Oral exposure to Mal d 1 affects the immune response in patients with birch pollen allergy

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Background: Antibodies and T cells specific for the major birch pollen allergen Bet v 1 cross-react with structurally related food allergens, such as Mal d 1 in apple.

Objective: We sought to evaluate the effects of oral uptake of Mal d 1 on the allergen-specific immune response in patients with birch pollen allergy.

Methods: Patients received 50 μg of rBet v 1 sublingually on 2 consecutive days outside of the birch pollen season. One year later, equal amounts of rMal d 1 were administered. Blood samples were collected before and after oral exposure, as well as before and after the intermediate birch pollen season. Allergen-specific IgE levels were determined by using ImmunoCAP. Proliferation of allergen-stimulated PBMCs was assessed, as well as the expression of IL-5, IL-13, IL-10, IFN-γ, and forkhead box protein 3 (Foxp3) in isolated T cells (real-time PCR). Allergen-specific T-cell lines were analyzed for epitope recognition.

Results: Orally administered Bet v 1 transiently reduced Bet v 1–specific serum IgE levels, as well as Bet v 1– and Mal d 1–induced T-cell proliferation, and enhanced the expression of IL-5, IL-10, and Foxp3. Orally applied Mal d 1 significantly decreased Bet v 1– and Mal d 1–specific IgE levels and induced IL-5 and IL-10 but no Foxp3 expression. In contrast to Bet v 1, Mal d 1 triggered IFN-γ production and T cells with a different epitope repertoire. Inhalation of birch pollen significantly enhanced allergen-specific IgE levels, T-cell proliferation, and IL-5, IL-10, IL-13, and Foxp3 expression.

Conclusion: Two sublingual administrations of 50 μg of Mal d 1 were well tolerated and induced transient immune responses seen during peripheral tolerance development. Thus recombinant Mal d 1 might be suitable and relevant for sublingual treatment of birch pollen–related apple allergy.
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Key words: *Birch pollen allergy, food allergy, oral allergy syndrome, Bet v 1, Mal d 1, IgE, T cells*

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More than 70% of patients with birch pollen allergy have allergic reactions to stone fruits, hazelnuts, certain vegetables, and legumes, making birch pollen–related food allergy a very common IgE-mediated plant food allergy among adolescents and adults in northern and central Europe, as well as North America.[1,2] Birch pollen–related food allergy mainly results from immunologic cross-reactivity between the major birch pollen allergen Bet v 1 and homologous dietary allergens, such as Mal d 1 in apple.[2,3] Bet v 1 and Mal d 1 share 65% amino acid (aa) sequence identity and the typical Bet v 1 fold.[4,5] As a consequence, a proportion of Bet v 1–specific IgE antibodies recognize Mal d 1, which can therefore trigger an allergic reaction on ingestion, with symptoms such as itching, burning, and edema formation typically confined to the oral cavity and directly at the site of contact (oral allergy syndrome [OAS]). Mal d 1 also activates Bet v 1–reactive T cells to proliferate and produce cytokines.[6,7] In contrast to IgE-cross-reactivity, T-cell cross-reactivity is not abolished by gastrointestinal degradation or heat treatment of the apple allergen.[8,9] Thus ingested Bet v 1–related food allergens might activate Bet v 1–specific T cells *in vivo* and induce their migration to target organs, such as the skin. In fact, consumption of such foods by patients with birch pollen allergy with atopic dermatitis can cause a worsening of their eczema, and Bet v 1–reactive $CD4^+$ T cells have been detected in skin biopsy specimens taken from patients with such flare-ups.[10]

The humoral and cellular cross-reactivity between Bet v 1 and its dietary homologs has been intensively studied *in vitro*, including the identification of cross-reactive IgE[11,12] and T-cell epitopes.[6,7,13,14] However, possible *in vivo* consequences of ingestion of cross-reactive food allergens on the immune response

Abbreviations used
aa: Amino acid
cpm: Counts per minute
Foxp3: Forkhead box protein 3
GMP: Good manufacturing practice
ISAC: Immuno-Solid phase Allergen Chip
ISU: ISAC Standardized Units
OAS: Oral allergy syndrome
SLIT: Sublingual immunotherapy
TCL: T-cell line
TT: Tetanus toxoid

to the major birch pollen allergen are largely unknown. Hypothetically, exposure to Bet v 1–related food allergens can boost Bet v 1–specific IgE and T-cell responses in the absence of birch pollen exposure, which is similar to those observed during the birch pollen season.[15-17]

To address this issue, we orally administered defined amounts of rMal d 1, a well-characterized and clinically relevant Bet v 1 homolog,[2,4,6] to patients with birch pollen allergy. Sublingual administration of equal amounts of rBet v 1 to the same subjects served as a control. Blood samples were collected before and at regular weekly intervals after sublingual allergen exposure, as well as before and after the birch pollen season, to monitor immune effects of natural respiratory exposure to Bet v 1. Bet v 1– and Mal d 1–specific antibody levels and allergen-induced proliferative and cytokine responses of T cells were assessed. In addition, T-cell epitope recognition was analyzed.

METHODS

Study population

In total, 24 patients with birch pollen allergy were included based on case history (rhinoconjunctivitis in spring), positive skin prick test responses to birch pollen (ALK-Abelló, Hørsholm, Denmark), and specific IgE levels to birch pollen extract, Bet v 1, and Mal d 1 (≥0.35 kU_A/L; ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden). Sera that contained greater than 100 kU/L Bet v 1–specific IgE were diluted 1:3 in PBS and reanalyzed. In addition, sera were analyzed by using the Immuno-Solid phase Allergen Chip (ISAC; Thermo Fisher Scientific). All but 4 patients (patients 2, 13, 15, and 24; Table I) reported experiencing OAS to apple. None of the patients had received allergen-specific immunotherapy during the past 5 years. The study was approved by the Ethics Committee of the Medical University of Vienna, and all patients provided informed written consent.

Antigens

Good manufacturing practice (GMP)-produced recombinant rBet v 1.0101 (termed GMP–rBet v 1 in the following) and Mal d 1.0108 (termed GMP–rMal d 1), as well as rBet v 2, were purchased from Biomay (Vienna, Austria). Recombinant allergens show similar IgE-binding and T cell–activating capacity as their natural counterparts.[4,6,18,19] rHev b 3 was kindly provided by H. Breiteneder (Vienna, Austria). Tetanus toxoid (TT) was purchased from Calbiochem (Darmstadt, Germany). Throughout the entire study, the same batches of antigens were used.

Study protocol

Patients were asked to avoid birch pollen–related foods during 2 months before and 1 month after allergen exposure. At the end of October 2008, 24 patients with birch pollen allergy were exposed to 50 μg of GMP–rBet v 1 (in 50 μL of 5 mmol/L phosphate buffer, pH 7.5) on 2 consecutive days. The allergen solution was kept under the tongue for 2 minutes and then swallowed. Patients were asked to score the intensity of symptoms from 0 to 3 (0 = no, 1 = mild, 2 = moderate, or 3 = severe symptoms). All patients were monitored for 60 minutes after allergen exposure. Blood samples were collected before and in weekly intervals after allergen exposure. Skin prick tests with titrated concentrations of each recombinant allergen were performed before and 6 weeks after oral exposure. From the same patients, sera were collected before (February 2009) and after (May 2009) the following birch pollen season. Six patients had withdrawn for reasons not connected with the study (3 moved away from Vienna, 2 started specific immunotherapy, and 1 had no time because of a new job). Thus in January 2010, 18 patients were exposed to 50 μg of GMP–rMal d 1 (in 75 μL of 20 mmol/L carbonate buffer, pH 9.0) on 2 consecutive days. Blood samples were again collected before and in weekly intervals after allergen exposure, and skin prick tests were performed.

Monitoring of allergen-specific IgE antibodies

Allergen-specific serum IgE levels were determined by using ImmunoCAP (Thermo Fisher Scientific) with a Phadia 250 instrument. All measurements were performed in the same assay run.

Allergen-specific proliferation of PBMCs

Freshly isolated PBMCs (2×10^5) were cultured in triplicates in serum-free Ultra Culture Medium (BioWhittaker, Walkersville, Md) supplemented with 2 mmol/L glutamine and 20 mmol/L β-mercaptoethanol in the absence or presence of GMP–rBet v 1 or GMP–rMal d 1 (each used at concentrations of 12.5 and 6.25 μg/mL), TT (0.25 μg/mL), or human rIL-2 (10 U/mL; Roche, Mannheim, Germany). After 6 days, proliferation was determined by means of tritiated thymidine incorporation. Data are expressed as Δ counts per minute (cpm), indicating cpm of stimulated cultures minus cpm of cultures left in medium alone. For each allergen, the concentration inducing optimum proliferative responses before sublingual exposure to GMP–rBet v 1 was individually determined and used for all subsequent time points.

Quantitative expression of cytokines and forkhead box protein 3

The expression of IL-4, IL-5, IL-13, IL-10, IFN-γ, and forkhead box protein 3 (Foxp3) was determined by using quantitative real-time PCR, as described previously.[20] Sufficient numbers of PBMCs at each investigated time point were available from 8 patients. Briefly, thawed PBMCs (10×10^6/4 mL) were cultured in 6-well plates (Costar, Corning, NY) in medium alone or in the presence of either GMP–rBet v 1 or GMP–rMal d 1 (both 5 μg/mL). After 6 hours, T cells were isolated from these cultures with anti-CD3 Dynal magnetic beads (Dynal, Hamburg, Germany). mRNA was isolated from purified T cells by using the RNeasy Mini Kit (Qiagen, Hamburg, Germany) and reverse transcribed with TaqMan reverse transcription reagents using random hexamers (Applied Biosystems, Foster City, Calif). Quantitative real-time PCR was performed with an ABI 7900 HT Sequence Detection System (Applied Biosystems) and cDNA-specific Assays-on-Demand to analyze 18S rRNA (control housekeeping gene), IL-4, IL-5, IL-13, IL-10, IFN-γ, and Foxp3 expression (Applied Biosystems). All amplifications were performed in duplicates. The cycle number at which the detected fluorescence exceeded the threshold (cycle threshold value) was determined. The fold change induction was calculated by using the software SDS 2.3 and DataAssist 2.0 (Applied Biosystems), comparing the samples collected at the indicated time points after allergen exposure with samples collected before exposure. The expression of cytokines was evaluated in allergen-stimulated cells, and Foxp3 expression was evaluated in unstimulated cells.

Allergen-specific T-cell lines and epitope mapping

Allergen-specific T-cell lines (TCLs) were generated from thawed PBMCs (1.5×10^6/well) and 10 μg/mL of either GMP–rBet v 1 or GMP–rMal d 1, as previously published.[21] TCLs were restimulated with GMP–rBet v 1 or GMP–rMal d 1 in the presence of irradiated (60 Gy) autologous PBMCs. rHev b 3 and

TABLE I. Characteristics of patients with birch pollen allergy

Patient no.	Sex	Age (y)	Total IgE (kU/L)	IgE specific for: Birch pollen (kU_A/L)*	rBet v 1 (kU_A/L)	Mal d 1 (kU_A/L)	rBet v 1 (ISU)†	rMal d 1 (ISU)	nAct d 8 (ISU)	rApi g 1 (ISU)	rAra h 8 (ISU)	rCor a 1.04 (ISU)	rDau c 1 (ISU)	rGly m 4 (ISU)	rPru p 1 (ISU)	Other allergies
1	F	25	129	5.1	4.7	3.5	8.9	3.8	0.0	0.0	0.4	4.4	0.0	0.0	3.4	Cat, HDM, ragweed
2	M	23	45.8	3.1	3.2	0.8	6.2	1.1	0.0	0.0	0.3	2.4	0.0	0.6	1.6	Mugwort, ragweed
3	F	23	338	13.3	12.2	6.2	23.0	9.4	3.0	1.7	2.6	9.4	0.4	1.8	12.2	Cat, dog
4	F	29	113	6.2	6.3	2.2	8.8	1.0	0.0	0.5	1.6	3.0	0.0	1.0	3.2	Grass, ragweed
5	M	30	44.1	8.8	5.9	4.5	8.6	5.1	0.0	0.0	0.0	5.2	0.0	1.0	6.7	HDM
6	F	23	354	8.0	3.8	1.9	15.8	7.8	0.0	1.8	2.3	5.2	0.0	1.3	1.5	Grass
7	M	22	361	20.9	18.0	4.0	49.6	7.0	0.0	3.0	0.7	9.8	0.4	5.2	13.3	Grass
8	F	23	301	10.2	11.4	1.3	34.5	3.0	0.0	0.0	1.8	3.8	0.0	0.5	11.0	None
9	F	45	253	83.9	80.2	19.6	35.3	4.6	0.0	0.4	4.6	16.4	0.0	3.6	26.0	None
10	F	23	50.2	4.6	3.9	1.2	6.3	3.0	0.0	0.0	1.1	3.3	0.0	2.0	2.1	Cat, grass, mold
11	F	52	394	37.0	34.0	16.1	53.0	27.5	0.0	0.8	13.0	22.0	0.0	15.0	10.0	Grass, rye
12	F	28	846	>100	82.5	22.1	31.2	14.3	0.0	3.0	10.1	19.2	0.0	12.4	5.5	Cat, grass, mold
13	F	23	43.5	28.0	27.1	1.4	22.5	0.7	0.0	0.7	0.0	9.1	0.0	0.0	3.8	Cat
14	F	25	260	8.2	7.5	1.7	13.1	3.4	0.0	0.0	0.0	4.3	0.0	0.0	6.8	Grass, HDM
15	M	27	234	37.5	38.1	7.8	60.1	23.0	0.0	0.0	6.2	3.5	0.0	15.5	23.0	Grass, HDM, rye
16	M	27	637	38.5	34.0	7.5	42.6	10.8	1.5	0.0	2.3	13.3	0.0	0.0	7.0	Grass
17	F	24	145	14.7	13.7	6.3	26.3	9.5	1.9	0.0	3.8	12.3	0.0	1.6	6.1	Cat, grass, rye
18	F	23	234	23.0	13.8	4.0	39.7	5.1	0.0	0.5	1.5	23.3	0.0	3.4	11.0	Cat, dog, grass, rye
19	M	30	124	2.2	2.1	0.5	6.0	0.5	0.0	0.0	0.0	0.6	0.0	0.0	0.8	Grass
20	F	22	1156	92.4	62.3	23.9	40.6	9.8	0.0	0.0	1.4	17.0	0.0	0.0	14.6	Grass, HDM
21	F	33	429	15.1	18.9	1.7	59.4	3.9	0.0	0.0	2.0	28.3	0.0	0.0	3.4	Mold
22	F	48	292	>100	193	42.9	76.2	34.4	16.4	31.2	9.1	43.3	4.2	33.2	45.1	Grass
23	F	21	488	69.1	53.7	20.5	51.9	16.1	0.0	0.0	11.0	29.1	0.0	12.3	15.4	HDM, rye
24	F	22	1231	43.1	34.7	6.6	24.2	2.2	0.0	0.0	0.0	6.0	0.0	1.2	1.4	Grass

F, Female; *HDM*, house dust mite; *M*, male.
*Specific IgE levels assessed by means of ImmunoCAP.
†Specific IgE levels assessed by means of ISAC.

rBet v 2 served as negative controls. For epitope mapping, a panel of 50 synthetic dodecapeptides (each 5 μg/mL), overlapping by 9 aa residues and together representing the complete aa sequence of Bet v 1.0101 (Mimotopes/Biotrend, Köln, Germany), was used.[21] A corresponding panel of 49 dodecapeptides, representing the complete aa sequence of Mal d 1.0108, was purchased from Intavis (Köln, Germany). After 48 hours, proliferation was evaluated. The stimulation index was calculated as the ratio between cpm obtained in cultures with or without allergen. A stimulation index of greater than 2 was considered positive.

Statistical analysis

Statistical analysis was performed with SPSS 14.0 software (SPSS, Chicago, Ill). Differences between parameters assessed at different time points were evaluated by using the Wilcoxon signed-rank test. *P* values of less than .05 were considered statistically significant.

RESULTS

Study population

In total, 24 adult Austrian patients with birch pollen allergy (median age, 24.5 years) were included (Table I). Four patients reported not having apple allergy. Sera of all patients were characterized with respect to total IgE levels (median, 276.0 kU/L), birch pollen–specific IgE levels (median, 18.0 kU_A/L), and Bet v 1– and Mal d 1–specific IgE levels (median, 15.9 kU_A/L and 4.3 kU_A/L, respectively), as determined by using ImmunoCAP. In addition, IgE reactivity to Bet v 1 and several Bet v 1–related food allergens was assessed by using ISAC (Table I). All patients displayed IgE to Bet v 1 (median, 28.7 ISAC Standardized Units [ISU]), Mal d 1 (median, 5.1 ISU), Cor a 1 from hazelnut (median, 9.3 ISU), and Pru p 1 from peach (median, 6.8 ISU; Table I). Nineteen patients showed IgE reactivity to Ara h 8 from peanut, 17 patients to Gly m 4 from soybean, 10 patients to Api g 1 from celeriac, 4 patients to Act d 8 from kiwi, and 3 patients to Dau c 1 from carrot. Twenty-two patients were additionally sensitized to allergen sources unrelated to birch pollen (Table I).

Clinical reactions to orally administered GMP–rBet v 1 and GMP–rMal d 1

Twenty-four patients with birch pollen allergy were orally exposed to 50 μg of GMP–rBet v 1 on 2 consecutive days outside of the pollen season. No significant differences between the first and second exposures were observed. All but 1 patient had OAS, which started within 1 to 10 minutes and ended within 20 and 60 minutes after each exposure. A summary of individual clinical symptoms is shown in Table II. Thirteen (54%) patients indicated mild itching in the oral cavity; 10 (42%) patients reported stronger local itching and burning plus either rhinoconjunctivitis or sensation of swelling of the lips, mouth, or throat without visible signs of edema. One of these patients had visible sublingual edema. Two patients additionally had gastric pain and headache for about

TABLE II. Clinical symptoms after oral exposure with rBet v 1 and rMal d 1

Patient no.	Challenge with GMP–rBet v 1				Challenge with GMP–rMal d 1			
	OAS	Symptoms	Localization	Intensity*	OAS	Symptoms	Localization	Intensity
1	Yes	Itch	SL, lips	1	Yes	Itch	SL	1
2	No	None	—	0	No	None	—	0
3	Yes	Itch	SL, throat	1	Yes	Burning, itch	Palate	1
4	Yes	Itch, sense of swelling	SL, lips, throat, eyes, skin	2	Yes	Itch, sense of swelling	Tongue, throat	2
5	Yes	Itch, blister	SL, throat, ear, lip	1	Yes	Itch	SL, palate, ear	1
6	Yes	Blister, sense of swelling	Lips, SL, throat	3	Yes	Burning, itch	SL, throat	1
7	Yes	Sense of swelling	Tongue, lips, ear	2	Yes	Itch	SL, ear	1
8	Yes	Itch	Throat	1	Yes	Itch	Throat	1
9	Yes	Itch	SL, throat	1	Yes	Itch	SL, throat	1
10	Yes	Sense of swelling, dysphagia	Tongue, throat	2	Yes	Burning, itch, sense of swelling	SL, lips, throat	2
11	Yes	Blister, burning, itch, sense of swelling	Lips, tongue, throat	2	Yes	Itch, tingling	SL	1
12	Yes	Sense of swelling	SL, throat	2†	Yes	Burning, itch, tingling	Throat	2
13	Yes	Dysphagia	Tongue, throat	2†	Yes	Itch, tingling	Throat	1
14	Yes	Itch	SL, throat	1†	Yes	Itch	Throat	1
15	Yes	Itch	SL	1	Yes	Hoarseness, itch, dysphagia	Throat	3†
16	Yes	Itch, gastric pain, sense of swelling	SL, throat, palate	2	Yes	Itch	Tongue	1
17	Yes	Itch, gastric pain, headache	SL, throat	1	Yes	Itch, sense of swelling, gastric pain, nausea	SL, tongue, throat, ear	2
18	Yes	Itch	SL, throat	1	No	None	—	0
19	Yes	Itch	SL, throat	1	ND	ND	ND	ND
20	Yes	Sense of swelling	Throat	2	ND	ND	ND	ND
21	Yes	Itch	Tongue, throat	1	ND	ND	ND	ND
22	Yes	Sense of swelling	Throat	1	ND	ND	ND	ND
23	Yes	Itch, sense of swelling	SL, throat	2	ND	ND	ND	ND
24	Yes	Itch	SL, throat, palate	1	ND	ND	ND	ND

The strongest reactions of 2 exposures are indicated.
ND, Not determined; *SL*, sublingual.
*Symptom intensity scores range from 0 to 3 (0 = no, 1 = mild, 2 = moderate, and 3 = severe symptoms).
†Patients were treated with antihistamines.

10 minutes, respectively. Two patients reported the formation of a blister on the lip a few hours after allergen exposure on the first day. Antihistamines were given to 3 patients to ameliorate their symptoms.

After 14 months, 18 of the 24 patients received 50 μg of GMP–rMal d 1 sublingually on 2 consecutive days. Again, no significant differences between the first and second exposures became evident. Eleven (61%) of 18 patients reported mild OAS (Table II). Local itching and burning and subjective edema or short-term gastric pain were reported by 4 (22%) of 18 patients. One patient had hoarseness and dysphagia after the second exposure and was treated with antihistamines. One patient had no allergic symptoms to GMP–rMal d 1, although he had responded to GMP–rBet v 1 with a mild OAS. The patient nonreactive to GMP–rBet v 1 did not react to GMP–rMal d 1 either (Table II). Oral exposure to either allergen did not alter the results of skin prick tests performed with GMP-produced recombinant allergens (data not shown).

Allergen-specific IgE responses to oral exposure with GMP–rBet v 1 and GMP–rMal d 1

Bet v 1– and Mal d 1–specific IgE antibody levels were measured before oral exposure to either GMP–rBet v 1 or GMP–rMal d 1 (B0 and M0, respectively) and at weekly intervals after exposure to either allergen (B1-B4 and M1-4, respectively), as well as before (BP0) and after (BP1) the birch pollen season. Within 1 week after oral exposure, Bet v 1–specific IgE levels decreased ($P = .054$) and then increased again to levels measured before exposure (Table III). No remarkable effects on Mal d 1–specific IgE levels were observed. Oral exposure to GMP–rMal d 1 significantly decreased Bet v 1–specific serum IgE levels at M1 ($P = .010$), M2 ($P = .012$), M3 ($P = .030$), and M4 ($P = .015$), as well as Mal d 1–specific IgE levels at M2 and M4 ($P = .045$ and $P = .004$, respectively). Respiratory exposure of birch pollen significantly increased both Bet v 1– and Mal d 1–specific IgE levels ($P = .048$ and $P = .046$, respectively; Table III).

Allergen-specific T-cell responses to oral exposure to GMP–rBet v 1 and GMP–rMal d 1

PBMCs could be isolated from 15 patients at each time point before and after allergen exposure (B0-B4, BP0-BP1, and M0-M4). These cells were stimulated with GMP–rBet v 1, GMP–rMal d 1, or TT as a control antigen. Bet v 1–induced proliferation was significantly lower at B1 than at B0 ($P = .048$) and increased again from B2 to B4 (B1:B3, $P = .048$; Fig 1). Similarly, Mal d 1–induced proliferation was significantly diminished at B1, with a tendency to increase thereafter (B1:B0, $P = .002$; B2:B0, $P = .008$; B3:B0, $P = .023$; and B4:B0, $P = .031$). Oral exposure to GMP–rMal d 1 also induced an early short-term

TABLE III. Allergen-specific IgE levels in sera of challenged patients

	Bet v 1–specific IgE (kU_A/L)												Mal d 1–specific IgE (kU_A/L)											
	B0	B1	B2	B3	B4	BP0	BP1	M0	M1	M2	M3	M4	B0	B1	B2	B3	B4	BP0	BP1	M0	M1	M2	M3	M4
1	4.7	4.7	4.9	4.6	5.2	3.4	10.8	4.9	4.2	4.3	3.8	4.6	3.5	3.0	3.3	3.0	3.2	1.9	5.8	2.5	2.1	2.1	2.2	2.2
2	3.2	2.4	3.3	3.0	2.7	1.5	2.5	1.0	1.2	1.4	0.9	0.9	0.8	0.6	0.8	0.8	0.7	0.5	0.7	0.3	0.3	0.3	0.2	0.3
3	12.2	10.0	10.3	11.5	12.2	6.7	10.1	NT	4.5	3.5	4.1	3.9	6.2	5.0	5.5	7.4	7.4	0.4	0.6	NT	2.7	2.6	2.2	2.6
4	6.3	7.1	7.3	6.5	6.2	6.2	8.5	5.4	5.3	4.8	5.1	4.6	2.2	2.1	2.2	2.1	2.2	1.7	2.3	1.3	1.7	1.4	1.6	0.9
5	5.9	6.2	6.9	8.8	7.4	6.2	5.9	6.4	4.7	5.4	5.9	5.4	4.5	5.1	5.2	2.1	2.0	4.4	4.3	3.6	2.4	3.1	3.6	3.3
6	3.8	3.4	4.0	3.9	3.8	2.5	6.4	3.8	2.1	2.7	3.1	2.5	1.9	2.2	2.1	1.9	1.9	1.3	3.3	1.8	1.5	1.4	NT	1.1
7	18.0	17.2	16.8	15.1	14.8	27.8	18.6	16.3	14.2	12.1	13.2	13.1	4.0	3.9	3.3	3.3	0.5	4.6	4.7	3.5	2.9	3.0	2.5	2.6
8	11.4	13.1	17.7	8.0	NT	13.6	22.0	10.6	8.1	9.6	9.1	8.2	1.3	1.1	1.9	5.4	NT	1.6	2.5	1.1	1.0	0.9	0.9	0.9
9	80.2	79.6	75.4	82.6	84.0	166.5	110.0	31.8	24.4	28.9	27.2	24.6	19.6	17.2	21.3	23.0	22.9	22.5	19.6	24.3	15.9	12.6	15.3	11.4
10	4.0	3.9	5.9	7.4	7.1	5.2	25.9	9.1	8.2	NT	8.2	8.3	1.2	1.3	1.6	1.9	2.1	1.7	6.8	NT	3.9	NT	3.6	3.4
11	34.0	33.7	35.1	33.0	30.1	25.2	27.2	23.2	26.7	19.0	18.4	18.4	16.1	12.4	17.4	15.3	16.4	14.4	15.7	14.9	11.6	11.8	9.4	11.2
12	82.5	92.4	92.3	164.5	144.0	84.2	82.3	65.4	65.1	62.1	67.8	56.4	22.1	28.6	38.2	32.0	35.6	24.3	19.6	15.9	17.9	18.7	19.7	12.7
13	27.1	15.2	13.9	17.5	15.1	15.5	12.7	9.0	5.9	9.7	7.8	13.2	1.4	0.7	0.8	1.3	1.0	0.9	1.1	0.7	0.7	0.8	0.7	0.9
14	7.5	6.6	6.1	6.3	5.7	5.4	7.9	3.2	3.2	2.7	2.0	2.7	1.7	1.8	1.6	1.8	1.5	1.3	3.4	1.0	0.7	0.8	0.7	0.9
15	38.1	36.8	94.8	94.7	98.1	72.9	58.3	41.4	38.9	42.8	45.6	37.7	7.8	7.9	56.7	54.2	55.0	28.2	21.6	9.0	6.5	8.1	7.6	6.4
16	34.0	30.8	31.5	30.2	25.8	24.0	52.7	28.1	NT	38.0	NT	31.2	7.5	5.7	5.7	4.7	5.4	5.6	13.6	7.3	NT	6.1	NT	6.5
17	13.7	11.6	16.1	18.9	20.6	16.8	21.0	13.0	11.5	12.2	10.9	12.1	6.3	5.6	7.6	6.4	7.8	7.4	10.1	5.9	5.5	4.6	4.4	5.2
18	13.8	10.3	9.0	10.7	8.4	8.0	9.0	5.5	4.5	4.0	4.3	4.2	4.0	3.2	2.9	2.9	2.8	1.6	2.2	1.2	1.2	1.1	1.2	1.0
19	2.1	2.2	2.4	2.6	2.6	2.4	3.7	NT	NT	NT	NT	NT	0.5	0.6	0.6	0.5	0.6	0.6	1.0	NT	NT	NT	NT	NT
20	62.3	54.7	54.7	59.8	60.4	46.4	71.7	NT	NT	NT	NT	NT	23.9	13.4	15.6	16.2	15.9	12.1	21.8	NT	NT	NT	NT	NT
21	18.9	25.3	24.7	20.3	24.0	23.0	28.9	NT	NT	NT	NT	NT	1.7	1.1	1.1	2.2	1.4	0.9	1.3	NT	NT	NT	NT	NT
22	193.5	95.1	87.4	183.0	190.5	79.3	82.7	NT	NT	NT	NT	NT	42.9	32.3	30.1	43.0	50.1	28.6	25.5	NT	NT	NT	NT	NT
23	53.7	55.0	61.7	56.6	56.0	43.6	NT	NT	NT	NT	NT	NT	20.5	23.8	25.6	30.0	31.4	24.8	NT	NT	NT	NT	NT	NT
24	34.7	26.7	23.7	26.8	27.9	22.8	40.0	NT	NT	NT	NT	NT	6.6	6.6	5.3	4.8	6.3	2.8	7.1	NT	NT	NT	NT	NT
Median	15.9	14.2	16.5	16.3	15.1	15.5	21.0*	9.0	5.9†	7.5*	7.8*	8.2*	4.5	4.9	4.2	4.0	3.2	2.4	4.7*	3.0	2.4	2.6*	2.4	2.6*

NT, Not tested.
*$P < .05$ and †$P < .01$, Wilcoxon signed-rank test.

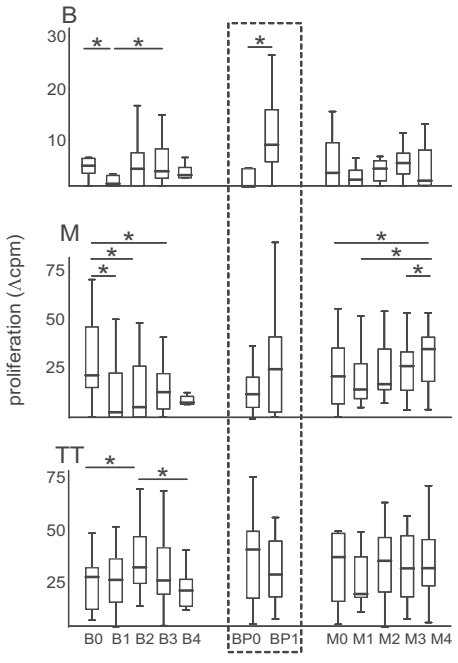

FIG 1. Lymphoproliferative responses of allergen-exposed patients. PBMCs (n = 15) collected before (B0, M0, and BP0) and in weekly intervals after oral exposure to GMP–rBet v 1 (B1-4) or GMP–rMal d 1 (M1-4) and after the birch pollen season (BP1) were stimulated with Bet v 1 *(B)*, Mal d 1 *(M)*, or TT. The Δcpm values for each patient were calculated at each time point and summarized in box plots. Background levels (median cpm) at the different time points were as follows: B0, 12,021; B1, 12,966; B2, 16,452; B3, 10,944; B4, 8,920; BP0, 7,314; BP1, 7,446; M0, 4,698; M1, 4,573; M2, 7,306; M3, 7,637; and M4, 3,854. *$P < .05$, Wilcoxon signed-rank test.

reduction in Bet v 1–induced T-cell proliferation, although differences did not reach statistical significance. At M4, Mal d 1–induced proliferation was significantly higher than at M0, M1, and M3 ($P = .015$, $P = .031$, and $P = .047$, respectively).

After the birch pollen season, Bet v 1–induced T-cell proliferation was significantly more pronounced than before ($P = .013$, Fig 1). Mal d 1–induced proliferation was also higher. TT-induced proliferation remained unchanged at all time points except for an increase 2 weeks after oral exposure to GMP–rBet v 1 (B0:B2, $P = .023$; B2:B4, $P = .006$; Fig 1).

Effects of allergen exposure on cytokine and Foxp3 expression of allergen-specific T lymphocytes

On the basis of the proliferation data, we analyzed cytokine and Foxp3 expression of T lymphocytes at B0, B1, B4, BP0, BP1, M0, M1, and M4. PBMCs sufficient to isolate mRNA from allergen-stimulated and nonstimulated $CD3^+$ T cells at each time point were available from 8 patients. One week after oral exposure to GMP–rBet v 1, Bet v 1–stimulated cells showed an increase in IL-5 and IL-10 expression, which returned to baseline levels at B4 (Fig 2). Mal d 1–stimulated T cells showed marginal IL-10 production. The expression of IL-13 and IFN-γ was not affected in cells stimulated with either allergen.

One week after exposure to GMP–rMal d 1, IL-5, IL-10, and IFN-γ mRNA levels were enhanced in Bet v 1–stimulated T cells and returned to baseline after 4 weeks. Mal d 1–stimulated T cells showed enhanced mRNA levels of IL-5 and IL-10 at M1 but not at M4. IL-13 levels were not altered. IFN-γ levels were decreased at M4.

After the birch pollen season, enhanced mRNA expression of IL-5, IL-13, and IL-10 was found in Bet v 1–stimulated T cells

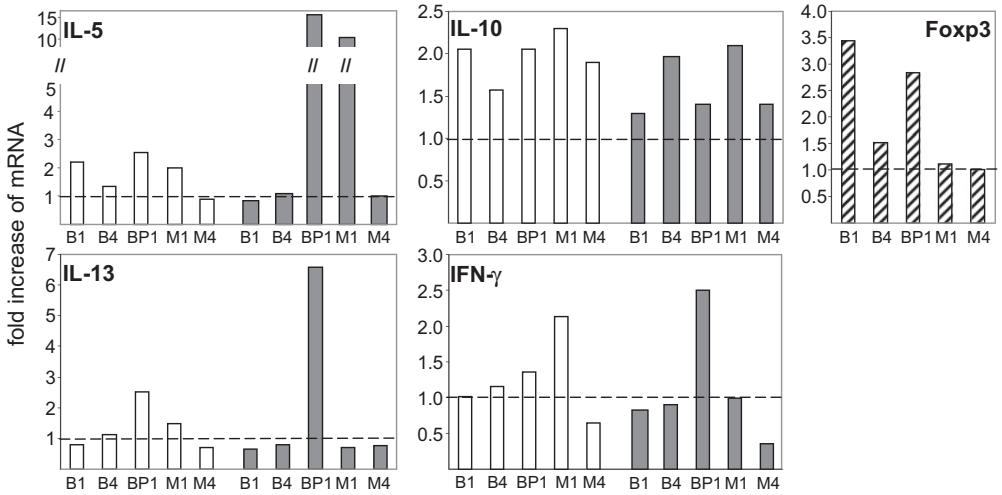

FIG 2. Allergen-specific cytokine response and Foxp3 expression of T cells from allergen-exposed patients. The relative levels of expression of IL-5, IL-13, IL-10, and IFN-γ mRNA in $CD3^+$ cells isolated from 8 patients observed at 1 and 4 weeks after oral exposure to GMP–rBet v 1 (B1 and B4, respectively) or GMP–rMal d 1 (M1 and M4, respectively) and after the birch pollen season (BP1) on stimulation with Bet v 1 *(white bars)* or Mal d 1 *(gray bars)* were individually calculated compared with mRNA expression levels before exposure. Foxp3 expression was analyzed in unstimulated $CD3^+$ cells *(hatched bars)*. Median values of 8 patients are shown.

(Fig 3). Mal d 1–stimulated T cells expressed noticeably higher levels of IL-5, IL-13, and IFN-γ. The expression of Foxp3 analyzed in nonstimulated T cells increased markedly 1 week after oral exposure to GMP–rBet v 1 and after the birch pollen season (Fig 2).

Epitope recognition of allergen-specific T lymphocytes after oral allergen exposure

From 5 patients, TCLs could be expanded with GMP–rBet v 1 or GMP–rMal d 1 from thawed PBMCs collected at B1 and M1. Bet v 1–induced cultures were mapped with dodecapeptides representing the complete aa sequence of Bet v 1 and recognized a higher number of peptides after oral exposure to GMP–rBet v 1 than to GMP–rMal d 1 (Fig 3). These data indicate that orally administered GMP–rBet v 1 triggered T cells with a broader epitope repertoire than GMP–rMal d 1. Mal d 1–induced TCLs were incubated with dodecapeptides representing the complete aa sequence of the apple allergen. Three of 5 TCLs responded to a higher number of Mal d 1–derived peptides after oral exposure to GMP–rBet v 1, and 2 TCLs reacted to more peptides after exposure to GMP–rMal d 1 (Fig 3).

DISCUSSION

Respiratory exposure to Bet v 1 during the birch pollen season boosts the allergen-specific immune response, as reflected by the induction of local and systemic IgE synthesis and $CD4^+$ memory T cells.[15,17,22,23] We sought to study whether oral uptake of Bet v 1–related food allergens might act in a similar manner because these proteins cross-react with Bet v 1–specific IgE antibodies and T cells.

To perform oral allergen exposure under defined experimental conditions, we used GMP–rMal d 1, a protein well characterized in regard to cross-reactivity with Bet v 1.[4,6-9,24] This approach allowed sublingual administration of precise amounts of pure apple allergen and the comparison with equal amounts of GMP–rBet v 1. We applied 50 μg of GMP–rMal d 1, which roughly corresponds to the amount of allergen contained in 1 apple.[25,26] This dose induced mild-to-moderate OAS. Only 1 patient additionally had transient nausea and gastric pain. Equal amounts of GMP–rBet v 1 also triggered mild-to-moderate OAS, and 2 patients additionally had short-term gastric pain. Six (33%) of 18 patients exposed to equal amounts of either allergen ranked the intensity of GMP–rBet v 1–induced symptoms higher than GMP–rMal d 1–induced reactions, and 1 patient had mild OAS to GMP–rBet v 1 but not to GMP–rMal d 1. Only 2 (11%) patients judged GMP–rMal d 1–induced symptoms to be stronger than GMP–rBet v 1–induced reactions. Taken together, sublingual administration of 50 μg of GMP–rMal d 1 was well tolerated and induced comparable clinical symptoms as equal amounts of GMP–rBet v 1.

Monitoring of allergen-specific IgE levels before and after the birch pollen season revealed a significant increase in Bet v 1–specific serum IgE levels. This expected effect was accompanied by a significant increase in Mal d 1–specific IgE levels, indicating that inhalation of birch pollen boosted a diversified Bet v 1–specific IgE response, including food-reactive antibodies. In contrast, oral exposure to either GMP–rBet v 1 or GMP–rMal d 1 induced an early decrease in allergen-specific IgE levels (Table III). We speculate that this observation results from *in vivo* capture of serum IgE antibodies by the administered allergen, as previously suggested by others.[27] This assumption implies that orally administered allergens must have been absorbed as intact IgE-binding proteins. Although Mal d 1 is readily degraded by gastric proteases and consequently loses its IgE-binding capacity in the stomach,[8,28] our patients have kept the allergen solutions under the tongue for 2 minutes before swallowing. The same procedure has been shown to lead to retention of nondegraded Par j 1, a major allergen of *Parietaria judaica*, in the sublingual mucosa.[29] Thus it might be important to hold GMP–rMal

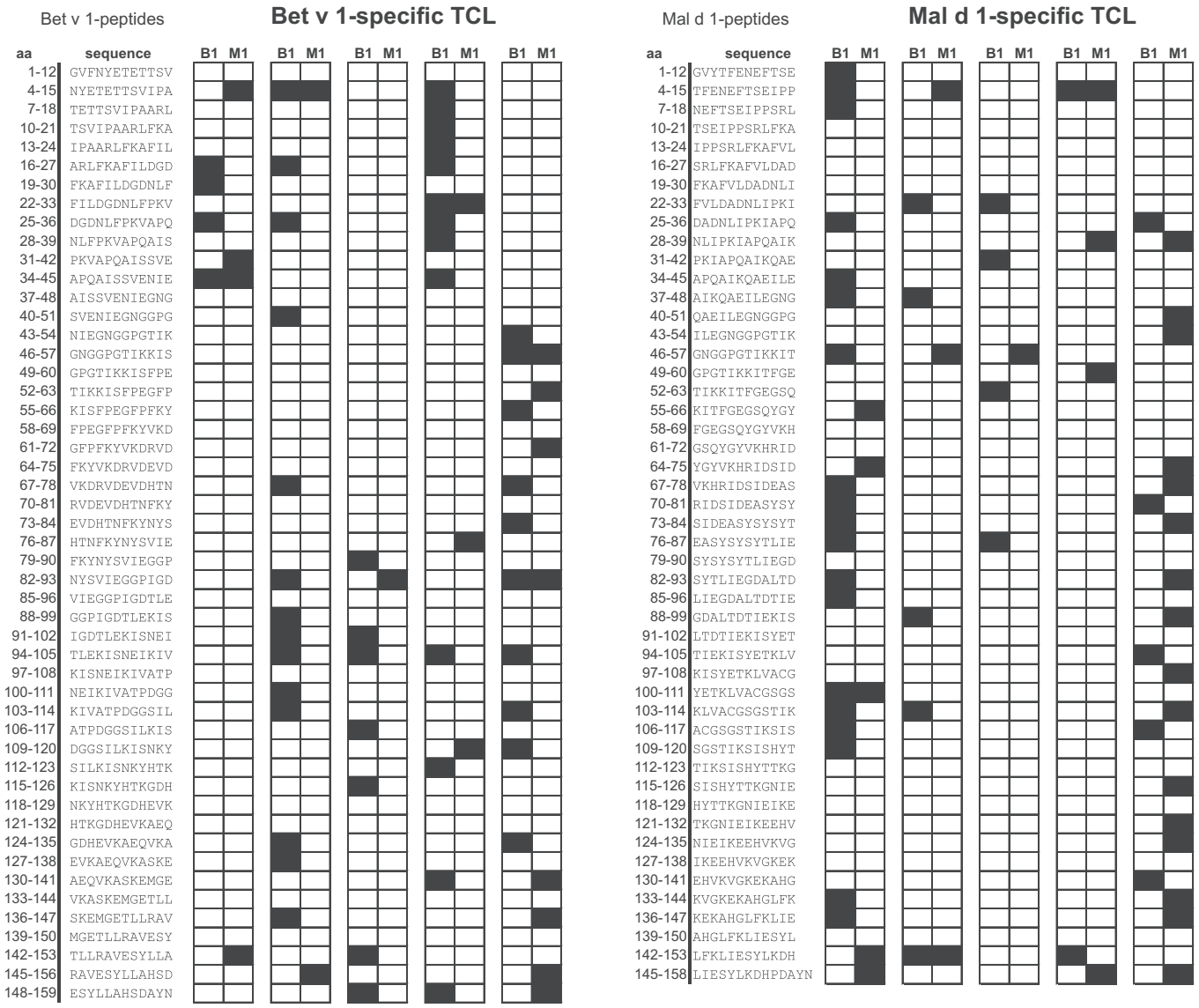

FIG 3. Epitope repertoire of allergen-specific TCLs from allergen-exposed patients. Allergen-specific TCLs were generated with either Bet v 1 or Mal d 1 from PBMCs collected 1 week after oral exposure to GMP–rBet v 1 (B1) or GMP–rMal d 1 (M1) from 5 patients and stimulated with overlapping peptides representing the entire aa sequence of either allergen. Peptides inducing stimulation indices of 2.0 or greater are indicated by *black boxes*.

d 1 sublingually for at least 2 minutes before swallowing to allow its absorption as intact allergen. Beyond that, our data indicate that 2 sublingual administrations of 50 μg of either GMP–rBet v 1 or GMP–rMal d 1 are not sufficient to boost allergen-specific IgE responses. We also exclude a potential "carryover" effect of oral administration of GMP–rBet v 1 on the later phases (birch pollen season, oral administration of GMP–rMal d 1) of our study.

After oral exposure to GMP–rBet v 1, an early short-term reduction in Bet v 1– and Mal d 1–induced T-cell proliferation became evident, accompanied by enhanced expression of IL-5, IL-10, and Foxp3 mRNA. We interpret these results as activation of allergen-specific T_H2 cells together with induction of transient peripheral tolerance, possibly mediated by IL-10^+Foxp3^+ T cells with suppressive capacity. This type of regulatory T cell has been found after sublingual administration of birch pollen extract.[20] Oral exposure to GMP–rMal d 1 induced similar effects. In contrast, exposure to birch pollen through the respiratory tract during the pollen season clearly enhanced Bet v 1– and Mal d 1–induced proliferation. This boost of allergen-specific T cells was also reflected by a marked increase in levels of the T_H2 cytokines IL-5 and IL-13 and accompanied by an increase in Foxp3 expression. Bet v 1–stimulated T cells produced enhanced levels of IL-10 after the birch pollen season. The apparent discrepancy (ie, enhanced allergen-induced proliferation despite increased IL-10 and Foxp3 expression) might be due to a stronger activation, higher proportion, or both of effector T_H2 than regulatory T cells because of the longer time period of respiratory exposure. Moreover, regulatory T cells in allergic patients have been shown to possess a reduced suppression capacity during the pollen season.[30]

Previous work has demonstrated that the majority of Mal d 1–reactive T cells are originally Bet v 1–specific cells that cross-react with the food allergen.[6] Only a minority of food-reactive T cells do not react with the birch pollen allergen. We observed that orally administered GMP–rMal d 1 triggered IFN-γ production in Bet v 1–stimulated T cells, whereas GMP–rBet v 1 did not. Furthermore, in contrast to Bet v 1–stimulated cells, Mal d 1–stimulated T cells expressed IFN-γ mRNA after the birch pollen season. These results suggested that different T cells responded to Mal d 1 and Bet v 1 *in vivo*. To further examine this assumption, we performed epitope mapping of cultures enriched for Bet v 1– or Mal d 1–specific T cells. A broader diversity of peptides was recognized in Bet v 1–generated TCLs after oral exposure to GMP–rBet v 1 than to GMP–rMal d 1, indicating that the apple allergen did not trigger T cells specific for all Bet v 1 epitopes. Also in Mal d 1–generated TCLs, peptide recognition differed after administration of either allergen. Of note, 4 (80%) of 5 rMal d 1–generated TCLs responded to peptides covering Mal d $1_{142\text{-}158}$ (LFKLIESYLKDHPDAYN) on exposure to the apple allergen. Thus T cells specific for this region were preferably activated by Mal d 1. Collectively, these data show that T-cell populations with different epitope repertoires and effector functions responded to orally applied birch pollen and apple allergen.

It has recently been reported that daily consumption of increasing amounts of fresh apple induces clinical tolerance to apple in patients with birch pollen-related apple allergy.[31] However, the authors could not detect immunologic changes in food-tolerant subjects. Because raw apples contain highly variable amounts of Mal d 1, we propose the use of pure GMP–rMal d 1 for sublingual immunotherapy (SLIT) of birch pollen–related food allergy. Two administrations of 50 μg of GMP–rMal d 1 kept under the tongue for at least 2 minutes were well tolerated and induced immune responses characteristic of peripheral tolerance. Continuous sublingual administration of GMP–rMal d 1 over a longer time period should induce allergen-specific T-cell tolerance, immune deviation, and regulatory T cells, as well as allergen-specific IgG_4 antibodies, as previously demonstrated for SLIT with birch pollen extract.[20] SLIT with birch pollen did not effectively alter T-cell and antibody responses to Mal d 1.[24,32] In view of our finding that orally administered GMP–rMal d 1 targeted different T cells than GMP–rBet v 1, SLIT with GMP–rMal d 1 might be a promising approach to cure birch pollen–related apple allergy.

Clinical implications: rMal d 1 can be considered for SLIT of birch pollen–related apple allergy.

REFERENCES

1. Eriksson NE, Formgren H, Svenonius E. Food hypersensitivity in patients with pollen allergy. Allergy 1982;37:437-43.
2. Geroldinger-Simic M, Zelniker T, Aberer W, Ebner C, Egger C, Greiderer A, et al. Birch pollen-related food allergy: clinical aspects and the role of allergen-specific IgE and IgG4 antibodies. J Allergy Clin Immunol 2011;127:616-22, e1.
3. Fernandez-Rivas M, Bolhaar S, Gonzalez-Mancebo E, Asero R, van Leeuwen A, Bohle B, et al. Apple allergy across Europe: how allergen sensitization profiles determine the clinical expression of allergies to plant foods. J Allergy Clin Immunol 2006;118:481-8.
4. Vanek-Krebitz M, Hoffmann-Sommergruber K, Laimer da Camara Machado M, Susani M, Ebner C, Kraft D, et al. Cloning and sequencing of Mal d 1, the major allergen from apple *(Malus domestica)*, and its immunological relationship to Bet v 1, the major birch pollen allergen. Biochem Biophys Res Commun 1995;214:538-51.
5. Radauer C, Lackner P, Breiteneder H. The Bet v. 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. BMC Evol Biol 2008;8:286.
6. Fritsch R, Bohle B, Vollmann U, Wiedermann U, Jahn-Schmid B, Krebitz M, et al. Bet v 1, the major birch pollen allergen, and Mal d 1, the major apple allergen, cross-react at the level of allergen-specific T helper cells. J Allergy Clin Immunol 1998;102:679-86.
7. Jahn-Schmid B, Radakovics A, Luttkopf D, Scheurer S, Vieths S, Ebner C, et al. Bet v 1142-156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens. J Allergy Clin Immunol 2005;116:213-9.
8. Schimek EM, Zwolfer B, Briza P, Jahn-Schmid B, Vogel L, Vieths S, et al. Gastrointestinal digestion of Bet v 1-homologous food allergens destroys their mediator-releasing, but not T cell-activating, capacity. J Allergy Clin Immunol 2005;116:1327-33.
9. Bohle B, Zwolfer B, Heratizadeh A, Jahn-Schmid B, Antonia YD, Alter M, et al. Cooking birch pollen-related food: divergent consequences for IgE- and T cell-mediated reactivity in vitro and in vivo. J Allergy Clin Immunol 2006;118:242-9.
10. Reekers R, Busche M, Wittmann M, Kapp A, Werfel T. Birch pollen-related foods trigger atopic dermatitis in patients with specific cutaneous T-cell responses to birch pollen antigens. J Allergy Clin Immunol 1999;104:466-72.
11. Mittag D, Batori V, Neudecker P, Wiche R, Friis EP, Ballmer-Weber BK, et al. A novel approach for investigation of specific and cross-reactive IgE epitopes on Bet v 1 and homologous food allergens in individual patients. Mol Immunol 2006;43:268-78.
12. Klinglmayr E, Hauser M, Zimmermann F, Dissertori O, Lackner P, Wopfner N, et al. Identification of B-cell epitopes of Bet v 1 involved in cross-reactivity with food allergens. Allergy 2009;64:647-51.
13. Bohle B, Radakovics A, Jahn-Schmid B, Hoffmann-Sommergruber K, Fischer GF, Ebner C. Bet v 1, the major birch pollen allergen, initiates sensitization to Api g 1, the major allergen in celery: evidence at the T cell level. Eur J Immunol 2003;33:3303-10.
14. Bohle B, Radakovics A, Luttkopf D, Jahn-Schmid B, Vieths S, Ebner C. Characterization of the T cell response to the major hazelnut allergen, Cor a 1.04: evidence for a relevant T cell epitope not cross-reactive with homologous pollen allergens. Clin Exp Allergy 2005;35:1392-9.
15. Gabrielsson S, Soderlund A, Paulie S, Rak S, van der Pouw Kraan TC, Troye-Blomberg M. Increased frequencies of allergen-induced interleukin-13-producing cells in atopic individuals during the pollen season. Scand J Immunol 1998;48:429-35.
16. Grindebacke H, Wing K, Andersson AC, Suri-Payer E, Rak S, Rudin A. Defective suppression of Th2 cytokines by CD4CD25 regulatory T cells in birch allergics during birch pollen season. Clin Exp Allergy 2004;34:1364-72.
17. Van Overtvelt L, Wambre E, Maillere B, von Hofe E, Louise A, Balazuc AM, et al. Assessment of Bet v 1-specific CD4+ T cell responses in allergic and non-allergic individuals using MHC class II peptide tetramers. J Immunol 2008;180:4514-22.
18. Ferreira FD, Hoffmann-Sommergruber K, Breiteneder H, Pettenburger K, Ebner C, Sommergruber W, et al. Purification and characterization of recombinant Bet v I, the major birch pollen allergen. Immunological equivalence to natural Bet v I. J Biol Chem 1993;268:19574-80.
19. Mothes-Luksch N, Spitzauer S, Hauswirth A, Krauth M, Valent P, Steiner R, et al. Comparison of in vivo allergenic activity of recombinant Bet v 1 with allergen-specific in vitro immune responses in birch pollen allergic patients. In: XXV Congress of the European Academy of Allergology and Clinical Immunology. Wiley-Blackwell; Vienna: European Academy of Allergology and Clinical Immunology; 2006. p. 294.
20. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. J Allergy Clin Immunol 2007;120:707-13.
21. Mutschlechner S, Egger M, Briza P, Wallner M, Lackner P, Karle A, et al. Naturally processed T cell-activating peptides of the major birch pollen allergen. J Allergy Clin Immunol 2010;125:711-8, e1-8.
22. Crimi E, Voltolini S, Gianiorio P, Orengo G, Troise C, Brusasco V, et al. Effect of seasonal exposure to pollen on specific bronchial sensitivity in allergic patients. J Allergy Clin Immunol 1990;85:1014-9.
23. Wosinska-Becler K, Plewako H, Hakansson L, Rak S. Cytokine production in peripheral blood cells during and outside the pollen season in birch-allergic patients and non-allergic controls. Clin Exp Allergy 2004;34:123-30.
24. Kinaciyan T, Jahn-Schmid B, Radakovics A, Zwolfer B, Schreiber C, Francis JN, et al. Successful sublingual immunotherapy with birch pollen has limited effects on

concomitant food allergy to apple and the immune response to the Bet v 1 homolog Mal d 1. J Allergy Clin Immunol 2007;119:937-43.
25. Matthes A, Schmitz-Eiberger M. Apple (*Malus domestica* L. Borkh.) allergen Mal d 1: effect of cultivar, cultivation system, and storage conditions. J Agric Food Chem 2009;57:10548-53.
26. Sancho AI, Foxall R, Browne T, Dey R, Zuidmeer L, Marzban G, et al. Effect of postharvest storage on the expression of the apple allergen Mal d 1. J Agric Food Chem 2006;54:5917-23.
27. Lofkvist T, Agrell B, Dreborg S, Svensson G. Effects of immunotherapy with a purified standardized allergen preparation of *Dermatophagoides farinae* in adults with perennial allergic rhinoconjunctivitis. Allergy 1994;49:100-7.
28. Schulten V, Lauer I, Scheurer S, Thalhammer T, Bohle B. A food matrix reduces digestion and absorption of food allergens in vivo. Mol Nutr Food Res 2011;55:1484-91.
29. Bagnasco M, Mariani G, Passalacqua G, Motta C, Bartolomei M, Falagiani P, et al. Absorption and distribution kinetics of the major *Parietaria judaica* allergen (Par j 1) administered by noninjectable routes in healthy human beings. J Allergy Clin Immunol 1997;100:122-9.
30. Grindebacke H, Larsson P, Wing K, Rak S, Rudin A. Specific immunotherapy to birch allergen does not enhance suppression of Th2 cells by CD4(+)CD25(+) regulatory T cells during pollen season. J Clin Immunol 2009;29:752-60.
31. Kopac P, Rudin M, Gentinetta T, Gerber R, Pichler C, Hausmann O, et al. Continuous apple consumption induces oral tolerance in birch-pollen-associated apple allergy. Allergy 2012;67:280-5.
32. Mauro M, Russello M, Incorvaia C, Gazzola G, Frati F, Moingeon P, et al. Birch-apple syndrome treated with birch pollen immunotherapy. Int Arch Allergy Immunol 2011;156:416-22.