

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

Luis Diego Archila, MSc,^a David Jeong, MD,^{b,f} Mariona Pascal, PhD,^{c,d} Joan Bartra, MD, PhD,^{d,e} Manel Juan, MD, PhD,^{c,d} David Robinson, MD,^b Mary L. Farrington, MD,^b and William W. Kwok, PhD^{a,f}
 Seattle, Wash, and Barcelona, Spain

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

From ^aBenaroya Research Institute at Virginia Mason, Seattle; ^bVirginia Mason Medical Center, Seattle; ^cServei d'Immunologia, CDB, Hospital Clínic de Barcelona; ^dInstitut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Universitat de Barcelona; ^eUnitat d'Al·lèrgia, Servei de Pneumologia, ICT, Hospital Clínic de Barcelona; and ^fthe Department of Medicine, University of Washington.

Supported by National Institutes of Health contract HHSN272200700046C.

Disclosure of potential conflict of interest: This study was funded by the National Institutes of Health (NIH). W. W. Kwok is an employee of the NIH. D. Jeong is employed by the Virginia Mason Medical Center and is on the advisory board for the Seattle Food Allergy Consortium. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 4, 2014; revised January 20, 2015; accepted for publication January 22, 2015.

Corresponding author: William W. Kwok, PhD, Benaroya Research Institute, 1201 9th Ave, Seattle, WA 98101-2795. E-mail: bkwok@benaroyaresearch.org.
 0091-6749/\$36.00

© 2015 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2015.01.029>

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 1. 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 synthesized 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 μ g/mL synthesized peptide pools and 1 μ g/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 (<i>DRB1</i> *)	slgE 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, 04:01	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	11:01, 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

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 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 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.

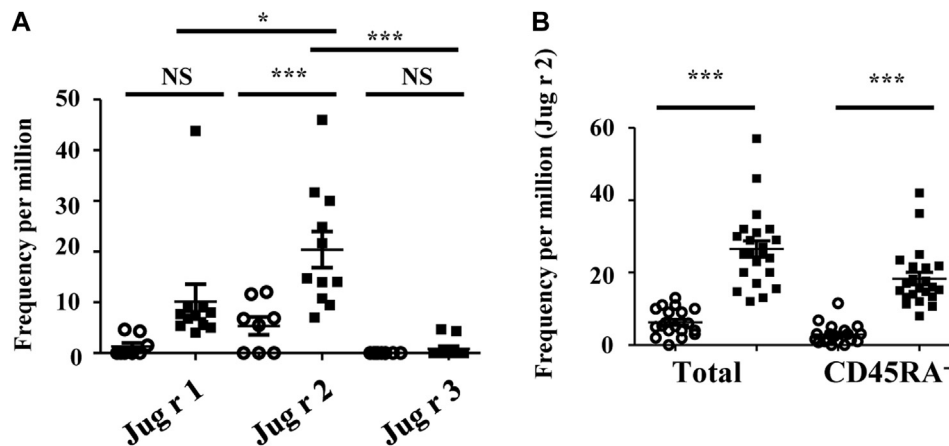


FIG 1. Frequencies of walnut allergen-reactive CD4⁺ T cells. **A**, Frequencies of Jug r 1-, Jug r 2-, and Jug r 3-reactive T cells in patients with walnut allergy ($n = 11$; solid squares) and nonallergic subjects ($n = 8$; open circles), as determined by using CD154 assays. Each data point represents 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 <i>DRB1</i> 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 2 ₁₅₂₋₁₇₁	EQQRHNPYYFHSQSIRSRH		•			•		•	•		•		
Jug r 2 ₁₈₄₋₂₀₃	FTTELLRGIENYRVVILD	•		•				•			•		•
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	QEMDVLVNYAEIKRGAMMVP						•	•			•		•
Jug r 2 ₄₁₆₋₄₃₅	KATVVVVYVEGTGRYEMACP								•				
Jug r 2 ₄₅₆₋₄₇₅	TGRFQKVITARLARGDIFVIP	•		•					•	•		•	•
Jug r 2 ₅₂₀₋₅₃₉	EAKELSFNMPREIEEIFES	•			•				•				
Jug r 2 ₅₃₆₋₅₅₅	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

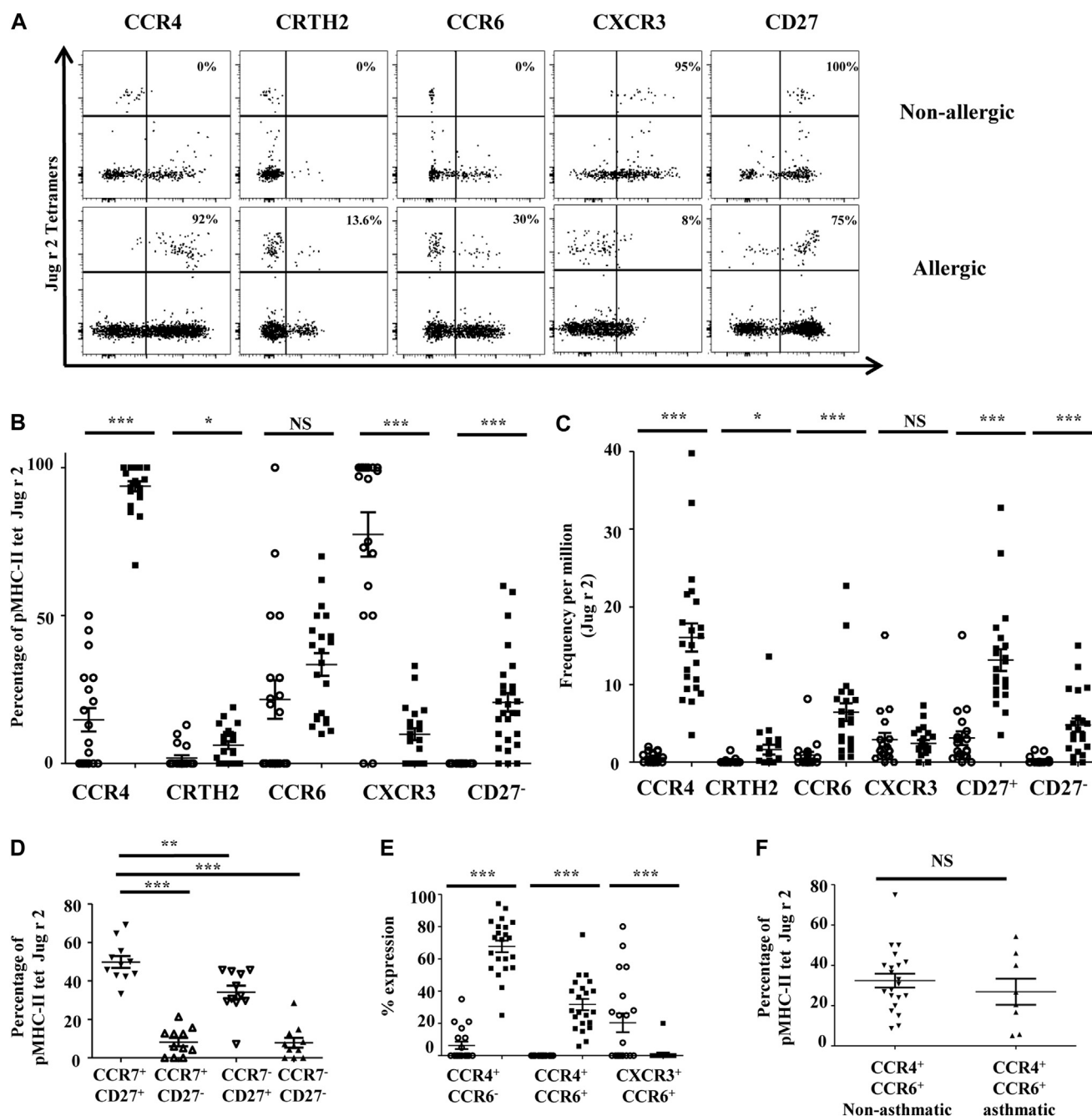


FIG 2. Phenotypes of Jug r 2-reactive T cells. **A**, First row, Profile in a *DRB1*15:01* nonallergic subject. Second row, Profile in a *DRB1*15:01* allergic patient. Percentages of surface markers expressed by Jug r 2-specific T cells are as indicated. **B**, Ex vivo expression of CCR4, CRTH2, CCR6, 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 of 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.

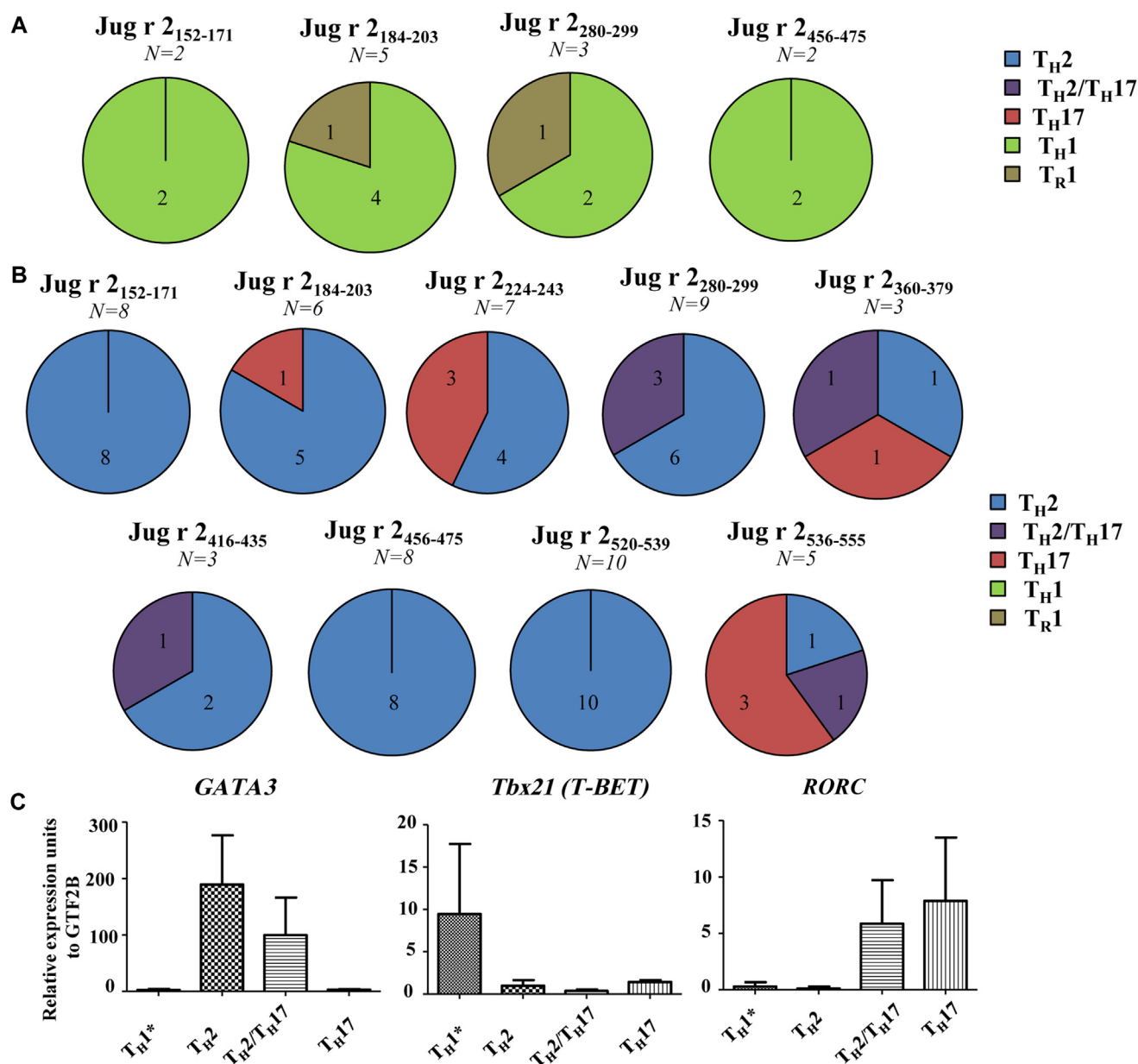


FIG 3. Jug r 2 T-cell subsets. **A** and **B**, Phenotype of TCCs derived from 6 nonallergic (Fig 3, A) and 8 allergic (Fig 3, B) subjects. Phenotype profiles were based on surface marker expression and cytokine production, the number of TCCs are as follows: T_H2, 45 TCCs; T_H2/T_H17, 6 TCCs; T_H17, 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

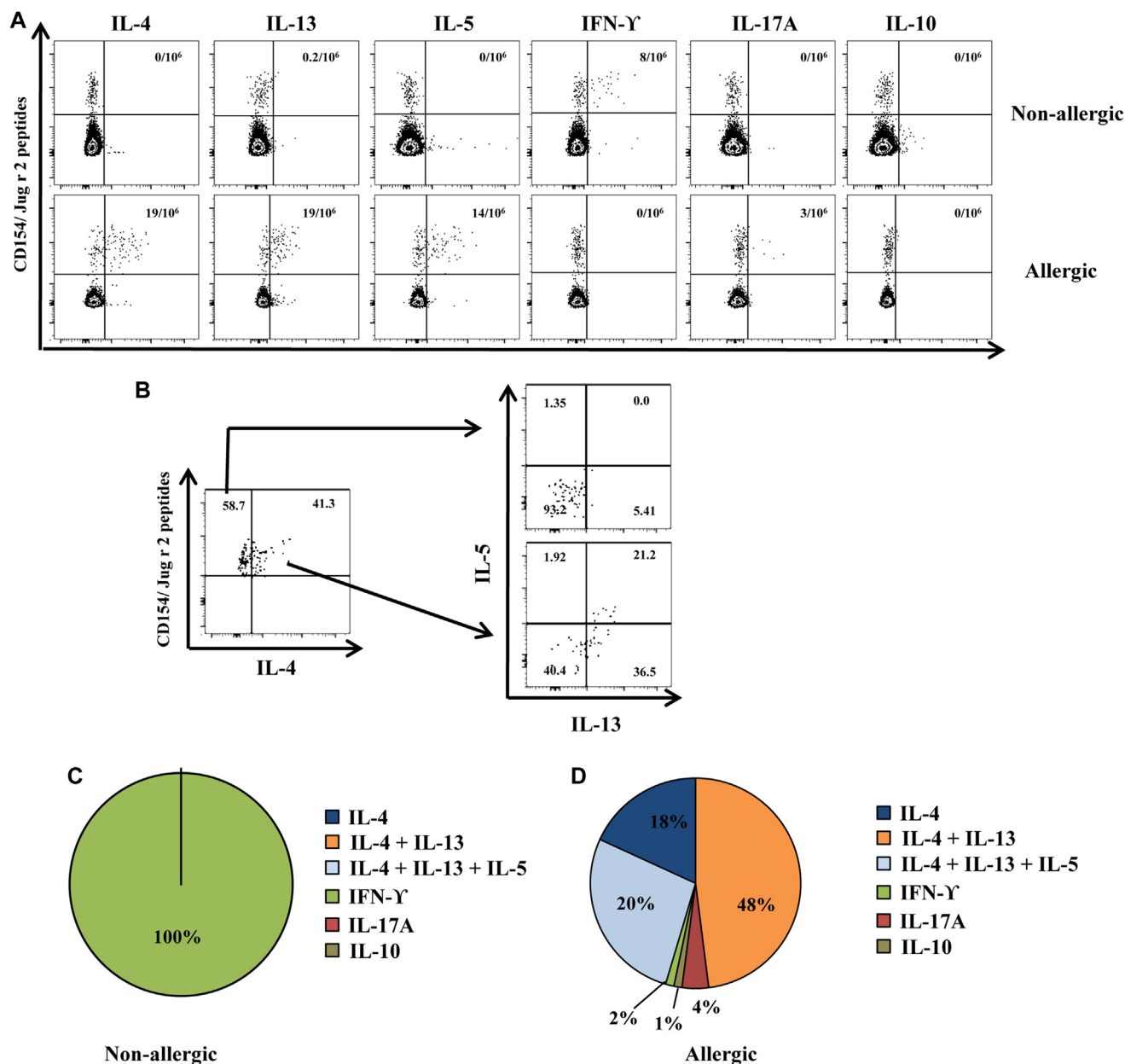


FIG 4. Cytokine profiles of Jug r 2-reactive T cells. **A**, First row, Cytokine profile in a DRB1*15:01 nonallergic subject. Second row, Cytokine profile in a DR15:01 allergic subject. Frequencies of Jug r 2-specific cytokine-producing T cells per million CD4⁺ T cells are as indicated. **B**, Gating 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 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 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

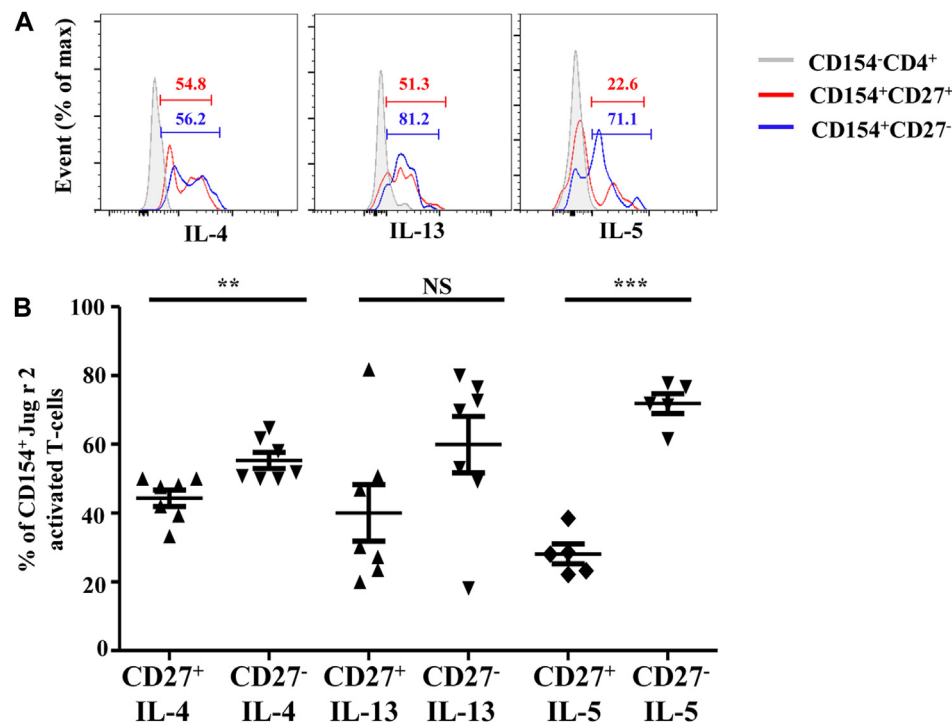


FIG 5. *Ex vivo* cytokine-producing capacities of CD27⁺ and CD27⁻ Jug r 2-reactive T cells. **A**, IL-4, IL-13, and IL-5 expression by CD27⁺ cells (red histogram), CD27⁻ cells (blue histogram), and CD154⁻ CD4⁺ cells as a control (gray histogram). **B**, Cytokine production by CD27⁺ and CD27⁻ CD154⁺ Jug 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 T_{eff} cells are capable of producing T_H2 cytokines (Fig 5, A). Interestingly, T_{eff} 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 T_{eff} 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-reactive¹⁸ and Pen m 2-reactive³⁹ 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 of 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 IgE⁴³ 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 al⁵⁰ 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.

We thank Lisa Myers for help with subject recruitment. We also thank members of our laboratory, especially Amedee Renand and I-Ting Chow, for discussions and suggestions.

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.

REFERENCES

1. Sicherer SH, Munoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol* 2010;125:1322–6.
2. Fleischer DM, Conover-Walker MK, Matsui EC, Wood RA. The natural history of tree nut allergy. *J Allergy Clin Immunol* 2005;116:1087–93.
3. Fleischer DM. The natural history of peanut and tree nut allergy. *Curr Allergy Asthma Rep* 2007;7:175–81.
4. Sicherer SH, Furlong TJ, Munoz-Furlong A, Burks AW, Sampson HA. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J Allergy Clin Immunol* 2001;108:128–32.
5. Sicherer SH, Sampson HA. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. *J Allergy Clin Immunol* 2014;133:291–307.
6. Muraro A, Roberts G, Clark A, Eigenmann PA, Halken S, Lack G, et al. The management of anaphylaxis in childhood: position paper of the European Academy of Allergy and Clinical Immunology. *Allergy* 2007;62:857–71.
7. Costa J, Carrapatoso I, Oliveira MB, Mafra I. Walnut allergens: molecular characterization, detection and clinical relevance. *Clin Exp Allergy* 2014;44:319–41.
8. Teuber SS, Dandekar AM, Peterson WR, Sellers CL. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 1998;101:807–14.
9. Teuber SS, Jarvis KC, Dandekar AM, Peterson WR, Ansari AA. Identification and cloning of a complementary DNA encoding a vicilin-like proprotein, jug r 2, from English walnut kernel (*Juglans regia*), a major food allergen. *J Allergy Clin Immunol* 1999;104:1311–20.
10. Wallowitz M, Peterson WR, Uratsu S, Comstock SS, Dandekar AM, Teuber SS. Jug r 4, a legumin group food allergen from walnut (*Juglans regia* Cv. Chandler). *J Agric Food Chem* 2006;54:8369–75.
11. Pastorello EA, Farioli L, Pravettoni V, Robino AM, Scibilia J, Fortunato D, et al. Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen. *J Allergy Clin Immunol* 2004;114:908–14.
12. Rosenfeld L, Shreffler W, Bardina L, Niggemann B, Wahn U, Sampson HA, et al. Walnut allergy in peanut-allergic patients: significance of sequential epitopes of walnut homologous to linear epitopes of Ara h 1, 2 and 3 in relation to clinical reactivity. *Int Arch Allergy Immunol* 2012;157:238–45.
13. Barre A, Sordet C, Culerrier R, Rance F, Didier A, Rouge P. Vicilin allergens of peanut and tree nuts (walnut, hazelnut and cashew nut) share structurally related IgE-binding epitopes. *Mol Immunol* 2008;45:1231–40.
14. Robotham JM, Teuber SS, Sathe SK, Roux KH. Linear IgE epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J Allergy Clin Immunol* 2002;109:143–9.
15. Woodfolk JA. T-cell responses to allergens. *J Allergy Clin Immunol* 2007;119:280–94.
16. Wambre E, James EA, Kwok WW. Characterization of CD4⁺T cell subsets in allergy. *Curr Opin Immunol* 2012;24:700–6.
17. Prickett SR, Voskamp AL, Dacumos-Hill A, Symons K, Rolland JM, O'Hehir RE. Ara h 2 peptides containing dominant CD4⁺T-cell epitopes: candidates for a peanut allergy therapeutic. *J Allergy Clin Immunol* 2011;127:608–15.
18. Delong JH, Simpson KH, Wambre E, James EA, Robinson D, Kwok WW. Ara h 1-reactive T cells in individuals with peanut allergy. *J Allergy Clin Immunol* 2011;127:1211–8.
19. Bacher P, Schink C, Teutschbein J, Knemeyer O, Assenmacher M, Brakhage AA, et al. Antigen-reactive T cell enrichment for direct, high-resolution analysis of the human naive and memory Th cell repertoire. *J Immunol* 2013;190:3967–76.

20. Novak EJ, Liu AW, Gebe JA, Falk BA, Nepom GT, Koelle DM, et al. Tetramer-guided epitope mapping: rapid identification and characterization of immunodominant CD4⁺ T cell epitopes from complex antigens. *J Immunol* 2001;166:6665-70.
21. Kwok WW, Roti M, Delong JH, Tan V, Wambre E, James EA, et al. Direct ex vivo analysis of allergen-specific CD4⁺ T cells. *J Allergy Clin Immunol* 2010;125:1407-9.
22. Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J Clin Invest* 1999;104:R63-7.
23. Kwok WW, Liu AW, Novak EJ, Gebe JA, Ettinger RA, Nepom GT, et al. HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2-infected individuals: direct detection of immunodominant antigen-responsive cells. *J Immunol* 2000;164:4244-9.
24. Oseroff C, Sidney J, Tripple V, Grey H, Wood R, Broide DH, et al. Analysis of T cell responses to the major allergens from German cockroach: epitope specificity and relationship to IgE production. *J Immunol* 2012;189:679-88.
25. Nagata K, Tanaka K, Ogawa K, Kemmotsu K, Imai T, Yoshie O, et al. Selective expression of a novel surface molecule by human Th2 cells in vivo. *J Immunol* 1999;162:1278-86.
26. Cosmi L, Annunziato F, Galli MIG, Maggi RME, Nagata K, Romagnani S. CCR2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease. *Eur J Immunol* 2000;30:2972-9.
27. Fritsch RD, Shen X, Sims GP, Hathcock KS, Hodes RJ, Lipsky PE. Stepwise differentiation of CD4 memory T cells defined by expression of CCR7 and CD27. *J Immunol* 2005;175:6489-97.
28. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004;22:745-63.
29. Rivino L, Messi M, Jarrossay D, Lanzavecchia A, Sallusto F, Geginat J. Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4⁺ central memory T cells. *J Exp Med* 2004;200:725-35.
30. Ito T, Carson WF, Cavassani KA, Connell JM, Kunkel SL. CCR6 as a mediator of immunity in the lung and gut. *Exp Cell Res* 2011;317:613-9.
31. Kunkel EJ, Campbell DJ, Butcher EC. Chemokines in lymphocyte trafficking and intestinal immunity. *Microcirculation* 2003;10:313-23.
32. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 2007;8:639-46.
33. Wambre E, Delong JH, James EA, LaFond RE, Robinson D, Kwok WW. Differentiation stage determines pathologic and protective allergen-specific CD4⁺ T-cell outcomes during specific immunotherapy. *J Allergy Clin Immunol* 2012;129:544-51.
34. Wambre E, Delong JH, James EA, Torres-Chinn N, Pflutzner W, Mobs C, et al. Specific immunotherapy modifies allergen-specific CD4(+) T-cell responses in an epitope-dependent manner. *J Allergy Clin Immunol* 2014;133:872-9.
35. Carballido JM, Carballido-Perrig N, Terres G, Heusser CH, Blaser K. Bee venom phospholipase A2-specific T cell clones from human allergic and non-allergic individuals: cytokine patterns change in response to the antigen concentration. *Eur J Immunol* 1992;22:1357-63.
36. Ebner C, Schenk S, Najafian N, Siemann U, Steiner R, Fischer GW, et al. Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J Immunol* 1995;154:1932-40.
37. Gett AV, Hodgkin PD. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc Natl Acad Sci U S A* 1998;95:9488-93.
38. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 2010;28:445-89.
39. Renand A, Newbrough S, Wambre E, Delong JH, Robinson D, Kwok WW. Arginine kinase Pen m 2 as an important shrimp allergen recognized by T2 cells. *J Allergy Clin Immunol* 2014;134:1456-9.e7.
40. Hintzen RQ, de Jong R, Lens SM, Brouwer M, Baars P, van Lier RA. Regulation of CD27 expression on subsets of mature T-lymphocytes. *J Immunol* 1993;151:2426-35.
41. Hamann D, Baars PA, Rep MH, Hooibrink B, Kerkhof-Garde SR, Klein MR, et al. Phenotypic and functional separation of memory and effector human CD8⁺ T cells. *J Exp Med* 1997;186:1407-18.
42. Mackenzie KJ, Nowakowska DJ, Leech MD, McFarlane AJ, Wilson C, Fitch PM, et al. Effector and central memory T helper 2 cells respond differently to peptide immunotherapy. *Proc Natl Acad Sci U S A* 2014;111:E784-93.
43. Milovanovic M, Drozdenko G, Weise C, Babina M, Worm M. Interleukin-17A promotes IgE production in human B cells. *J Invest Dermatol* 2010;130:2621-8.
44. Zhao Y, Yang J, Gao YD, Guo W. Th17 immunity in patients with allergic asthma. *Int Arch Allergy Immunol* 2010;151:297-307.
45. Ciprandi G, Filaci G, Battaglia F, Fenoglio D. Peripheral Th-17 cells in allergic rhinitis: new evidence. *Int Immunopharmacol* 2010;10:226-9.
46. Cosmi L, Maggi L, Santarlasci V, Capone M, Cardilicchia E, Frosali F, et al. Identification of a novel subset of human circulating memory CD4(+) T cells that produce both IL-17A and IL-4. *J Allergy Clin Immunol* 2010;125:222-30.
47. Wang YH, Voo KS, Liu B, Chen CY, Uygungil B, Spoede W, et al. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med* 2010;207:2479-91.
48. Irvin C, Zafar I, Good J, Rollins D, Christianson C, Gorska MM, et al. Increased frequency of dual-positive T2/T17 cells in bronchoalveolar lavage fluid characterizes a population of patients with severe asthma. *J Allergy Clin Immunol* 2014;134:1175-86.e7.
49. Blazquez AB, Knight AK, Getachew H, Bromberg JS, Lira SA, Mayer L, et al. A functional role for CCR6 on proallergic T cells in the gastrointestinal tract. *Gastroenterology* 2010;138:275-84.
50. Dhuban KB, d'Hennezel E, Ben-Shoshan M, McCusker C, Clarke A, Fiset P, et al. Altered T helper 17 responses in children with food allergy. *Int Arch Allergy Immunol* 2013;162:318-22.

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 μ L of 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). 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 $\times$ brefeldin A (eBioscience) for 5 hours at 37°C in a 5% CO₂ 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 μ g/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 ($\geq 10\%$), IL-5 ($\geq 10\%$), and IL-13 ($\geq 10\%$); 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 ($\geq 10\%$) and IL-17A ($\geq 10\%$) 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 ($\geq 10\%$) 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 ($\geq 10\%$), and expression of *T-bet* (*TBX21*). The T_R1 profile is exemplified by CXCR3 expression (some clones also coexpressed CD27), production of IFN- γ ($\geq 10\%$) and IL-10 ($\geq 10\%$), 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.

REFERENCES

- E1. Kwok WW, Roti M, Delong JH, Tan V, Wambre E, James EA, et al. Direct *ex vivo* analysis of allergen-specific CD4⁺ T cells. *J Allergy Clin Immunol* 2010;125:1407-9.
- E2. Archila LD, Delong JH, Wambre E, James EA, Robinson DM, Kwok WW. Grass-specific CD4(+) T-cells exhibit varying degrees of cross-reactivity, implications for allergen-specific immunotherapy. *Clin Exp Allergy* 2014;44: 986-98.

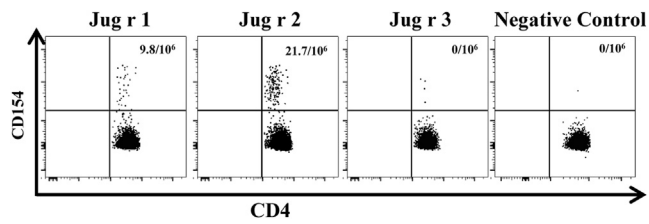


FIG E1. Frequencies of Jug r 1–, Jug r 2–, and Jug r 3–reactive T cells in a DR15:01 patient with walnut allergy. Frequencies of walnut allergen-reactive T cells per million CD4⁺ T cells are as indicated.

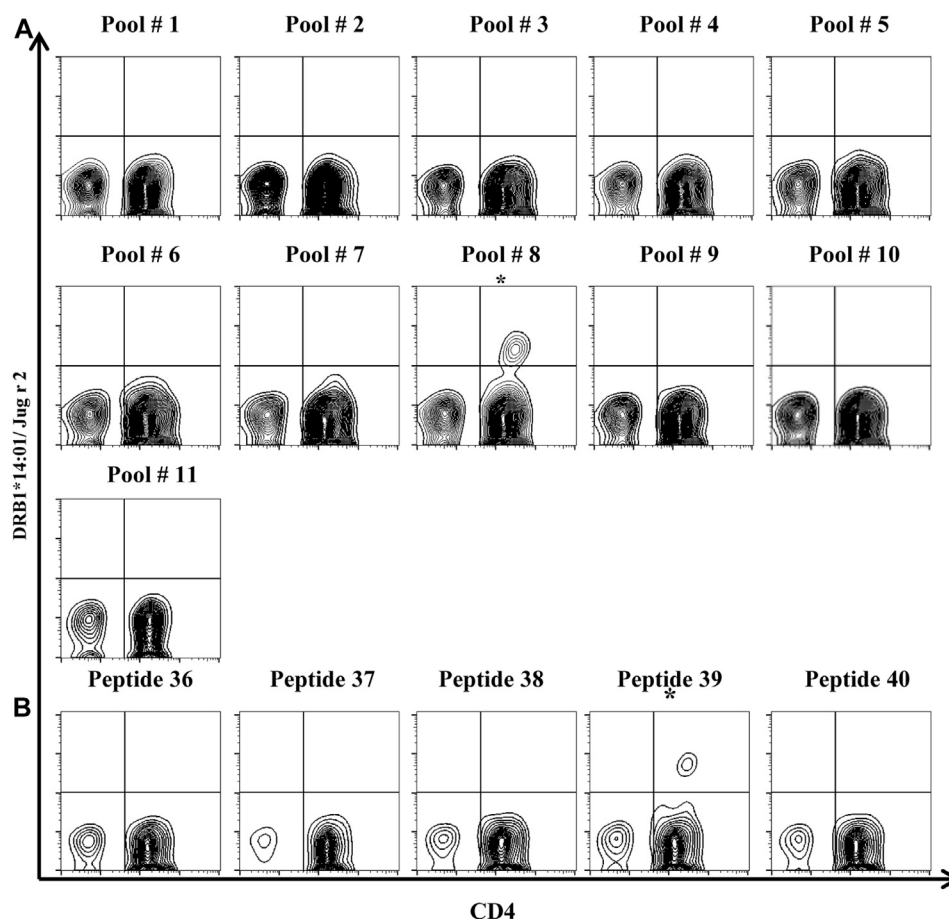


FIG E2. TGEM studies of DR14:01-restricted Jug r 2-reactive CD4⁺ T cells. **A**, PBMCs from a DR14:01 patient with walnut allergy were stimulated with 11 pools of Jug r 2 peptides for 2 weeks and subsequently stained with corresponding DR14:01/Jug r 2-pooled peptide tetramers. **B**, Cells stimulated with pool 8 were restained with individual peptides from the corresponding pool. The staining identified p39 (Jug r 2₄₅₆₋₄₇₅) as DR14:01-restricted Jug r 2 T-cell epitopes.

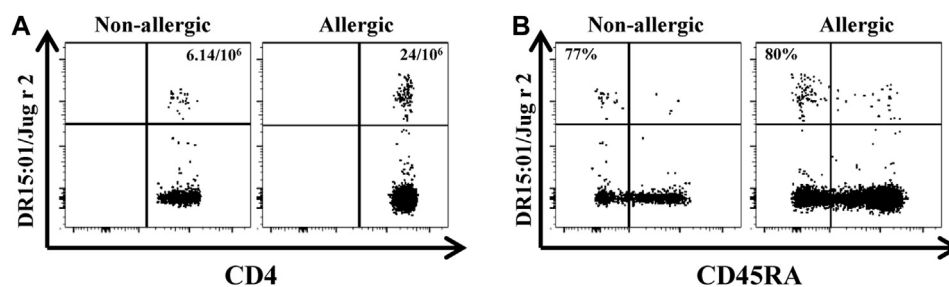


FIG E3. Frequencies of Jug r 2 epitope-reactive T cells. **A,** *Ex vivo* combined frequencies of Jug r 2₁₈₄₋₂₀₃⁻, Jug r 2₂₉₆₋₃₁₅⁻, Jug r 2₃₉₂₋₄₁₁⁻, and Jug r 2₄₅₆₋₄₇₅⁻-specific T cells in DR15:01 allergic and nonallergic subjects. Frequencies of Jug r 2-specific T cells per million CD4⁺ T cells are as indicated. **B,** *Ex vivo* percentages of memory (CD45RA⁻) Jug r 2-specific T cells in DR15:01 allergic and nonallergic subjects.

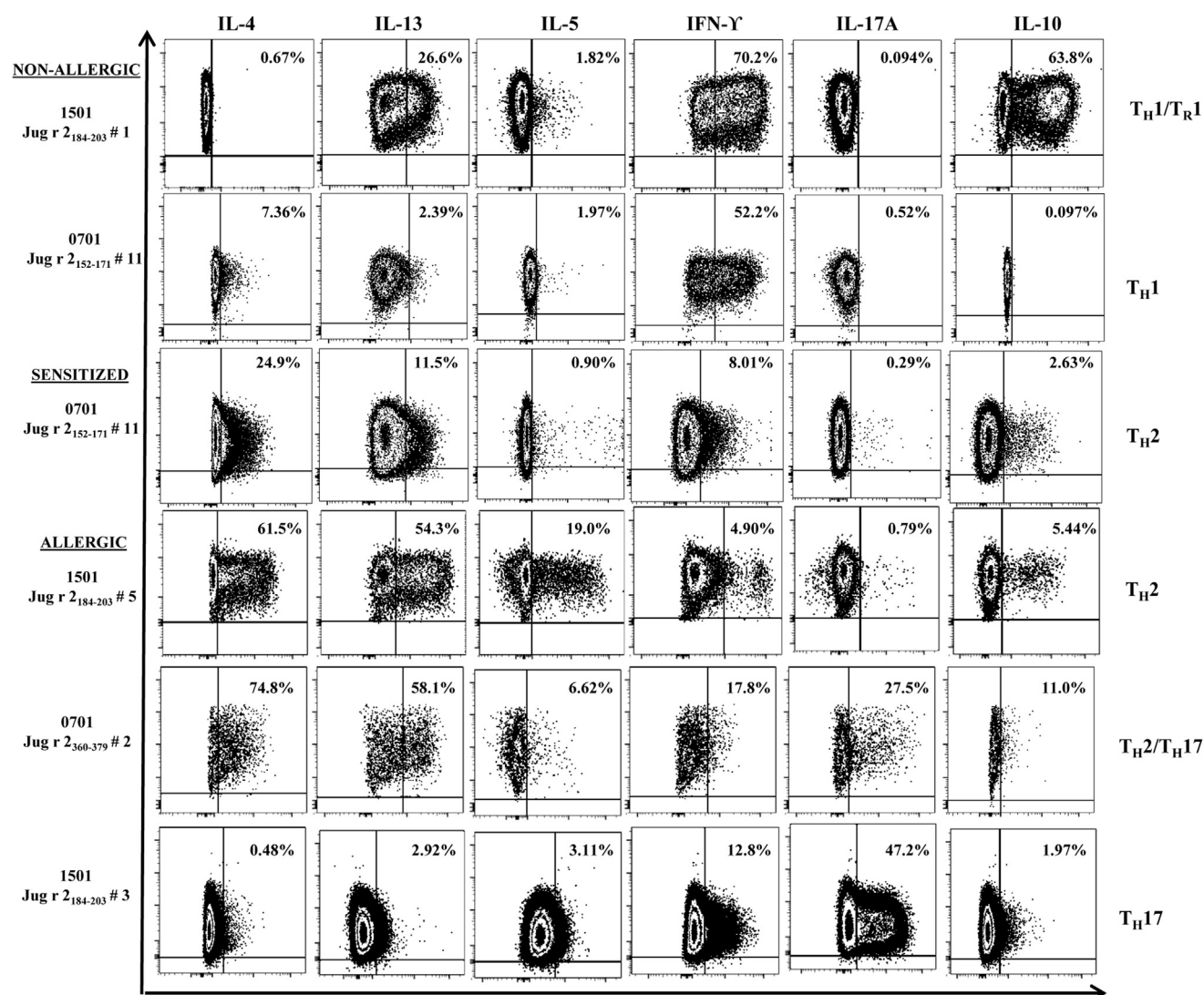


FIG E4. Cytokine profiles of *ex vivo* sorted TCCs generated from nonallergic (T_{H1}/T_{R1} [first row] and T_{H1} [second row]), sensitized (T_{H2} [third row]), and allergic (T_{H2} [fourth row], T_{H2}/T_{H17} [fifth row], and T_{H17} [sixth row]) subjects. Representative examples for Jug r 2₁₈₄₋₂₀₃-, Jug r 2₁₅₂₋₁₇₁-, and Jug r 2₃₆₀₋₂₇₉-reactive TCCs. Two different phenotypes are observed for the same specificity within the same subject.

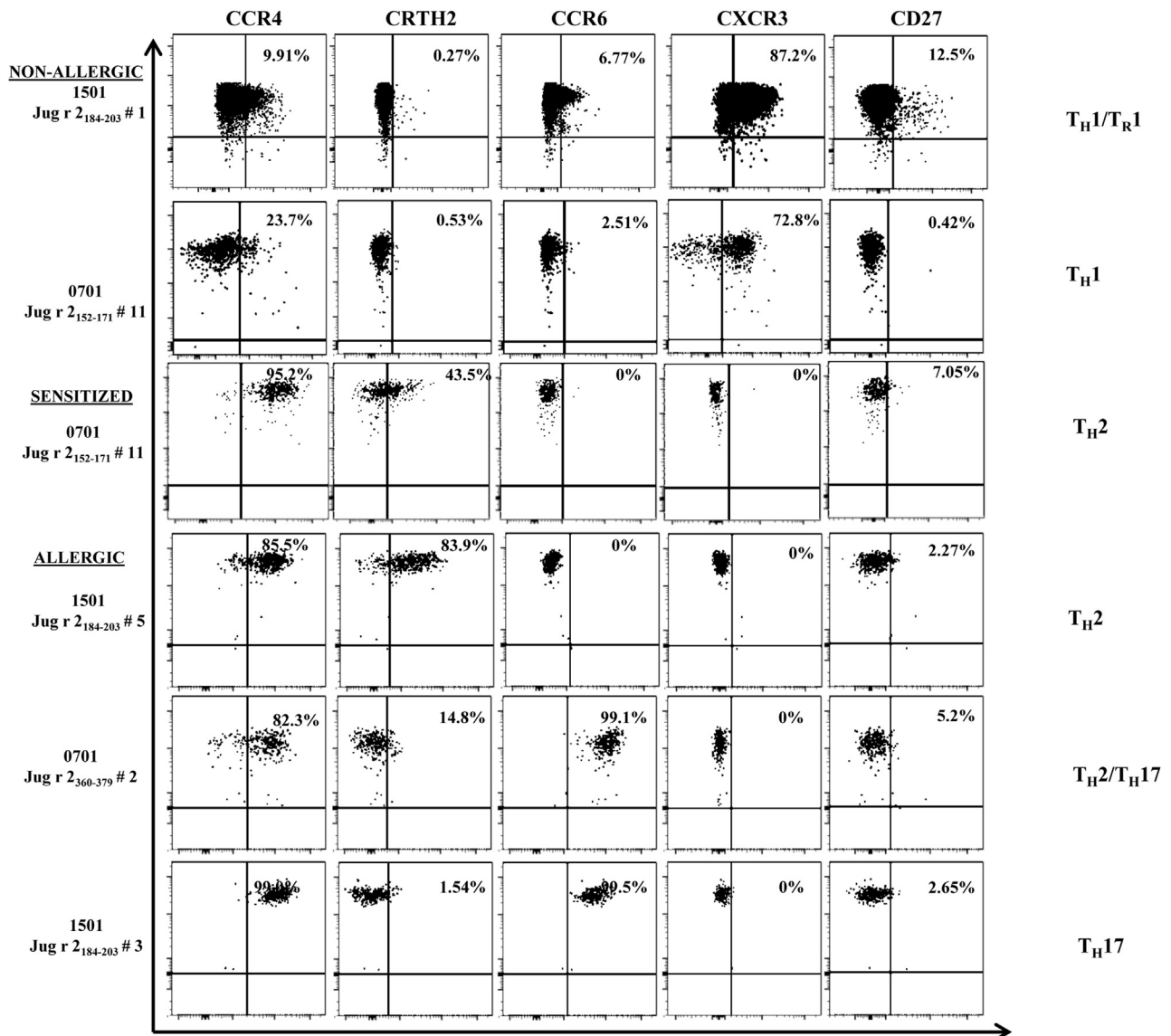


FIG E5. Surface phenotype of *ex vivo* sorted TCCs generated from nonallergic (T_H1/T_{R1} [first row] and T_H1 [second row]), sensitized (T_H2 [third row]), and allergic (T_H2 [fourth row], T_H2/T_{H17} [fifth row], and T_{H17} [sixth row]) subjects. Representative examples for Jug r 2₁₈₄₋₂₀₃-, Jug r 2₁₅₂₋₁₇₁-, and Jug r 2₃₆₀₋₃₇₉-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, 04:01	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 2₁₅₂₋₁₇₁; *II*, Jug r 2₁₈₄₋₂₀₃; *III*, Jug r 2₂₂₄₋₂₄₃; *IV*, Jug r 2₂₈₀₋₂₉₉; *V*, Jug r 2₂₉₆₋₃₁₅; *VI*, Jug r 2₃₆₀₋₃₇₉; *VII*, Jug r 2₃₉₂₋₄₁₁; *VIII*, Jug r 2₄₁₆₋₄₃₅; *IX*, Jug r 2₄₅₆₋₄₇₅; *X*, Jug r 2₅₂₀₋₅₃₉; and *XI*, Jug r 2₅₃₆₋₅₅₅.

†Specificities listed were combined per tube for analysis.