

Functional Analysis of Birch Pollen Allergen Bet v 1-Specific Regulatory T Cells¹

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Allergen-specific immunotherapy using peptides is an efficient treatment for allergic diseases. Recent studies suggest that the induction of CD4⁺ regulatory T (Treg) cells might be associated with the suppression of allergic responses in patients after allergen-specific immunotherapy. Our aim was to identify MHC class II promiscuous T cell epitopes for the birch pollen allergen Bet v 1 capable of stimulating Treg cells with the purpose of inhibiting allergic responses. Ag-reactive CD4⁺ T cell clones were generated from patients with birch pollen allergy and healthy volunteers by in vitro vaccination of PBMC using Bet v 1 synthetic peptides. Several CD4⁺ T cell clones were induced by using 2 synthetic peptides (Bet v 1_{141–156} and Bet v 1_{51–68}). Peptide-reactive CD4⁺ T cells recognized recombinant Bet v 1 protein, indicating that these peptides are produced by the MHC class II Ag processing pathway. Peptide Bet v 1_{141–156} appears to be a highly MHC promiscuous epitope since T cell responses restricted by numerous MHC class II molecules (DR4, DR9, DR11, DR15, and DR53) were observed. Two of these clones functioned as typical Treg cells (expressed CD25, GITR, and Foxp3 and suppressed the proliferation and IL-2 secretion of other CD4⁺ T cells). Notably, the suppressive activity of these Treg cells required cell-cell contact and was not mediated through soluble IL-10 or TGF- β . The identified promiscuous MHC class II epitope capable of inducing suppressive Treg responses may have important implication for the development of peptide-based Ag-specific immunotherapy to birch pollen allergy. *The Journal of Immunology*, 2007, 178: 1189–1198.

Birch pollen allergy is one of the most prevalent allergic diseases in northern Europe, North America, and northern Japan. The protein Bet v 1 is the major birch pollen allergen and is recognized by specific IgE Abs in 90% of patients with birch pollen allergy. In many patients with allergic diseases, therapy with antihistamines and antileukotrienes may alleviate their symptoms but these medications are not always effective and tend to cause unwanted secondary effects. In contrast, allergen-specific immunotherapy using therapeutic vaccines, including natural Ags, recombinant proteins, and peptides, appears to be a more effective way of controlling allergic diseases (1–3). The mechanism of allergen-specific immunotherapy in normalizing the allergic responses to allergens is not all that clear. Most believe that specific immunotherapy involves the modulation of allergen-specific CD4⁺ T cell responses, such as Th2 to Th1 polarization and/or the deletion (or induction of anergy) of effector T cells (3, 4). There is concern that administration of the intact natural Ag

(protein allergen) may cause unwanted side effects in some patients because allergen-IgE immune complexes formed on mast cells and basophils would induce the release of biological mediators such as histamine and leukotrienes. Therefore, the use of synthetic peptides corresponding to MHC class II-restricted T cell epitopes not containing conformational Ab epitopes for allergens could be an effective way to avoid IgE-mediated cell activation (3). Indeed, for cat and bee venom allergies, clinical studies with therapeutic peptide vaccination have already been done and were shown to reduce sensitivity to allergens (5–13). Moreover, it is clear that desirable peptides for therapeutic vaccines should be promiscuous T cell epitopes, which would be recognized by CD4⁺ T cells in the context of more than one MHC class II allele, allowing broad population coverage (3). Thus, the identification of therapeutically effective promiscuous MHC class II restricted T cell epitopes for Bet v 1 would be very useful to develop effective peptide-based Ag-specific immunotherapy for birch pollen allergy.

Recent studies suggest that the induction of CD4⁺ regulatory T (Treg)⁴ cells might also be associated with the suppression of allergic responses in allergy patients treated with specific immunotherapy (4, 14). CD4⁺ Treg cells can either be naturally occurring Treg cells, which originate in the thymus, and Ag-induced Treg cells, which are induced by certain conditions of Ag stimulation and/or cytokine stimulation from periphery (15, 16). It has been reported that Ag-induced Treg cells might consist of several types such as T regulatory-1, Th1-like, Th2-like, and Th3 cells, which produce IL-10, IFN- γ , IL-4, and TGF- β , respectively (17–21).

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Received for publication June 7, 2006. Accepted for publication October 30, 2006.

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¹ This study was supported by a grant from the Akiyama Foundation (to H.K.) and by National Institutes of Health Grants R01CA80782 and R01CA103921 (to E.C.).

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⁴ Abbreviations used in this paper: Treg, regulatory T; GITR, glucocorticoid-induced TNFR; Foxp3, Forkhead Box P3; RAST, radioallergosorbent test; L cell, mouse fibroblasts cell line; r-Bet v 1, recombinant Bet v 1; DC, dendritic cell; β_2 -M, β_2 -microglobulin; PADRE, pan DR epitope.

These Treg cells express several characteristic markers such as CD25, glucocorticoid-induced TNF receptor (GITR), and the Forkhead Box P3 (Foxp3) transcription factor (22–28). Although Foxp3 may be a relatively specific intracellular marker compared with other cell surface molecules, a definitive marker for Treg cells has not been found yet. The most important factor to decide the character of Treg cells is their ability to inhibit the function of effector T cells. It has been somewhat controversial whether Treg inhibition of effector T cells requires cell-cell contact or is mediated via soluble mediators, such as IL-10 and TGF- β (29). In addition, little is known about Ag-specificity of Treg cells, especially in allergic diseases. Thus, we believe that the examination for the phenotypes, cytokine profiles, and suppressive activities of Ag-specific CD4⁺ T cells generated by in vitro peptide stimulation would be important to clarify the association between induction of allergen-specific Treg cells and allergen-specific immunotherapy.

In the present study, we report that two synthetic peptides of Bet v 1 stimulated CD4⁺ T cell responses in patients with birch pollen nasal allergy and in healthy volunteers. One of these peptides (Bet v 1_{141–156}) behaved as a highly MHC class II promiscuous epitope since it was able to elicit T cell responses restricted by multiple MHC class II alleles (HLA-DR4, DR9, DR11, DR15, and DR53). Most importantly, Bet v 1 peptide-reactive Treg cells as well as helper T cells were generated in the same peptide-reactive CD4⁺ T cell cultures and these Treg cells were capable of suppressing the proliferation of effector helper T cells. This inhibitory activity required cell-cell contact and did not appear to be mediated by IL-10 or TGF- β . These results might open the door for the development of peptide-based Ag-specific immunotherapy for birch pollen allergy and give us important clues to clarify the mechanisms of this therapy.

Materials and Methods

Subjects

Three patients with birch pollen nasal allergy (P1, P2, and P3) and two healthy volunteers (H1 and H2) were studied. Type I allergy to birch pollen was confirmed by clinical symptoms, eosinophilic cells in the nasal discharge, positive skin test, and positive radioallergen sorbent tests (RAST). RAST against birch was conducted by SRL, and the titer obtained by RAST is listed in Table I. We considered positive RAST if the amount of IgE Ab was >0.70 U_A/ml. HLA typing was conducted using a standard method at the Japanese Red Cross Blood Center.

Cell lines

Mouse fibroblasts cell lines (L cells) transfected and expressing individual human MHC class II molecules were provided by Dr. R. W. Karr (Idera Pharmaceuticals, Cambridge, MA) and Dr. T. Sasazuki (Tokyo, Japan). The mouse cytotoxic T lymphocyte line CTLL-2, which proliferates in a dose-dependent manner with mouse IL-2 and IL-4 but proliferates only to human IL-2 or IL-15, was cultured in RPMI 1640 medium supplemented with 10% FBS and 10 IU/ml recombinant human IL-2.

Peptides and recombinant protein

Potential HLA-DR-restricted CD4⁺ T cell epitopes were selected from the amino acid sequence of the Bet v 1 protein using SYFPEITHI: database for MHC ligands and peptide motifs (30). The predicted peptide epitopes were synthesized by solid phase organic chemistry and purified by HPLC. The purity (>80%) and identity of peptides were assessed by HPLC and mass spectrometry, respectively. Recombinant Bet v 1 (r-Bet v 1) protein was purchased from BIOMAY.

Measurement of serum IgE reactive to Bet v 1 peptides and protein

ELISA was used to quantify serum levels of IgE reactive to the Bet v 1 peptides and protein as described previously (31). Briefly, immobilization of the peptides and r-Bet v 1 protein to 96-well ELISA plates (Nalge Nunc International) using disuccinimidyl suberate (Pierce) was performed according to the manufacturer's instructions. The plate of immobilized pep-

tides and r-Bet v 1 protein (2 μ g/well) was blocked with Block Ace (Yukiji-rushi), and washed with PBS-Tween (PBS containing 0.05% Tween 20). Then, 100 μ l/well of serum samples diluted with Block Ace was added to the plate. The plate was washed with PBS-Tween after incubating for 2 h at 37°C and additional incubated for 2 h at 37°C with 100 μ l of 1/1000-diluted rabbit anti-human IgE-conjugated HRP (DakoCytomation). After another washing, the substrate solution (BD Pharmingen) was added, and the reaction was terminated by added the stop solution (2 N H₂SO₄). The OD of each well was determined by using a microplate reader set to 450 nm.

In vitro induction of Ag-reactive CD4⁺ T cell clones with synthetic peptides

The procedure selected for the generation of Bet v 1-reactive CD4⁺ T cell clones using peptide-stimulated PBMC has been described in detail (32–36). Briefly, dendritic cells (DC) were produced in tissue culture from purified CD14⁺ monocytes (using Ab-coated magnetic microbeads from Miltenyi Biotec) that were cultured for 7 days at 37°C in a humidified CO₂ (5%) incubator in the presence of 50 ng/ml GM-CSF and 1000 IU/ml IL-4. Peptide-pulsed DC (1 μ g/ml for 2 h at room temperature) were irradiated (4200 rad) and cocultured with autologous purified CD4⁺ T cells (using Ab-coated magnetic microbeads from Miltenyi Biotec) in 96-well culture plates. One week later, the CD4⁺ T cells were restimulated with peptide-pulsed irradiated autologous PBMC (3 μ g/ml) and 2 days later, recombinant human IL-2 was added at a final concentration of 10 IU/ml. One week later, the T cells were tested for Ag reactivity using a cytokine-release assay as described below. Those cultures exhibiting a significant response of cytokine-release to peptides (at least 2.5-fold over background) were expanded in 24- or 48-well plates by weekly restimulation with peptides and irradiated autologous PBMC. T cell lines were cloned by limiting dilution and used for further characterization. Complete culture medium for all procedures consisted of AIM-V medium (Invitrogen Life Technologies) supplemented with 3% human male AB serum. All blood samples were obtained after the appropriate informed consent.

Measurement of Ag-reactive responses with CD4⁺ T cell clones

CD4⁺ T cells (3×10^4 /well) were mixed with irradiated APC in the presence of various concentrations of Ags (peptides and r-Bet v 1 protein) in 96-well culture plates. APC consisted of either autologous PBMC (1×10^5 /well) or HLA-DR-expressing L-cells (3×10^4 /well). Culture supernatants were collected after 48 h for measuring Ag-induced lymphokine (IFN- γ , IL-4, or IL-10) production by the CD4⁺ T cell clones using commercially available ELISA kits (BD Pharmingen). To show Ag specificity and MHC restriction, blocking of the Ag-induced response was assessed by adding anti-HLA-DR mAb L243 (IgG2a, prepared from supernatants of the hybridoma HB-55 obtained from the American Type Culture Collection), anti-HLA-DQ mAb SPVL3 (IgG2a, Beckman Coulter), anti-HLA class II (DR, DQ, and DP) mAb TU39 (IgG2a; BD Pharmingen), or anti-HLA-A, HLA-B, and HLA-C mAb W6/32 (IgG2a; American Type Culture Collection) at 10 μ g/ml throughout the 48-h incubation period. All assessments of ELISA were conducted at least in triplicate and results correspond to the mean values with the SD of the mean.

RT-PCR analysis

Total RNA was extracted from CD4⁺ T cell clones using the SV Total RNA Isolation system (Promega, Madison, WI). The RNA was reverse-transcribed for 60 min at 37°C using Moloney murine leukemia virus reverse transcriptase (GeneHunter) with oligo(dT) primers (Applied Biosystems), according to the manufacturer's protocols. The following primers were used for Foxp3 (sense, 5'-CCCCTTGCCCCACTTACA-3'; antisense, 5'-CTTCTCCTTCTCAGCACCA-3') and β_2 -microglobulin (β_2 -M; sense, 5'-TGTCTTTCAGCAAGGACTGG-3'; antisense, 5'-CCAGATTAACCACA ACCATG-3') (Sigma Genosys Japan). Hot start PCR was performed in a 10- μ l reaction mixture containing 4.95 μ l of H₂O, 1 μ l of 10 $\times$ PCR buffer (containing 15 mM MgCl₂), 1 μ l of 2 mM dNTP mixture, 1 μ l of 5 μ M sense primer, 1 μ l of 5 μ M antisense primer, 0.05 μ l of 5 U/ml AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 μ l of 100 ng/ μ l cDNA. The reaction was conducted as follows: initial denaturation at 94°C for 10 min; followed by 30 (β_2 -M) or 37 cycles (Foxp3) of 1 min at 94°C, 1 min at 57°C (β_2 -M) or 58°C (Foxp3), and 1 min at 72°C; and a final elongation step of 5 min at 72°C. The PCR products were separated by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

Flow cytometric analysis

T cell clones were maintained in the culture medium containing a low recombinant human IL-2 (50 IU/ml) for at least 10 days before flow cytometric analysis. Then, they were washed in cold PBS, centrifuged, and

Table I. MHC class II-restricted T cell responses to Bet v 1 peptides

Patients (P) and Healthy (H) Volunteers	Amount of IgE Ab (U _A /ml)	MHC Class II	Bet v 1-Reactive T Cell Clone	Bet v 1 Epitope	Restriction Molecule	Cytokine Profile	Phenotype
P1	4.7	DR4/15	P1-7F	Bet v 1 ₁₄₁₋₁₅₆	DR15	IL-4, IL-10	CD4 ⁺ CD25 ^{high} Foxp3 ⁺
			P1-11B	Bet v 1 ₁₄₁₋₁₅₆	DR4	IFN-γ	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
			P1-11D	Bet v 1 ₁₄₁₋₁₅₆	DR4/53	IFN-γ, IL-4	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
			P1-G1	Bet v 1 ₅₁₋₆₈	DR53	IFN-γ, IL-10	CD4 ⁺ CD25 ^{high} Foxp3 ⁺
			P1-A7	Bet v 1 ₅₁₋₆₈	DP	IL-4	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
P2	6.8	DR4/11	P2-D5	Bet v 1 ₁₄₁₋₁₅₆	DR11	IFN-γ, IL-4	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
			P2-H11	Bet v 1 ₁₄₁₋₁₅₆	DR11	IFN-γ, IL-4	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
P3	13.3	DR9/14	P3-4	Bet v 1 ₁₄₁₋₁₅₆	DR9	IL-4	NE ^a
			P3-2A	Bet v 1 ₁₄₁₋₁₅₆	DR9	IFN-γ, IL-4	CD4 ⁺ CD25 ^{low} Foxp3 ⁻
H1	<0.34	DR4/9	H1-3	Bet v 1 ₁₄₁₋₁₅₆	DR53	IFN-γ	NE
H2	<0.34	DR9/14	H2-6H	Bet v 1 ₁₄₁₋₁₅₆	DR9	IFN-γ, IL-10	NE

^a NE, Not examined.

resuspended in an appropriate volume of FACS staining buffer (PBS containing 0.1% NaN₃ and 2% FBS). Cells were incubated with Abs for 60 min in the dark at 4°C, and excess Abs were removed by washing the cells twice in cold staining buffer. The following Abs were used: PE-conjugated anti-CD4, PE-conjugated anti-CD25 (both from BD Pharmingen), and PE-conjugated anti-GITR (R&D Systems). For Foxp3 intracellular staining, the Cytofix/Cytoperm kit (BD Pharmingen) was used according to the manufacturer's protocols, and PE-conjugated anti-Foxp3 Ab was purchased from eBioscience. FACS and data analysis were conducted using the BD Biosciences FACSscan and accompanying CellQuest software, according to the manufacturer's protocols.

Proliferation assays

Helper T cell clones (1×10^5) were cultured for 66 h in 96-well round-bottom plates containing 5×10^4 CD3-depleted APC, 0.5 μg/ml anti-CD3 mAb, and different numbers of Treg or helper T cell clones, and during the final 18 h, each well was pulsed with 0.5 μCi/well [³H]thymidine (Amersham Biosciences). The radioactivity incorporated into DNA, which correlates with cell proliferation, was measured in a liquid scintillation counter after harvesting the cell cultures onto glass fiber filters. For some experiments, Abs against IL-10, TGF-β_{1, 2, 3}, or isotype controls (all from R&D Systems) were added in the assay at a final concentration of 10 μg/ml. All experiments were conducted in triplicate, and results corresponded to the mean cpm with the SD.

Transwell experiments were conducted in 24-well plates with pore size 0.4-μm cell culture inserts (BD Biosciences). Helper T cell clones (5×10^5) were cultured in the outer wells of 24-well plates in medium containing 0.5 μg/ml anti-CD3 mAb and 1×10^6 CD3-depleted APC. Equal numbers of Treg cell clones were added into the inner wells in the same medium containing 0.5 μg/ml anti-CD3 mAb and 1×10^6 CD3-depleted APC. After 48 h culture, the cells in the outer wells were harvested and transferred to 96-well round-bottom plates. [³H]Thymidine (0.5 μCi/well) was added, and the cells were cultured for an additional 18 h before being harvested for measuring the radioactivity with a liquid scintillation counter.

CTLL-2 cell proliferation assays

Helper T cell clones (1×10^5) and Treg cell clones (1×10^5) were cultured in 96-well round-bottom plates containing 1×10^5 irradiated autologous PBMC with 3 μg/ml peptides, 10 μg/ml r-Bet v 1 protein, and/or 0.5 μg/ml anti-CD3 mAb. Supernatants were harvested 12 h later, and IL-2 contents were measured by the proliferation of CTLL-2 cells. Supernatants (50 μl) was added to CTLL-2 cells (1×10^4) in 96-well round-bottom plates. After 24 h culture, [³H]thymidine (0.5 μCi/well) was added, and the cells were cultured for an additional 18 h before being harvested for measuring the radioactivity with a liquid scintillation counter.

Results

Prediction of Th or Treg peptide epitopes for Bet v 1

For the present studies, we selected two peptides from the Bet v 1 sequence (Bet v 1₁₄₁₋₁₅₆, GETLLRAVESYLLAHS and Bet v 1₅₁₋₆₈, PGTIKKISFPEGFPFKYV), based on an analysis using a computer-based algorithm that predicts the capacity of peptide se-

quences to bind to MHC molecules (30). However, before determining whether these peptides would be able to induce CD4⁺ T cell responses, we examined the reactivity of the peptides against antisera obtained from patients with birch pollen nasal allergy. The rationale for this assessment was that we wished to avoid using peptides reacting with anti-Bet v 1 IgE Abs as therapeutics against allergies due to the possibility of generating immune complexes that could potentially produce toxic effects. Sera from patients with allergy to Bet v 1 contained IgE Abs that reacted with r-Bet v 1 protein but not with peptides Bet v 1₁₄₁₋₁₅₆ and Bet v 1₅₁₋₆₈ (data not shown). These results indicate that peptides Bet v 1₁₄₁₋₁₅₆ and Bet v 1₅₁₋₆₈ do not appear to contain epitopes recognized by Bet v 1-specific IgE Abs and that potentially they could induce helper T cell or Treg responses.

Generation of peptide Bet v 1₁₄₁₋₁₅₆-reactive CD4⁺ T cell clones

Purified CD4⁺ T cells derived from three patients with birch pollen nasal allergy (P1, P2, and P3) and 2 healthy volunteers (H1 and H2) were stimulated in primary cell cultures using peptide Bet v 1₁₄₁₋₁₅₆-pulsed autologous DC as APC (as described in *Materials and Methods*), and restimulated for three weekly cycles with the peptide and freshly prepared irradiated autologous PBMC as APC. Seven days after the last stimulation, the T cell cultures were tested for their ability to produce cytokines (IFN-γ, IL-4, or IL-10) upon stimulation with the peptide and APC. Those cultures that exhibited at least a 2.5-fold increase production of any one of the cytokines to Ag compared with in the absence of the peptide (data not shown) were expanded and cloned by limiting dilution for further studies. For peptide Bet v 1₁₄₁₋₁₅₆, we obtained 3 CD4⁺ T cell clones from patient 1 (P1-7F, 11B, and 11D), two T cell clones from patient 2 (P2-D5 and H11), two T cell clones from patient 3 (P3-4 and 2A), one T cell clone from healthy volunteer 1 (H1-3), and one T cell clone from healthy volunteer 2 (H2-6H). Table I contains information pertaining some of the characteristics of these T cell clones and the individuals (patients and healthy volunteers), where these clones were derived from.

The T cell clones were first examined for their cytokine (IFN-γ, IL-4, and IL-10) production profiles. As shown in Table I, most of CD4⁺ T cell clones from patients and healthy volunteers produced IFN-γ as the result of antigenic stimulation. Six of the nine T cell clones produced IL-4. Two of the nine T cell clones (P1-7F and H2-6H) predominantly produced IL-10 compared with other T cell clones. Most of the clones, with the exception of three (P1-11B,

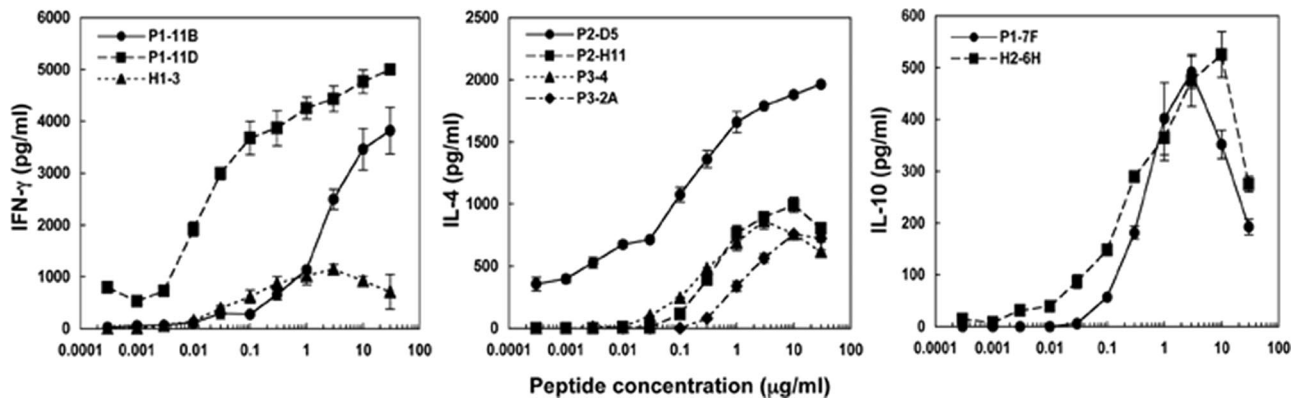


FIGURE 1. Peptide dose-response curves were done to estimate the avidity of CD4⁺ T cell clones for peptide Bet v 1_{141–156} using autologous PBMC as APC. These T cell clones were cocultured with autologous PBMC pulsed with peptide Bet v 1_{141–156} for 48 h. Supernatants were harvested to measure the secretion of IFN- γ , IL-4, and IL-10 using ELISA kits. No significant response was observed in the absence of peptide (data not shown). Values shown are triplicate determinations; error bars, SD.

P3-4, and H1-3), produced more than one of the lymphokines (Table I).

Next, we examined the response to Ag of CD4⁺ T cell clones by peptide titration curves using autologous PBMC as APC. The results in Fig. 1 show representative titration curves for a representative cytokine (IFN- γ , IL-4, or IL-10) produced by the

clones. The results revealed that Ag-mediated responses of the T cell clones occurred in a dose-dependent manner. Furthermore, the peptide concentration required to obtain half of the maximal response was in most of cases below 1 $\mu\text{g/ml}$, indicating that these clones had relatively high avidity for peptide Bet v 1_{141–156}.

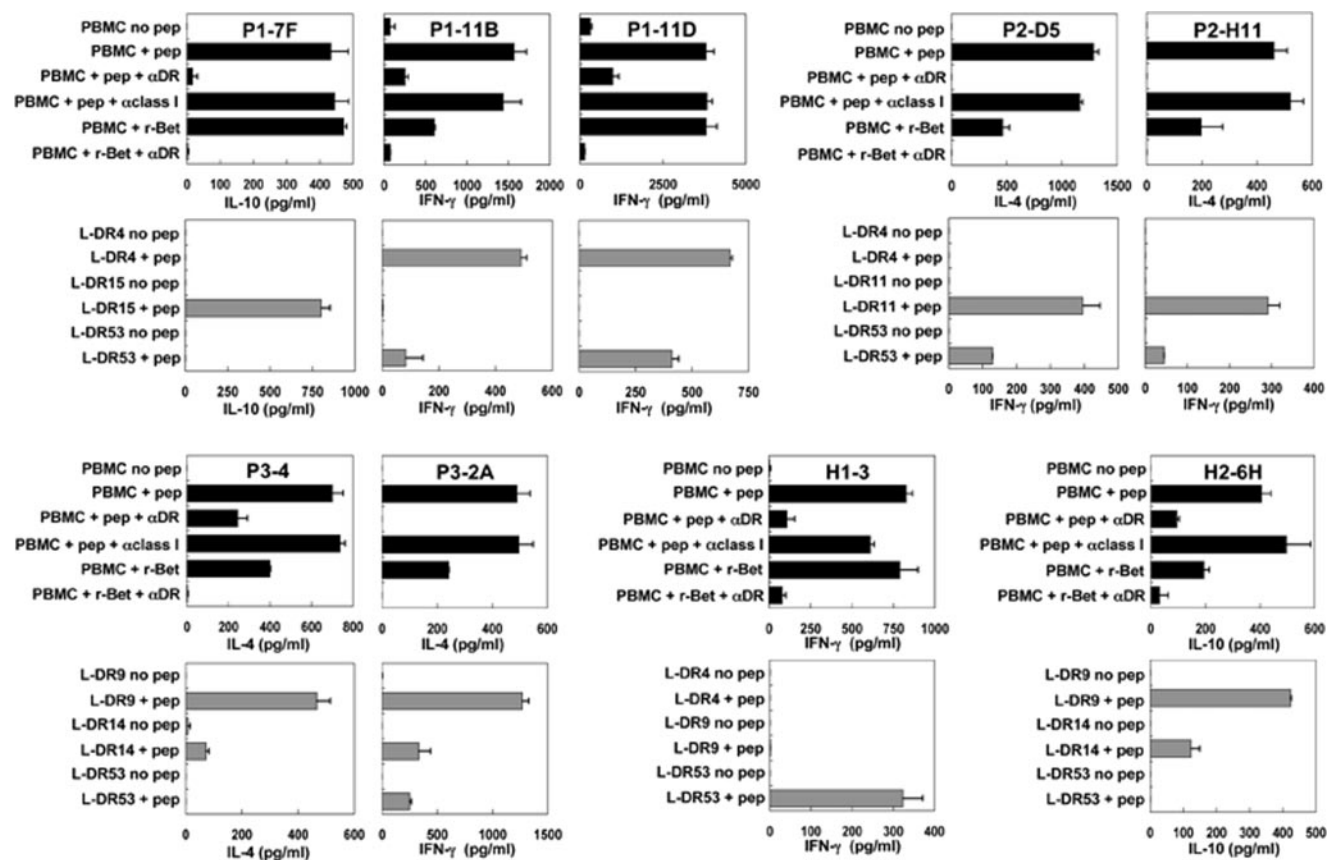


FIGURE 2. Specificity and MHC class II restriction analysis of peptide Bet v 1_{141–156}-reactive CD4⁺ T cell clones. T cell clones were isolated from an HLA-DR4/15 birch pollen allergic patient (P1-7F, 11B, and 11D), from an HLA-DR4/11 patient (P2-D5 and H11), from an HLA-DR9/14 patient (P3-4 and 2A), from an HLA-DR4/9 healthy volunteer (H1-3), and from an HLA-DR9/14 healthy volunteer (H2-6H) as described in *Materials and Methods*. Responses of T cell clones cocultured with autologous PBMC pulsed with peptide Bet v 1_{141–156} or r-Bet v 1 protein were inhibited by anti-HLA-DR mAb L243 but not by anti-HLA class I mAb W6/32 (both at used 10 $\mu\text{g/ml}$; top portion of each panel). When mouse fibroblasts cell lines (L cells) transfected with HLA-DR genes were used as APC (bottom portion of each panel), P1-7F, P1-11B, P1-11D, P2-D5, P2-H11, P3-4, P3-2A, H1-3, and H2-6H cells recognized the peptide in the context of HLA-DR15, DR4, DR4/53, DR11, DR11, DR9, DR9, DR53, and DR9, respectively. Values shown are triplicate determinations; error bars, SD.

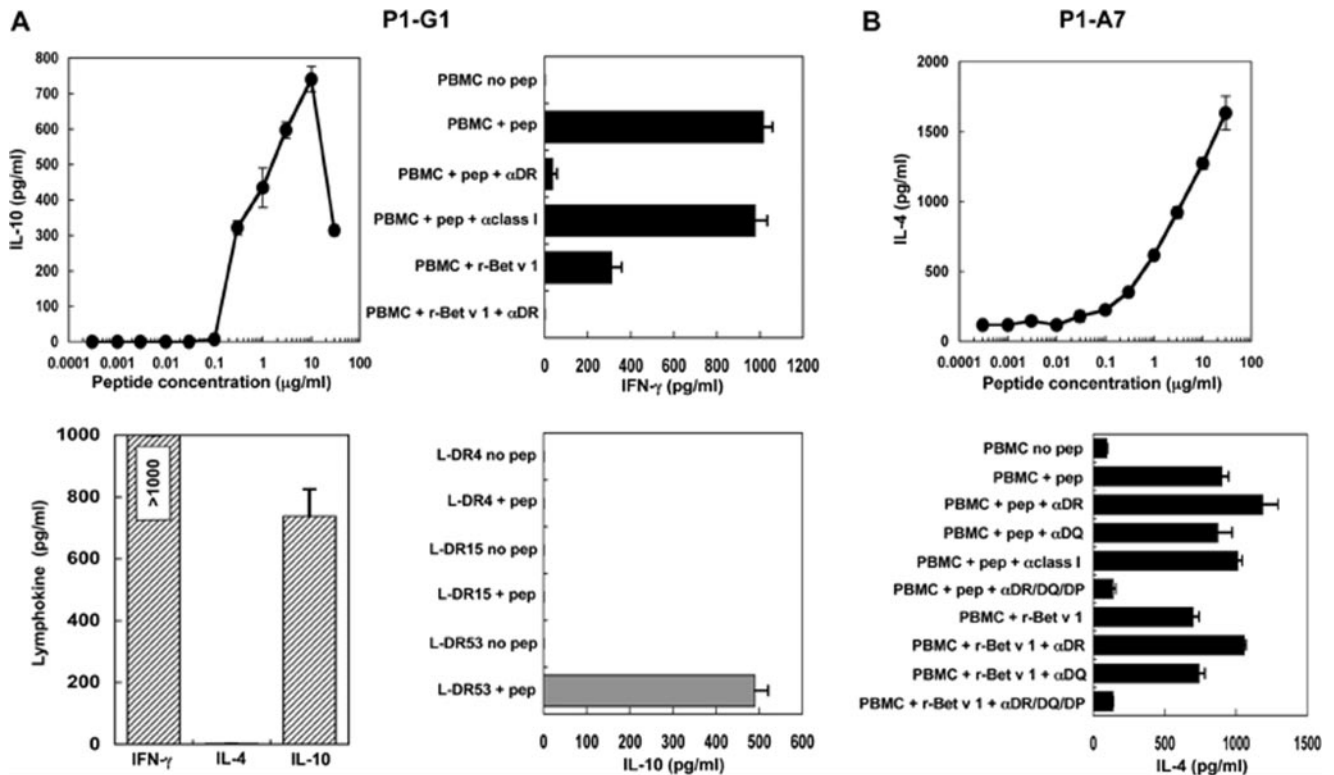


FIGURE 3. Ag specificity, MHC class II restriction, and cytokine profile of peptide Bet v $_{151-68}$ -reactive $CD4^+$ T cell clones. Two T cell clones (P1-G1 and P1-A7) were selected from an HLA-DR4/15 birch pollen allergic patient (patient 1) as described in *Materials and Methods*. *A, upper left panel*, Peptide dose-response curve was done to estimate the avidity of P1-G1 cells for peptide Bet v $_{151-68}$ using autologous PBMC as APC. *Upper right panel*, Response of P1-G1 cells cocultured with autologous PBMC pulsed with peptide Bet v $_{151-68}$ or r-Bet v 1 protein was inhibited by anti-HLA-DR mAb L243 but not by anti-HLA class I mAb W6/32 (both at used 10 μ g/ml). *Bottom right panel*, When mouse fibroblasts cell lines (L cells) transfected with *HLA-DR* genes were used as APC, P1-G1 cells recognized the peptide in the context of *HLA-DR53* allele. *Bottom left panel*, Cytokine profile of P1-G1 cells cocultured with autologous PBMC pulsed with peptide Bet v $_{151-68}$ for 48 h analyzed by ELISA. *B, upper panel*, Peptide dose-response curve was done to estimate the avidity of P1-A7 cells for peptide Bet v $_{151-68}$ using autologous PBMC as APC. *Bottom panel*, Response of P1-A7 cells cocultured with autologous PBMC pulsed with peptide Bet v $_{151-68}$ or r-Bet v 1 protein was inhibited by anti-HLA class II (DR, DQ and DP) mAb TU39 but not by anti-HLA-DR mAb L243, anti-HLA-DQ mAb SPVL3, or anti-HLA class I mAb W6/32 (all at used 10 μ g/ml).

Specificity and MHC class II restriction pattern of peptide Bet v $_{141-156}$ -reactive $CD4^+$ T cell clones

The nine Bet v $_{141-156}$ -reactive T cell clones were analyzed in more detail for their Ag specificity and MHC class II restriction pattern. First we assessed whether the epitope represented by peptide Bet v $_{141-156}$ could be produced by APC from protein Ag through the natural MHC class II Ag processing pathway. Thus, we tested the reactivity of the T cell clones against autologous APC that were fed r-Bet v 1 protein as a source of Ag. In all cases, the peptide-induced T cell clones were capable of recognizing the protein Ag presented by the APC (Fig. 2), indicating that epitope Bet v $_{141-156}$ is generated through Ag processing mechanisms. Moreover, the peptide and protein-induced response of all T cell clones was blocked by anti-HLA-DR mAb (L243) but not by anti-HLA class I mAb (W6/32), indicating that these T cells recognized Ag in the context of MHC class II molecules (Fig. 2).

Because the L243 mAb does not react with either HLA-DQ or HLA-DP, we assumed that HLA-DR molecules functioned as the MHC class II-restricting elements. To confirm this and to more specifically determine which DR alleles were responsible for peptide presentation, several L-cell fibroblast lines transfected with and expressing individual HLA-DR alleles were used as APC. The results in Fig. 2 show that five different HLA-DR alleles (HLA-DR4, -DR9, -DR11, -DR15, and -DR53) could present peptide Bet v $_{141-156}$ to the T cell clones (see Table I for summary of results). However, each clone, with the exception of P1-11D cells, prefer-

entially recognized the peptide in the context of one DR allele. These results indicate that the T cell epitope represented by peptide Bet v $_{141-156}$ is highly promiscuous with respect to its MHC class II restriction pattern.

Generation, Ag specificity, MHC class II restriction, and cytokine profile of Bet v $_{151-68}$ -reactive T cell clones

Following the same approach as described above, we were only able to generate 2 peptide Bet v $_{151-68}$ -reactive $CD4^+$ T cell clones (P1-G1 and P1-A7) from patient 1 (Fig. 3). While clone P1-G1 produced IFN- γ and IL-10 upon antigenic stimulation (Fig. 3A), clone P1-A7 secreted mostly IL-4 and little IFN- γ and IL-10 (Fig. 3B and Table I) as a result of activation with Ag and APC. Peptide titration curves revealed that Ag-mediated responses of these T cell clones occurred in a dose-dependent manner and that these T cell clones have relatively high avidities for the epitope Bet v $_{151-68}$. The two peptide Bet v $_{151-68}$ -reactive T cell clones responded to autologous PBMC pulsed with r-Bet v 1 protein, indicating that peptide Bet v $_{151-68}$ is also a naturally processed epitope. With respect to MHC class II restriction, as shown in Fig. 3A, only the L cells expressing HLA-DR53 were capable of presenting peptide Bet v $_{151-68}$ to P1-G1 cells. Furthermore, both peptide and protein-induced responses of this clone were inhibited by anti-HLA-DR mAb. On the other hand, clone P1-A7 was only inhibited by a broadly reactive anti-HLA class II (DR, DQ, and DP) mAb but not by more specific anti-HLA-DR or -DQ mAb

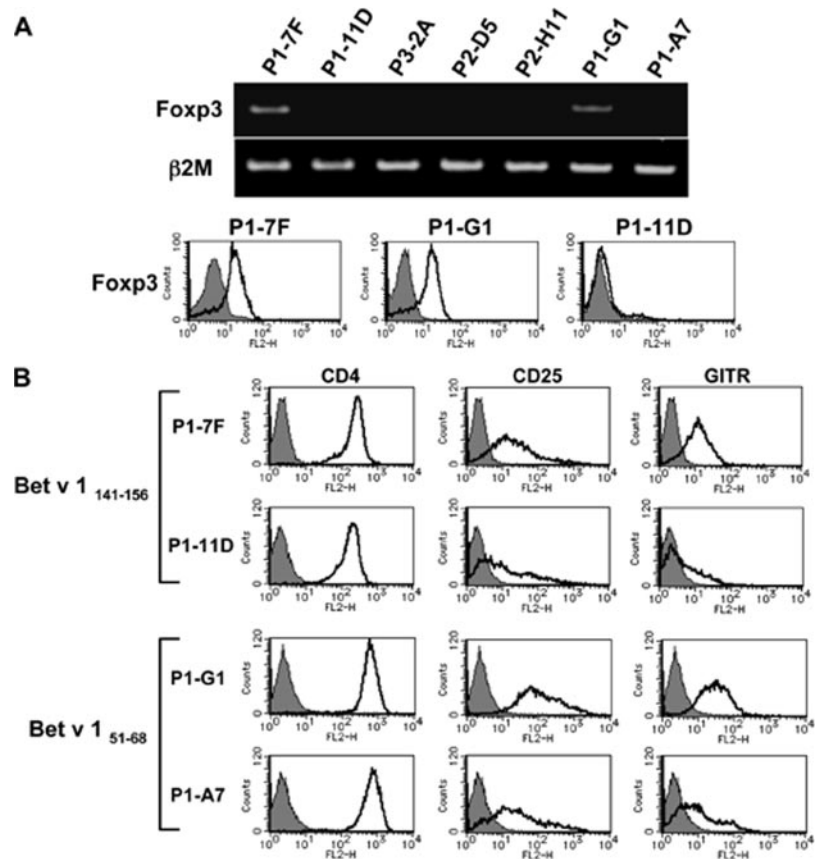


FIGURE 4. Phenotypic analysis of Bet v 1 peptide-reactive $CD4^+$ T cell clones. **A**, Expression of Foxp3 by RT-PCR and intracellular staining. Total RNA extracted from T cell clones was reverse transcribed and analyzed by PCR for the expression of Foxp3. P1-7F and P1-G1 cells were positive for Foxp3 mRNA, but other T cell clones were negative. As an internal control, β_2 -M cDNA was coamplified in each sample. For intracellular staining, cells were stained with PE-conjugated mAbs to Foxp3 (thick lines). Filled histograms, cells stained with isotype control Abs. P1-7F and P1-G1 cells expressed a significantly higher level of intracellular Foxp3 compared with P1-11D cells. **B**, Expression of cell surface molecules by flow cytometric analysis. Cells were stained with PE-conjugated mAbs to CD4, CD25, and GITR molecules (thick lines). Filled histograms, cells stained with isotype control Abs. Expression levels for CD25 and GITR was higher on the Foxp3 positive T cell clones as compared with the negative ones.

(Fig. 3B), suggesting that P1-A7 cells recognize Ag in the context of an HLA-DP molecule. However, at present we do not know the specific HLA-DP allele that restricts the response to Ag by clone P1-A7.

Phenotypic analysis of Bet v 1 peptide-reactive T cell clones

We observed that clones P1-7F, P1-G1, and H2-6H appeared to produce higher levels of IL-10 as compared with the other T cell clones (Table I), suggesting that these clones might represent Treg cells (14, 27). To test this hypothesis, we assessed the expression of Foxp3 (a transcription factor preferentially expressed by Treg cells) on these clones by RT-PCR and flow cytometric analysis. However, because H2-6H cells ceased to grow in tissue culture, we were not able to assess whether this cell clone expressed Foxp3. As shown in Fig. 4A, expression of Foxp3 mRNA was detected in P1-7F and P1-G1 cells, but not in several of the other T cell clones. Furthermore, in flow cytometric analysis, P1-7F and P1-G1 cells expressed a significantly higher level of intracellular Foxp3 compared with P1-11D cells.

Next, we examined the cell surface phenotypes of 4 T cell clones (which were selected as being representative) by flow cytometric analysis (Fig. 4B). As expected, all T cell clones were positive for CD4, but the expression of CD25 and GITR was significantly higher in the Foxp3 positive clones (P1-7F and P1-G1) as compared with the 2 Foxp3 negative clones (P1-11D and P1-A7).

Suppressive activity of Bet v 1 peptide-reactive T cell clones

The results presented above raised the possibility that T cell clones, P1-7F and P1-G1, might function as Treg cells, capable of inhibiting the effector function of other T lymphocytes. Thus, we examined the capacity of these T cell clones to suppress the proliferative responses of other Bet v 1 peptide-reactive $CD4^+$ T cell clones. For these experiments, we cocultured Foxp3-negative “re-

sponder” T cells (P1-11D or P1-A7) with the putative Foxp3-positive Treg clones (P1-7F or P1-G1) in medium containing autologous T cell-depleted APC and anti-CD3 mAb (to stimulate T cell proliferation). As shown in Fig. 5, P1-7F and P1-G1 (both Foxp3 positive) cells suppressed the proliferation of P1-11D cells in a dose-dependent manner. In contrast, P1-11B cells (a Foxp3-negative clone, data not shown) enhanced the proliferation of the responder P1-11D cells. Similarly, when we cocultured P1-A7 “responder” cells (Foxp3 negative) with P1-G1 “Treg” cells (Foxp3 positive), the proliferation of responder cells was inhibited in a dose-dependent manner (data not shown). It should be noted that Treg clones P1-7F and P1-G1 cells were anergic for the stimulation of anti-CD3 mAb (i.e., they failed to proliferate by themselves: 0:1 condition in Fig. 5). These results indicate that P1-7F and P1-G1 cells can be functionally and phenotypically considered as Treg cells.

Bet v 1-reactive Treg clones require cell-cell contact to exert their suppressive activity

We next examined how the Treg cell clones suppress the proliferation of helper T cell clones. The mechanism of suppression by Treg cells remains somewhat controversial with respect to whether it is mediated via cell-cell contact or soluble mediators, such as IL-10 and TGF- β (29). Thus, we first examined whether anti-IL-10 or anti-TGF- β Abs would be able to block the suppressive activity of the Treg cells. As shown in Fig. 6A, neither anti-IL-10 nor anti-TGF- β could block the suppressive effects of the P1-7F Treg cell clone on the proliferative response of the P1-11D helper T cell clone. Similarly, both Abs did not block the suppressive activity of the P1-G1 Treg cell clone on the proliferative response of P1-A7 helper T cell clone.

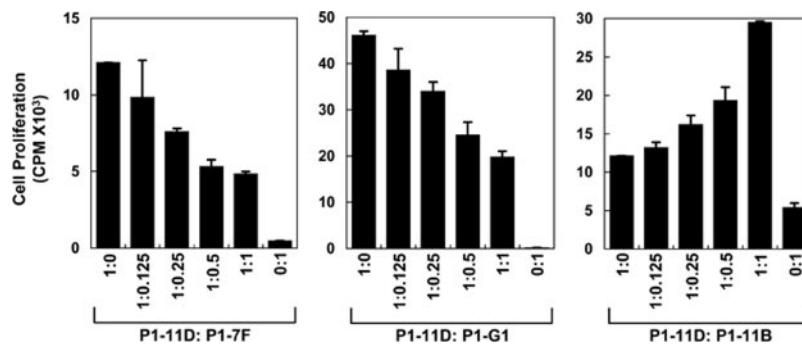


FIGURE 5. Functional analysis of Bet v 1 peptide-reactive Treg cell clones. Helper T cell clones (1×10^5) were cultured with CD3-depleted APC (5×10^4), anti-CD3 mAb ($0.5 \mu\text{g/ml}$), and different numbers of Treg or helper T cell clones for 66 h. The proliferation of helper T cell clones was measured by the direct incorporation of [^3H]thymidine added during the final 18 h. Bet v 1 peptide-reactive Treg cell clone, P1-7F and P1-G1, suppressed the proliferation of P1-11D cells in a dose-dependent manner. By contrast, P1-11B cells enhanced the proliferation of P1-11D cells. P1-7F and P1-G1 cells were anergic for the stimulation of anti-CD3 mAb. Values shown are triplicate determinations; error bars, SD.

To examine whether the Treg cell clones required cell-cell contact to exert their suppressive activity, we performed Transwell experiments. Helper T cell clones were cultured in the outer wells

in medium containing anti-CD3 mAb and T cell-depleted APC. Treg cell clones were added into the inner wells in the same medium containing anti-CD3 mAb and T cell-depleted APC. As a positive or negative control, we included a condition where both Treg and helper T cells were together in the outer well or only medium containing anti-CD3 mAb and T cell-depleted APC were added into the inner wells. As shown in Fig. 6B, neither P1-7F nor P1-G1 were able to suppress the proliferation of helper T cell clones when cocultured separately in a Transwell system. However, suppression occurred when both responder and Treg cells were situated together in the outer wells of the Transwell system. These results indicate that Bet v 1 peptide-reactive Treg cell clones generated in this study require cell-cell contact to exert their suppressive activity.

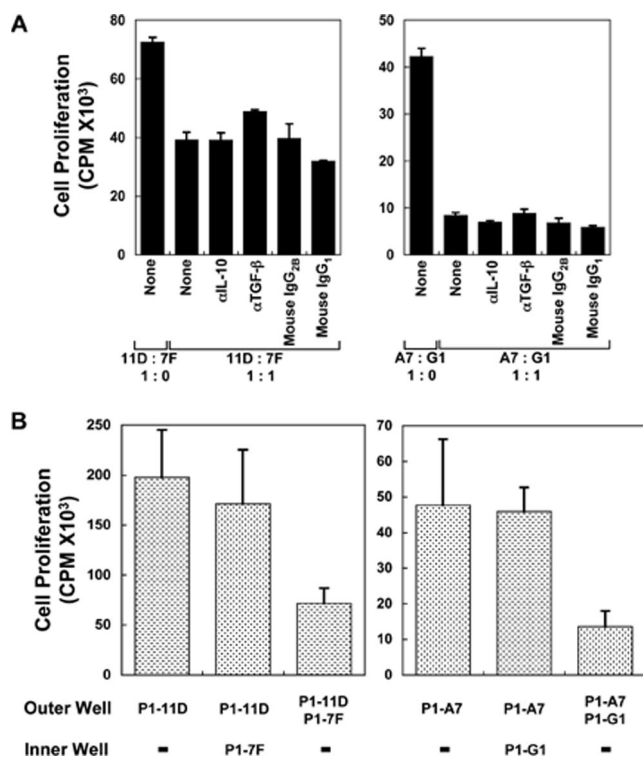


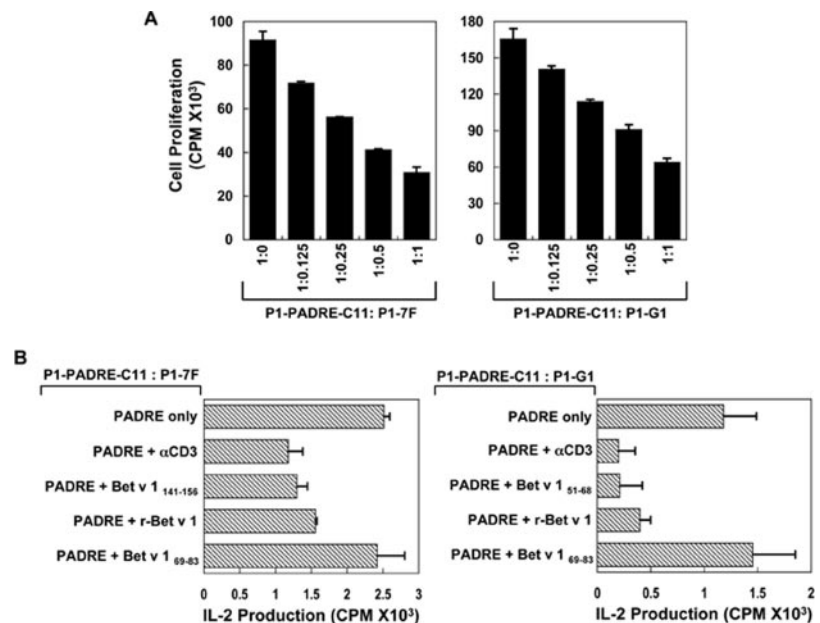
FIGURE 6. Bet v 1 peptide-reactive Treg cell clones require cell-cell contact to exert their suppressive activity. *A*, Helper T cell clones (1×10^5) were cultured with CD3-depleted APC (5×10^4), anti-CD3 mAb ($0.5 \mu\text{g/ml}$), and Treg cell clones (1×10^5) in the presence of anti-IL-10 ($\alpha\text{IL-10}$) or anti-TGF- β ($\alpha\text{TGF-}\beta$) mAb (both at $10 \mu\text{g/ml}$). Neither anti-IL-10 nor anti-TGF- β mAb could block the suppressive effects of Treg cell clones (P1-7F and P1-G1) on helper T cell clones (P1-11D and P1-A7). Mouse IgG_{2b} and IgG₁ served as isotype controls for anti-IL-10 and anti-TGF- β mAb, respectively. Values shown are triplicate determinations; error bars, SD. *B*, Transwell experiments were conducted in 24-well plates with pore size $0.4\text{-}\mu\text{m}$ cell culture inserts. Helper T cell clones (5×10^5) and Treg cell clones (5×10^5) were cultured in the outer wells and in the inner wells, respectively, with medium containing anti-CD3 mAb ($0.5 \mu\text{g/ml}$) and CD3-depleted APC (1×10^6). Treg cell clones (P1-7F and P1-G1) could not suppress the proliferation of helper T cell clones (P1-11D and P1-A7) when cocultured separately in a Transwell system. Assessments were conducted in three independent experiments. Columns, mean; error bars, SD.

Bet v 1 Treg cells inhibit IL-2 secretion by helper T cells and require ligand-specific activation for their suppressive activities

Although Ag-induced Treg cells require Ag exposure to acquire their suppressive activities, it has been reported that in some cases their inhibitory effects can be mediated in an Ag independent manner (29). Therefore, we examined whether our Bet v 1 peptide-reactive Treg cell clones required the presence of their own Ag to suppress the function of other Ag-reactive helper T cell clones. To perform these experiments, we generated a helper T cell clone reactive with the Pan DR Epitope (PADRE) peptide (37) (P1-PADRE-C11) from patient 1. First, we cocultured the P1-PADRE-C11 helper T cells with either the P1-7F or P1-G1 Treg cells in medium containing autologous T cell-depleted APC and anti-CD3 mAb. As shown in Fig. 7A, P1-7F and P1-G1 cells suppressed the proliferation of the P1-PADRE-C11 T cells in a dose-dependent manner, indicating that Bet v 1 peptide-reactive Treg cell clones could also suppress the proliferation of other Ag-reactive helper T cell clones.

We next tested whether Bet v 1-reactive Treg cell clones required ligand-specific activation to exhibit their suppressive activity against PADRE-reactive T cells. P1-7F and P1-PADRE-C11 cells were cultured with irradiated autologous PBMC pulsed with various peptide combinations. In these experiments, anti-CD3 mAb was used as a positive control to activate the P1-7F cells, while peptide Bet v 1₆₉₋₈₃ was used as an irrelevant antigenic peptide (negative control). Suppressive activity of P1-7F cells in these experiments was assessed by the inhibition of Ag mediated IL-2 production by the P1-PADRE-C11 Th cells since it is known that Treg cells typically suppress the proliferation of helper T cells by inhibiting IL-2 secretion (27, 28, 38, 39). P1-7F cells inhibited

FIGURE 7. Bet v 1 peptide-reactive Treg cell clones mediate suppression of IL-2 production by helper T cell clones and require ligand-specific activation to exert their suppressive activity. **A**, Bet v 1 peptide-reactive Treg cell clones (P1-7F and P1-G1) suppressed the proliferation of PADRE peptide-reactive CD4⁺ T cell clone (P1-PADRE-C11) in a dose-dependent manner. The culture conditions were identical to those in Fig. 5. **B**, Helper T cell clone (P1-PADRE-C11, 1×10^5) and Treg cell clones (P1-7F or P1-G1, 1×10^5) were cultured with irradiated autologous PBMC (1×10^5) in the presence of PADRE peptide and anti-CD3 (α CD3) mAb, Bet v 1 peptides, or r-Bet v 1 protein. Supernatants of cell cultures were harvested 12 h later, and IL-2 contents were measured by the proliferation of CTLL-2 cells. IL-2 secretion from P1-PADRE-C11 cells stimulated by PADRE peptide was suppressed by P1-7F or P1-G1 cells in the presence of Bet v 1 peptides (peptide Bet v 1₁₄₁₋₁₅₆ or Bet v 1₅₁₋₆₈, respectively) or r-Bet v 1 protein as well as anti-CD3 mAb used as a positive control. Peptide Bet v 1₆₉₋₈₃ was used as a negative control for the activation of Treg cell clones. Values shown are triplicate determinations; error bars, SD.



IL-2 secretion from P1-PADRE-C11 cells in the presence of peptide Bet v 1₁₄₁₋₁₅₆, or r-Bet v 1 protein as well as with anti-CD3 mAb (Fig. 7B, left panel). However, P1-7F cells could not inhibit IL-2 secretion from P1-PADRE-C11 cells in the presence of peptide PADRE alone or with the irrelevant peptide Bet v 1₆₉₋₈₃. Similar results were obtained with the P1-G1 Treg clone (Fig. 7B, right panel). These results demonstrate that Bet v 1 peptide-reactive Treg cell clones, P1-7F and P1-G1, require ligand-specific activation to exert their suppressive activity.

Discussion

Allergen-specific immunotherapy using therapeutic vaccines is an efficient treatment for allergic diseases including birch pollen allergy because it can affect the course of the disease and alleviate the symptoms. However, using the natural Ags in this therapy can cause severe reactions, including anaphylaxis. Therefore, peptide-based vaccines, such as defined T cell epitopes (but not B cell epitopes binding to Ag-specific IgE on mast cells or basophils), would be safe and useful in this type of therapy. In a previous study, we described some T cell epitopes of Bet v 1 and found that peptide Bet v 1₁₇₋₂₄ was recognized by Bet v 1-reactive T cells in the context of the HLA-DQ1 molecule (40). It is thought that desirable peptides for therapeutic vaccines should include promiscuous T cell epitopes, which can be recognized by CD4⁺ T cells in the context of more than one MHC class II allele, to offer a broad population coverage (3).

In this study, we first selected potential HLA-DR-restricted CD4⁺ T cell epitopes from the amino acid sequence of the Bet v 1 protein using a computer-based peptide/MHC binding algorithm (30) to identify potential promiscuous epitopes for this Ag. Two of the highest-ranking potentially promiscuous sequences that were identified by this analysis were Bet v 1₁₄₁₋₁₅₆ and Bet v 1₅₁₋₆₈ (data not shown). Bet v 1-specific IgE Abs in patients were not able to react to these two peptides, suggesting that two peptides do not contain epitopes recognized by Bet v 1-specific IgE Abs (data not shown). Interestingly, Bet v 1₁₄₁₋₁₅₆ is quite similar to the sequence that was reported previously by Ebner and his colleagues (41–43) that contains immunodominant T cell epitopes. These authors reported that the segment of Bet v 1 encompassing residues 145–158 contained T cell epitopes restricted by HLA-DR7

and DR15 (42). We also could elicit 9 different peptide Bet v 1₁₄₁₋₁₅₆-reactive CD4⁺ T cell clones and define the restriction of MHC molecules. The recognition of peptide Bet v 1₁₄₁₋₁₅₆ by these CD4⁺ T cell clones was restricted by HLA-DR4, DR9, DR11, DR15, or DR53 (Table I and Fig. 2). Our results and the data reported by Ebner et al. (42) clearly show that Bet v 1₁₄₁₋₁₅₆ includes a promiscuous epitope that can be presented to specific T cells by multiple HLA-DR molecules. In addition, we revealed that the recognition of peptide Bet v 1₅₁₋₆₈ by CD4⁺ T cells was restricted by HLA-DR53 and DP alleles. Bet v 1₁₄₁₋₁₅₆ and Bet v 1₅₁₋₆₈-reactive CD4⁺ T cell clones responded autologous PBMC pulsed with r-Bet v 1 protein, demonstrating that these T cell epitopes are naturally processed by APC via the exogenous MHC class II pathway.

The production of IFN- γ and IL-4 by Bet v 1 peptide-reactive CD4⁺ T cell clones revealed various patterns (Table I). Our results are in accordance with previous findings by Ebner et al. (44) and Sparholt et al. (45). However, Bet v 1 peptide-reactive CD4⁺ T cell clones generated from healthy volunteers produced low levels of IL-4, as compared with T cell clones generated from patients (Table I). To some extent these results were to be expected since Ag-specific IgE synthesis in type I allergic patients is regulated by IL-4, which is produced by Ag-specific CD4⁺ T cells. Similar findings have been reported by Ebner's group (43).

It has been observed that allergen-specific immunotherapy of allergic rhinitis and asthma results in increased numbers of IL-10-producing cells in peripheral blood (4, 14), suggesting that Treg cells may play an important role in this therapy. Accordingly, we investigated whether some of the Bet v 1 peptide-reactive T cell clones produced IL-10 and found that 3 clones (P1-7F, P1-G1, and H2-6H) secreted higher levels of IL-10 as compared with other CD4⁺ T cell clones (Table I). Moreover, our results indicate that 2 of these clones had other phenotypic characteristics of Treg cells: Foxp3⁺, CD25^{high}, and GITR⁺ (Fig. 4). More importantly, we were able to demonstrate that these T cell clones suppressed the proliferation of other helper T cell clones (Fig. 5) and that suppressor function required cell-cell contact and was not merely mediated via soluble IL-10 and TGF- β (Fig. 6). Our results also indicate that Bet v 1-reactive Treg clones required TCR stimulation to suppress the

activity of responder T cells (Fig. 7). Recently, Voo et al. (28) reported that EBV-encoded nuclear Ag 1 peptide-reactive Treg cells required cell-cell contact or an unidentified soluble factor (not IL-10 nor TGF- β) to exert their suppressive activity.

In our study, Bet v 1 Treg cells did not require IL-10 to suppress the proliferation of effector T cells in vitro though they secreted IL-10. However, there is a possibility that IL-10 produced by this type of Treg cells could have an important role in vivo. Allergic diseases are associated with high serum levels of allergen-specific IgE while normal response to allergens in healthy individuals is characterized by IgG Abs, especially of IgG4 class (46), which will compete with IgE Abs for binding to the allergen (47, 48). Several studies show that there are increases in the levels of serum allergen-specific IgG4 following allergen-specific immunotherapy (14, 49). Because IL-10 enhances IgG4 production (48–50), IL-10 producing Bet v 1-reactive Treg cells could increase the levels of allergen-specific IgG4 Ab levels as the result of peptide-based immunotherapy. Although naturally occurring Treg cells require cell-cell contact to exert their suppressive activity in vitro, some studies have shown that these Treg cells can also exert a suppressive effect through induction of IL-10 and/or TGF- β in vivo (48, 51–53). Thus, it is possible that Bet v 1-reactive Treg cells such as the ones we have described here could be capable of suppressing the proliferation of effector T cells through the IL-10 production in vivo.

Disclosures

The authors have no financial conflict of interest.

References

- Durham, S. R., S. M. Walker, E. M. Varga, M. R. Jacobson, F. O'Brien, W. Noble, S. J. Till, Q. A. Hamid, and K. T. Nouri-Aria. 1999. Long-term clinical efficacy of grass-pollen immunotherapy. *N. Engl. J. Med.* 341: 468–475.
- Valenta, R., T. Ball, M. Focke, B. Linhart, N. Mothes, V. Niederberger, S. Spitzauer, I. Swohoda, S. Vrtala, K. Westritschnig, and D. Kraft. 2004. Immunotherapy of allergic disease. *Adv. Immunol.* 82: 105–153.
- Larche, M., and D. C. Wraith. 2005. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat. Med.* 11: S69–S76.
- Francis, J. N., S. J. Till, and S. R. Durham. 2003. Induction of IL-10⁺CD4⁺CD25⁺ T cells by grass pollen immunotherapy. *J. Allergy Clin. Immunol.* 111: 1255–1261.
- Norman, P. S., J. L. Ohman, Jr., A. A. Long, P. S. Creticos, M. A. Geftter, Z. Shaked, R. A. Wood, P. A. Eggleston, K. B. Hafner, P. Rao, et al. 1996. Treatment of cat allergy with T-cell reactive peptides. *Am. J. Respir. Crit. Care Med.* 154: 1623–1628.
- Simons, F. E., M. Imada, Y. Li, W. T. Watson, and K. T. HayGlass. 1996. Fel d 1 peptides: effect on skin tests and cytokine synthesis in cat-allergic human subjects. *Int. Immunol.* 8: 1937–1945.
- Pene, J., A. Desroches, L. Paradis, B. Lebel, M. Farce, C. F. Nicodemus, H. Yssel, and J. Bousquet. 1998. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. *J. Allergy Clin. Immunol.* 102: 571–578.
- Muller, U., C. A. Akdis, M. Fricker, M. Akdis, T. Blesken, F. Bettens, and K. Blaser. 1998. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J. Allergy Clin. Immunol.* 101: 747–754.
- Maguire, P., C. Nicodemus, D. Robinson, D. Aaronson, and D. T. Umetsu. 1999. The safety and efficacy of ALLERVAX CAT in cat allergic patients. *Clin. Immunol.* 93: 222–231.
- Oldfield, W. L., A. B. Kay, and M. Larche. 2001. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J. Immunol.* 167: 1734–1739.
- Oldfield, W. L., M. Larche, and A. B. Kay. 2002. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 360: 47–53.
- Fellrath, J. M., A. Kettner, N. Dufour, C. Frigerio, D. Schneeberger, A. Leimgruber, G. Corradin, and F. Spertini. 2003. Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. *J. Allergy Clin. Immunol.* 111: 854–861.
- Alexander, C., M. Tarzi, M. Larche, and A. B. Kay. 2005. The effect of Fel d 1-derived T-cell peptides on upper and lower airway outcome measurements in cat-allergic subjects. *Allergy* 60: 1269–1274.
- Jutel, M., M. Akdis, F. Budak, C. Aebischer-Casaulta, M. Wrzyszc, K. Blaser, and C. A. Akdis. 2003. IL-10 and TGF- β cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur. J. Immunol.* 33: 1205–1214.
- Bluestone, J. A., and A. K. Abbas. 2003. Natural versus adaptive regulatory T cells. *Nat. Rev. Immunol.* 3: 253–257.
- Sakaguchi, S. 2005. Naturally arising Foxp3-expressing CD25⁺CD4⁺ regulatory T cells in immunological tolerance to self and non-self. *Nat. Immunol.* 6: 345–352.
- Roncarolo, M. G., and M. K. Levings. 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr. Opin. Immunol.* 12: 676–683.
- Bach, J. F. 2003. Regulatory T cells under scrutiny. *Nat. Rev. Immunol.* 3: 189–198.
- von Herrath, M. G., and L. C. Harrison. 2003. Antigen-induced regulatory T cells in autoimmunity. *Nat. Rev. Immunol.* 3: 223–232.
- Stock, P., O. Akbari, G. Berry, G. J. Freeman, R. H. Dekruyff, and D. T. Umetsu. 2004. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat. Immunol.* 5: 1149–1156.
- Kumar, V., and E. Sercarz. 1998. Induction or protection from experimental autoimmune encephalomyelitis depends on the cytokine secretion profile of TCR peptide-specific regulatory CD4 T cells. *J. Immunol.* 161: 6585–6591.
- Sakaguchi, S., N. Sakaguchi, J. Shimizu, S. Yamazaki, T. Sakihama, M. Itoh, Y. Kuniyasu, T. Nomura, M. Toda, and T. Takahashi. 2001. Immunologic tolerance maintained by CD25⁺CD4⁺ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol. Rev.* 182: 18–32.
- Wood, K. J., and S. Sakaguchi. 2003. Regulatory T cells in transplantation tolerance. *Nat. Rev. Immunol.* 3: 199–210.
- Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057–1061.
- Khatiri, R., T. Cox, S. A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin in CD4⁺CD25⁺ T regulatory cells. *Nat. Immunol.* 4: 337–342.
- Walker, M. R., D. J. Kaspricz, V. H. Gersuk, A. Benard, M. Van Landeghen, J. H. Buckner, and S. F. Ziegler. 2003. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4⁺CD25⁺ T cells. *J. Clin. Invest.* 112: 1437–1443.
- Wang, H. Y., D. A. Lee, G. Peng, Z. Guo, Y. Li, Y. Kuniwa, E. M. Shevach, and R. F. Wang. 2004. Tumor-specific human CD4⁺ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20: 107–118.
- Voo, K. S., G. Peng, Z. Guo, T. Fu, Y. Li, L. Frappier, and R. F. Wang. 2005. Functional characterization of EBV-encoded nuclear antigen 1-specific CD4⁺ helper and regulatory T cells elicited in vitro peptide stimulation. *Cancer Res.* 65: 1577–1586.
- Bluestone, J. A. 2005. Regulatory T-cell therapy: is it ready for the clinic? *Nat. Rev. Immunol.* 5: 343–349.
- Rammensee, H., J. Bachmann, N. P. Emmerich, O. A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50: 213–219.
- Nakatsura, T., S. Senju, M. Ito, Y. Nishimura, and K. Itoh. 2002. Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. *Eur. J. Cell. Biol.* 82: 826–836.
- Kobayashi, H., M. Wood, Y. Song, E. Appella, and E. Celis. 2000. Defining promiscuous MHC class II helper T-cell epitopes for the HER2/neu tumor antigen. *Cancer Res.* 60: 5228–5236.
- Kobayashi, H., R. Omiya, B. Sodey, M. Yanai, K. Oikawa, K. Sato, S. Kimura, S. Senju, Y. Nishimura, M. Tateno, and E. Celis. 2003. Identification of naturally processed helper T-cell epitopes from prostate-specific membrane antigen using peptide-based in vitro stimulation. *Clin. Cancer Res.* 9: 5386–5393.
- Kobayashi, H., T. Nagato, M. Yanai, K. Oikawa, K. Sato, S. Kimura, M. Tateno, R. Omiya, and E. Celis. 2004. Recognition of adult T-cell leukemia/lymphoma cells by CD4⁺ helper T lymphocytes specific for human T-cell leukemia virus type I envelope protein. *Clin. Cancer Res.* 10: 7053–7062.
- Kobayashi, H., T. Nagato, K. Oikawa, K. Sato, S. Kimura, N. Aoki, R. Omiya, M. Tateno, and E. Celis. 2005. Recognition of prostate and breast tumor cells by helper T lymphocytes specific for a prostate and breast tumor-associated antigen, TARP. *Clin. Cancer Res.* 11: 3869–3878.
- Kobayashi, H., T. Nagato, N. Aoki, K. Sato, S. Kimura, M. Tateno, and E. Celis. 2006. Defining MHC class II T helper epitopes for WT1 tumor antigen. *Cancer Immunol. Immunother.* 55: 850–860.
- Alexander, J., J. Sidney, S. Southwood, J. Ruppert, C. Oseroff, A. Maewal, K. Snok, H. M. Serra, R. T. Kubo, A. Sette, et al. 1994. Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides. *Immunity* 1: 751–761.
- Jiang, S., N. Camara, G. Lombardi, and R. I. Lechler. 2003. Induction of alloptide-specific human CD4⁺CD25⁺ regulatory T cells ex vivo. *Blood* 102: 2180–2186.
- Wang, H. Y., G. Peng, Z. Guo, E. M. Shevach, and R. F. Wang. 2005. Recognition of a new ARTC1 peptide ligand uniquely expressed in tumor cells by antigen-specific CD4⁺ regulatory T cells. *J. Immunol.* 174: 2661–2670.
- Abe, Y., S. Kimura, T. Kokubo, K. Mizumoto, M. Uehara, and M. Katagiri. 1997. Epitope analysis of birch pollen allergen in Japanese subjects. *J. Clin. Immunol.* 17: 485–493.
- Ebner, C., Z. Szeplafusi, F. Ferreira, A. Jilek, R. Valenta, P. Parronchi, E. Maggi, S. Romagnani, O. Scheiner, and D. Kraft. 1993. Identification of multiple T cell epitopes on Bet v I, the major birch pollen allergen, using specific T cell clones and overlapping peptides. *J. Immunol.* 150: 1047–1054.
- Breiteneder, H., O. Scheiner, R. Hajek, W. Hulla, R. Hutter, G. Fischer, D. Kraft, and C. Ebner. 1995. Diversity of TCRAV and TCRBV sequences used by human T-cell clones specific for a minimal epitope of Bet v I, the major birch pollen allergen. *Immunogenetics* 42: 53–58.

43. Ebner, C., S. Schenk, N. Najafian, U. Siemann, R. Steiner, G. W. Fischer, K. Hoffmann, Z. Szepefalusi, O. Scheiner, and D. Kraft. 1995. Nonallergic individuals recognize the same T cell epitopes of Bet v 1, the major birch pollen allergen, as atopic patients. *J. Immunol.* 154: 1932–1940.
44. Ebner, C., S. Schenk, Z. Szepefalusi, K. Hoffmann, F. Ferreira, M. Willheim, O. Scheiner, and D. Kraft. 1993. Multiple T cell specificities for Bet v I, the major birch pollen allergen, within single individuals: studies using specific T cell clones and overlapping peptides. *Eur. J. Immunol.* 23: 1523–1527.
45. Sparholt, S. H., J. N. Larsen, H. Ipsen, C. Schou, and R. J. van Neerven. 1997. Crossreactivity and T-cell epitope specificity of Bet v 1-specific T cells suggest the involvement of multiple isoallergens in sensitization to birch pollen. *Clin. Exp. Allergy* 27: 932–941.
46. Aalberse, R. C., R. van der Gaag, and J. van Leeuwen. 1983. Serologic aspects of IgG4 antibodies. I. Prolonged immunization results in an IgG4-restricted response. *J. Immunol.* 130: 722–726.
47. Akdis, C. A., T. Blesken, M. Akdis, S. S. Alkan, B. Wuthrich, C. H. Heusser, and K. Blaser. 1997. Induction and differential regulation of bee venom phospholipase A2-specific human IgE and IgG4 antibodies in vitro requires allergen-specific and nonspecific activation of T and B cells. *J. Allergy Clin. Immunol.* 99: 345–353.
48. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5: 271–283.
49. Till, S. J., J. N. Francis, K. Nouri-Aria, and S. R. Durham. 2004. Mechanisms of immunotherapy. *J. Allergy Clin. Immunol.* 113: 1025–1034.
50. Akdis, C. A., T. Blesken, M. Akdis, B. Wuthrich, and K. Blaser. 1998. Role of interleukin 10 in specific immunotherapy. *J. Clin. Invest.* 102: 98–106.
51. Asseman, C., S. Mauze, M. W. Leach, R. L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190: 995–1004.
52. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T. C. Barbosa, A. Cumano, and A. Bandeira. 2001. CD25⁺CD4⁺ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166: 3008–3018.
53. Green, E. A., L. Gorelik, C. M. McGregor, E. H. Tran, and R. A. Flavell. 2003. CD4⁺CD25⁺ T regulatory cells control anti-islet CD8⁺ T cells through TGF- β -TGF- β receptor interactions in type 1 diabetes. *Proc. Natl. Acad. Sci. USA* 100: 10878–10883.