# Assessment of Bet v 1-Specific CD4$^+$ T Cell Responses in Allergic and Nonallergic Individuals Using MHC Class II Peptide Tetramers[1]

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**In this study, we used HLA-DRB1*0101, DRB1*0401, and DRB1*1501 peptide tetramers combined with cytokine surface capture assays to characterize CD4$^+$ T cell responses against the immunodominant T cell epitope (peptide 141–155) from the major birch pollen allergen Bet v 1, in both healthy and allergic individuals. We could detect Bet v 1-specific T cells in the PBMC of 20 birch pollen allergic patients, but also in 9 of 9 healthy individuals tested. Analysis at a single-cell level revealed that allergen-specific CD4$^+$ T cells from healthy individuals secrete IFN-$\gamma$ and IL-10 in response to the allergen, whereas cells from allergic patients are bona fide Th2 cells (producing mostly IL-5, some IL-10, but no IFN-$\gamma$), as corroborated by patterns of cytokines produced by T cell clones. A fraction of Bet v 1-specific cells isolated from healthy, but not allergic, individuals also expresses CTLA-4, glucocorticoid-induced TNF receptor, and Foxp 3, indicating that they represent regulatory T cells. In this model of seasonal exposure to allergen, we also demonstrate the tremendous dynamics of T cell responses in both allergic and nonallergic individuals during the peak pollen season, with an expansion of Bet v 1-specific precursors from $10^{-6}$ to $10^{-3}$ among circulating CD4$^+$ T lymphocytes. Allergy vaccines should be designed to recapitulate such naturally protective Th1/regulatory T cell responses observed in healthy individuals.** *The Journal of Immunology,* **2008, 180: 4514–4522.**

Type I allergy is caused by an inappropriate Th2 response to allergens from the environment (1–3), leading to antigen-specific IgE production as well as recruitment and activation of proinflammatory cells (e.g., eosinophils and mast cells) in mucosal target organs (4–6). In contrast, under similar exposure conditions, tolerance to allergens is maintained in nonallergic individuals, possibly as a consequence of various nonexclusive mechanisms involving either 1) immune ignorance of nonpathogenic antigens, 2) anergy or deletion of allergen-reactive T cells, or 3) the induction of a protective allergen-specific CD4$^+$ T cell response. Specifically, a potential role of IL-10-producing T cells has been suggested in the induction and maintenance of peripheral tolerance to allergens, either in the context of natural responses in healthy individuals, or as a consequence of successful immunotherapy protocols (7–13). Understanding the nature of such CD4$^+$ T cells responses in healthy individuals is critical to improve current allergy vaccines, with the assumption that natural responses are protective against allergic inflammation, and thus that allergen-specific immunotherapy should recapitulate such immune responses (14). MHC class II peptide tetramer technology has been recently used succesfully in the field of allergy to characterize allergen-specific CD4$^+$ T cell responses (15–17). Soluble multimeric tetramers comprise fluorescently labeled recombinant MHC class II molecules, each bound to a high-affinity T cell epitope (18–21). Altogether, MHC class II peptide tetramers constitute high-avidity reagents allowing the detection of epitope-specific CD4$^+$ T lymphocytes.

Birch (*Betula verucosa*) pollen is causing one of the most common tree pollen allergies in Europe and North America. More than 95% of birch pollen (BP)[4] allergic patients exhibit IgEs against the major allergen Bet v 1, and up to 60% of these patients react solely to Bet v 1 (22, 23). To analyze in detail T cell responses to Bet v 1, we developed MHC class II peptide tetramers as a means to detect CD4$^+$ T lymphocytes specific for the Bet v $1_{141-155}$ immunodominant epitope following in vitro stimulation with Bet v $1_{141-155}$-Ii (invariant chain)-Key/epitope hybrid peptides (24). Using this approach, we demonstrate that specific T cells can be detected in PBMC of both allergic and nonallergic individuals, albeit with dramatically different patterns of T cell polarization.

## Materials and Methods

### *Patients and HLA-DR typing*

Citrated peripheral blood was obtained from BP or house dust mite (HDM) allergic patients recruited at Hôpital Béclère (Clamart, France), University

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[4] Abbreviations used in this paper: BP, birch pollen; DC, dendritic cell; ECD, PE-Texas red; GITR, glucocorticoid-induced TNF receptor; HDM, house dust mite; Ii, invariant chain; PC7, phycoerythrin-cyanine 7, PC7; rBet v 1, recombinant Bet v 1; TCC, T cell clone; Treg, regulatory T.

of Antwerp (Antwerpen, Belgium), and Vienna Hospital (Vienna, Austria). Blood samples from healthy donors with no history of atopy were obtained from Etablissement Français du Sang (Rungis, France). BP and HDM allergic patients were selected based on their clinical record, a positive skin prick test, as well as a positive in vitro basophil activation test (data not shown) performed with recombinant Bet v 1 (rBet v 1), produced in *Escherichia coli* (25). For HLA-DR typing, genomic DNA was extracted from blood samples using an Easy-DNA Kit (Invitrogen). Typing was performed by the PCR-sequence specific primers method using Dynal AllSet+ and CombiSet+ SSP family kits (Invitrogen) (26). Informed consent was obtained from all participants in the study. The latter was approved by the Comité Consultatif pour la Protection des Patients dans la Recherche Biomédicale (CCPPRB, Hôpital, Béclère).

### *Bioinformatic analysis and HLA-DR/DP peptide in vitro binding assays*

Immunodominant Bet v 1 epitopes restricted to HLA-DRB1 or HLA-DPB1 molecules were initially preselected by bioinformatic analyses performed using T-epitope and ProPred (http://www.imtech.res.in/raghava/propred) algorithms. Peptides spanning the whole Bet v 1 sequence and preserving putative anchor motifs were synthesized by NeoMPS with a purity grade >75% confirmed by HPLC. Human HLA-DR and HLA-DP molecules were purified from human EBV-transformed B cell lines homozygous for common human MHC class II molecules, as described elsewhere (27, 28), and dilutions were prepared in 10 mM phosphate, 150 mM NaCl, 1 mM *n*-dodecyl β-D-maltoside, 10 mM citrate, and 0.003% thimerosal buffer. Biotinylated reporter peptides were incubated in the presence of serial dilutions of each Bet v 1 peptide tested and appropriate purified MHC class II molecules. Specifically, the flu hemagglutinin HA306–318 peptide was used as a reporter peptide for binding to both the HLA-DRB1*0101 and HLA-DRB1*0401 molecules (using a 10 nM and 30 nM concentration, respectively). Two peptides from melanoma-associated Ag-3 (i.e., MAGE $3_{152–166}$ and MAGE $3_{213–236}$) were used as reporter peptides for binding to either the HLA-DRB1*1501 (at a 10 nM concentration) or HLA-DRB1*0301 molecule (at a 200 nM concentration). The $Oxy_{271–287}$ peptide was used as a reporter for binding to the HLA-DPB1*0401 molecule at a 10 nM concentration. After a 24-h incubation at 37°C (except for binding to HLA-DRB1*1501, which required a 72-h incubation) with the biotinylated reporter and Bet v 1 peptides, peptide-MHC class II complexes were added to 96-well plates coated with the L243 Ab directed to a monomorphic determinant of MHC class II molecules, and incubated for 2 h at room temperature. Bound biotinylated peptides were detected after adding a streptavidin-alkaline phosphatase conjugate (Amersham) and 4-methyl umbelliferyl phosphate as a substrate (Sigma-Aldrich) (28). Emitted fluorescence was measured at 450 nm upon excitation at 365 nM on a Fluorolite 1000 fluorometer (Dynex). Data were expressed as peptide concentrations inhibiting 50% binding of biotinylated reporter peptides ($IC_{50}$) (28).

### *In vitro expansion of Bet v 1-specific T cells*

PBMC were isolated from peripheral blood by centrifugation over Ficoll-Paque PLUS (Amersham Biosciences), washed three times in PBS (Cambrex), and resuspended in complete RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated human AB serum (Sigma-Aldrich), 2 mM L-glutamine, 20 μg/ml gentamicin, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50 μM 2-ME, and 1% nonessential amino acids (all from Invitrogen). A Bet v $1_{141–155}$-Ii-Key conjugate peptide mix was used as a restimulation reagent in most experiments. This conjugate peptide mix comprises complete or truncated versions of the Bet v $1_{141–155}$ peptide conjugated with a polymethylene linker to the Ii-Key moiety of the MHC class II-associated invariant chain (24).

Freshly isolated PBMC ($5 \times 10^6$/ml) were stimulated in 12-well plates with either the conjugate peptide mix (10 μg/ml), Bet v $1_{141–155}$ peptide (10 μg/ml), or purified rBet v 1 (50 μg/ml) in 2 ml complete RPMI 1640 medium. After 5 days at 37°C, 10 U/ml IL-2 (Roche) was added to cell cultures every 3 days. On day 14, cells were restimulated with Ag-pulsed autologous PBMC plus IL-2. In selected experiments, this stimulation procedure was repeated twice.

### *Staining with MHC class II peptide tetramers*

HLA-DRB1*0101, DRB1*1501, and DRB1*0401 iTAg peptide tetramers used in this study were made by Beckman Coulter. Briefly, the α-chain and β-chain of HLA-DRB1 molecules were produced as a fusion to biotin in a baculovirus expression system as described elsewhere (29). Tetramers made using streptavidin were complexed with either Bet v $1_{141–155}$ (ETLL RAVESYLLAHS) or the CLIP (LPKPPKPVSKMRMATPLLMQALPM) as a negative control. All tetramers were conjugated to PE and obtained at a 100 μg/ml concentration.

For tetramer staining, $10^6$ cells were incubated with 10 μg/ml MHC class II peptide tetramers for 2 h at 37°C in PBS buffer (i.e., PBS + 1% FCS or PBS + 1% BSA). After washing in cold PBS, Abs (i.e., FITC-labeled anti-CD4, PE-Texas red (ECD)-labeled anti-CD14, phycoerythrin-cyanine 7 (PC7)-labeled anti-CD3, all from Beckman Coulter) and ViaProbe reagent (BD Biosciences) were added for 20 min in the dark at 4°C. Cells were stained with corresponding isotype-matched Ab as controls. After washing, samples were analyzed using a FC500 flow cytometer (Beckman Coulter), after excluding dead cells and $CD14^+$ monocytes. In selected experiments, cells were further incubated with Abs directed against various surface markers (i.e., CD62L, CD45 RO, CCR7, CXCR3, CCR4, and CCR5 from BD Biosciences; CD25 and CD69 from Beckman Coulter; CTLA-4 and glucocorticoid-induced TNF receptor (GITR) from R&D Systems) or corresponding isotype-matched Ab before FACS analysis. For Foxp 3 intracellular staining, $tetramer^+$ T cells were fixed and permeabilized with Fix/Perm buffer (eBioscience, San Diego, CA), washed with a permeabilization buffer (eBioscience), and stained in 200 μl permeabilization buffer with FITC-conjugated anti-Foxp3 (clone PCH101, eBioscience) or corresponding isotype-matched mAbs. After 30 min at 4°C, cells were washed and immediately analyzed by flow cytometry.

### *Cytokine surface capture assays*

Cells were stained with 5 μg/ml MHC class II Bet v $1_{141–155}$ tetramer at 37°C in PBS buffer for 30 min. Cells ($2.5 \times 10^6$) were subsequently washed and stimulated in RPMI 1640 complete medium with $2.5 \times 10^5$ autologous PBMC pulsed with Bet v $1_{141–155}$. After 3 h at 37°C, cells were harvested and labeled in ice-cold RPMI 1640 medium with 50 μg/ml of either anti-IFN-γ/CD45, anti-IL-5/CD45, or anti-IL-10/CD45 Ab-Ab conjugates (Miltenyi Biotec) for 10 min at 4°C. Cells were resuspended in complete culture medium and incubated 45 min at 37°C to allow cytokine secretion. Cells were then washed, resuspended in ice-cold buffer containing 0.5% BSA and 5 mM EDTA, and stained with PC7-labeled anti-CD4, ECD-labeled anti-CD14, ViaProbe reagent, and either FITC-labeled anti-IFN-γ or APC-labeled anti-IL-10 or anti-IL-5 mAbs. After 30 min at 4°C, cells were washed and immediately analyzed by flow cytometry.

### *PCR analysis of purified MHC class II Bet v $1_{141–155}$ $tetramer^+CD4^+$ T cells*

Bet v $1_{141–155}$ tetramer positive and negative $CD4^+$ T cells were sorted from cultures obtained from both BP allergic and nonallergic individuals, using a MoFlo (Dako). Total RNA was extracted from the various cell populations with RNeasy Kit (Qiagen), and cDNAs were synthesized using a TaqMan Reverse Transcription Reagent kit (Applied Biosystems) according to the manufacturer's instruction. Messenger RNA expression was evaluated by quantitative PCR on a 7300 Real-Time PCR System (Applied Biosystems) using predesigned TaqMan Gene Expression reagents according to the manufacturer's instructions (Applied Biosystems). To assess T cell polarization, the following genes were monitored: T-bet (Hs00203436), GATA-3 (Hs00231122), Foxp3 (Hs00203958), CTLA-4 (Hs00175480), GITR (Hs00188346), IFN-γ (Hs00174143), IL-4 (Hs00174122), IL-13 (Hs00174379), and IL-10 (Hs00174086). Data were obtained after normalizing the target gene amplification value with an endogenous control (β-actin). The relative amount of target genes in each sample was assessed in comparison with $tetramer^-CD4^+$ T cells from the same donor.

### *Generation of T cell clones (TCC)*

Bet v 1-specific TCC were generated as described (30). Each TCC was mapped for epitope recognition using a panel of 50 synthetic 12-mer peptides representing the entire amino acid sequence of Bet v 1 (30). Cytokine secretion was determined in culture supernatants harvested 48 h after stimulation of the TCC with 5 μg/ml Bet v 1 and autologous irradiated PBMC, using an ELISA with Endogen Matched Ab Pairs (Endogen) according to the manufacturer's instructions. Limits of detection were: IL-4, 9 pg/ml; IFN-γ, 9.5 pg/ml; IL-10, 4.7 pg/ml) (31). TCC were assigned to Th subsets as follows: Th2, ratio IL-4/IFN-γ of >5; Th1, ratio IFN-γ/IL-4 of >5; and Th0, ratio IFN-γ/IL-4 between 0.2 and 5.

### *Statistical analyses*

Data were analyzed for statistical significance using a Student's *t* test. A $p$ value $<0.05$ was considered to be statistically significant.

Table I. *Binding capacities of Bet v 1 peptides to immunopurified HLA-DR and HLA-DP molecules*[a]

| Peptides | Sequences | HLA-DRB1 Alleles 0101 | 0401 | 1501 | 0301 | HLA-DPB1 Allele 0401 |
|---|---|---|---|---|---|---|
| P2–16 | VFNYETETTSVIPAA | **155** | **447** | — | — | 4,215 |
| P9–22 | TTSVIPAARLFKAFI | **283** | 5,244 | **214** | **572** | >10,000 |
| P27–41 | DNLFPKVAPQAISSV | 2,345 | — | 20,000 | — | >10,000 |
| P63–77 | PFKYVKDRVDEVDHT | 3,000 | 17,748 | 23,452 | — | >10,000 |
| P77–91 | TNFKYNYSVIEGGPI | **91** | 1,470 | 65,000 | — | 1,366 |
| P99–113 | SNEIKIVATPDGGSI | 2,646 | 3,464 | **650** | — | >10,000 |
| P111–125 | GSILKISNKYHTKGD | **145** | 1,597 | **280** | 2,164 | >10,000 |
| P141–155 | ETLLRAVESYLLAHS | **1** | **78** | **2** | 1,058 | 1,461 |
| $HA_{306–318}$ | | **2** | **38** | | | |
| $MAGE3_{152–166}$ | | | | **30** | | |
| $MAGE3_{213–236}$ | | | | | **383** | |
| $Oxy_{271–287}$ | | | | | | **5** |

[a] $IC_{50}$ values (means) are expressed in nanomolars and have been evaluated from at least two to three independent experiments. Each binding experiment was conducted using reporter peptides such as $HA_{306–318}$, $MAGE3_{152–166}$, $MAGE3_{213–236}$, or $Oxy_{271–287}$ as described in *Materials and Methods*. $IC_{50}$ values <1,000 nM are in bold type; —, $IC_{50}$ values >100,000 nM.

## Results

### *Design of MHC class II peptide tetramers*

To design MHC class II peptide tetramers to assess CD4$^+$ T cell responses against the Bet v 1 major allergen from BP, we first identified T cell epitopes within Bet v 1 exhibiting a high-affinity binding for HLA-DRB1 or HLA-DPB1 molecules. To this end, a bioinformatic search for HLA class II anchoring motifs within the Bet v 1 sequence was performed using both T-epitope and ProPred algorithms. From this information, a set of overlapping peptides encompassing the entire Bet v 1 sequence was synthesized. In vitro binding experiments were conducted using purified MHC class II molecules corresponding to four common HLA-DRB1 alleles (i.e., HLA-DRB1*0101, DRB1*0301, DRB1*0401, DRB1*1501) covering altogether up to 50% of the Caucasian population. Binding to the HLA-DPB1*0401 molecule (expressed in 76% of the Caucasian population) was also tested in parallel. MHC class II molecules were purified from homozygous EBV-transformed cell lines, and competitive binding assays were conducted to evaluate relative binding affinities of Bet v 1 peptides in comparison with known labeled reporter peptides. High-affinity Bet v 1 peptides were defined as the ones with an affinity <1000 nM for MHC class II molecules (27, 28). As shown in Table I, the Bet v $1_{141–155}$ peptide binds efficiently to three HLA-DRB1 molecules (i.e., DRB1*0101, DRB1*0401, DRB1*1501) with an $IC_{50}$ concentration between 1 and 78 nM. Two peptides (Bet v $1_{9–22}$ and Bet v $1_{111–125}$) also demonstrated a good binding affinity for the DRB1*0101 and DRB1*1501 alleles, whereas Bet v $1_{2–16}$ binds significantly to HLA-DR1*0101 and HLA-DR1*0401. Only peptides with a low or intermediate affinity for HLA-DRB1*0301 and HLA-DP1*0401 were identified (Table I). Based on these data, the Bet v $1_{141–155}$ peptide was selected to engineer tetramers with each of the HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*1501 molecules. This Bet v 1 epitope has been shown to be recognized by T cells from both allergic and healthy individuals, thus confirming that it represents an immunodominant T cell epitope (30–32).

### *Detection of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells in allergic and nonallergic individuals*

PBMC were isolated from individuals PCR-typed for DRB1*0101, DRB1*1501, or DRB1*0401 expression. In most circumstances, the frequency of circulating Bet v $1_{141–155}$ peptide-specific CD4$^+$ T cells is too low to be detected without in vitro stimulation. Thus, PBMC were stimulated with a conjugate peptide mix comprising both complete and serially truncated versions of the Bet v $1_{141–155}$ peptide coupled with a polymethylene linker to the Ii-Key peptide. The latter interacts with an allosteric site on HLA class II molecules, thereby facilitating peptide exchange and binding (18). We confirmed that this conjugate peptide mix is substantially more potent than the Bet v $1_{141–155}$ peptide alone or rBet v 1 in expanding Bet v 1-specific CD4$^+$ T cells from peripheral blood (33). Under such in vitro stimulation conditions, we could routinely detect MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells in allergic patients within 1–4 wk of culture (Fig. 1 and Table II). Interestingly, using this method, we could also

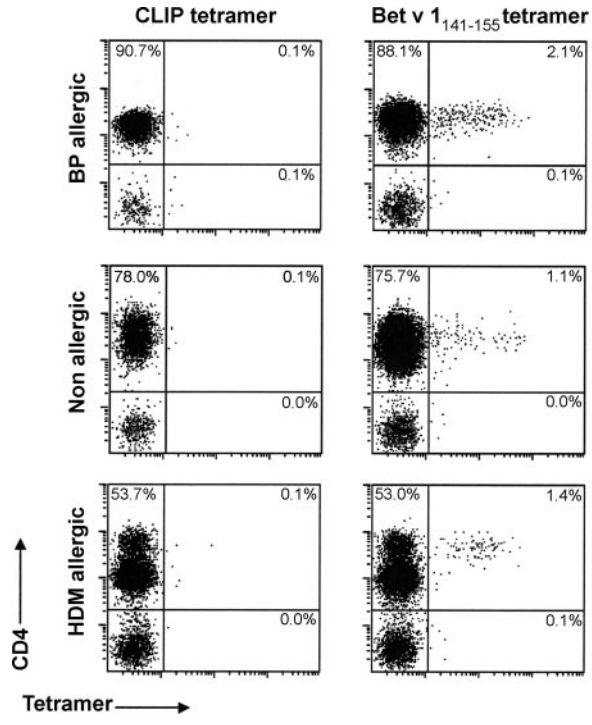


**FIGURE 1.** Detection of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells. Freshly isolated PBMC from BP allergic, HDM allergic, or nonallergic individuals, all expressing the DRB1*1501 haplotype, were stimulated with a Bet v $1_{141–155}$-Ii-Key peptide mix (10 $\mu$g/ml) for 21 days. Cells were then assessed by flow cytometry with either the Bet v $1_{141–155}$ tetramer or the CLIP tetramer as a negative control. For flow cytometry analysis, lymphocytes were gated by forward and sideways light scattering. Viable CD3$^+$ T cells were selected based on both CD3 expression and absence of staining with ViaProbe and an anti-CD14 Ab.

Table II. *Detection of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells in allergic and nonallergic individuals*[a]

| Status of Donors | Identification of Donors | HLA-DRB1 | MHC Class II Tetramer$^+$CD4$^+$ T cells (%) | | Month of Blood Collection | No. of Days of In Vitro Stimulation |
|---|---|---|---|---|---|---|
| | | | CLIP | Bet v $1_{141–145}$ | | |
| BP allergic | BP1 | 1501 | 0.1 | 0.5 | April | 0 |
| | BP1 | 1501 | 0.1 | 3.8 | April | 21 |
| | BP1 | 1501 | 0.1 | 1.8 | November | 24 |
| | BP1 | 1501 | 0.1 | 2.1 | December | 21 |
| | BP2 | 1501 | 0.2 | 1.1 | July | 21 |
| | BP3 | 1501 | 0.1 | 2.3 | July | 21 |
| | BP4 | 1501 | 0.1 | 2.1 | March | 21 |
| | BP5 | 1501 | 0 | 0.9 | May | 15 |
| | BP6 | 1501 | 0 | 3.1 | May | 15 |
| | BP7 | 1501 | 0.1 | 1.1 | March | 14 |
| | BP8 | 1501 | 0.1 | 2.1 | March | 21 |
| | BP9 | 0401 | 0.2 | 1.1 | November | 32 |
| | BP10 | 0401 | 0.1 | 1.4 | November | 30 |
| | BP11 | 0401 | 0.1 | 0.7 | June | 21 |
| | BP12 | 0401 | 0.1 | 0.6 | July | 21 |
| | BP14 | 0401 | 0.1 | 0.3 | July | 17 |
| | BP14 | 0401 | 0.1 | 2.1 | May | 15 |
| | BP15 | 0101 | 0.1 | 0.3 | July | 11 |
| | BP16 | 0101 | 0.1 | 13.7 | June | 30 |
| | BP17 | 0101 | 0 | 2.9 | June | 21 |
| | BP18 | 0101 | 0.1 | 1.2 | July | 23 |
| | BP19 | 0101 | 0 | 0.8 | November | 21 |
| | BP20 | 0101 | 0.1 | 14.9 | March | 18 |
| Nonallergic | H1 | 1501 | 0.1 | 1.8 | May | 28 |
| | H1 | 1501 | 0 | 0.3 | April | 0 |
| | H1 | 1501 | 0.1 | 1.2 | January | 18 |
| | H2 | 1501 | 0.2 | 1.8 | September | 32 |
| | H3 | 1501 | 0 | 2.1 | March | 27 |
| | H5 | 0401 | 0.1 | 2.4 | February | 30 |
| | H6 | 0101 | 0.1 | 1.9 | January | 27 |
| | H7 | 0101 | 0 | 0.9 | March | 30 |
| | H8 | 0101 | 0.1 | 1.5 | March | 30 |
| | H9 | 0101 | 0.1 | 1.0 | October | 27 |
| HDM allergic | HDM1 | 1501 | 0.3 | 0.9 | July | 21 |
| | HDM2 | 1501 | 0.1 | 0.9 | July | 18 |
| | HDM3 | 0401 | 0.1 | 0.7 | November | 21 |
| | HDM4 | 0101 | 0.1 | 2.1 | March | 45 |
| | HDM4 | 0101 | 0.1 | 0.7 | July | 21 |
| | HDM5 | 0101 | 0.2 | 1.3 | October | 21 |

[a] Blood samples were obtained from individuals in Austria, Belgium, and France. The numbers of days of in vitro stimulation with the conjugate peptide mix needed to detect Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells are shown.

detect Bet v $1_{141–155}$-specific CD4$^+$ T cells in all healthy individuals tested, as well as in patients allergic to HDM but not BP, with either a HLA-DRB*0101, DRB*0401 or DRB*1501 background (Fig. 1 and Table II).

The Bet v $1_{141–155}$ class II tetramer reactivity observed in the previous experiments is clearly specific, because 1) only background staining ($\leq$0.3%) is usually detected with corresponding MHC class II tetramers made with the irrelevant CLIP peptide (Fig. 1 and Table II), and 2) only CD4$^+$, but not CD4$^-$, cells are labeled with Bet v $1_{141–155}$ tetramers (Fig. 1). Also, no staining is observed with CD4$^+$ T lymphocytes from a BP allergic patient expressing a nonrelevant HLA class II haplotype, nor with Der p 1-specific T cells expanded from a HDM allergic HLA-DRB1*0101 patient (data not shown). As shown in Table II, Bet v 1-specific CD4$^+$ T cells had usually to be stimulated for longer periods (i.e., 2–3 wk) with the Bet v $1_{141–155}$-Ii-Key conjugate peptide mix to be detectable in healthy individuals in comparison to BP allergic patients.

### *Single-cell phenotypic and functional characterization of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells in allergic and nonallergic individuals*

Using multiple color labeling and FACS analysis, the surface phenotype of tetramer$^+$CD4$^+$ T cells was assessed in both allergic and nonallergic individuals. As shown in Fig. 2*A*, MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells from BP allergic patients are CD25$^+$, CD69$^+$, CD45 RO$^+$, CXCR3$^-$, and CCR5$^-$. These cells are also CD62L$^-$ and CCR7$^-$, suggesting an effector memory phenotype. Interestingly, MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells from healthy individuals are also activated (CD25$^+$, CD69$^+$, CD45 RO$^+$), but they rather exhibit a central memory profile (CD62L$^+$CCR7$^+$). They also express CXCR3, a chemokine receptor that has been reported to enhance allergen-specific IFN-$\gamma$ production (34). Furthermore, Bet v $1_{141–155}$ - specific T cells in nonallergic individuals express CTLA-4$^+$ (which may provide regulatory signals during an immune response) (35), and a fraction of them (i.e., 8–10%) exhibit high levels of GITR. Although both samples contain Foxp3$^{low}$ cells (likely representing activated T cells), Foxp3$^{bright}$ cells are only detected within tetramer$^+$ T cells from healthy ($n = 4$, mean $\pm$ SEM: 25.3 $\pm$ 2.1%), but not BP allergic individuals ($n = 4$, 1.0 $\pm$ 0.2%) ($p < 0.01$). Such Foxp3$^{bright}$CD4$^+$ T cells exhibit a high avidity for MHC class II Bet v $1_{141–155}$ tetramers (Fig. 2*B*).

The cytokine profile of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells was subsequently determined using a cytokine surface capture assay. To prevent TCR down-regulation during tetramer peptide labeling, tetramer staining was performed

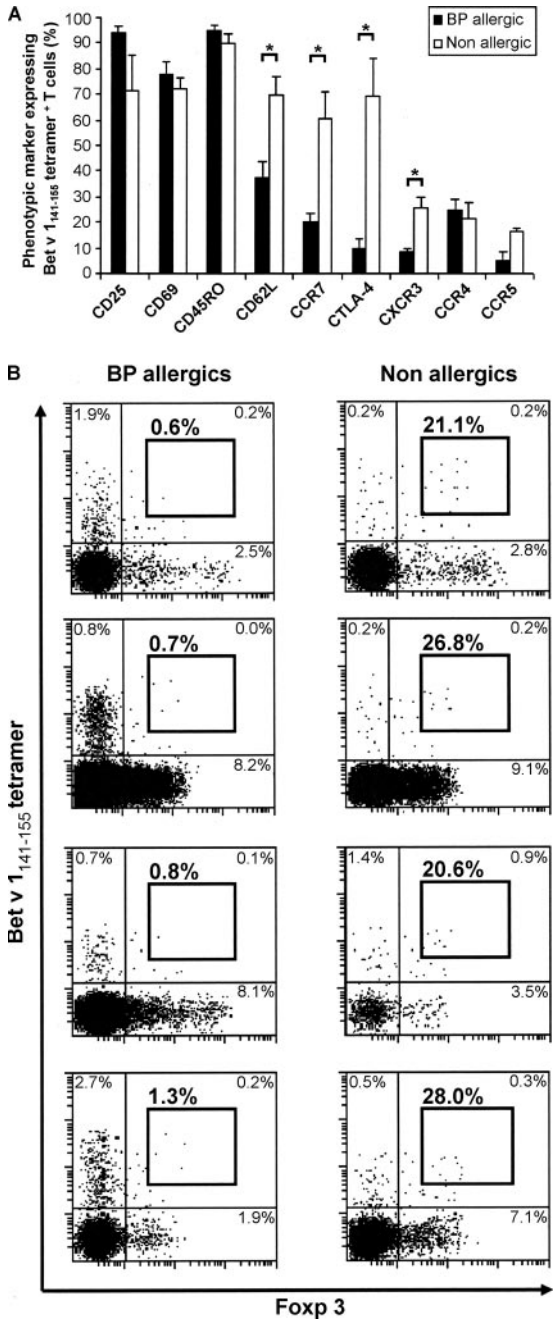


**FIGURE 2.** Phenotypic analysis of MHC class II Bet v1$_{141–155}$ tetramer$^+$CD4$^+$ T cells. *A*, Expression of cell-surface molecules by flow cytometry analysis. Freshly isolated PBMC from BP allergic ($n = 7$) or nonallergic ($n = 4$) individuals were stimulated with a Bet v 1$_{141–155}$-Ii-Key peptide mix (10 $\mu$g/ml) for at least 10 days. Cells were then stained with appropriate Bet v 1$_{141–155}$ tetramers and with a combination of Abs directed against the indicated surface markers. Results are expressed as mean percentages of MHC class II Bet v 1$_{141–155}$ tetramer$^+$CD4$^+$ T cells expressing the various surface markers. Error bars represent SEM. Statistical significance was determined using the Student *t* test (*, $p < 0.05$). *B*, Analysis of Foxp3 expression by intracellular staining. Cells obtained from four BP allergic (*left panels*) or four nonallergic individuals (*right panels*) were stained with Bet v 1$_{141–155}$ tetramer or the CLIP tetramer as a negative control and then with a FITC-conjugated anti-Foxp3 Ab or an isotype control after mild permeabilization. Percentages of Foxp3$^{bright}$ cells within Bet v 1$_{141–155}$ tetramer$^+$ cells are indicated in bold type.

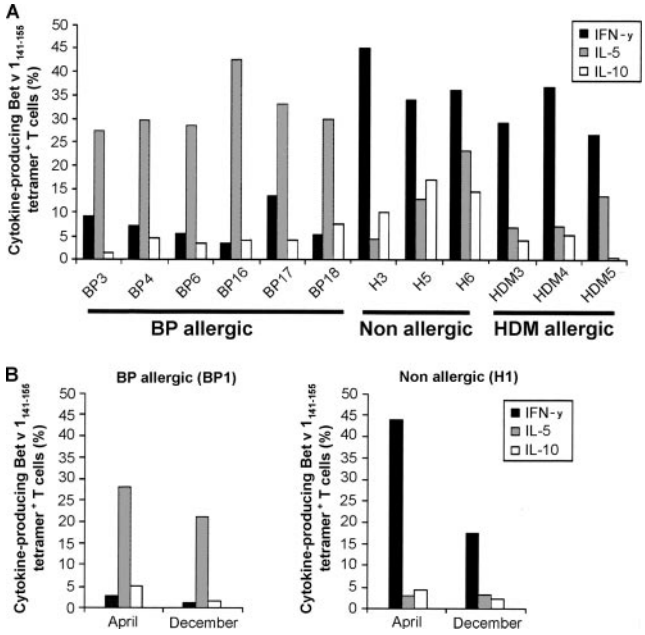


**FIGURE 3.** Cytokine production by MHC class II Bet v 1$_{141–155}$ tetramer$^+$CD4$^+$ T cells. *A*, Freshly isolated PBMCs from BP allergic, HDM allergic, or nonallergic individuals were stimulated with a Bet v 1$_{141–155}$-Ii-Key conjugate peptide mix (10 $\mu$g/ml) for at least 10 days. Cells were subsequently stained with MHC class II Bet v 1$_{141–155}$ tetramer, stimulated with autologous PBMC pulsed with Bet v 1$_{141–155}$ for 3 h at 37°C. Cells were then stained with either anti-IFN-$\gamma$/CD45, anti-IL-5/CD45, or anti-IL-10/CD45 Ab-Ab conjugates, and incubated in complete culture medium for 45 min at 37°C to allow cytokine secretion. Cells were washed and stained with PC7-labeled anti-CD4, ECD-labeled anti-CD14, ViaProbe reagent, and either FITC-labeled anti-IFN-$\gamma$ or APC-labeled anti-IL-10 or anti-IL-5 Ab. After 30 min at 4°C, cells were washed and immediately analyzed by flow cytometry. Results are expressed as percentages of MHC class II Bet v 1$_{141–155}$ tetramer$^+$CD4$^+$ T cells producing a given cytokine after Bet v 1$_{141–155}$-Ii-Key conjugate peptide mix restimulation. *B*, Patterns of cytokine production are maintained over time. PBMC isolated during (April) or outside (December) the pollen season from BP allergic (BP1) or non allergic (H1) individuals were stimulated with a Bet v 1$_{141–155}$-Ii-Key peptide mix (10 $\mu$g/ml) for at least 10 days. Cells were subsequently stained as described above and analyzed by flow cytometry. Results are expressed as percentages of MHC class II Bet v 1$_{141–155}$ tetramer$^+$CD4$^+$ T cells producing a given cytokine after Bet v 1$_{141–155}$-Ii-Key conjugate peptide mix restimulation.

under suboptimal conditions (i.e., at room temperature for 30 min, using a 5 $\mu$g/ml tetramer concentration). Under these conditions, the vast majority (i.e., >90%) of CD4$^+$ T cells producing cytokines following allergen stimulation are found in the Bet v 1$_{141–155}$ tetramer$^+$ cell population, thereby further confirming the specificity of tetramer staining. As shown in Fig. 3*A*, we found that most MHC class II Bet v 1$_{141–155}$ tetramer$^+$ cells from BP allergic patients ($n = 7$) produce IL-5 (mean ± SEM percentages of tetramer$^+$ cells producing this cytokine: 30.3 ± 2.7%), and to a lower extent IL-10 (3.9 ± 0.8%) or IFN-$\gamma$ (6.5 ± 1.5%). This pattern is representative of a Th2 response known to occur in allergic patients. Importantly, these results are in agreement with data obtained with a panel of TCC generated from PBMC of BP allergic patients and tested for their capacity to produce IL-4, IL-10, or IFN-$\gamma$ (Table III). The latter results also confirm that a similar pattern of cytokines is produced by T cells directed to various T cell epitopes within Bet v 1.

In contrast, Bet v 1-specific CD4$^+$ T cells from healthy individuals ($n = 4$) secrete mostly IFN-$\gamma$ (mean ± SEM: 39.7 ± 2.7%, $p < 0.01$ vs allergics), little IL-5 (10.9 ± 4.6%, $p < 0.02$ vs allergics), and, in some donors, they also produce IL-10 (11.5 ± 1.7%, $p = 0.07$ vs allergics) in response to allergen stimulation

Table III. *Characterization of Bet v 1-specific T cell clones form BP allergic patients*[a]

| Bet v 1-Reactive TCC | Bet v 1 Epitope (aa) | Th Subsets | Cytokine Profile | | |
|---|---|---|---|---|---|
| | | | IL-4 (pg/ml) | IFN-γ (pg/ml) | IL-10 (pg/ml) |
| RR 9 | 4–18 | Th2 | 334 | <9.5 | 261 |
| WD 36 | 4–18 | Th2 | 176 | 15 | 9.1 |
| WF 2 | 112–123 | Th2 | 630 | 120 | 260 |
| RR 48 | 139–150 | Th2 | 826 | 100 | 14 |
| SD 334 | 139–156 | Th2 | 434 | <9.5 | 263 |
| HPR 111 | 142–153 | Th1 | 26 | 2910 | 112 |
| WD 14 | 142–153 | Th0 | 0 | <9.5 | 63 |
| HPR 77 | 142–156 | Th2 | 856 | 100 | <4.7 |
| HPR 17 | 142–156 | Th2 | 464 | 30 | 32 |
| HPR 31 | 142–156 | Th2 | 502 | <9.5 | 567 |
| HPR 49 | 142–156 | Th2 | 220 | <9.5 | 67 |
| SUG 1 | 145–159 | Th0 | 528 | 235 | 84.7 |

[a] Bet v 1-specific TCC isolated from six different BP allergic individuals and specific for different Bet v 1 epitopes were stimulated with 5 μg/ml rBet v 1 and autologous irradiated PBMC. The cytokine profile was determined in culture supernatants by ELISA. TCC were assigned to T helper subsets subsets as follows: Th2, ratio IL-4/IFN-γ > 5; Th1, ratio IFN-γ/IL-4 > 5; and Th0, ratio IFN-γ/IL-4 between 0.2 and 5.

(Fig. 3*A*). Interestingly, a comparable pattern of cytokine production was observed in Bet v $1_{141–155}$-specific CD4$^+$ T cells obtained from HDM allergic patients unsensitized to BP ($n = 3$, mean ± SEM: 30.9 ± 2.9% and 9.1 ± 2.1% for IFN-γ and IL-5, respectively, with $p < 0.005$ and $p < 0.01$ when compared with BP allergic individuals). Little IL-10 production (3.3 ± 1.5%) was found in the latter patient population after Bet v $1_{141–155}$-Ii-Key peptide stimulation. No significant differences in patterns of cytokines produced by Bet v 1-specific T cells obtained within or outside the pollen season were observed in both allergic and nonallergic individuals, even if usually fewer tetramer$^+$ T cells produce

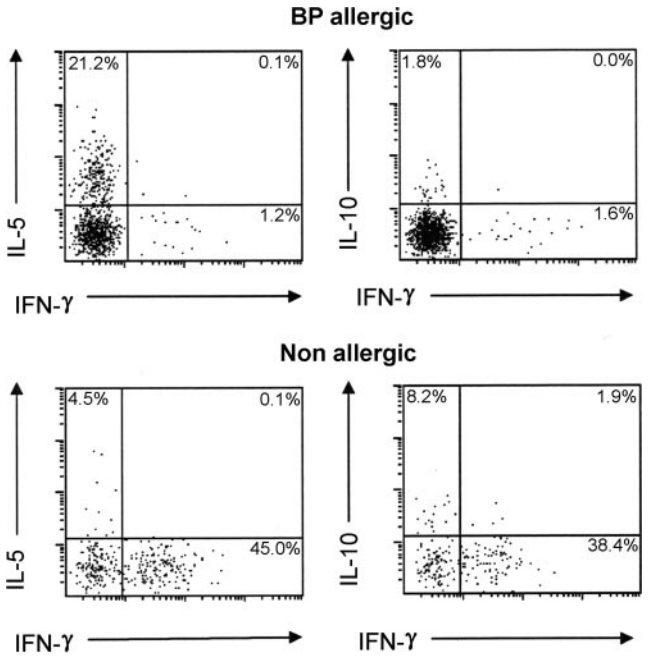


**FIGURE 4.** Mutually exclusive secretion of IL-10 or IFN-γ by MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells. Freshly isolated PBMC from BP allergic (*upper panels*) or nonallergic (*lower panels*) individuals, both expressing the DRB1*1501 haplotype, were stimulated with a Bet v $1_{141–155}$-Ii-Key conjugate peptide mix (10 μg/ml) for 12–17 days. Cells were subsequently stained as described in Fig. 3 and analyzed by flow cytometry. FACS plots are gated on MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells, and percentages of Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells expressing the respective cytokine are indicated in each plot.

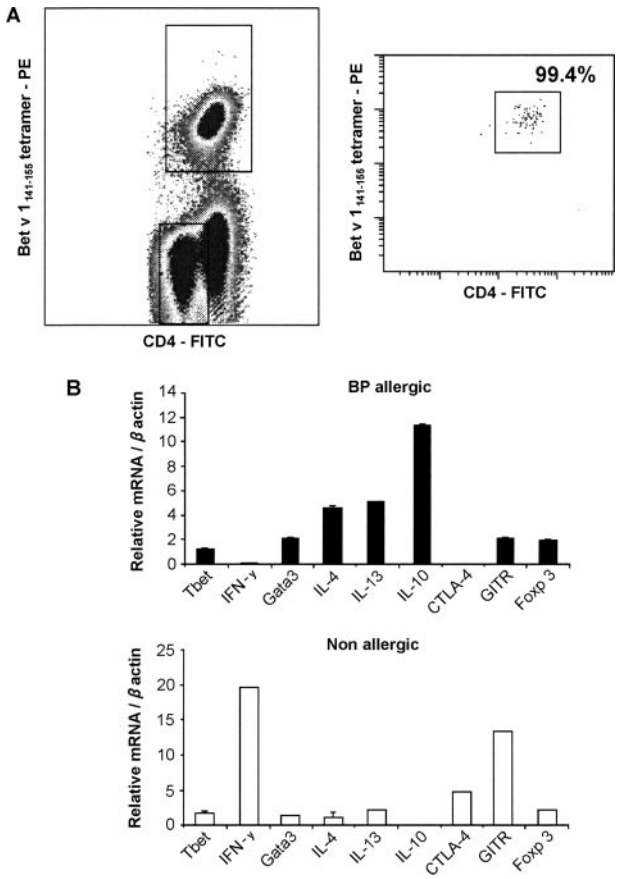


**FIGURE 5.** Analysis of patterns of T cell differentiation in sorted MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells. *A*, Cell sorting of MHC class II Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells was performed using PBMC from BP allergic and nonallergic individuals using a MoFlo. Reanalysis of sorted cells (right panel) confirmed that purity was >99%. *B*, Patterns of gene expression in sorted Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells from allergic and nonallergic individuals. Gene expression was analyzed by quantitative PCR as described in the *Materials and Methods*. Data are expressed as relative amounts of mRNA in sorted Bet v $1_{141–155}$ tetramer$^+$CD4$^+$ T cells in comparison with sorted Bet v $1_{141–155}$ tetramer$^-$CD4$^+$ T cells from the same donor. Data are normalized to amounts of β-actin. Data shown are representative of experiments conducted on samples obtained from two BP allergic and two non-BP allergic donors.

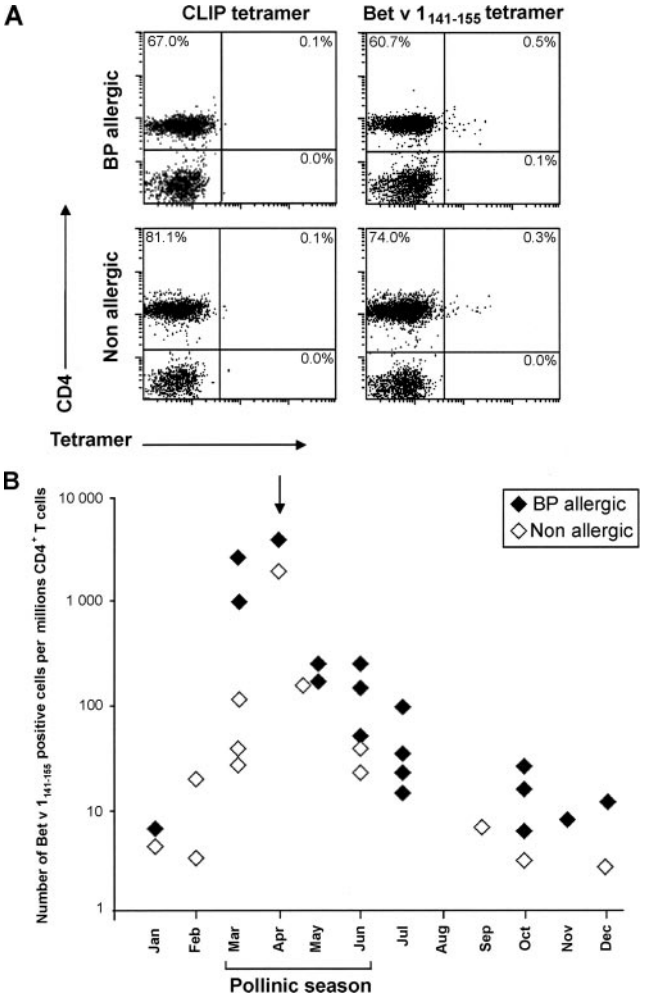


**FIGURE 6.** Frequency of MHC class II Bet v $1_{141-155}$ tetramer$^+$CD4$^+$ T cells. *A*, Freshly isolated PBMC from DRB1*1501 BP allergic or nonallergic individuals at the peak of the pollen season were directly assessed (without any ex vivo stimulation) by flow cytometry with either Bet v $1_{141-155}$ tetramer or CLIP tetramer as a control. All other samples were processed after in vitro Ag stimulation as described in the *Materials and Methods*. For flow cytometry analysis, live lymphocytes were gated by forward and sideways light scattering, and CD14$^+$ cells monocytes were excluded. *B*, Using CFSE-labeled cells, in vitro stimulation with Bet v $1_{141-155}$-Ii-Key conjugate peptide mix was estimated to induce CD4$^+$ T cell proliferation every 24 h during the 10 initial days in culture. Based on those assumptions and the number of days required to stimulate Bet v 1-specific CD4$^+$ T cells to allow tetramer detection, an estimate of Bet v 1-specific T cell frequencies is provided in relation to sampling time and birch pollen exposure.

cytokines in wintertime following Bet v $1_{141-155}$-Ii-Key peptide stimulation (Fig. 3*B*). As shown in Fig. 4, triple labeling reveals that IL-10 and IFN-$\gamma$ are clearly produced by distinct cell subsets among MHC class II Bet v $1_{141-155}$ tetramer$^+$CD4$^+$ T cells detected in healthy individuals.

Collectively, these results are consistent with the presence of both Th1 (IFN-$\gamma^+$) and regulatory T cells (IL-10$^+$, CTLA-4$^+$, GITR$^+$, Foxp3$^{bright}$) within Bet v $1_{141-155}$-specific CD4$^+$ T cells in healthy individuals. These results were further confirmed on sorted (99% pure) MHC class II Bet v $1_{141-155}$ tetramer$^+$CD4$^+$ T cells obtained from BP and HDM allergic patients, respectively (Fig. 5*A*). RNA was extracted from tetramer$^+$CD4$^+$ T cells, cDNAs were reverse transcribed, and patterns of T cell differentiation were assessed by quantitative PCR. This analysis revealed either a clear Th2 (IL4$^+$, IL13$^+$, IL10$^+$) or Th1/regulatory T (Treg) cell profile (IFN-$\gamma^+$, CTLA-4$^+$, GITR$^+$) for BP allergic or nonallergic individuals, respectively (Fig. 5*B*).

### *Frequency of Bet v $1_{141-155}$-specific T cells in peripheral blood of allergic and nonallergic individuals*

Whereas in vitro stimulation is usually required to detect Bet v 1-specific T cells, we were able to detect MHC class II Bet v $1_{141-155}$ tetramer$^+$ T cells without any ex vivo expansion from the blood of both an allergic and a nonallergic individual with an HLA-DRB1*1501 background, at the peak exposure during the BP season (i.e., late April–early May) (cf. Table II and Fig. 6*A*). Bet v $1_{141-155}$-specific T cells represented 0.5 and 0.3% CD4$^+$ T cells in those blood samples, respectively.

The frequency of Bet v 1-specific T cells was further estimated outside of the pollen season on the basis of cell divisions required during in vitro stimulation to expand tetramer$^+$CD4$^+$ T cells to a level where they could be detected. We estimate that stimulation with the Bet v $1_{141-155}$-Ii-Key conjugate peptide mix leads to the doubling of allergen-specific CD4$^+$ T cells every 24 h during the 10 initial days of the culture. Based on those assumptions as well as the number of days required to expand Bet v 1-specific CD4$^+$ T cells, we estimate the frequency of Bet v 1-specific cells to be in the range of $10^{-6}$ to $10^{-5}$ circulating CD4$^+$ T cells outside birch pollen seasonal exposure, for both allergic and nonallergic individuals. These data establish the dramatic (2–3 log) expansion of allergen-specific T cells during the pollen season, which, interestingly, occurs in both allergic and nonsensitized individuals (Fig. 6*B*).

## Discussion

A deficit in immune tolerance is considered to cause allergy to otherwise harmless allergens in predisposed individuals. Suggested mechanisms involved in maintaining peripheral tolerance to allergens include 1) anergy or deletion of allergen-specific T cells, for example, as a consequence of a low level of costimulation beyond TCR engagement, following the interaction of immature dendritic cells (DCs) with naive T cells; and 2) induction of either Th1, CD25$^+$ Foxp3$^+$, or IL-10-producing Tr1 regulatory T lymphocytes inhibiting both allergen-specific Th2 responses and effector mechanisms associated with allergic inflammation (36–44). Th1 CD4$^+$ T cells are classically stimulated when high doses of antigen are presented by IL-12-producing DCs (45). Induction of regulatory T cells rather occurs as a consequence of Ag presentation in the presence of cytokines such as IL-10 and TGF-$\beta$, or following engagement of ICOS, CTLA-4, or PD-1 receptors with their cognate ligands, during T cell–DC interaction (38, 46, 47).

To better understand cellular mechanisms associated with allergen tolerance in healthy individuals, we developed MHC class II peptide tetramers to assess CD4$^+$ T cell responses to the BP major allergen Bet v 1. The immunodominant Bet v $1_{141-155}$ epitope was found to bind with a very high (1–78 nM) affinity to three common HLA-DRB1 class II haplotypes (DRB1*0101, DRB1*0401, DRB1*1501). This observation is in agreement with other studies demonstrating that Bet v $1_{141-155}$ is a major epitope recognized by T cells from both allergic and healthy individuals (30–32, 48, 49). Using such MHC class II peptide tetramers, we were able to detect circulating Bet v 1-specific T cells in all blood samples tested. We consistently found fewer Bet v 1-specific T cells in healthy vs BP allergic individuals, and therefore longer in vitro stimulation protocols were required to assess T cell responses in the former population. MHC class II peptide tetramer staining was clearly specific, identifying bona fide Bet v 1-specific T cells, because only

$CD4^+$, but not $CD8^+$, T cells were stained. Also, following stimulation with the allergen, most (i.e., >90%) T cells secreting cytokines are MHC class II Bet v $1_{141-155}$ $tetramer^+$. Our results are in agreement with several studies demonstrating that healthy individuals can mount allergen-specific T cell responses (49–52). In contrast, a recent study suggested that MHC class II peptide tetramers could detect T cell responses to the Lol p 1 grass pollen allergen in allergic but not in healthy individuals (15). Whereas it cannot be excluded that distinct seasonal allergens may elicit different types of immune responses, we can also possibly explain this discrepancy by the fact that we used in our study a potent in vitro stimulation protocol to expand rare allergen-specific T cell progenitors, relying on a Bet v $1_{141-155}$-Ii-Key conjugate peptide mix. This stimulation protocol takes advantage of the capacity of the Ii-Key/epitope hybrid to facilitate peptide exchange and direct binding to MHC class II molecules, as a consequence of an interaction between the Ii-Key moiety and an allosteric site on the MHC class II molecule (24). In our case, the Bet v $1_{141-155}$-Ii-Key peptide preparation is ~10-fold more potent in expanding Bet v 1-specific progenitors than is the Bet v $1_{141-155}$ peptide alone or the purified recombinant protein (33). One drawback of long-term in vitro stimulation protocols is that they could lead to the selective expansion of T cell subsets. We think this to be very unlikely in the present study, given that the phenotype and cytokine secretion profile of T cells obtained both outside or during the peak pollen season (i.e., after long- or short-term stimulation, respectively) were identical, thus suggesting that stimulation conditions used in the present study did not skew T cell populations.

Surface phenotyping of MHC class II Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells reveals that such cells are effector memory T cells ($CD45\ RO^+$, $CD62L^-$, $CCR7^-$) in BP allergic patients, whereas they are rather central memory T cells ($CD62L^+$, $CCR7^+$) in nonallergic individuals. Interestingly, only in healthy, but not in allergic individuals was a fraction of $tetramer^+CD4^+$ T cells found to be $CTLA\text{-}4^+$, $GITR^{bright}$, and $Foxp3^{bright}$, with the later molecules being considered as markers associated with regulatory T cells (53–55). MHC class II Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells were further assessed at a single-cell level for the cytokines they produce after allergen restimulation. Bet v 1-specific T cells isolated from BP allergic patients secrete, as expected, mostly IL-5 and IL-10, but not IFN-γ. These data are consistent with other studies (15, 32, 50, 56), as well as with our analysis identifying predominantly Th2 clones in the peripheral blood of allergic patients (Table III). In contrast, Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells detected in healthy individuals are mainly IFN-γ-producing Th1 cells and likely IL-10-producing Treg cells. These dominant patterns were further confirmed by quantitative PCR analysis in 99% pure MHC class II Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells obtained by cell sorting from BP and HDM allergic patients, respectively. These data also confirm the allergen specificity of Th2 responses observed in allergic patients. Interestingly, IL10-producing T cells are distinct from the ones secreting IFN-γ, as shown by multiple cytokine capture in MHC class II Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells. Altogether, these data are consistent with a mixed Th1/Treg cell response to allergens in healthy individuals, in agreement with several studies conducted under various exposure conditions to seasonal or perennial allergens such as Bet v 1 or Der p 1, respectively (12, 30, 50, 51). The magnitude of the Th1 vs Treg cell response to Bet v 1 observed in the present study was unexpected, but it supports a predominant role of immune deviation toward IFN-γ production to establish allergen-specific tolerance, as proposed recently by others (57).

One interesting feature of the BP allergy model is the seasonal exposure to the major Bet v 1 allergen, allowing investigation of the dynamics of allergen-specific responses. We observed a progressive expansion of Bet v 1-specific T cells starting from February, likely due to exposure to Bet v 1 cross-reactive allergens from hazel or alder. During the peak birch pollen exposure (April–May), we could detect MHC class II Bet v $1_{141-155}$ $tetramer^+CD4^+$ T cells ex vivo (i.e., without any in vitro expansion) from the blood of both healthy or BP allergic individuals, with measured frequencies in the range of 0.3–0.5% circulating $CD4^+$ T cells. We consistently observed that in vitro stimulation was required outside the pollen season to detect $tetramer^+$ T cells in blood samples. Based on cell proliferation characteristics assessed in CFSE-labeled cells stimulated with the Bet v $1_{141-155}$-Ii-Key conjugate peptide mix, we estimate that Bet v 1-specific T cells are present at much lower frequencies (i.e., between $10^{-6}$ and $10^{-5}$ circulating $CD4^+$ T cells) during wintertime. Thus, Bet v 1-specific cells undergo a dramatic expansion, from $10^{-6}$ to $10^{-3}$ $CD4^+$ T cells during the pollen season, whereas the pattern of cytokines produced remains the same throughout the year, for both BP allergic and nonallergic individuals.

Collectively, our results demonstrate that tolerance to the Bet v 1 seasonal allergen in healthy individuals is associated with the presence and expansion of allergen-specific $CD4^+$ T lymphocytes. In contrast to BP allergic patients, those likely "protective" T cells comprise Th1- and likely IL-10-producing regulatory T cells. This observation has a significant implication in allergy vaccine design, in that strategies to induce IFN-γ and IL-10 production by naive T cells (e.g., using specific immunization schemes, mucosal routes, Th1/Treg cell adjuvants) appear valid to pursue in future immunotherapy protocols.

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## Disclosures

Several coauthors (L.V.O., E.W., E.v.H., C.L., and P.M.) are employees in biopharmaceutical companies.

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