

Characterization of the T-Cell Epitopes of the Major Peach Allergen Pru p 3

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Key Words

Peach allergy · Lipid transfer protein · Pru p 3 allergen ·
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Abstract

Background: Pru p 3 is the major peach allergen recognized by more than 90% of peach-allergic individuals of the Mediterranean area. Identification of the dominant Pru p 3 T-cell epitopes can improve our understanding of the immune responses against this protein and could be helpful in the development of hypoallergenic immunotherapy. For this purpose, we examined the phenotypes, specificities and cytokine secretion profiles of proliferating T cells in response to Pru p 3 in peach-allergic individuals. **Methods:** Peripheral blood mononuclear cells from 15 peach-allergic patients were incubated with Pru p 3. The proliferation of antigen-specific T-cell lines (TCLs) was assessed by tritiated methylthymidine incorporation. T-cell epitopes were identified by analyzing the reactivity of TCLs against 8 overlapping peptides spanning the entire length of Pru p 3. We characterized the phenotype of Pru-p-3-specific TCLs by flow cytometry and analyzed their production of interleukin (IL) 4 and γ-interferon (IFN-γ) by ELISA. **Results:** Ninety-two Pru-p-3-specific TCLs were isolated (stimulation index ≥5). These TCLs proliferated mainly in response to Pru p 3_{12–27} and Pru p

3_{57–72}. Pru-p-3-specific TCLs were mainly CD4+ (81%) and expressed cell surface CD30. In addition, TCLs produced high levels of IL-4 and low levels of IFN-γ, indicating a Th2 phenotype. **Conclusions:** Two immunodominant T-cell-reactive regions of Pru p 3 were identified: Pru p 3_{12–27} and Pru p 3_{57–72}. These peptides showed a differential ability to elicit a Th2 response. Taken together, our results provide a better understanding of the immunological T-cell reactivity against Pru p 3.

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Introduction

Peach allergy is usually associated with birch or mugwort pollen allergies, but in the Mediterranean area it is often independent from any kind of pollinosis. The members of the Prunoideae botanical subfamily (peach, apricot, plum, cherry etc.) are the most frequently consumed fruits of the Rosaceae family in Mediterranean countries, especially peach (*Prunus persica*), and are the fruits most frequently involved in food allergies [1–3]. Many peach-allergic reactions are severe [4, 5], involving systemic symptoms, but mild-grade oral allergy syndrome (OAS) is commonly reported [6].

Previously, we and others sequenced [7] and identified [2, 8] the major peach allergen Pru p 3 as a molecule with

a molecular weight of 9 kDa belonging to the group of lipid transfer proteins (LTPs). In the Mediterranean area, Pru p 3 is recognized by specific serum IgE antibodies in the majority of peach-allergic subjects [2].

Plant LTPs constitute a family of basic 9-kDa proteins (90–95 amino acids long) that are widely distributed in plant-derived foods and pollens and are involved in plant defense against pathogens [9]. The strong resistance of LTPs to heat and simulated pepsin digestion [10–12] makes these proteins potentially severe food allergens. The regions of Pru p 3 bound by IgE antibodies have been previously identified [13], and recombinant Pru p 3 has been produced [14]. However, few primary data have been published that identify T-cell epitopes of Pru p 3 [15, 16].

The identification of Pru p 3 T-cell epitopes is important in order to better understand the immunological reactivity of this allergen. In addition, such knowledge would provide us with important information regarding the cellular mechanisms underlying the shift from food tolerance to allergy. Moreover, studies of food-allergen-associated T-cell epitopes could reveal other relevant epitopes shared by different foods (belonging to related botanical families) or pollen allergens.

Furthermore, there is considerable interest in this field because the use of helper epitopes may improve immune responses to synthetic vaccine constructs [17, 18].

Evidence for the efficacy of T-cell-epitope-based peptide immunotherapy has been documented for both bee venom [19, 20] and cat dander [21, 22] allergies. In fact, the identification and retention of critical T-cell epitopes in the preparation of hypoallergenic immunotherapies [23, 24] is crucial to T-cell-targeted strategies.

The main purpose of this study was to identify immunodominant Pru p 3 T-cell epitopes. Here, we present an analysis of T-cell epitopes of the major peach allergen Pru p 3 as identified in a group of Italian patients with peach-induced clinical reactions. Pru-p-3-specific oligoclonal T-cell lines (TCLs) were stimulated with a set of overlapping Pru p 3 peptides representing the entire sequence of this peach LTP, and proliferation and cytokine production by these TCLs were measured.

Materials and Methods

Patients

We selected peach-allergic patients referred to the Clinical Allergy and Immunology Unit, Foundation IRCCS Ca' Granda Ospedale Maggiore Policlinico, and to the Unit of Allergology and Immunology, Niguarda Ca' Granda Hospital, in Milan, Italy.

Patients with a history of peach allergy only, without any pollinosis, were selected based on the following criteria: (1) a positive skin test to fresh peach fruit performed using the prick + prick technique [25]; (2) the presence of peach-specific IgE antibodies in serum; (3) a positive history of first-grade OAS, mild-grade OAS with gastrointestinal or cutaneous symptoms confirmed by positive open food challenge [6] or a documented history of severe-grade OAS to peach (patients with a history of severe reactions were not challenged), and (4) a positive reaction exclusively to the 9-kDa band of raw peach extract and positive IgE binding to purified Pru p 3 by SDS-PAGE/IgE immunoblotting. We also included volunteer control subjects who were chosen on the basis of a completely negative clinical history of allergy to food and airborne allergens, a negative skin prick test and a lack of specific IgE antibodies against common pollens and foods.

The selected patients were enrolled in the study after providing informed consent. The study protocol was approved by the Medical Ethics Committee of the University of Milan and Niguarda Ca' Granda Hospital. None of the patients had previously received any form of specific immunotherapy or glucocorticoid treatment for 3 months prior to blood donation.

Determination of Peach-Specific IgE Levels in Serum

Peach-specific IgE levels in serum were determined for all the patients using the Immunocap System according to the manufacturer's instructions (Phadia, Uppsala, Sweden). The limit of detection was 0.35 kUA/l.

Allergen and Reagents

Pru-p-3-specific TCLs were cultured in RPMI 1640 medium (Euro Clone Ltd., UK) supplemented with 10% pooled heat-inactivated human serum obtained from healthy male donors of the AB blood type (Blood Transfusion Service, Ospedale Maggiore IRCCS, Milan, Italy), 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (Euro Clone Ltd.).

Pru p 3 had previously been separated and purified from fresh raw peaches according to the methods of Pastorello et al. [7]. Pru-p-3-derived peptides were prepared by automatic solid-phase synthesis on a peptide synthesizer (model 433A, Applied Biosystems, Foster City, Calif., USA) by using fluorenylmethoxycarbonyl chemistry.

Final endotoxin levels, determined using the Limulus amoebocyte lysate assay after endotoxin depletion using Endotrap-based affinity chromatography (Cambrex Bioscience, Milan, Italy), were <0.3 EU/mg protein.

Recombinant human IL-2 (rIL-2) was obtained from Chiron BV, Amsterdam, The Netherlands.

Phycoerythrin-conjugated anti-human CD4, fluorescein-isothiocyanate-conjugated anti-human CD8 and anti-human CD30 monoclonal antibodies (Becton Dickinson, San Jose, Calif., USA) were used to analyze the phenotype of the Pru-p-3-specific TCLs using a FACScan flow cytometer (Becton Dickinson) by double antibody staining.

SDS-PAGE and IgE Immunoblotting

IgE immunoblotting was performed as previously described [7]. Briefly, raw peach extract and in-house purified Pru p 3 were separated using a 7.5–20% SDS-PAGE gradient gel that was blotted onto a nitrocellulose membrane. The membrane was incubated overnight at 4°C with a 1:4 dilution of patient sera, pooled

serum from nonallergic individuals as a control or buffer alone. After incubation with ^{125}I -labeled anti-human IgE antibody (Radim Spa, Rome, Italy), binding of IgE was detected by autoradiography.

Pru p 3 Peptide Synthesis and Purification

Eight peptides, 16–18 amino acids in length with 5 overlapping residues, were synthesized based on the Pru p 3 sequence [8] from the N- to the C-terminal end (1–16, 12–27, 23–38, 34–49, 44–61, 57–72, 68–82, 74–91). Peptides were assembled on a model 433A Applied Biosystem peptide synthesizer, using a stepwise solid-phase fluorenylmethoxycarbonyl method [26, 27], onto a preloaded 2-chlorotriethyl chloride resin from Novabiochem (Läufelfingen, Switzerland). The peptide solution was resuspended in 5% aqueous acetic acid and lyophilized. Analytical and semipreparative reverse-phase high-performance liquid chromatography was carried out on a Tri Rotar-VI HPLC system. Linear gradients of acetonitrile in water/0.1% trifluoroacetic acid were used to elute bound peptides. Matrix-assisted laser desorption/ionization time-of-flight analyses were performed on a Voyager-RP Biospectrometry Workstation (PE Biosystem Inc.). Observed experimental values for peptide masses were in agreement with theoretically calculated values.

Establishment of Pru-p-3-Specific TCLs

TCLs were established on the same day, when heparinized venous blood was collected from the patients. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using Lymphoprep discontinuous density gradients (Nicomed Pharma AS, Oslo, Norway) and plated in complete culture medium (CM) on glass Petri dishes (Greiner Labortechnik) for 1 h at 37°C in a humidified atmosphere of 5% CO_2 . Nonadherent cells were removed by rinsing CM with a Pasteur pipette, and the adherent cells were harvested in cold calcium- and magnesium-free phosphate-buffered saline (PBS; Euro Clone Ltd.). Adherent cells, comprised of more than 95% CD14+ cells, were cultured at a density of $10^7/\text{ml}$ in CM and pulsed with $50\text{ }\mu\text{g/ml}$ Pru p 3 for 4 h at 37°C at 5% CO_2 . This dose had been found to be optimal in preliminary experiments where a range of 10–100 $\mu\text{g/ml}$ Pru p 3 was tested. After incubation, monocytes were washed and mixed with the nonadherent cell fraction containing T lymphocytes. Cells were cultured at a density of $2 \times 10^5/200\text{ }\mu\text{l/well}$ in 96-well round-bottom plates (Falcon Becton Dickinson & Co., Lincoln Park, N.J., USA) for 13 days. On days 5 and 8, rIL-2 at a concentration of 20 U/ml was added to the cultures.

Proliferation Assay for Pru-p-3-Specific TCLs

On day 13, each TCL was tested for reactivity to Pru p 3. For this purpose, autologous PBMCs, preincubated with $50\text{ }\mu\text{g/ml}$ Pru p 3 (or with no peptide as a control) for 2–4 h, were used as antigen-presenting cells. For each TCL, 1×10^4 cells were cocultured in duplicate with 2.5×10^4 irradiated (5,000 rad), autologous PBMCs for 72 h in 96-well round-bottom plates and pulsed with tritiated methylthymidine (^3H -TdR, $2\text{ }\mu\text{Ci/well}$; Amersham, UK) during the last 18 h of culture. Cell proliferation was determined by analyzing the extent of ^3H -TdR incorporation, as measured by scintillation counting using a β -counter (Minimaxi Tri-carb, Canberra Packard). The results are expressed as a stimulation index (SI), which is defined as the ratio between mean counts per minute of antigen-stimulated cultures and unstimulated cul-

tures. TCLs with an $\text{SI} \geq 3$ were considered to be specific for Pru p 3. TCLs that proliferated in response to Pru p 3 were cultured in CM supplemented with 40 U/ml of rIL-2 every 3–4 days.

Proliferation Assay for Pru p 3 Single-Peptide-Specific TCLs

To identify the epitope(s) responsible for the response of lymphocytes to peach allergen, Pru-p-3-specific TCLs were assessed for their ability to proliferate in response to 8 synthetic peptides spanning the 91 residues of Pru p 3. Autologous PBMCs, previously incubated for 2–4 h with $50\text{ }\mu\text{g/ml}$ of the different synthetic peptides, were used as antigen-presenting cells. 2×10^4 cells from each Pru-p-3-specific TCL were cocultured in duplicate with 2.5×10^4 irradiated (5,000 rad), peptide-pulsed PBMCs, for 72 h in 96-well round-bottom plates and pulsed with ^3H -TdR ($2\text{ }\mu\text{Ci/well}$) during the last 18 h of culture. Cell proliferation was determined by analyzing the extent of ^3H -TdR incorporation. The results are expressed as an SI, and Pru-p-3-specific TCLs with an $\text{SI} \geq 3$ were considered to be specific for each peach peptide.

Analysis of Cell Surface Marker Expression by Pru-p-3-Specific TCLs

Phycoerythrin-labeled anti-human CD4, fluorescein-isothiocyanate-labeled anti-human CD8 and anti-human CD30 mouse monoclonal antibodies were used to analyze the phenotype of Pru-p-3-specific TCLs by antibody staining. After 20 days of culture, Pru-p-3-specific TCLs (1×10^5 cells/experiment) were harvested from the culture plates, washed, resuspended in PBS, labeled with $25\text{ }\mu\text{l}$ (diluted 1:5 with PBS) of anti-CD4 and anti-CD8 and $10\text{ }\mu\text{l}$ of undiluted anti-CD30 and incubated in the dark for 25 min at 4°C . Cells were then washed in 1 ml of PBS by centrifugation for 10 min at 1,500 rpm and 4°C . The final pellet was resuspended in 0.5 ml of PBS. For flow cytometry, 5×10^3 cells were acquired using a FACScan instrument. Dot blots were generated with phycoerythrin on the y-axis and fluorescein isothiocyanate on the x-axis. Results are expressed as percentage of positive cells.

Determination of Cytokine Production by Pru-p-3-Specific TCLs

To analyze the cytokine secretion profiles of Pru-p-3-specific TCLs, 15–20 days after initial stimulation, 2.5×10^4 cells/well were restimulated in triplicate with 5×10^4 irradiated PBMCs, previously pulsed with $50\text{ }\mu\text{g/ml}$ Pru p 3, in 96-well round-bottom plates at a final volume of $200\text{ }\mu\text{l/well}$. After 36 h, the culture plates were centrifuged at 1,390 rpm for 10 min at room temperature. Supernatants were collected, pooled and stored at -20°C for later determination of cytokine concentrations. The reactivity of TCLs upon restimulation was confirmed by analyzing ^3H -TdR incorporation in parallel cultures. IL-4 and γ -interferon (IFN- γ) secreted in the culture supernatants were quantified by using commercial ELISA kits (Quantikine, R&D Systems Europe Ltd., Abingdon, UK) according to the manufacturer's instructions. The sensitivity of the assays was 10 pg/ml for IL-4 and 8 pg/ml for IFN- γ . Allergen-specific TCLs were distinguished into Th subsets according to their cytokine profiles in response to Pru p 3 stimulation. TCLs releasing at least 5 times more IL-4 than IFN- γ were classified as Th2 TCLs, whereas those releasing at least 5 times more IFN- γ than IL-4 were classified as Th1 TCLs. TCLs producing similar levels of IL-4 and IFN- γ were classified as Th0 [28].

Table 1. Demographic data, clinical manifestations of peach allergy, results of the peach skin test and levels of peach-specific IgE antibodies for study participants

Patient	Age years	Sex	Clinical history	SPT	Peach IgE kUA/l
1	36	F	A	+++	23.5
2	37	F	OAS	++	6.0
3	33	M	U	++	9.2
4	39	F	GI	+++	18.9
5	35	M	OAS	++	6.1
6	26	F	A	+++	24.0
7	24	F	U, AE	+	8.7
8	40	F	A	+++	21
9	37	F	U, AE	+++	18.2
10	18	F	GI, AE	++++	59.5
11	23	M	AD, OAS	++++	45.8
12	35	F	OAS	++	6.1
13	55	M	U	++	4.7
14	26	F	A	+++	25.3
15	39	F	OAS	++	5.7

SPT = Skin prick test; AD = atopic dermatitis, A = anaphylaxis, U = urticaria, AE = angioedema, GI = gastrointestinal symptoms.

HLA Typing

HLA class II DRB1, DQA1 and DQB1 alleles and haplotypes were determined by using the INNO-Lipa HLA typing tests (Innogenetics, Belgium), which are based on reverse-hybridization principles. The nomenclature of the HLA-DR and HLA-DQ alleles is in accordance with the WHO Nomenclature Committee for Factors of the HLA System [29].

Statistical Analysis

One-way analysis of variance (ANOVA), the χ^2 test with Yates' correction for continuity or Pearson's correlation test were used as appropriate. SPSS software (v. 12.0, SPSS Inc., Chicago, Ill., USA) was employed for analysis. *p* values <0.05 were considered to be significant.

Results

Patients

Fifteen peach-allergic patients (11 female and 4 male; mean age: 36 years, age range: 18–55 years) fit the inclusion criteria. Although these patients presented clinical symptoms only to peach, they also showed positive skin tests and specific IgE antibodies for other fruits of the Prunoideae subfamily, such as apricot, plum and cherry. Table 1 shows demographic data, clinical symptoms, the

Table 2. Number and percentage (in parentheses) of Pru-p-3-specific TCLs in each patient

Patient No.	Number of Pru-p-3-specific TCLs	SI (min.–max.)
1	23/33 (70)	3.1–32.1
2	12/30 (40)	3–68.6
3	10/42 (24)	3–18.9
4	7/38 (18)	3–52
5	10/28 (36)	3.3–110
6	4/34 (12)	3.2–9
7	20/43 (46)	3–64.1
8	2/30 (7)	3.4–5
9	4/33 (12)	3.1–24.1
10	10/39 (26)	3.4–17.1
11	4/41 (10)	3–9.7
12	5/35 (14)	3–6.3
13	22/43 (51)	3–75
14	12/40 (30)	3.6–85.6
15	11/38 (29)	4.7–14.8

Minimum and maximum SIs for TCLs were established using Pru p 3 as the initial stimulus.

results of the peach skin tests and the levels of peach-specific IgE antibodies for each patient.

Pru-p-3-Specific TCLs

In order to compare the reactivity of Pru-p-3-specific TCLs in the study participants, we selected the TCLs with the highest SI; 92 Pru-p-3-specific TCLs with SI ≥ 5 were selected from the PBMCs of the 15 patients. All subjects exhibited a proliferative response against Pru p 3. The percentage of Pru-p-3-specific TCLs in each patient ranged from 7 to 70% of the total cultures, and these lines showed a specific proliferative response (SI range 3–110) against Pru p 3 (table 2). The extent of the T-cell response did not correlate with the levels of Pru-p-3-specific IgE antibodies present in the serum. Pru-p-3-specific TCLs were not established from the PBMCs of 5 negative controls (negative for atopy and peach allergy).

T-Cell Epitopes Recognized by Pru-p-3-Specific TCLs

Table 3 shows the frequency of TCL recognition of each of the 8 Pru p 3 peptides for each patient, indicating the number of TCLs that reacted to a single peptide and those that reacted to more than one peptide. TCLs were not reactive towards neighboring peptides in all but 1 case (patient No. 5); therefore TCLs were considered to be

Table 3. Number of TCLs established for all 15 patients with respect to the 8 synthetic Pru p 3 peptides

Pa- tient No.	Total of TCLs	Pru p 3 synthetic peptides																
		TCLs that reacted to a single peptide								TCLs that reacted to more than one peptide								
		1-16	12-27	23-38	34-49	44-61	57-72	68-82	74-91	12-27 34-49	12-27 44-61	12-27 57-72	34-49 57-72	44-61 74-91	57-72 74-91	12-27 34-49	12-27 57-72	34-49 57-72
1	18						6									12		
2	8		1				1			2		1	1				2	
3	7		4				3											
4	5		1									2					2	
5	6	1	5															
6	1		1															
7	13	1					3		1			1			2		1	4
8	1		1															
9	3						1					2						
10	5					2			2						1			
11	3		1						2									
12	4		1				2				1							
13	4		4															
14	11						11											
15	3						3											

specific for an epitope within the region of the recognized peptide. Fifty-eight specific TCLs (63%) recognized a single peptide with the majority reacting to peptide Pru p 3₁₂₋₂₇ (19 TCLs, 21%) or peptide Pru p 3₅₇₋₆₂ (30 TCLs, 33%). Thirty-four lines (37%) recognized 2 or 3 different peptides (right side of table 3). None of the TCLs exhibited an SI = 3 for peptides Pru p 3₂₃₋₃₈ and Pru p 3₆₈₋₈₂. The phenotypes, the peptides recognized and the cytokine profiles of these Pru-p-3-specific TCLs are shown in table 4.

Figure 1 shows the pattern of recognition of the TCLs from the selected patients for the 8 synthetic peptides based on whether they reacted to a single peptide or multiple peptides. The results clearly show that Pru p 3₁₂₋₂₇ and Pru p 3₅₇₋₇₂ were the 2 immunodominant peptides since they were recognized by TCLs from 12 (80%) and 9 (60%) of the 15 peach-allergic patients, respectively.

Expression of Surface Markers by Pru-p-3-Specific TCLs and Their Production of IL-4 and IFN- γ

Flow-cytometric analysis of Pru-p-3-specific TCLs showed that the majority of these TCLs expressed the CD4 antigen. In addition, TCLs could be classified into 3 groups. The first group was comprised mainly of CD4+ T cells, representing 81% of all TCLs analyzed for the ex-

pression of surface markers. The second group was comprised mainly of CD8+ TCLs, accounting for 8% of total TCLs. The last group was comprised of a heterogenic population of CD4+, CD8+, CD4+CD8+ and CD4-CD8- T cells, accounting for 11% of total TCLs (table 5).

The surface marker phenotype CD30, commonly expressed in the context of atopic diseases [30, 31], was also analyzed (table 4). Approximately 51% of the Pru-p-3-specific TCLs expressed a considerable amount of cell surface CD30 (>40%).

Secretion of IL-4 and IFN- γ by TCLs responding to Pru p 3 synthetic peptides was measured in the culture supernatants after 36 h of stimulation (table 4). The amount of IL-4 produced by TCLs ranged from undetectable (<10 pg/ml) to 2,272 pg/ml, while the levels of IFN- γ ranged from undetectable (<8 pg/ml) to 2,014 pg/ml. The levels of IL-4 produced by TCLs correlated with the expression levels of CD30 ($p = 0.001$; Pearson's correlation). Pru-p-3-specific TCLs with an IL-4/IFN- γ ratio of >5 were characterized as having a Th2 profile. In contrast, an IFN- γ /IL-4 ratio of >5 indicated a Th1 profile; similar or undetectable levels of both of these cytokines indicated a Th0 profile [28]. To confirm their Th profile, we analyzed those TCLs that responded to a single peptide.

Table 4. Phenotype, synthetic-peptide-specific responses and IL-4 and IFN- γ production after stimulation with Pru p 3 for 36 h, for individual TCLs derived from each patient

Pa- tient No.	TCLs	Phenotype, %			Pru p 3 peptides			Cytokine levels after Pru p 3 stimulation, pg/ml		Pure line profile		
		CD4+	CD8+	CD30+	p12-27	p57-72	others	IL-4	IFN- γ	Th1	Th0	Th2
1	1	53.1	1.1	24.7				263	16			
	2	93.2	0.6	55.6	+	+		411	0			
	3	80.9	17.1	54.2	+	+		232	0			
	4	81.1	17.3	56.7		+		200	0			+
	5	81.6	7.9	5.8		+		244	0			+
	6	95.5	0.1	49.0	+	+		275	225			
	7	90.4	1.0	15.6	+	+		0	0			
	8	96.3	0.9	8.9	+	+		636	0			
	9	94.2	2.0	34.5		+		84	0			+
	10	98.6	0.2	69.6		+		101	0			+
	11	99.3	0.0	84.2	+	+		203	0			
	12	98.5	0.2	84.1	+	+		2,272	0			
	13	88.8	4.9	73.3	+	+		1,492	0			
	14	95.7	0.5	55.4	+	+		0	0			
	15	97.6	1.2	52.2		+		195	0			+
	16	94.7	4.3	90.6	+	+		329	0			
	17	96.9	2.0	87.7	+	+		281	0			
	18	96.0	2.1	85.3		+		0	0		+	
2	1	93.8	2.8	23.1	+	+	34-49	307	0			
	2	30.2	37.5	11.8		+	34-49	524	49			
	3	52.2	1.1	23.4	+	+	34-49	190	1,213			
	4	52.3	3.6	31.8	+			281	0			+
	5	86.2	3.5	27.1	+	+		0	0			
	6	16.9	79.1	3.9	+		34-49	35	168			
	7	11.7	30.2	17.4	+		34-49	57	108			
	8	44.3	42.5	15.1		+		0	0		+	
3	1	78.5	4.1	12.2	+			0	0		+	
	2	71.9	19.4	1.1	+			0	0		+	
	3	79.9	4.1	13.4	+			0	0		+	
	4	70.7	11.9	46.4		+		334	0			+
	5	83.7	10.4	51.2	+			185	16			+
	6	79.1	11.3	39.4		+		360	58			+
	7	34.1	42.7	33.6		+		14	525	+		
4	1	53.3	3.6	30.5	+	+		0	0			
	2	35.1	41.7	32.9	+	+	74-91	0	0			
	3	0.1	98.3	0.8	+	+	74-91	30	325			
	4	79.1	8.9	60.2	+	+		405	2,014			
	5	86.6	1.6	52.6	+			378	226		+	
5	1	93.7	1.5	71.7	+			125	0			+
	2	98.7	0.1	90.6	+			151	0			+
	3	1.9	96.3	2.5	+			0	0		+	
	4	63.3	9.8	44.8	+			0	0		+	
	5	92.7	2.2	75.5			1-16	216	0			+
	6	75.7	13.6	42.4	+			128	0			+
6	1	82.6	7.9	48.6	+			0	0		+	
7	1	91.3	2.3	42.7		+		333	0			+
	2	96.1	1.1	75.8		+	34-49 74-91	245	0			

Table 4 (continued)

Pa- tient No.	TCLs	Phenotype, %			Pru p 3 peptides			Cytokine levels after Pru p 3 stimulation, pg/ml		Pure line profile		
		CD4+	CD8+	CD30+	p12-27	p57-72	others	IL-4	IFN- γ	Th1	Th0	Th2
	3	91.7	2.7	63.0		+		27	10		+	
	4	90.3	3.3	43.4		+	34-49 74-91	29	32			
	6	51.2	16.5	13.8		+	34-49 74-91	76	137			
	7	96.7	0.7	67.5		+	74-91	327	0			
	9	92.3	1.3	52.7			1-16	351	0			+
	12	62.3	10.8	41.5		+	74-91	0	0			
	13	54.2	2.1	26.9	+	+	74-91	0	0			
8	1	47.3	12.7	18.2	+			0	0			
9	1	58.3	19.2	39.4		+		907	0			+
	2	97.7	0.3	84.1	+	+		1,409	0			
	3	37.4	38.7	20.0	+	+		0	0			
10	1	87.6	3.3	50.6			44-61 74-91	154	0			
	2	54.4	0.4	28.0			44-61	27	21		+	
	3	88.2	0.3	55.9			74-91	566	0			+
	4	84.1	2.4	32.7			74-91	230	0			+
	5	79.5	0.6	20.8			44-61	114	0			+
11	1	21.1	69.9	5.8			74-91	0	21		+	
	2	57.3	20.2	38.4			74-91	174	0			+
	3	42.7	13.7	19.2	+			260	0			+
12	1	10.1	86.2	0.3		+		15	66		+	
	2	77.8	13.9	44.7	+		44-61	0	0			
	3	69.7	12.9	45.4		+		0	0		+	
	4	1.7	82.9	1.4	+			0	215	+		
13	1	94.2	2.0	34.5	+			262	0			+
	2	75.7	13.6	42.4	+			0	0		+	
	3	74.8	1.3	39.9	+			0	0		+	
	4	87.4	0.3	57.8	+			64	0			+
14	1	76.9	10.8	56.9		+		1,081	0			+
	2	85.5	6.7	59.6		+		707	0			+
	3	39.1	42.7	23.6		+		0	0		+	
	4	73.7	12.7	18.2		+		0	0		+	
	5	15.7	84.2	10.5		+		0	159	+		
	6	69.6	0.1	41.9		+		1,470	0			+
	7	37.4	38.7	20.0		+		0	0		+	
	8	58.3	19.2	39.4		+		333	59			+
	9	84.1	0.2	44.6		+		148	23			+
	10	60.1	39.6	53.2		+		265	0			+
	11	55.4	20.2	35.7		+		735	0			+
15	1	88.8	4.9	73.3		+		111	0			+
	2	70.7	16.9	46.4		+		137	0			+
	3	81.7	11.9	52.8		+		68	0			+

Th profiles are shown only for those TCLs that responded to a single peptide. Gray-shaded rows indicate dominant CD4+ TCLs; only these TCLs were considered for determining the correlation between the peptide and the cytokines produced.

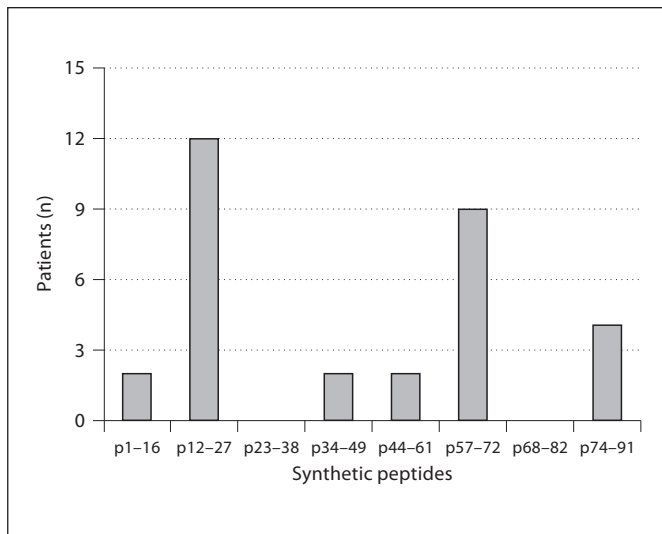


Fig. 1. Reactivity of patient TCLs that recognize single or multiple peptides against single Pru p 3 peptides.

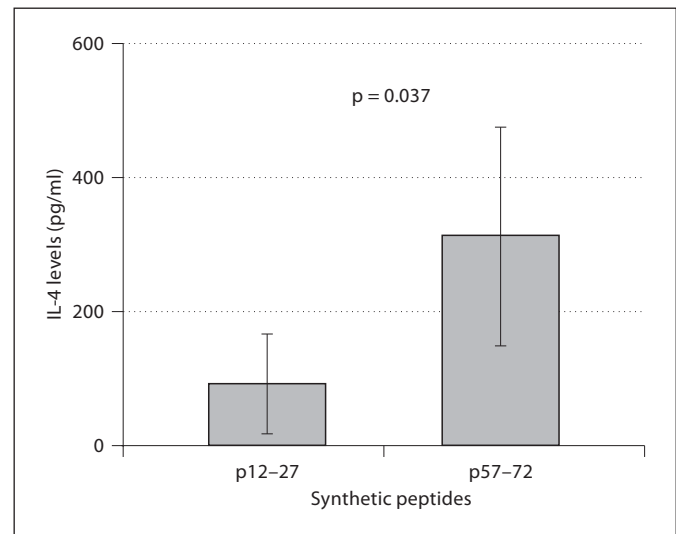


Fig. 2. Production of IL-4 by TCLs that react to Pru p 3₁₂₋₂₇ or Pru p 3₅₇₋₇₂ expressed as the mean concentration $\pm$ 95% confidence interval. Differences between the two peptides are statistically significant ($p < 0.037$; ANOVA).

Table 5. Cell surface phenotype of Pru-p-3-specific TCLs and the percentage of each phenotype with respect to the total number of TCLs ($n = 92$) established from peach-allergic patients

Surface phenotype	Number of Pru-p-3-specific TCLs	Percent	Number of Pru-p-3-specific TCLs reacting to a single peptide	Profile		
				Th1	Th0	Th2
CD4+CD8–	75	81	46	–	13	33
CD4+CD8+/CD4–CD8–	10	11	5	1	3	1
CD4–CD8+	7	8	5	2	3	–
CD4+CD30+	47	51	28	–	7	21

Th Profile of TCLs Reacting to Single Pru p 3 Synthetic Peptides

To determine whether the different peptides analyzed differed in their capacity to elicit a Th2 response, the Th profile of Pru-p-3-specific TCLs that responded exclusively to a single peptide was analyzed. This analysis was possible because even if cytokine levels were determined after Pru p 3 stimulation, for those lines reacting to a single peptide we assumed that cytokines were produced in response to that single peptide. Only specific TCLs that were 50% positive for CD4 were considered for further analyses (table 4, gray highlighted rows); only the TCLs that responded to peptides Pru p 3₁₂₋₂₇ ($n = 15$) or Pru p

3₅₇₋₇₂ ($n = 23$) were suitable in number to be compared. We observed that TCLs responding to a single peptide, namely Pru p 3₁₂₋₂₇ or Pru p 3₅₇₋₇₂, originated mainly from individual patients. For example, patients No. 5 and 13 produced TCLs specific for Pru p 3₁₂₋₂₇, while patients No. 14 and 15 produced TCLs specific only for Pru p 3₅₇₋₇₂. As shown in figure 2, TCLs recognizing peptide Pru p 3₁₂₋₂₇ produced a significantly lower amount of IL-4 (mean value = 91.4 pg/ml; 95% confidence interval = 74.6) than TCLs responding to Pru p 3₅₇₋₇₂ (mean value = 312.8 pg/ml; 95% confidence interval = 164.3; $p = 0.037$; ANOVA). As shown in figure 3, Pru-p-3₁₂₋₂₇-specific TCLs mostly displayed a Th0 profile when compared

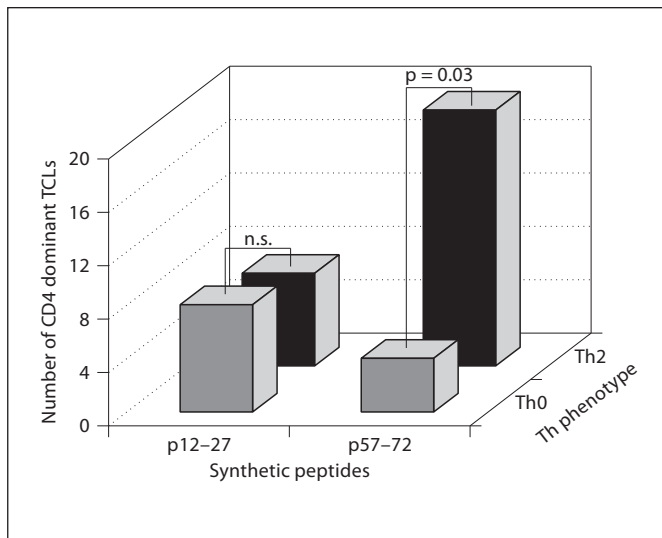


Fig. 3. Number of dominant CD4⁺ TCLs that react only to Pru p 3₁₂₋₂₇ or Pru p 3₅₇₋₇₂. Pru-p-3₁₂₋₂₇-reactive TCLs exhibit a mixed Th0/Th2 phenotype, while Pru-p-3₅₇₋₇₂-reactive TCLs exhibit a Th2 phenotype (χ^2 test with Yates' correction).

to Pru-p-3₅₇₋₇₂-specific TCLs, which mostly displayed a Th2 profile ($p = 0.03$; χ^2 test with Yates' correction for continuity). Despite the fact that only analysis of individual T cell clones could confirm their Th profiles, we found that only the Th0 profile of Pru p 3₁₂₋₂₇ was consistent because all but 3 Th0 TCLs produced undetectable cytokine levels. The other TCLs that recognized only Pru p 3₁₋₁₆, Pru p 3₄₄₋₆₁ or Pru p 3₇₄₋₉₁ secreted 283 ± 95 , 70 ± 61 and 348 ± 180 pg/ml of IL-4, respectively. All these lines exhibited a Th2 profile, with the exception of TCLs from 1 patient (patient No. 10, line 2).

HLA Typing

HLA-DR/DQ typing was performed for 14 of the study participants. We observed a high frequency of HLA-DRB1*11 (86%) haplotype in these patients, but this increased frequency did not reach statistical significance when using the χ^2 test with Yates' correction for continuity.

Discussion

Peach allergens are frequently involved in allergic reactions in the adult population of the Mediterranean area. Pru p 3 is a major peach allergen recognized by serum IgE antibodies in many peach-allergic individuals

[7]. While there are reports in the literature on the existence of peach-specific B-cell epitopes [13], data on peach-specific T-cell epitopes have only recently emerged [15, 16]. Moreover, allergenic Pru p 3 homologues are present in several foods, and cross-reactivity among various fruit and vegetable allergens belonging to different botanical families has been demonstrated [32–35]. The identification of Pru-p-3-specific T-cell epitopes provides important information on the characteristics of this allergen as well as on the cellular mechanisms underlying the shift from food tolerance to allergy. In addition, results obtained from studies of T-cell epitopes from other food allergens can help elucidate the relevant T-cell epitopes that are shared with pollen allergens [36].

In the present study, we identified novel Pru-p-3-specific T-cell epitopes by analyzing PBMCs from peach-allergic patients and sensitized only to Pru p 3. The high SIs of the TCLs analyzed and their large numbers are peculiar findings in this peach-allergic population. Oligoclonal Pru-p-3-specific TCLs were analyzed after stimulation with Pru p 3, and their expression of CD4, CD8 and CD30, as well as their production of IL-4 and IFN- γ , were determined. After stimulation with a panel of 8 overlapping synthetic peptides (each composed of 16–18 amino acids) that spanned the entire length of the 91-amino-acid-long Pru p 3, we analyzed the peptide-specific T-cell response. Since we considered only TCLs that reacted to a single peptide, we assumed that cytokine production was due to TCL recognition of that single peptide.

Five out of the 8 Pru p 3 synthetic peptides were recognized by the patients' TCLs. In particular, the TCLs from 12/15 patients (80%) recognized Pru p 3₁₂₋₂₇ whereas 9/15 patients (60%) reacted to Pru p 3₅₇₋₇₂. Several single TCLs reacted to Pru p 3₅₇₋₇₂, thus allowing us to clearly identify cell lines with a Th2 profile. This type of reactivity is typical of most known aeroallergens and food allergens, and suggests that this epitope is important in triggering sensitization in these subjects. In contrast, despite a greater number of patients reacting to Pru p 3₁₂₋₂₇, only a small number of TCLs from these patients showed a Th2 profile. This effect in turn hindered our ability to identify an appropriate Th subset, even when many of the TCLs from these patients were Th0. The frequency of the T-cell responses to both of these peptides suggests that within their protein sequence lie immunodominant T-cell epitopes for Pru p 3.

The peptides investigated here overlap by only 5 amino acids, and this could have hampered the identification of other immunogenic peptides since a 9-residue overlap has been shown to be more suitable for the identification

of immunogenic peptides [37]. However, we followed a preliminary approach to identify relevant T-cell peach peptide epitopes. We believe that, even if biased by a limited overlap, the epitopes that we have identified are of interest given the reproducibility of our findings. In addition, our results are consistent with those of 2 recently published studies where Pru p 3_{65–80} was identified as the major Th2 immunogenic peptide [15, 16]. In the Spanish study, TCL recognition after amino acid 75 is low [15]. Hence the immunopeptide appears to correspond to the epitope that we have detected here. This finding has also been confirmed by Schulten et al. [16], where T cells from 9/18 patients (50%) recognized Pru p 3_{61–75}, thus indicating a Th2 profile. They also found that Pru p 3_{13–27} was one of the most actively recognized regions by T-cell clones. Only 1/4 T-cell clones reacting to this peptide showed a Th2 phenotype, which is in agreement with our results (i.e. 50% of the Pru-p-3_{12–27}-specific TCLs were Th0). Pru p 3_{13–27} was not identified by Tordesillas et al. [15], probably because of the length of the peptide or the selected allergic patient population.

We found that Pru p 3 contains multiple T-cell epitopes, similar to other allergenic food proteins, such as α ₁-casein found in milk, Gal d 1 and Gal d 2 found in eggs, Api g 1 found in celery, Ara h 2 found in peanuts, Cor a 1.04 found in hazelnuts, Ber e 1 found in Brazil nuts, and bovine serum albumin found in beef. All of these proteins have been previously mapped and were shown to contain many T-cell epitopes within their sequences [38, 39]. Future studies performed on highly selected patients will allow us to identify the clinical role that the T-cell epitopes identified here have in different patients.

Phenotypic and functional analyses of Pru-p-3-specific TCLs revealed a typical Th2 T-cell response (58.6%) similar to that observed for other previously characterized allergens. In contrast to other allergen-specific TCLs, Pru-p-3-specific TCLs exhibited a high frequency of cells with a Th0 phenotype (34.5%) as determined by the different cytokine profiles displayed by these TCLs. TCLs that recognized Pru p 3_{12–27} produced a significantly lower amount of IL-4 than lines responding to Pru p 3_{57–72}, which are classified as Th0 cell lines (i.e. low producers of IL-4). Pru p 3_{57–72} appears to be associated with a Th2 response, whereas Pru p 3_{12–27} does not seem to induce a significant production of IL-4 by T lymphocytes, which is necessary for IgE synthesis, eosinophil activation and subsequent development of clinical IgE-mediated allergic responses. IL-4 is the main cytokine that triggers isotype switching to the IgE isotype; this cytokine stimulates

HLA class II and accessory molecules and directs naïve Th0 cells towards a Th2 phenotype. In addition, IL-4 is important in maintaining allergic immune responses by preventing T-cell apoptosis [39]. Analysis of the expression of surface markers by Pru-p-3-specific TCLs revealed a typical CD4⁺ cell surface phenotype (81%). Moreover, 51% of these lines expressed the surface antigen CD30, which has been associated with atopic diseases since decreased levels of this molecule may be indicative of acquired tolerance [30, 31].

Interestingly, HLA typing of patients with T-cell reactivity to Pru p 3 revealed a remarkably high frequency of the HLA-DRB1*11 haplotype (86%) as compared to the normal distribution of this haplotype in the Italian general population (55%) [40]. This finding is only of descriptive value since we provide no evidence supporting the notion that Pru-p-3-derived peptides are presented on HLA-DR molecules. The HLA class II alleles involved in peptide presentation have not been characterized.

The use of synthetic peptides for the identification of the precise T-cell epitopes involved in type I allergy has important implications for the development of specific immunotherapy as has been done for bee venom and cat dander allergies [18, 41]. However, peptide immunotherapy has not been shown to be promising for many allergens that exhibit multiple T-cell epitopes and that are individually different. The determination of the number of immunodominant T-cell epitope(s) of an allergen can help investigators understand which allergen(s) are more amenable for the development of immunotherapeutic peptide strategies.

The IgE binding epitopes of Pru p 3 have been identified. This knowledge can be useful in the development of recombinant hypoallergenic molecules, such as low- or nonbinding IgE isoforms [42], and future immunotherapeutic strategies.

In our study we identify Pru p 3_{12–27} and Pru p 3_{57–72} as 2 important T-cell epitopes. The identification of T-cell epitopes for foods that are cross-reactive with peach in vitro may help clarify which epitopes are involved in the sensitization that leads to an immune response. Once the key T-cell epitopes that lead to Pru p 3 sensitization are identified, it will be possible to improve our understanding regarding the clinical manifestations provoked by this allergen as well as to clarify the problems regarding cross-reactivity.

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