Clinical allergy : basophils, T cells, and therapeutic design

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ARA H 1 PEPTIDES COMPRISING DOMINANT CD4+ T-CELL EPITOPES: CANDIDATES FOR A PEANUT ALLERGY THERAPEUTIC

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ABSTRACT

Background: Peanut allergy is a life-threatening condition; there is currently no cure. While whole allergen extracts are used for specific immunotherapy for many allergies, they can cause severe reactions and even fatalities in peanut allergy. **Objective:** To identify short, HLA-degenerate CD4+ T-cell epitope-based peptides of the major peanut allergen Ara h 1 that target allergen-specific T cells without causing IgE-mediated inflammatory cell activation, as candidates for safe peanut-specific immunotherapy. **Methods:** Ara h 1-specific CD4+ T-cell lines (TCL) were generated from peripheral blood mononuclear cells (PBMC) of peanut-allergic subjects using CFSE-based methodology. Dominant T-cell epitopes were identified using CFSE and thymidine-based proliferation assays. Epitope HLA-restriction was investigated using blocking antibodies, HLA-genotyping and epitope prediction algorithms. Functional peanut-specific IgE reactivity to peptides was assessed by basophil activation assay. **Results:** 145 Ara h 1-specific TCL were generated from 18 HLA-diverse peanut-allergic subjects. The TCL recognized 20-mer peptides throughout Ara h 1. Nine 20-mers were selected as containing dominant epitopes and their recognition confirmed in 18 additional peanut-allergic subjects. Ten core epitopes were mapped within these 20-mers. These were HLA-DQ and/or HLA–DR restricted, with each presented on at least two different HLA-molecules. Seven short (≤20 aa) non-basophil-reactive peptides encompassing all core epitopes were designed and validated in peanut-allergic donor PBMC T-cell assays. **Conclusions and Clinical Relevance:** Short CD4+ T-cell epitope-based Ara h 1 peptides were identified as novel candidates for a safe, T-cell targeted peanut-specific immunotherapy for HLA-diverse populations.

INTRODUCTION

Peanut allergy is the leading cause of food-induced anaphylactic fatalities world-wide[1, 2]. It is a major health care problem affecting 1- 2% of the population[2-4], with clinical symptoms ranging from mild oropharyngeal irritation to life-threatening anaphylaxis. Unlike egg and milk food allergy that is present in infants and typically resolve by school age, peanut allergy is life-long in 80% of cases. This significantly impairs the quality of life of afflicted individuals and their families[5-7], with further impact on the wider community through efforts to manage this severe condition[2, 8]. There is currently no cure for peanut allergy. Avoidance is the only means of control, with epinephrine as emergency treatment for anaphylaxis. Even with diligent precautions, most peanut-allergic subjects have accidental exposures which can have severe or even fatal consequences[1, 4, 5, 9].

Whole allergen extracts are currently used for specific immunotherapy for respiratory and insect venom allergies, but are unavailable in clinical practice for treatment of food allergy due to risks of severe side effects or even death in the case of peanut allergy. The limited studies on specific immunotherapy for peanut allergy provide encouragement that desensitization is feasible, but the observed adverse reactions highlight major safety concerns[10-18]. These risks are especially pertinent for peanut allergy since peanut allergens may induce anaphylaxis at minute doses with little correlation between previous severity of reactions and a person's first anaphylactic episode[11, 19]. Consequently, there is an urgent need to develop a safe, disease-modifying therapeutic for peanut-allergic individuals.

Anaphylaxis results from the release of inflammatory cell mediators triggered by binding and cross-linking of cell-bound allergen-specific IgE by the relevant allergen. During specific immunotherapy, stimulation of appropriate T-cell responses is considered essential for successful desensitization and the subsequent reduction and/or inhibition of allergen-specific IgE[20-22]. Although conventional immunotherapy administers whole allergen extracts, studies on cat allergy[23-27] and bee venom allergy[28, 29] clearly demonstrate that short T-cell epitope-based peptides of major allergens are sufficient for effective desensitization without causing adverse IgE-mediated reactions. Importantly, targeting T cells specific for immunodominant epitopes of major allergens can alter responses to whole allergen extracts (linked suppression). Many studies reporting successful peptide immunotherapy in murine models of allergy demonstrated that administration of immunodominant T-cell epitope peptides of major allergens induced tolerance not only to those peptides, but also to purified allergen and whole allergen extracts[30-35]. More recently, clinical administration of Fel d 1 T-cell epitope peptides in humans altered T-cell responses to those peptides, other non-related Fel d 1 peptides, and whole cat allergen extract[25].

We aimed to design peptides based on most reliably recognized CD4+ T-cell epitopes of major peanut allergens for a T-cell-targeted immunotherapy for peanut allergy as a safe (non-IgE reactive) and effective alternative to whole allergens. Of eleven peanut allergens identified (Ara h 1-11)[36], Ara h 1 and Ara h 2 are the two designated major allergens whose recognition is most consistently reported in >50% of cohorts tested[4, 37, 38]. Although a number of studies have

indicated Ara h 2 to be the more potent of these two allergens[39-41], Ara h 1 also plays a major role in the pathogenesis of peanut allergy, with numerous studies reporting strong correlations between symptom severity and IgE reactivity to both Ara h 1 and Ara h 2[42-47]. Ara h 1 is the most abundant major allergen in peanut, accounting for 12-16% of total peanut protein[48]. This is an important consideration for driving linked epitope suppression in allergen immunotherapy, since inducing T-cell suppressor activity against abundant major allergens will undoubtedly facilitate reduced responses to whole allergen extracts. We recently designed a panel of T-cell epitope-based Ara h 2 peptides for inclusion in a peptide therapeutic[49]. In a single report of sequences of T-cell-reactive peptides from Ara h 1 using predictive tetramer-based epitope mapping[50], core epitopes were not determined and only ten HLA-DR tetramers were used, preventing detection of epitopes presented on other HLA-types. Here we provide a comprehensive report of precise core T-cell epitopes of Ara h 1 based on analysis of full T-cell repertoires from a large cohort of HLA-diverse peanut-allergic subjects. We reveal novel HLA-DQ-restricted epitopes as well as epitopes within previously reported T-cell reactive Ara h 1 20-mers[50]. We also demonstrate presentation of the latter epitopes on additional HLA-molecules to those previously reported[50]. Using these sequences we designed a panel of HLA-degenerate, T-cell reactive Ara h 1 peptides to combine with those we identified previously from Ara h 2[49], providing a broadly acting therapeutic to take forward for pre-clinical and clinical testing to treat HLA-diverse peanut-allergic populations.

METHODS

Subjects

Peanut-allergic adult subjects were recruited from The Alfred Allergy Clinic, Melbourne, Australia (Table S1). All subjects had clinical symptoms of IgE-mediated peanut allergy and peanut-specific IgE CAP score ≥1 (≥0.49 kU_A/l; Pharmacia CAP System™, Pharmacia Diagnostics, Uppsala, Sweden). Subjects used for T-cell line (TCL) generation were genotyped (HLA-DRB1, -DQB1 and -DPB1, exon 2) by the Victorian Transplantation and Immunogenetics Service (Table S2). 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[49, 51]. Ara h 1 and Ara h 2 were enriched from CPE by liquid chromatography as described[49]. Endotoxin contents were 1.7, 4.0 and 78.0 EU/mg for CPE, Ara h 1 and Ara h 2 respectively (Endpoint Chromogenic LAL assay, Lonza, Walkersville, USA). Ara h 1 peptides (Mimotopes, Victoria, Australia and GenScript USA Inc, New Jersey, USA; Table S3) were reconstituted at 2 mg/mL in 10% dimethyl sulfoxide/PBS (20-mers and truncated peptide sets) or PBS alone (custom-synthesized core epitope peptides). All antigens were confirmed to be

neither mitogenic nor toxic as described[52].

Generation of Ara h 1-specific $CD4^+$ T-cell lines (TCL)

Ara h 1-specific oligoclonal TCL were generated from peripheral blood mononuclear cells (PBMC) of peanut-allergic subjects using 5,6-carboxyfluorescein diacetate succinimidylester (CFSE)-based methodology[53] as described[49], with CPE (100 µg/mL), Ara h 1 (10 µg/mL) or 20-mer peptides spanning the Ara h 1 sequence (11 amino acid (aa) overlap (17 aa overlap for the last peptide); Table S3; 10 µg/mL/peptide) as the driving antigens. All TCL were tested for specificity (proliferation) to individual Ara h 1 20-mers (10 µg/mL) as well as CPE (100 µg/mL) and/or Ara h 1 (10 µg/mL). Core epitope sequences were mapped within selected 20-mers using peptide sets truncated from the N- or C-terminus of the 20-mer as described[49].

T-cell assays

All culturing was performed in RPMI-1640 containing 2 mM L-glutamine, 100 IU/mL penicillin-streptomycin and 5% heat-inactivated human AB serum (cRPMI; Sigma-Aldrich, St Louis, USA). Antigen-induced TCL proliferation was assessed by ^{3}H-thymidine (^{3}H-TdR) uptake assays as described[49]. A stimulation index (SI; cpm antigen-stimulated T cells/cpm unstimulated T cells) ≥2.5 was considered positive and all positive responses confirmed in ≥2 assays. HLA-restriction of epitope recognition by TCL was assessed using monoclonal antibodies (mAb) against HLA-DR (L243), HLA-DQ (SVP-L3) or HLA-DP (B7/21) to block epitope presentation as described[49]. To allow detection of peptide-induced $CD4^+$ T-cell proliferation within whole PBMC, 7-day cultures of CFSE-labelled PBMC were set up as described for TCL generation[49]. At least 10,000 $CD4^+$ T cells were analyzed per sample and SI calculated as percentage of $CD4^+CFSE^{lo}$ (proliferated) cells with antigen/percentage of $CD4^+CFSE^{lo}$ cells without antigen (background). The detection threshold for a specific response in this assay was assessed by expanding peptide-specific TCL from proliferated $CD4^+$ cells over a range of SI values for three subjects. Specific TCL could be generated from divided T cells with SI as low as 1.1 in all three subjects (data not shown) allowing designation of an SI≥1.1 as positive.

Basophil activation test

Basophil activation was assessed by CD63 upregulation detected by flow cytometry as described [54]. Positive controls were rabbit anti-human IgE antibody (7.5 µg/mL; DAKO Corporation, CA, USA), N-formyl-methionine-leucine-phenylalanine (fMLP) (0.4 µg/mL; Sigma) and CPE. CPE, Ara h 1 and peptides were tested over a 3-log concentration range (5, 0.5 and 0.05 µg/mL).

RESULTS

Selection of Ara h 1 20-mer peptides containing dominant CD4+ T-cell epitopes most reliably recognized by peanut-allergic subjects

A total of 145 Ara h 1-specific T-cell lines (TCL) were generated from PBMC of 18 HLA-diverse peanut-allergic subjects (Table S1 and S2) by isolating and expanding antigen-specific (proliferated) $CD4^+CFSE^{lo}$ T cells from 7-day CFSE-labelled PBMC cultures stimulated with CPE, Ara h 1 or pools of Ara h 1 20-mer peptides collectively spanning the Ara h 1 sequence (Table S3). The 20-mer peptide(s) recognized (SI≥2.5) by each subject are shown in Table 1 and data summarized in Figure 1. For some subjects, CPE or Ara h 1 stimulation generated most TCL whilst for others it was the peptide pools. Where TCL were generated from a given subject using different antigen preparations (CPE, Ara h or peptide pools), TCL 20-mer specificities were comparable. Overall, there was no bias in the TCL 20-mer specificity generated depending on antigen preparation.

Table 1. Proliferative responses (thymidine uptake) of T-cell lines to Ara h 1 20-mer peptides.

No.	TCL	Ara h 1 20-mer peptide residues	Subject 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
			1	3	3	3	3	4	4	6	7	7	7	8	10	11	12	14	21	21
1	1-20																	4.3		3.6
2	10-29																	3.4		
3	19-38														4.1			3.8		
4	28-47																			6.3
5	37-56																			3.7
6	46-65														4.3		4.3			4.2
7	55-74												20			3.3				
8	64-83				2.8															
9	73-92												3.5							
10	82-101												2.8							
11	91-110												3.7							
12	100-119												6.5							
13	109-128												7.2							
14	118-137												8.2				2.7			
15	127-146									3.7				5.0				2.4		
16	136-155													14		29				
17	145-164																			
18	154-173								11											
19	163-182					5.3										6.6				
20	172-191					4.6										11				
21	181-200					4.8														
22	190-209																4.0			16
23	199-218				142					7.2				15						30
24	208-227		21	4.7								78		110						
25	217-236													3.5						
26	226-245								2.5	2.9			3.8		2.7			8.6		
27	235-254								4.6									3.3		
28	244-263								6.4											5.0
29	253-272																	5.2	26	
30	262-281															3.4			47	2.8
31	271-290									2.6						78			43	
32	280-299							30						3.2					33	
33	289-308													179					6.3	
34	298-317													141				3.5		9.0
35	307-326																		2.7	
36	316-335					3.9														
37	325-344					7.3										11		3.3		
38	334-353									3.4						3.5	3.1		3.2	
39	343-362															2.5		7.1		
40	352-371					141								5.0	8.1	58				
41	361-380		6.7						21						9.0	29				
42	370-389												14						46	19
43	379-398												6.2							4.2
44	388-407												8.1		19		6.1	3.0		
45	397-416												16				11	13		3.1
46	406-425												10				12	47		2.5
47	415-434												15				3.6	25		12
48	424-443												22		2.5			2.7		4.7
49	433-452												28				15	22		31
50	442-461						150		5.2	18				136				5.9	4.1	
51	451-470						156				43			147		2.7		5.7	12	
52	460-479																	14		25
53	469-488															84		49	23	
54	478-497																	27		
55	487-506																	3.8		47
56	496-515															34		24		26
57	505-524						3.3			6.0					11				16	39
58	514-533																2.5			
59	523-542											4.8							14	
60	532-551										6.2									
61	541-560									4.0									3.2	17
62	550-569									19									2.7	
63	559-578									2.8										7.6
64	568-587					8.2														
65	577-596					32								7.8						4.3
66	586-605																			
67	595-614																			
68	604-623																			
69	607-626																			25

TCL, T-cell line. Only positive stimulation indices (SI ≥2.5) are shown. For subjects with multiple TCL specific for a given 20-mer, the highest SI is shown. SIs above 10 have been rounded to the nearest whole number. Dark grey, SI ≥2.5<5.0; Black, SI ≥5.0.

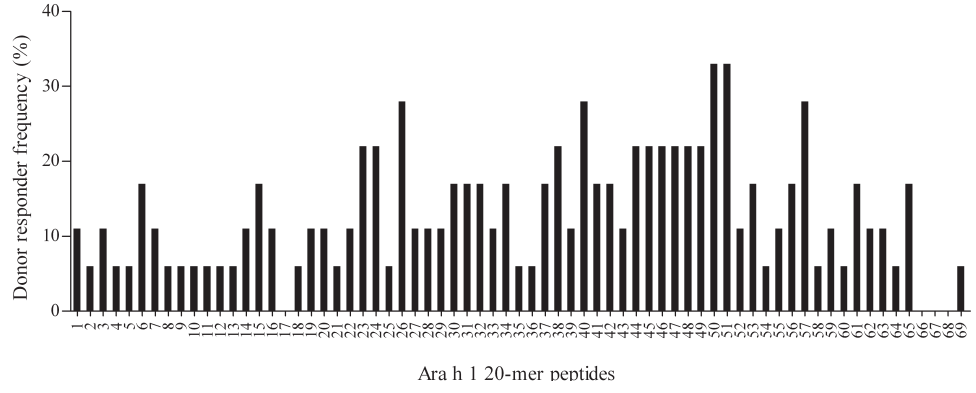

Figure 1: Donor responder frequency profile for Ara h 1 20-mer peptides.
Donor responder frequencies for TCL recognition of Ara h 1 20-mer peptides (n= 18 peanut-allergic subjects).

The 145 TCL collectively recognized epitopes throughout the entire Ara h 1 sequence, with only four of the sixty-nine 20-mers failing to stimulate any TCL. Fourteen 20-mers (23, 24, 26, 38, 40, 44-51 and 57) were each recognized by four (22%) or more subjects, with peptides 50 and 51 having the most responders (six subjects; 33%; Figure 1). Although dominant allergen epitopes are most simply defined as being those peptides/regions most frequently recognized within the respective allergen sequence [55-59], we considered a number of factors in addition to TCL responder frequencies to further define and refine our selection of epitopes for inclusion in a therapeutic. These included the magnitude of TCL response, number of specific TCL per subject, reproducibility of specific TCL response and ability to target specific T cells in PBMC. Based on these parameters, nine of the fourteen 20-mers (peptides 23, 24, 40, 46, 47, 49, 50, 51 and 57) were selected for subsequent analyses. These nine 20-mers were collectively recognized by 16 of the 18 subjects (89%) in this cohort, and typically induced strong and consistent proliferative responses in specific TCL, with the majority of SI over five and many considerably higher (Table 1). Furthermore, each of these 20-mers was recognized by multiple TCL from many responders reflecting a prevalence of T cells specific for these peptides among the subjects' T-cell repertoires. To assess recognition in a wider cohort, PBMC from an additional 20 peanut-allergic subjects were screened by CFSE assay for $CD4^+$ T-cell proliferation in whole PBMC following seven days stimulation with each peptide (Table 2, upper panel and Figure S1). This assay provided a sensitive and accurate screen for detecting the full repertoire of peptide-specific $CD4^+$ T cell proliferative responses within whole PBMC. All 20 subjects showed PBMC T-cell proliferation to CPE or a combination of enriched Ara h 1 and Ara h 2. The 20-mers were collectively recognized by 18 (90%) of these subjects, with 40-79% responding to each 20-mer. Analysis of four subjects from the original cohort used for TCL generation confirmed they also had T cells specific for other 20-mers in addition to those recognized by their TCL (Table 2, lower panel).

Table 2. CFSE-based detection of peanut-allergic donor CD4+ T-cell proliferation in response to selected Ara h 1 20-mers.

		Stimulation Indices (SI)										+ve 20-mers			
			Ara h 1 20-mers									SI>1.1		SI>1.5	
Subject	**No Antigen***	**CPE**	**23**	**24**	**40**	**46**	**47**	**49**	**50**	**51**	**57**	**No.**	**%**	**No.**	**%**
19	0.22	91.6	3.5	0.7	3.3	nt	nt	nt	0.7	3.2	36.0	4/6	67	4/6	67
20	0.08	1.4	1.1	1.4	0.0	nt	nt	nt	1.0	69.3	0.0	3/6	50	1/6	17
21	0.45	7.0	0.4	2.8	0.5	nt	nt	nt	0.3	1.0	0.4	1/6	17	1/6	17
22	0.27	54.6	0.4	0.9	0.2	nt	nt	nt	1.7	0.5	0.2	1/6	17	1/6	17
23	3.02	5.9	0.6	0.8	1.0	nt	nt	nt	1.2	0.4	2.0	2/6	33	1/6	17
24	0.26	6.8	0.5	0.5	0.6	nt	nt	nt	2.8	2.0	0.7	2/6	33	2/6	33
25	0.10	152.0	2.2	1.2	0.6	23.4	1.9	3.1	0.9	0.4	0.7	5/9	56	4/9	44
26	0.07	122.8	2.3	5.8	0.9	0.6	1.3	12.7	4.2	4.4	2.7	7/9	78	6/9	67
27	0.17	1.4	0.6	0.8	0.9	1.0	0.7	1.3	0.6	1.1	1.5	3/9	33	1/9	11
28	0.06	37.5	5.6	8.9	6.0	18.0	1.7	2.5	3.0	12.6	29.5	9/9	100	9/9	100
29	1.87	2.9	1.7	1.7	1.3	0.7	1.1	1.6	1.6	1.7	1.8	8/9	89	6/9	67
30	0.11	2.1	1.5	2.6	1.7	0.9	0.9	1.2	0.4	2.1	0.5	5/9	56	4/9	44
31	0.08	10.3	1.9	2.1	1.2	2.5	9.0	1.9	1.5	1.3	2.0	9/9	100	7/9	78
32	0.06	5.8	0.8	2.4	1.3	1.2	1.6	2.3	10.2	0.9	1.3	7/9	78	4/9	44
33	1.30	2.3	1.8	0.4	0.8	1.5	1.2	0.9	0.8	0.1	0.2	3/9	33	2/9	22
34	1.04	1.6^	2.1	1.0	2.8	2.4	2.3	1.5	0.3	2.0	4.1	6/9	66	6/9	66
35	0.08	8.2^	1.0	0.8	1.3	0.5	3.3	5.3	0.3	0.8	2.0	4/9	44	3/9	33
36	0.35	6.1^	1.6	1.4	1.6	1.7	4.8	2.6	0.8	1.2	1.5	8/9	89	7/9	78
37	1.05	7.8	0.3	0.3	0.3	0.6	0.2	0.9	0.7	0.9	0.3	0/9	0	0/9	0
38	0.78	1.3	0.8	0.9	0.8	0.7	0.9	0.9	0.7	0.7	0.8	0/9	0	0/9	0
Responders w SI>1.1	**#**	20/20	11/20	10/20	8/20	7/14	10/14	11/14	8/20	11/20	11/20				
	%	100	55	50	40	50	71	79	40	55	55				
Responders w SI>1.5	**#**	17/20	10/20	7/20	4/20	6/14	7/14	9/14	7/20	8/20	10/20				
	%	85	50	35	20	43	50	64	35	40	50				
1	0.17	7.1^	2.4	2.7	1.8	1.1	1.7	2.5	0.4	0.5	1.2	7/9	78	5/9	56
2	0.19	83.5	1.8	10.3	1.6	1.8	1.0	1.6	3.4	1.9	2.9	8/9	89	8/9	89
4	0.62	12.3	5.2	1.8	4.2	nt	nt	nt	4.7	6.5	9.7	6/6	100	6/6	100
10	0.23	44.4	14.4	5.3	5.3	nt	nt	nt	14.2	8.1	4.4	6/6	100	6/6	100
Responders w SI>1.1	**#**	24/24	15/24	14/24	12/24	9/16	11/16	13/16	11/24	14/24	15/24				
	%	100	63	58	50	56	69	81	46	58	63				
Responders w SI>1.5	**#**	21/24	14/24	11/24	8/24	7/16	8/16	11/16	10/24	11/24	13/24				
	%	88	58	46	33	44	50	69	42	46	54				

Upper panel shows new peanut-allergic donor cohort; lower panel shows four subjects from peanut-allergic donor cohort used for TCL generation with combined totals from upper and lower panels. CPE, crude peanut extract; +ve, positive; nt, not tested (peptide stocks not available at time of testing); Grey, stimulation indices ≥1.1<2.5; Black, stimulation indices ≥2.5;

* Background proliferation with no antigen, % $CD4^+CFSE^{lo}$ T cells of total $CD4^+$ T cells ^A combination of enriched Ara h 1 and Ara h 2 (10 µg/mL of each) was used instead of CPE for these subjects.

Combined totals for all 24 subjects tested with the CFSE assay showed 46-81% responded to each 20-mer. If a higher SI of 1.5 was used as a positive cut off, the frequency of responders per 20-mer was only slightly reduced to 33-69%. Overall, T-cell recognition of one or more of the selected panel of nine 20-mers was confirmed in 35 (92%) of 38 subjects analyzed using either cut-off.

Mapping core T-cell epitopes within selected Ara h 1 20-mer peptides

Minimal length peptides decrease risk of cross-linking cell-bound IgE on inflammatory cells during clinical administration and facilitate therapeutic production. The minimum T-cell stimulatory sequence (core epitope) within each selected 20-mer was determined by testing proliferation of reactive TCL from different subjects to truncated peptide sets (e.g. Figure 2 and Table 3). The number of residues required to induce maximal T-cell proliferation varied from 6-19 aa between different TCL and/or subjects (Table 3), consistent with previous reports for CD4+ T-cell epitopes[60, 61]. Due to variation in the number of flanking-residues required for optimal epitope recognition[61], TCL were considered to recognize the same epitope if peptides containing a common core sequence induced recognition. Based on this criterion, ten distinct CD4+ T-cell epitopes were identified ('consolidated epitopes', Table 3), with common cores varying from 5-12 aa (underlined sequences, Table 3). 'Consolidated epitope' sequences were selected to encompass residues required for maximal stimulation of all specific TCL tested to ensure broadest possible recognition.

At least one epitope was found within each of the nine 20-mers, with 20-mers 50 and 51 each containing two distinct but overlapping T-cell epitopes: one unique to each 20-mer ((442-458) and (452-470)), and the other within the overlap sequence ((451-461), Table 3 and Figure 2). No single TCL responded to both epitopes within either 20-mer, further confirming the distinction of these epitopes (data not shown). HLA-epitope prediction algorithms[62, 63] also highlighted one or more strong HLA class II (HLA-II) binding motifs within each of our minimal-stimulatory sequences. Data are shown for the Propred[62] HLA-DR binding algorithm in Table S4. This algorithm did not predict HLA-DR epitopes within peptide 40, but algorithms of the Immune Epitope Database (IEDB) and Analysis Resource [63] predicted epitopes within this peptide to bind most strongly to HLA-DP and/or -DQ molecules.

Finally, to avoid unnecessary sequence duplication and to minimize peptide numbers for a therapeutic, six of the consolidated epitopes (comprising three overlapping epitope pairs) were combined into three single peptides of 20 aa or less ((206-225), (409-427) and (451-470); grey shading, Table 3). The combined epitope peptides efficiently stimulated TCL specific for either epitope (data not shown) and together with the remaining four consolidated epitopes ((353-371), (436-452), (442-458) and (507-524)), provided a panel of seven candidate peptides for further characterization (see asterisks, Table 3). CFSE-based screening of nine subjects from our cohorts confirmed that these peptides could each directly target detectable numbers of Ara h 1-specific T cells among whole PBMC of peanut-allergic subjects (Table 4). In a few cases the T cell response of a given subject to the original 20-mer and

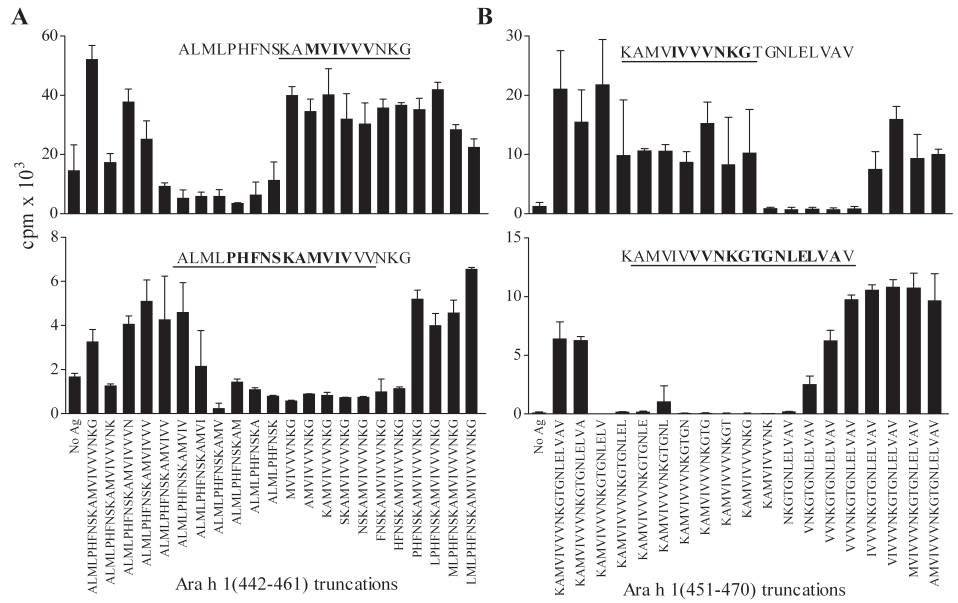

Figure 2: Mapping core T-cell epitopes within Ara h 1 20-mer peptides 50 and 51.
20-mer-specific TCL proliferation to truncated peptide sets. Representative TCL shown for peptides 50 (A) and 51 (B) (mean cpm replicate wells +SD). Upper panels indicate the epitope in overlap between the 20-mers (n = 2; 3 TCL). Lower panels indicate epitopes unique to each 20-mer; A) n = 3; 6 TCL. B) n = 4; 7 TCL. Epitope sequences recognized by represented TCL are bolded and 'consolidated epitopes' recognized by all specific TCL are underlined.

the corresponding candidate peptide differed. Where responses to candidates were reduced as compared to the 20-mer, flanking residue(s) required for optimal T cell recognition or HLA-binding may have been removed. It is well recognized that flanking residues can stabilize peptide binding to HLA class II molecules. In contrast, improved responses could reflect generation of additional new epitopes, improved epitope purity (cores were synthesized at high purity (>95%) whilst 20-mers were produced as peptide sets with a minimum estimated purity of 70%) or alteration of epitopes (or flanking residues) to enable better interaction with HLA and/or T cell receptor (TCR) molecules. Indeed, this was considered to be the case where responses to candidate peptide (442-458) were much stronger than to 20-mer 50. Since this region contains multiple adjacent hydrophobic residues, even single residue changes could significantly alter the charge and structure of this peptide, thus affecting its biochemical properties and interactions with HLA and/or TCR molecules. Nonetheless, most responses to candidates were comparable or improved compared to responses to the original 20-mers.

Table 3. Core T-cell epitope sequences mapped within selected Ara h 1 20-mers.

20-mer peptide		Minimum sequence required for T-cell recognition		Consolidated epitope (common core underlined)		Confirmed Responders	
#	Residues	Residues	Sequence	Residues/ aa	Sequence	TCL	Sub-jects
23	(199-218)	(206-213)	FQNLQNHR	(206-215) 10 aa	FQNLQNHRIV	6	3
		(206-215)	FQNLQNHRIV				
24	(208-227)	(213-222)	RIVQIEAKPN	(213-225) 13 aa	RIVQIEAKPNTLV	6	3
		(213-225)	RIVQIEAKPNTLV				
		(214-219)	IVQIEA				
		Overlapping epitopes combined		(206-225) 20 aa	FQNLQNHRIVQIEAKPNTLV*	12	6
40	(352-371)	(353-371)	WSTRSSENNEGVIVKVSKE	(353-371) 19 aa	WSTRSSENNEGVIVKVSKE*	3	3
		(359-371)	ENNEGVIVKVSKE				
		(361-370)	NEGVIVKVSK				
46	(406-425)	(409-418)	NNFGKLFEVK	(409-425) 17 aa	NNFGKLFEVKPDKKNPQ	3	2
		(409-425)	NNFGKLFEVKPDKKNPQ				
		(411-418)	FGKLFEVK				
47	(415-434)	(416-427)	EVKPDKKNPQLQ	(416-427) 12 aa	EVKPDKKNPQLQ	2	1
		Overlapping epitopes combined		(409-427) 19 aa	NNFGKLFEVKPDKKNPQLQ*	3	2
49	(433-452)	(436-445)	VEIKEGALML	(436-452) 17 aa	VEIKEGALMLPHFNSKA*	5	2
		(436-449)	VEIKEGALMLPHFN				
		(440-452)	EGALMLPHFNSKA				
50	(442-461)	(442-458)	ALMLPHFNSKAMVIVVV	(442-458) 17 aa	ALMLPHFNSKAMVIVVV*	6	3
		(443-457)	LMLPHFNSKAMVIVV				
		(446-456)	PHFNSKAMVIV				
51	(451-470)	(451-459)	KAMVIVVVN	(451-461) 11 aa	KAMVIVVVNKG	3	2
		(452-461)	AMVIVVVNKG				
		(455-461)	IVVVNKG				
		(452-467)	AMVIVVVNKGTGNLEL	(452-470) 19 aa	AMVIVVVNKGTGNLELVAV	7	4
		(452-468)	AMVIVVVNKGTGNLELV				
		(457-469)	VVNKGTGNLELVA				
		(457-470)	VVNKGTGNLELVAV				
		Overlapping epitopes combined		(451-470) 20 aa	KAMVIVVVNKGTGNLELVAV*	10	6
57	(505-524)	(507-524)	GDVFIMPAAHPVAINASS	(507-524) 18 aa	GDVFIMPAAHPVAINASS*	12	4
		(509-524)	VFIMPAAHPVAINASS				
		(510-521)	FIMPAAHPVAIN				
		(511-517)	IMPAAHP				
		(511-521)	IMPAAHPVAIN				

Grey shading indicates overlapping consolidated epitope pairs combined into single peptides for further analyses as outlined in the text.
*The seven candidate peptides proposed for a therapeutic

Determining HLA class II restriction specificity of Ara h 1 T-cell epitopes

There is no identified HLA-II association with peanut allergy[64], therefore peptides selected for therapy must bind diverse HLA-II molecules for wide applicability. To determine the HLA-II type presenting each epitope, anti-HLA-DR, -DP or -DQ mAbs were used to block individual epitope presentation to T cells. For each TCL tested, epitope recognition was prevented by one or more HLA-mAb in a dose-dependent manner (e.g. Figure S2, Supporting information) and the same mAb blocked recognition of CPE (data not shown), demonstrating consistency for presentation of naturally processed and synthetic epitope forms. At least two subjects and/or TCL were tested per epitope (Table 5). Consistent with predictions of the HLA-II algorithms described above[62, 63], anti-HLA-DR blocked recognition of all but one epitope, (353-371), which was blocked by anti-HLA-DQ in both subjects tested. For epitopes (436-452) and (507-524), recognition was blocked by anti-HLA-DR for some TCL but by anti-HLA-DQ for others, confirming HLA-binding degeneracy for these epitopes.

Table 4. CFSE-based detection of peanut-allergic donor CD4+ T-cell proliferation in response to selected Ara h 1 candidate peptides.

		Stimulation Indices (SI)								+ve peptides			
		Candidate peptides								SI > 1.1		SI > 1.5	
Subject	No Ag*	CPE	206-225	353-371	409-427	436-452	442-458	451-470	507-524	No.	%	No.	%
1	0.17	7.1^	2.3	2.0	1.3	0.9	1.6	0.5	2.7	5/7	71	4/7	57
30	0.11	2.1	1.4	0.3	2.0	6.8	0.3	2.1	0.6	4/7	57	3/7	43
31	0.08	10.3	1.7	0.8	2.0	3.2	2.9	1.3	1.4	6/7	86	4/7	57
32	0.06	5.8	2.3	4.2	1.5	7.0	3.6	0.9	0.8	5/7	71	5/7	71
33	1.30	2.3	0.7	0.7	1.3	0.5	0.4	0.1	0.5	1/7	14	0/7	0
34	1.04	1.6^	2.2	2.0	2.0	1.2	4.0	2.0	2.8	7/7	100	6/7	86
35	0.08	8.2^	1.0	13.5	3.1	2.8	1.7	0.8	1.4	5/7	71	4/7	43
36	0.35	6.1^	1.9	1.4	2.5	4.4	2.1	1.2	2.4	7/7	100	5/7	71
Responders w SI>1.1	No.	8/8	6/8	5/8	8/8	6/8	6/8	4/8	5/8				
	%	100	75	63	100	75	75	50	63				
Responders w SI>1.5	No.	8/8	5/8	4/8	6/8	5/8	6/8	2/8	3/8				
	%	100	63	50	75	63	75	25	38				

CPE, crude peanut extract; +ve, positive; Grey, stimulation indices ≥1.1<2.5; Black, stimulation indices ≥2.5.
* Background proliferation with no antigen, % $CD4^+CFSE^{lo}$ T cells of total $CD4^+$ T cells
^A combination of enriched Ara h 1 and Ara h 2 (10 µg/mL of each) was used instead of CPE for these subjects.

Table 5. HLA class II restriction of core epitope peptides.

20-mer	Epitope	Subject	HLA-restriction	Corresponding HLA-allele(s)	
23	(206-215)	18 3	HLA-DR HLA-DR	DRB1 04:05 DRB1 03:01	DRB1 15:01 DRB1 08:01
24	(213-225)	12 10	HLA-DR HLA-DR	DRB1 08:01 DRB1 11:01	DRB1 10:01 DRB1 15:01
40	(353-371)	4 13 14	HLA-DQ HLA-DQ nt	DQB1 03:01 DQB1 03:01 DQB1 06:09	DQB1 06:02 DQB1 06:02
46	(409-425)	16 15	HLA-DR nt	DRB1 04:04 DRB1 03:01P	DRB1 13:01 DRB1 04:01
47	(416-427)	16 15	HLA-DR nt	DRB1 04:04 DRB1 03:01P	DRB1 13:01 DRB1 04:01
49	(436-452)	18	HLA-DQ HLA-DR	DQB1 03:02 DRB1 04:05	DQB1 06:02 DRB1 15:01
50	(442-458)	17 9	HLA-DR HLA-DR	DRB1 11:04 DRB1 09:01	DRB1 15:01 DRB1 13:01
50+51	(451-461)	12 6	HLA-DR HLA-DR	DRB1 08:01 DRB1 04:01	DRB1 10:01 DRB1 04:04
51	(452-470)	10 14	HLA-DR nt	DRB1 11:01 DRB1 13:02	DRB1 15:01
57	(507-524)	17 13	HLA-DR HLA-DQ	DRB1 11:04 DQB1 03:01	DRB1 15:01 DQB1 06:02

nt = not tested (TCL not available); Grey shading indicates overlapping epitope pairs combined into single peptides for further analyses as outlined in the text.

To assess HLA-binding degeneracy of epitopes whose recognition was blocked by a single HLA-mAb, the respective HLA-alleles of at least two subjects with TCL specific for that epitope were compared (Table S2 and Table 5). The absence of shared *HLA-DRB1* or *HLA-DQB1* alleles between subjects recognizing HLA-DR- or HLA-DQ-restricted epitopes respectively confirmed that each epitope was presented on at least two different HLA-molecules. The HLA-binding algorithms further supported these data, with each epitope containing motifs predicted to bind multiple HLA-molecules[62, 63] (e.g. Table S4).

Testing candidate peptides for basophil activation

To provide a safe alternative to whole allergens, peptides must not bind and cross-link cell-bound IgE. Basophil reactivity to peptides was assessed in fresh blood from seven of the peanut-allergic subjects recruited for this study (Figure 3). All seven subjects showed high levels of basophil activation to CPE over a concentration range. Whilst responses to Ara h 1 varied between subjects at the lowest dose, the highest concentration induced high activation in all subjects. However, none of the candidate peptides induced activation at any concentration tested. One subject showed a very low response (8%) to peptide (409-427), but this was below

the threshold of positive activation[65] and was negligible compared to the activation induced by Ara h 1 (80-90%) or CPE (74-76%) in this subject.

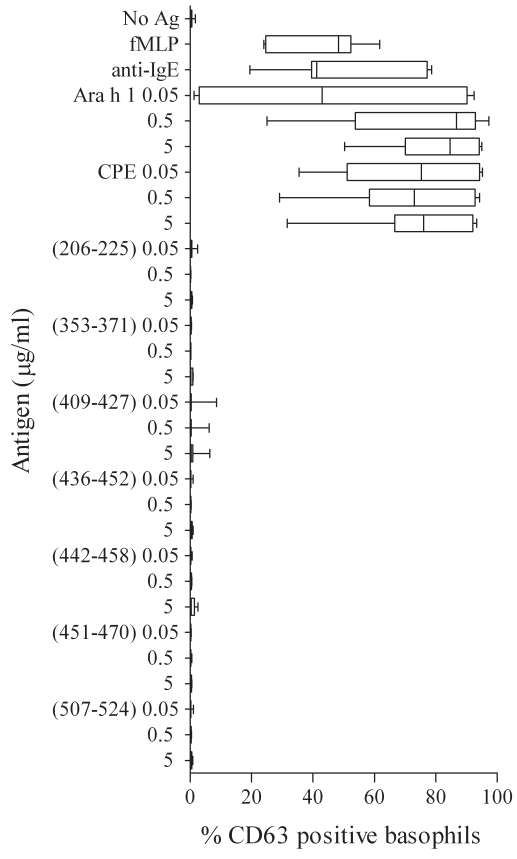

Figure 3: Basophil activation in response to candidate Ara h 1 peptides.
Box-and-whiskers plot showing percentage of activated ($CD63^{hi}$) basophils (IgE^{hi}) in response to Ara h 1 or candidate peptides for seven peanut-allergic subjects. Negative control was no antigen (unstimulated) and positive controls were anti-IgE, fMLP and CPE. Whiskers show minimum to maximum values.

DISCUSSION

An appropriately selected T-cell-targeted peptide immunotherapeutic will provide a safe treatment option for peanut-allergic individuals. Candidate peptides must comprise HLA-degenerate CD4+ T-cell epitopes of the major peanut allergens recognized by HLA-diverse peanut-allergic individuals, without cross-linking cell-bound IgE and activating inflammatory cells. In order to maximize the population coverage and efficacy of a therapeutic, we designed a peptide set containing T-cell epitopes from the two major allergens, Ara h 1 and Ara h 2. Following on from our previous study of Ara h 2[49], we now provide the first report of core sequences of CD4+ T-cell epitopes of the most abundant major peanut allergen, Ara h 1. We identified and characterized ten, HLA-diverse CD4+ T-cell epitopes of Ara h 1, and used these sequences to design candidate Ara h 1 peptides to combine with our candidate Ara h 2 peptides for therapeutic development.

To commence this study we selected nine 20-mers of Ara h 1 containing most frequently recognized epitopes based on responses of 145 TCL from 18 HLA-diverse peanut-allergic subjects. The cohort HLA profile was typical of Caucasian populations[66] in countries where peanut allergy is prevalent[67]. We further validated our 20-mer selection by demonstrating their collective recognition by PBMC T cells directly *ex vivo* from an additional 18 peanut-allergic subjects (Table 2), resulting in a total responder frequency of 92% for the 38 subjects analyzed. Although we could not confirm T-cell recognition of these 20-mers in four subjects, it is possible that specific T cells went undetected for two of these subjects (5 and 7), as data were only obtained from three and four TCL respectively (Table 1).

The minimum T-cell stimulatory sequences identified within our selected 20-mers varied from 6 to 19 aa (Table 3), consistent with reports of different peptides processed for HLA-II presentation and/or required for HLA- and/or TCR-binding both within and between subjects[60, 61]. As we used oligoclonal TCL, it is possible that the longer sequences contained more than one epitope. Indeed, algorithms[62, 63] predicted up to three HLA-binding motifs within some of our consolidated epitopes (Table S4). Seven of our epitopes showed overlap with T-cell-reactive Ara h 1 20-mers recently identified using HLA-DR tetramers[50], providing further support for recognition of these peptides in larger peanut-allergic populations. In addition, we confirmed presentation of five of these peptides on additional HLA-molecules to those used for the tetramer mapping.[50] However, epitopes (353-371), (436-452) and (442-458) were unique to our study. Consistent with this observation, we showed these epitopes were either presented on HLA-DQ molecules (commonly observed for allergen T-cell epitopes[68-72]), or HLA-DR types for which no tetramer-specific T cells were detected.[50] Inclusion of HLA-DQ-restricted epitopes is particularly advantageous for therapeutics as these alleles are less variable and thus more prevalent in mixed populations than HLA-DR alleles[73]. However, we confirmed HLA-binding degeneracy for all epitopes identified (both HLA-DR and -DQ-restricted) (Table 5), with further degeneracy predicted by algorithms[62, 63] (e.g. Table S4), emphasizing their collective suitability for targeting HLA-diverse peanut-allergic populations.

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, 12-14, 19, 37, 74]. Mapping the core sequences of T-cell epitopes enables refined peptide design for a therapeutic, but selecting optimal peptide combinations is a balance between peptide length and number. Longer peptides will increase population coverage by encompassing more T-cell epitopes, and being fewer in number, will reduce the complexity of therapeutic standardization compared to using a greater number of shorter peptides. However, the main concern with longer peptides is the increased potential for IgE binding and cross-linking, resulting in adverse reactions. We opted to combine overlapping epitopes into peptides up to 20 aa in length. Of over 23 linear IgE epitopes reported for Ara h 1[75-77], only two minor epitopes (409-418 and 461-470) fell within our candidate peptides[75]. Most importantly, none of these peptides caused activation of peanut-reactive basophils in all of the seven peanut-allergic subjects tested (Figure 3) emphasizing the potential for these peptides to provide a safe alternative to whole allergen extract for immunotherapy.

In summary we report the novel identification of ten dominant CD4+ T-cell epitopes of Ara h 1 that collectively show diverse HLA class II-restriction. We incorporated these epitopes into a panel of seven short (≤20 aa), HLA-degenerate peptides that can target T cells within PBMC of HLA-diverse allergic individuals without causing activation of peanut-allergic donor basophils. The combination of these Ara h 1 peptides with our three T-cell epitope-based Ara h 2 peptides [49] provides strong candidates for a broad acting and safe peptide-therapeutic to treat peanut allergy.

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SUPPLEMENTARY TABLES

Table S1. Subject demographics

Subject	Sex	Age	Atopic*	Asthma	Peanut CAP kU_A/l (score)	Anaph-ylaxis	Use of patient samples			
							TCL	20-mer CFSE	Core CFSE	BAT
1	M	39	Yes	No	2.18 (2)	Yes	X	X	X	
2	M	34	Yes	Yes	0.78 (2)	Yes	X	X		
3	F	53	Yes	as a child	83.90 (5)	Yes	X			
4	F	19	Yes	No	98.90 (5)	Yes	X	X		
5	F	22	Yes	No	4.72 (3)	Yes	X			
6	M	30	Yes	No	17.00 (4)	Yes	X			
7	M	42	No	No	15.40 (3)	Yes	X			
8	M	36	Yes	Yes	56.60 (5)	Yes	X			
9	M	30	Yes	Yes	30.60 (4)	Yes	X			
10	M	37	Yes	Yes	42.70 (4)	Yes	X	X		X
11	F	26	Yes	Yes	2.82 (2)	Yes	X			
12	F	23	Yes	Yes	>100 (6)	Yes	X			
13	M	30	Yes	No	>100 (6)	Yes	X			
14	M	30	Yes	Yes	36.60 (4)	Yes	X			X
15	F	31	Yes	No	84.30 (5)	No	X			
16	F	20	Yes	Yes	1.16 (2)	Yes	X			
17	F	25	Yes	No	2.12 (2)	Yes	X			X
18	M	35	Yes	Yes	1.23 (2)	No	X			
19	M	27	Yes	Yes	6.19 (3)	Yes		X		
20	F	25	Yes	Yes	87.2 (5)	Yes		X		
21	F	53	Yes	No	1.43 (2)	No		X		
22	F	28	Yes	Yes	9.53 (3)	na		X		
23	F	37	Yes	No	6.94 (3)	Yes		X		
24	M	38	Yes	Yes	2.42 (2)	Yes		X		
25	M	28	Yes	Yes	>100 (6)	Yes		X		
26	F	70	No	No	2.18 (2)	Yes		X		X
27	F	26	Yes	No	1.37 (2)	No		X		
28	F	35	Yes	No	SPT 14mm	Yes		X		
29	F	23	na	No	2.37 (2)	na		X		
30	F	28	Yes	Yes	9.2 (3)	No		X	X	
31	F	30	Yes	Yes	10.20 (3)	Yes		X	X	
32	M	53	Yes	No	2.01 (2)	Yes		X	X	
33	M	26	Yes	Yes	12.00(3)	Yes		X	X	
34	M	43	Yes	Yes	1.63 (2)	No		X	X	X
35	F	33	Yes	na	0.49 (1)	No		X	X	
36	M	28	Yes	na	0.72 (2)	no		X	X	
37	F	21	Yes	Yes	1.51 (2)	Yes		X		
38	M	28	Yes	Yes	1.43(2)	Yes		X		
39	M	29	Yes	No	31.80 (4)	Yes				X
40	F	52	Yes	Yes	7.23 (3)	Yes				X

* Atopic is defined by specific IgE to one or more of a panel of common aeroallergens either by RAST or skin prick test. TCL, T cell line; 20-mer CFSE, screen for T cell reactivity to selected Ara h 1 20-mers; Core CFSE, screen for T cell reactivity to candidate Ara h 1 peptides; BAT, basophil activation test; na, data not available; SPT, skin-prick test (RAST not available for this subject).

Table S2. HLA genotyping for subjects used for T-cell line generation

Subject	HLA-genotypes					
	DRB1		DQB1		DPB1	
1	07:01	15:01	02:01	06:02	04:01	
2	01:01	03:01	05:01	06:02	04:01	04:02
3	03:01	08:01	02:01P	04:02	03:01P	04:01
4	11:01	15:01	03:01P	06:02	04:01	
5	11:01	15:01	03:01P	06:02	03:01P	04:01
6	04:01	04:04	03:02	04:02	13:01P	04:01
7	07:01	08:01	03:03	04:02	04:01	06:01
8	01:03	04:01	03:02	05:01	03:01P	02:01
9	09:01	13:01	03:03	06:03	03:01P	04:02P
10	11:01	15:01	03:01P	06:02	04:01	
11	03:01	13:02	02:01P	06:09	01:01	04:01
12	08:01	10:01	04:02	05:01	03:01P	04:01
13	12:01P	15:01	03:01	06:02	13:01P	04:01
14	13:02		06:09		05:01	04:02P
15	03:01P	04:01	04:01P		02:01P	03:01P
16	04:04	13:01	03:02	06:03	02:01	04:01
17	11:04	15:01	03:01P	06:02	02:01	14:01
18	04:05	15:01	03:02	06:02	03:01P	04:01

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 'P' represent groups of alleles that share common sequences in exon 2 (**http://hla.alleles.org/alleles/p_groups.html**).

Table S3. Ara h 1 20-mer peptides

Pool	No.	Residues	Sequence
1	1	1-20	MRGRVSPLMLLLGILVLASV
	2	10-29	LLLGILVLASVSATHAKSSP
	3	19-38	SVSATHAKSSPYQKKTENPC
	4	28-47	SPYQKKTENPCAQRCLQSCQ
	5	37-56	PCAQRCLQSCQQEPDDLKQK
	6	46-65	CQQEPDDLKQKACESRCTKL
	7	55-74	QKACESRCTKLEYDPRCVYD
2	8	64-83	KLEYDPRCVYDPRGHTGTTN
	9	73-92	YDPRGHTGTTNQRSPPGERT
	10	82-101	TNQRSPPGERTRGRQPGDYD
	11	91-110	RTRGRQPGDYDDDRRQPRRE
	12	100-119	YDDDRRQPRREEGGRWGPAG
	13	109-128	REEGGRWGPAGPREREREED
	14	118-137	AGPREREREEDWRQPREDWR
3	15	127-146	EDWRQPREDWRRPSHQQPRK
	16	136-155	WRRPSHQQPRKIRPEGREGE
	17	145-164	RKIRPEGREGEQEWGTPGSH
	18	154-173	GEQEWGTPGSHVREETSRNN
	19	163-182	SHVREETSRNNPFYFPSRRF
	20	172-191	NNPFYFPSRRFSTRYGNQNG
	21	181-200	RFSTRYGNQNGRIRVLQRFD
4	22	190-209	NGRIRVLQRFDQRSRQFQNL
	23	199-218	FDQRSRQFQNLQNHRIVQIE
	24	208-227	NLQNHRIVQIEAKPNTLVLP
	25	217-236	IEAKPNTLVLPKHADADNIL
	26	226-245	LPKHADADNILVIQQGQATV
	27	235-254	ILVIQQGQATVTVANGNNRK
	28	244-263	TVTVANGNNRKSFNLDEGHA
5	29	253-272	RKSFNLDEGHALRIPSGFIS
	30	262-281	HALRIPSGFISYILNRHDNQ
	31	271-290	ISYILNRHDNQNLRVAKISM
	32	280-299	NQNLRVAKISMPVNTPGQFE
	33	289-308	SMPVNTPGQFEDFFPASSRD
	34	298-317	FEDFFPASSRDQSSYLQGFS
	35	307-326	RDQSSYLQGFSRNTLEAAFN
6	36	316-335	FSRNTLEAAFNAEFNEIRRV
	37	325-344	FNAEFNEIRRVLLEENAGGE
	38	334-353	RVLLEENAGGEQEERGQRRW
	39	343-362	GEQEERGQRRWSTRSSENNE
	40	352-371	RWSTRSSENNEGVIVKVSKE
	41	361-380	NEGVIVKVSKEHVEELTKHA
	42	370-389	KEHVEELTKHAKSVSKKGSE
7	43	379-398	HAKSVSKKGSEEEGDITNPI
	44	388-407	SEEEGDITNPINLREGEPDL
	45	397-416	PINLREGEPDLSNNFGKLFE
	46	406-425	DLSNNFGKLFEVKPDKKNPQ
	47	415-434	FEVKPDKKNPQLQDLDMMLT
	48	424-443	PQLQDLDMMLTCVEIKEGAL
	49	433-452	LTCVEIKEGALMLPHFNSKA
8	50	442-461	ALMLPHFNSKAMVIVVVNKG
	51	451-470	KAMVIVVVNKGTGNLELVAV
	52	460-479	KGTGNLELVAVRKEQQQRGR
	53	469-488	AVRKEQQQRGRREEEEDEDE
	54	478-497	GRREEEEDEDEEEEGSNREV
	55	487-506	DEEEEGSNREVRRYTARLKE
	56	496-515	EVRRYTARLKEGDVFIMPAA
9	57	505-524	KEGDVFIMPAAHPVAINASS
	58	514-533	AAHPVAINASSELHLLGFGI
	59	523-542	SSELHLLGFGINAENNHRIF
	60	532-551	GINAENNHRIFLAGDKDNVI
	61	541-560	IFLAGDKDNVIDQIEKQAKD
	62	550-569	VIDQIEKQAKDLAFPGSGEQ
	63	559-578	KDLAFPGSGEQVEKLIKNQK
10	64	568-587	EQVEKLIKNQKESHFVSARP
	65	577-596	QKESHFVSARPQSQSQSPSS
	66	586-605	RPQSQSQSPSSPEKESPEKE
	67	595-614	SSPEKESPEKEDQEEENQGG
	68	604-623	KEDQEEENQGGKGPLLSILK
	69	607-626	QEEENQGGKGPLLSILKAFN

Table S4. Predicted HLA-DR binding motifs in selected Ara h 1 20-mers

HLA molecule	Ara h 1 20-mer peptide 23 (199-218)	24 (208-227)	46 (406-425)	47 (415-434)	49 (433-452)	50 (442-461)	51 (451-470)	57 (505-524)
DRB1_0101	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0102	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0301	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0305	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0306	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0307	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0308	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0309	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0311	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0401	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0402	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0404	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0405	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0408	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0410	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0421	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0423	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0426	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0701	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0703	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0801	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0802	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0804	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0806	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0813	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_0817	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1101	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1102	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1104	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1106	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1107	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1114	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1120	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1121	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1128	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1301	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1302	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1304	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1305	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1307	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1311	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1321	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1322	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1323	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1327	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1328	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1501	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1502	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB1_1506	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB5_0101	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS
DRB5_0105	FDQRSRQFQNLQNHRIVQIE	NLQNHRIVQIEAKPNTLVLP	DLSNNFGKLFEVKPDKKNPQ	FEVKPDKKNPQLQDLDMMLT	LTCVEIKEGALMLPHFNSKA	ALMLPHFNSKAMVIVVVNKG	KAMVIVVVNKGTGNLELVAV	KEGDVFIMPAAHPVAINASS

HLA-DR binding motifs (grey shading) were predicted using the ProPred algorithm published by Singh H, Raghava GP, et al. ProPred: prediction of HLA-DR binding sites. Bioinformatics. 2001;17:1236-7., (http://www.immuneepitope.org; accessed 30th January 2012). Predicted primary anchor residues are bolded and underlined. Peptide 40 (352-371) is not shown as no HLA-DR binding motifs were predicted for this peptide by this algorithm.

SUPPLEMENTARY FIGURES

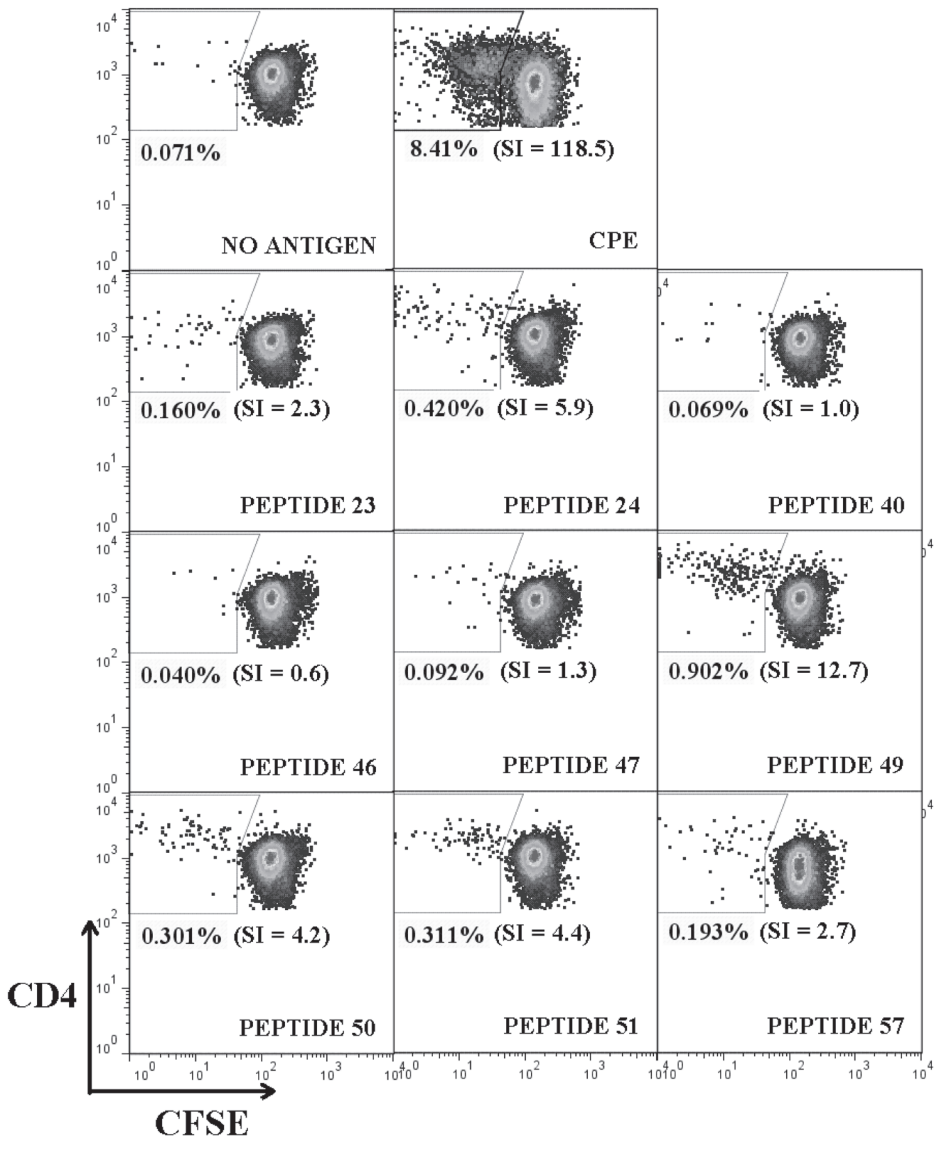

Figure S1. Representative CFSE-based assay for detecting CD4+ T-cell proliferation in PBMC.
Proliferation of CFSE-labelled PBMC from peanut-allergic subject 26 following 7 days stimulation with selected Ara h 1 20-mer peptides. Medium alone (No Antigen) or crude peanut extract (CPE) provided negative and positive controls respectively. At least 10,000 live $CD4^+$ T cells were analyzed per sample. Gates indicate percentage $CD4^+CFSE^{lo}$ (proliferating) T cells of total $CD4^+$ T cells with stimulation indices (SI) in parentheses.

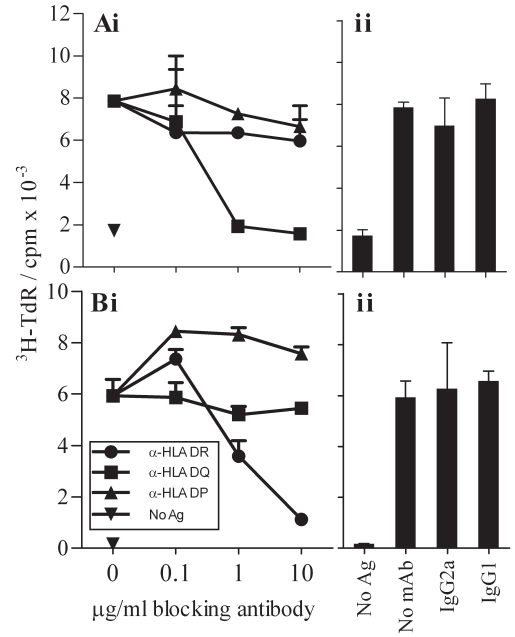

Figure S2. Representative HLA class II restriction specificity of T-cell epitope recognition.
Proliferation of specific TCL to selected epitopes in the presence of HLA-DR (circles), -DQ (squares) or -DP (triangles) mAbs (Ai and Bi) or isotype control antibodies (10 µg/ml) (Aii and Bii), (mean cpm replicate wells +SD). Graphs show sample data for an HLA-DR-restricted epitope (442-458) (A) and an HLA-DQ restricted epitope (507-524) (B).