

Ara h 2 peptides containing dominant CD4⁺ T-cell epitopes: Candidates for a peanut allergy therapeutic

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Background: Peanut allergy is a life-threatening condition; there is currently no cure. Although whole allergen extracts are used for specific immunotherapy for many allergies, they can cause severe reactions, and even fatalities, in peanut allergy.

Objective: This study aimed to identify short, T-cell epitope-based peptides that target allergen-specific CD4⁺ T cells but do not bind IgE as candidates for safe peanut-specific immunotherapy.

Methods: Multiple CD4⁺ T-cell lines specific for the major peanut allergen Ara h 2 were generated from PBMCs of 16 HLA-diverse subjects with peanut allergy by using 5,6-carboxyfluorescein diacetate succinimidylester-based methodology. Proliferation and ELISPOT assays were used to identify dominant epitopes recognized by T-cell lines and to confirm recognition by peripheral blood T cells of epitope-based peptides modified for therapeutic production. HLA restriction of core epitope recognition was investigated by using anti-HLA blocking antibodies and HLA genotyping. Serum-IgE peptide-binding was assessed by dot-blot.

Results: Five dominant CD4⁺ T-cell epitopes were identified in Ara h 2. In combination, these were presented by HLA-DR, HLA-DP, and HLA-DQ molecules and recognized by T cells from all 16 subjects. Three short peptide variants containing these T-cell epitopes were designed with cysteine-to-serine substitutions to facilitate stability and therapeutic production. Variant peptides showed HLA-binding degeneracy, did not bind peanut-specific serum IgE, and could directly target T_H2-type T cells in peripheral blood of subjects with allergy.

Conclusion: Short CD4⁺ T-cell epitope-based Ara h 2 peptides were identified as novel candidates for a T-cell-targeted peanut-specific immunotherapy for an HLA-diverse population.

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Key words: Peanut allergy, Ara h 2, T-cell epitope, peptide, immunotherapy

Abbreviations used

aa:	Amino acid
CFSE:	5,6-Carboxyfluorescein diacetate succinimidylester
CPE:	Crude peanut extract
cpm:	Counts per minute
EBV-B cells:	EBV-transformed B cells
HLA-II:	HLA class II
nAra h 2:	Natural Ara h 2
r:	Recombinant
SI:	Stimulation index
TBP:	TRIS-bis-propane
TCL:	T-cell line
TT:	Tetanus toxoid

Peanut allergy is a major health care problem affecting 1% to 2% of the population,¹⁻³ with clinical symptoms ranging from mild oropharyngeal irritation and vomiting to life-threatening systemic anaphylaxis. It is the leading cause of food-induced anaphylactic fatalities worldwide.^{2,4} Unlike egg and milk food allergy that presents in infancy and typically resolves by school age, peanut allergy persists through adulthood in 80% of cases, significantly impairing the quality of life of afflicted individuals and their families.⁴⁻⁷

There is currently no cure for peanut allergy, leaving avoidance as the only preventative option and adrenaline for anaphylactic crises. Even with diligent precautions, most subjects with peanut allergy have accidental exposures that can result in severe and sometimes fatal reactions.^{4,8}

Research trials of conventional allergen desensitization, or more recently oral immunotherapy using whole peanut extract, provide encouragement on feasibility, but the potential for adverse reactions highlights major safety concerns.⁹⁻¹⁵ This risk is especially pertinent with peanut allergens because they may induce anaphylaxis at minute doses, even in individuals who previously experienced only mild symptoms.^{13,16} Consequently, there is an urgent need to develop a safe, disease-modifying therapeutic for individuals with peanut allergy.

Mechanistic studies on clinically effective immunotherapy with other allergens demonstrate immune deviation from a predominantly T_H2 to T_H1 immune response and induction of regulatory T cells that downregulate T_H1 and T_H2 responses. Specific immunotherapy is also accompanied by decreased specific IgE, increased protective specific IgG₄ antibody levels, and reduced number and activation of mast cells, eosinophils, and basophils.¹⁷⁻²² Stimulation of appropriate T-cell responses during allergen-specific immunotherapy is necessary for effective desensitization.²² Conventional immunotherapy administers whole

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allergen extracts, but peptides containing T-cell epitopes of major allergens provide an effective, safer (non-IgE-reactive) alternative.^{23,24} In proof-of-concept studies, T-cell epitope-based peptides from the major allergens phospholipase A₂ and Fel d 1 have been used to treat bee venom^{25,26} and cat allergy,²⁷⁻³¹ respectively, without causing IgE-mediated reactions. Importantly, there is evidence for linked suppression using peptide immunotherapy. In *in vitro* and murine studies, the dominant T-cell epitope peptide of the house dust mite major allergen Der p 1 induced tolerance not only to this peptide but also to whole Der p 1 and house dust mite extract.^{32,33} Clinical administration of selected Fel d 1 peptides altered T-cell responses to those peptides, other nonrealted Fel d 1 peptides, and whole cat allergen extract.²⁹

We aim to develop a T-cell-targeted peptide immunotherapy as a safe treatment option for individuals with peanut allergy. However, data on CD4⁺ T-cell epitopes of peanut allergens are limited. Only a few reports document CD4⁺ T-cell responses to individual peptides spanning whole peanut allergens. These include analyses of CD4⁺ T-cell proliferation to unsequenced digestion products of the major peanut allergens Ara h 1 and Ara h 2,^{34,35} and 2 reports, including our own pilot study, of T-cell reactivity to overlapping 15-mer or 20-mer peptides spanning Ara h 2.^{36,37} Although these latter studies implicated broad regions of CD4⁺ T-cell reactivity within Ara h 2, core epitopes were not determined, population sizes were small, and analyses were limited. Other studies identifying peanut allergen peptides with therapeutic value focused on identifying short peptides that bound IgE from individuals with peanut allergy, and subsequently developing non-IgE-binding variants that retained T-cell reactivity.^{36,38-40} Given the pivotal role of CD4⁺ T cells in driving the immune response to allergen, we took the more direct strategy of identifying the full repertoire of dominant CD4⁺ T-cell epitopes of a major peanut allergen recognized by proliferative T cells from an HLA-diverse peanut-allergic cohort and subsequently determining their therapeutic potential. Here we present definitive data identifying and characterizing 5 dominant CD4⁺ T-cell epitopes of Ara h 2, the most widely recognized and potent allergen for individuals with peanut allergy.⁴¹⁻⁴⁵ We design peptides containing the identified epitopes and demonstrate key properties that make them prime candidates for a specific immunotherapy for subjects with peanut allergy.

METHODS

Subjects

Sixteen Caucasian adult subjects with peanut allergy and 3 healthy, nonatopic control (negative skin prick testing to common allergens) Caucasian adult subjects were recruited from The Alfred Allergy Clinic, Melbourne, Australia (see this article's Table E1 in the Online Repository at www.jacionline.org). Subjects with peanut allergy had clinical symptoms of IgE-mediated peanut allergy and peanut-specific IgE CAP score ≥ 2 (≥ 1.16 kU_A/L; Pharmacia CAP System; Pharmacia Diagnostics, Uppsala, Sweden) and were genotyped (HLA-DRB1, HLA-DQB1, and HLA-DPB1, exon 2) by the Victorian Transplantation and Immunogenetics Service. The study was approved by The Alfred and Monash University Ethics Committees, and informed written consent obtained from each subject.

Antigens

Crude peanut extract (CPE) was prepared from commercial unsalted, dry-roasted peanuts as described,⁴⁶ dialyzed against PBS, and filter-sterilized (0.2 μ m). Natural Ara h 2 (nAra h 2) was enriched from CPE based on

published methodology.⁴⁷ Briefly, CPE was buffer exchanged into 20 mmol/L TRIS-bis-propane (TBP), pH 7.2, by using Vivaspin columns (Sartorius Stedim Biotech SA, Aubagne, France) and applied onto a 5-mL Mono-Q 10/10 column (FPLC System; Pharmacia, St Albans, United Kingdom) equilibrated with TBP. After washing with TBP, a linear gradient of 30 mL 0 to 1 mol/L NaCl/TBP was applied to elute bound proteins (1 mL/min). Fractions, 0.5 mL, were analyzed by SDS-PAGE and those containing nAra h 2 with minimal other proteins pooled and dialyzed against PBS. Endotoxin contents were 1.7 and 78 endotoxin units/mg for CPE and nAra h 2, respectively (Endpoint Chromogenic LAL assay; Lonza, Walkersville, Md).

Ara h 2 peptides (Mimotopes, Clayton, Australia; see this article's Table E2 in the Online Repository at www.jacionline.org) were reconstituted at 2 mg/mL 10% dimethyl sulfoxide/PBS. All antigens were confirmed to be neither mitogenic nor toxic.

Generation of Ara h 2-specific CD4⁺ T-cell lines

Ara h 2-specific oligoclonal T-cell lines (TCLs) were generated from PBMCs of subjects with peanut allergy by using 5,6-carboxyfluorescein diacetate succinimidylester (CFSE)-based methodology.⁴⁸ Briefly, culturing was performed in RPMI-1640 containing 2 mmol/L L-glutamine, 100 IU/mL penicillin-streptomycin, and 5% human blood group AB serum (cRPMI; Sigma-Aldrich, St Louis, Mo). PBMCs were labeled with 0.1 μ mol/L CFSE (Molecular Probes, Eugene, Ore) and cultured (2.5×10^6 /mL) with cRPMI alone, CPE (100 μ g/mL), nAra h 2 (10 μ g/mL), Ara h 2 20-mer-peptide pools (see this article's Table E3 in the Online Repository at www.jacionline.org; 10 μ g/mL/peptide), or as a control, tetanus toxoid (TT; 10 limit of flocculation units/mL; Statens Serum Institute, Copenhagen, Denmark) for 7 days at 37°C. After staining with CD4-phycoerythrin and 7-amino-actinomycin D (BD Pharmingen, San Diego, Calif), CD4⁺CFSE^{dim}7-amino-actinomycin D⁺ cells were sorted (10 cells/well) into 96-U-well plates containing irradiated allogeneic feeder cells, anti-CD3 (OKT-3), recombinant (r) IL-2 (Cetus, Emeryville, Calif) and Fungizone (Invitrogen, Carlsbad, Calif) as described.⁴⁸ Cells were fed with rIL-2 as required, and after 10 to 14 days, they were transferred to 48-well plates and tested for proliferation to nAra h 2 (10 μ g/mL). Ara h 2-positive TCLs were expanded with anti-CD3 and rIL-2⁴⁸ in T25 culture flasks (BD, Franklin Lakes, NJ) for 10 to 12 days, then tested for specificity (proliferation) to overlapping 20-mer peptides spanning Ara h 2 (10 μ g/mL).

Proliferation assays

Proliferation assays were performed on 72-hour duplicate or triplicate cultures in 96-U-well plates containing 1×10^4 T cells/well, 1×10^4 irradiated (5000 rad) autologous EBV-transformed PBMCs (EBV-B cells) as antigen-presenting cells and antigens as specified. Negative control was cRPMI alone. Cells were pulsed with ³H-thymidine (0.5 μ Ci/well) for the last 16 hours and uptake recorded as mean counts per minute (cpm) of replicate cultures. A stimulation index (SI; cpm antigen-stimulated T cells/cpm unstimulated T cells) ≥ 2.5 was considered positive, and all positive responses were confirmed in ≥ 2 assays.

HLA class II blocking assays

T cells and irradiated EBV-B cells (1×10^4 of each) were incubated with 0.1 to 10 μ g/mL blocking mAb against HLA-DR (L243, BD Pharmingen), HLA-DQ (SVP-L3), or HLA-DP (B7/21; gifts from S. Mannering, St Vincent's Research Institute, Melbourne, Australia) or isotype-control antibodies (IgG_{2a}, BD Pharmingen; IgG₁, BioLegend, San Diego, Calif) for 1 hour at 37°C before addition of peptides (2-10 μ g/mL) or CPE (100 μ g/mL) and testing of the proliferative response as discussed.

Cytokine ELISPOT assays

MAIP ELISPOT plates (Millipore, Billerica, Mass) were coated overnight at 4°C with 10 μ g/mL IL-4, IFN- γ , or IL-5 antibodies (eBioscience, San Diego, Calif) in PBS. Wells were blocked (cRPMI, 1 hour, 37°C), and then PBMCs (3.5×10^5) or T cells and irradiated EBV-B cells (1×10^4 of each) were added in duplicate 100 μ L cultures with CPE (100 μ g/mL), nAra h 2

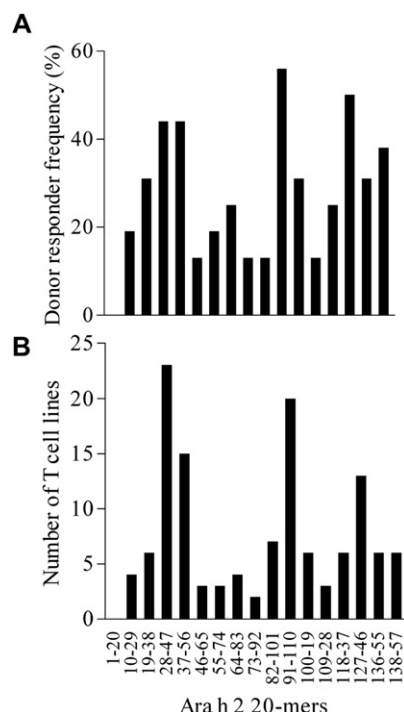


FIG 1. Responder frequency profiles for Ara h 2 20-mer peptides. Aggregate donor responder frequencies (**A**) and total numbers of specific TCLs (**B**) are shown for each Ara h 2 20-mer peptide for all 16 subjects with peanut allergy.

(10 μ g/mL), or peptides (10 μ g/mL). Controls were cRPMI alone, TT (10 LfU/mL), and phytohemagglutinin (1 μ g/mL; Sigma-Aldrich). After 48 hours of culture at 37°C, plates were incubated with biotinylated IL-4, IL-5, or IFN- γ antibodies (eBioscience; 1 μ g/mL PBS, 2 hours) followed by ExtrAvidin alkaline phosphatase (Sigma-Aldrich; 1/3000 PBS, 2 hours) before developing with alkaline phosphatase substrate (Bio-Rad, Hercules, Calif). When spots appeared in positive control wells, plates were washed, air-dried, and read (AID ELISPOT 4.0 h reader; Autoimmun Diagnostika, Strassberg, Germany).

Dot-blot IgE-binding assay

Nitrocellulose membrane (0.2 μ m pore size) was dotted with 10 μ g CPE or 20 μ g peptide, blocked, incubated with individual subject sera (diluted 1:10), and washed as described.⁴⁹ Membranes were then incubated with rabbit polyclonal antihuman IgE antibody (1:500; DAKO, Glostrup, Denmark) followed by horseradish peroxidase-labeled goat antirabbit IgG (1:1000; Promega, Madison, Wis) for 1 hour each. IgE binding was detected by using substrate 4-chloro-1-naphthol (Sigma-Aldrich) and hydrogen peroxide.⁴⁶

RESULTS

Selection of Ara h 2 20-mer peptides containing dominant CD4⁺ T-cell epitopes recognized by subjects with peanut allergy

A total of 69 Ara h 2-specific TCLs were generated from PBMCs of 16 subjects with peanut allergy by isolating and expanding antigen-specific (proliferated) CD4⁺CFSE^{dim} T cells from 7-day CFSE-labeled PBMC cultures stimulated with CPE, nAra h 2 or, for 6 subjects, pools of Ara h 2 20-mer peptides.

The 20-mer peptides recognized (SI $\geq$ 2.5) by each TCL are shown in Table E3, and data are summarized in Fig 1. As reported for other allergens,⁵⁰ patterns of T-cell peptide recognition were more restricted for some subjects (eg, 8 and 9) than others (eg, 1 and 10). Nonetheless, certain 20-mers were consistently

recognized by multiple subjects. Ara h 2(91-110), (127-146), (28-47), and (37-56) had the most responders, recognized by 9 (56%), 8 (50%), 7 (44%), and 7 (44%) of the 16 subjects with peanut allergy, respectively (Fig 1, A). At least 1 TCL from each of the 16 subjects (in total, 80% of all TCL) recognized 1 or more of these four 20-mers, with reactive TCL typically showing strong proliferative responses (SI > 8). Furthermore, each of these 20-mers was recognized by multiple TCLs from many responders, reflecting a prevalence of T cells specific for these peptides among the subjects' T-cell repertoires. Indeed, 13 of 14 (93%) TCLs generated from PBMCs stimulated with Ara h 2 peptide pools were specific for 1 or more of these 20-mers, further demonstrating that these 20-mers could directly target Ara h 2-specific T cells among PBMCs of individuals with peanut allergy and preferentially over other Ara h 2 20-mers. Because Ara h 2(28-47), (37-56), (91-110), and (127-146) collectively showed the highest donor and TCL responder frequencies, strongest T-cell responses, and preferential ability to target PBMC T cells, these 4 peptides were identified as containing dominant Ara h 2 T-cell epitopes.

Mapping core T-cell epitopes of dominant Ara h 2 20-mer peptides

Keeping immunotherapeutic peptides as short as possible minimizes risk of cross-linking cell-bound IgE on inflammatory cells and facilitates therapeutic production. The minimum T-cell stimulatory sequence (core epitope) within each dominant 20-mer was determined by testing proliferation of reactive TCLs to peptide sets truncated from the N-terminus or C-terminus of the 20-mer (Table E2). Truncations of Ara h 2(37-56) were not required because most TCLs that recognized this peptide also responded with comparable magnitude to Ara h 2(28-47) (Table E3), and mapping by using Ara h 2(28-47) truncations revealed that the core epitope recognized by these lines lay within the overlap between these 2 peptides.

In total, 5 core CD4⁺ T-cell epitopes were identified (Fig 2). Ara h 2(28-47) and (91-110) each contained 2 distinct but overlapping T-cell epitopes: (32-44) and (37-47) (Fig 2, A, upper and lower panels, respectively), and (91-102) and (95-107) (Fig 2, B, upper and lower panels, respectively). No single TCL responded to both epitopes of an overlapping pair.

For Ara h 2(32-44)-specific TCL, the lack of response to epitope (37-47) is demonstrated in Fig 2, A (truncation 37-47, upper panel). Similarly, for Ara h 2(95-107)-specific TCLs, the lack of response to truncation (91-102) is evident in Fig 2, B (lower panel). Only a single epitope, (128-141), was found within Ara h 2(127-146) (Fig 2, C).

For each epitope, the number of residues required to induce maximal TCL proliferation varied from 9 to 15 aa between subjects (Fig 2, boldface sequences), but there was a minimum core sequence of 9 to 11 aa required for recognition by every reactive TCL (Fig 2, underlined sequences). In selecting epitope sequences for further analyses, residues required for maximal stimulation of all TCLs tested were incorporated to ensure the broadest possible recognition.

Determining HLA class II restriction specificity of dominant Ara h 2 T-cell epitopes

There is no confirmed HLA class II (HLA-II) association with peanut allergy⁵¹; therefore, peptides selected for therapy must

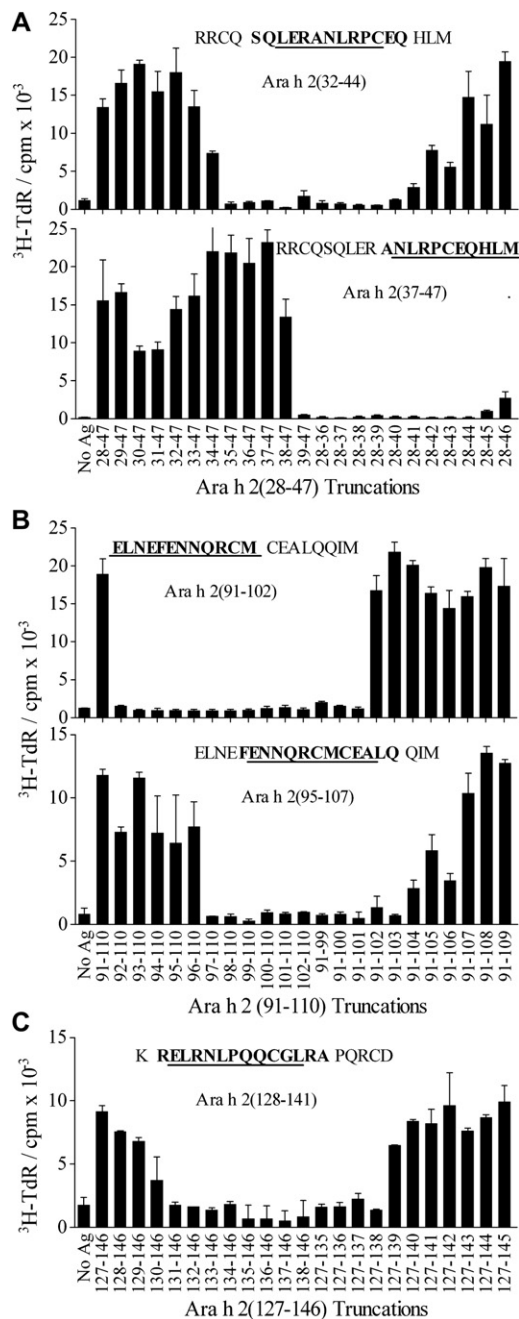


FIG 2. Mapping core T-cell epitopes within dominant Ara h 2 20-mer peptides. Dominant 20-mer-specific TCL proliferation to truncated peptide sets. Representative data show each epitope identified (mean cpm replicate wells + SD). Sequences required for recognition by all tested subjects are boldface; conserved cores are underlined. **A**, Ara h 2(28-47); upper panel, n = 3 (5 TCLs); lower panel, n = 4 (7 TCLs). **B**, Ara h 2(91-110); upper panel, n = 3 (6 TCLs); lower panel, n = 3 (5 TCLs). **C**, Ara h 2(127-146); n = 5 (6 TCLs). $^3\text{H-TdR}$, ^3H -thymidine; Ag, antigen.

bind diverse HLA-II molecules for wide applicability. To determine the HLA-II type presenting each epitope, anti-HLA-DR, anti-HLA-DP, or anti-HLA-DQ mAbs were used to block epitope presentation to T cells.

For each TCL tested, epitope recognition was prevented by 1 or more HLA mAbs in a dose-dependent manner (Fig 3). Isotype control antibodies (10 $\mu\text{g/mL}$) had no effect (data not shown).

Each HLA mAb prevented recognition of 1 or more epitopes, collectively indicating presentation by HLA-DR, HLA-DP, and HLA-DQ. In each case, the same mAb blocked recognition of CPE (data not shown), demonstrating consistency for presentation of naturally processed and synthetic epitope forms. In all subjects tested, T-cell recognition of epitope (32-44) was consistently blocked by anti-HLA-DP (Fig 3, A), epitope (95-107) by anti-HLA-DQ (Fig 3, E), and epitopes (91-102) and (128-141) by anti-HLA-DR (Fig 3, D and F). In contrast, recognition of epitope (37-47) was blocked by anti-HLA-DR in subjects 8 and 13 (Fig 3, B) and anti-HLA-DQ in subjects 9 and 11 (Fig 3, C). Each epitope of an overlapping pair was blocked by a different HLA mAb, further demonstrating their distinction.

The ability to block recognition of a given epitope by the same HLA mAb in multiple subjects indicated preferential presentation by a specific HLA type. To assess potential for degeneracy of epitope presentation by allelic variants of the respective HLA type, HLA alleles of subjects recognizing a given epitope were compared (Table E1). The absence of a shared *HLA-DQB1* allele among all subjects for whom recognition of epitope (95-107) was blocked by anti-HLA-DQ indicated that this epitope must be presented by multiple HLA-DQB1 molecules. Similarly, the diversity in *HLA-DRB1* alleles among subjects for whom recognition of epitopes (127-141) or (37-47) was blocked by anti-HLA-DR indicated binding degeneracy of both epitopes for multiple HLA-DRB1 molecules. Epitope (37-47) was also presented by HLA-DQB1*06:09 because both subjects (9 and 11) who recognized this epitope in the context of HLA-DQ had this allele, and for subject 9, it was the only *DQB1* allele present. Because *DPB1**04:01 or *DRB1**15:01 alleles were present in all subjects recognizing epitopes (32-44) (blocked by anti-HLA-DP) or (95-107) (blocked by anti-HLA-DR), respectively, degeneracy of these epitopes could not be determined. However, because *DPB1**04:01 and *DRB1**15:01 are prevalent in populations worldwide,⁵² epitopes presented by these HLA molecules would still be broadly recognized.

Testing T-cell reactivity of cysteine-substituted Ara h 2 T-cell epitope peptides

To minimize the number of peptides for a therapeutic and avoid unnecessary sequence duplication, each overlapping epitope pair was combined into a single peptide—Ara h 2(32-47) and Ara h 2(91-107). The resultant peptides were less than 18 aa and efficiently stimulated T cells specific for either epitope (Fig 4). Together with Ara h 2(128-141), this provided a panel of 3 HLA-degenerate peptides. However, each peptide contained 1 or more cysteine residues, which are prone to oxidation and disulphide bridge formation that can hinder synthesis, stability, and bioavailability of therapeutic peptides. To avoid these problems, cysteines were replaced with structurally conserved but less reactive serines (Table E2). The cysteine-substituted peptides stimulated comparable T-cell proliferation (Fig 4) and cytokine production (see this article's Fig E1 in the Online Repository at www.jacionline.org) to the parent peptides. Sequence analysis and synthesis of these modified peptides confirmed water solubility and ease of generation at high purity (Mimotopes).

Testing candidate peptides for serum-IgE binding

To provide a safe alternative to whole allergens, peptides must not bind and cross-link cell-bound IgE. Using methodology

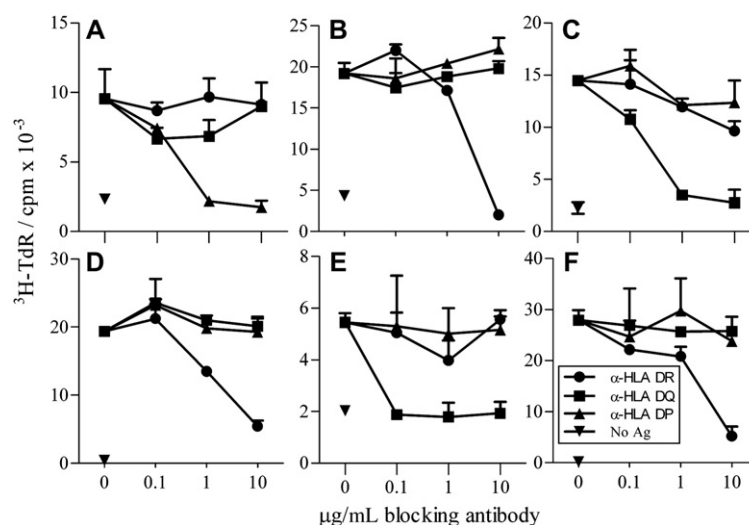


FIG 3. HLA-II restriction specificity of T-cell recognition of dominant epitopes. Proliferation of specific TCLs to each epitope in the presence of HLA-DR (circles), HLA-DQ (squares), or HLA-DP (triangles) mAbs. Graphs show representative TCLs (mean cpm replicate wells + SD). **A**, Ara h 2(32-44), *n* = 2 (4 TCLs). **B**, Ara h 2(37-47), *n* = 2 (4 TCLs). **C**, Ara h 2(37-47), *n* = 3 (4 TCLs). **D**, Ara h 2(91-102), *n* = 2 (3 TCLs). **E**, Ara h 2(95-107), *n* = 3 (4 TCLs). **F**, Ara h 2(128-141), *n* = 3 (3 TCLs). $^3\text{H-TdR}$, ^3H -thymidine; Ag, antigen.

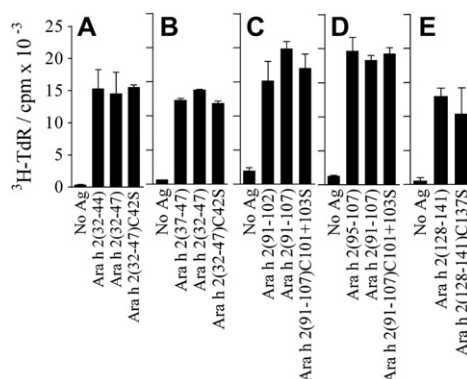


FIG 4. T-cell recognition of peptides containing cysteine-to-serine substituted epitopes. TCL proliferation in response to "parent" or cysteine-substituted Ara h 2 peptides. Graphs show representative TCLs for each epitope (mean cpm replicate wells + SD). **A**, Ara h 2(32-44), *n* = 2 (3 TCLs). **B**, Ara h 2(37-47), *n* = 3 (5 TCLs). **C**, Ara h 2(91-102), *n* = 3 (3 TCLs). **D**, Ara h 2(95-107), *n* = 2 (2 TCLs). **E**, Ara h 2(128-141), *n* = 3 (3 TCLs). $^3\text{H-TdR}$, ^3H -thymidine; Ag, antigen.

optimized for Ara h 2 peptides,⁴⁹ serum from each subject with peanut allergy was tested for IgE binding to cysteine-substituted (candidate) peptides (Fig 5). Although all subjects with peanut allergy showed clear IgE reactivity to CPE, no IgE binding was detected to any candidate peptide (Fig 5, A). Serum from a subject with latex allergy with known IgE reactivity to peptides of major latex allergen Hev b 5 provided a positive control for peptide-IgE binding (Fig 5, B).

Validation of candidate peptides for targeting peanut-specific T cells in PBMCs of subjects with peanut allergy

Successful allergen desensitization mandates a decreased $\text{T}_\text{H}2$ -response to allergen. To demonstrate that candidate peptides could target pathogenic $\text{T}_\text{H}2$ -type T cells directly in whole blood from

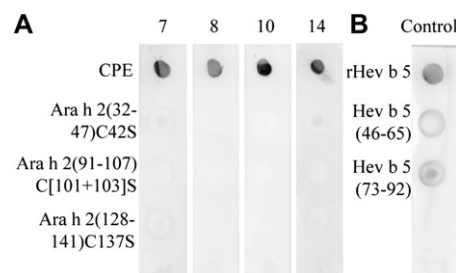


FIG 5. IgE reactivity to T-cell epitope-derived peptides. Dot-blots showing binding of CPE or candidate peptides by IgE from peanut-allergic sera (**A**; representative blots for 4 of 16 subjects). Positive control blot for peptide-binding (**B**) using serum of a subject with latex allergy with known IgE reactivity to Hev b 5 peptides.

individuals with peanut allergy, ELISPOT cytokine assays were performed on PBMCs from a panel of subjects with peanut allergy (donors 6, 12, and 15). Because the hallmark of allergen-specific T cells from subjects with allergy is elevated production of $\text{T}_\text{H}2$ -type cytokines in response to specific allergen compared with subjects without peanut allergy, PBMCs from subjects without peanut allergy were analyzed as controls (Fig 6). In response to the control antigen TT, IL-4, IL-5, and IFN- γ were produced by PBMCs from both subject groups (Fig 6). In contrast, only PBMCs from donors with peanut allergy secreted cytokines in response to peanut allergens or candidate peptides, with prominent IL-4 and IL-5 production and a detectable but limited IFN- γ response induced by 1 or more peptides for each subject with peanut allergy tested. Responses to parent and cysteine-substituted candidate peptides were comparable and reflected those of specific TCLs (Fig E1). These data further confirmed that TCLs reflected the peripheral blood repertoire of pathogenic T cells in individuals with peanut allergy, and that candidate peptides were recognized by, and could target, pathogenic $\text{T}_\text{H}2$ -type T cells in PBMCs of these subjects.

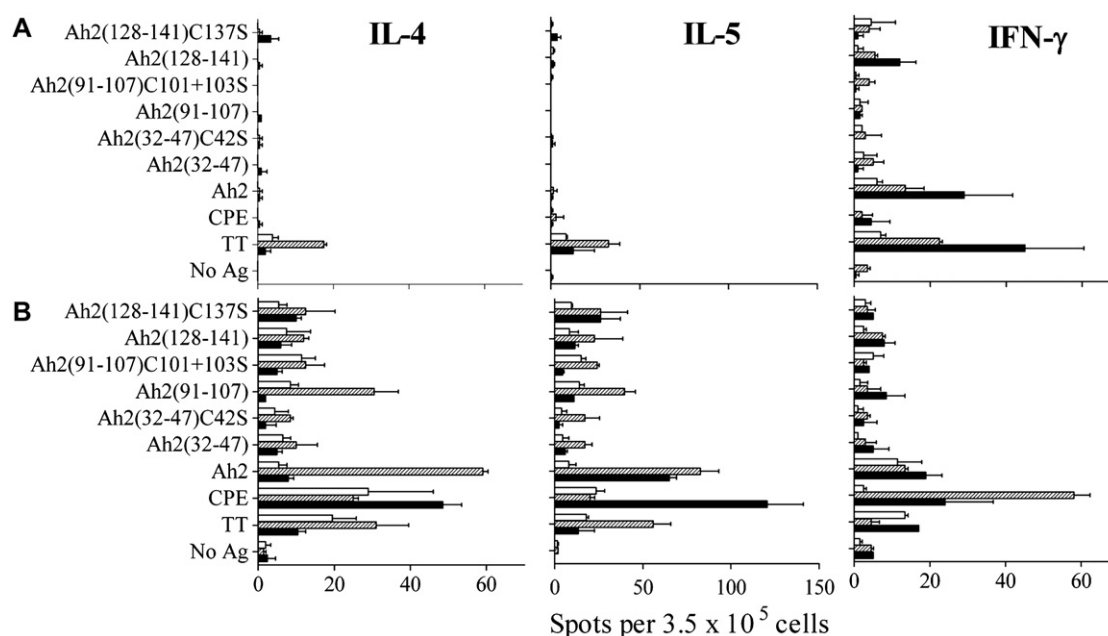


FIG 6. PBMC cytokine secretion in response to T-cell epitope-derived peptides. PBMCs from 3 nonatopic control subjects (**A**) and 3 subjects with peanut allergy (**B**) stimulated with CPE, nAra h 2, candidate peptides, or TT (control). IL-4, IL-5, and IFN- γ secretion determined by ELISPOT. Mean spots of replicate wells (+SD) shown for each subject. Ag, Antigen.

DISCUSSION

An appropriately selected T-cell-targeted peptide immunotherapeutic will provide a safe treatment option for individuals with peanut allergy. Candidate peptides must (1) contain dominant CD4⁺ T-cell epitopes of major peanut allergens recognized by multiple individuals with peanut allergy, (2) be presented on different HLA-II molecules to target T cells in genetically diverse individuals, (3) not bind and cross-link cell-bound IgE, and (4) be scalable and amenable to therapeutic production. This comprehensive study is the first to report core sequences of dominant CD4⁺ T-cell epitopes of a major peanut allergen. We have identified and characterized 5 HLA-diverse CD4⁺ T-cell epitopes of Ara h 2, the most potent and widely recognized peanut allergen among individuals with peanut allergy. Using these sequences, we have designed candidate peptides that meet all these criteria for therapeutic development.

To commence this study, we selected 4 “dominant” 20-mers of Ara h 2 on the basis of responses of multiple TCLs from each of 16 HLA-diverse subjects with peanut allergy. Two of these 20-mers differed from those we reported previously.³⁷ Importantly, however, when data from both studies were pooled, this panel of four 20-mers remained the most frequently recognized among TCLs from the combined cohort of 24 subjects with peanut allergy, further validating our final 20-mer selection for core-epitope mapping. Although our approach may have excluded analysis of nonproliferative but functional T cells, proof-of-concept studies indicate epitopes recognized by proliferative T_H2 cells are sufficient for effective therapy.⁵³

Despite varied patterns of peptide recognition by TCLs between subjects (influenced by many factors including subject genetics and patterns of allergen encounter⁵⁰), 1 or more of the 5 epitopes identified within these 20-mers was recognized by each

of the 16 subjects with peanut allergy in this study. Together with their collective presentation by HLA-DR, HLA-DQ, and HLA-DP molecules (commonly observed for allergen T-cell epitopes⁵⁴⁻⁵⁸), this emphasized their broad applicability, particularly because the HLA-allele profile of our cohort is typical of Caucasian populations⁵² in countries with the highest incidence of peanut allergy.⁵⁹ Each candidate peptide contains at least 1 HLA-degenerate epitope for presentation by multiple HLA molecules, with Ara h 2(32-47) alone containing epitopes presented on all 3 HLA types and at least 4 different HLA molecules. Inclusion of HLA-DQ and HLA-DP-restricted epitopes in a therapeutic is particularly advantageous because these alleles are less variable and thus more prevalent in mixed populations than HLA-DR alleles.⁶⁰

The main rationale for developing a peptide immunotherapy for peanut allergy is to identify an effective allergen preparation that does not invoke the adverse effects seen with whole peanut extract.^{10-14,17,61} Consistent with the absence of reported linear IgE epitopes^{36,38,49,62} within our candidate peptides, we were unable to detect peptide binding by IgE from peanut-allergic sera, despite detection of CPE-reactive IgE in all samples. This emphasizes the potential for these peptides to provide a safe alternative to whole allergen extract for immunotherapy.

In summary, we report the novel identification of 5 dominant CD4⁺ T-cell epitopes of Ara h 2 that collectively show diverse HLA-II restriction. We incorporated cysteine-free variants of these epitopes into a combinatorial panel of 3 short (≤ 17 aa), HLA-degenerate peptides that can target pathogenic T cells in HLA-diverse individuals with allergy without binding peanut-reactive serum IgE. Because immunologic suppression to whole allergen can be induced by administration of isolated T-cell epitopes from the allergen sequence,²⁹ these peptides are prime candidates for a therapeutic for peanut allergy.

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Clinical implications: This study identifies 3 short peptides containing 5 dominant T-cell epitopes of Ara h 2 as novel and suitable candidates for T-cell targeted immunotherapy for peanut allergy.

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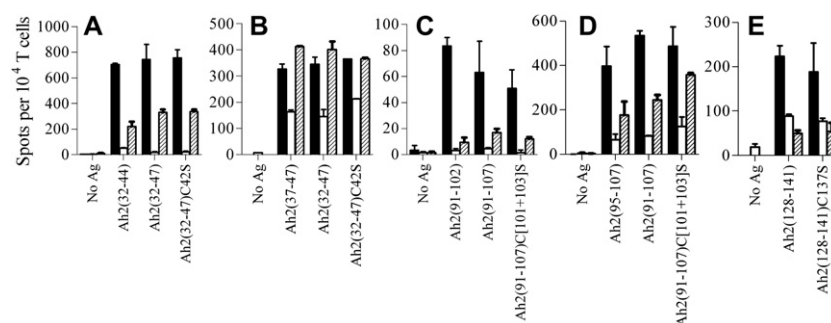


FIG E1. T-cell recognition of peptides containing cysteine-to-serine substituted epitopes. TCL cytokine secretion in response to “parent” or cysteine-substituted Ara h 2 peptides determined by ELISPOT. *Graphs* show representative TCL specific for each epitope (mean spots of replicate wells + SD). IL-4, *black bars*; IL-5, *hatched bars*; IFN-γ, *white bars*. **A**, Ara h 2(32-44), n = 2 (3 TCLs). **B**, Ara h 2(37-47), n = 3 (5 TCLs). **C**, Ara h 2(91-102), n = 3 (3 TCLs). **D**, Ara h 2(95-107), n = 2 (2 TCLs). **E**, Ara h 2(128-141), n = 3 (3 TCLs). Ag, Antigen.

TABLE E1. Demographics and genotype of subjects with peanut allergy

Donor	Sex	Age (y)	Atopic	Asthma	Anaphylaxis	Peanut CAP kU _A /L (score)	HLA genotypes					
							DRB1		DQB1		DPB1	
PA 1	F	32	Yes	Yes	Yes	10.60 (3)	07:01	15:01	02:01G	06:02	02:01	04:01
PA 2	F	53	Yes	Yes	Yes	83.90 (5)	03:01	08:01	02:01G	04:02	03:01/104:01	04:01
PA 3	M	30	Yes	No	Yes	17.00 (4)	04:01	04:04	03:02	04:02	13:01/107:01	04:01
PA 4	F	22	Yes	No	Yes	4.72 (3)	11:01	15:01	03:01G	06:02	03:01/104:01	04:01
PA 5	F	25	Yes	No	Yes	2.12 (2)	11:04	15:01	03:01G	06:02	02:01	14:01
PA 6	M	37	Yes	Yes	Yes	42.70 (4)	11:01	15:01	03:01G	06:02	04:01	
PA 7	M	36	Yes	Yes	Yes	56.60 (5)	01:03	04:01	03:02	05:01	03:01/104:01	02:01
PA 8	M	30	Yes	No	Yes	>100 (6)	12:01G	15:01	03:01G	06:02	13:01/107:01	04:01
PA 9	M	30	Yes	Yes	Yes	36.60 (4)	13:02		06:09		05:01	04:02/105:01
PA 10	F	23	Yes	Yes	Yes	>100 (6)	08:01	10:01	04:02	05:01	03:01/104:01	04:01
PA 11	F	26	Yes	Yes	Yes	2.82 (2)	03:01	13:02	02:01G	06:09	01:01	04:01
PA 12	M	35	Yes	Yes	No	1.23 (2)	04:05	15:01	03:02	06:02	03:01/104:01	04:01
PA 13	M	30	Yes	Yes	Yes	30.60 (4)	09:01	13:01	03:03	06:03	03:01/104:01	04:02/105:01
PA 14	F	19	Yes	No	Yes	98.90 (5)	11:01	15:01	03:01G	06:02	04:01	
PA 15	F	36	Yes	No	Yes	6.94 (3)	04:01	04:04	03:01G	03:02	02:01	04:01
PA 16	F	20	Yes	Yes	Yes	1.16 (2)	04:04	13:01	03:02	06:03	02:01	04:01
NA 1	F	32	No	No	No	0.00 (0)						
NA 2	F	24	No	No	No	0.03 (0)						
NA 3	M	24	No	No	No	0.00 (0)						

F, Female; M, male; NA, nonatopic control; PA, peanut allergy.

All HLA abbreviations comply with recent changes to allele nomenclature (<http://hla.alleles.org/announcement.html> and <http://www.ebi.ac.uk/imgt/hla/>).

Alleles followed by a “G” represent groups of alleles that share common sequences in exon 2 (http://hla.alleles.org/alleles/g_groups.html).

TABLE E2. Ara h 2 peptides*

Residues	Sequence	Residues	Sequence	Residues	Sequence
20-mer peptides spanning Ara h 2 with 11 aa overlap		28-41	RRCQSQLERANLRP	132-146	NLPQQCGLRAPQRCD
		28-40	RRCQSQLERANLR	133-146	LPQQCGLRAPQRCD
1-20	LTILVALALFLAAHASARQ	28-39	RRCQSQLERANL	134-146	PQQCGLRAPQRCD
10-29	FLAAHASARQQWELQGDRR	28-38	RRCQSQLERAN	135-146	QQCGLRAPQRCD
19-38	RQQWELQGDRCQSQLERAN	28-37	RRCQSQLERA	136-146	QCGLRAPQRCD
28-47	RRCQSQLERANLRPCEQHLM	28-36	RRCQSQLER	137-146	CGLRAPQRCD
37-56	ANLRPCEQHLMQKIQRDEDS			138-146	GLRAPQRCD
46-65	LMQKIQRDEDSYERDPYSPS	N- and C-terminal truncations of peptide (91-110)		138-146	GLRAPQRCD
55-74	DSYERDPYSPSQDPYSPSPY			128-145	KRELRLNPQQCGLRAPQRC
64-83	PSQDPYSPSPYDRRGAGSSQ	92-110	LNEFENNQRMCCEALQQIM	128-144	KRELRLNPQQCGLRAPQR
73-92	PYDRRGAGSSQHQRCCNEL	93-110	NEFENNQRMCCEALQQIM	128-143	KRELRLNPQQCGLRAPQ
82-101	SQHQRCCNELNEFENNQRC	94-110	EFENNQRMCCEALQQIM	128-142	KRELRLNPQQCGLRAP
91-110	ELNEFENNQRMCCEALQQIM	95-110	FENNQRMCCEALQQIM	128-141	KRELRLNPQQCGLRA
100-119	RCMCEALQQIMENQSDRLQG	96-110	ENNQRMCCEALQQIM	128-140	KRELRLNPQQCGLR
109-128	IMENQSDRLQGRQQEQQFKR	97-110	NNQRMCCEALQQIM	128-139	KRELRLNPQQCGL
118-137	QGRQQEQQFKRELRLNPQQC	98-110	NQRMCCEALQQIM	128-138	KRELRLNPQQCG
127-146	KRELRLNPQQCGLRAPQRCD	99-110	QRCMCEALQQIM	128-137	KRELRLNPQQC
136-155	QCGLRAPQRCDLDVESGGRD	100-110	RCMCEALQQIM	128-136	KRELRLNPQQ
138-157	CDLDVESGGRDRY	101-110	CMCEALQQIM	128-135	KRELRLNPQ
		102-110	MCEALQQIM		
N- and C-terminal truncations of peptide (28-47)		91-109	ELNEFENNQRMCCEALQOI	Core epitope peptides	
		91-108	ELNEFENNQRMCCEALQQ	32-44	SQLERANLRPCEQ
29-47	RCQSQLERANLRPCEQHLM	91-107	ELNEFENNQRMCCEALQ	37-47	ANLRPCEQHLM
30-47	CQSQLERANLRPCEQHLM	91-106	ELNEFENNQRMCCEAL	91-102	ELNEFENNQRMC
31-47	QSQLERANLRPCEQHLM	91-105	ELNEFENNQRMCCEA	95-107	FENNQRMCCEALQ
32-47	SQLERANLRPCEQHLM	91-104	ELNEFENNQRMCCE	128-141	RELRLNPQQCGLRA
33-47	QLERANLRPCEQHLM	91-103	ELNEFENNQRMC		
34-47	LERANLRPCEQHLM	91-102	ELNEFENNQRMC	Peptides containing overlapping epitope pairs	
35-47	ERANLRPCEQHLM	91-101	ELNEFENNQRC		
36-47	RANLRPCEQHLM	91-100	ELNEFENNQR	32-47	SQLERANLRPCEQHLM
37-47	ANLRPCEQHLM	91-99	ELNEFENNQ	91-107	ELNEFENNQRMCCEALQ
38-47	NLRPCEQHLM				
39-47	LRPCEQHLM	N- and C-terminal truncations of peptide (128-147)		Cysteine-to-serine substituted therapeutic candidate peptides	
28-46	RRCQSQLERANLRPCEQHL				
28-45	RRCQSQLERANLRPCEQH	128-146	RELRLNPQQCGLRAPQRCD	32-47 C42S	SQLERANLRPSEQHLM
28-44	RRCQSQLERANLRPCEQ	129-146	ELRLNPQQCGLRAPQRCD	91-107 C[101+103]S	ELNEFENNQRSMSEALQ
28-43	RRCQSQLERANLRPCE	130-146	LRNLPQQCGLRAPQRCD		
28-42	RRCQSQLERANLRPC	131-146	RNLPQQCGLRAPQRCD	128-141 C137S	RELRLNPQQSGLRA

*Peptide sequences based on Ara h 2 sequence published by Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, et al. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. Arch Biochem Biophys 1997;342:244-53.

TABLE E3. Proliferative responses of T-cell lines of subjects with peanut allergy to Ara h 2 20-mer peptides

				Ara h 2 20-mer peptides																	
				Pool 1			Pool 2			Pool 3			Pool 4			Pool 5			Pool 6		
Subject	Driving antigen	TCL	cpm of unstimulated TCL (mean ± SD)	1-20	10-29	19-38	28-47	37-56	46-65	55-74	64-83	73-92	82-101	91-110	100-119	109-128	118-137	127-146	136-155	138-157	
A	CPE	1	282 ± 6		3.0																
	CPE	2	1031 ± 301			2.8															
	CPE	3	292 ± 110							3.4											
	CPE	4	131 ± 23	209.7										210.5							
	CPE	5	234 ± 18															214.6			
	CPE	1	1123 ± 1106							3.1											
	CPE	2	279 ± 86	4.9	11.6	3.9															
	CPE	1	99 ± 33			2.5															
	CPE	2	154 ± 62				6.6														
	CPE	3	651 ± 59							4.3				3.6							
	Ah2	1	182 ± 4											2.5							
	Ah2	1	2257 ± 716											3.3						3.5	
	Ah2	1	418 ± 85											40.2	55.3						
	Ah2	2	1067 ± 122											2.5	2.5						
	Ah2	3	563 ± 14											6.0	13.5						
	Ah2	4	1202 ± 113															2.7	3.0		
	Ah2	5	895 ± 201															7.0	9.7		
	Ah2	1	3071 ± 669				2.6	3.2		2.6				2.5	3.6						
	CPE	1	2245 ± 160													3.4					
	CPE	2	2323 ± 281											3.2	3.0	3.3				3.1	
	Ah2	3	747 ± 117											7.4	13.4						
	Ah2	4	969 ± 107												17.3						
	Ah2	5	740 ± 107												18.0						
	Ah2	6	4811 ± 1072					16.1	18.7												
	Ah2	7	3310 ± 395					5.0													
	Ah2	8	3401 ± 75					7.4													
	Ah2	9	4968 ± 687					6.7													
	Ah2	9	4968 ± 687					8.3	7.2												
	CPE	10	1419 ± 145					9.8	15.5												
	Ah2	11	887 ± 156					21.3	21.3												
	CPE	1	951 ± 51					24.6	18.4												
	CPE	2	1042 ± 15					24.8	19.8												
CPE	3	1005 ± 4					46.8	41.9													
CPE	4	474 ± 11																	2.7		
CPE	5	877 ± 283														4.8					
Ah2	6	647 ± 243											3.9	28.6	3.5		3.0				
CPE	1	3795 ± 1863						8.6	6.7												
CPE	2	943 ± 154					19.9														
CPE	3	1630 ± 421					11.9														
CPE	4	871 ± 527	3.6	3.2		25.4	2.8	4.8				3.0				3.3			2.6		
CPE	5	1763 ± 630				4.9				3.1											
CPE	6	1567 ± 188			2.8	3.5								3.0	4.0	3.1	2.8		2.7		
Ah2	1	5153 ± 1153													4.3						
Ah2	1	727 ± 176															3.0	12.8			
Ah2	1	110 ± 34															2.9				
Ah2	2	1113 ± 1											18.2				2.9	3.3			
Ah2	3	1019 ± 83				8.7	9.2											7.1			
Ah2	4	333 ± 211																9.0	7.1	10.2	
CPE	1	285 ± 32																73.4			
CPE	2	191 ± 66								2.5								2.5			
CPE	1	582 ± 154																3.4	8.6		
CPE	2	6305 ± 1904																	4.1	2.5	
Ah2	3	607 ± 206							2.5												
Ah2	4	2215 ± 169			3.6																
Ah2	1	4927 ± 367																		2.5	
Ah2	2	1456 ± 21																8.6			

(Continued)

TABLE E3. (Continued)

				Ara h 2 20-mer peptides																
				Pool 1			Pool 2			Pool 3			Pool 4		Pool 5			Pool 6		
Subject	Driving antigen	TCL	cpm of unstimulated TCL (mean ± SD)	1-20	10-29	19-38	28-47	37-56	46-65	55-74	64-83	73-92	82-101	91-110	100-119	109-128	118-137	127-146	136-155	138-157
B	7	Pool 4	2	2257 ± 5										14.9						
		Pool 3	10	2483 ± 1667							10.7									
		Pool 2	11	2095 ± 384				4.9	7.0											
		Pool 2	12	1725 ± 235				4.8												
	8	Pool 2	13	1297 ± 560				3.3												
		Pool 4	14	2494 ± 286											6.0					
		Pool 4	15	3038 ± 1236										5.1	4.5					
		Pool 4	16	2868 ± 84										5.4	4.8					
		Pool 4	6	732 ± 78											16.6					
	9	Pool 4	7	1001 ± 30											2.6					
		Pool 2	8	9614 ± 4631				4.3	4.5											
	11	Pool 2	2	638 ± 61				33.3	46.6											
12	Pool 5	2	268 ± 26.4															9.6		
16	Pool 5	3	661 ± 62															16.8		

A_h2, Natural Ara h 2.Only positive simulation indices (≥ 2.5) are shown: gray, ≥ 2.5 , ≤ 5.0 ; black, > 5.0 .

A, Allergen-driven TCL; B, peptide-driven TCL.