

Characterization of the allergic T-cell response to Pru p 3, the nonspecific lipid transfer protein in peach

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Background: Pru p 3, the nonspecific lipid transfer protein from peach, is an important plant food allergen that frequently induces systemic reactions.

Objective: We sought to analyze the allergic T-cell response to Pru p 3.

Methods: PBMCs from Italian and Spanish patients with peach allergy were stimulated with purified natural Pru p 3. Allergen-specific T-cell lines were used to identify T-cell epitopes of Pru p 3. Pru p 3-specific T-cell clones (TCCs) were analyzed for allergen-induced secretion of IL-4, IFN- γ , and IL-10 and expression of the integrin $\beta 7$, a receptor critical for gut homing. **Results:** No difference in T-cell responses of Italian and Spanish patients was found. Among several T cell-activating regions, Pru p 3₁₃₋₂₇, Pru p 3₃₄₋₄₈, Pru p 3₄₃₋₅₇, and Pru p 3₆₁₋₇₅ were most frequently recognized in 18 Pru p 3-specific T-cell lines. The majority of 32 Pru p 3-specific TCCs belonged to the T_H2 subset. In contrast to TCCs specific for other plant food and pollen allergens, only a limited number of Pru p 3-specific TCCs produced significant amounts of IL-10. The expression of integrin $\beta 7$ on Pru p 3-specific TCCs was comparable with that observed on peanut-specific TCCs and higher compared with that seen in different pollen-specific TCCs.

Conclusion: The T-cell response to Pru p 3 is dominated by T_H2 cells presumably primed in the gut. The identification of relevant T cell-activating regions provides a basis for engineering hypoallergenic variants of Pru p 3 with less IgE binding and retained T-cell stimulatory capacity for safe

immunotherapy of peach allergy. (*J Allergy Clin Immunol* 2009;124:100-7.)

Key words: Peach allergy, food allergy, nonspecific lipid transfer protein, Pru p 3, T cells, T_H1/T_H2, IL-10

IgE-mediated food allergy in adults is often triggered by plant foods, in particular fruits from the Rosaceae family, such as apples and peaches. In the Mediterranean population nonspecific lipid transfer proteins (nsLTPs) have been identified as predominant allergens in Rosaceae fruits, whereas they are of minor relevance in Central and Northern Europe.¹⁻⁴ nsLTPs are also contained in various other foods, such as nuts, citrus fruits, grapes, and various vegetables (eg, tomatoes, maize, asparagus, cabbage, and lettuce).⁵ In addition to the oral allergy syndrome, nsLTPs frequently induce more severe systemic reactions, including urticaria and life-threatening anaphylaxis.^{4,6,7} This has been linked to their high resistance to protease treatment, as well as to technologic and thermal processing.⁸⁻¹²

Pru p 3, the major peach allergen in the Mediterranean area, is among the best-characterized allergenic members of the nsLTP family. Pru p 3 has been cloned, sequenced, and produced as a recombinant allergen in different expression systems.¹³⁻¹⁷ IgE-binding epitopes of Pru p 3 have been investigated, and its 3-dimensional crystal structure has been described.¹⁸⁻²⁰ However, despite this detailed characterization, information on the T-cell response to nsLTP is limited. Allergen-specific T lymphocytes play a pivotal role in the induction and maintenance of type I allergy. To understand the sensitization process to an allergen, it is therefore important to analyze its immune reactivity at the level of specific T cells.

The aim of this study was to characterize the allergic T-cell response to Pru p 3. For this purpose, PBMCs isolated from Italian and Spanish patients with peach allergy were tested for proliferative responses to purified natural Pru p 3.²¹ PBMCs from patients with pollen allergy and peach tolerance served as control subjects. Pru p 3-specific T-cell lines (TCLs) and T-cell clones (TCCs) isolated from patients with peach allergy were used to identify T-cell epitopes by using 12-mer peptides representing the entire amino acid (aa) sequence of Pru p 3.¹³ The cytokine production patterns of Pru p 3-specific TCCs in response to allergen-specific stimulation were analyzed.

Because of their high resistance to gastric digestion, nsLTPs have been considered true food allergens that cause sensitization through the gastrointestinal route.^{12,17,22,23} However, a possible inhalant sensitization to Pru p 3 has also been discussed.^{24,25} To investigate the means of sensitization to Pru p 3, we analyzed the expression of integrin $\alpha 4\beta 7$ on Pru p 3-specific TCCs. Integrin $\alpha 4\beta 7$ binds to the key intestinal mucosal addressin cell adhesion

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Abbreviations used

aa: Amino acid
CLA: Cutaneous lymphocyte antigen
FITC: Fluorescein isothiocyanate
nsLTP: Nonspecific lipid transfer protein
SI: Stimulation index
TCC: T-cell clone
TCL: T-cell line

molecule 1 and is critical for lymphocyte gut homing.^{26,27} Integrin expression on Pru p 3–specific TCCs was compared with that on TCCs specific for other true plant food allergens (ie, Ara h 1 from peanut) and TCCs specific for different pollen allergens.

METHODS

Allergenic proteins and peptides

Natural Pru p 3 was purified as previously described.²¹ For epitope-mapping experiments, a panel of 27 synthetic dodeka-peptides overlapping for 9 aa residues and representing the complete aa sequence of Pru p 3.0101 (Mimotopes/Biotrend, Köln, Germany) was used.¹³ Natural Ara h 1 was a kind gift from Clare Mills (IFR Norwich, Norwich, United Kingdom) and purified as previously described.²⁸ Single batches of Pru p 3 and Ara h 1, both containing less than 0.03 EU/100 µg of protein, as determined with the Limulus Amoebocyte Lysate assay (BioWhittaker, Walkersville, Md), were used throughout the study.

Patients

In total, 18 patients with peach allergy (12 female and 6 male patients with a mean age of 29 years) were included in the study. All patients provided written consent before enrollment in the study, which was approved by institutional ethical committees. Peach-specific and Pru p 3–specific IgE values were evaluated by means of CAP/RAST (Phadia, Uppsala, Sweden). IgE values and clinical symptoms to peach of all patients are summarized in Table I. After consumption of peach, 6 patients had experienced solely local reactions (ie, the oral allergy syndrome), and 12 patients had systemic reactions, such as urticaria, angioedema, or rhinitis. All patients had positive skin prick test responses to commercial peach extract and positive prick-to-prick test responses with fresh peaches. Four Austrian patients with grass pollen allergy and peach tolerance (CAP/RAST to peach and Pru p 3, <0.35 kU/L; data not shown) served as control subjects.

Proliferation assays

PBMCs (2×10^5) were cultured in triplicate in 96-well plates (Nunc; Nunc, Roskilde, Denmark) in 200 µL of serum-free Ultra Culture Medium (BioWhittaker) supplemented with 2 mmol/L glutamine and 2×10^{-5} mol/L 2-mercaptoethanol in medium alone or in the presence of titrated concentrations of Pru p 3 for 7 days. Proliferation was measured by adding tritiated thymidine during the last 16 hours of culture.

Pru p 3–specific TCLs

Pru p 3–specific TCLs were generated by stimulating PBMCs (1.5×10^6) with 25 µg/mL Pru p 3 in 24-well flat-bottomed culture plates (Costar, Cambridge, Mass) under conditions as described above. Cultures without Pru p 3 served as controls. After 5 days, suboptimal doses of human rIL-2 (10 U/mL; Boehringer Ingelheim, Mannheim, Germany) were added. At day 7, T-cell blasts were harvested by means of density gradient centrifugation and subjected to limiting dilution to isolate TCCs. The remaining T-cell blasts of TCLs established with or without Pru p 3 were expanded by using irradiated PBMCs and IL-2. Ten days after the last feeding, TCLs were stimulated in duplicate with Pru p 3 (10 µg/mL) in the presence of 1×10^5 irradiated (60 Gy)

autologous PBMCs. Pru p 3–reactive TCLs were stimulated with each of the twenty-seven 12-mer peptides (5 µg/mL) to identify T-cell epitopes. After 48 hours, proliferation was evaluated as described above. The stimulation index (SI) was calculated as the ratio between counts per minute obtained in cultures with TCLs plus autologous PBMCs plus stimulus and counts per minute obtained in cultures containing TCLs and PBMCs.

Pru p 3–specific TCCs

T-cell blasts from Pru p 3–reactive TCLs were seeded by means of the limiting dilution technique in 96-well round-bottom plates (Nunc) in the presence of 2×10^5 irradiated PBMCs, 0.5% vol/vol PHA (Gibco, Grand Island, NY), and IL-2 (4 U per well) in the medium described above. The specificity of grown microcultures was assessed in proliferation assays by using 10 µg/mL Pru p 3. Thereafter, Pru p 3–reactive TCCs were expanded by alternating turns of stimulation with irradiated PBMCs plus allergen or IL-2. Epitope-mapping experiments were performed as described above. For induction of cytokine secretion, TCCs were washed and incubated with irradiated autologous PBMCs in the presence of Pru p 3 (10 µg/mL) in duplicate for 48 hours. Cultures containing TCCs and PBMCs in medium alone served as negative controls. Cytokine levels in supernatants were measured by means of ELISA with matched antibody pairs (Endogen, Woburn, Mass), according to the manufacturer's instructions (limits of detection: IL-4, 9 pg/mL; IFN-γ, 9.5 pg/mL; IL-10, 4.7 pg/mL). Cytokine levels in control cultures were subtracted from those determined in nonstimulated cultures. TCCs were assigned to T_H subsets as follows: T_H2, IL-4/IFN-γ, greater than 5; T_H1, IL-4/IFN-γ, less than 0.2; and T_H0, IL-4/IFN-γ, 0.2 to 5.

Cryopreserved TCCs specific for different pollen allergens were available from previous studies.^{29–32} These TCCs were thawed, expanded, and stained for integrin expression. Peanut allergen-specific TCCs were obtained by means of limiting dilution from TCLs generated by stimulating PBMCs from patients with peanut allergy and sensitization with Ara h 1 (25 µg/mL), according to the protocol described above. Specificity was tested by using Ara h 1 (5 µg/mL).

Flow cytometry

Allergen-specific TCCs were stained with CD4–fluorescein isothiocyanate (FITC), CD8–phycoerythrin, CD3–peridinin-chlorophyll-protein, TCR α/β–FITC, cutaneous lymphocyte antigen (CLA)–FITC, and anti-human integrin β7–phycoerythrin (all from PharMingen, San Diego, Calif) and CD49d–FITC and CD103–FITC (both from Coulter Immunotech, Marseille, France). Isotype-matched antibodies were used as controls. Analysis was performed on a FACStar plus (Beckton Dickinson, San Jose, Calif) with CELLquest Software, version 3.3. In pilot experiments some TCCs were stained 24 hours and 7 days after allergen-specific stimulation. No difference in the expression of CLA, integrin β7, CD49d, and CD103 was observed, indicating an independence of the activation state. Thereafter, all TCCs were stained 7 days after allergen-specific stimulation.

RESULTS

Patients with peach allergy

The clinical data of the 10 Italian (I1–I10) and 8 Spanish (S1–S8) patients with peach allergy are summarized in Table I. All patients experienced allergic reactions to consumption of peach and showed positive CAP/RAST results to peach. None of the individuals was exclusively sensitized to peach. The majority of patients were allergic to other foods, respiratory allergens, or both. Sensitization to peach nsLTP was confirmed by means of Pru p 3–specific CAP/RAST (Table I).

Primary responses to Pru p 3

PBMCs from 10 patients with peach allergy were incubated with titrated concentrations of Pru p 3 (Fig 1). Four Austrian

TABLE I. Clinical data of the patient population

Patient ID	Sex	Age (y)	IgE (kU/L) specific for:		Symptoms to peach*	Other sensitizations†	
			Peach	nPru p 3		Respiratory	Food
I1	Female	16	44.0	54.0	OAS, URT	G	eg, le, to
I2	Female	23	26.0	29.6	AE, URT	A, J	le, me, to, wa
I3	Female	44	0.8	3.1	OAS, URT	G	ar, le, to, wa
I4	Female	24	59.5	24.6	AE, URT	T	ar, ha, le
I5	Male	36	6.9	7.6	OAS	C, G, J, O	ar, to
I6	Male	14	0.9	10.1	OAS	B, C, G, J, M, O	ap, ce, ha, pe
I7	Female	26	4.8	6.8	OAS, URT	A, D, G, J, M	ha, le, or, to
I8	Female	26	11.3	4.6	OAS	C, D, G, J, O, P	ar, pl
I9	Male	14	13.5	4.2	URT	G, J, M	no
I10	Female	31	7.5	1.9	URT	G	ha, eg, pn
S1	Female	43	10.5	11.7	OAS	A, D, G, M	eg, fi, gr, le, to
S2	Male	46	0.6	0.5	AE, URT	G, J, M, O	ap, ha, pe
S3	Female	30	12.0	8.7	OAS, URT	O	me
S4	Male	30	2.9	1.3	URT	J, T, O	ha
S5	Female	23	5.5	4.3	OAS	no	ba, to
S6	Female	26	6.4	4.0	URT	G, T	f
S7	Female	41	0.8	0.4	RHIN	J	no
S8	Male	35	3.7	3.7	OAS	no	ap, pe

*OAS, Oral allergy syndrome; URT, urticaria; AE, angioedema; RHIN, rhinitis.

†G, Grasses; eg, eggplant; le, lettuce; to, tomato; A, *Alternaria* species; J, cypress; me, melon; wa, walnut; ar, apricot; T, plane; ha, hazelnut; C, cat; O, olive; B, birch/hazel; M, mugwort; ap, apple; ce, celery; pe, pear; D, mites; or, orange; P, *Parietaria* species; pl, plum; pn, peanuts; fi, fig; gr, grape; ba, banana; f, fish.

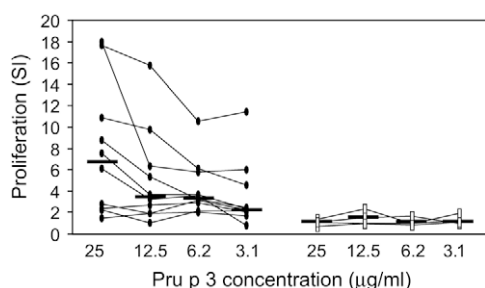


FIG 1. Lymphoproliferation of PBMCs from patients with (solid circles) and without (open circles) peach allergy to titrated concentrations of Pru p 3. SIs are shown. The symbols represent individual patients, and the horizontal lines indicate the median.

patients with grass pollen allergy but without peach allergy served as control subjects. In donors with peach allergy, a dose-dependent response to Pru p 3 was observed, and 25 µg/mL induced the strongest proliferation. Control subjects showed very weak proliferative responses (Fig 1). By using 25 µg/mL Pru p 3, proliferation ranged from 5,210 to 48,676 cpm (median, 21,894 cpm) in patients with peach allergy and from 2,078 to 2,451 cpm (median, 2,166 cpm) in control subjects. Background levels ranged from 604 to 14,454 cpm in allergic subjects and from 1,536 to 1,819 in control subjects.

T-cell epitopes of Pru p 3

Eighteen Pru p 3-specific TCLs (SI >2) obtained from Italian and Spanish patients with peach allergy were stimulated with 27 overlapping 12-mer peptides covering the complete sequence of Pru p 3. None of the control cultures generated in parallel without Pru p 3 proliferated in response to the peach allergen. Considering an SI of greater than 2.5 as positive, multiple T-cell epitopes distributed over the entire peach allergen were identified (Fig 2). The peptides Pru p 3₂₈₋₃₉ and Pru p 3₇₆₋₈₇ were recognized by

1 patient each. The peptides Pru p 3₁₋₁₂, Pru p 3₄₋₁₅, Pru p 3₁₀₋₂₁, Pru p 3₁₉₋₃₀, Pru p 3₅₅₋₆₆, Pru p 3₅₈₋₆₉, and Pru p 3₇₀₋₈₁ were recognized by 2 patients each. Three patients harbored T cells specific for epitopes located at Pru p 3₇₋₁₈, Pru p 3₄₉₋₆₃, Pru p 3₅₈₋₆₉, and Pru p 3₆₇₋₇₈. Four patients recognized an epitope located at Pru p 3₃₁₋₄₂ and Pru p 3₄₀₋₅₁. In 6 of 18 TCLs, the peptides Pru p 3₁₃₋₂₇ and Pru p 3₄₃₋₅₇ were recognized, and 8 of 18 patients harbored T cells specific for Pru p 3₃₄₋₄₈. Amino acid stretches 22 to 36 and the C-terminal part (aa range 73 to 91; except for peptide Pru p 3₇₆₋₈₇ for 1 individual) seemed not to be involved in T-cell activation. Nine patients recognized an epitope located at Pru p 3₆₁₋₇₅. Except for patient I4, each patient recognized more than 1 epitope (Fig 2). No differences in epitope recognition between Italian (I1-I10) and Spanish (S1-S8) patients were observed.

Phenotype and epitope recognition of Pru p 3-specific TCCs

In total, 32 Pru p 3-specific TCCs were isolated from 11 patients with peach allergy (Table II). All TCCs were CD4⁺TCRαβ⁺ (data not shown). Analysis of cytokine patterns in response to allergen-specific stimulation revealed that 16 of 32 TCCs belonged to the T_H2 subset, 4 of 32 to the T_H1 subset, and 12 of 32 to the T_H0 subset. Epitope-mapping experiments could be performed with 21 Pru p 3-specific TCCs from 8 patients (Table II). Two TCCs were reactive with an epitope located within Pru p 3₄₋₁₅ (GVSSALAPCIP), 4 within Pru p 3₁₃₋₂₇ (CI PYVRGGGAVPPAC), 1 within Pru p 3₃₄₋₄₈ (VNNLARTTPDR QAAC), 1 within Pru p 3₃₇₋₄₈ (LARTTPDRQAAC), 3 within Pru p 3₅₈₋₇₂ (VPGVNPNNAAAL), 1 within Pru p 3₆₁₋₇₂ (VNPNNAAALPGK), and 1 within Pru p 3₆₄₋₇₅ (NNAALPGKCGV). The results at the clonal level confirmed that the most prevalent T cell-activating regions of Pru p 3 are located at aa sequence 13 to 27 (reactive with 4 TCCs from 3 different patients), aa sequence 34 to 48 (reactive with 2 TCCs from 2 different patients), and aa sequence 58 to 75 (reactive with 5 TCCs from 3

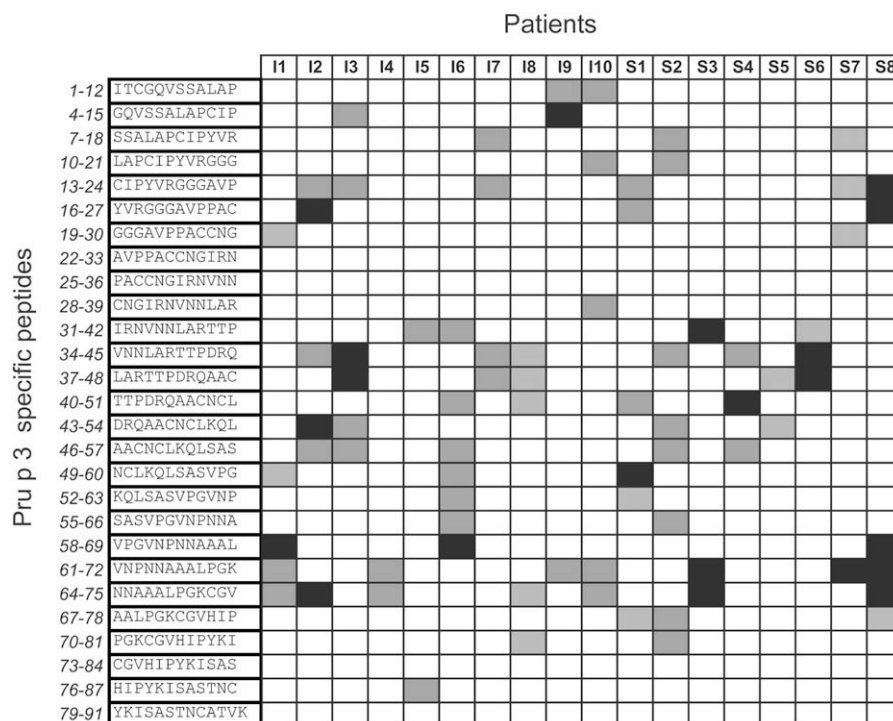


FIG 2. T-cell epitopes of Pru p 3. One Pru p 3-specific TCL from each Italian (I1-I10) and Spanish (S1-S8) donor with peach allergy was stimulated with 27 overlapping peptides representing the aa sequence of the allergen. Peptides inducing SIIs of greater than 2.5 are shown as gray boxes, and peptides inducing SIIs of greater than 5 are shown as black boxes.

different patients). Eight Pru p 3-specific TCCs from 4 different patients did not react with any of the 12-mer peptides tested despite strong reactivity with the entire peach protein. These data indicated the existence of additional T-cell epitopes that were not detectable by using synthetic 12-mer peptides representing the aa sequence of the isoform Pru p 3.0101.¹³

IL-10 production of Pru p 3-specific TCCs

We noted that the frequency of Pru p 3-specific TCCs that synthesized noticeable IL-10 in response to allergen-specific stimulation was by far lower (1/16 T_H2, 0/12 T_H0, and 1/4 T_H1 clones; Table II) compared with that previously reported for TCCs specific for the major birch pollen allergen Bet v 1 (11/19 T_H2, 5/13 T_H0, and 0/1 T_H1 clones³³ and 4/9 T_H2, 0/2 T_H0, and 1/1 T_H1 clones³⁴) and the major celery allergen Api g 1 (3/7 T_H2, 0/3 T_H0, and 1/3 T_H1 clones).³⁵ Cytokine responses of 10 TCCs specific for the major allergen from hazelnut Cor a 1 and not cross-reactive with birch and hazel pollen allergens³⁶ and those of 10 TCCs specific for the major mugwort pollen allergen Art v 1³⁰ available from previous studies were analyzed to further investigate the general relevance of this finding (Table III). Based on the data obtained for Pru p 3-specific TCCs, we arbitrarily defined the synthesis of 100 pg/mL IL-10 as a cutoff level for IL-10 positivity (Table II). Overall, 60% of Cor a 1-specific TCCs (3/3 T_H2, 1/4 T_H0, and 2/3 T_H1 clones) and 40% of Art v 1-specific TCCs (1/4 T_H2, 2/4 T_H0, and 1/2 T_H1 clones) produced IL-10 in response to allergen-specific stimulation. These data confirmed that the number of IL-10-producing Pru p 3-specific TCCs was much lower compared with the number of TCCs specific for other plant food and respiratory allergens.

Expression of integrin β7 on Pru p 3-specific TCCs

In total, 17 Pru p 3-specific TCCs from 10 patients with peach allergy were analyzed for their surface expression of integrin α4 (CD49d), integrin β7, and CD103, a member of the integrin family expressed on intraepithelial lymphocytes.³⁷ In addition, we stained for the CLA-identifying T lymphocytes functionally involved in the pathogenesis of different T cell-mediated cutaneous diseases.³⁸ Furthermore, 2 TCCs specific for Ara h 1 from peanut (a true food allergen), 3 TCCs specific for Bet v 1, 3 TCCs specific for Art v 1, and 1 TCC specific for Phl p 2 from timothy grass (all respiratory allergens) were analyzed. All TCCs were highly positive for integrin α4 and none for CD103 or CLA (data not shown). The fluorescence intensity of integrin β7 on representative examples of allergen-specific TCCs is summarized in Fig 3. In general, Pru p 3-specific TCCs expressed integrin β7 with an intensity comparable with that of TCCs specific for Ara h 1, whereas TCCs specific for respiratory allergens expressed integrin β7 with less intensity.

DISCUSSION

We sought to evaluate the allergic T-cell response to Pru p 3 to gain more insights into the sensitization process of this important food allergen. For this purpose, we analyzed epitope recognition, cytokine responses, and the expression of integrin β7 of peripheral Pru p 3-specific T cells derived from patients with peach allergy.

In total, 18 T cell-activating regions of Pru p 3 were identified by using oligoclonal Pru p 3-specific T-cell cultures from Italian and Spanish patients with peach allergy and synthetic peptides

TABLE II. Phenotype and epitope recognition of Pru p 3-specific TCCs

	TCCs	IL-4 (pg/mL)	IFN- γ (pg/mL)	IL-10 (pg/mL)	Subset	Recognized peptide	aa Sequence
1	I1 21	20.4	57.9	<4.7	T _H 0	ND	
2	I1 95	609	<9.5	<4.7	T _H 2	VPGVNPNNAALPGK	58-72
3	I2 25	460	20.8	577	T _H 2	ND	
4	I2 42	9.7	56.5	<4.7	T _H 1	Neg	
5	I2 52	122	1480	108	T _H 1	CIPYVRGGGAVPPAC	13-27
6	I2 162	<9.0	<9.5	<4.7	T _H 0	NNAAALPGKCGV	64-75
7	I2 190	583	<9.5	5.0	T _H 2	Neg	
8	I3 14	461	<9.5	<4.7	T _H 2	ND	
9	I3 28	706	<9.5	<4.7	T _H 2	LARTTPDRYAAC	37-48
10	I3 29	142	<9.5	<4.7	T _H 2	GQVSSALAPCIP	4-15
11	I3 178	<9.0	<9.5	<4.7	T _H 0	ND	
12	I4 29	95.0	15.3	<4.7	T _H 2	VNPNNAALPGK	61-72
13	I4 55	79.7	22.8	<4.7	T _H 0	VPGVNPNNAALPGK	58-72
14	I4 57	206	<9.5	<4.7	T _H 2	ND	
15	I4 84	465	64	<4.7	T _H 2	VPGVNPNNAALPGK	58-72
16	I5 100	667	<9.5	9.2	T _H 2	Neg	
17	I5 109	425	<9.5	<4.7	T _H 2	Neg	
18	I5 162	476	<9.5	<4.7	T _H 2	ND	
19	I6 31	53.3	<9.5	<4.7	T _H 2	Neg	
20	I6 74	22.2	96.5	9.5	T _H 0	Neg	
21	I6 225	<9.0	<9.5	<4.7	T _H 0	ND	
22	I7 2	311	13.6	<4.7	T _H 2	VNNLARTTPDRQYYC	34-45
23	I7 8	35.0	<9.5	<4.7	T _H 2	CIPYVRGGGAVPPAC	13-27
24	I7 170	<9.0	179	<4.7	T _H 1	ND	
25	I8 63	<9.0	<9.5	<4.7	T _H 0	ND	
26	I9 18	<9.0	<9.5	<4.7	T _H 0	GQVSSALAPCIP	4-15
27	I9 45	246	<9.5	<4.7	T _H 2	Neg	
28	I9 123	32.5	24.7	<4.7	T _H 0	Neg	
29	I9 135	<9.0	47.5	<4.7	T _H 1	CIPYVRGGGAVPPAC	13-27
30	I9 147	28.9	74.0	<4.7	T _H 0	CIPYVRGGGAVPPAC	13-27
31	I10 4	<9.0	<9.5	<4.7	T _H 0	ND	
32	S1 57	<9.0	<9.5	<4.7	T _H 0	ND	

ND, Not determined; Neg, no proliferative response to any peptide under investigation.

TABLE III. Cytokine profiles of TCCs specific for plant food and pollen allergens

	TCCs	IL-4 (pg/mL)	IFN- γ (pg/mL)	IL-10 (pg/mL)	Subset*	Specific for†:
1	P1 2	<9.0	>1,000	174	T _H 1	Cor a 1
2	P1 55	82.6	21.3	381	T _H 0	Cor a 1
3	P2 32	263	37.6	430	T _H 2	Cor a 1
4	P3 32	255	<9.5	>1,000	T _H 2	Cor a 1
5	P4 4	268	57.6	484	T _H 2	Cor a 1
6	P4 30	31.1	85.2	13.9	T _H 0	Cor a 1
7	P5 134	<9.0	>1,000	237	T _H 1	Cor a 1
8	P6 98	137	791	79.1	T _H 1	Cor a 1
9	P7 16	<9.0	<9.5	<4.7	T _H 0	Cor a 1
10	P7 28	8.5	<9.5	<4.7	T _H 0	Cor a 1
11	P8 96	<9.0	<9.5	200	T _H 0	Art v 1
12	P8 104	<9.0	88	260	T _H 1	Art v 1
13	P8 280	208	13.5	16.2	T _H 2	Art v 1
14	P9 41	526	309	240	T _H 0	Art v 1
15	P10 142	336	<9.5	<4.7	T _H 2	Art v 1
16	P10 218	<9.0	<9.5	<4.7	T _H 0	Art v 1
17	P11 84	190	<9.5	<4.7	T _H 2	Art v 1
18	P11 295	<9.0	474	87.3	T _H 1	Art v 1
19	P12 24	387	15.1	>1,000	T _H 2	Art v 1
20	P12 41	509	502	99.1	T _H 0	Art v 1

*Subsets were defined as described in the Methods section.

†Cor a 1.04 is the major allergen in hazelnuts, and Art v 1 is the major mugwort pollen allergen.

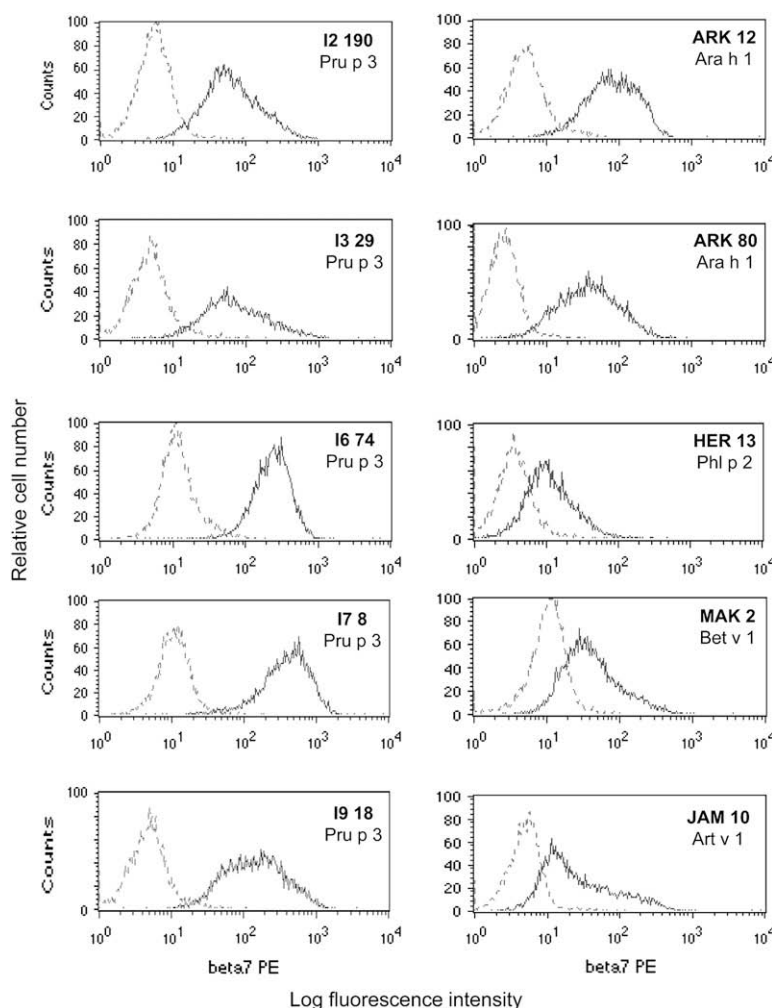


FIG 3. Expression of integrin $\beta 7$ on allergen-specific T cells. TCCs specific for allergens in peach (Pru p 3), peanut (Ara h 1), and pollen from timothy grass (Phl p 2), birch (Bet v 1), and mugwort (Art v 1) were stained for integrin $\beta 7$. The x-axis of each histogram shows staining with anti-human integrin $\beta 7$ (solid line) and the matched isotype control (dashed line) in a gate set on $CD4^+$ cells.

representing the aa sequence of isoform Pru p 3.0101 (Fig 2).¹³ No differences in epitope recognition patterns between Italian and Spanish patients were found (Fig 2). Of note, 17 of 18 patients under investigation recognized more than 1 T-cell epitope, although Pru p 3 consists of only 91 aa residues. Three regions (aa regions 13-27, 34-48, and 43-57) were recognized by more than 30% and Pru p 3₆₁₋₇₅ was recognized by 50% of the 18 patients under investigation. The aa region 61 to 75 is identical among the different Pru p 3 isoforms.^{13,14,16} Therefore Pru p 3₆₁₋₇₅ can be considered the immunodominant T cell-activating region of Pru p 3. Analyzing the peptide recognition of Pru p 3-specific TCCs confirmed that the defined aa regions contained relevant T-cell epitopes (Table II). Some TCCs expanded in response to purified natural Pru p 3 showed strong reactivity with the entire allergen but did not respond to any of the 12-mer peptides used for epitope mapping (Table II). This suggests the existence of additional T-cell epitopes in other isoforms of Pru p 3.

The IL-4 and IFN- γ levels produced by Pru p 3-specific TCCs after allergen-specific stimulation reflected a T_H2 -dominated response characteristic for type I hypersensitivity (Table II). Interestingly, only 6% of Pru p 3-specific TCCs synthesized the

immunosuppressive cytokine IL-10. This observation strikingly contrasted to findings in TCCs specific for other plant food allergens, such as Api g 1 and Cor a 1, or major pollen allergens, such as Bet v 1 and Art v 1, of which 40% to 60% synthesized more than 100 pg/mL IL-10 (Table III).^{34,35} In allergic individuals allergen-specific IL-10-producing $CD4^+$ T cells have been mainly considered regulatory T cells induced by means of repeated allergen administration during specific immunotherapy³⁹⁻⁴¹ or during natural allergen exposure through repeated bee stings.⁴² Usually, regulatory T cells do not proliferate in response to antigen-specific stimulation *in vitro*. However, all our IL-10-producing allergen-specific TCCs easily expanded from PBMCs because they proliferated well in response to allergens. Thus these cultures do not show typical features of regulatory T cells but rather appear to be allergen-specific IL-10-secreting effector T cells. We speculate that this T-cell subset is induced on repeated allergen exposure, such as through daily inhalation of pollen allergens during the pollen season and the annual occurrence of the pollen season. Actually, enhanced numbers of IL-10⁺ lymphocytes during the birch pollen season have been detected in patients with birch pollen allergy.⁴³ The lack of Pru p 3-specific IL-10-producing

effector T cells in patients with peach allergy might result from the fact that these individuals had not been exposed to peach for a longer time period. Indeed, all patients under investigation had been advised to strictly avoid the consumption of peach to prevent possible severe anaphylactic reactions.⁴⁴

Peripheral lymphocytes usually recirculate to the site of activation (eg, T cells primed in intestinal sites preferentially migrate to the gut), which is guided by specific homing receptors.⁴⁵ Integrins of the $\beta 7$ family are upregulated on gut-homing effector and memory cells.^{27,46,47} It has been shown that peripheral T lymphocytes specific for milk allergens expressed high levels of $\alpha 4\beta 7$.⁴⁸ To determine whether peripheral Pru p 3-specific T cells were prone to gut homing, we analyzed the expression of integrin $\beta 7$ on Pru p 3-specific TCCs. Their staining intensity was similar to the expression of integrin $\beta 7$ on T cells specific for peanut allergens (true plant food allergens) and higher compared with TCCs specific for different respiratory allergens (Fig 3). It has previously been shown that CD4⁺ T cells from patients with milk-induced urticaria expressed CLA on *in vitro* stimulation with milk allergens.^{49,50} All Pru p 3-specific TCCs were negative for CLA, including those isolated from patients with peach-induced urticaria. We conclude that nsLTP-specific T lymphocytes preferentially migrate to intestinal sites, where they presumably have been primed. These findings support that nsLTPs are true food allergens sensitizing individuals through the gastrointestinal route.

In summary, the allergic T-cell response to the nsLTP from peach was dominated by TH2 cells that had presumably been primed in the gut. Previously, Pru p 3₂₃₋₃₆, Pru p 3₃₉₋₄₄, and Pru p 3₈₀₋₉₁ have been suggested as potential antibody recognition sites,¹⁸ and the aa residues L₃₇R₃₉T₄₀P₄₂D₄₃R₄₄A₄₆P₇₀S₇₆P₇₈Y₇₉ have been identified as a conformational IgE epitope recognized by individuals with peach-induced hypersensitivity reactions.²⁰ In this study we identified several T-cell epitopes, among which Pru p 3₁₃₋₂₇, Pru p 3₄₃₋₅₇, Pru p 3₃₄₋₄₈, and Pru p 3₆₁₋₇₅ were most relevant. Together, these data might form the basis for engineering Pru p 3 variants with reduced IgE reactivity and retained T cell-activating capacity. Such hypoallergens should not induce IgE-mediated side effects and thus could be used for specific immunotherapy of peach allergy, which, at present, does not exist.⁵¹

Clinical implications: The knowledge of T-cell epitopes is important to retain the T-cell stimulatory capacity of novel vaccines designed for safe and efficient specific immunotherapy.

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