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

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Abstract

Background—Allergic reactions to walnut can be life threatening. While IgE epitopes of walnut have been studied, CD4⁺ T-cell specific epitopes for walnut remain uncharacterized. Particularly, 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 non-allergic subjects. Particularly, the relationship of phenotypes and frequencies of walnut specific T-cells with the disease.

Methods—CD154 up-regulation assay was used to examine CD4⁺ T-cell reactivity towards walnut allergens. Jug r 1, Jug r 2 and Jug r 3. Tetramer-Guided epitope mapping approach was utilized to identify HLA-restricted CD4⁺ T-cells epitopes in Jug r 2. Direct *ex vivo* staining with peptide-major histocompatibility complex class II (pMHC-II) tetramers enabled the comparison of frequency and phenotype of Jug r 2-specific CD4⁺ T-cells between allergic and non-allergic subjects. Jug r 2-specific T-cell-clones were also generated and mRNA transcription factor levels were assessed by RT qPCR. Intracellular cytokine staining (ICS) assays were performed for further phenotypical analyses.

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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⁺ T_{CM} (central memory) phenotype. A subset of these T-cells express CCR4⁺CCR6⁺ irrespectively of the asthmatic status of the allergic subjects. ICS 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 expressed either GATA3 and RORC, or RORC, 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 subjects 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 non-asthmatic and asthmatic subjects with walnut allergy. Understanding this T-cell heterogeneity may render better understanding of the disease manifestation.

Keywords

Food allergy; walnut; Jug r 2; T-cells; MHC class II tetramers; epitopes

INTRODUCTION

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 tree nut allergic subjects (34%)(4) and both cashew and walnut accounted for the majority of life-threatening anaphylactic reactions due to 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 common 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 US(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).

While 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 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(17;18). Jug r 3 was also studied since we have a small cohort of samples from Spain, where LTP 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 CD154 activation assay(19). 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)(20). 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*. 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. Understanding this T-cell heterogeneity may improve our understanding of disease manifestation.

RESULTS

Jug r 2-reactive CD4⁺ T-cells dominate the immune response in walnut allergy

Up-regulation of CD154 in CD4⁺ T-cells after 3 h stimulation of PBMC 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 non-allergic subjects. For non-allergic 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) (Figure 1A and Figure E1). 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), even amongst 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 Jug r 1 T-cell immune responses in allergic subjects and 4 fold greater than Jug r 2 T-cell immune responses in non-allergic subjects. Thus, Jug r 2-reactive CD4⁺ T-cells dominate the walnut allergen specific T-cell repertoire in subjects 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-B cell cooperation, which the latter has been observed in 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 (Figure E2). 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 2). Peptides Jug r 2₁₅₂₋₁₇₁, Jug r 2₁₈₄₋₂₀₃, Jug r 2₂₂₄₋₂₄₃, Jug r 2₃₉₂₋₄₁₁, Jug r 2₄₅₆₋₄₇₅ and Jug r 2₅₂₀₋₅₃₉ were presented by 3 or more different DRB1 alleles. Identical epitopes were identified in allergic and non-allergic 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 direct *ex vivo* staining with Jug r 2-tetramers (Figure 1B and Figure E3). Each subject was stained with a panel of tetramers corresponding to the HLA of the subject (Table E1). In non-allergic 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-cell in allergic subjects was 26.53 ± 2.26 per 10^6 , which was at least 4-fold higher compared to non-allergic subjects. The average frequency within the CD45RA⁻ compartment was 18.34 ± 1.72 reactive CD4⁺ T-cells per 10^6 . This tetramer staining frequency data agree with the results from the CD154 assays and confirm that Jug r 2-reactive CD4⁺ T-cells are present in higher frequencies in PBMC of allergic compared to non-allergic subjects.

Surface phenotype of Jug r 2 specific CD4⁺ T-cells

The surface phenotypes of Jug r 2-specific T-cells were determined by direct *ex vivo* staining of PBMC (Figure 2A). A higher percentage of the tetramer positive cells in non-allergic group expressed CXCR3 (T_H1 marker) compared to the allergic group (Figure 2B). However, because of the higher frequency of total Jug r 2-specific T-cells in the allergic group compared to the non-allergic group, the average frequency of T_H1 allergen specific T-cells in both groups was similar (Figure 2C). Conversely, a higher percentage of tetramer positive cells in the allergic group expressed CCR4 and CRTH2 (T_H2 markers)(25;26) compared to the non-allergic group (Figure 2B). Significant difference in percentage of Jug r 2-specific T-cells that lost CD27 expression was also observed between the two groups, with CD27⁻ Jug r 2-specific T-cells being 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-cells (T_{eff}) compared to the non-allergic group (Figure 2C). Though CD27⁻ Jug r 2-specific T_{eff} were present, there were still higher percentages of CD27⁺ Jug r 2-reactive T-cells compared to CD27⁻ Jug r 2-specific cells in the allergic group. The majority of these tetramer positive CD27⁺ T-cells also co-expressed CCR7 and CD62L, suggesting these CCR4⁺CD27⁺CCR7⁺ cells are central memory T-cells (T_{CM})(27-29) (Figure 2D **and data not shown**). It should also be noted that most Jug r 2-reactive T-cells in allergic subjects were CRTH2⁻. Though there was no difference in 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 two groups was different (Figure 2E). 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 non-allergic subjects. Also of interest, T_H17 Jug r 2-reactive T-cells were detected in both non-asthmatic and asthmatic subjects with walnut allergy (Figure 2F), 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 non-allergic subjects which was significantly higher compared to allergic subjects (Figure 2E). No difference 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 history of walnut ingestion. However, significant difference was observed in memory T-cell frequencies (19.46 ± 1.96 vs. 11.98 ± 1.67 per 10^6) among these two groups.

Cytokine profiles of Jug r 2-specific CD4⁺ T-cells in non-allergic and allergic subjects

Jug r 2-specific T-cells were single cell sorted for generation of TCC. In non-allergic subjects, a total of 12 TCCs were generated. All TCCs from non-allergic subjects elicited a distinct protective T_H1/T_R1 (IFN- γ and IL-10) or T_H1 (IFN- γ) response (Figure 3A and

Figure E4 and E5). 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 (Figure 3B and Figure 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 IL17A (T_H2/T_H17) and was detected for three specificities only, Jug r 2₂₈₀₋₂₉₉, Jug r 2₃₆₀₋₃₇₄ and Jug r 2₅₃₆₋₅₅₅. The third profile is exemplified by the production of IL-17A only (T_H17). In total 23.7% of the T-cell clones obtained from allergic subjects were capable of producing IL-17A. Transcript levels of 3 transcription factors of these clones were also assessed by RT quantitative PCR (Figure 3C). In accordance with cytokine production profile, T_H1 clones derived from non-allergic subjects expressed the highest levels of T-bet (Tbx21). In allergic subjects, T_H2 clones mainly expressed GATA-3. As expected, T_H2/T_H17 TCCs expressed GATA-3 and RORC, indicating their ability to produce IL-4 and IL17A. Finally, T_H17 TCCs mainly expressed RORC.

The T_H1 responses in non-allergic subjects and T_H2 and T_H17 responses in allergic subjects were also confirmed by direct *ex vivo* staining. For these experiments, PBMC were co-stained for cytokine expression and CD154 after 6 hours of peptide stimulation. Non-allergic 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 individuals. On the other hand, T_H2 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 80.3% \pm 3.84 and 52.5% \pm 2.15 in non-allergic and allergic subjects, respectively). Examples of these *ex vivo* experiments are shown in Figure 4A. Amongst 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. Gating strategy for identifying double and triple producers is shown in Figure 4B. Results of experiments from 8 non-allergic subjects and 11 allergic subjects are summarized in Figure 4C and Figure 4D. Both T_{CM} and T_{eff} cells are capable of producing T_H2 cytokines (Figure 5A). Interestingly, T_{eff} cells are more capable of producing IL-4 and IL-5 compared to T_{CM} cells (Figure 5B). These data are in agreement with earlier studies which suggest that loss of CD27 correlates with an increase of IL-4 production and T_{eff} cells are more terminally differentiated cells(27;33;34). In contrast to the TCC data, we failed to observe a T_H1/TR1 profile in non-allergic subjects, or a T_H2/T_H17 profile in allergic subjects. Different kinetics of cytokine production for these cell types, and the rarity of these cells, may account for these different outcomes by these two approaches.

DISCUSSION

Allergic reactions to walnut account for the vast majority of severe reactions in tree-nut allergic subjects in the US(1). Contributions of CD4⁺ T-cells to this disease remain elusive. In particular, walnut specific CD4⁺ T-cell epitopes have not been identified. The frequency and the phenotype of walnut allergen specific T-cells have not been examined *ex vivo*. In this study, both class II tetramer assay and CD154 up-regulation assay 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 aa 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 subjects with walnut allergy.

Consistent with previous studies (18;33-36), allergen-tolerant subjects and allergic subjects recognize identical allergen-derived epitopes. However, Jug r 2-reactive T-cells are present at substantially lower frequencies in non-allergic subjects (Figure 1). In addition, surface phenotypes and functional properties of these T-cells are distinct in non-allergic and allergic subjects. Through direct *ex vivo* analysis of PBMC and analysis of TCC, CXCR3⁺ Jug r 2-specific T-cells with predominant IFN- γ and low IL-10 production were observed in non-allergic 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 non-allergic subjects at a very low frequency which is below the threshold of the detection method. Results obtained from either the tetramer assay or CD154 up-regulation assays are compatible. Antigen specific T-cells which were cytokine non-producers were also detected with both assays. 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 pollen allergic subjects 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 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 show CCR4⁺T_{CM} in humans 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-(18) and Pen m 2-(39)reactive T-cells, and these cells were capable to produce IL-4. Accumulating evidence proposes that CD27 is lost after repetitive antigenic stimulation (40;41) 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(18). Food avoidance in walnut allergic subjects may have resulted in the accumulation of CRTH2⁻ T_{CM} in peripheral blood of allergic subjects. The results from this study do not contradict to our previous pollen studies(33;34), 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(42).

It has been demonstrated that IL-17A can promote class switch to IgE(43) and IL-17A producing CD4⁺ T-cells are more frequent on allergic subjects(43-45). T_H2/T_H17 cells are also observed in subjects with allergic asthma(46-48). However, the involvement of T_H17 cells in food allergy remains obscure. In the current study a sub-population 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 non-asthmatic walnut allergic individuals. These results are consistent with previous studies with food allergens(18;39), 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. This is indeed a possibility as CCR6 is also a GALT-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 food allergic children and that lack of T_H17 responses may play a potential role in food tolerance. 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.

METHODS ONLINE

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 PBMC in 200 μ L T-cell culture medium were stained with 20 μ g/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, Bergisch Gladbach, Germany). 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, San Jose, CA) for 10 minutes at 4°C before flow-cytometry. Data were analyzed utilizing FlowJo (Tree Star, Ashland, Ore) gating on forward scatter/side scatter and excluding $CD14^+$, $CD19^+$ and Viaprobe populations. Frequency was calculated as previously described (E1). For phenotyping studies, antibodies were used against markers of interest; CCR4 (R&D systems, Minneapolis, MN), $CD45RA$ (eBioscience, San Diego, CA) and $CD38$ (eBioscience, San Diego, CA).

Intracellular cytokine staining

For *ex-vivo* intracellular cytokine staining (ICS) combined with $CD154$ activation assay, BD GolgiStopTM was added during stimulation (BD biosciences, East Rutherford, NJ) according to the manufacturer's instructions. *In vitro* ICS combined with tetramer staining was performed as previously described(E2). Briefly, T-cell lines or TCC were restimulated with 50 ng/mL phorbol 12-myristate-13-acetate and 1 mg/mL ionomycin in the presence of 1 \times brefeldin-A (eBiosciences, San Diego, CA) for 5 hours at 37°C, 5% CO₂. After 10 minutes at room temperature, cells were then fixed with fixation buffer (eBioscience, San Diego, CA) and washed twice with a permeabilization buffer (eBioscience, San Diego, CA). Cells were then stained with a panel of antibodies directed against cytokines (eBioscience, San Diego, CA and BD biosciences, East Rutherford, NJ) of interest for 20 minutes at room temperature; cells were washed and immediately analyzed in 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 by using a FACS Aria (at single-cell purity). Cells were and expanded in a 96-well plate in the presence of 1.0×10^5 irradiated PBMC and 2 µg/ml of PHA (Remel, Lenexa, KS). T-cells were re-screened 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 (Figure 3 and Figure E4 and E5): T_H2 profile is exemplified by CCR4⁺ with or without CRT2 expression, production of IL-4 (10%), IL-5(10%), and IL-13 (10%), and expression of GATA3; T_H2/T_H17 profile is characterized by co-expression CCR4 and CCR6 and show the ability to produce both IL-4 (10%), IL- and IL17A (10%) but no IFN-γ and IL-5 and express both GATA3 and ROR_γ. The T_H17 profile is exemplified by co-expression of CCR4 and CCR6, production of IL-17A (10%) and sometimes low IFN-γ (=10%) and expression of ROR_γ; the T_H1 profile is exemplified by CXCR3 expression (some clones also co-expressed CD27), production of IFN-γ (10%) and expression of T-bet (TBX21); and the T_R1 profile is exemplified by CXCR3 expression (some clones also co-expressed 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 T-cell-clones (TCC) derived from non-allergic and allergic subjects with Gene elute™ Mammalian total RNA miniprep kit (Sigma-aldrich, St. Louis, MO) and reverse transcribed as cDNAs using the TaqMan Reverse Transcription Reagent kit (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions. Messenger RNAs were assessed by quantitative PCR using pre-designed Taqman Gene Expression reagents (Applied Biosystems, Foster city, CA). Data were expressed as relative amounts of cytokine mRNA and were normalized based on relative amounts of GTF2B mRNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of nonstandard abbreviations used

HLA	Human histocompatibility leukocyte antigen
MHC	Major histocompatibility complex
PBMC	Peripheral blood mononuclear cell

Jug r	<i>Juglans regia</i>
PE	Phytoerythrin
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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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 central memory phenotype.
- 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.

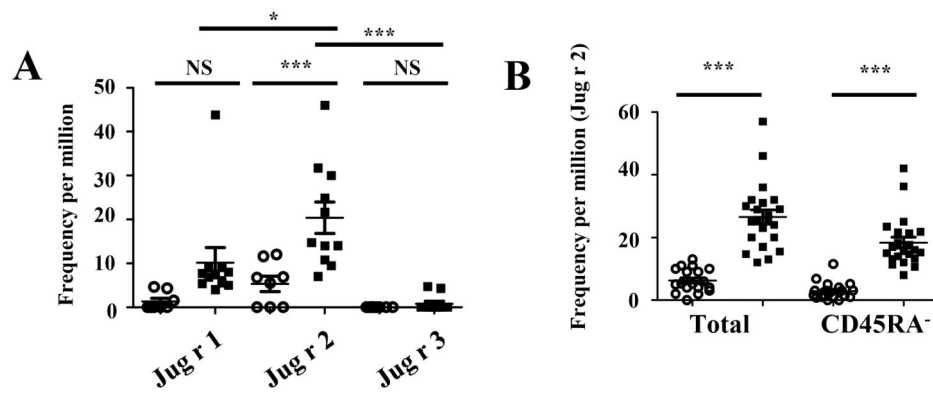


Figure 1.

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 subjects with walnut allergy (n=11; filled squares) and non-allergic subjects (n=8; opened circles) with CD154 assays. Each data point represents the frequency of T-cells reactive to each allergen. An ANOVA test (with Bonferroni correction) was used to compare all columns in the statistical analysis. **B**, Frequencies of Jug r 2 epitope-reactive T-cells in subjects with walnut allergy (n=17; filled squares) and subjects without walnut allergy (n=19; opened circles) with tetramer assays. Each data point represents the frequency of T-cells specific to a combination of epitopes in Jug r 2. A Student *t* test was used in the statistical analysis. **P*<0.05, ***P*<0.001, ****P*<0.0001. NS. Not significant.

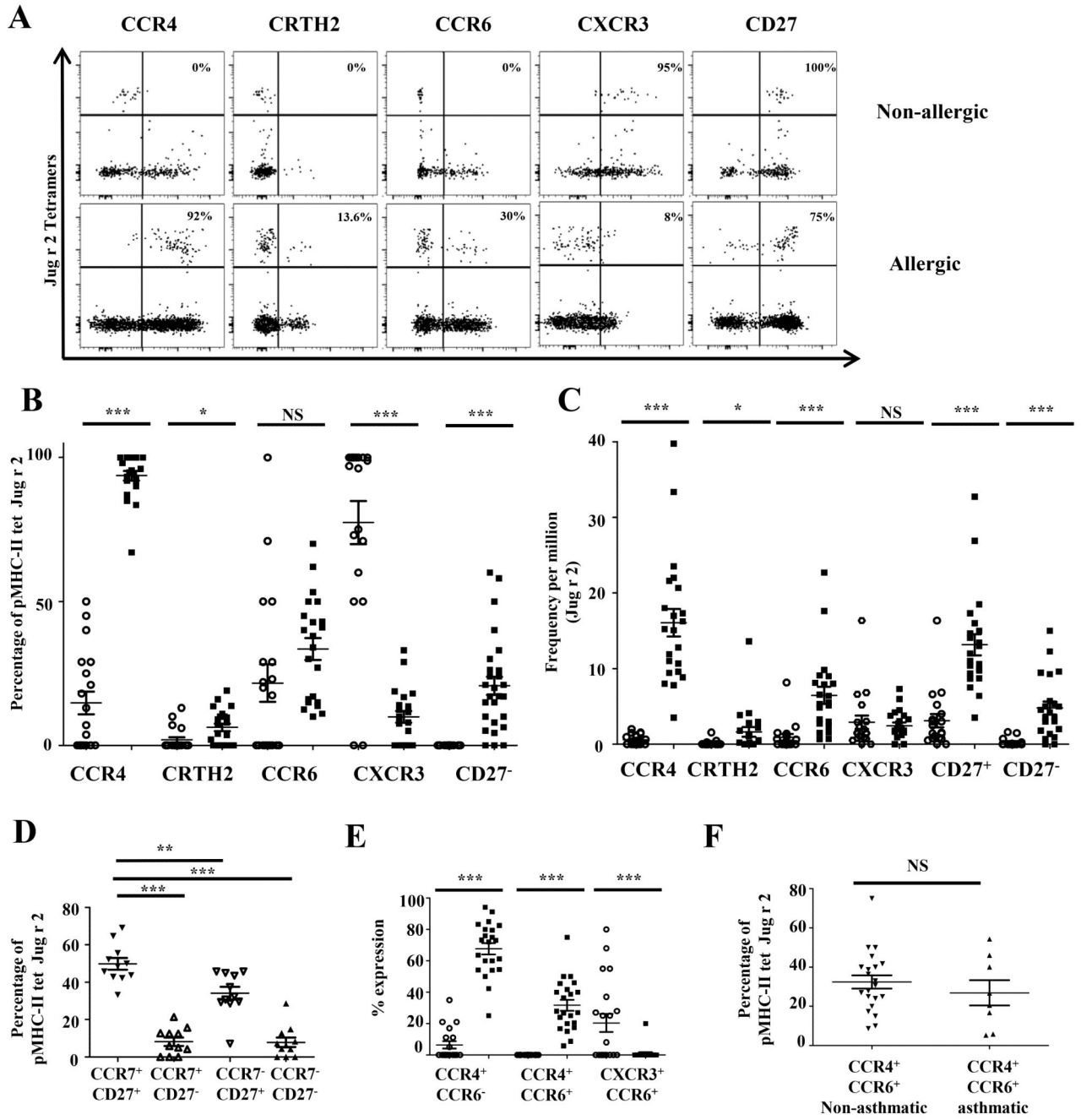


Figure 2. Phenotypes of Jug r 2 reactive T-cells. **A**, *First row*, profile in a DRB1*15:01 non-allergic subject. *Second row*, profile in a DRB1*15:01 allergic subject. The percentages of surface markers expressed by Jug r 2-specific T-cells are as indicated. **B**, *Ex vivo* expression of CCR4, CRTH2, CCR6, CXCR3 and CD27 of Jug r 2-specific T-cells in subjects with walnut allergy (n=17; filled square) and subjects without walnut allergy (n=19; opened circle). 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 subjects with walnut allergy (n=17; filled square) and subjects without walnut allergy (n=19; opened circle). 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; CXCR3 and CCR6. Each data point represents results for surface expression in tetramer positive T-cells from 17 subjects with walnut allergy (filled square) and 19 subjects without walnut allergy (opened circle). **F**, Surface expression of CCR4 and CCR6 was analyzed on tetramer positive T-cells from non-asthmatic and asthmatic walnut allergic subjects. A Student *t* test was used in the statistical analysis for Figure 1B, C, E and F. An ANOVA test (with Bonferroni correction) was used to compare all columns in the statistical analysis for Figure 1D. **P*<0.05, ***P*<0.001, ****P*<0.0001. NS. Not significant.

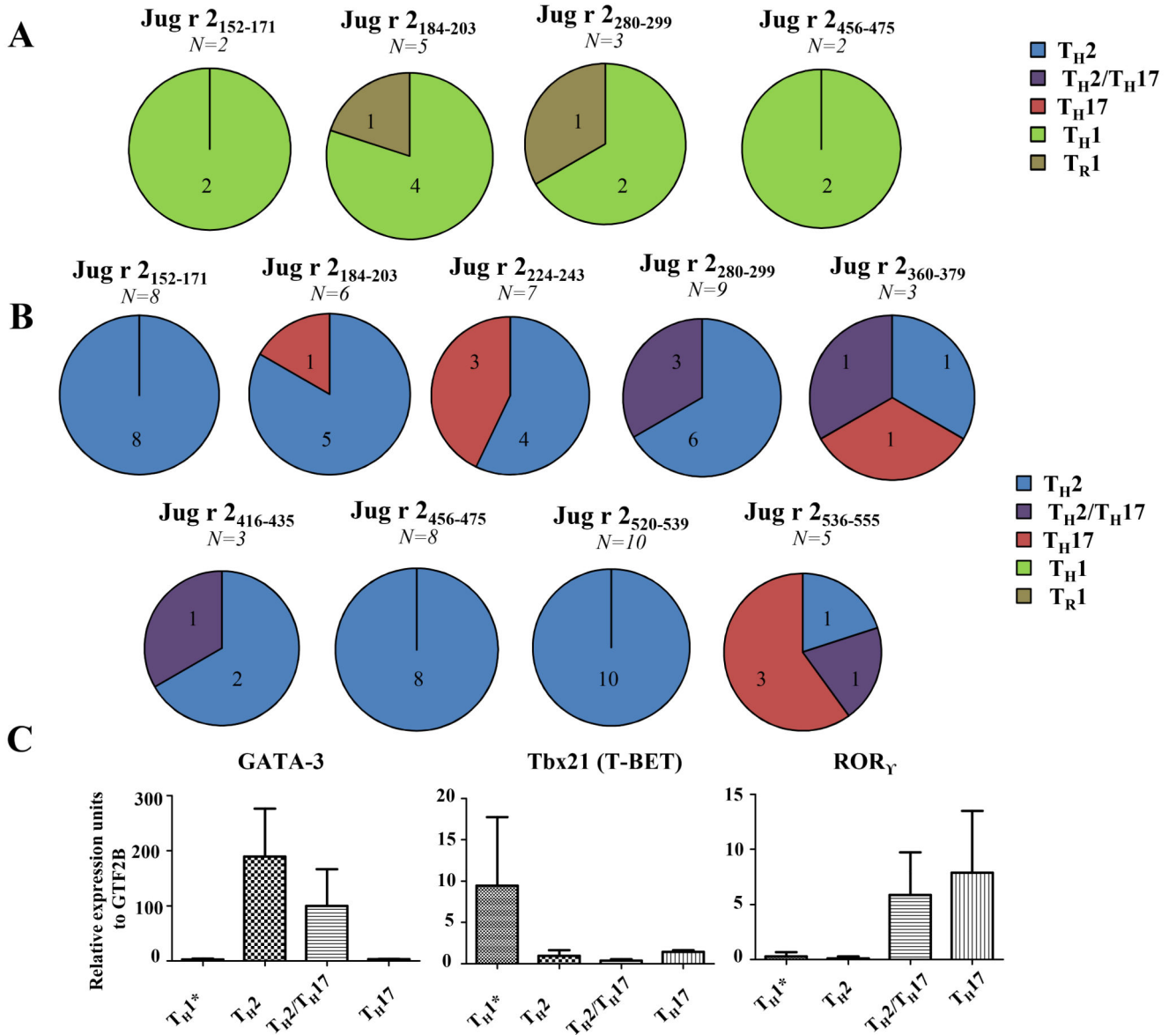


Figure 3. Jug r 2 T-cell subsets. **A** and **B**, Phenotype of T-cell clones derived from 6 non-allergic (Figure 3, A) and 8 allergic (Figure 3, B) subjects. Phenotype profiles based on surface marker expression and cytokine production in T_H2= 45 T_H2/T_H17=6, T_H17=8 T_H1*=12 T-cell clones. *Derived from non-allergic subjects. The numbers of T-cell clones 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 GATA-3, TBX21, and RORC were assessed by quantitative PCR. Data were expressed as relative amounts of cytokine mRNA in Jug r 2 epitope-specific T-cell clones derived from 6 non-allergic and 8 allergic subjects. Data were normalized based on relative amounts of GTF2B mRNA.

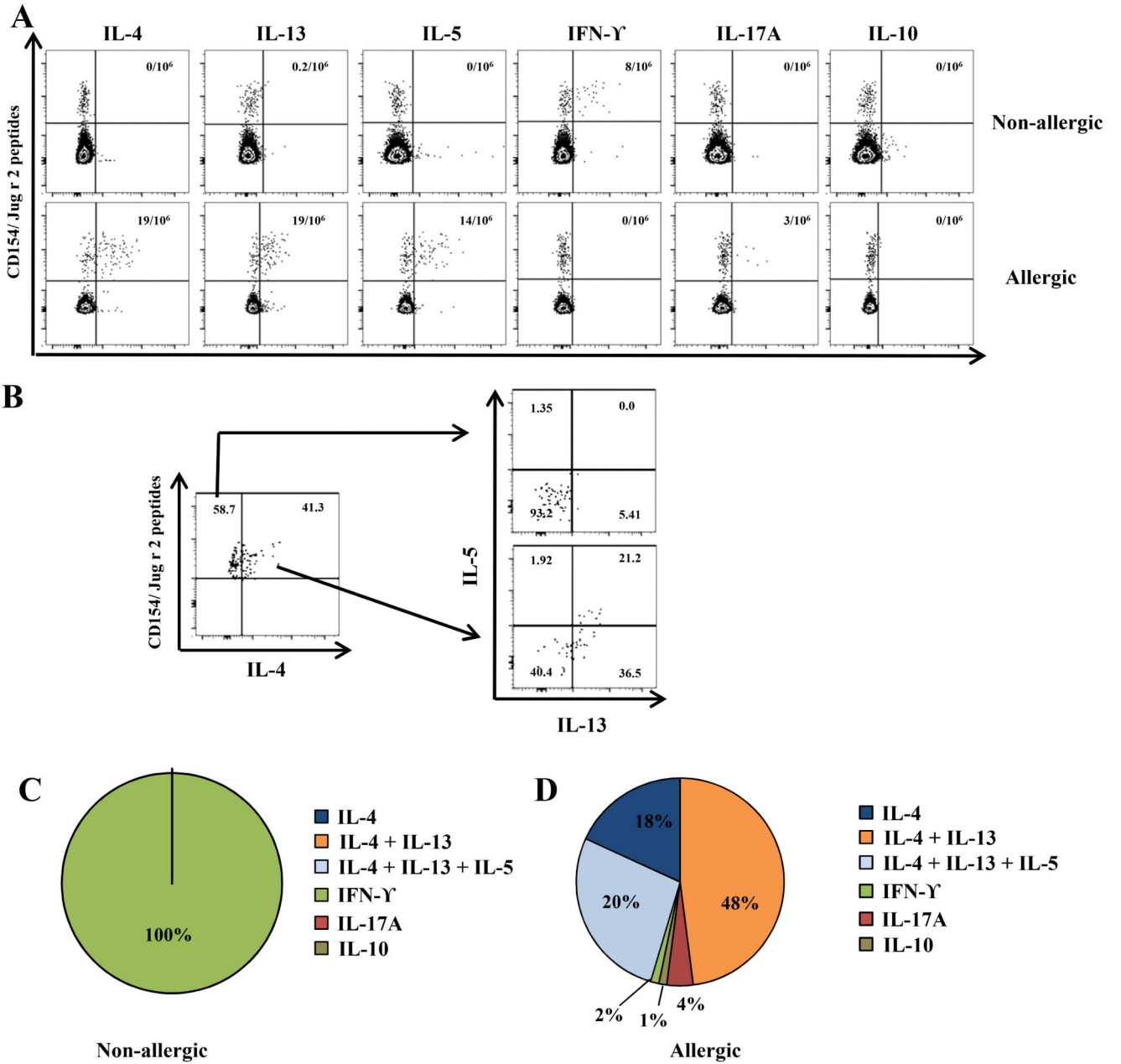


Figure 4. Cytokine profiles of Jug r 2-reactive T-cells. **A**, *First row*, Cytokine profile in a DRB1*15:01 non-allergic subject. *Second row*, Cytokine profile in a DR15:01 allergic subject. The frequencies of Jug r 2-specific cytokine producing T-cells per million CD4⁺ T-cells are as indicated. **B**, Gating strategy for identifying IL-4, IL-4+IL-13 and IL-4+IL-13+IL-5 Jug r-2 reactive T-cells, in this subject IL-4= 40.4%, IL-4+IL-13=36.5%, IL4+IL13+IL-5=21.2%. **C** and **D**, Cytokine profiles of Jug r 2-reactive T-cells in non-allergic and allergic subjects. Data are representative of 11 subjects with walnut allergy and 8 non-allergic subjects and are presented as the mean frequency of cytokine producing T-cells from each group in pie charts.

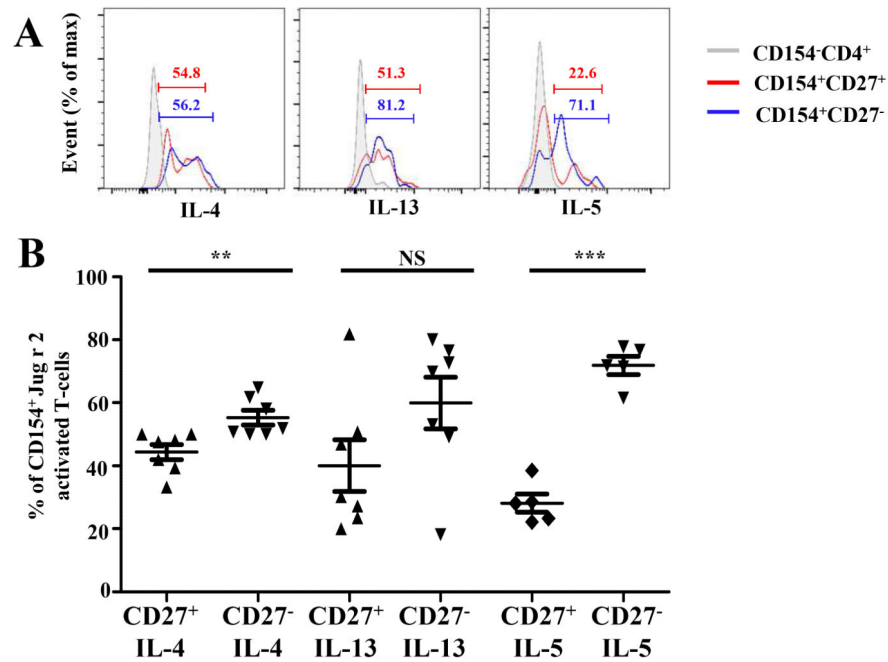


Figure 5. *Ex vivo* cytokine producing capacities of CD27⁺ and CD27⁻ Jug r 2-reactive T-cells. **A**, IL-4, IL-13 and IL-5 expression by CD27⁺ (red histogram), CD27⁻ (blue histogram) and CD154⁻CD4⁺ as control (grey histogram). **B**, Cytokine production by CD27⁺ and CD27⁻ CD154⁺Jug r 2-reactive T-cells in allergic subjects. Data are representative of 7 allergic subjects. A Student *t* test was used 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 walnut (256) kU/L	Skin Prick Test	Jug r			Jug r 3 ISAC ISU	Symptoms to Walnut	Asthma
						1 ISAC ISU	2 ISAC ISU	0			
Walnut allergies											
1	26	F	07:01, 14:01	38.6	Not tested	12.46	6.82	0	0	I, III, V, VII, VIII	yes
2	9	F	07:01, 15:01	26.1	Not tested	3.11	1.42	0	0	I, II, V	no
3	10	M	15:01, 09:01	81.7	6 × 4 mm	48.6	1.58	0	0	II, III, V	no
4	10	M	15:01, 14:02	23.4	6 × 10 mm	43.9	1.64	0.28	0	II, IV, VI, VII	yes
5	10	M	04:01, 15:01	37	9 × 9 mm	11.26	1.88	0	0	I, III	yes
6	34	F	04:04, 11:01	2.29	Not tested	0.26	3.81	0	0	I, II, III, VIII	no
7	23	F	01:01, 15:01	8.86	Not tested	3.69	0	0.06	0	I, II, III, IV, VI	no
8	10	F	01:01, 08:01	97.3	15 × 9 mm	81.5	1.46	0	0	III, IV, V	no
9	8	F	13:01, 15:01	1.51	7 × 7 mm	4.77	0	0	0	I, II, III	no
10	34	F	07:01, 15:01	77.7	15 × 10 mm	43.9	1.9	0.02	0	I, II, III, IV, V	no
11	27	F	15:01, 16:01	1.91	Not tested	0	0	1.18	0	II, III, IV, V, VII	no
12	14	F	04:05, 15:01	4.97	Not tested	0	4.36	7.3	0	II, IV, V	yes
Walnut sensitized subjects											
13	36	F	01:01, 04:04	0.39	12 × 12 mm	0.34	0	0	0		no
14	18	F	01:01, 13:01	0.58	5 × 5 mm	0	0	0.30	0		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	0		no
17	24	M	04:03, 07:01	1.07	Not tested	0	0	0.95	0		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, 04:01	0	Not tested	—	—	—	—	Absent	no
22	26	M	04:03, 15:01	0	Not tested	—	—	—	—	Absent	no

ID	Age	Sex	HLA (DRB1*)	sIgE walnut (256) kU/L	Skin Prick Test	Jug r			Symptoms to Walnut	Asthma
						ISAC ISU	ISAC ISU	ISAC ISU		
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	11:01 , 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

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

Jug r 2 CD4+ T-cell epitopes

Jug r 2	Amino acid sequence	HLA DRB1 Restriction											
		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 2 ₁₅₂₋₁₇₁	EQRHNPYYFHSQSIRSRH		•			•		•		•			•
Jug r 2 ₁₈₄₋₂₀₃	FTERTELLRGIENYRVVILD	•		•			•						•
Jug r 2 ₂₂₄₋₂₄₃	TRGRATLTLVSQETRESFNL						•			•			
Jug r 2 ₂₈₀₋₂₉₉	PGQFREYYAAGAKSPDQSYL			•									•
Jug r 2 ₂₉₆₋₃₁₅	QSYLRVFSNDILVAALNTPR												•
Jug r 2 ₃₆₀₋₃₇₉	SGGPISLKSESPSYSNQFGQ												•
Jug r 2 ₃₉₂₋₄₁₁	QEMDVLVNYAEIKRKGMMVVP												•
Jug r 2 ₄₁₆₋₄₃₅	KATVVVYVVEGTGRYEMACP												•
Jug r 2 ₄₅₆₋₄₇₅	TGRFQKVTARLARGDIFVIP	•		•									•
Jug r 2 ₅₂₀₋₅₃₉	EAKELSFNMPREEIEEIFES												•
Jug r 2 ₅₃₆₋₅₅₅	IFESQMESYFVPTERQSRRG												•