

Letter to the Editor

Arginine kinase Pen m 2 as an important shrimp allergen recognized by T_H2 cells

To the Editor:

Patients with shrimp allergy have IgE that binds tropomyosin, the major shrimp allergen.^{1,2} Tropomyosin could also induce CD4⁺ T-cell proliferation in patients with shrimp allergy.^{2,3} Other shrimp allergens, including arginine kinase,^{4,5} sarcoplasmic calcium-binding protein,⁶ and myosin light chain,⁷ have also been identified. However, little is known about specific T-cell responses toward these allergens. In this report we examined T-cell responses toward tropomyosin and arginine kinase in patients with shrimp allergy and nonallergic subjects.

A total of 9 adults with a history of allergic symptoms to shrimp and positive ImmunoCAP results to shrimp extract were recruited for this study (see the Methods section in this article's Online Repository at www.jacionline.org). Subjects recruited with shrimp allergy were associated with either the HLA-DRB1*03:01 (DR3) or HLA-DRB1*15:01-DRB5*01:01-DQB1*0602 (DR15) haplotype. Twelve adults without shrimp allergy, including 7 non-atopic control subjects and 5 patients with peanut allergy without shrimp allergy, with matched HLA class II restriction were also recruited as control subjects (see Table E1 in this article's Online Repository at www.jacionline.org). The tetramer-guided epitope mapping approach was used to identify a CD4⁺ T-cell epitope for Pen m 1 and Pen m 2 (tropomyosin and arginine kinase from the black tiger shrimp *Penaeus monodon*, respectively). Pen m 1₉₋₂₈ and Pen m 2₈₁₋₁₀₀ were identified as DRB1*03:01-restricted T-cell epitopes. Pen m 1₁₆₁₋₁₈₀ and Pen m 2₁₉₃₋₂₁₂ were identified as DRB5*01:01-restricted T-cell epitopes. Finally, Pen m 1₉₇₋₁₁₆ and Pen m 2₂₆₅₋₂₈₄ were identified as DQA*01:02/DQB*06:02-restricted T-cell epitopes (see Fig E1 and Table E2 in this article's Online Repository at www.jacionline.org).

The anti-phycoerythrin (PE) magnetic bead tetramer approach was used to detect shrimp allergen-reactive T cells *ex vivo* in PBMCs. Pen m 1 and Pen m 2 epitope-specific memory (CD45RA⁻) CD4⁺ T cells were detected in allergic but not nonallergic adults *ex vivo* (Fig 1, A and B, and see Fig E2, A and B, in this article's Online Repository at www.jacionline.org). However, the frequency of Pen m 1 epitope-specific CD4⁺ T cells was low and not significantly different between adults with or without shrimp allergy (Fig 1, B). In contrast, the frequency of Pen m 2 epitope-specific CD4⁺ T cells was significantly higher in adults with shrimp allergy compared with nonallergic adults (Fig 1, B). Regarding each subject individually, Pen m 1 and Pen m 2 epitope-specific memory T cells were detected in 1 of 9 and 4 of 9 allergic subjects, respectively, with a frequency of greater than 5 cells per million CD4⁺ T cells (Fig 1, C). These allergen-specific T cells were not detected in the naive T-cell compartment in allergic or nonallergic subjects (see Fig E2, C). Detectable Pen m 1- and Pen m 2-specific CD4⁺ T cells in allergic subjects were associated with the memory phenotype CD45RA⁻ and were CCR4⁺CXCR3^{low}, suggesting a T_H2 phenotype (Fig 1, D and E). Variable expressions of CCR6, chemoattractant receptor-homologous molecule expressed on T_H2 cells (CRTH2), and CD27 were observed (Fig 1, E) and T cells with CRTH2 expression were associated with low expression of CD27, which correspond to a terminally differentiated T_H2 phenotype.⁸

The CD154 upregulation assay was also used to examine the overall T-cell responses toward Pen m 1 and Pen m 2 in 7 of 9 patients with shrimp allergy. Strong Pen m 1- and Pen m 2-specific responses correlated with our peptide MHC class II tetramer staining in patients with shrimp allergy (see Fig E3 in this article's Online Repository at www.jacionline.org). These results confirmed that, in contrast to Pen m 1-derived epitopes, Pen m 2-derived epitopes are strongly immunogenic *ex vivo* in patients with shrimp allergy.

Pen m 1- and Pen m 2-specific T-cell lines were generated by stimulating PBMCs from allergic subjects with antigenic Pen m 1 or Pen m 2 peptides for 2 weeks. Pen m 2-specific T-cell lines were successfully generated only from allergic subjects (Fig 2 and see Fig E4 in this article's Online Repository at www.jacionline.org). All these specific T-cell lines obtained produced IL-4 and IL-5, confirming the observed *ex vivo* T_H2 phenotype (Fig 2 and see Fig E4). Although IL-4 was the dominant response, there were Pen m 2-specific T-cell lines that produced IL-17.

We were also able to generate Pen m 1-specific T-cell lines from allergic and nonallergic adults (Fig 2, B, and see Fig E4). Only Pen m 1-specific T-cell lines derived from allergic adult 9 (who presented a significant accumulation of specific T cells *ex vivo*) produced IL-4 and IL-5, confirming the observed *ex vivo* T_H2 phenotype (Fig 1, E). All other Pen m 1-specific T-cell lines derived from either allergic or nonallergic adults produced IFN- γ , IL-10, or both (Fig 2, B, and see Fig E4).

Recent publications show that tropomyosin can induce CD4⁺ T-cell proliferation in patients with shrimp allergy.^{2,3} However, it is unclear whether these proliferating cells are T_H2 cells. Pen m 1 epitopes described in the current work include similar amino acid sequences as seen in these previous studies. Only 1 allergic subject in our cohort presented with detectable Pen m 1-specific T cells with a T_H2 profile. The T_H1-like profile of Pen m 1-specific T-cell lines from the majority of patients with shrimp allergy and nonallergic subjects suggests Pen m 1-specific T cells might not play a major role in mediating the T_H2 responses in shrimp allergy in subjects with either the DR3 or DR15 haplotype. This T_H1-like profile was previously observed in the T-cell response against alder allergen in nonallergic subjects, which suggests a nonallergic response.⁸

Pen m 1 from the black tiger shrimp *P monodon* has high amino acid sequence homology to other shrimp tropomyosin (Lit v 1, Pen a 1, and Met e 1) and other crustacean tropomyosins. Moreover, shrimp tropomyosin has high sequence homology (>80%) to tropomyosin from cockroaches and house dust mites. IgE cross-reactivity between shrimp and insect/dust mite tropomyosin was reported.⁹ Interestingly, all adults with shrimp allergy in our cohort were also sensitized to house dust mite (see Table E1). Hence it is possible that insect/dust mite tropomyosin sensitizes subjects to shrimp allergen in the absence of Pen m 1-reactive CD4⁺ T cells.

The current study suggests an important pathogenic role of Pen m 2-reactive T cells in patients with shrimp allergy with either the DR3 or DR15 haplotype. For further study of T-cell responses to shrimp allergens, it is essential to examine T-cell responses to tropomyosin, arginine kinase, sarcoplasmic calcium-binding protein, and myosin light chain in a larger cohort. Understanding

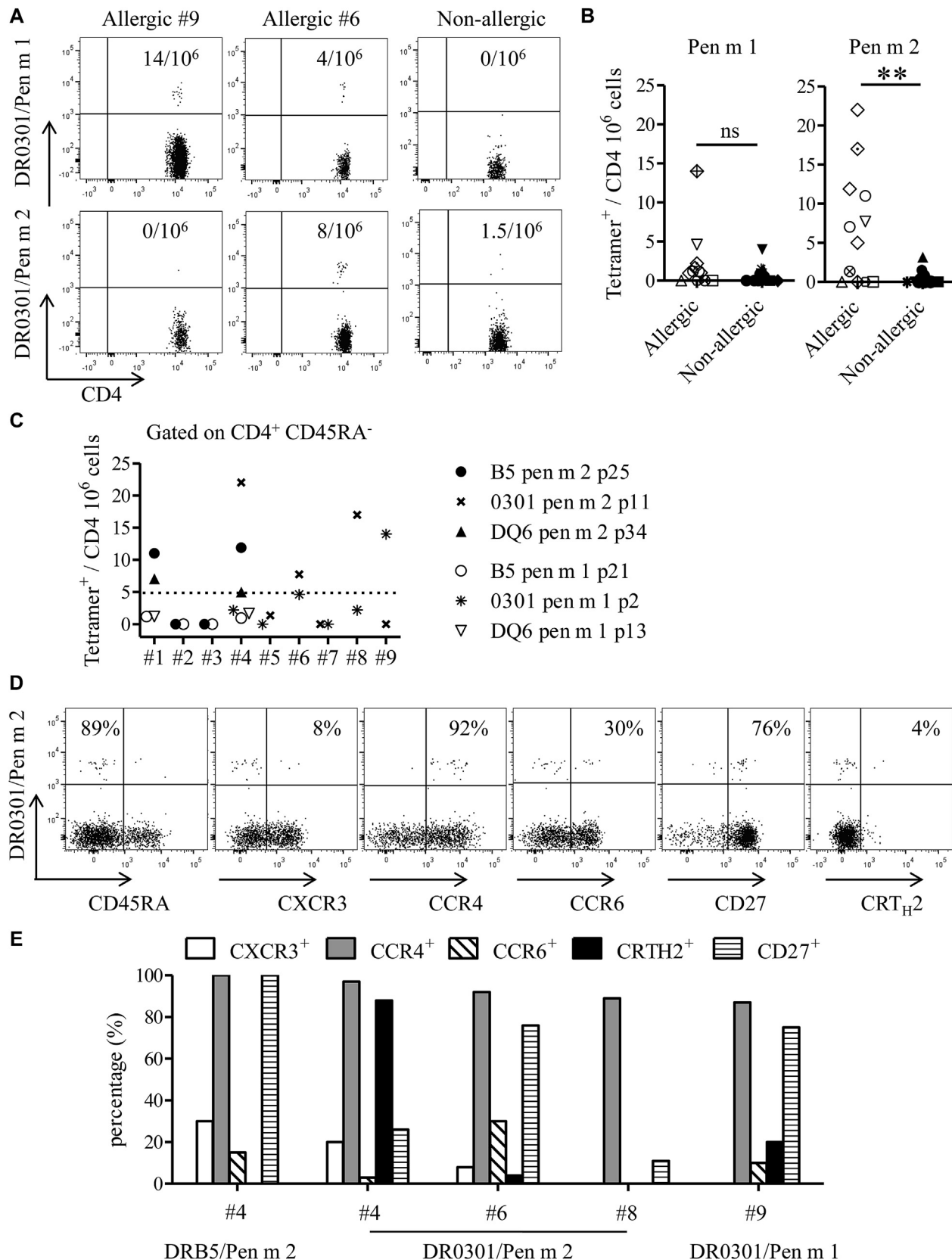


FIG 1. Frequency and phenotype of Pen m 1 and Pen m 2 epitope-specific CD4⁺ T cells. **A**, Ex vivo frequency of Pen m 1 and Pen m 2 epitope-specific CD45RA⁻ CD4⁺ T cells in adults with or without shrimp allergy. Frequencies of specific T cells per million CD4⁺ T cells are as indicated. **B**, Comparison of Pen m 1- and Pen m 2-specific CD4⁺ T-cell frequency for each epitope described (data point) between 9 allergic adults and 12 nonallergic adults. Different symbols represent different subjects. **C**, Frequencies of memory Pen m 1- and Pen m 2-specific CD4⁺ T cells for 9 allergic subjects. **D**, PBMCs of an allergic adult were stained with PE-labeled DRB1*03:01/Pen m 2₈₁₋₁₀₀ tetramers and a panel of antibodies. **E**, Phenotype of Pen m 2- and Pen m 1-specific T cells for 4 allergic adults. Numbers indicate (y-axis) the percentage of tetramer-positive cells that expressed a specific surface marker. Data were compared by using the Student unpaired *t* test. ***P* ≤ .01. ns, Not significant.

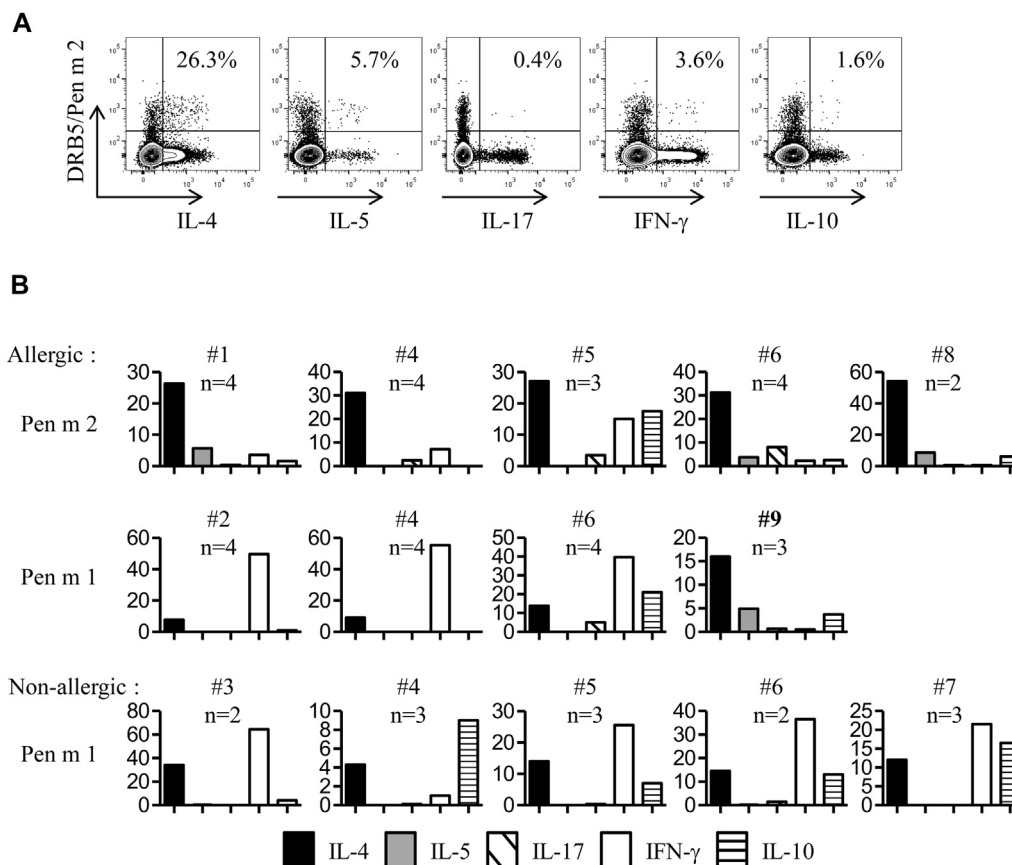


FIG 2. Intracellular cytokine staining of Pen m 1 and Pen m 2 epitope-specific T-cell lines. **A**, Cytokine profile of a DRB5*01:01/Pen m 2 epitope-specific T-cell line generated from allergic adult PBMCs. Percentages of tetramer-positive cells that expressed the cytokine of interest are as indicated. **B**, Graphs represent cytokine profiles of Pen m 1 and Pen m 2 epitope-specific T-cell lines from allergic adults (*top 2 rows*) and nonallergic subjects (*bottom row*). Numbers indicate the number of allergen-specific cell lines examined in each subject, and the average percentage of tetramer-positive cells that produced the indicated cytokine is shown (*y-axis*).

T-cell responses to all these shrimp allergens is a prerequisite for rational design of a future immunotherapy vaccine.

We thank Jennifer Heaton, Lisa Myers Bulmash, and Gladys Doronio for help with subject recruitment. We also thank Diana Sorus for assistance in preparing the article. Finally, we thank Nadia Torres-Chinn for help with blood processing.

Amedee Renand, PhD^a
Sally Newbrough, MD, PhD^b
Erik Wambre, PhD^a
Jonathan H. DeLong, BSc^a
David Robinson, MD^c
William W. Kwok, PhD^{a,b}

From ^athe Benaroya Research Institute at Virginia Mason; ^bthe Department of Medicine, University of Washington; and ^cthe Virginia Mason Medical Center, Seattle, Wash. E-mail: bkwok@benaroyaresearch.org.

Supported by US National Institutes of Health contract HHSN272200700046C.

Disclosure of potential conflict of interest: A. Renand has received research support from the US National Institutes of Health and is employed by Benaroya Research Institute. S. Newbrough has received travel support from the American Academy of Allergy, Asthma & Immunology and is employed by the University of Washington. J. H. DeLong has received research support from the US National Institutes of Health. W. W. Kwok has received research support from the US National Institutes of Health. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

- Shanti KN, Martin BM, Nagpal S, Metcalfe DD, Rao PV. Identification of tropomyosin as the major shrimp allergen and characterization of its IgE-binding epitopes. *J Immunol* 1993;151:5354-63.
- Wang S, Delgado JC, Ravkov E, Eckels DD, Georgelas A, Pavlov IY, et al. *Penaeus monodon* tropomyosin induces CD4 T-cell proliferation in shrimp-allergic patients. *Hum Immunol* 2012;73:426-31.
- Ravkov EV, Pavlov IY, Martins TB, Gleich GJ, Wagner LA, Hill HR, et al. Identification and validation of shrimp-tropomyosin specific CD4 T cell epitopes. *Hum Immunol* 2013;74:1542-9.
- Yu CJ, Lin YF, Chiang BL, Chow LP. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J Immunol* 2003;170:445-53.
- Garcia-Orozco KD, Aispuro-Hernandez E, Yepiz-Plascencia G, Calderon-de-la-Barca AM, Sotelo-Mundo RR. Molecular characterization of arginine kinase, an allergen from the shrimp *Litopenaeus vannamei*. *Int Arch Allergy Immunol* 2007;144:23-8.
- Shiomi K, Sato Y, Hamamoto S, Mita H, Shimakura K. Sarcoplasmic calcium-binding protein: identification as a new allergen of the black tiger shrimp *Penaeus monodon*. *Int Arch Allergy Immunol* 2008;146:91-8.
- Ayuso R, Grishina G, Bardina L, Carrillo T, Blanco C, Ibanez MD, et al. Myosin light chain is a novel shrimp allergen, Lit v 3. *J Allergy Clin Immunol* 2008;122:795-802.
- 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, e1-7.
- Lopata AL, O'Hehir RE, Lehrer SB. Shellfish allergy. *Clin Exp Allergy* 2010;40:850-8.

METHODS

Human subjects

Subjects were recruited from the Virginia Mason Medical Center Allergy Clinic and Benaroya Research Institute. Patients with allergy and a documented record of shrimp allergy symptoms and positive ImmunoCAP scores for shrimp-specific IgE (Phadia AB, Uppsala, Sweden) were recruited. Nonallergic subjects with no clinical symptoms of allergy and negative ImmunoCAP scores for shrimp 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 E1.

Tetramer-guided epitope mapping

Biotinylated HLA-DR proteins were purified, as previously described.^{E1} A total of 34 Pen m 1 peptides (p1 to p34), which were 20 amino acids in length with a 12-amino-acid overlap spanning the entire Pen m 1 sequence (including the signal peptide), were synthesized (Mimotopes, Notting Hill, Australia). A total of 41 Pen m 2 peptides (p1 to p41), which were 20 amino acids in length with a 12-amino-acid overlap spanning the entire Pen m 2 sequence, were also synthesized. Pen m 1 peptides were divided into 6 pools of 5 peptides each plus a seventh pool of 4 peptides. Pen m 2 peptides were divided into 8 pools of 5 each plus a ninth pool of 3 peptides. These peptide mixtures were loaded into the biotinylated HLA-DR proteins to generate pooled tetramers, as described.^{E1} Cells were cultured for 14 days and then stained with pooled peptide tetramers. Cells from wells that produced positive staining were stained again by using tetramers loaded with each individual peptide from the positive pool.

Ex vivo analysis of Pen m 1– and Pen m 2–reactive CD4⁺ T cells

For tetramer staining, 30 million PBMCs in 200 μ L of T-cell culture medium were stained with 20 μ g/mL PE-labeled tetramers at room temperature for 100 minutes. Cells were washed and incubated with anti-PE magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) at 4°C for 20 minutes, and a one-tenth fraction was saved for analysis. The other fraction was passed through a magnetic column (Miltenyi Biotec). After enrichment, cells were stained with peridinin-chlorophyll-protein complex (PerCP)–Cy5.5 anti-CD14 (HCD14; BioLegend, San Diego, Calif), PerCP–Cy5.5 anti-CD19 (HIB19, BioLegend), V500 anti-CD4 (RPA-T4; BD Biosciences, San Jose, Calif), Alexa Fluor 700 anti-CD45RA (HI100, BD Biosciences), Alexa Fluor 488 anti-CD183 (CXCR3; G025H7, BioLegend), PE–Cy7 anti-CD194 (CCR4; TG6/CCR4, BioLegend), Alexa Fluor 647 anti-CD294 (CRTH2; BM16, BD Biosciences), allophycocyanin–Cy7 anti-CD27

(O323, BioLegend), and Brilliant Violet 421 anti-CD196 (CCR6; G034E3, BioLegend) antibodies and with cell viability solution (BD Via-Probe, BD Biosciences). Data were acquired on a FACS LSR II flow cytometer (BD Biosciences) by using FACSDiva software (BD Biosciences) and analyzed with FlowJo 8.8.2 software (TreeStar, Ashland, Ore). The frequency of Pen m 1– and Pen m 2–specific T cells was determined by using the following formula:

$$F = n/N,$$

where n designates the number of tetramer-positive cells in the bound fraction after enrichment, and N is the total number of CD4⁺ T cells calculated as $10 \times$ the number of CD4⁺ T cells in a one-tenth pre-enriched fraction that was saved for analysis.

Intracellular cytokine staining

For *in vitro* intracellular cytokine staining of IFN- γ , IL-4, IL-17, IL-5, and IL-10, PBMCs were stimulated for 2 weeks with specific peptide and then stained with the corresponding PE-labeled tetramers for 60 minutes at 37°C. Cells were then restimulated with 25 ng/mL phorbol 12-myristate 13-acetate and 1 μ g/mL ionomycin in the presence of 10 μ g/mL Brefeldin-A for 4 hours at 37°C. After stimulation, cells were stained with allophycocyanin–Cy7 anti-CD4 (OKT3, BioLegend), Alexa Fluor 488 anti-IL-4 (8D4-8; eBioscience, San Diego, Calif), Alexa Fluor 700 anti-IFN- γ (4S.B3, BioLegend), Alexa Fluor 647 anti-IL-21 (3A3-N2.1, BD Biosciences), PerCP–Cy5.5 anti-IL-17 (BL168, BioLegend), and PE–Cy7 anti-IL-10 (JES3-9D7, BioLegend) antibodies and with fixable viability stain 450 (BD Biosciences). Intracellular staining were performed with fixation and permeabilization buffer (eBioscience). Data were acquired on a FACS LSR II flow cytometer (BD Biosciences) by using FACSDiva software (BD Biosciences) and were analyzed with FlowJo 8.8.2 software (TreeStar).

Statistical analysis

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

REFERENCES

- E1. 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.
- E2. Chattopadhyay PK, Yu J, Roederer M. A live-cell assay to detect antigen-specific CD4⁺ T cells with diverse cytokine profiles. *Nat Med* 2005;11:1113-7.

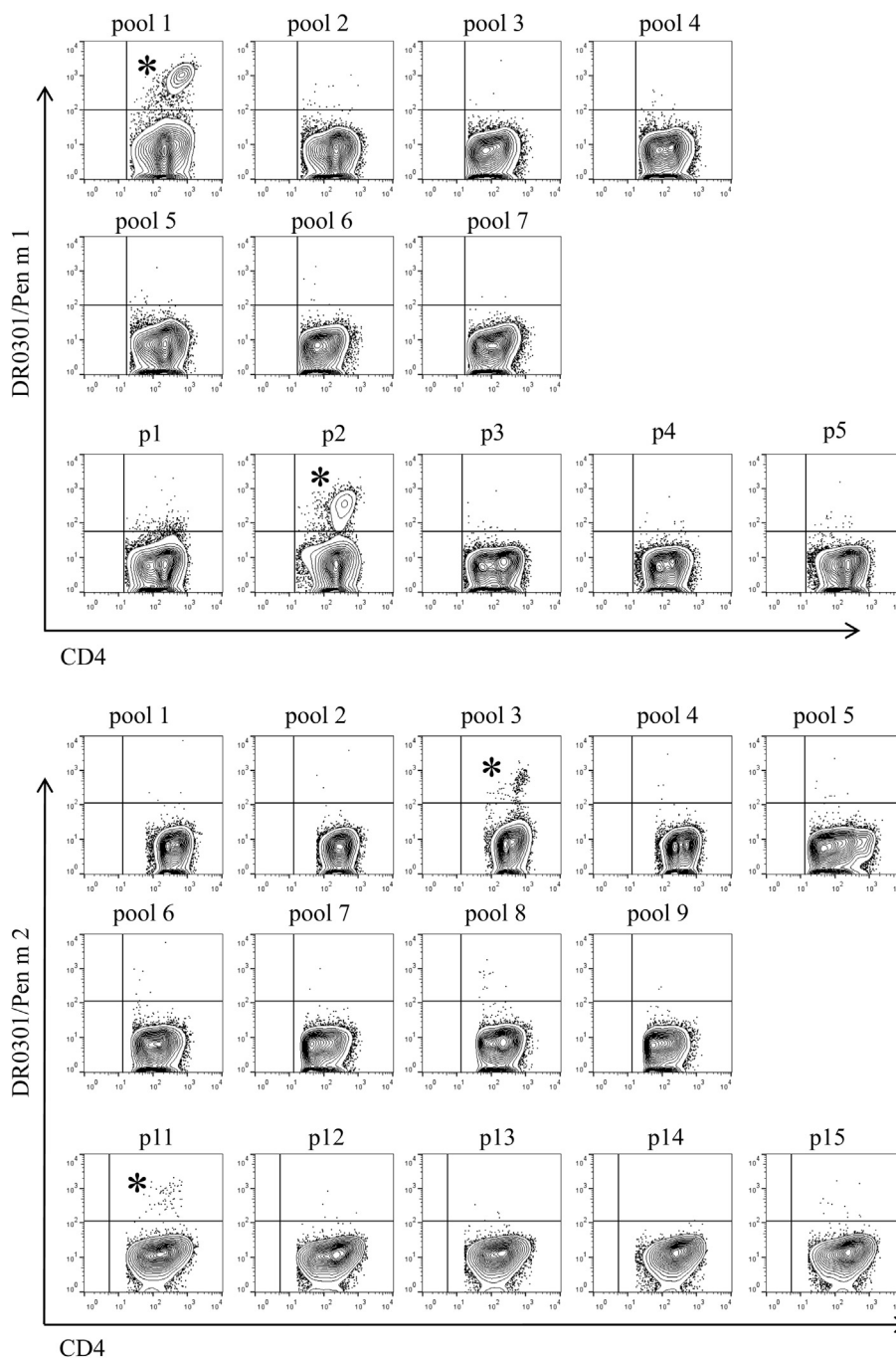


FIG E1. Tetramer-guided epitope mapping studies of DR0301-restricted Pen m 1- and Pen m 2-reactive CD4⁺ T cells. PBMCs from a DR0301 subject with shrimp allergy were stimulated with 7 pools of Pen m 1 peptides and 9 pools of Pen m 2 peptides for 2 weeks and subsequently stained with corresponding DR0301/Pen m 1- or DR0301/Pen m 2-pooled peptide tetramers (top 2 rows of *top* and *bottom* panels, respectively). Cells that were stimulated with Pen m 1 pool 1 or Pen m 2 pool 3 were restained with individual peptides from the corresponding pool (*third* row of *top* and *bottom* panels, respectively). The staining identified Pen m 1 p2 (Pen m 1₉₋₂₈) and Pen m 2 p11 (Pen m 2₈₁₋₁₀₀) as DR0301-restricted Pen m 1 and Pen m 2 T-cell epitopes.

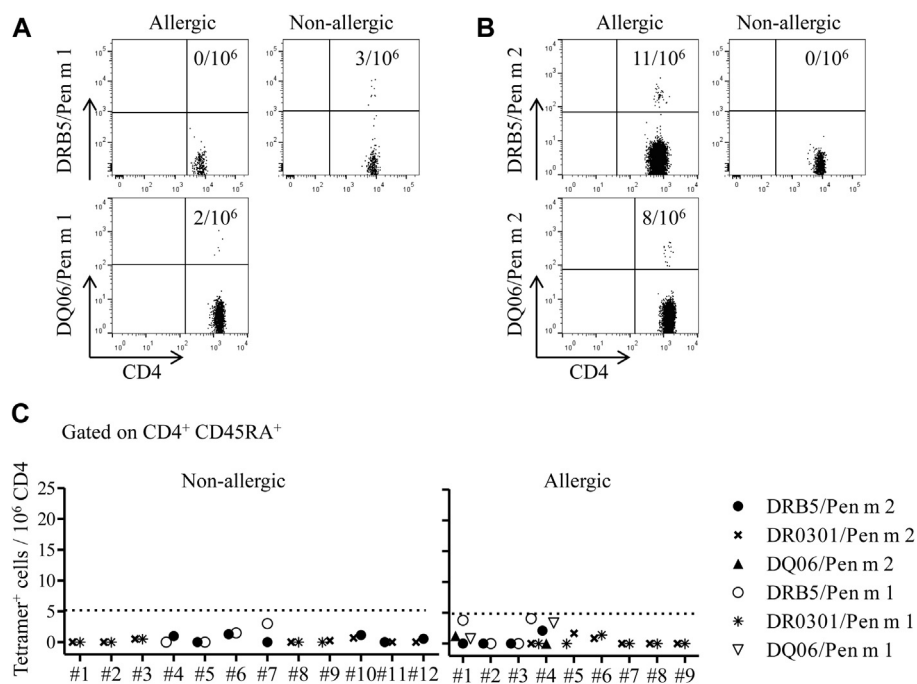


FIG E2. Frequency of Pen m 1 and Pen m 2 epitope-specific CD4⁺ T cells in each subject. **A** and **B**, *Ex vivo* frequency of Pen m 1 (Fig E2, A) and Pen m 2 (Fig E2, B) epitope-specific CD4⁺ T cells in DRB5*01:01-DQA*01:02/DQB*06:02 adults with or without shrimp allergy. Frequencies of specific T cells per million CD4⁺ T cells are as indicated. **C**, Frequencies of naive Pen m 1 and Pen m 2 epitope-specific CD4⁺ T cells for 9 allergic (*right*) and 12 nonallergic (*left*) subjects. Each symbol denotes a DRB1*03:01, DRB5*01:01, or DQA*01:02/DQB*06:02-restricted T-cell epitope, as indicated on the figure.

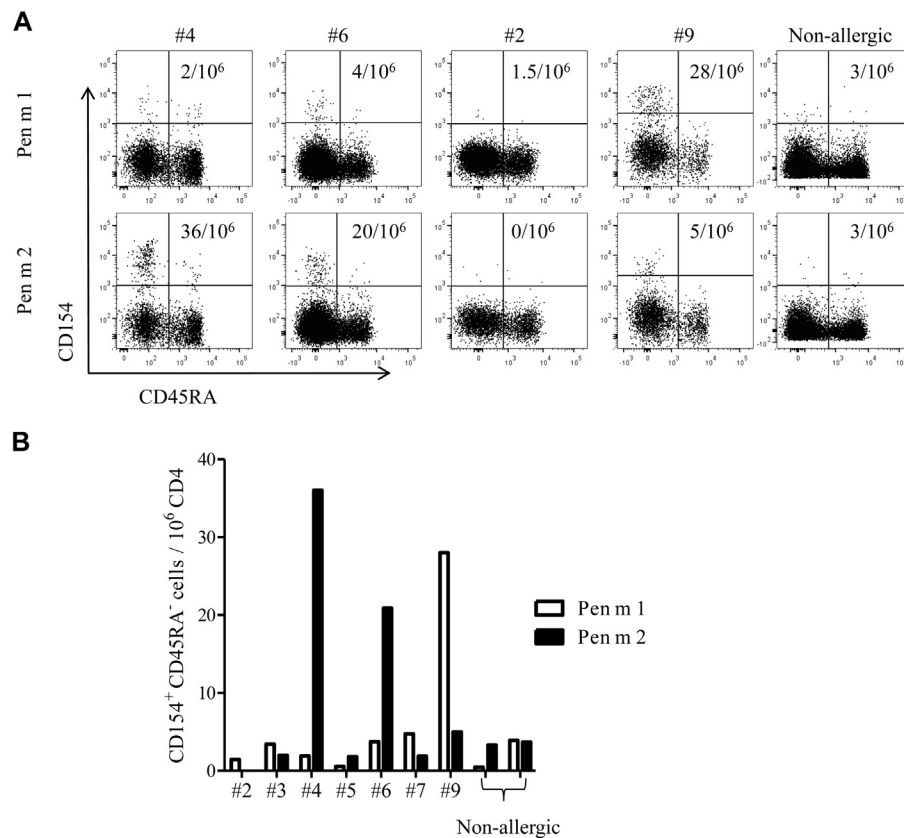


FIG E3. Pen m 1- and Pen m 2-specific CD4⁺ T-cell responses in allergic subjects with CD154 assays. For the CD154 expression assay,^{E2} 30×10^6 PBMCs (at 6×10^6 cells/mL) were stimulated for 3 hours at 37°C with 5 μ g/mL synthesized peptide pools (Mimotopes) spanning the entire Pen m 1 or Pen m 2 sequences in the presence of 1 μ g/mL anti-CD40 (HB14, Miltenyi Biotec). After stimulation, PBMCs were labeled with PE-conjugated CD154, and CD154⁺ cells were enriched with anti-PE magnetic beads (Miltenyi Biotec). **A**, CD154 expression on gated CD4⁺ T cells after 3 hours of PBMC stimulation with Pen m 1 or Pen m 2. Results shown are after anti-PE bead enrichment. Numbers indicate frequency of CD154⁺ CD45RA⁻ CD4⁺ T cells per 10⁶ CD4⁺ T cells. **B**, Frequency of total CD154⁺ CD45RA⁻ CD4⁺ T cells after Pen m 1 (*open bar*) or Pen m 2 (*solid bar*) stimulation of PBMCs in 7 allergic and 2 nonallergic subjects.

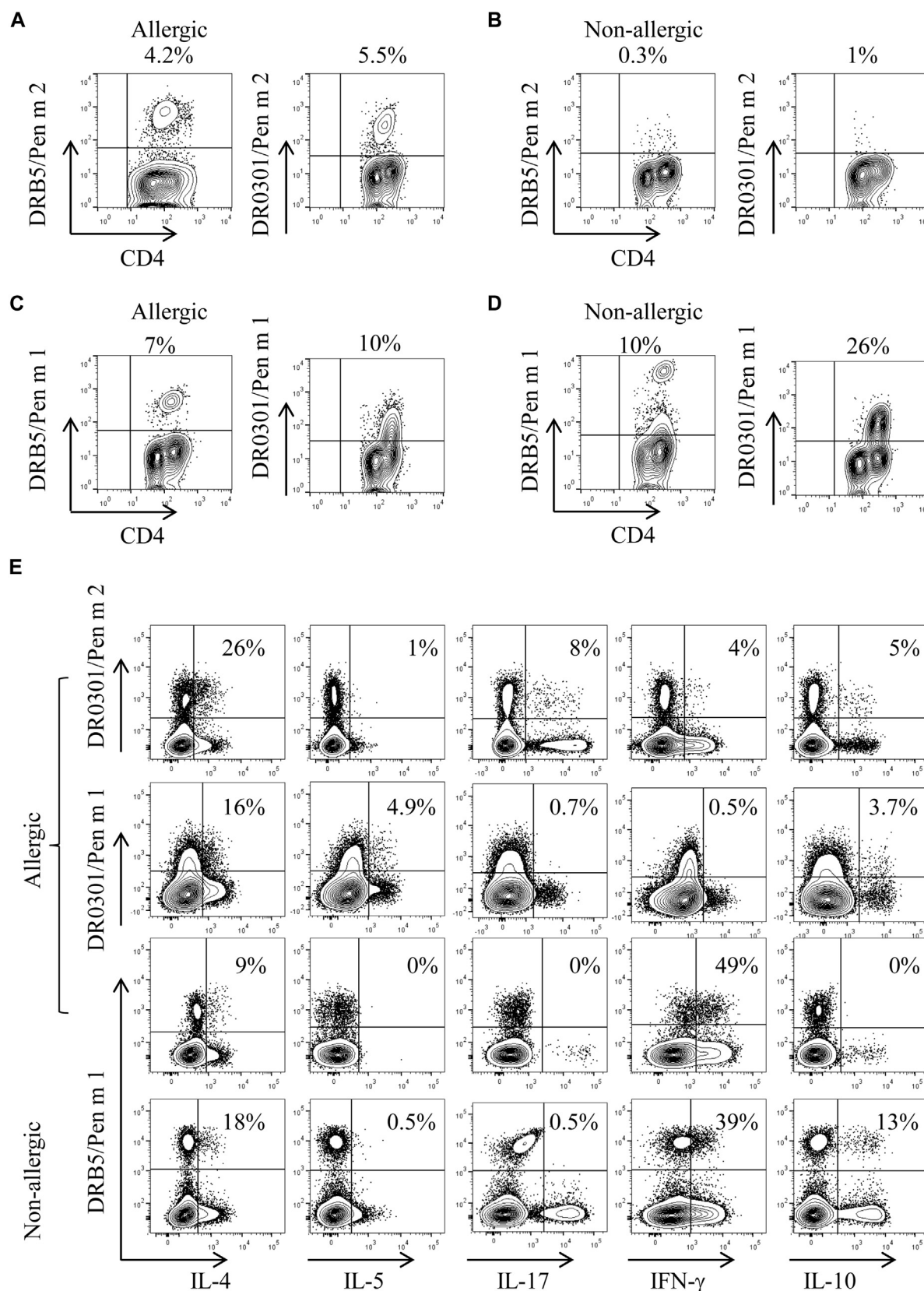


FIG E4. DRB1*03:01 and DRB5*01:01/Pen m 1- and Pen m 2-specific T-cell lines. **A** and **B**, DRB1*03:01 and DRB5*01:01/Pen m 2-specific T-cell lines were analyzed by means of tetramer staining after stimulation of PBMCs from allergic (Fig E4, **A**) or nonallergic (Fig E4, **B**) adults with antigenic Pen m 2 peptides for 2 weeks. **C** and **D**, DRB1*03:01 and DRB5*01:01/Pen m 1-specific T-cell lines were analyzed after stimulation of PBMCs from allergic (Fig E4, **C**) or nonallergic (Fig E4, **D**) adults with antigenic Pen m 1 peptides for 2 weeks. **E**, Examples of cytokine profile of DRB1*03:01/Pen m 2-, DRB1*03:01/Pen m 1-, and DRB5*01:01/Pen m 1-specific T-cell lines from allergic (*top*) and nonallergic (*bottom*) adults.

TABLE E1. Study subjects' characteristics

Sample	HLA-DR	ImmunoCAP score		
		Shrimp IgE	Dust mite P IgE	Symptoms
Shrimp allergy				
1	1501/1115	3	3	Hives
2	1501/0101	3	3	Hives
3	1501/1501	1	1	Anaphylaxis
4	1501/0301	3	4	Wheezing
5	0101/0301	2	1	Anaphylaxis
6	0301/0301	2	2	Anaphylaxis
7	0301/0301	2	2	Anaphylaxis
8	0301/0404	3	4	Anaphylaxis
9	0301/1104	3	2	Anaphylaxis
Not shrimp allergic				
1	0301/0401	0	0	
2	0301/0401	0	0	
3	0301/0401	0	0	
4	1501/07*	0	0	
5	1501/1501	0	0	
6	0401/1501	0	0	
7	0101/1501	0	0	
8	0301/0701	0	0	Peanut-induced anaphylaxis
9	0301/0701	0	0	Peanut-induced anaphylaxis
10	0401/1501	0	0	Peanut-induced anaphylaxis
11	0701/1501	0	3	Peanut allergy
12	0101/1501	0	0	Peanut-induced anaphylaxis

ImmunoCAP score: 0, <0.35 IU/mL; 1, 0.35-0.69 IU/mL; 2, 0.70-3.49 IU/mL; 3, 3.50-17.49 IU/mL; and 4, 17.50-49.99 IU/mL.

*All subjects with the DR1501 haplotype also have the DRB5*01:01-DQA*01:02/DQB*06:02 haplotype.

TABLE E2. Pen m 1 and Pen m 2 T-cell epitopes

HLA restriction	Epitope	AA sequence
DRB1*03:01	Pen m 1 ₉₋₂₈	QAMKLEKDNAMDRADTLEQQ
DRB1*03:01	Pen m 2 ₈₁₋₁₀₀	LFDPIEDYHVGFKQTDKHP
DRB5*01:01	Pen m 1 ₁₆₁₋₁₈₀	KYDEVARKLAMVEADLERAE
DRB5*01:01	Pen m 2 ₁₉₃₋₂₁₂	RFLQAANACRYWPAGRGIYH
DQA*01:02/ DQB*06:02	Pen m 1 ₉₇₋₁₁₆	EDLERSEERLNTATTKLAEA
DQA*01:02/ DQB*06:02	Pen m 2 ₂₆₅₋₂₈₄	LGFLTFCPTNLGTTVRASVH