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

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Abstract

Background—Allergies to cashew are increasing in prevalence, with clinical symptoms ranging from oral pruritus to fatal anaphylactic reaction. Yet, cashew-specific T-cell epitopes and T-cell cross-reactivity amongst cashew and other tree nut allergens in humans remain uncharacterized.

Objectives—In this study, we characterized cashew specific T-cell responses in cashew allergic subjects and examined cross-reactivity of these cashew specific cells toward other tree nut allergens.

Methods—CD154 up-regulation assay was used to determine immunodominance hierarchy among cashew major allergens at the T cell level. 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.

Results—CD4⁺ T-cell responses were directed towards cashew allergens Ana o 1 and Ana o 2. Multiple Ana o 1 and Ana o 2 T-cell epitopes were then identified. These epitopes elicited either TH2 or TH2/TH17 responses in allergic subjects, which were 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.

Conclusions—Phylogenetically diverse tree nut allergens can activate cashew reactive T-cells and elicit a TH2 type response at an epitope specific level.

Clinical relevance—Lack of cross-reactivity between walnut and cashew suggest that cashew peptide immunotherapy approach may not be most effective for walnut.

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Keywords

Food allergy; cashew; tree nuts; Ana o 1; Ana o 2; cross-reactivity; T-cells; MHC class II tetramers; epitopes

INTRODUCTION

Tree nut allergies, like peanut allergies, are increasing in prevalence. They now 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, occur 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 (*Anacardium occidentale*) (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 is known to be allergenic in other tree nuts [12]. Furthermore, amino acid sequence alignments of vicilins, legumins and albumins indicate a high degree of amino acid identity 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 (specific IgE) level, owing to their botanic relatedness [15–17]. This phenomenon has also been demonstrated 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. Our results showed that allergic subjects have a predominant T_H2 (T_H T-helper) phenotype, however, T_H2/T_H17 responses were also detected. T-cell clones (TCC) specific to these epitopes were generated to assess cross-reactivity by tetramer co-staining and proliferation experiments. We found that TCC specific to cashew allergen derived epitopes could readily proliferate with hazelnut and pistachio, but not with walnut allergen derived peptides.

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, based on history of an acute reaction to cashew plus a positive ImmunoCAP score for cashew extract (>0.35 kU/L) (Phadia AB, Uppsala, Sweden), were recruited for this study. As an inclusion criteria, subjects with a low sIgE score to cashew need to have a large wheal size in the skin prick test (≥ 8 mm $\times$ 8 mm). Twelve non-atopic and 6 atopic subjects with no clinical symptoms to cashew, a negative ImmunoCAP score and HLA (Human histocompatibility leukocyte antigen)-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™ 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 synthesized 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].

Ex-vivo analysis of cashew-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 7×10^6 cells/mL) in culture medium (RPMI 1640 (Gibco) + 10% pooled human serum + 1% PenStrep) were stimulated with 5 μ g/mL of synthesized peptide pools (at a final concentration of 3 nM for Ana o 1 and Ana o 2 and 13 nM for Ana o 3), and 1 μ g/mL anti-CD40 (Miltenyi Biotec, Auburn, CA) for 3 hours (for frequency and surface phenotype) at 37°C. Cells were also mock stimulated with DMSO (0.05% final concentration) as negative control. After stimulation, cells were stained with PE (phycoerythrin)-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. To set gates for each phenotypic marker, T-cells were gated within the naïve compartment, as these markers are not expressed on naïve T-cells. Appropriate isotype antibody staining was also included to confirm positive staining of the marker used (Supplemental Figure 3A and 3B). 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 (Peptide/MHC class 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 µg/mL): Cashew (*Anacardium occidentale*, Ana o), Pistachio (*Pistacia vera*, Pis v), Hazelnut (*Corylus americana*, Cor a), Walnut (*Juglans regia*, Jug r) (Greer Laboratories, Lenoir, NC, USA) and mixture containing all tree nut extracts and simultaneously stained with anti-CD3 (eBioscience, San Diego, CA, USA), anti-CD203 (Beckman Coulter, Pasadena, CA, USA), anti-CRTH2 (Chemoattractant receptor-homologous molecule expressed on T_H2 cells) (BD Biosciences, Franklin Lakes, NJ, USA) for 25 min at 37°C. Basophils were identified as CD3-CRTH2⁺ cells, and activation status was assessed following the detection of CD203c in the presence of the allergens tested [24]. 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 FACS Aria (at single-cell purity). Expansion was done in a 96-well plate in the presence of 1.0×10^5 irradiated PBMC and 2 µg/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) or tree nut extract (2 µg/ml), in the presence of autologous PBMC (peripheral blood mononuclear cells) as APC (Antigen presenting cells). 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.

Phenotypical analysis of T cell clones

TCC ICS (intracellular cytokine staining) combined with tetramer staining was performed as previously described [25]. Cells were stained with a panel of antibodies directed against cytokines of interest, IL-4 (eBiosciences), IL-5 (Biolegend), IL-13 (Biolegend), IL-17A (Biolegend), IFN-γ (Biolegend) and IL-10 (BD Biosciences) for 20 minutes at room temperature. For phenotype analysis TCC were stained with a panel of antibodies directed against markers of interest, CCR4 (Biolegend), CRTH2 (BD Biosciences), CCR6 (Biolegend), CXCR3 (BD Biosciences) and CD27 (BD Biosciences). Two profiles (T_H2 and T_H2/T_H17) of TCCs were arbitrarily defined as follows (Figure 6): T_H2 profile was 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 was characterized by co-expression CCR4 and CCR6 (data not shown) and co-production of both IL-4 and IL17A (10%) but no IFN-γ and IL-5 by individual cells within the clone.

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. Fifty 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 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 played 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⁻) 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 generate detectable T-cell responses *ex vivo* in cashew allergic subjects.

Chemokine receptor and differentiation marker expression of Ana o 1- and Ana o 2-specific T-cells were analyzed by *ex vivo* staining of PBMC 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 suggest a T_H2 effector phenotype (T_{eff}) for the Ana o 1- and Ana o 2-specific CD4⁺ T cells. The expression of CCR4 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 β 7 expression was detected, indicating the capacity of some cells homing 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 3C). In conclusion, for allergic subjects, both Ana o and Ana o 2-specific cells expressed conventional T_H2 markers. We did not observe a significant difference in the expression of different surface markers 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 identity 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, peptides resembling homologous regions of cashew derived epitopes for other tree nut species were identified by selecting sequences with high amino acid sequence identity to Ana o 1 and Ana o 2 epitopes utilizing Blast alignments (Supplementary Table 1) [26]. Overall, the majority of Ana o 1 and Ana o 2 homologs from different tree nut species showed amino acid sequence identity 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 **directly *ex-vivo*** 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 2). As several TCC with identical specificity were obtained from one subject (e.g. six 09:01 Ana o 1₂₈₁₋₃₀₀-specific TCC) by *ex vivo* single cell sorting, it is still possible some of these TCC may in fact have been derived from the same identical clone *in vivo* and the some results described below should be interpreted with this caveat in mind. 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 low affinity clones that did not co-stained but proliferated upon peptide specific stimulation. For example, 2 out of 9 TCC DRB1*15:01 restricted Ana o 2₃₂₁₋₃₄₀ and 2 out of 4 DRB1*07:01 restricted Ana o 1₂₈₁₋₃₀₀ did not co-stain with pistachio loaded tetramers, but these clones proliferated upon peptide specific activation. Low affinity CD4⁺ T-cells, below detection level with pMHC-II tetramers, with pathogenic properties have been previously observed for autoimmune disease[27]. Results

from all these proliferation assays with peptides derived from different tree nut proteins were summarized in Figure 4D and Supplementary Table 2. At the epitope level, 7/8 of cashew epitopes screened could elicit cross-reactive response to pistachio, and 5/8 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, and 53.8% of the clones did not cross react to hazelnut or pistachio but did exclusively respond to cashew. To confirm our observations, TCC were stimulated with tree nut extracts and proliferation was analyzed. Similar profiles were observed for TCC tested (Figure 4E). Nonetheless, Ana o 1₄₃₃₋₄₅₂ specific DRB5*01:01 restricted TCC proliferated to pistachio extract while it did not proliferate to pistachio peptide stimulation. We speculate that antigen processing of the pistachio extract leads to the generation of peptides of different lengths that are more potent stimuli compared to the 20mers used in the synthetic peptide stimulation. Notably, proliferation to walnut peptides and extract was not observed for all the TCC with different specificities.

The cytokine profiles of 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 elicited almost identical cytokine profile when stimulated with peptides or tree nut extract (Supplemental Figure 4). TCC did not elicit cytokine responses to walnut peptides and extract. Collectively, these results suggest that cashew-specific T-cells can readily react to extract and T-cell epitopes with amino acid sequence identity 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 both walnut peptides and extract suggested that walnut reactive T-cells represented a distinct population of T-cells, as most of these allergic subjects had 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 the class II tetramer and CD154 upregulation assays were used to examine cashew allergen-specific T-cells. The CD154 upregulation assay has the advantage that it enables the detection of global responses to an allergen in the absence of HLA-restriction information and epitope identification which is the prerequisite for class II tetramer-based experiments. On the other hand, tetramer assays are more specific and sensitive compared to the CD154 assay. In this study, both Ana o 1 and Ana o 2 were identified as the predominant cashew allergens that elicit CD4⁺ T-cell responses. T cell reactivity to Ana o 3 was essentially absent. As we did not assay for Ana o 3 sIgE, it remains a possibility that the selected subjects may not have been sensitized to Ana O 3. 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 had been previously detected in both asthmatic and non-asthmatic subjects with peanut [28], walnut [22] and shrimp allergies [29]. ICS showed that the majority of TCC clones derived from cashew epitopes mainly produced 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⁺β7⁺CCR7⁺ T-cells as they have the capacity to home to the GALT [30–33]. 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 [34]. 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 epitopes with amino acid sequence identity from other species of tree nuts. We also found that the 46% of cashew-specific TCC from Ana o 1 and Ana o 2 reacted to peptides with amino acid identity from hazelnut and pistachio via proliferation assays. Importantly, these peptides with amino acid identity were able to elicit identical T_H2 cytokine in cashew reactive T cells. Our observations inferred that phylogenetic relatedness among tree nuts reflects cross-reactivity. 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 almost identical

in their aa sequences (78%, 80%, and 70%, respectively) and have a high degree of IgE-binding cross-reactivity [16;35]. 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 amino acid sequence identity is found in similar ranges (60%) between Ana o 2 and their corresponding allergens Jug r 4 in walnut, neither T-cell proliferation (peptide and/or extract) or class II tetramer co-staining were 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 was 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 1) walnut allergens contained species-specific T-cell epitopes and 2) walnut should be categorized within a tree nut allergens family that is distinct from cashew. Both walnut and pecan belong to the family of Juglandaceae [36] and their allergens are highly homologous. For example, Jug r 2 and Car i (*Carya illinoensis*) 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 published 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].

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 [38] 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 [39]. Additionally, Kulis et al [40] demonstrated that cashew immunotherapy prevented allergic responses to both cashew and pistachio in a cashew sensitized murine model as well as 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 [40]. We speculate that these cross-reactive epitopes between cashew and walnut are epitopes with low affinity to MHC-molecules. Low affinity cross-reactive epitopes may also be present in humans, but remain undetected in our tetramer approach. Indeed, 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. Nonetheless, our results suggest that immunotherapy for cashew will be able to alleviate reactions toward pistachio and hazelnut.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of nonstandard abbreviations used

sIgE	specific IgE
HLA	Human histocompatibility leukocyte antigen
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell
Ana o	<i>Anacardium occidentale</i>
Pis v	<i>Pistacia vera</i>
Cor a	<i>Corylus americana</i>
Car i	<i>Carya illinoensis</i>
Jug r	<i>Juglans regia</i>
PE	Phycoerythrin
pMHCII	Peptide/MHC class II
T_H	T helper
CRTH2	Chemoattractant receptor-homologous molecule expressed on T _H 2 cells
TGEM	Tetramer-guided epitope mapping
ICS	Intracellular cytokine staining
TCL	T-cell line
TCC	T-cell clone

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Figure 1.

Frequencies of cashew allergen reactive CD4⁺ T-cells with CD154 upregulation assay. **A**, Frequencies of Ana o 1-, Ana o 2- and Ana o 3-reactive T-cells within the memory compartment (CD45RA⁻) in a DRB1*01:01 subject with cashew allergy. Negative control was without peptide stimulation, and isotype control staining was with peptide stimulation. The frequencies of cashew allergen reactive T-cells per million CD4⁺ T-cells are as indicated. **B**, 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. **C**, 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 Dunnett's 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.

Frequencies of cashew allergen reactive CD4⁺ T cells with tetramer assay. **A**, Frequencies 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 CXCR3 of 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. 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 Allophycocyanin-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 1₂₈₁₋₃₀₀, Ana o 1₄₃₃₋₄₅₂, Ana o 1₅₁₃₋₅₃₂, Ana o 2₂₃₃₋₂₅₂, Ana o 2₃₂₉₋₃₄₈ and Ana o 2₃₂₁₋₃₄₀-specific T cell clones using APCs from their respective DRB1* specificities. Cells were stimulated with specific or irrelevant control peptide. **E**, Summarized proliferation results for Ana o 1₂₈₁₋₃₀₀, Ana o 1₄₃₃₋₄₅₂ and Ana o 1₅₁₃₋₅₃₂-specific T cell clones using autologous APCs. Cells were stimulated with different tree nut extract. For **D** and **E**. 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.

IgE and T-cells directed against other tree nut allergens. 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	Date of first reaction	Date of last reaction
Cashew allergies												
1	28	F	07:01, 14:01	>100	Not tested	58.9	>100	38.6	I, III, V, VII, VIII	yes	2005 (walnut)	2007 (pistachio/cashew)
2	11	F	09:01, 15:01	31.4	Not tested	5.62	28.6	17.1	I, III, IV, V	yes	2013 (cashew)	No known ingestions since
3	19	F	01:01, 13:01	1.73	12 × 12 mm	0.67	2.2	0.58	I, IV, V	yes	1998 (cashew)	No known ingestions since
4	24	F	01:01, 13:01	0.5	9 × 9 mm	<0.35	0.77	<0.35	I, II, III	yes	2000 (cashew)	2012 (walnut)
5	8	F	15:01, 04:04	>100	Not tested	26.9	>100	6.61	IV, V	yes	2009 (cashew/pistachio)	No known ingestions since
6	24	F	01:01, 15:01	32.6	Not tested	3.69	37	8.86	I, II, III, IV, VI	no	2013 (cashew)	No known ingestions since
7	21	M	03:01, 15:01	19	10 × 10 mm	61.2	25.4	28.2	I, II, IV, V	yes	2012 (unknown nut)	2013 (pistachio/cashew)
8	11	M	01:01, 10:01	9.78	15 × 15 mm	1.03	13.9	6.79	III, IV	no	2012 (pecan)	2014 (pecan/cashew)
9	34	F	07:01, 15:01	7.66	15×10 mm	21.3	6.58	77.7	I, II, III, IV, V	no	1984 (unknown nut)	2014 (cashew)
10	8	F	07:01, 11:01	2.95	8 × 8 mm	7.38	7.4	3.93	I, II, III, IV	no	2007 (cashew)	2010 (pistachio)
11	11	M	01:01, 13:02	41.6	Not tested	0.72	4.94	15.8	I, IV, V	yes	2006 (cashew)	2010 (cashew)
12	36	F	01:01, 04:04	0.38	15×15 mm	0.38	0.36	0.69	II, III, IV	no	1984 (walnut)	2014 (cashew)
13	10	F	01:01, 04:04	>100	5 × 5 mm	40.5	>100	97.3	III, IV, V	no	2005 (walnut)	2009 (walnut/cashew)
14	15	F	11:01, 13:01	57.3	Not tested	87.02	65.9	>100	I, III	yes	2001 (pecan/hazelnut/cashew)	No known ingestions since
Nonatopic subjects												
18	29	M	01:01, 03:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
19	31	F	07:01, 07:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
20	41	F	04:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
21	34	M	07:01, 13:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
22	32	F	15:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction

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	Date of first reaction	Date of last reaction
23	10	F	07:01, 11:01	0	Not tested	Not tested	Not tested	Not tested	Absent	No	No known reaction	No known reaction
24	30	F	01:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
25	31	F	04:04, 16: 01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
26	23	M	01:01, 07:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
27	22	F	07:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
28	23	M	07:01, 13:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
29	31	M	01:01, 04:04	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
Atopic subjects without cashew and other tree nut allergy												
30 [*]	10	M	09:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
31 [*]	6	M	10:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
32 [*]	63	M	01:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction
33	41	F	09:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
34	26	F	04:04, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	yes	No known reaction	No known reaction
35	8	F	01:01, 15:01	0	Not tested	Not tested	Not tested	Not tested	Absent	no	No known reaction	No known reaction

I Itchy mouth, lips and / or pharynx

II Abdominal discomfort and / or diarrhea

III Nausea or vomiting

IV Acute or Severe skin itching, or hives, or angioedema

VR 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 1 281-300	GPGGENPESFYRAFSWEILE	●		●	●					
Ana o 1 385-404	MVVSYANITKGGMSVPFYNS			●						
Ana o 1 433-452	HPSYKKLRARIRKDTVFIVP	●			●					●
Ana o 1 513-532	VFGKQDEEFFQGPPEWRKEK									●
Ana o 2 233-252	KVKDDELVRIPRSQSERG	●	●							
Ana o 2 289-308	PARADIYTPVEVGRLLTTLNSL		●							
Ana o 2 297-316	PEVGRLLTTLNSLNLPLKWL		●							
Ana o 2 321-340	EKGVLTKNALVLPWNLNSH							●		
Ana o 2 329-348	ALVLPWNLNSHSIIYGCKG		●						●	
Ana o 2 385-404	QNEAVVKRAREERFEWISFK				●		●			