HLA-typed using Dynal Unitray^{TM} SSP Kits (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

TGEM

Peptide libraries were generated based on Ana o 1 and Ana o 2 sequences. The libraries consisted of overlapping peptides spanning the entire allergen, which were 20 amino acids in length with a 12 amino acid overlap synthetized by Mimotopes (Clayton, Australia). Peptide-loaded HLA-DR proteins were generated, as previously described(19,20) . The tetramer-guided epitope-mapping procedure was conducted as previously described(21). Briefly, PBMC were cultured for 14 days and subsequently stained with pooled peptide tetramers. Cells from wells that gave positive staining were re-screened utilizing tetramers loaded with each individual peptide from that positive pool.

Ex-vivo analysis of walnut-specific $CD4^+$ T-cells

$CD154^+$ detection assay, was carried out as previously described(22). Briefly, for detection of $CD154^+$-reactive T-cells, 35 million PBMC (at 7 x 10^6 cells/mL) in culture medium (RPMI 1640 (Gibco) + 10%

pooled human serum + 1% PenStrep) were stimulated with 5µg/mL of synthesized peptide pools and 1 µg/ml anti-CD40 (Miltenyi Biotec, Auburn, CA) for 3 hours (for frequency and surface phenotype) and 6 hours (for ICS) at 37°C. Cells were also mock stimulated with DMSO (0.05% final concentration) as negative control. After stimulation, cells were stained with PE-conjugated CD154 (Miltenyi Biotec, Auburn, CA) and labeled with anti-PE magnetic beads (Miltenyi Biotec, Auburn, CA) for 20 minutes at 4°C. A 1/100 fraction of cells was saved for analysis. The other fraction was passed through a Miltenyi magnetic column; magnetically enriched cells were next stained with a panel of antibodies of interest for 20 minutes at room temperature. After staining, cells were stained again with Via-probe^{+} (BD Biosciences, East Rutherford, NJ) for 10 minutes at 4°C before flow-cytometry. Data acquisition was performed using a LSR II flow cytometer and data were analyzed utilizing FlowJo (Tree Star, Ashland, Ore). Frequency was calculated as previously described for tetramer analysis(23).

Ex vivo analysis with pMHC-II tetramers was carried out as previously described(23).

Basophil stimulation tests

Basophil activation was measured as previously described(24). Briefly, heparinized whole blood from cashew allergic subjects was incubated with tree nut extract (2 lg/mL): Cashew (*Anacardium occidentale*, Ana o), Pistachio (*Pistacia vera*, Pis v), Hazelnut (*Corylus americana*, Cor a), and Walnut (*Juglans regia*, Jug r) (Greer Laboratories, Lenoir, NC, USA) and simultaneously stained with anti-CD3 (eBioscience, San Diego, CA, USA), anti-CD203 (Beckman Coulter, Pasadena, CA, USA), anti-CRTH2 (BD Biosciences, Franklin Lakes, NJ, USA) for 25 min at 37°C. Basophils were identified as CD3 CRTH2^{+}, and activation status was assessed following the detection of CD203c in the presence of the allergens tested(24). Tree nut extracts are sterile solutions containing the extractables of tree nut allergens (20 000 BAU/mL), 0.5% Sodium Chloride, 0.275% Sodium Bicarbonate, and 50% Glycerine by volume as preservative. Whole blood stimulated with buffer without allergen and a mixture of tree nut extract were used as negative and positive controls, respectively.

T-cell cloning procedure and proliferation analysis

T-cell clones were generated by staining T-cells with tetramer directly *ex-vivo* and sorting gated tetramer-positive $CD4^+$ and $CD45RA^-$ cells using a FACSAria (at single-cell purity). Expansion was done in a 96-well plate in the presence of 1.0×10^5 irradiated PBMC and 2 mg/ml PHA (Remel, Lenexa, KS). T-cells were re-screened with tetramers loaded with antigenic epitopes to assess positivity for the corresponding specificity. T-cells were stimulated in parallel with the corresponding peptides (1 and 10 μg/ml), adding HLA-DR-matched irradiated PBMC as APC. After 48 hours, each well was pulsed for an additional 16 hours with 1 mCi [3H]thymidine (Amersham Biosciences, Piscataway, NJ), and uptake was measured with a scintillation counter to assess proliferation.

Intracellular cytokine staining

TCC intracellular cytokine staining combined with tetramer staining was performed as previously described(25). Briefly, TCC were restimulated with 50 ng/mL phorbol 12-myristate-13-acetate and 1 mg/mL ionomycin in the presence of 1x brefeldin-A (eBiosciences) for 5 hours at 37°C, 5% CO2. After 10 minutes at room temperature, cells were then fixed with fixation buffer (eBioscience) and washed twice with a permeabilization buffer (eBioscience). Cells were then stained with a panel of antibodies directed against cytokines of interest for 20 minutes at room temperature; cells were washed and immediately analyzed in LSR-II flow cytometer. Two profiles (T_H2 and T_H2/T_H17) of TCCs were arbitrarily defined as follows (**Figure 6**):T_H2 profile is exemplified by CCR4+ with or without CRTH2 expression (data not shown) and production of IL-4 (≥10%), IL-5(≥10%), and IL-13 (≥10%); T_H2/T_H17 profile is characterized by co-expression CCR4 and CCR6 (data not shown) and co-produce IL-4 (≥10%) and IL17A (≥10%) but no IFN-ϒ and IL-5.

Cytokine detection with ELISA

For cytokine ELISA, IL-4 (clone 8D4-8) and IFN-γ (clone MD-1) capturing antibodies (BioLegend) were coated onto 96-well round bottom plates. 50 µl of supernatants from co-cultures of T cell clones with autologous APC were collected after 48 h stimulation and added to each well. After overnight incubation, bound cytokines were detected by biotinylated, α-IL-4 (clone MP4-25D2) and α-IFN-γ (clone 4s.B3) antibodies (BioLegend) and quantified using a Victor2 D time resolved fluorometer (Perkin Elmer).

Statistical analysis

Statistical analysis was performed using the tests indicated in the figure legends utilizing Prism 5.0 software (Graphpad).

RESULTS

Ana o 1 and Ana o 2 as predominant cashew allergens recognized by $CD4^+$ T-cells

The CD154 up-regulation assay was used to examine Ana o 1, Ana o 2 and Ana o 3 reactive $CD45RA^-$ $CD4^+$ memory T-cell responses in cashew non-allergic and allergic subjects. For non-allergic subjects, Ana o 1, Ana o 2 and Ana o 3 $CD4^+$ T-cell responses were low (average frequencies of 6.87 ± 1.5, 3.88 ± 2.6 and <1 per million $CD4^+$ T-cells, respectively)**.** Conversely, in allergic subjects, strong Ana o 1, Ana o 2 and weak Ana o 3-specific responses were observed (average frequencies 23.81 ± 6.8, 22.70 ± 5.4 and 3.32 ± 1.1 per million $CD4^+$ T-cells for Ana o 1, Ana o 2, and Ana o 3 respectively) **(Figure 1A, Figure 1B)**. The average frequency of Ana o 1 and Ana o 2 responses was 6 fold and 5 fold greater, respectively than Ana o 3 T-cell immune responses in allergic subjects. No statistical differences were observed between Ana o 1 and Ana o 2 T-cell responses. Collectively, Ana o 1 and Ana o 2-reactive $CD4^+$ T-cells play a predominant role in subjects with cashew allergy.

Both Ana o 1 and Ana o 2-specific $CD4^+$ T-cells have a T_H2 and T_H2/T_H17 phenotypes

The TGEM approach was utilized to identify $CD4^+$ T-cell epitopes within cashew allergens Ana o 1 and Ana o 2 (**Supplemental Figure 1**). For Ana o 1, a total of 4 immunogenic epitopes restricted to DRB1*01:01, DRB1*07:01, DRB1*09:01 and DRB5*01:01 were identified and for Ana o 2, a total of 6 immunogenic epitopes restricted to DRB1*04:04, DRB1*07:01, DRB1*09:01, DRB1*15:01 and DRB4*01:01 were identified (**Table 2**).

Frequency of Ana o 1 and Ana o 2-specific $CD4^+$ T-cells was examined by direct *ex vivo* staining with Ana o 1- and Ana o 2-tetramers (**Figure 2A and Figure 2B**). For non-allergic subjects, $CD4^+$ T-cells specific for Ana o 1- and Ana o 2 were barely detectable by tetramers (around 1 per million $CD4^+$ T-cells). Conversely, in allergic subjects the average frequency within the memory compartment ($CD45RA^-$), the average frequency was 16.42 ± 1.7 and 15.41 ± 2.8 per million $CD4^+$ T-cells, respectively (**Figure 2A and**

Figure 2B). These data confirmed the results from the CD154 assays that both Ana o 1 and Ana o 2 are strongly immunogenic *ex vivo* in cashew allergic subjects.

The surface phenotypes of Ana o 1- and Ana o 2-specific T-cells were analyzed by *ex vivo* staining of PBMC to determine whether there were differences in epitope-specific T-cell responses in allergic subjects (**Figure 3**). A high percentage of Ana o 1- and Ana o 2-specific $CD4^+$ T-cells expressed CCR4, while a low percentage expressed CXCR3. These data suggested a T_H2 effector phenotype (T_{eff}) for the Ana o 1- and Ana o 2-specific $CD4^+$ T cells. The expression of CRTH2 and CD27 in these cells were heterogeneous. Although T-cells that lost CD27 expression were present, there were still higher percentages of T-cells that expressed CD27 for both Ana o 1 and Ana o 2-specific $CD4^+$ T-cells. Additionally, the majority of these tetramer positive $CD27^+$ T-cells also co-expressed CCR7, suggesting that a proportion of these T-cells have a central memory phenotype (T_{CM}) (**Supplemental Figure 2A**). Variable expression of CCR6 with low β7 expression was detected, indicating the capacity of some cells to home to gut. In addition, all $CCR6^+$ T-cells co-expressed CCR4 suggesting that a subset of T-cells are T_H17 and/or T_H2/T_H17 (**Supplemental Figure 2B**). Conversely, for the non-allergic subjects with appreciable frequency, the main phenotype observed was characterized by $CXCR3^+CCR6^+CD27^+$ (**Supplemental Figure 3**). In conclusion, for allergic subjects, both Ana o and Ana o 2-specific cells were T_H2 liked, we did not observe a significant difference in surface phenotypes between Ana o 1 and Ana o 2.

Cashew-specific $CD4^+$ T-cells in allergic subjects are capable of cross-reacting with hazelnut and pistachio but not to walnut

It is likely that due to amino acid sequence homology among Ana o 1 with Cor a 11, Pis v 3 and Jug r 2 (44%, 78% and 36.6% respectively) and Ana o 2 with Cor a 9, Pis v 5 and Jug r 4 (60%, 80.5%, and 63.2% respectively) (http://fermi.utmb.edu/SDAP/), cross-reactivity between different tree nut allergens may be present at the T-cell level. For this purpose, potential epitopes for other tree nut species were identified by selecting sequences homologous to selected Ana o 1 and Ana o 2 epitopes utilizing Blast alignments (**Table**

3). Overall, the majority of Ana o 1 and Ana o 2 homologs from different tree nut species show sequence homology to cashew derived epitopes, suggesting these are plausible T-cell epitopes.

On account of these findings, Ana o 1- and Ana o 2-specific T-cells were single cell sorted for generation of TCC. TCC were obtained from 7 out of the 12 cashew epitopes identified. A total of 36 Ana o 1 and 16 Ana o 2 TCC were obtained and T-cell cross-reactivity was first evaluated by co-staining experiments using tetramers **(Figure 4A, 4B, 4C and Supplementary Table 1)**. Three profiles were observed: (i) cross-reactivity with hazelnut and pistachio; (ii) cross-reactivity with hazelnut or pistachio; and (iii) no cross-reactivity with hazelnut or pistachio. Cross-staining with walnut was not observed. These profiles were later confirmed utilizing proliferation assay, where an SI>3 was defined as cut-off for positivity. Though clones that were co-stained by hazelnut tetramers and pistachio tetramers were also positive in proliferative assay, there were clones that did not co-stained but proliferated upon peptide specific stimulation. For examples, 2 out of 9 TCC DRB1*15:01 restricted Ana o $2_{321\text{-}340}$ and 2 out of 4 DRB1*07:01 restricted Ana o $1_{281\text{-}300}$ did not co-stain with pistachio loaded tetramers, but these clones proliferated upon peptide specific activation. Results from all these proliferation assays were summarized in **Figure 4D** and **Supplementary Table 1**. At the epitope level, 7/9 of cashew epitope screened could elicit cross-reactive response to pistachio, and 5/9 of cashew epitopes identified could elicit cross-reactive response to hazelnut. At the T cell level, 19.2% of the cashew reactive clones isolated were cross-reactive to both hazelnut and pistachio, 15.4 % of the clones were cross-reactive to pistachio and not to hazelnut, 11.5% of the clones were cross-reactive to hazelnut and not to pistachio, 53.8% of the clones did not cross react to hazelnut and pistachio and exclusively responded towards cashew.

The functionality of these TCC was evaluated with ICS in addition to tetramer staining (**Figure 5**). In support of phenotyping experiments, both Ana o 1 and Ana o 2-specific $CD4^+$ T-cells produced T_H2 associated cytokines, including abundant IL-4, IL-5 and IL-13, while others only produced IL-4. TCCs that co-produced IL-4 and IL-17A with small amounts of IFN-γ were also observed. Cross-reactive epitopes

also elicit almost identical cytokine profile (**Supplemental Figure 4**). Collectively, this results suggested that cashew-specific T-cells can readily react to homologous T-cell epitopes from other tree-nut species.

Cashew- and Walnut-reactive CD4$^+$ T-cells are detected in cashew allergic subjects that have IgE sensitivity to other tree nuts

Majority of cashew allergic subjects had a positive Immunocap score for hazelnut, pistachio and walnut extract. Subjects were also evaluated for IgE reactivity to these tree nuts utilizing a basophil activation assay (**Figure 6A and 6B**). This confirmed that these cashew allergic subjects are sensitized to other tree nut species.

The observation that not all TCC co-stain and most lacked T-cell reactivity to walnut peptides suggested that walnut reactive T-cells represent a distinct population of T-cells, as most of these allergic subjects have a positive Immunocap score to walnut and positive basophil activation test. For this objective, we used CD154 assay and compared frequencies of Cashew- and Walnut-reactive T-cells in our cohort (**Figure 6C and 6D**). No differences in frequencies for walnut- and cashew-reactive T-cells were observed. These results suggested that these subjects are sensitized to both cashew and walnut and their respective allergen-reactive T-cells are distinct populations of T cells.

DISCUSSION

Although allergic reactions to cashew nut are increasing in prevalence and adverse reactions may be more severe than peanut allergy(3-5), the role of $CD4^+$ T-cell responses in cashew allergy has not yet been studied. In this study, both Ana o 1 and Ana o 2 were identified as the predominant cashew allergens that elicit $CD4^+$ T-cell responses. Similar to walnut allergen-specific T-cells(22), we observed a T_H2 dominant population (including T_{CM} and T_{eff}), while a sub-population of T-cells that co-expressed CCR4 and CCR6 (T_H2/T_H17 and T_H17-like) was also detected. This sub-population has been previously detected in both asthmatic and non-asthmatic subjects with peanut(26), walnut(22) and shrimp allergies(27). ICS showed that the majority TCC clones derived from cashew epitopes mainly produce IL-4, IL-13 and IL-5, while a minority of TCC co-produced IL-4 and IL-17A implicating that these $CCR4^+CCR6^-$ and $CCR4^+CCR6^+$ cells were *bona fide* T_H2 and T_H2/T_H17 cells, respectively. Compared with peanut allergy, cashew nut allergy causes more gastro-intestinal symptoms(4). Indeed these symptoms may be attributed to the presence of $CCR6^+\beta7^+CCR7^+$ T-cells as they have the capacity to home to the GALT(28-31). The association of CCR6 expression with food allergen specific T cells in both asthmatic and non-asthmatic allergic subjects may be unique as allergen specific T cells for aeroallergens in non-asthmatic subjects do not expressed CCR6 (Kwok WW, unpublished). The role of $CCR6^+$ T-cells play a role in gastrointestinal allergic disease has also been documented in a murine model(32). On the other hand, T-cell responses in non-allergic subjects were nearly absent in most subjects tested, for subjects with detectable frequency, the phenotype was $CXCR3^+CCR6^+CD27^+$.

It has been estimated that the majority of cashew allergic subjects are poly-sensitized to different tree nuts(5). Prior studies showed that vicilins and legumins from different tree nuts exhibit moderate cross-reactivity at the IgE level(15-18). In contrast, cross-reactivity at the T-cell level in humans has not been thoroughly documented. In the present study, we detected basophil responses towards different tree nuts in subjects recruited on the basis of cashew allergy, confirming a poly-sensitization status. We also identified cashew derived epitopes and looked for cross-reactivity to homologous epitopes from other

species of tree nuts. We found that the 46% of cashew-specific TCC from Ana o 1 and Ana o 2 reacted to homologous peptides from hazelnut and pistachio via proliferation assays. Importantly, these homologous peptides are able to elicit identical T_H2 cytokine in cashew reactive T cells. Our observations infer that phylogenetic relatedness among tree nuts reflects cross-reactivity as we observed that most of cashew derived T-cell epitopes cross-reacted with pistachio epitopes. This is in agreement with the fact that cashew allergens Ana o 1, Ana o 2 and Ana o 3 and their corresponding allergens Pis v 1, Pis v 2 and Pis v 3 of the closely-related pistachio nut are highly homologous in the aa sequences (78%, 80%,and 70%, respectively) and have a high degree of IgE-binding cross-reactivity(16,33). Although Ana o 1 and the hazelnut allergen Cor a 11 are only 44% homologous, we were still able to detect cross-reactive epitopes between cashew and hazelnut. In contrast, though sequence homology is found in similar ranges (60%) between Ana o 2 and their corresponding allergens Jug r 4 in walnut, neither T-cell proliferation nor class II tetramer co-staining was observed for all of the walnut homologous peptides. Additionally, all of the previously identified Jug r 2 epitopes(22) did not fall into the regions identified for Ana o 1 cashew derived epitopes. This is expected as Jug r 2 and Ana o 1 have low sequence homology 36%. Nonetheless, walnut T-cell responses were still detected in these subjects, suggesting that walnut allergens contains species-specific T-cell epitopes, and this places walnut in a group of allergens within the tree nut allergens family that is distinct from cashew. Both walnut and pecan belong to the family of Juglandaceae(34) and walnut and pecan allergens are highly homologous. For example, Jug r 2 and Car i 2 are 92% homologous, thus, we expect there will be extensive T cell cross reactivity between walnut and pecan. Similar profiles of IgE cross-reactivity have been reported by Goetz et al (15). They reported that cashew, pistachio and hazelnut form a group of tree nuts which were distinct from walnut and pecan(15). However, a low degree of cross-reactivity between cashew and walnut was still observed. In addition to the cross-reactive T cell epitopes as discussed above, TCC derived from Ana o $1_{433-452}$ did not co-stain with class II tetramers nor proliferate to homologous peptides from other tree nut species suggesting this as a species-specific epitope for cashew. It is also possible that Ana o $1_{433-452}$ is conserved in other tree nut species not tested in our study.

From an immunotherapeutic standpoint, the presence of cross-reactive T-cell epitopes raises the possibility that single species peptide immunotherapy might be effective in modulating or depleting(35) these cross-reactive populations with both T_H2 and T_H2/T_H17 phenotype. Indeed, linked epitope suppression has been previously shown where peptide immunotherapy with selected epitopes from a single allergen resulted in suppression of responses to other epitopes(36). Additionally, Kulis et al(37) demonstrated that cashew immunotherapy prevented allergic responses to both cashew and pistachio in a cashew sensitized murine model and to cashew and walnut in a multisensitized murine model. Kulis et al also reported T cell cross-reactivity between walnut and cashew in a cashew sensitized mouse model(37). It is likely that these cross-reactive epitopes are epitopes with low affinity to MHC-molecules. Low affinity cross-reactive epitopes may also be present in humans which are overlooked in our tetramer approach. However, the presence of walnut specific T-cell populations in walnut sensitized subjects of our cashew allergic cohort and their lack of cross-reactivity with cashew suggest that cashew peptide immunotherapy approach may not be most effective for walnut.

Overall, the T cell cross-reactivity between tree nuts as reported here together with the extent of IgE cross-reactivity amongst different tree nuts as reported earlier are direct evidence that exposure to one tree nut can lead to allergic immune response related to other tree nuts. Thus subjects whom have clinical reaction to one tree nut should avoid all true nuts as a precaution.

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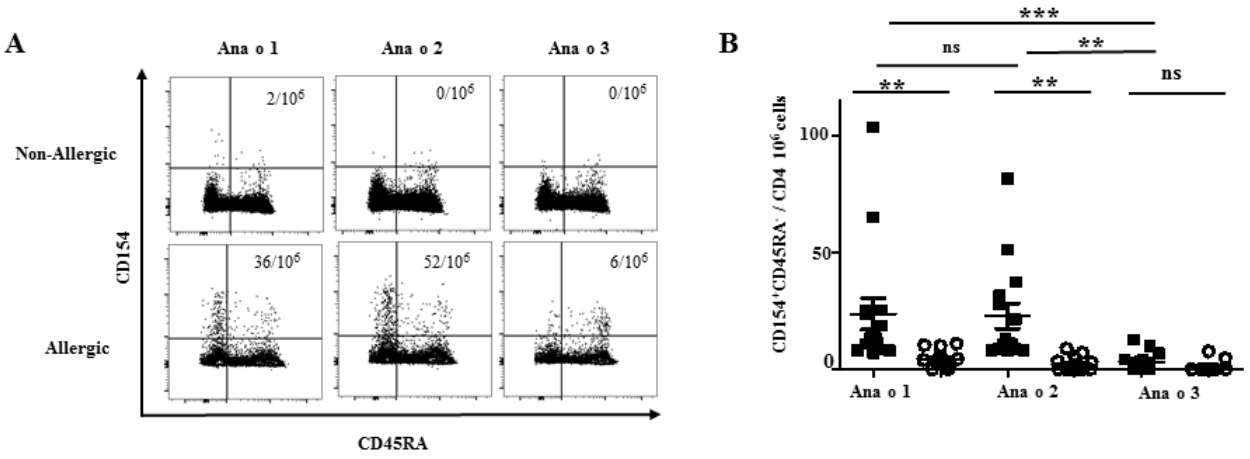


Figure 1. Frequencies of cashew allergen reactive $CD4^+$ T-cells. **A,** Frequencies of Ana o 1-, Ana o 2- and Ana o 3-reactive T-cells within the memory compartment ($CD45RA^-$) in a DRB1*07:01 subject without allergy and a DRB1*07:01 subject with cashew allergy. The frequencies of cashew allergen reactive T-cells per million $CD4^+$ T-cells are as indicated. **B,** Frequencies of $CD154^+CD45RA^-$ Ana o 1-, Ana o 2- and Ana o 3-reactive T-cells in subjects with cashew allergy (n=14; filled squares) and non-allergic subjects (n=18; opened circles). Each data point represents the frequency of T-cells reactive to each allergen. An ANOVA test (with Dunnets correction) was used to compare all columns in the statistical analysis $*P<0.05$, $**P<0.001$, $***P<0.0001$. NS. Not significant.

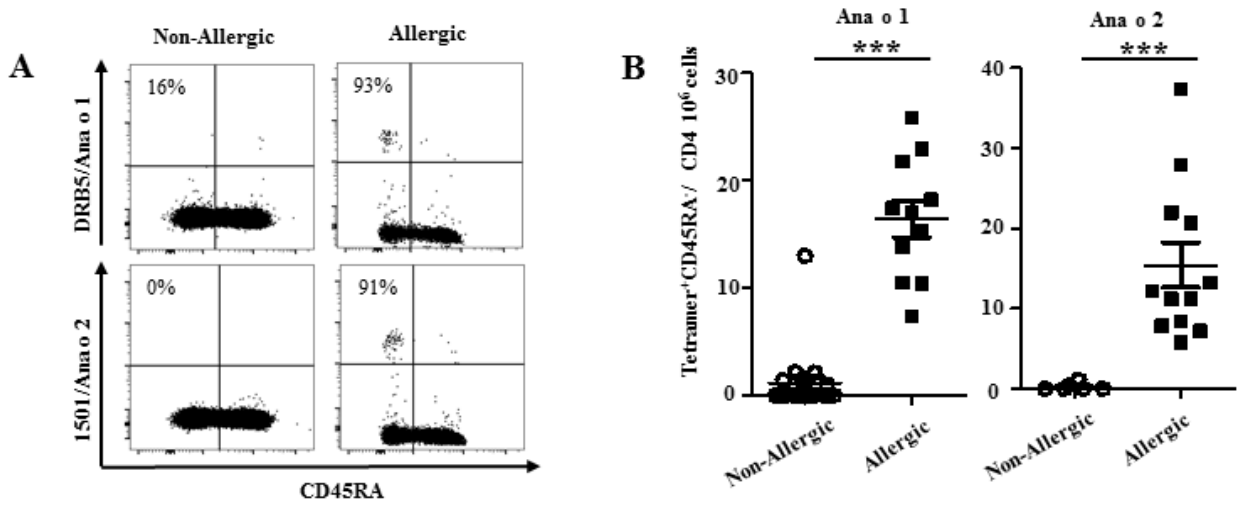


Figure 2. A, Percentages of Ana o 1- and Ana o 2-specific T-cells within the memory compartment ($CD45RA^-$) in a DRB1*15:01-DRB5*01:01 subject without allergy and a subject with cashew allergy. **B,** Frequencies of $Tetramer^+CD45RA^-$ Ana o 1- and Ana o 2-specific T-cells in subjects with cashew allergy (n=12; filled squares) and non-allergic subjects (n=12; opened circles). Each data point represents the frequency of T-cells specific to each allergen. A Student *t* test was used in the statistical analysis. **P*<0.05, ***P*<0.001, ****P*<0.0001. NS. Not significant.

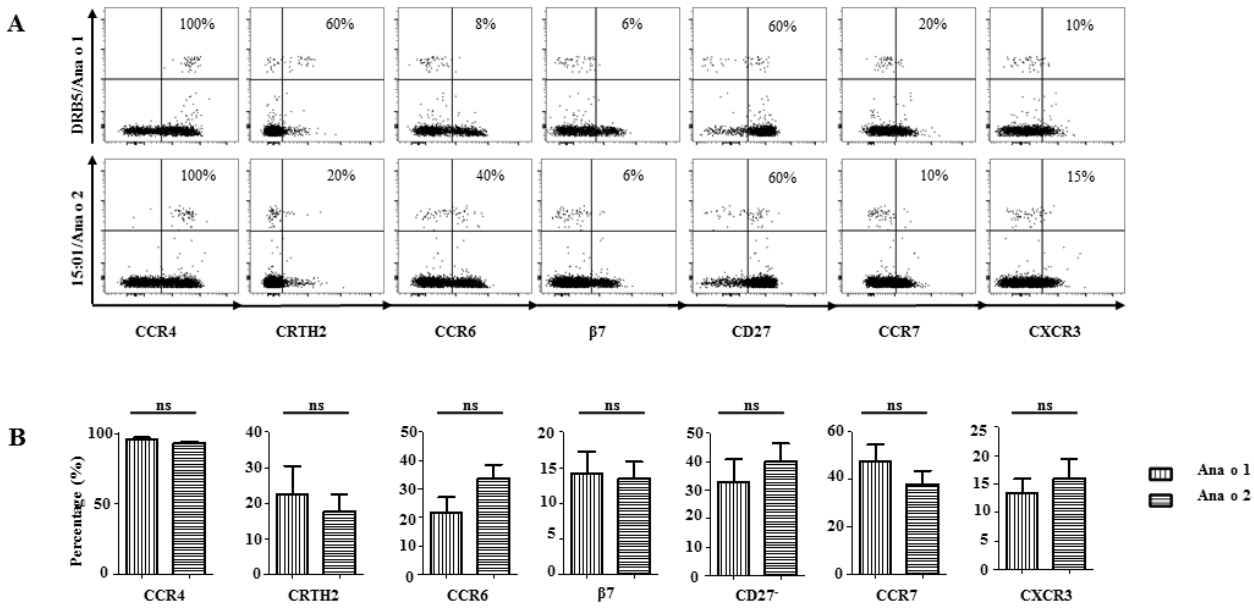


Figure 3. Phenotypes of Ana o 1- and Ana o 2-reactive T-cells. **A,** *First row,* profile for Ana o 1 in a DRB5*01:01 allergic subject. *Second row,* Ana o 2 profile in a DRB1*15:01 allergic subject. The percentages of surface markers expressed by Ana o 1- and Ana o 2-specific T-cells are as indicated. **B,** *Ex vivo* expression of CCR4, CRTH2, CCR6, β7,$CD27^-$, CCR7 and CXCR3of Ana o 1- and Ana o 2- specific T-cells in subjects with cashew allergy (n=12) Each bar represents the percentage of tetramer positive T-cells with marker expression for each allergen. A Student *t* test was used to compare expression of each marker in the statistical analysis. **P*<0.05, ***P*<0.001, ****P*<0.0001. NS. Not significant.

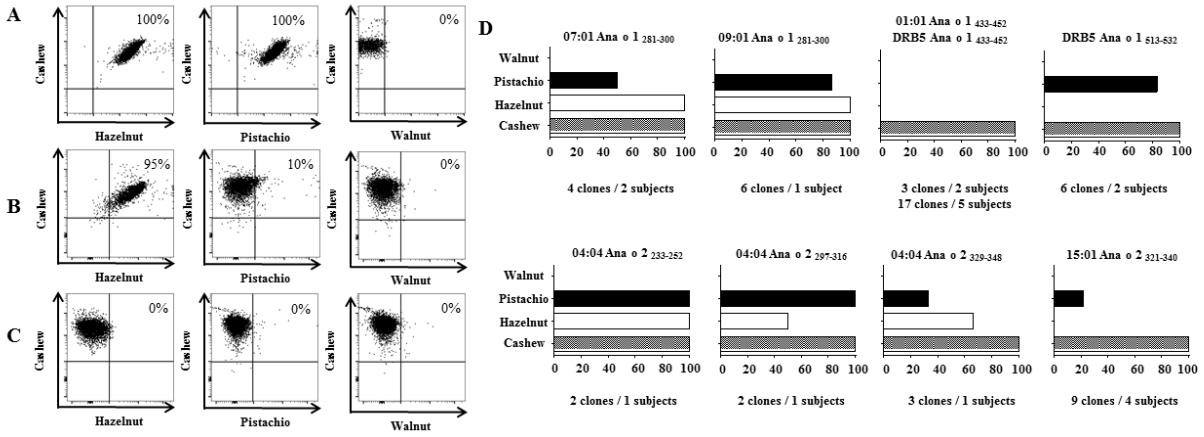


Figure 4. Cashew-specific $CD4^+$ T-cells in allergic subjects are capable of cross-reacting with hazelnut and pistachio but not to walnut. T-cell clones isolated by DRB1*01:01, *07:01, *09:01, DRB5*01:01, *04:04 and *15:01 tetramers were assayed for specificity and functionality. The plots in **A, B,** and **C,** show representative results for different cross-reactivity profiles for the cashew-derived epitopes in Ana o 1 and Ana o 2, cells were co-stained with APC-labelled DRB1*/Ana o peptide loaded tetramers (*y* axis) and with PE-labelled DRB1*/homologous tree nut peptide-loaded tetramers (*x* axis). **A,** cross-reactivity to hazelnut and pistachio **B,** cross-reactivity to hazelnut or pistachio **C,** no cross-reactivity to hazelnut or pistachio. **D,** Summarized proliferation results for Ana o $1_{281\text{-}300}$, Ana o $1_{433\text{-}452}$, Ana o $1_{513\text{-}532}$, Ana o $2_{233\text{-}252}$, Ana o $2_{329\text{-}348}$ and Ana o $2_{321\text{-}340}$-specific T cell clones using APCs from their respective DRB1*specificities. Cells were stimulated with specific or irrelevant control peptide. SI: stimulation index; cpm of specific peptide divided by cpm of irrelevant peptide. a SI>3 was defined as cut-off for positivity. Bars represent percentage of clones that had a SI>3. The number of clones and subjects from whom TCC were derived from are indicated under each graph.

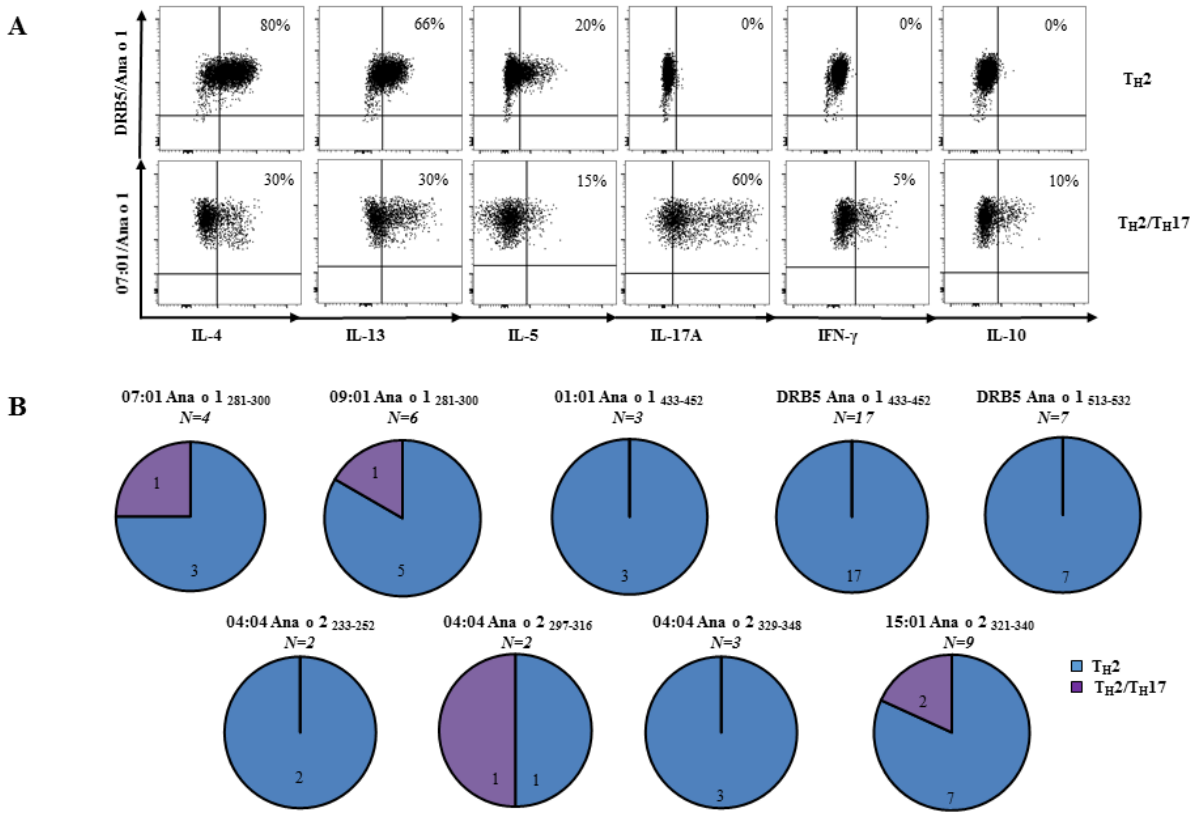


Figure 5. Cytokine profiles of Ana o 1 and Ana o 2-specific T-cell clones. **A.** ICS staining of a T_H2 clone and a T_H2/T_H17 clone, and **B,** Phenotype of 53 T-cell clones derived from 9 allergic subjects. The numbers of T-cell clones for each profile and specificity are as indicated.

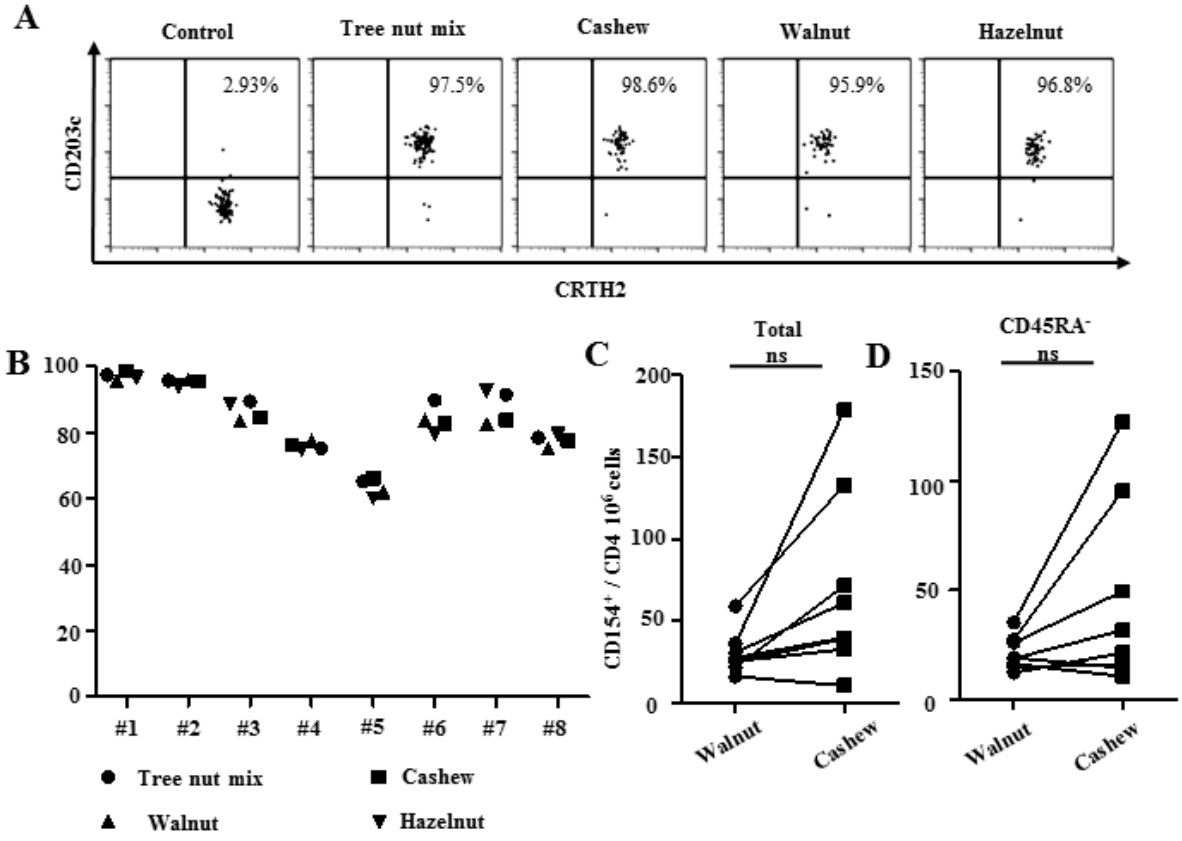


Figure 6. Cashew allergic subjects have IgE sensitivity to hazelnut, pistachio and walnut. Up-regulation of CD203c indicated activation of basophils. **A,** Representative results from a DRB1*15:01 cashew allergic subject. **B,** Summarized results for 8 cashew allergic subjects. Each data point represents the percentages of CD203c positive cells on stimulated basophils for each tree nut allergen. **C,** Frequencies of walnut-(filled circles) and cashew-reactive (filled squares) T-cells in subjects with cashew allergy (n=8). **D,** Frequencies of $CD154^{+}CD45RA^{-}$ walnut-(filled circles) and cashew-reactive (filled squares) T-cells in subjects with cashew allergy (n=8; filled squares) and non-allergic subjects (n=8). Each data point represents the frequency of T-cells reactive to each allergen. A Student *t* test was used to compare expression of each marker in the statistical analysis. *P<0.05, **P<0.001, ***P<0.0001. NS. Not significant.

Table 1. HLA and allergic status of recruited subjects

ID	Age	Sex	HLA (DRB1*)	sIgE cashew (f202) kU/L	Skin Prick Test to cashew	sIgE hazelnut (f17) kU/L	sIgE pistachio (f203) kU/L	sIgE walnut (f256) kU/L	Symptoms after cashew ingestion	Asthma
Cashew allergics										
1	28	F	07:01 , 14:01	>100	Not tested	58.9	>100	38.6	I, III, V, VII , VIII	yes
2	11	F	09:01 , 15:01	31.4	Not tested	5.62	28.6	17.1	I, III, IV, V	yes
3	19	F	01:01 , 13:01	1.73	12 x 12 mm	0.67	2.2	0.58	I, IV, V	yes
4	24	F	01:01 , 13:01	0.5	9 x 9 mm	<0.35	0.77	<0.35	I, II, III	yes
5	8	F	15:01 , 04:04	>100	Not tested	26.9	>100	6.61	IV, V	yes
6	24	F	01:01 , 15:01	32.6	Not tested	3.69	37	8.86	I, II, III, IV,VI	no
7	21	M	03:01 , 15:01	19	10 x 10 mm	61.2	25.4	28.2	I, II, IV, V	yes
8	11	M	01:01 , 10:01	9.78	15 x 15 mm	1.03	13.9	6.79	III, IV	no
9	34	F	07:01 , 15:01	7.66	15x10 mm	21.3	6.58	77.7	I, II, III, IV, V	no
10	8	F	07:01 , 11:01	2.95	8 x 8 mm	7.38	7.4	3.93	I, II, III, IV	no
11	11	M	01:01 , 13:02	41.6	Not tested	0.72	4.94	15.8	I, IV, V	yes
12	36	F	01:01 , 04:04	0.38	15x15 mm	0.38	0.36	0.69	II, III, IV	no
13	10	F	01:01 , 04:04	>100	5 x 5 mm	40.5	>100	97.3	III, IV, V	no
14	15	F	11:01 , 13:01	57.3	Not tested	87.02	65.9	>100	I, III	yes
Nonatopic subjects										
18	29	M	01:01 , 03:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
19	31	F	07:01 , 07:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
20	41	F	04:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
21	34	M	07:01 , 13:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
22	32	F	15:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
23	10	F	07:01 , 11:01	0	Not tested	Not tested	Not tested	Not tested	Absent	No
24	30	F	01:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
25	31	F	04:04, 16: 01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
26	23	M	01:01, 07:01	0	Not tested				Absent	yes

27	22	F	07:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
28	23	M	07:01 , 13:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
29	31	M	01:01 , 04:04	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
Atopic subjects without cashew allergy										
30 *	10	M	09:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
31 *	6	M	10:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
32 *	63	M	01:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no
33	41	F	09:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
34	26	F	04:04 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes
35	8	F	01:01 , 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no

I Itchy mouth, lips and / or pharynx

II Abdominal discomfort and / or diarrhea

III Nausea or vomiting

IV Severe skin itching or hives, acute or angioedema

V Rhinitis and / or conjunctivitis and / or respiratory compromise

VI Dizziness (feeling loss of consciousness)

VII Syncope (loss of consciousness)

VIII Desaturation with respiratory compromise

* Subjects also had history of peanut and positive IgE ImmunoCAP for peanut

Table 2. Ana o 1 and Ana o 2 CD4⁺ T-cell epitopes

		HLA DRB1 Restriction								
Ana o	Amino acid sequence	01:01	04:04	07:01	09:01	11:01	13:01	15:01	DRB4	DRB5
Ana o 1 $_{281\text{-}300}$	GPGGENPESFYRAFSWEILE	•		•	•					
Ana o 1 $_{385\text{-}404}$	MVVSYANITKGGMSVPFYNS			•						
Ana o 1 $_{433\text{-}452}$	HPSYKKLRARIRKDTVFIVP	•			•					•
Ana o 1 $_{513\text{-}532}$	VFGKQDEEFFFQGPEWRKEK									•
Ana o 2 $_{233\text{-}252}$	KVKDDELRVIRPSRSQSERG	•	•							
Ana o 2 $_{289\text{-}308}$	PARADIYTPEVGRLTTLNSL		•							
Ana o 2 $_{297\text{-}316}$	PEVGRLTTLNSLNLPILKWL		•							
Ana o 2 $_{321\text{-}340}$	EKGVLYKNALVLPHWNLNSH							•		
Ana o 2 $_{329\text{-}348}$	ALVLPHWNLNSHSIIYGCKG		•						•	
Ana o 2 $_{385\text{-}404}$	QNFAVVKRAREERFEWISFK				•		•			

Table 3. Cashew and tree nut homolog $CD4^+$ T-cell epitopes.

Group Allergen	Tree nut species	‡ Amino acid sequence
Ana o 1$_{281\text{-}300}$	Ana o 1	GPGGENPESFYRAFSWEILE
	Pis v 3	GPGGENPESFYRAFS**R**E**V**LE
	Cor a 11	G**A**GGE**D**PESFYRAFS**W**E**V**LE
	Jug r 2	**A**AG**AKS**P**DQS**Y**LRVFSNDIL**
Ana o 1$_{281\text{-}300}$	Ana o 1	GPGGENPESFYRAFSWEILE
	Pis v 3	GPGGENPESFYRAFS**R**E**V**LE
	Cor a 11	G**A**GGE**D**PESFYRAFS**W**E**V**LE
	Jug r 2	**A**AG**AK**SP**DQ**SY**LRVFSNDIL**
Ana o 1 $_{385\text{-}404}$	Ana o 1	MVVSYANITKGGMSVPFYNS
	Pis v 3	**IM**VSY**V**NITKGGMS**G**PFYNS
	Cor a 11	**LM**VS**FA**NITKG**S**M**AG**P**Y**YNS
	Jug r 2	**VL**VN**NYAE**I**KR**G**A**MM**V**P**H**YNS
Ana o 1 $_{433\text{-}452}$	Ana o 1	HPSYKKLRARIRKDTVFIVP
	Pis v 3	**G**PSYKKL**SSS**IR**T**D**SVFV**VP
	Cor a 11	YQK**IS**AR**L**R**RGV**VFV**APAGH**
	Jug r 2	**TGRFQ**K**VT**AR**LA**R**GDI**F**VI**P
Ana o 1 $_{513\text{-}532}$	Ana o 1	VFGKQDEEFFFQGPEWRKEK
	Pis v 3	VFGKQDEEFFFQG**PK**WR**QH**
	Cor a 11	**IFKN**QD**QA**FFF**P**GP**NKQQ**E**E**
	Jug r 2	**IFES**Q**M**E**SY**F**VPTERQS**R**RG**
Ana o 2 $_{233\text{-}252}$	Ana o 2	KVKDDELRVIRPSRSQSERG
	Pis v 5	KVK**G**D-L**Q**VIRP**P**R**R**QSERG

	Cor a 9	**EGR**L**LQ**V**V**R**PE**R**S**R**RQ**E**WERQ**
	Jug r 4	**R**V**EGRQ**L**LQ**V**I**R**P**RW**S**REEQE**
Ana o 2 **289-308**	Ana o 2	PARADIYTPEVGRLTTLNSL
	Pis v 5	P**S**R**S**DIYTPEVGR**I**IT**S**LNSL
	Cor a 9	**RS**RADIYT**EQ**VGR**I**N**TV**N**S**N**
	Jug r 4	P**S**RADIYT**E**E**A**GR**I**S**T**V**N**S**H**
Ana o 2 **297-316**	Ana o 2	PEVGRLTTLNSLNLPILKWL
	Pis v 5	PEVGR**I**IT**S**LNSLNLPILKWL
	Cor a 9	**EQ**VGR**I**N**TV**N**S**N**T**LP**V**L**R**WL**
	Jug r 4	**RIST**V**SHTLPV**L**RW**L**QLSA**
Ana o 2 **321-340**	Ana o 2	EKGVLYKNALVLPHWNLNSH
	Pis v 5	E**R**GVL**QN**NAL**MV**PHWN**F**N**AS**
	Cor a 9	E**R**G**D**L**QREG**L**YV**PHWNLN**A**H**
	Jug r 4	**LYSD**AL**YV**PHWNLN**AHSVVY**
Ana o 2 **329-348**	Ana o 2	ALVLPHWNLNSHSIIYGCKG
	Pis v 5	AL**M**VPHWN**F**N**A**HSI**V**YGCKG
	Cor a 9	**G**L**Y**VPHWNLN**A**HS**VV**Y**AIR**G
	Jug r 4	PHWNLN**AH**S**VV**YA**LR**G**RAEV**
Ana o 2 **385-404**	Ana o 2	QNFAVVKRARREERFEWISFK
	Pis v 5	QNFAVVKRAR**GQ**RFEWISFK
	Cor a 9	QNFAV**A**KRA**ES**E**G**FEW**VA**FK
	Jug r 4	QNFAVVKRAR**NE**G**F**EW**V**SFK

‡ Homologous sequences of amino acids for each epitope region. Bold letters depict variant residues. Underlined amino acids depict DRB1*04:04, *07:01, *09:01, *15:01 and DRB5*01:01 motifs P1, P4, P6, P7 and P9. The MHC-II binding predictions were made on 6/4/2015 using the IEDB analysis resource Consensus tool [1][2].

Supplementary Table 1. Cross-reactivity between Ana o and Tree nut homologous epitopes.

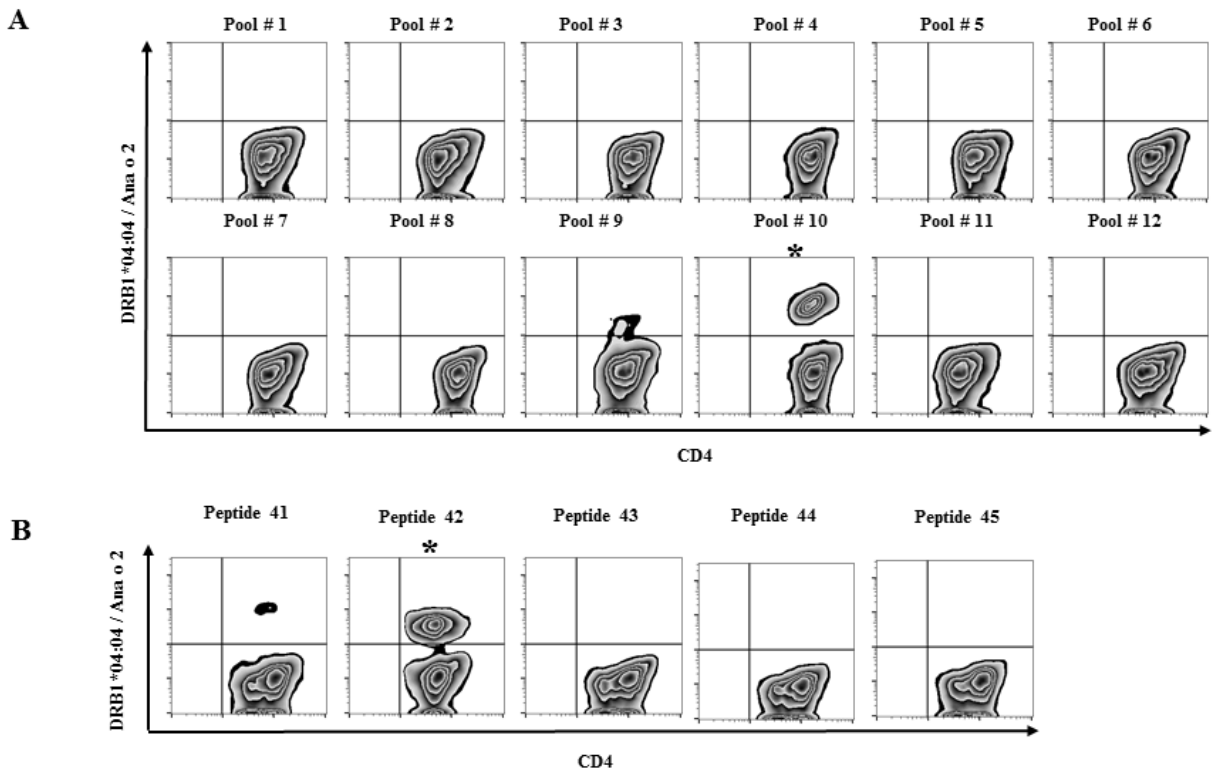
Subject	Clone	Hazelnut X Cashew Pistachio X Cashew	Hazelnut X Cashew	Pistachio X Cashew	Cashew only
1	07:01 Ana o 1 $_{281-300}$ # 1	●			
2	07:01 Ana o 1 $_{281-300}$ # 2	*			
2	07:01 Ana o 1 $_{281-300}$ # 3		●		
2	07:01 Ana o 1 $_{281-300}$ # 4		●		
3	09:01 Ana o 1 $_{281-300}$ # 1	●			
3	09:01 Ana o 1 $_{281-300}$ # 2	*			
3	09:01 Ana o 1 $_{281-300}$ # 3	*			
3	09:01 Ana o 1 $_{281-300}$ # 4		●		
3	09:01 Ana o 1 $_{281-300}$ # 5	●			
3	09:01 Ana o 1 $_{281-300}$ # 6	●			
4	01:01 Ana o 1 $_{433-452}$ # 1				●
5	01:01 Ana o 1 $_{433-452}$ # 2				●
5	01:01 Ana o 1 $_{433-452}$ # 3				●
1	DRB5 Ana o 1 $_{433-452}$ # 1				●
6	DRB5 Ana o 1 $_{433-452}$ # 2				●
6	DRB5 Ana o 1 $_{433-452}$ # 3				●
6	DRB5 Ana o 1 $_{433-452}$ # 4				●
6	DRB5 Ana o 1 $_{433-452}$ # 5				●
6	DRB5 Ana o 1 $_{433-452}$ # 6				●
6	DRB5 Ana o 1 $_{433-452}$ # 7				●
6	DRB5 Ana o 1 $_{433-452}$ # 8				●
6	DRB5 Ana o 1 $_{433-452}$ # 9				●
6	DRB5 Ana o 1 $_{433-452}$ # 10				●
7	DRB5 Ana o 1 $_{433-452}$ # 11				●
7	DRB5 Ana o 1 $_{433-452}$ # 12				●
7	DRB5 Ana o 1 $_{433-452}$ # 13				●
7	DRB5 Ana o 1 $_{433-452}$ # 14				●
7	DRB5 Ana o 1 $_{433-452}$ # 15				●
8	DRB5 Ana o 1 $_{433-452}$ # 16				●
9	DRB5 Ana o 1 $_{433-452}$ # 17				●
6	DRB5 Ana o 1 $_{513-532}$ # 1				●
6	DRB5 Ana o 1 $_{513-532}$ # 2			●	
7	DRB5 Ana o 1 $_{513-532}$ # 3			*	
7	DRB5 Ana o 1 $_{513-532}$ # 4			●	
7	DRB5 Ana o 1 $_{513-532}$ # 5			●	
7	DRB5 Ana o 1 $_{513-532}$ # 6			*	
5	04:04 Ana o 2 $_{233-252}$ # 1	●			
5	04:04 Ana o 2 $_{233-252}$ # 2	●			
5	04:04 Ana o 2 $_{297-316}$ # 1	●			
5	04:04 Ana o 2 $_{297-316}$ # 2		●		
5	04:04 Ana o 2 $_{329-348}$ # 1			●	
5	04:04 Ana o 2 $_{329-348}$ # 2		*		
5	04:04 Ana o 2 $_{329-348}$ # 3		*		
1	15:01 Ana o 2 $_{321-340}$ # 1			●	
3	15:01 Ana o 2 $_{321-340}$ # 2			●	
3	15:01 Ana o 2 $_{321-340}$ # 3				●
6	15:01 Ana o 2 $_{321-340}$ # 4				●

6	15:01 Ana o 2 $_{321\text{-}340}$ # 5	●
6	15:01 Ana o 2 $_{321\text{-}340}$ # 6	●
7	15:01 Ana o 2 $_{321\text{-}340}$ # 7	●
7	15:01 Ana o 2 $_{321\text{-}340}$ # 8	●
7	15:01 Ana o 2 $_{321\text{-}340}$ # 9	●

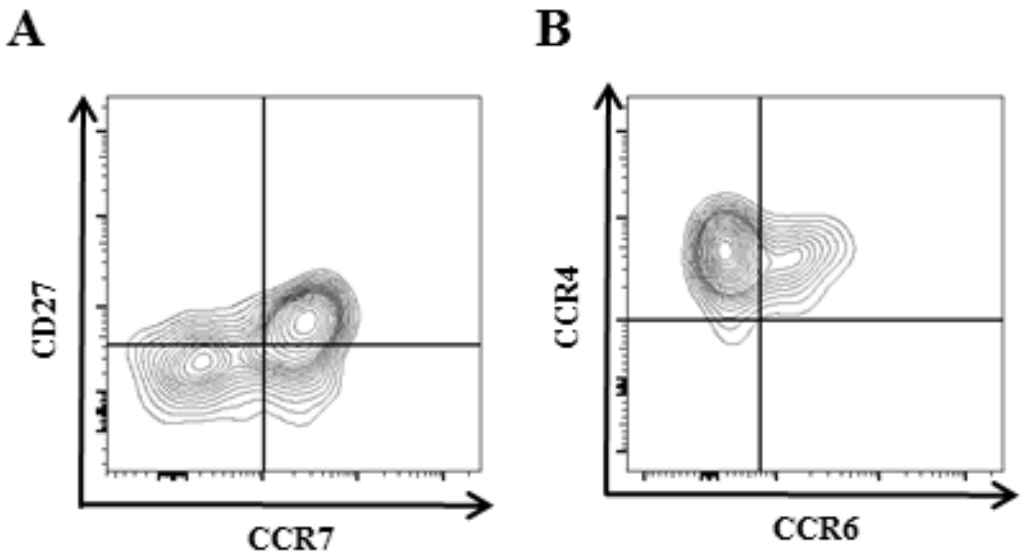
● Cross-reactivity as confirmed by tetramer co-staining and proliferation assay

* Cross-reactivity as confirmed by proliferation assay only

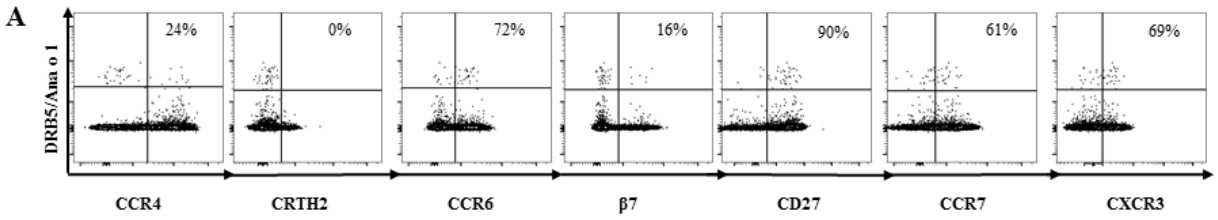
Supplemental Figures



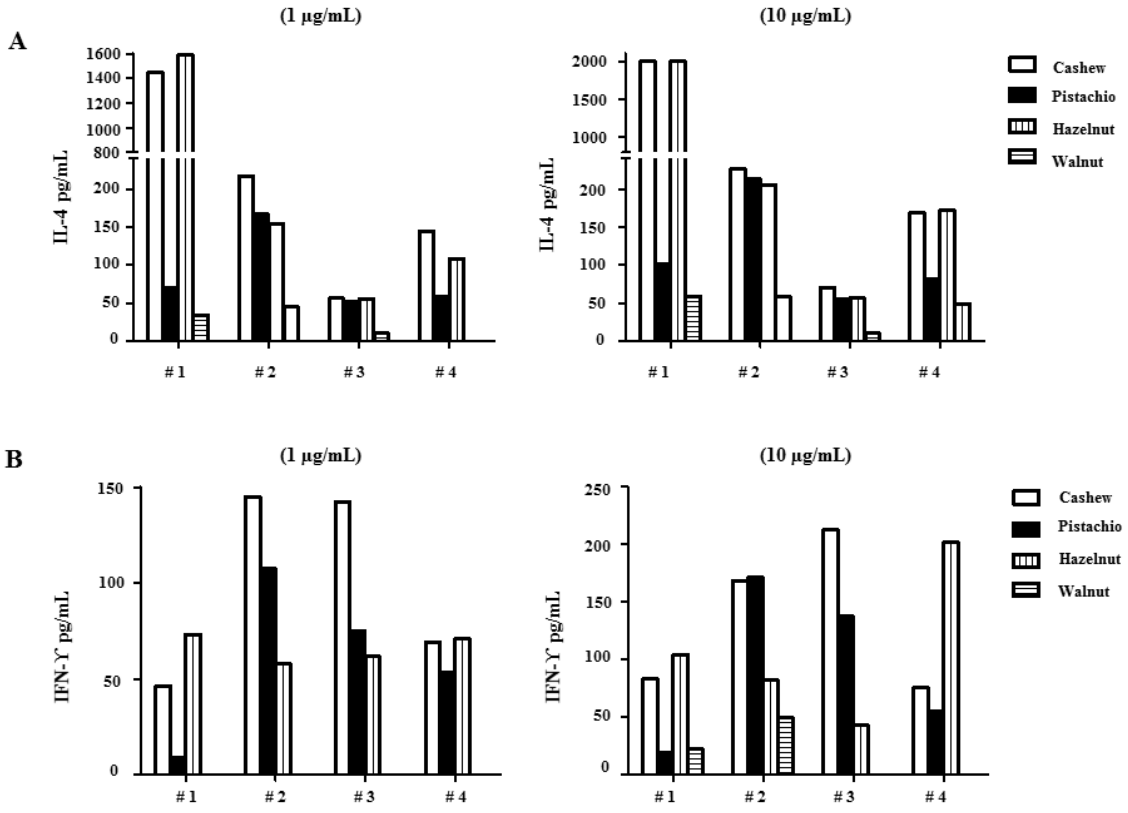
Supplemental Figure 1. TGEM studies of DR04:04-restricted Ana o 2-reactive $CD4^+$ T-cells. **A.** PBMC from a DR04:04 patient with cashew allergy were stimulated with 12 pools of Ana o 2 peptides for 2 weeks and subsequently stained with corresponding DR04:04/Ana o 2-pooled peptide tetramers. **B.** Cells stimulated with pool 10 were restained with individual peptides from the corresponding pool. The staining identified p42 (Ana o $2_{329\text{-}348}$) as DR04:04-restricted Ana o 2 T-cell epitopes.



Supplemental Figure 2. Phenotypes of Ana o 1-reactive T cells in allergic subjects. **A.** Tetramer-positive CD45RA- T cells were gated against CCR7 and CD27. **B.** Tetramer-positive CD45RA- T cells were gated against CCR4 and CCR6. The percentages of surface markers expressed by DRB1*07:01 Ana o $1_{281\text{-}300}$-specific T cells are as indicated.



Supplemental Figure 3. Phenotypes of Ana o 1-reactive T-cells in a non-allergic subject. **A,** Profile for Ana o 1 in a DRB5*01:01 allergic subject. The percentages of surface markers expressed by Ana o 1-specific T-cells are as indicated.



Supplemental Figure 4. Cytokine profiles of Ana o $1_{281\text{-}300}$-specific TCCs. 4 different Ana o $1_{281\text{-}300}$-specific TCCs were co-cultured with autologous APC in presence of 1 µg/mL and 10 µg/mL of Ana o $1_{281\text{-}300}$ or homologous tree nut peptides. **A.** Comparison of IL-4 (pg/mL) supernatant production in two different settings. **B.** Comparison of IFN-ϒ (pg/mL) supernatant production in two different settings. Representative results from experiments using 4 clones are shown. Average of triplicates is presented.

Jug r 2–reactive CD4$^+$ T cells have a dominant immune role in walnut allergy

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Background: Allergic reactions to walnut can be life-threatening. Although IgE epitopes of walnut have been studied, CD4$^+$ T cell–specific epitopes for walnut remain uncharacterized. In particular, the relationship of both phenotype and frequency of walnut-specific T cells to the disease have not been examined.

Objectives: We sought to provide a thorough phenotypic analysis for walnut-reactive T cells in allergic and nonallergic subjects, particularly the relationship of phenotypes and frequencies of walnut-specific T cells with the disease.

Methods: The CD154 upregulation assay was used to examine CD4$^+$ T-cell reactivity toward the walnut allergens Jug r 1, Jug r 2, and Jug r 3. A tetramer-guided epitope mapping approach was used to identify HLA-restricted CD4$^+$ T-cell epitopes in Jug r 2. Direct *ex vivo* staining with peptide–major histocompatibility complex class II tetramers enabled comparison of the frequency and phenotype of Jug r 2–specific CD4$^+$ T cells between allergic and nonallergic subjects. Jug r 2–specific T-cell clones were also generated, and mRNA transcription factor levels were assessed by using quantitative RT-PCR. Intracellular cytokine staining assays were performed for further phenotypic analyses.

Results: Jug r 2 was identified as the major allergen that elicited CD4$^+$ T-cell responses. Multiple Jug r 2 T-cell epitopes were identified. The majority of these T cells in allergic subjects have a CCR4$^+$ phenotype. A subset of these T cells express CCR4$^+$CCR6$^+$ irrespective of the asthmatic status of the allergic subjects. Intracellular cytokine staining confirmed these T_H2-, T_H2/T_H17-, and T_H17-like heterogenic profiles. Jug r 2–specific T-cell clones from allergic subjects mainly expressed *GATA3*, nonetheless, a portion of T-cell clones both *GATA3* and RAR-related orphan receptor C *(RORC)* or *RORC* alone, confirming the presence of T_H2, T_H2/T_H17, and T_H17 cells.

Conclusions: Jug r 2–specific responses dominate walnut T-cell responses in patients with walnut allergy. Jug r 2 central memory CD4$^+$ cells and terminal effector T cells were detected in peripheral blood, with the central memory phenotype as the most prevalent phenotype. In addition to conventional T_H2 cells, T_H2/T_H17 and T_H17 cells were also detected in nonasthmatic and asthmatic patients with walnut allergy. Understanding this T-cell heterogeneity might render better understanding of the disease manifestation. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Food allergy, walnut, Jug r 2, T cells, MHC class II tetramers, epitopes*

Allergic reactions to tree nuts, including walnut, cashew, and almond, are common, affecting approximately 1.1% of children younger than 18 years and 0.5% of adults in the United States.[1] Similar to peanut allergy, tree nut allergy generally has an onset in early childhood and persists throughout life. It is estimated that only 9% of patients outgrow tree nut allergy.[2,3] In the United States walnut allergy is the most frequent among patients with tree nut allergy (34%),[4] and both cashew and walnut accounted for the majority of life-threatening anaphylactic reactions caused by tree nuts.[2] Food avoidance is the only therapeutic option; however, the ubiquity of these foods in the diet makes avoidance difficult, and accidental ingestion is a common occurrence.[5,6]

The most commonly consumed walnut species is *Juglans regia*. Currently, 5 allergens have been reported.[7] Jug r 1 (2S albumin), Jug r 2 (7S vicilin–like protein), and Jug r 4 (11S legumin–like protein) have been described as important major allergens in the United States.[8-10] On the other hand, Jug r 3 (lipid transfer protein) has been proposed as a major allergen in the Mediterranean area.[11] Jug r 5 is a profilin, and its role as a walnut allergen is limited.[7]

Although IgE epitopes of walnut allergens have been studied,[12-14] CD4$^+$ T cell–specific epitopes for walnut allergens remain uncharacterized. It is now established that allergen-specific T cells play an important role in allergic inflammation.[15,16] In this study we examined T-cell reactivity toward Jug r 1 and Jug r 2 because their corresponding allergens in peanut, 2S albumin (Ara h 2) and 7S vicilin–like seed storage protein (Ara h 1), respectively, are highly immunogenic in patients with peanut allergy.[17,18] Jug r 3 was also studied because we have a small cohort of samples from Spain, where lipid transfer protein is the major plant food allergen.[11] We initially investigated Jug r 1 Jug r 2– and Jug r 3–specific T-cell responses using the CD154 activation assay.[19] Jug r 2, but neither Jug r 1 nor Jug r 3, elicited dominant T-cell responses in allergic patients. Several Jug r 2–derived epitopes were then identified by using tetramer-guided epitope mapping (TGEM).[20] The magnitude and phenotype of the response of Jug r 2–specific CD4$^+$ T cells in allergic and

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Abbreviations used
CRTH2: Chemoattractant receptor–homologous molecule expressed on T_H2 cells
ICS: Intracellular cytokine staining
PE: Phycoerythrin
RORC: RAR-related orphan receptor C
TCC: T-cell clone
T_{CM}: Central memory T
Teff: Effector T
TGEM: Tetramer-guided epitope mapping

nonallergic subjects were determined directly *ex vivo*. Results show that allergic patients have a predominant T_H2 phenotype; however, T_H17 responses in some subjects were also observed. T cells with $CCR4^+CD27^+$, $CCR4^+CD27^-$, $CCR4^+CCR6^+$, and $CCR4^+CCR6^-$ surface phenotypes were detected in allergic patients. T cells from nonallergic subjects have T_H1 and T_H1/T_R1 phenotypes characterized by surface expression of CXCR3. Understanding this T-cell heterogeneity might improve our understanding of disease manifestation.

METHODS

Subjects

Subjects were recruited from Hospital Clinic de Barcelona (Barcelona, Spain), the Virginia Mason Medical Center Allergy Clinic, and Benaroya Research Institute (Seattle, Wash). All subjects were recruited with informed consent and institutional review board approval (title "Allergen and T-cell reagent resources for the study of allergic diseases," approval no. IRB7109). Twelve subjects (2 Spanish and 10 US subjects) with a history of an acute reaction to walnut ingestion, a positive ImmunoCAP score for walnut extract (≥0.35 kU/L; Thermo Fisher Scientific, Uppsala, Sweden), and, in some cases, a skin test response of 4 mm or greater were recruited for this study. The individual sera of all recruited patients were tested with the allergen microarray immunoassay ImmunoCAP ISAC (Phadia, Thermo Fisher Scientific), according to the manufacturer's protocol. In addition, 5 sensitized subjects with a skin test response of 4 mm or greater, an ImmunoCAP score of 0.35 kU/L or greater, or both who were avoiding eating walnut but had no history of ingestion were also recruited for this study. The clinical features of these subjects are presented in Table I. Seven nonatopic and 12 atopic subjects with no history of clinical symptoms to walnut and a negative ImmunoCAP score and who were HLA matched were also recruited as control subjects for this study. DNA samples were HLA typed with Dynal Unitray SSP Kits (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions.

Ex vivo analysis of walnut-specific $CD4^+$ T cells

A peptide library was generated based on Jug r 1, Jug r 2, and Jug r 3 sequences. The library consisted of overlapping peptides spanning the entire allergen, which were 20 amino acids in length with a 12-amino-acid overlap synthetized by Mimotopes (Clayton, Australia). Peptides were dissolved in dimethyl sulfoxide. For detection of $CD154^+$-specific T cells, 35 million PBMCs (at 7×10^6 cells/mL) in culture medium (RPMI 1640 [Gibco, Carlsbad, Calif] plus 10% pooled human serum plus 1% PenStrep) were stimulated with 5 μg/mL synthesized peptide pools and 1 μg/mL anti-CD40 (Miltenyi Biotec, Auburn, Calif) for 3 (for frequency and surface phenotype) and 6 (for intracellular cytokine staining [ICS]) hours at 37°C. Cells were also mock stimulated with dimethyl sulfoxide (0.05% final concentration) as a negative control. After stimulation, cells were stained with phycoerythrin (PE)–conjugated CD154 (Miltenyi Biotec) and labeled with anti-PE magnetic beads (Miltenyi Biotec) for 20 minutes at 4°C. A 1:100 fraction of cells was saved for analysis. The other fraction was passed through a Miltenyi magnetic column; magnetically enriched cells were next stained with a panel of antibodies of interest for 20 minutes at room temperature. After staining, cells were stained again with Via-probe$^+$ (BD Biosciences, East Rutherford, NJ) for 10 minutes at 4°C before flow cytometry. Data acquisition was performed with an LSR II flow cytometer, and data were analyzed with FlowJo software (Tree Star, Ashland, Ore). Frequency was calculated as previously described for tetramer analysis.[21]

Ex vivo analysis with peptide–major histocompatibility complex class II tetramers was carried out, as previously described.[21] For more information, see the Methods section in this article's Online Repository at www.jacionline.org.

TGEM

Peptide-loaded HLA-DR proteins were generated, as previously described.[22,23] The TGEM procedure was conducted, as previously described.[20] Briefly, PBMCs were stimulated with peptide pools, cultured for 14 days, and then stained with pooled peptide tetramers. Cells from wells that produced positive staining were rescreened with tetramers loaded with each peptide from the positive pool.

For more information on ICS, T-cell cloning procedures, and RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR, see the Methods section in this article's Online Repository.

Statistical analysis

Statistical analysis was performed with the tests indicated in the figure legends and Prism 5.0 software (GraphPad Software, La Jolla, Calif).

RESULTS

Jug r 2–reactive $CD4^+$ T cells dominate the immune response in patients with walnut allergy

Upregulation of CD154 in $CD4^+$ T cells after 3 hours of stimulation of PBMCs with Jug r 1, Jug r 2, and Jug r 3 peptide libraries was used to evaluate frequencies of walnut allergen–reactive $CD4^+$ T cells in allergic and nonallergic subjects. For nonallergic subjects, magnitudes of Jug r 1, Jug r 2, and Jug r 3 T-cell responses were low (average frequencies of 1.3 ± 0.6, 4.8 ± 1.7, and <1 per million $CD4^+$ T cells, respectively; Fig 1, *A*, and see Fig E1 in this article's Online Repository at www.jacionline.org). In the allergic group stimulation with Jug r 1 peptide libraries induced $CD154^+CD4^+$ T cells (average frequency of 10.2 ± 3.4 per million $CD4^+$ T cells). Jug r 3 responses were nearly absent (average frequency of 0.8 ± 1.8 per million $CD4^+$ T cells), even among the Spanish cohort. In contrast, Jug r 2 responses were strong (average 20.4 ± 3.6 per million $CD4^+$ T cells). The average frequency of Jug r 2 responses was 2-fold greater than that of Jug r 1 T-cell immune responses in allergic subjects and 4-fold greater than that of Jug r 2 T-cell immune responses in nonallergic subjects. Thus Jug r 2–reactive $CD4^+$ T cells dominate the walnut allergen–specific T-cell repertoire in patients with walnut allergy. A correlation between Jug r 1–, Jug r 2–, and Jug r 3–specific IgE with their respective allergen-reactive T-cell frequencies was not observed (data not shown). Lack of correlation might be due to high cross-reactivity at the IgE level between tree nuts or unlinked cognate T-cell/B-cell cooperation, the latter of which has been observed with other allergens.[24]

Identification of $CD4^+$ T-cell epitopes in walnut allergen Jug r 2

The TGEM approach was used to identify $CD4^+$ T-cell epitopes within the dominant walnut allergen Jug r 2 (see Fig E2

TABLE I. HLA and allergic status of recruited subjects

ID	Age (y)	Sex	HLA (*DRB1**)	sIgE to walnut (f256 [kU/L])	Skin prick test	Jug r 1 (ISAC ISU)	Jug r 2 (ISAC ISU)	Jug r 3 (ISAC ISU)	Symptoms to walnut	Asthma
Patients with walnut allergy										
1	26	F	07:01, 14:01	38.6	Not tested	12.46	6.82	0	I, III, V, VII, VIII	Yes
2	9	F	07:01, 15:01	26.1	Not tested	3.11	1.42	0	I, II, V	No
3	10	M	15:01, 09:01	81.7	6 × 4 mm	48.6	1.58	0	II, III, V	No
4	10	M	15:01, 14:02	23.4	6 × 10 mm	43.9	1.64	0.28	II, IV, VI, VII	Yes
5	10	M	04:01, 15:01	37	9 × 9 mm	11.26	1.88	0	I, III	Yes
6	34	F	04:04, 11:01	2.29	Not tested	0.26	3.81	0	I, II, III, VIII	No
7	23	F	01:01, 15:01	8.86	Not tested	3.69	0	0.06	I, II, III, IV, VI	No
8	10	F	01:01, 08:01	97.3	15 × 9 mm	81.5	1.46	0	III, IV, V	No
9	8	F	13:01, 15:01	1.51	7 × 7 mm	4.77	0	0	I, II, III	No
10	34	F	07:01, 15:01	77.7	15 × 10 mm	43.9	1.9	0.02	I, II, III, IV, V	No
11	27	F	15:01, 16:01	1.91	Not tested	0	0	1.18	II, III, IV, V, VII	No
12	14	F	04:05, 15:01	4.97	Not tested	0	4,36	7.3	II, IV, V	Yes
Walnut-sensitized subjects										
13	36	F	01:01, 04:04	0.39	12 × 12 mm	0.34	0	0		No
14	18	F	01:01, 13:01	0.58	5 × 5 mm	0	0	0.30		Yes
15	15	F	11:01, 13:01	>100	Not tested	—	—	—		Yes
16	26	M	15:01, 03:01	22.5	Not tested	0	0	5.5		No
17	24	M	04:03, 07:01	1.07	Not tested	0	0	0.95		No
Nonatopic subjects										
18	28	M	04:01, 15:01	0	Not tested	—	—	—	Absent	No
19	29	M	01:01, 03:01	0	Not tested	—	—	—	Absent	No
20	31	F	07:01, 07:01	0	Not tested	—	—	—	Absent	No
21	37	M	03:01, 0401	0	Not tested	—	—	—	Absent	No
22	26	M	04:03, 15:01	0	Not tested	—	—	—	Absent	No
23	34	M	07:01, 13:01	0	Not tested	—	—	—	Absent	No
24	32	F	15:01, 15:01	0	Not tested	—	—	—	Absent	No
25	31	F	07:01, 08:01	0	Not tested	—	—	—	Absent	No
Atopic subjects without walnut allergy										
26*	26	F	04:01, 15:01	0	Not tested	—	—	—	Absent	Yes
27	56	M	11:0, 15:01	0	Not tested	—	—	—	Absent	No
28*	20	M	13:02, 07:01	0	Not tested	—	—	—	Absent	Yes
29	41	F	09:01, 15:01	0	Not tested	—	—	—	Absent	Yes
30	28	M	01:01, 07:01	0	Not tested	—	—	—	Absent	Yes
31	47	F	09:01, 15:01	0	Not tested	—	—	—	Absent	Yes
32*	63	M	15:01, 01:01	0	Not tested	—	—	—	Absent	Yes
33*	56	F	07:01, 03:01	0	Not tested	—	—	—	Absent	Yes
34	53	M	11:01, 15:01	0	Not tested	—	—	—	Absent	No
35	48	F	11:01, 15:01	0	Not tested	—	—	—	Absent	No

Symptoms to walnut: *I*, Itchy mouth, lips, and/or pharynx; *II*, abdominal discomfort and/or diarrhea; *III*, nausea or vomiting; *IV*, severe skin itching or hives, acute or angioedema; *V*, rhinitis and/or conjunctivitis and/or respiratory compromise; *VI*, dizziness (feeling loss of consciousness); *VII*, syncope (loss of consciousness); and *VIII*, desaturation with respiratory compromise.

*Subjects also had a history of peanut and a positive IgE ImmunoCAP result for peanut.

in this article's Online Repository at www.jacionline.org). A total of 11 immunogenic epitopes restricted to *DRB1*01:01*, *DRB1*01:03*, *DRB1*03:01*, *DRB1*04:01*, *DRB1*04:02*, *DRB1*04:04*, *DRB1*07:01*, *DRB1*09:01*, *DRB1*11:01*, *DRB1*14:01*, and *DRB1*15:01* were identified (Table II). The peptides Jug r $2_{152\text{-}171}$, Jug r $2_{184\text{-}203}$, Jug r $2_{224\text{-}243}$, Jug r $2_{392\text{-}411}$, Jug r $2_{456\text{-}475}$, and Jug r $2_{520\text{-}539}$ were presented by 3 or more different *DRB1* alleles. Identical epitopes were identified in allergic and nonallergic subjects (data not shown).

High frequencies of Jug r 2–reactive $CD4^+$ T cells in peripheral blood of allergic subjects

Frequency of Jug r 2–specific $CD4^+$ T cells was also examined by means of direct *ex vivo* staining with Jug r 2 tetramers (Fig 1, *B*, and see Fig E3 in this article's Online Repository at www.jacionline.org). Each subject was stained with a panel of tetramers corresponding to the HLA of the subject (see Table E1 in this article's Online Repository at www.jacionline.org). In nonallergic subjects the frequency of Jug r 2–specific $CD4^+$ T-cell responses was low, with an average frequency of 6.3 ± 0.8 per 10^6 $CD4^+$ T cells. Within the memory compartment ($CD45RA^-$), the average frequency was 2.9 ± 0.6 per 10^6 $CD4^+$ T cells. Conversely, the average frequency of Jug r 2–specific $CD4^+$ T cells in allergic subjects was 26.53 ± 2.26 per 10^6 $CD4^+$ T cells, which was at least 4-fold higher compared with that seen in nonallergic subjects. The average frequency within the $CD45RA^-$ compartment was 18.34 ± 1.72 reactive $CD4^+$ T cells per 10^6 $CD4^+$ T cells. These tetramer staining frequency data agree with results from the CD154 assays and confirm that Jug r 2–reactive $CD4^+$ T cells are present in higher frequencies in PBMCs of allergic compared with nonallergic subjects.

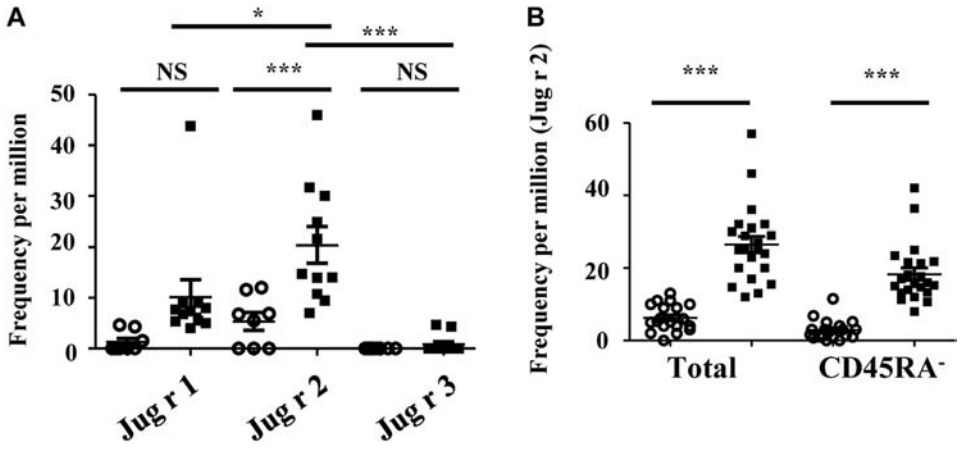

FIG 1. Frequencies of walnut allergen–reactive $CD4^+$ T cells. **A,** Frequencies of Jug r 1–, Jug r 2–, and Jug r 3–reactive T cells in patients with walnut allergy (n = 11; *solid squares*) and nonallergic subjects (n = 8; *open circles*), as determined by using CD154 assays. Each data point represents the frequency of T cells reactive to each allergen. ANOVA (with the Bonferroni correction) was used to compare all columns in statistical analysis. **B,** Frequencies of Jug r 2 epitope–reactive T cells in patients with walnut allergy (n = 17; *solid squares*) and subjects without walnut allergy (n = 19; *open circles*), as determined by using tetramer assays. Each data point represents the frequency of T cells specific to a combination of epitopes in Jug r 2. The Student *t* test was used in statistical analysis. $*P < .05$ and $***P < .0001$. *NS*, Not significant.

TABLE II. Jug r 2 $CD4^+$ T-cell epitopes

		HLA *DRB1* restriction											
Jug r 2	Amino acid sequence	01:01	01:02	01:03	03:01	04:01	04:02	04:04	07:01	09:01	11:01	14:01	15:01
Jug r $2_{152\text{-}171}$	EQQRHNPYYFHSQSIRSRH		•			•		•	•		•		
Jug r $2_{184\text{-}203}$	FTERTELLRGIENYRVVILD	•		•				•			•		•
Jug r $2_{224\text{-}243}$	TRGRATLTLVSQETRESFNL						•	•	•				
Jug r $2_{280\text{-}299}$	PGQFREYYAAGAKSPDQSYL			•					•				
Jug r $2_{296\text{-}315}$	QSYLRVFSNDILVAALNTPR												•
Jug r $2_{360\text{-}379}$	SGGPISLKSESPSYSNQFGQ								•				
Jug r $2_{392\text{-}411}$	QEMDVLVNYAEIKRGAMMVP						•	•			•		•
Jug r $2_{416\text{-}435}$	KATVVVYVVEGTGRYEMACP								•				
Jug r $2_{456\text{-}475}$	TGRFQKVTARLARGDIFVIP	•		•					•	•		•	•
Jug r $2_{520\text{-}539}$	EAKELSFNMPREEIEEIFES	•			•				•				
Jug r $2_{536\text{-}555}$	IFESQMESYFVPTERQSRRG				•								

Surface phenotype of Jug r 2–specific $CD4^+$ T cells

The surface phenotypes of Jug r 2–specific T cells were determined by means of direct *ex vivo* staining of PBMCs (Fig 2, *A*). A higher percentage of tetramer-positive cells in the nonallergic group expressed CXCR3 (T_H1 marker) compared with those in the allergic group (Fig 2, *B*). However, because of the higher frequency of total Jug r 2–specific T cells in the allergic group compared with the nonallergic group, the average frequency of T_H1 allergen-specific T cells in both groups was similar (Fig 2, *C*). Conversely, a higher percentage of tetramer-positive cells in the allergic group expressed CCR4 and chemoattractant receptor–homologous molecule expressed on T_H2 cells (CRTH2; T_H2 markers)[25,26] compared with the nonallergic group (Fig 2, *B*). A significant difference in the percentage of Jug r 2–specific T cells that lost CD27 expression was also observed between the 2 groups, with $CD27^-$ Jug r 2–specific T cells present only in the allergic group. Thus in the allergic group there were higher frequencies of $CCR4^+$, $CRTH2^+$, and $CD27^-$ Jug r 2–specific effector T (Teff) cells compared with the nonallergic group (Fig 2, *C*). Although $CD27^-$ Jug r 2–specific Teff cells were present, there were still higher percentages of $CD27^+$ Jug r 2–reactive T cells compared with $CD27^-$ Jug r 2–specific cells in the allergic group. The majority of these tetramer-positive $CD27^+$ T cells also coexpressed CCR7 and CD62 ligand, suggesting these $CCR4^+CD27^+CCR7^+$ cells are central memory T (T_{CM}) cells (Fig 2, *D*, and data not shown).[27-29] It should also be noted that most Jug r 2–reactive T cells in allergic subjects were $CRTH2^-$. Although there was no difference in the percentage of Jug r 2–specific T cells that were $CCR6^+$ (T_H17 subset marker and gut-homing marker)[30-32] between the 2 groups, the percentage of $CCR4^+CCR6^+$ (T_H17 subset marker) Jug r 2–specific cells between the 2 groups was different (Fig 2, *E*). On average, 32.6% of the Jug r 2–specific T cells from the allergic subjects had a T_H17 phenotype and were essentially absent in nonallergic subjects.

Also of interest, T_H17 Jug r 2–reactive T cells were detected in both nonasthmatic and asthmatic patients with walnut allergy (Fig 2, *F*), suggesting there is no link between asthmatic status and the appearance of this cell type. In contrast, 20.4% of Jug r 2–reactive T cells expressed $CXCR3^+CCR6^+$ (T_H1/T_H17 subset markers) in nonallergic subjects, which was significantly higher than that seen in allergic subjects (Fig 2, *E*). No difference

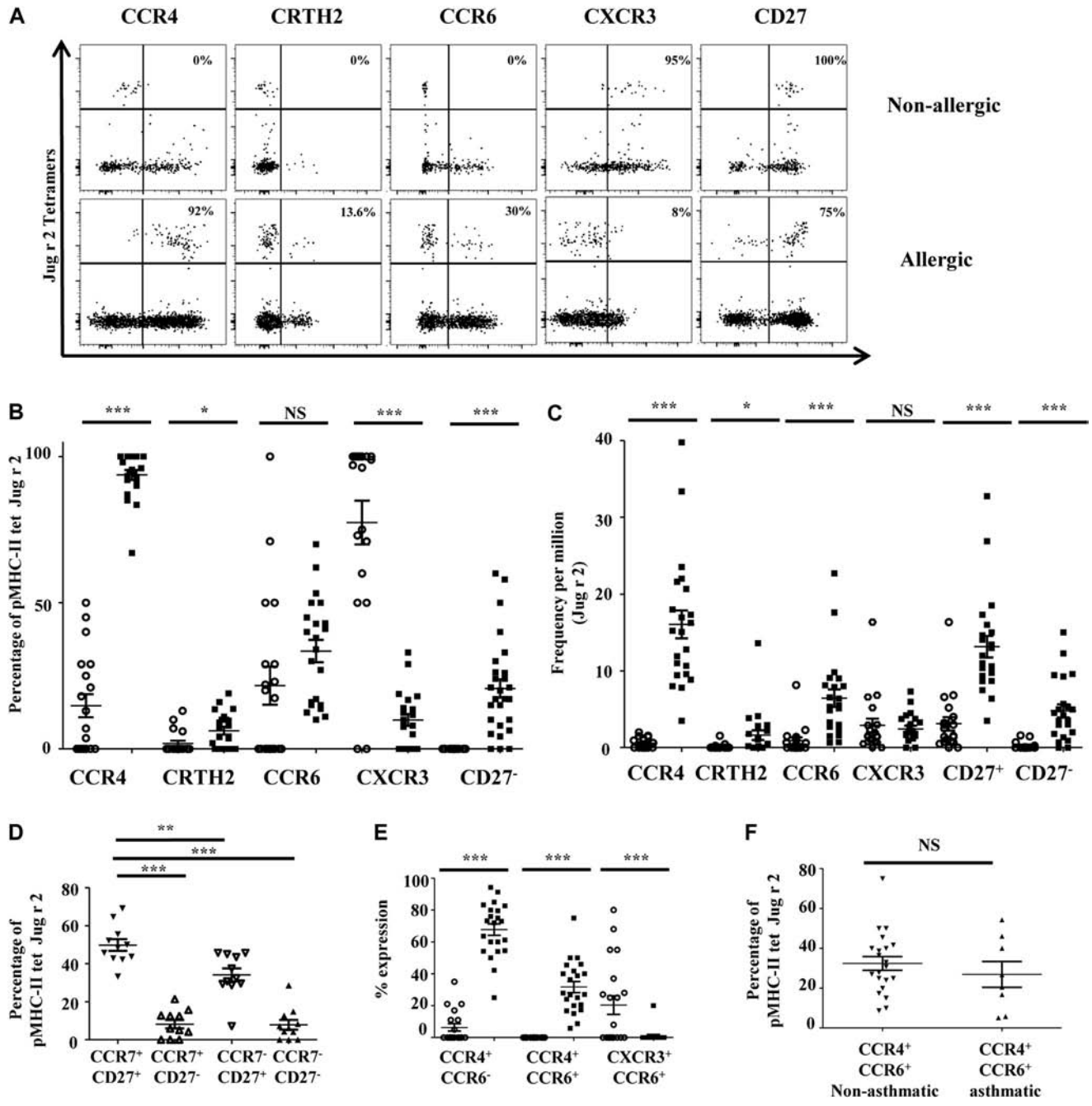

FIG 2. Phenotypes of Jug r 2–reactive T cells. **A,** *First row*, Profile in a *DRB1*15:01* nonallergic subject. *Second row*, Profile in a *DRB1*15:01* allergic patient. Percentages of surface markers expressed by Jug r 2–specific T cells are as indicated. **B,** *Ex vivo* expression of CCR4, CRTH2, CCR6, CXCR3, and CD27 of Jug r 2–specific T cells in patients with walnut allergy (n = 17; *solid squares*) and patients without walnut allergy (n = 19; *open circles*). Each data point represents the percentage of tetramer-positive T cells with marker expression. **C,** *Ex vivo* frequencies of CCR4, CRTH2, CCR6, CXCR3, and CD27 expressing Jug r 2–specific T cells per million $CD4^+$ T cells in patients with walnut allergy (n = 17; *solid squares*) and subjects without walnut allergy (n = 19; *open circles*). Each data point represents the frequency of T cells specific to a combination of epitopes in Jug r 2. **D,** Tetramer-positive $CD45RA^-$ T cells were gated against CCR7 and CD27. Data are representative of 8 allergic subjects. **E,** Tetramer-positive $CD45RA^-$ T cells were gated against CCR4 and CCR6 and CXCR3 and CCR6. Each data point represents results for surface expression in tetramer-positive T cells from 17 patients with walnut allergy *(solid squares)* and 19 subjects without walnut allergy *(open circles)*. **F,** Surface expression of CCR4 and CCR6 was analyzed on tetramer-positive T cells from nonasthmatic subjects and asthmatic patients with walnut allergy. The Student *t* test was used in the statistical analysis for Fig 2, *B*, *C*, *E*, and *F*. ANOVA (with the Bonferroni correction) was used to compare all columns in the statistical analysis for Fig 1, *D*. **P* < .05, ***P* < .001, and ****P* < .0001. *NS*, Not significant; *pMHC-II*, peptide–major histocompatibility complex class II.

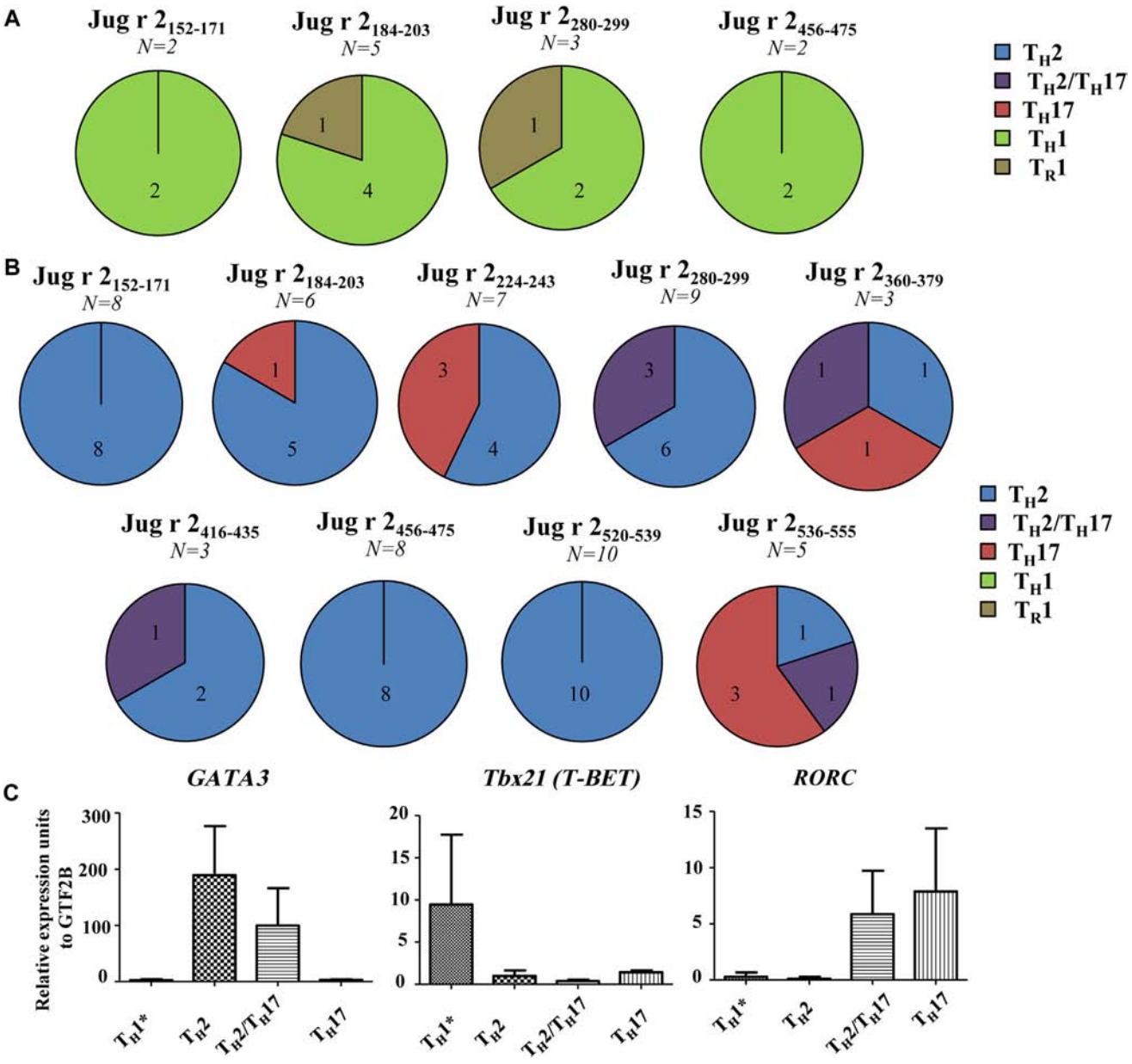

FIG 3. Jug r 2 T-cell subsets. **A** and **B,** Phenotype of TCCs derived from 6 nonallergic (Fig 3, *A*) and 8 allergic (Fig 3, *B*) subjects. Phenotype profiles were based on surface marker expression and cytokine production, the number of TCCs are as follows: T_H2, 45 TCCs; T_H2/T_H17, 6 TCCs; T_H17, 8; and T_H1 (derived from nonallergic subjects), 12 TCCs). Numbers of TCCs for each profile and specificity are as indicated. Percentages of clones for each specificity are presented as mean values from each group in pie charts. **C,** mRNA levels corresponding to *GATA3*, *TBX21*, and *RORC* were assessed by using quantitative PCR. Data were expressed as relative amounts of cytokine mRNA in Jug r 2 epitope–specific TCCs derived from 6 nonallergic and 8 allergic subjects. Data were normalized based on relative amounts of *GTF2B* mRNA.

was observed in total T-cell frequencies (27.5 ± 2.5 vs 19.91 ± 3.4 per 10^6) and T-cell phenotypes (data not shown) between subjects with or without a history of walnut ingestion. However, a significant difference was observed in memory T-cell frequencies (19.46 ± 1.96 vs 11.98 ± 1.67 per 10^6) between these 2 groups.

Cytokine profiles of Jug r 2–specific $CD4^+$ T cells in nonallergic and allergic subjects

Jug r 2–specific T cells were single cell sorted for generation of T-cell clones (TCCs). In nonallergic subjects a total of 12 TCCs were generated. All TCCs from nonallergic subjects elicited a distinct protective T_H1/T_R1 (IFN-γ and IL-10) or T_H1 (IFN-γ) response (Fig 3, *A*, and see Figs E4 and E5 in this article's Online Repository at www.jacionline.org). For subjects sensitized or allergic to walnut, a total of 59 TCCs were generated. All these clones were $CCR4^+CD27^-$. Three heterogenic profiles (T_H2, T_H2/T_H17, and T_H17 like) were observed (Fig 3, *B*, and see Figs E4 and E5). The first profile is exemplified by production of T_H2 cytokines (IL-4, IL-5, and IL-13). This T_H2 profile was detected for all epitopes tested. TCCs with the second profile show the ability to produce both IL-4 and IL-17A (T_H2/T_H17) and were

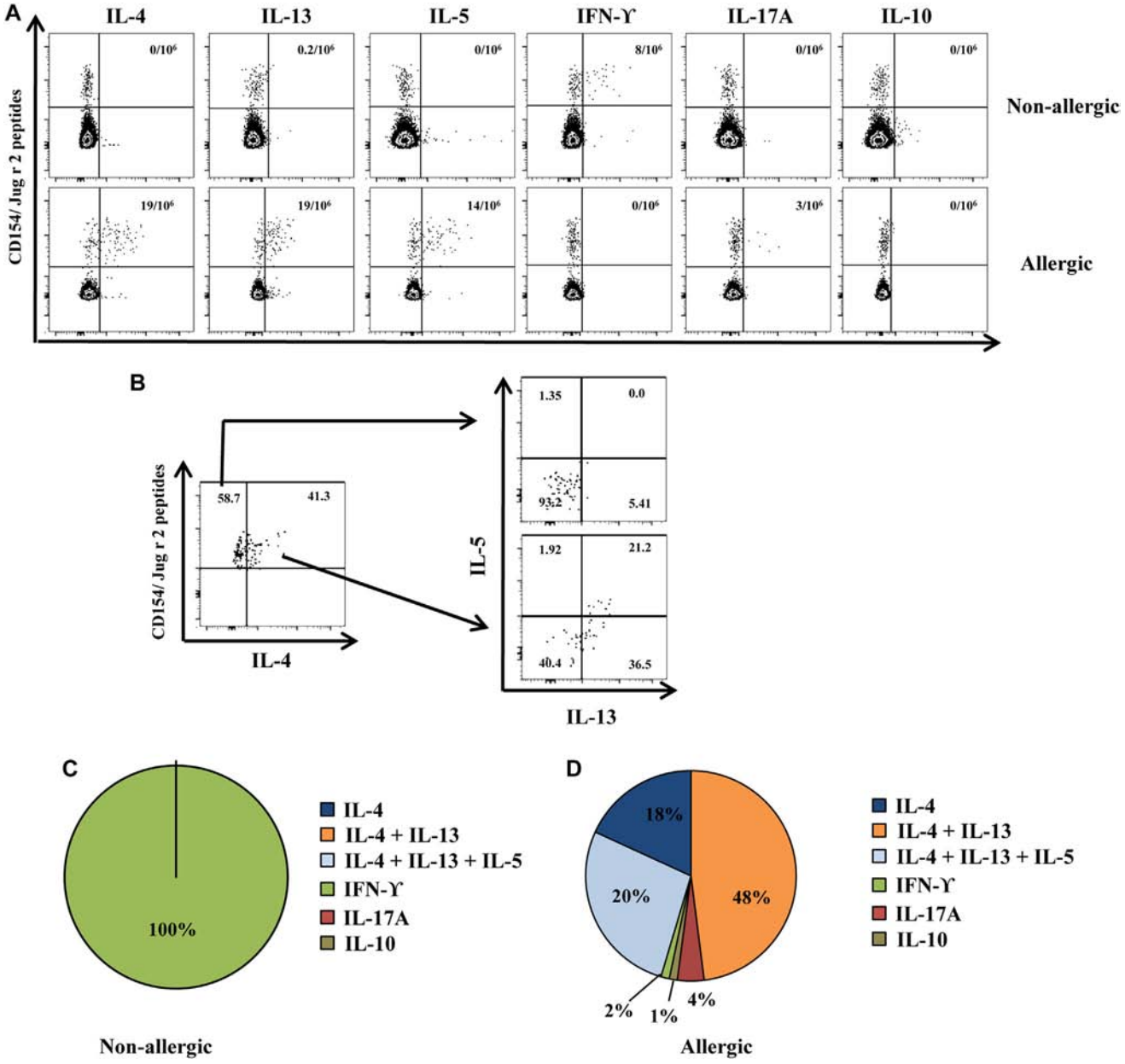

FIG 4. Cytokine profiles of Jug r 2–reactive T cells. **A,** *First row*, Cytokine profile in a *DRB1*15:01* nonallergic subject. *Second row*, Cytokine profile in a DR15:01 allergic subject. Frequencies of Jug r 2–specific cytokine-producing T cells per million $CD4^+$ T cells are as indicated. **B,** Gating strategy for identifying IL-4, IL-4 plus IL-13, and IL-4 plus IL-13 plus IL-5 Jug r 2–reactive T cells in this subject: IL-4, 40.4%; IL-4 plus IL-13, 36.5%; and IL-4 plus IL-13 plus IL-5, 21.2%. **C** and **D,** Cytokine profiles of Jug r 2–reactive T cells in nonallergic and allergic subjects. Data are representative of 11 patients with walnut allergy and 8 nonallergic subjects and are presented as the mean frequency of cytokine-producing T cells from each group in pie charts.

detected for 3 specificities only: Jug r $2_{280\text{-}299}$, Jug r $2_{360\text{-}374}$, and Jug r $2_{536\text{-}555}$. The third profile is exemplified by the production of IL-17A only (T_H17). In total, 23.7% of the TCCs obtained from allergic subjects were capable of producing IL-17A.

Transcript levels of 3 transcription factors of these clones were also assessed by using quantitative RT-PCR (Fig 3, *C*). In accordance with cytokine production profile, T_H1 clones derived from nonallergic subjects expressed the highest levels of *T-bet (TBX21)*. In allergic patients T_H2 clones mainly expressed *GATA3*. As expected, T_H2/T_H17 TCCs expressed *GATA3* and RAR-related orphan receptor C *(RORC)*, indicating their ability to produce IL-4 and IL-17A. Finally, T_H17 TCCs mainly expressed *RORC*.

The T_H1 responses in nonallergic subjects and T_H2 and T_H17 responses in allergic patients were also confirmed by means of direct *ex vivo* staining. For these experiments, PBMCs were costained for cytokine expression and CD154 after 6 hours of peptide stimulation. Nonallergic Jug r 2–reactive $CD4^+$ T-cell responses were dominated by production of IFN-γ; production of both IL-4 and IL-17A were absent in these subjects. On the other hand, T_H2

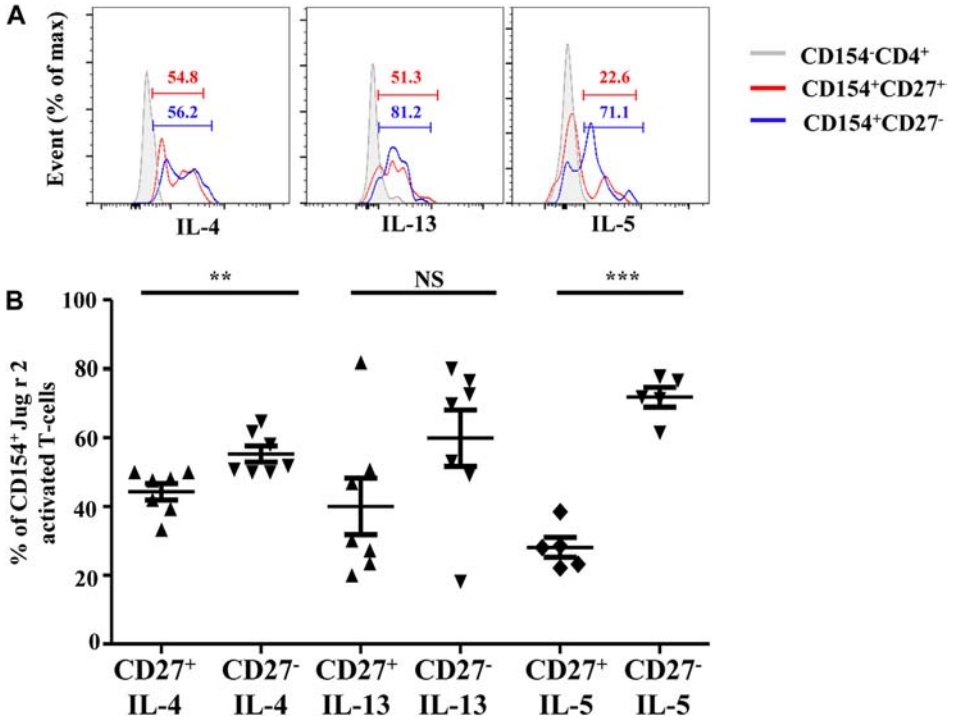

FIG 5. *Ex vivo* cytokine-producing capacities of $CD27^+$ and $CD27^-$ Jug r 2–reactive T cells. **A,** IL-4, IL-13, and IL-5 expression by $CD27^+$ cells *(red histogram)*, $CD27^-$ cells *(blue histogram)*, and $CD154^-CD4^+$ cells as a control *(gray histogram)*. **B,** Cytokine production by $CD27^+$ and $CD27^-CD154^+$ Jug r 2–reactive T cells in allergic subjects. Data are representative of 7 allergic subjects. The Student *t* test was used in statistical analysis. $^{**}P < .001$ and $^{***}P < .0001$. *NS,* Not significant.

and T_H17 responses were detected in allergic subjects, confirming our *in vitro* observations with TCCs. Interestingly, we also observed $CD154^+$ Jug r 2 T cells that were incapable of producing cytokines (average of 80.3% ± 3.84% and 52.5% ± 2.15% in nonallergic and allergic subjects, respectively). Examples of these *ex vivo* experiments are shown in Fig 4, *A*. Among the T_H2-producing cells, cells can also be classified as IL-4 producers, IL-4 and IL-13 producers, and IL-4, IL-5, and IL-13 producers. We did not detect cells that produced IL-13 alone. The gating strategy for identifying double and triple producers is shown in Fig 4, *B*. Results of experiments from 8 nonallergic subjects and 11 allergic patients are summarized in Fig 4, *C* and *D*.

Both T_{CM} and Teff cells are capable of producing T_H2 cytokines (Fig 5, *A*). Interestingly, Teff cells are more capable of producing IL-4 and IL-5 compared with T_{CM} cells (Fig 5, *B*). These data are in agreement with earlier studies, which suggest that loss of CD27 correlates with an increase in IL-4 production and that Teff cells are more terminally differentiated cells.[27,33,34] In contrast to the TCC data, we did not observe a T_H1/T_R1 profile in nonallergic subjects or a T_H2/T_H17 profile in allergic patients. Different kinetics of cytokine production for these cell types and the rarity of these cells might account for these different outcomes using these 2 approaches.

DISCUSSION

Allergic reactions to walnut account for the vast majority of severe reactions in patients with tree nut allergy in the United States.[1] Contributions of $CD4^+$ T cells to this disease remain elusive. In particular, walnut-specific $CD4^+$ T-cell epitopes have not been identified. Frequency and phenotype of walnut allergen–specific T cells have not been examined *ex vivo*. In this study both the class II tetramer and CD154 upregulation assays were used to examine walnut allergen–specific T cells. Jug r 2 was identified as the major walnut allergen that elicits $CD4^+$ T cell responses. Hot spots with promiscuous Jug r 2 $CD4^+$ T-cell peptides presented by multiple *DRB1* alleles were identified. Six of the Jug r 2 peptides (20 amino acids each) can be presented by at least 3 different *DRB1* alleles, including 3 peptides that can be presented by at least 5 different *DRB1* alleles. These 6 promiscuous epitope regions should be good candidates for peptide vaccine to desensitize patients with walnut allergy.

Consistent with previous studies,[18,33-36] allergen-tolerant subjects and allergic patients recognize identical allergen-derived epitopes. However, Jug r 2–reactive T cells are present at substantially lower frequencies in nonallergic subjects (Fig 1). In addition, surface phenotypes and functional properties of these T cells are distinct in nonallergic and allergic subjects. Through direct *ex vivo* analysis of PBMCs and analysis of TCCs, $CXCR3^+$ Jug r 2–specific T cells with predominant IFN-γ and low IL-10 production were observed in nonallergic subjects. On the other hand, $CCR4^+$ T cells that produced T_H2 and T_H17 cytokines were exclusively observed in allergic subjects. It remains a possibility that walnut allergen–specific T_H2 cells are also present in nonallergic subjects at a very low frequency, which is less than the threshold of the detection method. Results obtained from either the tetramer assay or CD154 upregulation assays are compatible. Antigen-specific T cells, which were cytokine nonproducers, were also detected with both assays.

A different differentiation state and stage of cell cycle should be responsible for the heterogeneous cytokine production capacity.[37,38]

We have previously demonstrated that only terminally differentiated ($CD27^-$) allergen-specific T cells from patients with pollen allergy display a T_H2 phenotype[33,34] and that lack of CD27 expression coincides with CRTH2 expression.[34] In the present study Jug r 2–specific terminally differentiated T_H2 T cells were present in allergic patients and were essentially absent in nonallergic subjects. In addition, both $CD27^+CRTH2^-CCR4^+$ T_{CM} and $CD27^-CRTH2^+CCR4^+$ Teff cells were present in allergic patients with T_{CM} as the most prevalent phenotype. We also demonstrated that both T_{CM} and Teff cells were capable of producing T_H2 cytokines. A previous report showed that $CCR4^+$ T_{CM} cells in human subjects are capable of producing IL-4, even though they are not fully differentiated.[29] Variable expressions of CRTH2 have been previously observed for Ara h 1–reactive[18] and Pen m 2–reactive[39] T cells, and these cells were capable of producing IL-4. Accumulating evidence proposes that CD27 is lost after repetitive antigenic stimulation,[40,41] and loss of CD27 after TCC generation suggests that occasional antigen stimulation is essential for the expression of CRTH2 in food allergen–specific T cells.[18] Food avoidance in patients with walnut allergy might have resulted in the accumulation of $CRTH2^-$ T_{CM} cells in peripheral blood of allergic subjects. The results from this study do not contradict those our previous pollen studies[33,34] because patients with pollen allergy are subjected to annual challenges of high doses of pollens during the pollen season. The presence of allergen-specific T_{CM} cells as a consequence of food avoidance in patients with food allergy might complicate the treatment of food allergy because T_{CM} cells are less susceptible to deletion by means of allergen-specific immunotherapy, as shown in a murine model.[42]

It has been demonstrated that IL-17A can promote class-switching to IgE[43] and that IL-17A–producing $CD4^+$ T cells are more frequent on allergic patients.[43-45] T_H2/T_H17 cells are also observed in patients with allergic asthma.[46-48] However, the involvement of T_H17 cells in patients with food allergy remains obscure. In the current study a subpopulation of $CCR4^+CCR6^+$ Jug r 2 T cells, which produced IL-17A alone or IL-4 and IL-17A, was detected in both asthmatic and nonasthmatic patients with walnut allergy. These results are consistent with previous studies with food allergens[18,39] in which T_H2/T_H17 allergen-specific T cells have been previously described. These data implicate a direct association of $CCR4^+CCR6^+$ antigen-specific $CD4^+$ T_H2/T_H17 cells with food allergy, disregarding asthmatic status. This is a possibility because CCR6 is also a gut-associated lymphoid tissue–associated homing marker.[30,31] A murine model also suggests that CCR6 plays a role in the development of gastrointestinal allergic disease.[49] On the other hand, Dhuban et al[50] recently suggested that T_H17 responses are impaired in children with food allergy and that lack of T_H17 responses might play a potential role in food tolerance. The presence of this population in allergic subjects raises important questions about the pathophysiologic role of these $CCR4^+CCR6^+$ food allergen–specific $CD4^+$ T cells in patients with food allergy in general. Future effort should be committed to examine whether $CCR4^+CCR6^+$ allergen-specific cells are more prevalent in patients with food allergy than in patients with airborne allergy.

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Key messages

- **Jug r 2 is the dominant walnut allergen recognized by T cells.**
- **The predominant phenotype for Jug r 2–reactive T cells is the central memory phenotype.**
- **Walnut-specific T cells with T_H2, T_H17, and T_H2/T_H17 phenotypes could be detected in nonasthmatic and asthmatic patients with walnut allergy.**

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METHODS

Ex vivo analysis of walnut-specific CD4$^+$ T cells

The frequency of Jug r 2–specific T cells was measured, as previously described.[E1] Briefly, 30 million PBMCs in 200 μL of T-cell culture medium were stained with 20 μg/mL PE-labeled tetramers (tetramers being used are shown in Table E1) for 100 minutes. Cells were then washed and incubated with anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 minutes at 4°C, and a 1:100 fraction was saved for analysis; the other fraction was passed through a magnetic column (Miltenyi Biotec). Bound PE-labeled cells were flushed and collected. Cells in the bound and precolumn fractions were stained with a panel of antibodies of interest for 20 minutes at room temperature. After staining, cells were stained with Via-probe$^+$ (BD Biosciences) for 10 minutes at 4°C before flow cytometry. Data were analyzed by using FlowJo software (Tree Star), gating on forward scatter/side scatter and excluding CD14$^+$, CD19$^+$, and Via-probe populations. Frequency was calculated, as previously described.[E1] For phenotyping studies, antibodies were used against markers of interest: CCR4 (R&D systems, Minneapolis, Minn), CD45RA (eBioscience, San Diego, Calif), and CD38 (eBioscience).

ICS

For *ex vivo* ICS combined with the CD154 activation assay, BD GolgiStop was added during stimulation (BD Biosciences), according to the manufacturer's instructions. *In vitro* ICS combined with tetramer staining was performed, as previously described.[E2] Briefly, T-cell lines or TCCs were restimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 1 mg/mL ionomycin in the presence of 1× brefeldin A (eBioscience) for 5 hours at 37°C in a 5% CO_2 atmosphere. After 10 minutes at room temperature, cells were then fixed with fixation buffer (eBioscience) and washed twice with a permeabilization buffer (eBioscience). Cells were then stained with a panel of antibodies directed against cytokines (eBioscience and BD Biosciences) of interest for 20 minutes at room temperature; cells were washed and immediately analyzed in an LSR II flow cytometer.

T-cell cloning procedure

T-cell lines were generated by staining T cells with tetramer directly *ex vivo*, and tetramer-positive CD4$^+$ and CD45RA$^-$ cells were sorted with a FACSAria (at single-cell purity). Cells were expanded in a 96-well plate in the presence of 1.0×10^5 irradiated PBMCs and 2 μg/mL PHA (Remel, Lenexa, Kan). T cells were rescreened with tetramers loaded with antigenic epitopes to assess positivity for the corresponding specificity. Five profiles (T_H2, T_H2/T_H17, T_H17-like, T_H1, and T_R1) of TCCs were arbitrarily defined as follows (Figs 3, E4, and E5): the T_H2 profile is exemplified by CCR4$^+$ with or without CRTH2 expression; production of IL-4 (≥10%), IL-5 (≥10%), and IL-13 (≥10%); and expression of *GATA3*. The T_H2/T_H17 profile is characterized by coexpression CCR4 and CCR6 and shows the ability to produce both IL-4 (≥10%) and IL-17A (≥10%) but no IFN-γ and IL-5 and to express both *GATA3* and *RORC*. The T_H17 profile is exemplified by coexpression of CCR4 and CCR6, production of IL-17A (≥10%) and sometimes low IFN-γ levels (10%), and expression of *RORC*. The T_H1 profile is exemplified by CXCR3 expression (some clones also coexpressed CD27), production of IFN-γ (≥10%), and expression of *T-bet (TBX21)*. The T_R1 profile is exemplified by CXCR3 expression (some clones also coexpressed CD27), production of IFN-γ (≥10%) and IL-10 (≥10%), and expression of *T-bet (TBX21)*.

RNA isolation, cDNA synthesis, and real-time quantitative RT-PCR

Total RNA was extracted from Jug r 2–specific TCCs derived from nonallergic and allergic subjects with the GenElute Mammalian total RNA Miniprep kit (Sigma-Aldrich, St Louis, Mo) and reverse transcribed as cDNAs with the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster City, Calif), according to the manufacturer's instructions. mRNAs were assessed by using quantitative PCR with predesigned TaqMan Gene Expression reagents (Applied Biosystems). Data were expressed as relative amounts of cytokine mRNA and normalized based on relative amounts of *GTF2B* mRNA.

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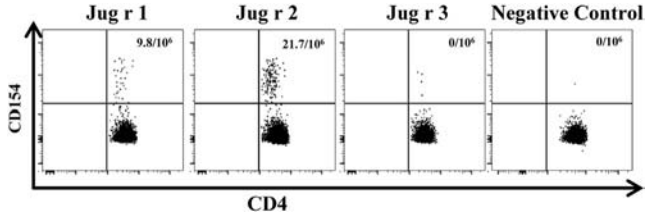

FIG E1. Frequencies of Jug r 1–, Jug r 2–, and Jug r 3–reactive T cells in a DR15:01 patient with walnut allergy. Frequencies of walnut allergen–reactive T cells per million $CD4^+$ T cells are as indicated.

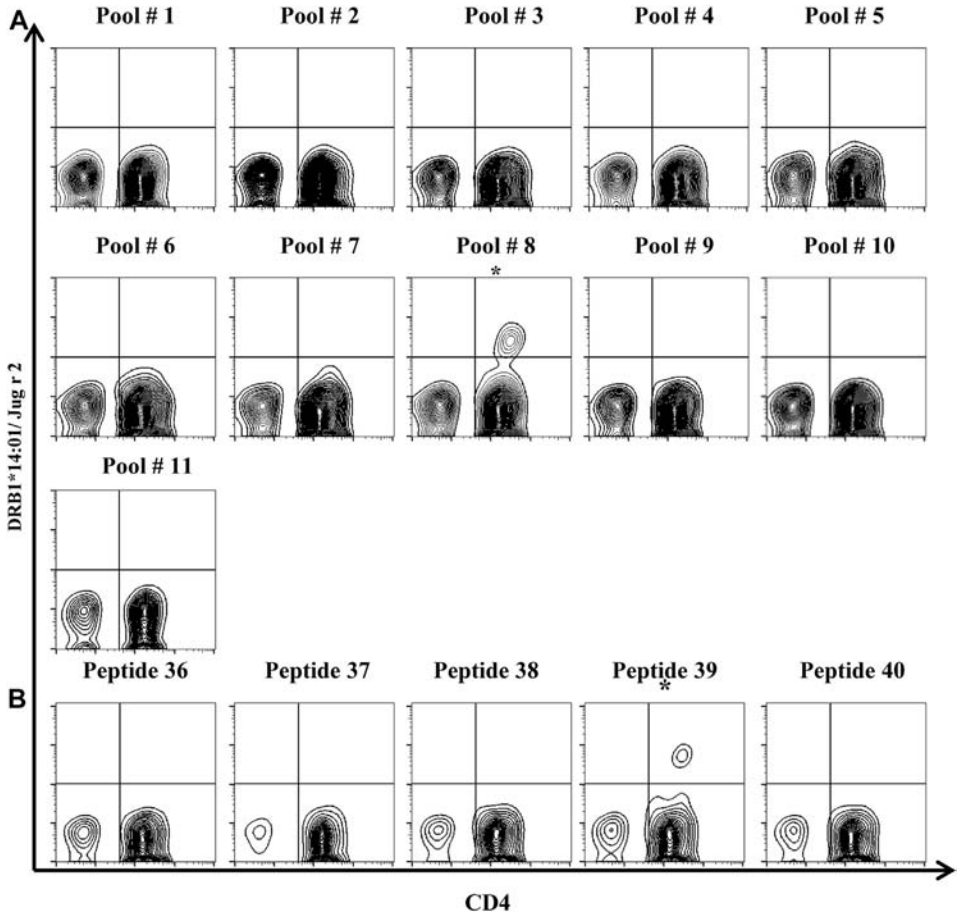

FIG E2. TGEM studies of DR14:01-restricted Jug r 2–reactive $CD4^+$ T cells. **A,** PBMCs from a DR14:01 patient with walnut allergy were stimulated with 11 pools of Jug r 2 peptides for 2 weeks and subsequently stained with corresponding DR14:01/Jug r 2–pooled peptide tetramers. **B,** Cells stimulated with pool 8 were restained with individual peptides from the corresponding pool. The staining identified p39 (Jug r $2_{456\text{-}475}$) as DR14:01-restricted Jug r 2 T-cell epitopes.

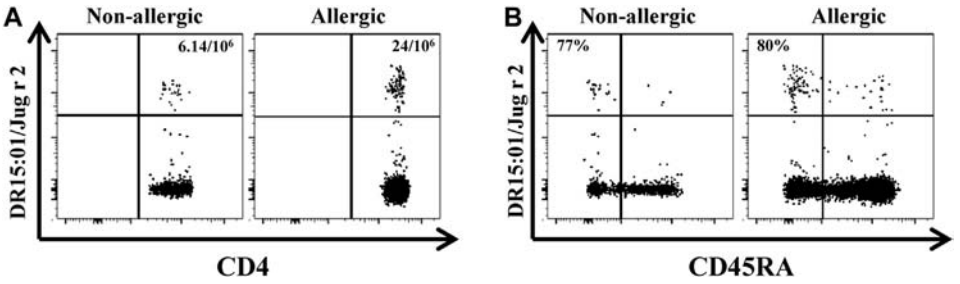

FIG E3. Frequencies of Jug r 2 epitope–reactive T cells. **A,** *Ex vivo* combined frequencies of Jug r $2_{184\text{-}203}$–, Jug r $2_{296\text{-}315}$–, Jug r $2_{392\text{-}411}$–, and Jug r $2_{456\text{-}475}$–specific T cells in DR15:01 allergic and nonallergic subjects. Frequencies of Jug r 2–specific T cells per million $CD4^+$ T cells are as indicated. **B,** *Ex vivo* percentages of memory ($CD45RA^-$) Jug r 2-specific T cells in DR15:01 allergic and nonallergic subjects.

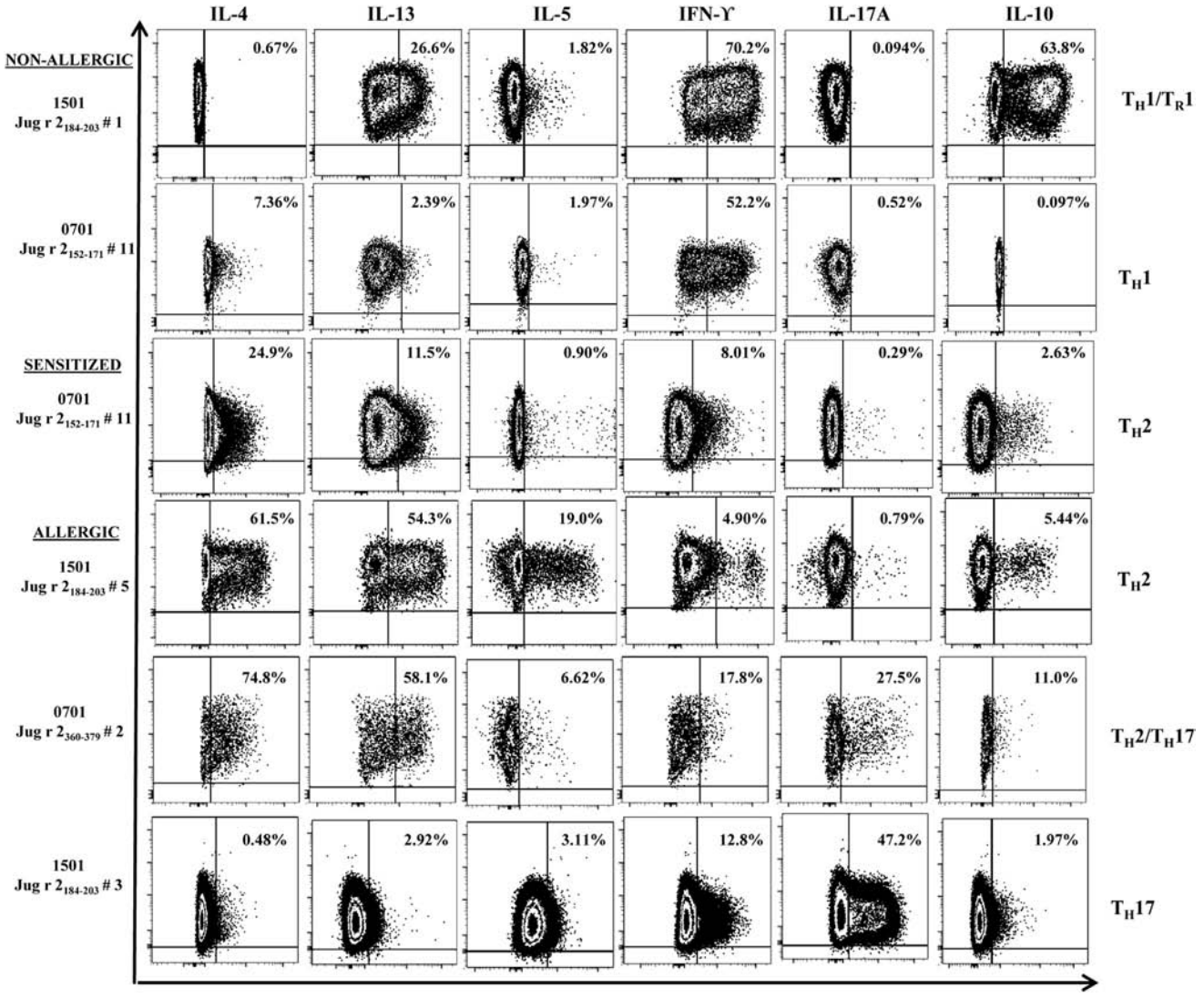

FIG E4. Cytokine profiles of *ex vivo* sorted TCCs generated from nonallergic (T_H1/T_R1 *[first row]* and T_H1 *[second row]*), sensitized (T_H2 *[third row]*), and allergic (T_H2 *[fourth row]*, T_H2/T_H17 *[fifth row]*, and T_H17 *[sixth row]*) subjects. Representative examples for Jug r $2_{184-203}$–, Jug r $2_{152-171}$–, and Jug r $2_{360-279}$–reactive TCCs. Two different phenotypes are observed for the same specificity within the same subject.

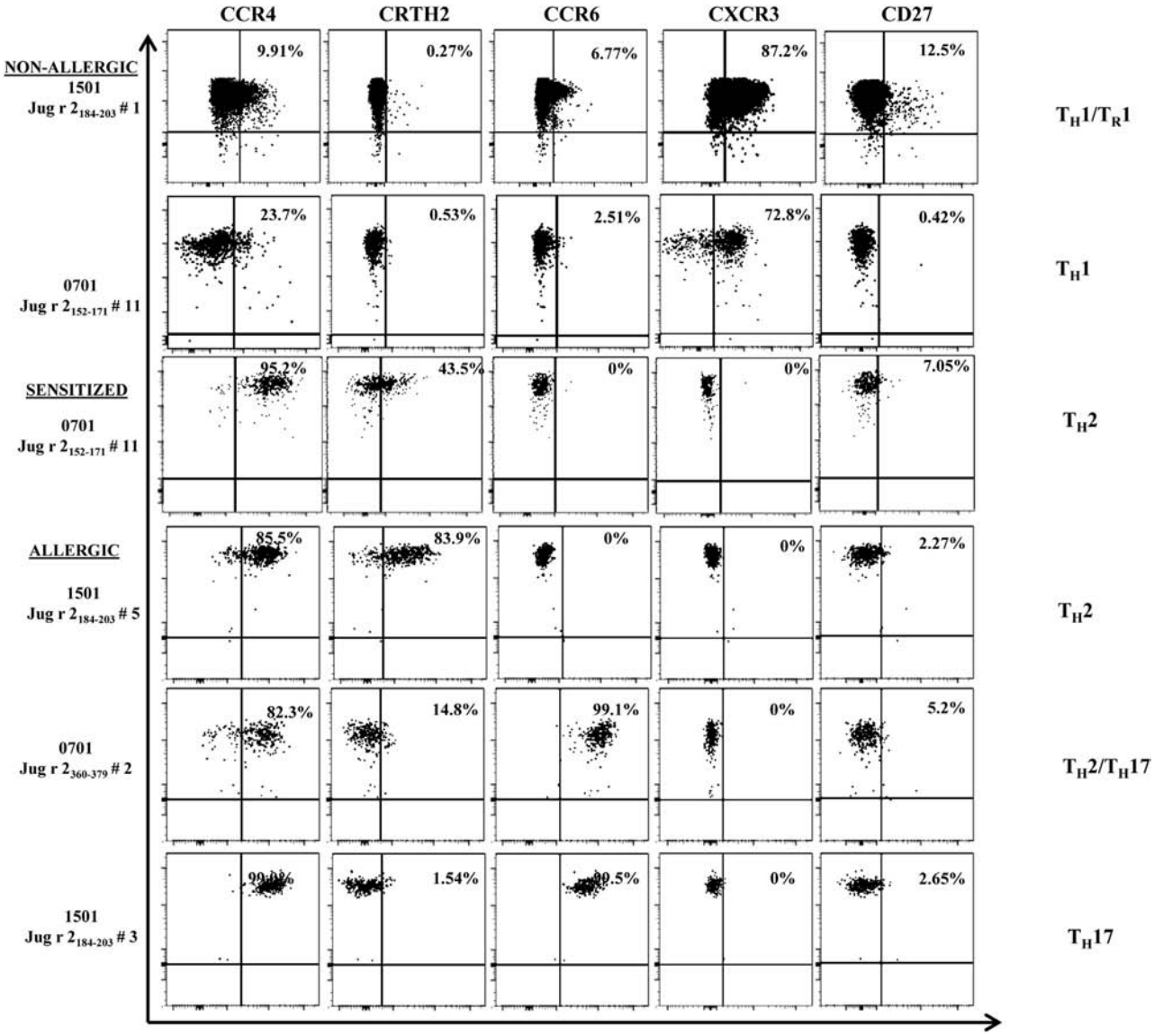

FIG E5. Surface phenotype of *ex vivo* sorted TCCs generated from nonallergic (T_H1/T_R1 *[first row]* and T_H1 *[second row]*), sensitized (T_H2 *[third row]*), and allergic (T_H2 *[fourth row]*, T_H2/T_H17 *[fifth row]*, and T_H17 *[sixth row]*) subjects. Representative examples for Jug r $2_{184-203}$–, Jug r $2_{152-171}$–, and Jug r $2_{360-379}$–reactive TCCs. Two phenotypes are observed for the same specificity within the same subject.

TABLE E1. MHC-II tetramers used in *ex vivo* analysis

Subject	HLA	Epitopes*	Tubes†
Patients with walnut allergy			
1	07:01, 14:01	0701/I, III, IV, VI, VIII, IX, X	1
2	07:01, 15:01	0701/I, III, IV, VI, VIII, IX, X	1
		1501/II, V, VII, IX	2
3	15:01, 09:01	1501/II, V, VII, IX	1
4	15:01, 14:02	1501/II, V, VII, IX	1
5	04:01, 15:01	1501/II, V, VII, IX	1
6	04:04, 11:01	0404/I, II, III, VII	1
		1101/I, II, VI	2
7	01:01, 15:01	0101/II, IX, X	1
		1501/II, V, VII, IX	2
8	01:01, 08:01	0101/II, IX, X	1
9	13:01, 15:01	1501/II, V, VII, IX	1
10	07:01, 15:01	0701/I, III, IV, VI, VIII, IX, X	1
		1501/II, V, VII, IX	2
11	15:01, 16:01	1501/II, V, VII, IX	1
12	04:05, 15:01	1501/II, V, VII, IX	1
Walnut-sensitized subjects			
13	01:01, 04:04	0101/II, IX, X	1
		0404/I, II, III, VII	2
14	01:01, 13:01	0101/II, IX, X	1
15	11:01, 13:01	1101/I, II, VI	1
16	15:01, 03:01	0301/X, XI	1
		1501/II, V, VII, IX	2
17	04:03, 07:01	0701/I, III, IV, VI, VIII, IX, X	1
Nonatopic subjects			
18	04:01, 15:01	1501/II, V, VII, IX	1
19	01:01, 03:01	0101/II, IX, X	1
		0301/X, XI	2
20	07:01, 07:01	0701/I, III, IV, VI, VIII, IX, X	1
21	03:01, 0401	0301/X, XI	1
22	04:03, 15:01	1501/II, V, VII, IX	1
23	07:01, 13:01	0701/I, III, IV, VI, VIII, IX, X	1
24	15:01, 15:01	1501/II, V, VII, IX	1
25	07:01, 08:01	0701/I, III, IV, VI, VIII, IX, X	1
Atopic subjects without walnut allergy			
26	04:01, 15:01	1501/II, V, VII, IX	1
27	11:01, 15:01	1101/I, II, VI	1
		1501/II, V, VII, IX	2
28	13:02, 07:01	0701/I, III, IV, VI, VIII, IX, X	1
29	09:01, 15:01	1501/II, V, VII, IX	1
30	01:01, 07:01	0101/II, IX, X	1
		0701/I, III, IV, VI, VIII, IX, X	2
31	09:01, 15:01	1501/II, V, VII, IX	1
32	15:01, 01:01	0101/II, IX, X	1
		1501/II, V, VII, IX	2
33	07:01, 03:01	0301/X, XI	1
		0701/I, III, IV, VI, VIII, IX, X	2
34	11:01, 15:01	1101/I, II, VI	1
		1501/II, V, VII, IX	2
35	11:01, 15:01	1101/I, II, VI	1
		1501/II, V, VII, IX	2

*Each roman number depicts an epitope. Epitopes used per tube are listed below: *I*, Jug r $2_{152\text{-}171}$; *II*, Jug r $2_{184\text{-}203}$; *III*, Jug r $2_{224\text{-}243}$; *IV*, Jug r $2_{280\text{-}299}$; *V*, Jug r $2_{296\text{-}315}$; *VI*, Jug r $2_{360\text{-}379}$; *VII*, Jug r $2_{392\text{-}411}$; *VIII*, Jug r $2_{416\text{-}435}$; *IX*, Jug r $2_{456\text{-}475}$; *X*, Jug r $2_{520\text{-}539}$; and *XI*, Jug r $2_{536\text{-}555}$.

†Specificities listed were combined per tube for analysis.