T-cell epitopes of the major peach allergen, Pru p 3: Identification and differential T-cell response of peach-allergic and non-allergic subjects

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ABSTRACT

Lipid transfer proteins (LTPs), particularly peach Pru p 3, are the most relevant plant food allergens in the South of Europe, and, therefore, their allergic properties have been extensively studied. However, neither T-cell epitopes nor their effect on the patients' T-cell response has been investigated in any member of the LTP panallergen family. The objective of the present study was to map the major T-cell epitopes of Pru p 3, as well as to evaluate their induced T-cell response in peach-allergic versus control subjects. Thus, peripheral blood mononuclear cells (PBMCs) from 18 peach-allergic patients and Pru p 3-specific T-cell lines (TCLs) from 9 of them were cultured with Pru p 3 and with a panel of 17 derived peptides (10-mer overlapping in 5 amino acids representing the full sequence of Pru p 3). Proliferation in 5-day assays was carried out via tritiated-thymidine incorporation, while IL4 and IFNγ production was assessed via sandwich enzyme-linked immunosorbent tests (ELISA) of TCL culture supernatants. The results were compared to those obtained from 10 non-peach allergic control volunteers. Two consecutive peptides showed the highest activation capacity. About 74% of PBMCs and TCLs recognized them, forming a single T-epitope: Pru p 3_{65-80}. Additionally, other specific T-cell epitopes were observed. Pru p 3_{25-35} was detected by more than 60% of TCLs from peach-allergic patients, and Pru p 3_{45-55} only activated PBMCs from control subjects. Interestingly, TCLs from patients were associated with a Th2-type, whereas control TCLs presented a Th1-type cytokine response. The major immunogenic T-cell epitope identified in Pru p 3, Pru p 3_{65-80}, is a good candidate to develop new vaccines for hypersensitivity reactions associated with LTP allergens from Rosaceae fruits.

1. Introduction

Peach is the major plant food involved in IgE-mediated reactions affecting the adult population from Southern European countries (Cuesta-Herranz et al., 1998; Kanny et al., 2001; Fernandez-Rivas et al., 2003). Accordingly, Pru p 3, its major allergen and lipid transfer protein (LTP) (Sanchez-Monge et al., 1999; Salcedo et al., 2007), has been identified as the food allergen showing the highest prevalence of sensitization (positive-specific IgE) in the Spanish adult population with plant food allergy (Red Vegetalia, Instituto de Salud Carlos III, MSC, Spain; unpublished results). Clinically relevant peach allergy is associated to Pru p 3 (Fernandez-Rivas et al., 2003), and this allergen also mediates cross-reactions between peach and some pollens, such as *Artemisia vulgaris* (Lombardero et al., 2004; Salcedo et al., 2007; Zuidmeer and van Ree, 2007) and *Platanus acerifolia* (Laurer et al., 2007), a relevant issue considering that most peach-allergic patients (around 80% in Spain) also suffer pollinosis (Cuesta-Herranz et al., 1999).

Plant non-specific LTPs (type 1) are 9 kDa basic polypeptides, with a very compact and conserved 3-D structure stabilized by four disulphide bridges (Salcedo et al., 2007). This protein family is widely distributed throughout the plant kingdom, and has been related to defence mechanisms against plant pathogens and abiotic

stress (Salcedo et al., 2007). Allergenic LTPs are highly resistant to protease digestion and heat treatments. They are usually associated to severe and systemic clinical symptoms and can be primary sensitizers by ingestion. They are also the main allergens of several fruits, vegetables and nuts in the Mediterranean area (Salcedo et al., 2007). Altogether, these characteristics have led to propose LTPs, particularly Pru p 3, as a model of true food allergens (van Ree, 2002; Salcedo et al., 2007).

Cross-reactivity between Pru p 3 and other plant food LTP allergens has been demonstrated (Diaz-Perales et al., 2000), and a recombinant form with immunological equivalence to natural Pru p 3 has been developed to be used in allergen component-based in vitro diagnosis (Diaz-Perales et al., 2003). Furthermore, both sequential and conformational IgE epitopes have been located in the 3-D structure of Pru p 3, and a potential hypoallergenic variant has been produced by site-directed mutagenesis (Garcia-Casado et al., 2003; Pacios et al., 2008). In contrast, no T-cell epitope of either Pru p 3 or any other allergenic LTP from plant foods has been identified so far.

In spite of the high number of cross-reactions which have been related with peach (over 20), the only prescription for affected patients is the non-consumption of the foods involved. The scarce knowledge of food allergy mechanism prevents the development of new strategies for immunotherapy. Therefore, further research work is necessary to improve the diagnosis and treatment of this kind of hypersensitivity. The identification of major T-cell epitopes of the main plant food allergens is crucial for the development of T-cell targeted strategies (Glaspole et al., 2005). In the present study, PBMCs and TCLs from peach-allergic patients and non-peach-allergic controls were stimulated with the allergen and with a set of overlapping peptides representing the full sequence of Pru p 3. The induced proliferation responses and cytokine production were measured.

2. Materials and methods

2.1. Peach-allergic patients and non-peach-allergic controls

Peripheral blood from 18 peach-allergic patients sensitised to Pru p 3 and from 10 control volunteers non-allergic to peach was collected for this study. Patients were selected at the Servicio de Alergia, Fundacion Jimenez Diaz (Madrid) based on the following criteria: (1) a convincing clinical history of allergic reactions after peach ingestion. The presence of peach allergy was further confirmed in all selected patients by an open challenge test to peach, following a method previously described (Cuesta-Herranz et al., 1998); (2) a positive skin prick test (SPT; EAACI, subcomittee of Skin Tests Allergen Standarization, 1989) response to a commercial peach extract (ALK-Abello, Madrid, Spain); and (3) sensitization to Pru p 3, ascertained by a positive SPT response to purified natural Pru p 3 (20 μg/ml) and a significant specific IgE level to recombinant Pru p 3 (5 μg/ml in solid phase; range: 0.304–2.757 OD units; cut-off value: mean + 3 × S.D. = 0.189 to bovine serum albumin used as negative control) determined by an ELISA assay as previously reported (Diaz-Perales et al., 2003). Fourteen out of the 18 selected patients showed oral allergy syndrome (OAS), 3 had systemic reactions and associated OAS and 1 had exclusively systemic symptoms. According to their lack of reaction after peach ingestion, a negative SPT to a commercial peach extract (ALK-Abello, Madrid, Spain), and non-sensitized to Pru p 3, the 10 non-allergic to peach control volunteers confirmed non-IgE to recombinant Pru p 3 (range: 0.000–0.088 OD units), determined by the ELISA assay (see above). Seven out of the 10 control subjects were non-atopic, 2 were allergic to grass (Phleum pratense) pollen and 1 was allergic to wasp venom.

A written INformed consent was obtained from both patients and controls and the study was approved by the Ethics Committee of Fundacion Jimenez Diaz (Madrid).

2.2. Purified Pru p 3 and synthetic Pru p 3-derived peptides

Natural Pru p 3 was isolated from peach peel and its recombinant form from cultured supernatants of transformed Pichia pastoris cells as described by Diaz-Perales et al. (2003).

A panel of 17 decapeptides was synthesized by following the amino acid sequence of Pru p 3. Peptides of 10-mer (overlapping in 5 amino acids) were produced by EZBiolabs (Westfield, IN, USA). The stock of each peptide was suspended in DMSO (10 mg/ml), and then diluted to 1 mg/ml in PBS buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4) before added to the cell cultures. DMSO or PBS buffer did not affect the proliferation of PBMC or TCL cultures (data not shown).

2.3. Proliferation assays with PBMCs

PBMCs were freshly isolated from 100 ml of blood subjected to density gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). Cultures were established in triplicate in 96-well plates (Costar, NY, USA) at 5×10^5 cells per well for proliferation analysis in a total volume of 200 μl of RPMI media (Invitrogen, Paisley, UK), supplemented with 10% (v/v) of fetal calf serum (Invitrogen), 0.02 mM mercaptoethanol, 2 mM of glutamine, and 10 mM HEPES, in the presence of 10 μg/ml of Pru p 3 or 1 μg/ml of each peptide, for 5 days at 37 °C, in a 5% CO_2 humidified atmosphere. During the last 16 h, [^{3}H]-thymidine (0.5 μCi/well) was added, and the incorporated radioactivity was measured by scintillation counting. The concentrations of Pru p 3 and synthetic peptides were determined by dose–response experiments using PBMCs from two different patients and 0–50 μg/ml of Pru p 3 or each peptide. These concentrations were similar to those previously published for other allergens (Ebner et al., 1995; Zeiler et al., 1999; Jahn-Schmid et al., 2002). Phytohemagglutinin-L from Phaseolus vulgaris (PHA; 1 pg/ml; Roche, Mannheim, Germany) was used as a positive control to evaluate the proliferative response of PBMC cultures.

The stimulation index (SI) was calculated as the ratio between counts of antigen-stimulated cultures and counts without any activator. A SI ≥ 2 was considered a positive value for PBMCs.

2.4. Pru p 3-specific TCLs

PBMCs (2×10^6 cells per well; 24-well plates) were treated with 10 μg/ml of Pru p 3 in a RPMI supplemented media (see above). After 5 days, a half-volume media was removed and suboptimal doses of rIL2 (10 ng/ml; Biosource, Invitrogen, UK) were added. Cultures were continued for an additional 7 days. On the 12th day, fresh media supplemented with rIL2 was added in the presence of 10^5 autologous-mitomycin-treated PBMCs. Finally (on the 19th day of culture) Pru p 3-TCLs (5×10^4 cells per well) and their mitomycin-treated PBMCs (10^5 cells per well) were seeded in 96 wells. Pru p 3 (10 μg/ml) or each peptide (1 μg/ml) were added to the TCL culture in triplicate wells for 48 h. Within the last 16 h, [^{3}H]-thymidine (0.5 μCi/well) was added, and the incorporated radioactivity was measured by scintillation counting. The stimulation index was calculated as described above. In this case, a SI ≥ 3 was considered a positive value for TCLs.

Before counting, supernatants were recovered to quantify the cytokines (IL4 and IFNγ) by ELISA assays.
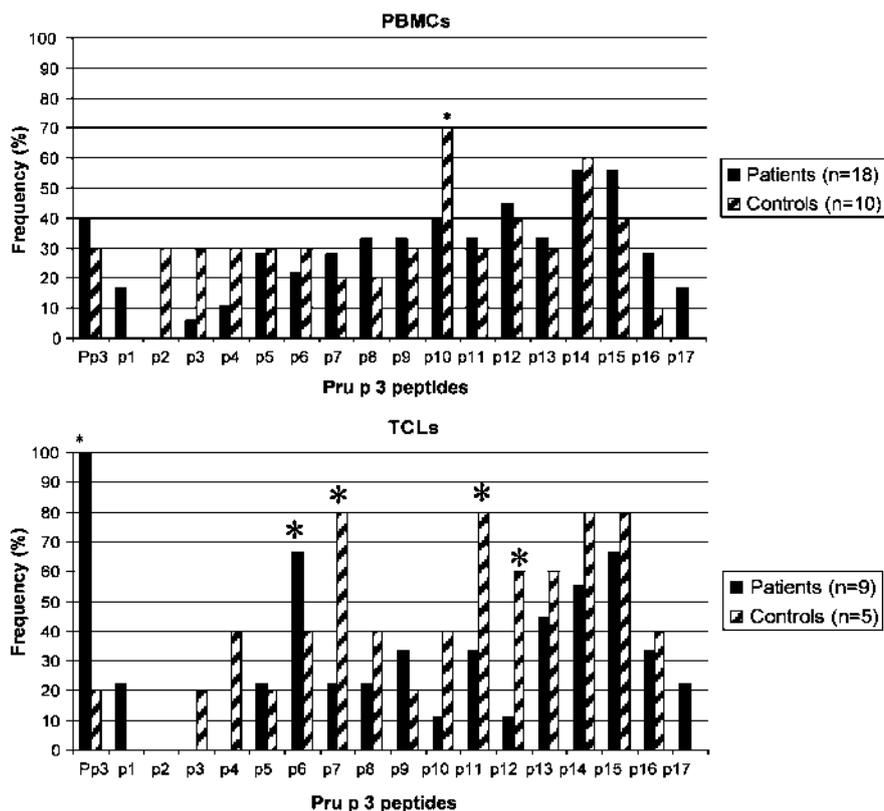
Fig. 1. Frequency (%) of positive responses of PBMCs and TCLs from patients and control subjects to Pru p 3 (Pp3) and its 17 synthetic derived peptides (p1–p17). Significant differences (*p* < 0.05) between both groups are marked (*).

2.5. Location of T-cell epitopes on the 3-D structure of Pru p 3

Chain A of the X-ray structure, PDB code 2B5S (Pasquato et al., 2006), was used to locate peptides on the three dimensional structure and surface of Pru p 3. Molecular graphics in Fig. 1 were prepared with PyMOL 1.0 (pymol.sourceforge.net).

2.6. Determination of IL4 and IFNγ levels in TCL cultures

Fifty microliters of TCL-supernatants were used to quantify the cytokine levels by using matched antibody-pairs according to the manufacturer's instructions (sensitive limits: IL4, 0.6 pg/ml; IFNγ, 2 pg/ml; Immuno Tools, Friesoythe, Germany). Cultures containing TCLs with mitomycin-treated PBMCs alone served as negative controls. IFNγ/IL4 ratios over 10, between 2 and 10 and less than 2, were respectively considered as Th1-, Th0- and Th2- responses.

2.7. Phenotypic analysis of TCLs

To analyze T-cell phenotypes, cells were stained with 20 μL of anti-CD4-fluorescein isothiocyanate, anti-CD8 phycoerithrin (PE) and anti-CD3-Cy5-PE reagent mixture (Affinity BioReagents, CO, USA) for 20 min in the dark (300 × g, 5 min) and washing with PBS, cells were suspended in 300 μL PBS buffer, and analyzed in a FACSort flow cytometer with the CellQuest Pro software (BD Biosciences, Erembodegen, Belgium).

2.8. Statistical analysis

Proliferative responses (SI) to each antigen (Pru p 3 and each of the derived peptides) of patients and control subjects were compared (Fig. 2) by Mann–Whitney non-parametric U-test. Lev-

els of IL4 and IFNγ in supernatants of TCLs from patients and controls treated with Pru p 3 and each of the 17 derived peptides (joint analysis of the 17 samples; Fig. 3) were compared by Krustal–Wallis one-way nonparametric ANOVA. A level of significance <5% (*p* < 0.05) was considered to be significant in both analysis.

3. Results

3.1. T-cell epitope mapping: patients versus controls

PBMCs from 18 peach-allergic patients sensitized to Pru p 3 and 10 non-peach-allergic controls without Pru p 3 sensitization were cultured in the presence of the complete allergen or each of its 17-derived synthetic peptides. Overall, the proliferative response observed against these antigens were roughly more similar in patients (mean: 9714 cpm; range: 2440–23,327 cpm; background: 832 cpm) than in control subjects (mean: 11,325; range: 1331–20,005 cpm; background: 1859 cpm). The PBMC responses were analyzed by comparing the SI obtained with each antigen. The results are summarized in Table 1 and Fig. 2. Pru p 3 activated 7 out of 18 (39%) and 3 out of 10 (30%) of PBMCs from patients and controls, respectively, resulted similarly to those reported for other allergens and groups of patients (Glaspole et al., 2005; Tanabe, 2007). A fault in the cultures can be discarded, because commercial phytohemagglutinin (PHA) induced high responses in all PBMCs (mean: 37,787; range: 22,956–89,723 cpm; SI range: 27.6–107.8).

PBMCs from patients displayed different responses to the 17-derived synthetic peptides. Peptides 14 and 15 triggered the highest rate of activation (positive SI to at least one of them in 72% – 13 out of 18 – of PBMCs), being the only one reaching stimulation in more than 50% of the patients analyzed. Both contiguous peptides can
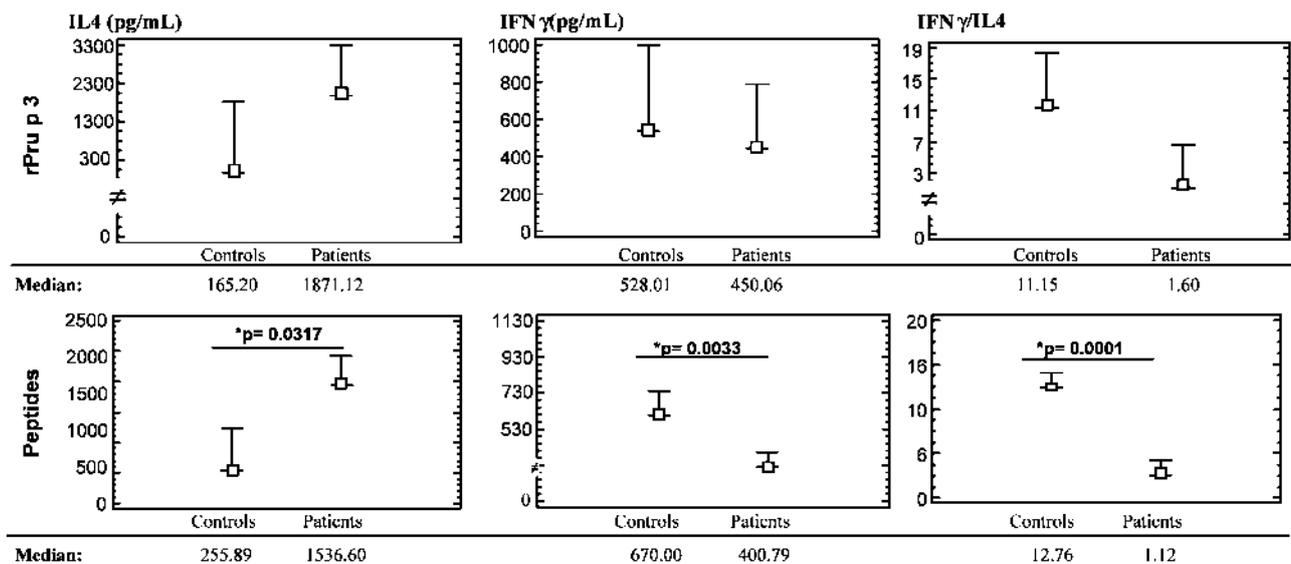
Fig. 2. Frequency (%) of positive levels of IL4 and IFNγ in TCL supernatants from patients and control subjects treated with rPru p 3 or its derived peptides (pooled data of the 17 synthetic peptides). Medians (□) and S.D.s (bars) were calculated from $n = 14$ for Pru p 3 and $n = 238$ for peptides. Significant differences ($p < 0.05$) are indicated (*).

(A)

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        1         10            20              30              40          50
        ITCGQVSSSLAPCIPYVRGGGAVPPACCNGIRNVNNLARTTPDRQAACNC
        51        60            70              80          91
        LKQLSASVPGVNPNNAAALPGKCGVSIPYKISASTNCATVK
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Table 1
Positive responses (stimulation index, SI) of PBMCs and TCLs from peach-allergic patients to purified Pru p 3 and synthetic peptides (1–17) covering its full amino acid sequence. The positive results are remarked by shadowings.

Peptide no./sequence	Patients no.	PBMCs 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	a*	TCLs 6	7	8	9	10	11	12	13	14	b*
	Pru p 3	2,1	2,0				2,3		2,7		2,0	2,9	2,2							39	3,8	3,1	3,4	3,1	3,1	3,5	3,1	3,5	3,1	100
1	ITCGQVSSSL								2,7						2,1	2,9				17		3,6	3,1							22
2	VSSSLAPCIP																			0										0
3	APCIPYVRGG												2,7							6										0
4	YVRGGGAVPP									3,1			2,5							11										0
5	GAVPPACCNG		2,2								2,0		2,0					2,2	2,2	28		3,3							3,5	22
6	ACCNGIRNVN		2,2							2,2			2,1			2,4				22		3,0		3,7		3,5	3,2	4,0	3,5	67
7	IRNVNNLART	2,1	2,3				3,2			2,8			2,0							28		5,9		3,4						22
8	NLARTTPDRQ		2,1				2,9				2,3			2,4						22		3,1		3,8						22
9	TPDRQAACNC							2,0	2,0		2,2			2,6	2,2	2,1				33	3,0	3,6		3,8						33
10	AACNCLKQLS	2,0				2,8	3,5	2,3		2,4					2,6	2,4				39									3,3	11
11	LKQLSASVPG					2,1	2,4	2,4			2,0			2,3		2,0				33			3,2			3,3			3,3	33
12	ASVPGVNPNN	2,2		2,0		2,8					2,2			2,3		2,3	2,3	2,0		45									3,7	11
13	VNPNNAAALP	2,4			2,2				2,4		2,4				3,1	2,4				33		5,1				3,7		3,1	3,1	44
14	AAALPGKCGV	3,0				3,3	2,6		2,3		2,0	4,0		2,5		2,7	8,3	9,8		56		5,5		3,5	3,9		4,0	3,7		56
15	GKCGVSIPYK	3,8	2,4			3,3	3,0	2,6			2,3	2,4	2,3		2,6			2,9		56		3,6	3,1	3,5	3,2	3,5		3,7		67
16	SIPYKISAST					3,9	2,5						2,1	2,8				2,5		28				3,7	3,0				3,1	33
17	ISASTNCATVK			2,3		2,9	3,2													17			3,2	3,0						22

A SI ≥ 2 for PBMCs and SI ≥ 3 for TCLs was considered positive, *a and *b are the frequency (%) of positive responses to Pru p 3 or to each peptide for PBMCs or TCLs, respectively.

IL4 (pg/mL)

3300
2300
1300
300
0

rPru p 3

≠

Controls   Patients

IFN γ(pg/mL)

1000
800
600
400
200
0

Controls   Patients

IFN γ/IL4

19
15
11
7
3
0

≠

Controls   Patients

Median: 165.20   1871.12   528.01   450.06   11.15   1.60

2500
2000
1500
1000
500
0

Peptides

*p= 0.0317

Controls   Patients

1130
930
730
530
0

*p= 0.0033

Controls   Patients

20
16
10
6
0

*p= 0.0001

Controls   Patients

Median: 255.89   1536.60   670.00   400.79   12.76   1.12

Fig. 3. Location on the 3-D structure of Pru p 3 of T-cell epitopes Pru p 3$_{25-35}$ (blue) and Pru p 3$_{65-80}$ (green). (A) Amino acid sequence of Pru p 3, remarking the T-epitopes described in the present study. (B) Ribbon diagram showing amino acid side chains. The labels denote the four helices and C terminus. (C) Molecular surface (wireframe) showing the areas covered by the T-cell epitopes (solid) at the orientation in B.

be considered as a single T-cell epitope: Pru p 3$_{65-80}$. The rest of peptides developed positive responses from 45% to 0% of PBMCs (Table 1). A similar pattern was found in the control group for most of the synthetic peptides, except for peptide 10 (Fig. 2, PBMCs), which provoked stimulation (SI $\geq$ 2) in 70% of control subjects, but only in 39% of PBMCs from patients. In spite of the statistical significance of this difference ($p = 0.0083$), the number of non-peach allergic subjects analyzed should be increased to define Pru p 3$_{45-55}$ (peptide 10) as a non-allergic T-cell epitope. On the other hand, Pru p 3$_{65-80}$ (peptides 14 and 15) was also recognized by approximately 90% of PBMCs from control subjects.

TCLs were produced by a long-time culture with Pru p 3 of PBMCs from nine patients and five controls subjects. Their proliferative response induced by the full allergen and its derived peptides are summarized in Table 1 (TCLs) and Fig. 2 (TCLs). The quantitative response was similar in both groups (patients, mean: 8813 cpm, range: 2493–30,304 cpm, background: 1048 cpm; controls, mean: 9120 cpm, range: 1232–41,638 cpm, background: 1253), as well as the T-cell phenotype, which showed the same CD4+/CD8+ ratio (65%/35%). In contrast with the behaviour exhibited by PBMCs, all the patients' TCLs were activated by Pru p 3 (100%), while only 20% were activated in control subjects.

Two main T-cell epitopes were identified analyzing the TCLs from patients: Pru p 3$_{65-80}$ (SI > 3 in 78% of lines), previously located in the screening of PBMCs, and Pru p 3$_{25-35}$ by TCLs and PBMCs (67% versus 22%), indicated a higher specificity and a lower activity than Pru p 3$_{65-80}$. The latter also stimulated all five TCLs from control subjects, which were also activated by Pru p 3$_{30-40}$ (peptide 7) and Pru p 3$_{50-60}$ (peptide 11) in four out of five (80%) samples. Interestingly, peptide 7 is contiguous and shares 5 amino acid residues with Pru p 3$_{25-35}$, the second T-cell epitope identified by analysis of TCLs from patients (see above). The same is true for peptide 11 with respect to Pru p 3$_{45-55}$ (peptide 10), which had the highest stimulation rate to PBMCs from control subjects.

Both of major T-cell epitopes of Pru p 3 were located on the 3-D structure of the peach allergen (Fig. 1) in the immediate vicinity of the IgE-binding regions described before (Garcia-Casado et al., 2003; Pacios et al., 2008). Pru p 3$_{25-35}$ spans virtually all the helix H2 (25–36) and hence, it shows alternating buried and exposed residues. Pru p 3$_{65-80}$ spans part of helix H4 (63–73) and a large seg-

ment of the C-terminal coil (74–91). Contrarily to Pru p 3$_{25-35}$, the latter T-cell epitope is mostly composed of exposed residues even though it shows predominance of hydrophobic amino acids. However, four residues (A66, L69, I77, and Y79) are almost completely buried.

3.2. IL4 and IFNγ levels in TCL cultures indicate a differential type of T-cell response to Pru p 3 in patients and control subjects

Supernatants of TCL cultures treated with Pru p 3 or its derived peptides were recovered to quantify the production of IL4 and IFNγ, used as molecular markers of a Th2 or Th1 response, respectively (Fig. 3). Patients' TCLs produced mainly IL4, with an IFNγ/IL4 ratio lower than 2. In contrast, control subjects yielded a high amount of IFNγ and an IFNγ/IL4 ratio higher than 11. These differences between patients and controls were non-statistically significant for Pru p 3, probably due to the limited number of samples analyzed, but were clearly significant ($p < 0.05$) for both IL4 and IFNγ levels, as well as the IFNγ/IL4 ratio, considering the pooled data observed in TCL cultures stimulated with each synthetic peptide (Fig. 3). This scenario was further confirmed by the analysis of cytokine levels in TCL supernatants treated with Pru p 3 (Table 2), indicated a Th2 type response in most patients, and a Th1 type in most control subjects. A Th0 response was detected in two cases from each the patient and control group.

4. Discussion

Allergies are on the rise, becoming an important health problem in western countries. Over 25% of the population suffers from IgE-mediated allergic symptoms (Burks and Ballmer-Weber, 2006). The search of new tools for diagnosis and treatment would improve the life-quality of patients. In the last decade, new concepts of specific immunotherapy have evolved based on recombinant expression of allergens. Hypoallergenic isoforms or mutants with reduced IgE-binding capacity would enable the use of a specific, safer and more efficient immunotherapy in the future (Woodfolk, 2007; Wagner et al., 2008).

The involvement of CD4+ T-lymphocytes in the hypersensitive type I reactions is well established (Abbas et al., 1996; Thomas et

Table 2
IL4 and INFγ levels in TCL supernatants from patients and control subjects after stimulation with rPru p 3 Cytokine.

Cytokine production[a]			
TCL derived from:	IFN-γ (pg/ml)	IL4 (pg/ml)	Th subset[b]
Patient 6	40	2,500	2
Patient 7	200	3,000	2
Patient 8	50	2,000	2
Patient 9	300	2,000	2
Patient 10	2200	10,000	2
Patient 11	800	1,000	2
Patient 12	50	50	0
Patient 13	110	3,000	2
Patient 14	300	40	0
Control 3	1100	26	1
Control 4	1600	150	1
Control 5	5000	700	1
Control 6	340	100	0
Control 7	100	50	0

[a] Means ($n = 3$).

[b] An INFγ/IL4 ratio >10, from 2 to 10, and <2 was considered a Th1, Th0, and Th2 response, respectively.

al., 2004). T-cells also contribute to induce tolerance to specific immunotherapy. During the vaccination process, a typical allergic Th2 response changes to a Th1, and, finally, a suppression of allergen-specific T-cell responses has been observed (Bohle, 2006, 2007; Woodfolk, 2007). Evaluation of immunotherapies using peptides is generating great expectations. The challenge with T-peptide epitopes would result in stimulation of the T-cells, but no activation of mast cells and basophiles (Haselden et al., 2001; Smith and Larche, 2004). Therefore, it seems crucial to identify the dominant T-cell epitopes of the major peach allergen Pru p 3, in order to develop a T-cell targeted vaccine to be used for allergic patients to this fruit.

The aim has been approached by proliferation assays of PBMCs and TCLs from clinically verified peach-allergic patients sensitized to Pru p 3. These PBMCs exhibited a moderate Pru p 3-specific activation (39%). This low-cellular response could be explained as a result of suppression mediated by immunoregulatory mechanism (Zeiler et al., 1999; Glaspole et al., 2005). However, a further step selecting Pru p 3-specific TCLs from some of the same patients, rendered CD4+ T-cell clones, most with a Th2 cytokine profile, and all of them activated by Pru p 3. Taking into account the results of the whole set of proliferation assays testing the synthetic peptides which covered the full Pru p 3 amino acid sequence, two T-cell epitopes have been uncovered. Pru p 3_{65-80}, defined by two consecutive immunodominant peptides, emerged as a major epitope recognized by 72% of PBMCs and over 78% of TCLs from patients. Pru p 3_{25-35} represents a second T-cell epitope, pointed out exclusively by TCLs (67% of positive responses). Remarkably, both sequential regions are localized in the 3-D structure of the peach allergen near its IgE epitopes previously described (Garcia-Casado et al., 2003; Pacios et al., 2008). Thus, Pru p 3_{65-80} overlaps a conformational IgE epitope (Pacios et al., 2008), whereas Pru p 3_{25-35} covers the first sequential (positions 23–36) epitope identified by Garcia-Casado et al. (2003). Pru p 3_{25-35} comprises alternating buried and exposed residues, whereas Pru p 3_{65-80} mostly faces the clashes with the solvent spite its hydrophobic residue predominance. However, the significance of buried residues in both T-cell epitopes suggests that sequential issues instead of precise surface location might be more important in the activation of the T-cell response.

Interestingly, the analysis of PBMCs and TCLs from 10 control volunteers without peach allergy non-sensitized to Pru p 3, roughly detected the same reactive synthetic peptides than those described above for the patient group. Pru p 3_{65-80} was the more active sequential region activating PBMCs and TCLs from the control group, and a second active amino acid region, Pru p 3_{30-40}, overlaps with the second T-cell epitope localized when assaying patients' TCLs. Additionally, Pru p 3_{50-60} was recognized by control subjects, but not by PBMCs or TCLs derived from patients. Potential T-cell epitopes of other allergens, such as Art v 1 and Bet v 1, have been reported to also stimulate T-cell from both patients and control subjects (Ebner et al., 1995; Haselden et al., 2001; Jahn-Schmid et al., 2002; Glaspole et al., 2005). This fact suggests that the switch to allergic tolerance must not operate in the antigen-T-cell recognition, but in a subsequent step. A higher IFNγ/IL4 ratio was observed in the controls in comparison with the patients' results. The majority of allergen-specific TCLs derived from allergic individuals displayed a Th2-like pattern of cytokine production, while non-allergic TCLs showed a Th1-like pattern of cytokines. This difference could explain the response of controls versus patients: since the former could tolerate Pru p 3, the latter would react by inducing an allergic response (Schade et al., 2000; Turcanu et al., 2008). The difference in cytokine responses between control and peach-allergic subjects does not seem to be based on different genetic backgrounds, because TCLs from both groups displayed a similar CD4+/CD8+ rate, and two control individuals allergic to grass pollen (control 5 and control 7 in Table 2) showed a Th1 and Th0 response, respectively, according to their IFNγ/IL4 ratio.

Summarizing, a limited number of T-cell epitopes are present in the peach allergen Pru p 3, the sequential Pru p 3_{65-80} being particularly relevant. These epitopes lead to activation of different Th subtypes in patients (Th2- response) and in peach non-allergic control subjects (Th1- response). The characterization of T-cell epitopes has implications for future developments in specific immunotherapy for type 1 allergy. The immunodominant T-cell epitope identified in Pru p 3 may be a suitable candidate for peptide treatment of peach allergy.

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