

Ara h 1-reactive T cells in individuals with peanut allergy

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Background: Effective immunotherapy for peanut allergy is hampered by a lack of understanding of peanut-reactive CD4⁺ T cells.

Objective: To identify, characterize, and track Ara h 1-reactive cells in subjects with peanut allergy by using Ara h 1-specific class II tetramers.

Methods: Tetramer-guided epitope mapping was used to identify the antigenic peptides within the peanut allergen Ara h 1. Subsequently, HLA class II/Ara h 1-specific tetramers were used to determine the frequency and phenotype of Ara h 1-reactive T cells in subjects with peanut allergy. Cytokine profiles of Ara h 1-reactive T cells were also determined.

Results: Multiple Ara h 1 epitopes with defined HLA restriction were identified. Ara h 1-specific CD4⁺ T cells were detected in all of the subjects with peanut allergy tested. Ara h 1-reactive T cells in subjects with allergy expressed CCR4 but did not express CCR2. The percentage of Ara h 1-reactive cells that expressed the $\beta 7$ integrin was low compared with total CD4⁺ T cells. Ara h 1-reactive cells that secreted IFN- γ , IL-4, IL-5, IL-10, and IL-17 were detected.

Conclusion: In individuals with peanut allergy, Ara h 1-reactive T cells occurred at moderate frequencies, were predominantly CCR4⁺ memory cells, and produced IL-4. Class II tetramers can be readily used to detect Ara h 1-reactive T cells in the peripheral blood of subjects with peanut allergy without *in vitro* expansion and would be effective for tracking peanut-reactive CD4⁺ T cells during immunotherapy. (*J Allergy Clin Immunol* 2011;127:1211-8.)

Key words: Food allergy, peanut, Ara h 1, T cells, class II tetramers

Peanut allergy is relatively common, affecting approximately 1% of children in the United States and the United Kingdom. Peanut sensitivity typically presents early in life and is often permanent, because only 20% of young children resolve their food allergy by adulthood.^{1,2} In addition, peanut allergy is associated with a significant risk of fatality. Currently, the only standard treatment options for patients with peanut allergy are vigilant avoidance and administration of epinephrine in the event of an accidental

Abbreviations used

CLA: Cutaneous lymphocyte antigen

CRTH2: Chemoattractant receptor-homologous molecule expressed on T_H2 cells

ICS: Intracellular cytokine staining

TGEM: Tetramer-guided epitope mapping

ingestion. Both of these are associated with considerable psychological morbidity. As a consequence, multiple approaches to induce tolerance to peanut have been carried out. Studies initiated more than 15 years ago demonstrated that desensitization is possible for peanut allergy by injection of crude peanut extract.^{3,4} However, these injections were associated with frequent episodes of anaphylaxis.⁵ More recently, peanut oral immunotherapy trials were attempted, and the outcomes were encouraging.⁶⁻⁸ However, even in these recent oral immunotherapy trials, treatment was associated with side effects that required medication.⁶

Peanut-specific CD4⁺ T cells are known to be involved in the pathophysiology of peanut allergy and could act as a useful biomarker for evaluating the progress of peanut oral immunotherapy.^{9,10} Ideally, a T-cell biomarker should distinguish between desensitization and tolerance induction. It would also be useful to predict the likelihood of relapse after induction of tolerance. In response to the need for a reliable biomarker for monitoring, the current study sought to develop and apply class II tetramer reagents to monitor peanut-reactive T cells. Identification of T-cell epitopes for the common peanut allergens is crucial for the development of such reagents. Currently, 11 peanut allergens have been identified (<http://www.allergen.org>). Sensitization to Ara h 1 and Ara h 2 has been found in 80% to 95% of North American patients with peanut allergy.^{11,12} However, sensitization to Ara h 1 seems to be lower in subjects with peanut allergy in European countries, ranging from 30% to 75%.¹²⁻¹⁴ Sensitization to Ara h 6 was high in some European populations, and sensitization to Ara h 9 was found to be the most prevalent in Mediterranean populations.^{12,13} Multiple IgE epitopes have been identified in Ara h 1,¹⁵ but no primary data have been published on Ara h 1 T-cell epitopes. In the current study, we first used tetramer-guided epitope mapping (TGEM) to identify CD4⁺ T-cell epitopes in Ara h 1 and subsequently used these class II tetramer reagents to study CD4⁺ T cells specific for Ara h 1 in individuals with peanut allergy and without allergy. Ara h 1 T-cell epitopes restricted by 8 HLA class II alleles were identified. By using tetramers corresponding to these epitopes, we examined the frequency and phenotype of peanut-specific CD4⁺ T cells in individuals with and without allergy. In addition, cytokine analysis was performed on Ara h 1-specific T-cell clones and lines from individuals with peanut allergy.

METHODS

Human subjects

Subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute. Subjects with allergy and a documented

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TABLE I. HLA and allergic status of recruited subjects

Subject no.	Sex	Age (y)	HLA	Asthma	Peanut IgE ImmunoCAP
Individuals with peanut allergy					
1	F	60	DR0401/DR1501-B5	No	3
2	M	34	DR0404/DR0803	No	5
3	M	23	DR0401/DR1302	No	4*
4	F	24	DR0701/DR1401	Yes	3
5	F	49	DR1101/DR1302	Yes	4
6	M	36	DR0101/DR1501-B5	Yes	3†
7	M	22	DR0101/DR0701	No	3†
8	M	26	DR0101/DR1301	Yes	2
9	M	20	DR0301/DR0701	Yes	5
10	M	35	DR0901/DR1101	No	4
11	F	19	DR0701/DR1502-B5	Yes	6
12	F	33	DR0301/DR0701	Yes	5
Nonatopic subjects					
13	F	30	DR0101/DR0103	No	0
14	F	30	DR0101/-	No	0
15	M	39	DR0401/DR1101	No	0
16	F	30	DR0401/DR1401	No	0
17	F	28	DR1501-B5/-	No	0
18	M	29	DR0401/DR1501-B5	No	0
Atopic subjects without peanut allergy					
19	M	38	DR0401/DR1401	No	0‡
20	M	Unknown	DR0401/DR1501-B5	No	0*†
21	M	50	DR1101/DR1501-B5	Yes	0*†
22	F	26	DR0901/DR1502-B5	No	0†
23	F	33	DR0301/DR0401	No	0*

F, Female; M, male.

*Subjects also had history of timothy grass allergy and positive IgE ImmunoCAP for timothy grass.

†Subjects also had history of alder pollen allergy and positive IgE ImmunoCAP for alder.

‡Subject also had history of cat allergy and positive IgE ImmunoCAP for cat.

record of peanut anaphylaxis and positive ImmunoCAP scores for peanut-specific IgE (Phadia AB, Uppsala, Sweden) were recruited. A subset of these subjects also had documented seasonal allergy. Atopic subjects without peanut allergy and nonatopic subjects with no clinical symptoms of allergy and negative ImmunoCAP scores for timothy grass pollen, cat, dust mite, and peanut were also recruited. DNA samples were HLA-typed by using Dynal Unitray SSP Kits (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. All subjects were recruited with informed consent and institutional review board approval. The attributes of these human subjects are summarized in Table I.

TGEM

Biotinylated HLA-DR proteins were purified as described.¹⁶ A total of 77 peptides (p1 to p77), which were 20 amino acids in length with a 12 amino acid overlap spanning the entire Ara h 1 sequence (including the signal peptide), were synthesized (Mimotopes, Clayton, Australia). These peptides were divided into 14 pools of 5 peptides each plus a 15th pool of 7 peptides. These peptide mixtures were loaded into the biotinylated HLA-DR proteins to generate pooled tetramers as described.¹⁷ Cells were cultured for 14 days and then stained with pooled peptide tetramers. Cells from wells that gave positive staining were stained again by using tetramers loaded with each individual peptide from the positive pool.

Ex vivo analysis of Ara h 1 and pollen-reactive CD4⁺ T cells

The frequency of Ara h 1-specific T cells was measured as previously described.^{18,19} Briefly, 30 million PBMCs in 200 μ L T-cell culture medium were stained with 20 μ g/mL phycoerythrin-labeled tetramers at room temperature for 100 minutes. Cells were then stained with anti-CD3 fluorescein isothiocyanate (eBioscience), anti-CD4 allophycocyanin (eBioscience), anti-CD14 peridinin chlorophyll protein (BD Biosciences, San Diego, Calif) and anti-CD19

peridinin chlorophyll protein (BD Biosciences) for 20 minutes at 4°C. Cells were washed and incubated with anti-phycoerythrin magnetic beads (Miltenyi Biotec) at 4°C for 20 minutes and washed again, and a 1/100 fraction was saved for analysis. The other fraction was passed through a magnetic column (Miltenyi Biotec, Bergisch Gladbach, Germany). Bound, phycoerythrin-labeled cells were flushed and collected. Cells in the bound and precolumn fractions were stained with Via-Probe (BD Biosciences) for 10 minutes before flow cytometry. Data were analyzed by using FlowJo (Tree Star, Ashland, Ore) gating on forward scatter/side scatter and excluding CD14⁺ and CD19⁺ populations and Via-Probe⁺ (dead) cells. Frequency was calculated as previously described.¹⁹

For phenotyping studies, antibodies against markers of interest were used instead of anti-CD3. Staining for Aln g 1 and Phl p 1-reactive T cells was carried out with DR0701/Aln g 1₁₃₇₋₁₅₆, DR1501/Aln g 1₁₃₇₋₁₅₆, and DR0401/Phl p 1₁₂₀₋₁₃₉ tetramers.

Intracellular cytokine staining

For intracellular staining of IFN- γ , IL-4, IL-17, IL-5, and/or IL-10, PBMCs were stimulated for 2 weeks with specific peptide and then stained with the corresponding phycoerythrin-labeled tetramers for 60 minutes at 37°C. Cells were then restimulated with 50 ng/mL phorbol 12-myristate 13-acetate and 1 μ g/mL ionomycin in the presence of 10 μ g/mL monensin in 1 mL complete media for 6 hours at 37°C, 5% CO₂. After restimulation, cells were stained with anti-CD4 (BD Biosciences), anti-CD3 (eBioscience), and a combination of anti-CD14 (BD Biosciences), anti-CD19 (Dako, Glostrup, Denmark) and ViViD reagent (Invitrogen) to exclude nonspecific tetramer staining. After 30 minutes at room temperature, cells were fixed with fixation buffer (eBioscience), washed twice with a permeabilization buffer (eBioscience), and stained in 200 μ L permeabilization buffer with various combinations of anti-IFN- γ , anti-IL-17, anti-IL-10 (all from Biolegend), anti-IL-4 (eBioscience) and anti-IL-5 (Miltenyi Biotec). After 30 minutes at 4°C, cells were washed and immediately analyzed by flow cytometry.

TABLE II. Ara h 1 CD4⁺ T-cell epitopes

HLA	Ara h 1	Amino acid sequence
DRB1*0101	Ara h 1 (201-220)	QRSRQFQNLQNHRIVQIEAK
	Ara h 1 (233-252)	DNILVIQQGQATVTVANGNN
	Ara h 1 (505-524)	KEGDVFIMPAAHPVAINASS
DRB1*0301	Ara h 1 (409-428)	NNFGKLFVVKPDKKNPQLQD
DRB1*0401	Ara h 1 (201-220)	QRSRQFQNLQNHRIVQIEAK
	Ara h 1 (329-348)	FNEIRRVLLEENAGGEQEER
	Ara h 1 (505-524)	KEGDVFIMPAAHPVAINASS
DRB1*0404	Ara h 1 (577-596)	QKESHFVSARPSQSQSPPSS
	Ara h 1 (329-348)	FNEIRRVLLEENAGGEQEER
	Ara h 1 (449-468)	NSKAMVIVVVKGTGNLELV
DRB1*1101	Ara h 1 (169-188)	TSRNNPFYFSPRRFSTRYGN
	Ara h 1 (321-340)	LEAAFNAEFNEIRRVLLEEN
	Ara h 1 (457-476)	VVNKGTGNLELVAVRKEQQQ
DRB1*1401	Ara h 1 (465-484)	LELVAVRKEQQQRGRREEEE
	Ara h 1 (321-340)	LEAAFNAEFNEIRRVLLEEN
	Ara h 1 (329-348)	FNEIRRVLLEENAGGEQEER
DRB1*1502	Ara h 1 (201-220)	QRSRQFQNLQNHRIVQIEAK
DRB5	Ara h 1 (209-228)	LQNHRIVQIEAKPNTLVLPK
	Ara h 1 (369-388)	SKEHVEELTKHAKSVSKKGS
	Ara h 1 (489-508)	EEEGSNREVRRTARLKEGD

RESULTS

Identification of CD4⁺ T-cell epitopes in Ara h 1

A total of 12 subjects with peanut allergy with a history of anaphylaxis to peanut were recruited for this study. Six nonatopic subjects and 5 atopic subjects without peanut allergy were also recruited as control subjects. The TGEM approach was used to identify CD4⁺ T-cell epitopes for Ara h 1 in subjects with peanut allergy as described in Methods. Detailed results for one of these experiments with a representative DR1101 subject are shown in this article's Fig E1 in the Online Repository at www.jacionline.org. Ara h 1₁₆₉₋₁₈₈, Ara h 1₃₂₁₋₃₄₀, Ara h 1₄₅₇₋₄₇₆, and Ara h 1₄₆₅₋₄₈₄ were identified as DR1101-restricted T-cell epitopes. A complete summary of results from all epitope identification experiments is shown in Table II. In summary, a total of 20 epitopes were identified, restricted by DR0101, DR0301, DR0401, DR0404, DR1101, DR1401, DR1502, and DRB5. It is likely that pairs of consecutive peptides, such as Ara h 1₄₅₇₋₄₇₆/Ara h 1₄₆₅₋₄₈₄ (restricted by DR1101) and Ara h 1₃₂₁₋₃₄₀/Ara h 1₃₂₉₋₃₄₈ (restricted by DR1401) contain an identical minimum epitope. In using TGEM, we failed to identify any Ara h 1 epitopes restricted by DR0701 and DR1501. However, each subject with a DR0701 or DR1501 haplotype recognized Ara h 1 epitopes restricted by another class II allele. Therefore, it is possible that there are no DR1501 and DR0701-restricted Ara h 1 epitopes. Alternatively, DR0701 and DR1501-restricted Ara h 1-specific T cells may be of low avidity, making them difficult to detect by using tetramers.

Ex vivo detection and cell surface phenotyping of Ara h 1-specific CD4⁺ T cells

To detect Ara h 1-specific T cells directly *ex vivo*, we used anti-phycoerythrin magnetic beads to enrich for phycoerythrin-labeled tetramer-positive cells. This approach enabled us to characterize the phenotype and frequency of Ara h 1-reactive T cells in the peripheral blood without expanding PBMCs *in vitro*. Representative results for a DR1101 subject with peanut allergy and a DR1101 nonatopic subject are shown in Fig 1, A. The results of additional experiments are summarized in Fig 1, B. These results

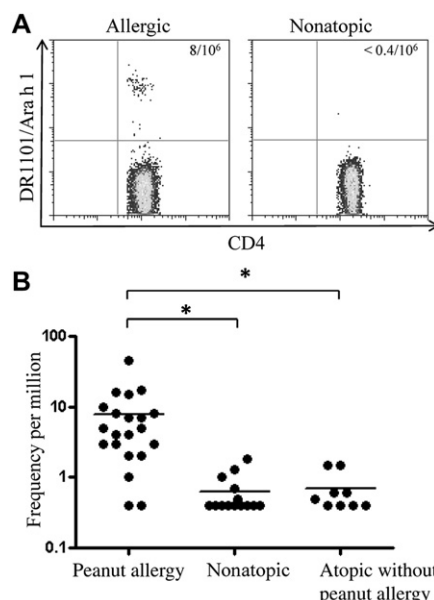


FIG 1. Frequencies of Ara h 1 epitope-reactive T cells. **A**, Frequencies of Ara h 1₃₂₁₋₃₄₀-specific T cells in a DR1101 subject with allergy and a DR1101 subject without allergy. The frequencies of Ara h 1-specific T cells per million CD4⁺ T cells are as indicated. **B**, Frequencies of Ara h 1 epitope-reactive T cells in 11 subjects with peanut allergy, 6 nonatopic subjects, and 5 atopic subjects without peanut allergy. Each data point represents the frequency of T cells specific for a single epitope in Ara h 1. A Student *t* test was used in the statistical analysis. **P* < .05.

indicated that the average frequency of Ara h 1-reactive T cells in subjects with peanut allergy was approximately 9 cells per million, whereas the average frequency in nonatopic subjects and atopic subjects without peanut allergy was less than 1 cell per million. *Ex vivo* tetramer staining of Ara h 1-reactive T cells also allowed direct examination of cell surface phenotypes for Ara h 1-reactive T cells in subjects with allergy by using surface markers such as CD45RA (a naive T-cell marker), CD45RO (a memory T cell marker), chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2) and CCR4 (T_H2 markers),^{20,21} and cutaneous lymphocyte antigen (CLA) and β 7 integrin (T-cell homing markers).^{22,23} The expression of each of these markers on Ara h 1-reactive T cells was compared with that of total CD4⁺ T cells. Representative results for 1 of these experiments are shown in Fig 2, A. Complete results from multiple subjects are summarized in Fig 2, B. In total, the data indicate that Ara h 1-reactive T cells in subjects with peanut allergy were memory T cells and expressed the T_H2 marker CCR4. The majority of these cells did not express CRTH2, and only a small fraction of these cells expressed the gut-homing marker β 7 integrin. The majority of Ara h 1-reactive T cells also expressed CD25. The frequency of Ara h 1-reactive T cells in subjects without allergy was very low, which precluded the examination of their phenotypes. As indicated in Table I, 3 subjects with peanut allergy also had a seasonal allergy to timothy grass or alder pollen. Because we had also developed appropriate tetramer reagents to study these pollen-reactive T cells, we examined the phenotype of pollen-specific T cells in these subjects with peanut allergy. This allowed a comparison of Ara h 1-reactive T cells with Aln g 1 (alder pollen allergen) or Phl p 1 (timothy grass pollen allergen)-reactive T cells within the same subjects. As shown in this

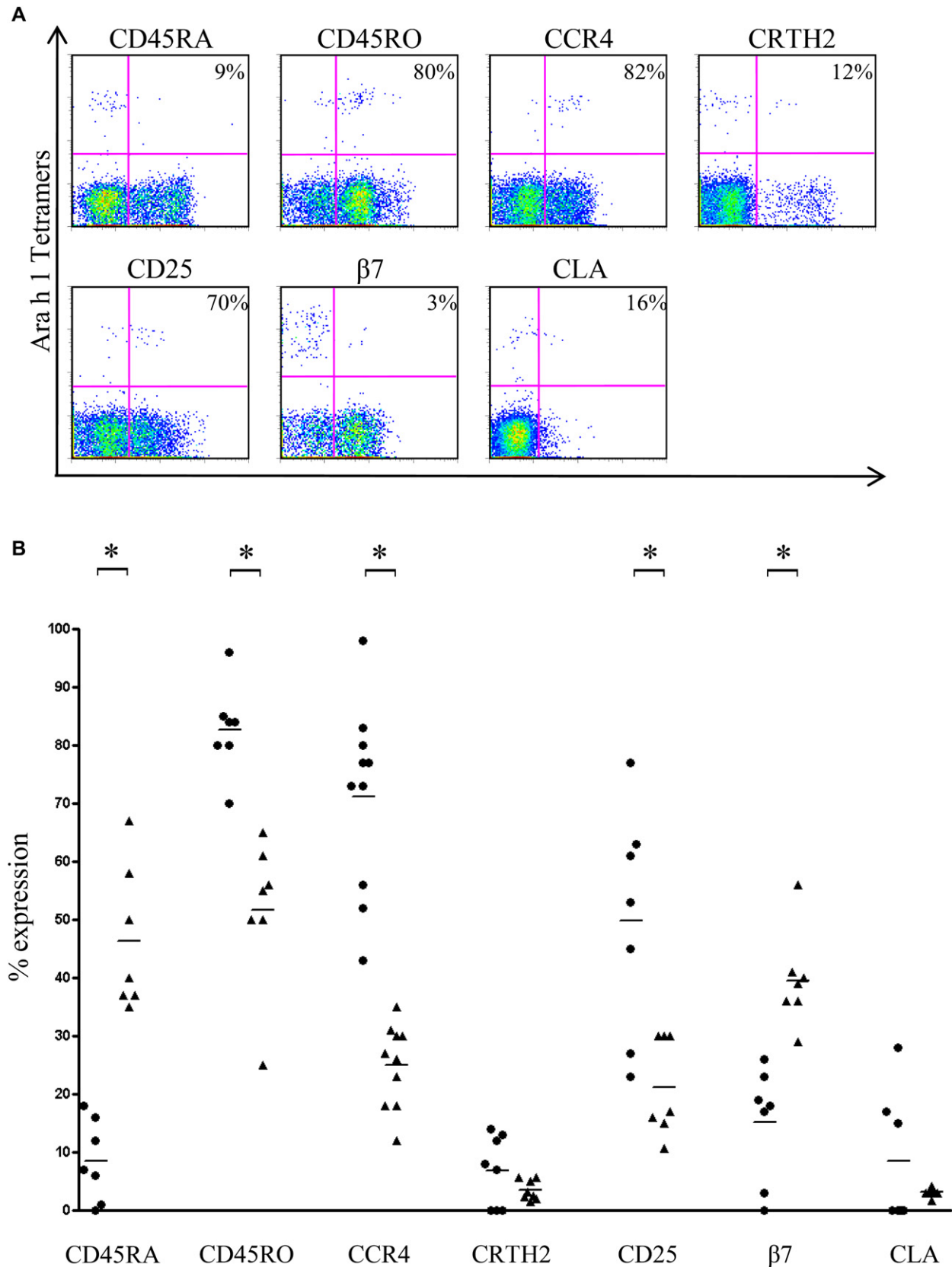


FIG 2. Phenotype of Ara h 1-reactive T cells. **A**, PBMCs of a DR1101 subject with peanut allergy were stained with phycoerythrin-labeled DR1101/Ara h 1₃₂₁₋₃₄₀ tetramers and a panel of antibodies. **B**, Comparison of *ex vivo* phenotypes of Ara h 1-reactive and total CD4⁺ T cells for multiple subjects with peanut allergy. Ara h 1-reactive cells are denoted by circles, total CD4⁺ T cells by triangles. A Student ttest was used in the statistical analysis. **P* < .05.

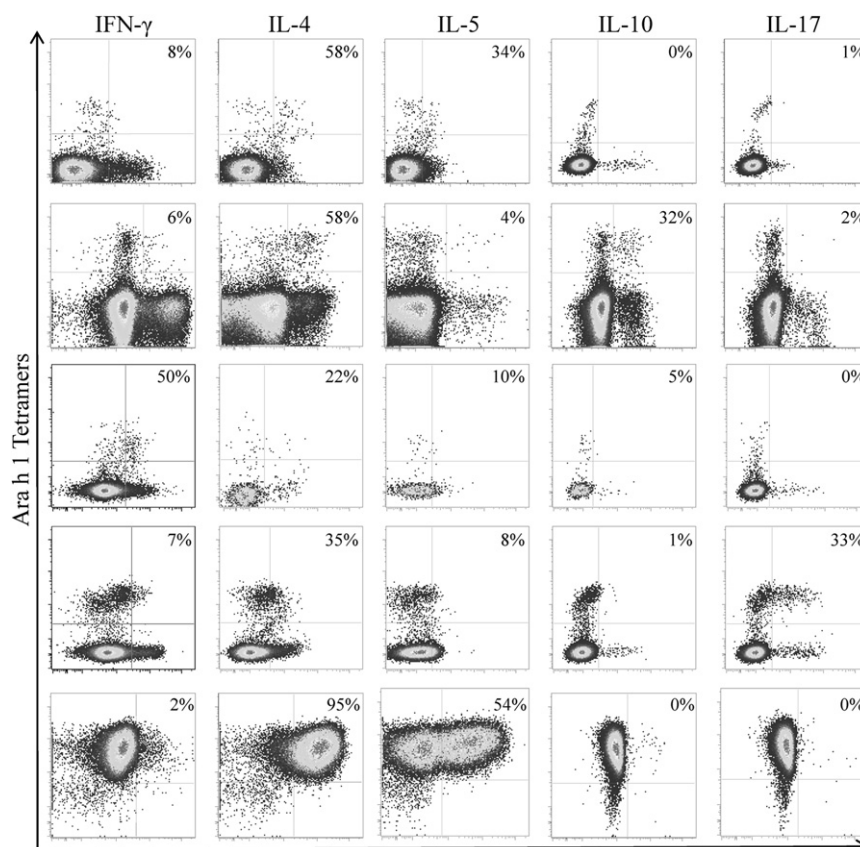


FIG 3. Intracellular cytokine staining of Ara h 1-reactive cell lines. *First row*, An IL-4 and IL-5–producing DR1101-restricted Ara h 1_{169–188}–stimulated cell line. *Second row*, An IL-4 and IL-10–producing DR0401-restricted Ara h 1_{329–348}–stimulated cell line. *Third row*, An IFN-γ–producing DR1401-restricted Ara h 1_{329–340}–stimulated cell line. *Fourth row*, An IL-4 and IL-17–producing DR0404-restricted Ara h 1_{329–348}–stimulated cell line. *Fifth row*, An IL-4 and IL-5–producing DR0401-restricted Ara h 1_{577–596}–specific T-cell clone. Each percentage shown indicates the percentage of cytokine-producing cells from the tetramer-positive population.

article's Fig E2 in the Online Repository at www.jacionline.org, the results of these experiments indicated that while the majority of T cells reactive to Ara h 1, Aln g 1, and Phl p 1 expressed CCR4, only T cells reactive to Aln g 1 and Phl p 1 expressed CRTH2.

Cytokine profiles of Ara h 1-reactive T cells

The CCR4 surface phenotype of Ara h 1-reactive T cells indicated that these T cells belong to the T_H2 lineage. The T_H2 phenotype of these cells was further confirmed by examining the cytokine profiles of Ara h 1-specific T-cell lines and clones derived from subjects with peanut allergy. Ara h 1-specific cell lines were generated by stimulating the PBMCs of subjects with peanut allergy with antigenic Ara h 1 peptides for 2 weeks. Ara h 1-reactive T-cell clones were isolated by sorting single Ara h 1 tetramer-positive T cells from Ara h 1 lines and subsequently expanding them with phytohemagglutinin. Cytokine profiles were examined by tetramer staining in conjunction with intracellular cytokine staining (ICS). Representative results of these assays are shown in Fig 3. Additional data for multiple subjects are summarized in Table III. We observed that all of the Ara h 1-reactive cell lines and clones examined produced IL-4. At least one third of the lines also produced IL-5. More than half of the cell lines produced a low amount of IFN-γ. As shown in

Fig 4, multicolor ICS identified cell lines that produced IL-4 and IL-5 simultaneously or IL-4 individually. Cell lines that produced IL-10 or IL-17 individually or in combination with IL-4 were also observed (Fig 4), although the percentage of IL-4 and IL-17 coproducers was minimal. Release of IL-5 and IL-13 by Ara h 1-specific lines was confirmed by measuring cytokine in the supernatants of our Ara h 1-stimulated T-cell lines using the MesoScale Discovery multiplex kit (Gaithersburg, Md) (see this article's Fig E3 in the Online Repository at www.jacionline.org). These experiments indicated that T-cell lines stimulated with Ara h 1 peptides produced at least 8-fold more IL-5 and IL-13 than IFN-γ. In total, these data indicated that the majority of Ara h 1-reactive CD4⁺ T cells in subjects with peanut allergy were T_H2 cells but also confirmed the existence of Ara h 1-reactive cells that produced IFN-γ, IL-10, and IL-17.

DISCUSSION

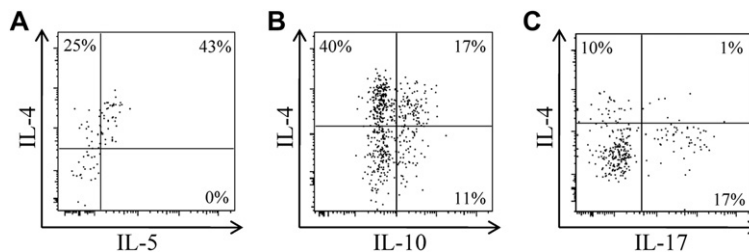
Although basic and clinical research have been ongoing for a number of years, CD4⁺ T cells specific for food allergens remain poorly characterized. In particular, the phenotype of these cells has not been examined *ex vivo*. Therefore, it is unknown whether food allergen-specific cells differ from other allergen specific CD4⁺ T cells. In this study, we developed class II tetramer reagents to identify CD4⁺ epitopes for a major peanut allergen, Ara h 1. We

TABLE III. Cytokine profiles of Ara h 1 clones and lines

HLA restriction	Epitope	Cell line/clone	IFN- γ	IL-4	IL-5	IL-10	IL-17
DR0101	Ara h 1 ₂₀₁₋₂₂₀	Line 1	–	++	–/+	–	–
DR0101	Ara h 1 ₂₀₁₋₂₂₀	Line 2	–	++	ND	ND	–
DR0401	Ara h 1 ₂₀₁₋₂₂₀	Clone 9	–	++	++	–	–
DR0401	Ara h 1 ₃₂₉₋₃₄₈	Clone 3	–/+	++	++	–	–
DR0401	Ara h 1 ₃₂₉₋₃₄₈	Clone 7	–	++	–	–	–
DR0401	Ara h 1 ₅₇₇₋₅₉₆	Clone 19	–	++	++	–	–
DR0401	Ara h 1 ₃₂₉₋₃₄₈	Line 1	+	ND	ND	++	–
DR0401	Ara h 1 ₃₂₉₋₃₄₈	Line 2	+	++	–	+	–
DR0401	Ara h 1 ₃₂₉₋₃₄₈	Line 3	–/+	++	–	++	–
DR0404	Ara h 1 ₃₂₉₋₃₄₈	Line 1	–	++	–	–	–
DR0404	Ara h 1 ₃₂₉₋₃₄₈	Line 2	–/+	++	–/+	–	++
DR1101	Ara h 1 ₁₆₉₋₁₈₈	Line 1	–	++	++	–	–
DR1101	Ara h 1 ₁₆₉₋₁₈₈	Line 2	–/+	++	++	–	–
DR1101	Ara h 1 ₁₆₉₋₁₈₈	Line 3	+	++	++	+	–
DR1101	Ara h 1 ₃₂₁₋₃₄₀	Line 1	–/+	++	++	–/+	–
DR1401	Ara h 1 ₃₂₁₋₃₄₀	Line 1	++	+	–/+	–	–

ND, Not determined.

–, Less than 5% positive in ICS staining; –/+, 6% to 10%; +, 11% to 30%; ++, greater than 30%.

**FIG 4.** Dual cytokine analysis of Ara h 1-reactive T-cell lines. PBMCs were stimulated with Ara h 1 peptide for 2 weeks and stained with tetramers. Tetramer-positive cells were gated, and cytokine profiles were analyzed by ICS staining. **A**, IL-4 and IL-5–producing DR1101-restricted Ara h 1₁₆₉₋₁₈₈-reactive T cells from allergic subject 5 (Table I). **B**, IL-4 and IL-10–producing DR0401-restricted Ara h 1₃₂₉₋₃₄₈-reactive T cells from allergic subject 1. **C**, IL-4 and IL-17–producing DR0404-restricted Ara h 1₃₂₉₋₃₄₈-reactive T cells from allergic subject 2. Each percentage shown indicates the percentage of cytokine-producing cells from the tetramer-positive population. Data shown are representative of at least 3 independent experiments.

further used these tetramer reagents to examine directly the frequency and phenotype of Ara h 1-reactive T cells in adult subjects who had a documented history of peanut anaphylaxis. Ara h 1-specific T cells could be readily detected in the PBMCs of subjects with peanut allergy. However, their mean frequency (approximately 9 cells per million) was slightly lower than the frequency of alder or timothy grass pollen-specific T cells within the same subjects (data not shown). Using a similar approach, Ara h 1 epitope-specific T cells were barely detectable in subjects without peanut allergy (the average frequency was less than 1 cell per million). The scarcity of detectable Ara h 1-reactive T cells in the majority of subjects without peanut allergy suggests that these cells exist at low frequencies. Alternatively, it is possible that Ara h 1-reactive T cells in subjects without allergy are of low avidity and therefore difficult to detect by using tetramers.²⁴ By using ICS assays, Prussin et al²⁵ reported that the frequency of peanut-reactive T cells in subjects with peanut allergy was 100 to 200 per million CD4⁺ T cells. The frequencies observed here were 10-fold lower. However, much of this discrepancy can be explained by differences in methodology. The frequencies observed in this report we determined for individual antigenic epitopes from a single peanut protein restricted by a single HLA. In contrast, Prussin et al²⁵ stimulated with peanut extract, which has multiple peanut allergens, each containing multiple epitopes. In addition, most subjects

have T cells restricted by multiple class II alleles. Taking these factors into account, the frequency of peanut epitope-specific CD4⁺ T cells seems comparable for these 2 studies.

All of the subjects with peanut allergy recruited for this study had a history of anaphylactic reaction to peanut. Given this severity of disease, it could be considered surprising that the frequency of Ara h 1 epitope-specific T cells was lower than pollen epitope-reactive T cells in the same subjects. It could be argued that a long period of peanut avoidance for this group of subjects may have reduced the frequency of Ara h 1-reactive T cells. In support of this notion, it has been previously observed that the frequency of pollen-reactive T cells can be several-fold lower out of the pollen season than within the peak pollen season.²⁶ Alternatively, it could be that the most frequent peanut-reactive T cells are directed toward peanut allergens other than Ara h 1. To address this question further, it would be quite interesting to examine the frequency of Ara h 1-reactive T cells in subjects who have been recently diagnosed with peanut allergy. It would also be fruitful to investigate T cells specific for other peanut allergens such as Ara h 2. Indeed, data from a recent study in France suggested that sensitization to Ara h 2 is more predictive for peanut allergy diagnosis than sensitization to Ara h 1.¹³ Because sensitization to Ara h 1 is high in the North American population, monitoring both Ara h 1 and Ara h 2-reactive T cells may

be essential for examining cellular immune responses toward peanut in subjects with peanut allergy in North America.

In addition to measuring frequencies, the surface phenotypes of Ara h 1-specific T cells in subjects with peanut allergy were examined in this study. Although the scope of this study did not allow us to correlate cell phenotype with any clinical characteristics, such as severity of peanut allergy, patterns were evident across our subject population as a whole. As expected, Ara h 1-specific cells expressed the memory T-cell marker CD45RO and did not express CD45RA, a naive marker. The majority also expressed CCR4, a T_H2 -associated cell trafficking marker. In contrast, only a minority of these cells expressed CRTH2, which is thought to be the most definitive T_H2 marker. This result was clearly not because of a technical failure to label CRTH2 in our assay, because pollen-specific T cells from multiple subjects with peanut allergy were shown to express CCR4 and CRTH2. Interestingly, a higher percentage Ara h 1-reactive T cells expressed CD25 than did the total PBMCs in the same subject. The CD25 expression of Ara h 1-reactive T cells occurred in the absence of peanut exposure and is probably correlated with their T_H2 phenotype.²⁰ Despite the fact that CD25 expression is also linked to suppressive T-cell function,²⁷ some caution is warranted in concluding that allergen-specific $CD4^+CD25^+$ T cells have regulatory function, because these cells were shown to produce robust levels of cytokine (as discussed here). Expression of the skin-homing marker CLA was not statistically different on Ara h 1-reactive T cells compared with total $CD4^+$ T cells. In contrast, examination of the gut homing marker $\alpha4\beta7$ on Ara h 1-reactive T cells indicated that these cells express significantly less $\beta7$ integrin than do the total $CD4^+$ T cells. This outcome was unexpected, because Ara h 1-reactive T cells would be likely to first encounter peanut allergens in the gastrointestinal system, which is accessed by $\alpha4\beta7$ expression. One possible explanation is that $\beta7^+$ Ara h 1-reactive T cells are sequestered within the gut and secondary lymphoid tissue and do not circulate in the peripheral blood.

Cytokine profiles of peanut-reactive T cells were also examined in this study. As might be expected, the vast majority of Ara h 1-specific T-cell lines and clones examined produced T_H2 cytokines such as IL-4 and IL-5. These included cell lines that produced IL-4 only and cell lines that produced IL-4 and IL-5 simultaneously. Analysis of culture supernatants indicated that IL-5-producing cell lines could also produce IL-13. In addition, cells that produced IL-10 were also observed. One powerful application of tetramers is the antigen specific analysis of the coproduction of cytokines on the single cell level. We found IL-4 and IL-10 coproducers as well as cells that produced only IL-10. It is likely the IL-10 produced by IL-4/IL-10-coproducing cells functions in an autocrine fashion to downregulate IL-4 production. Ara h 1-reactive T cells that produced IL-17 were also observed. Among the IL-17 producers, a very small percentage produced both IL-4 and IL-17. The presence of IL-4 and IL-17 coproducers in subjects with asthma has been previously reported by Cosmi et al.²⁸ Oseroff et al.²⁹ also reported the presence of timothy grass-specific IL-17-producing cells in subjects with timothy grass allergy. Both groups indicated IL-17-producing cells represented a small subset of the cells in their studied cell population. These previous findings concur with the current data in that only 1 of the 16 Ara h 1-reactive lines examined produced IL-17. Somewhat surprisingly, Ara h 1-specific IFN- γ -producing cells were also observed. IFN- γ has been implicated in the downregulation of T_H2 responses and also in the recruitment of T_H2 cells to

sites of inflammation.^{30,31} The current study did not address the various roles of these different T_H subsets. Nevertheless, these current findings demonstrate that Ara h 1-specific $CD4^+$ T cells have multifunctional capacities in subjects with peanut allergy. Among these, T_H2 -type $CD4^+$ T cells are likely to act as the dominant players, with T_H1 , T_H17 , and regulatory cells of identical antigen specificity also playing important roles.

As mentioned, most of the Ara h 1-specific cells examined did not express CRTH2, the most definitive T_H2 marker. However, CCR4 staining results and cytokine secretion profiles clearly indicated that these cells are functionally T_H2 -like. The fact that the subjects from this study have avoided contact with peanut allergens for a long period may have resulted in a lack of CRTH2 expression by Ara h 1-reactive T cells. This would suggest that occasional antigen stimulation is essential for the expression of CRTH2 by T cells. This notion is supported by our observation that 75% (6/8) of Ara h 1-specific T-cell clones expressed various levels of CRTH2 after activation and subsequent resting (data not shown). However, it is also possible that these Ara h 1-specific cells could be CRTH2-negative for a functional reason and may play a role in the pathophysiology of allergy distinct from CRTH2-positive T_H2 cells.

This study highlights the use of tetramer reagents to detect and characterize Ara h 1-reactive $CD4^+$ T cells. Its findings demonstrate the feasibility of using Ara h 1-specific tetramers as a specific biomarker for monitoring peanut-specific $CD4^+$ T cells in various settings. Although the frequency of Ara h 1-reactive $CD4^+$ T cells is relatively low in subjects with peanut allergy, there was a significant difference in the frequency of Ara h 1 $CD4^+$ -reactive T cells in subjects with allergy compared with subjects without allergy. More importantly, tetramer reagents can be used to monitor the surface phenotype of peanut-reactive T cells. As such, immune monitoring with tetramers can provide unambiguous data regarding the function, frequency, and phenotype of allergenic reactive T cells. These reagents, used independently or in conjunction with other assays, may open up new approaches to monitor changes in the phenotype and frequency of food allergen-reactive $CD4^+$ T cells during immunotherapy.

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Key messages

- Peanut-specific T cells could be detected in subjects with peanut allergy by using class II tetramers.
- Most of these were T_H2 cells, but cells that secreted IFN- γ , IL-10, and IL-17 were also detected.

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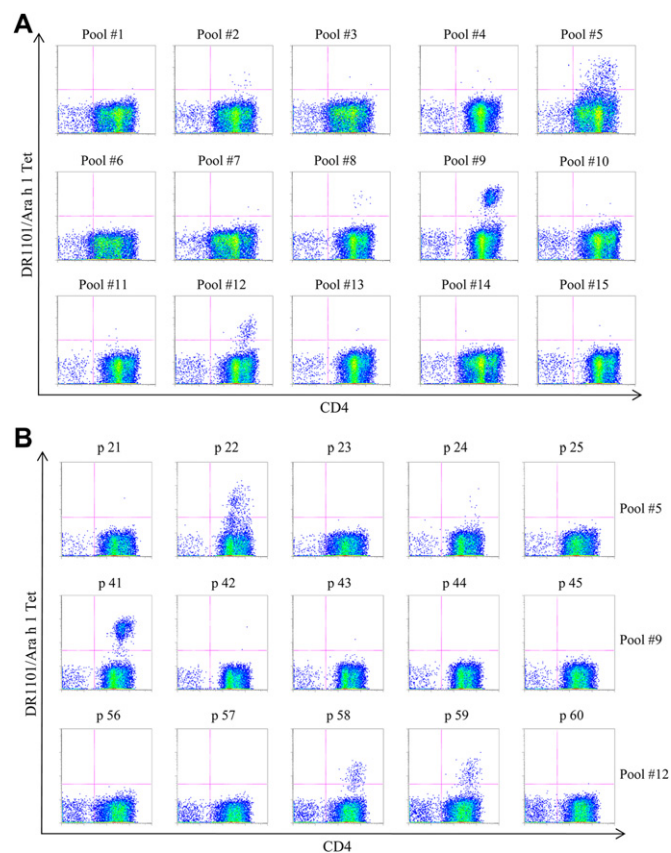


FIG E1. TGM studies of DR1101-restricted Ara h 1-reactive CD4⁺ T cells. **A**, PBMCs from a DR1101 subject with peanut allergy were stimulated with 15 pools of Ara h 1 peptides for 2 weeks and subsequently stained with corresponding DR1101/Ara h 1 pooled peptide tetramers (*Tet*). **B**, Cells that were stimulated with pools #5, #9, and #12 were restained with individual peptides from the corresponding pool. The staining identified p22 (Ara h 1₁₆₉₋₁₈₈), p41 (Ara h 1₃₂₁₋₃₄₀), p58 (Ara h 1₄₅₇₋₄₇₆), and p59 (Ara h 1₄₆₅₋₄₈₄) as DR1101-restricted Ara h 1 T-cell epitopes.

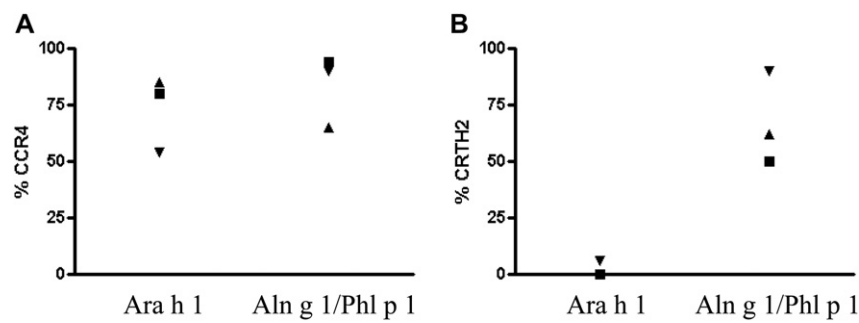


FIG E2. Surface phenotype of Ara h 1 peanut-reactive T cells and Aln g 1 or Phl p 1 pollen-reactive cells in subjects with peanut allergy. **A**, CCR4 expression of Ara h 1 and Aln g 1 or Phl p 1-specific T cells in subjects with peanut allergy. **B**, CRTH2 expression of Ara h 1 and Aln g 1 or Phl p 1-specific T cells in subjects with peanut allergy. Each symbol represents staining of a unique epitope for a particular subject.

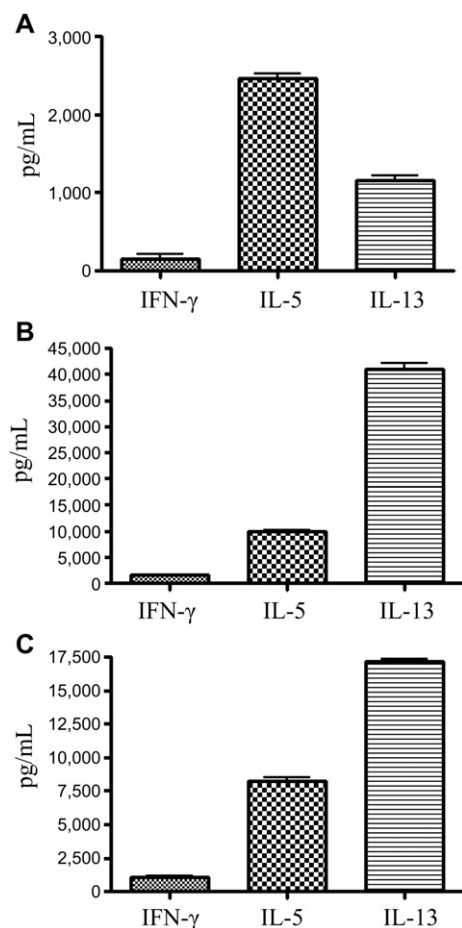


FIG E3. Cytokine profiles of Ara h 1-reactive CD4⁺ T cells. **A**, PBMCs from a DR0101 subject were stimulated with peptide Ara h 1₂₀₁₋₂₉₀. On day 14, cells were transferred in triplicate to wells that had been precoated with DR0101/Ara h 1₂₀₁₋₂₉₀ tetramers, and supernatants were harvested after an overnight culture. Cytokines were assayed by the MesoScale Discovery multiplex kit. **B**, PBMCs from a DR0401 subject were stimulated with peptide Ara h 1₅₀₅₋₅₂₄ for 2 weeks, subsequently activated with DR0401/Ara h 1₅₀₅₋₅₂₄ tetramers, and then analyzed as described. **C**, PBMCs from a DR1501/B5 subject were stimulated with peptide Ara h 1₂₀₉₋₂₂₈ for 2 weeks, subsequently activated with DRB5/Ara h 1₂₀₉₋₂₂₈ tetramers, and then analyzed as described.