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Single Cell Assessment of Allergen-Specific T Cell Responses with MHC Class II Peptide Tetramers: Methodological Aspects

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Key Words
Bet v 1 · MHC class II tetramers · T cell tolerance

Abstract
Background: We report herein critical methodological principles for assessing, at a single cell level, allergen-specific T cell responses using MHC class II peptide tetramers. ***Methods:*** We developed MHC class II peptide tetramers to monitor T cell responses against the immunodominant Bet v $1_{141–155}$ peptide in individuals with either an HLA-DRB1*0101, DRB1*0401 or DRB1*1501 background. In vitro stimulation was performed with serially truncated versions of the Bet v $1_{141–155}$ epitope chemically conjugated to the Ii-Key peptide. ***Results:*** Identification of Bet v $1_{141–155}$ as a high-affinity epitope for multiple HLA-DRB1 allotypes led to the development of corresponding tetramers detecting Bet v $1_{141–155}$-specific T cells with a high specificity and sensitivity. Stimulation with Bet v $1_{141–155}$ Ii-Key conjugate peptides is the most efficient procedure to expand Bet v $1_{141–155}$-specific CD4+ T cells, allowing to detect such cells in both allergic and healthy individuals. MHC class II Bet v $1_{141–155}$ tetramer-positive T cells produce IFN-γ and IL-10 in healthy individuals, and IL-5 in allergic patients. Frequencies of Bet v 1-specific CD4+ T cells circulating in the blood of allergic or nonallergic individuals range from approximately 10^{-5} to 10^{-3} CD4+ T cells, outside or within the pollen season, respectively. ***Conclusions:*** MHC class II peptide tetramers are valuable tools to assess allergen-specific T cell responses, both qualitatively and quantitatively. Selection of a high-affinity T cell epitope, as well as optimization of in vitro stimulation conditions to expand rare T cell progenitors are critical success factors in those analyses.

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Introduction

Allergen-specific CD4+ T cells are thought to play a central role in both the pathogenesis and regulation of allergic inflammation, depending on which cytokines they produce [1, 2]. Excessive production of Th2 cytokines is the hallmark of allergic T cell responses [3–6], whereas IL-10-producing regulatory T cells as well as IFN-γ-secreting Th1 cells are rather associated with the establishment of tolerance, both in the course of natural immune responses [7] as well as during successful immunotherapy [2, 8–10]. Tools are needed to analyze at a single cell level allergen-specific CD4+ T lymphocytes, in both allergic and nonallergic individuals, (1) in order to

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dissect cellular immune mechanisms linked with peripheral tolerance, and (2) to monitor allergen-specific immunological changes during immunotherapy protocols.

The identification within common allergens of distinct immunodominant epitopes restricted to various HLA-DR allotypes [11–14] facilitates the design and development of MHC class II peptide tetramers as reagents to investigate allergen-specific T cell responses mediated by CD4+ T lymphocytes [15, 16]. Such tetramers classically consist of fluorescently labeled complexes of 4 MHC class II heterodimers assembled in presence of a peptide representing a high-affinity T cell epitope [17]. MHC molecules coupled to biotin are subsequently multimerized with labeled streptavidin, providing high-avidity reagents to detect epitope-specific T cells by flow cytometry. Although MHC class I peptide tetramers have been used successfully in numerous studies to identify, quantify and characterize antigen-specific CD8+ T cells, it has been more difficult to detect CD4+ T cells with MHC class II peptide tetramers, largely due to their low frequency within peripheral blood mononuclear cells (PBMCs) [18, 19]. In this context, in vitro expansion of rare allergen-specific T cell progenitors is required prior to tetramer staining [20–22].

We document herein experimental conditions needed to ensure efficient and specific detection of rare allergen-specific CD4+ T cells using MHC class II peptide tetramer technology. Focusing on the Bet v 1 major allergen from birch pollen as a target, we demonstrate that MHC class II peptide tetramers made with the high-affinity immunodominant Bet v $1_{141–155}$ T cell epitope can be used to detect Bet v 1-specific CD4+ T cells in both allergic and nonallergic individuals. We also describe methods to characterize at a single level the pattern of cytokines produced by such cells. We conclude that critical success factors to apply this technology to the assessment of allergen-specific T cell responses include (1) the identification of immunodominant T cell epitope(s) with a high affinity for common MHC class II haplotypes and (2) efficient in vitro stimulation conditions.

Material and Methods

Cells and HLA Typing

Blood samples were obtained after informed consent from either patients allergic to birch pollen or from healthy donors with no history of atopy. Allergic patients were diagnosed by positive skin prick testing. Samples were obtained from the Etablissement Français du Sang (Rungis, France), the Hopital Antoine Beclere (Clamart, France) and the Department of Allergology, University of Antwerp, Belgium. The study was approved by the Comité Consultatif pour la Protection des Patients dans la Recherche Biomédicale of Bicêtre Hospital, Paris. Of 64 individuals PCR typed for their HLA-DRB1 allotypes, 14 individuals were selected in this study based on their HLA-DRB1*0101 (3 allergic and 3 healthy individuals), HLA-DRB1*0401 (1 allergic and 1 healthy individual) or HLA-DRB1*1501 (3 allergic and 3 healthy individuals) background.

PBMCs were isolated by centrifugation over a Ficoll-Paque Plus density gradient (Amersham Biosciences, Orsay, France) and cultured in RPMI 1640 supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml L-glutamine, 50 μM β-mercaptoethanol (all from Invitrogen, Cergy Pontoise, France) plus 10% heat-inactivated human AB serum (Sigma-Aldrich, St. Louis, Mo., USA). Genomic DNA was extracted from blood samples using an Easy DNA kit (Invitrogen, Carlsbad, Calif., USA). HLA-DRB1 low-resolution (2-digit) typing was performed by PCR sequence-specific primers using a Histo Type/DNA-DR Mini kit (Biologische Analysensystem GmbH, Lich, Germany). High-resolution (4-digit) typing was achieved by PCR sequence-specific primers using a Dynal All Set™ kit (Invitrogen, Cergy-Pontoise, France) to identify patients expressing HLA-DRB1*0101, DRB1*0401 and DRB1*1501 alleles [23, 24].

In vitro Peptide Binding Assays with Purified HLA-DR Molecules

T cell epitopes derived from Bet v 1 containing potential anchor motifs for either HLA-DRB1*0101, DRB1*0401 or DRB1*1501 molecules were predicted using T-epitope and ProPred (http://www.imtech.res.in/raghava/propred) algorithms. Bet v 1 candidate peptides were synthesized by NeoSystem (Strasbourg, France) and used with a purity grade >75% confirmed by HPLC. Peptides were tested in an HLA-DR peptide in vitro binding assay, as described elsewhere [25]. Briefly, HLA-DRB1*0101, DRB1*0401 and DRB1*1501 molecules were immunopurified from the human Epstein-Barr virus-transformed B cell lines HOM-2, BOLETH and SCHU using the L243 antibody directed against a monomorphic determinant (American Type Culture Collection, Manassas, Va., USA) coupled to protein A-Sepharose CL 4B gel (GE Healthcare, Aulnay-sous-Bois, France). Purified HLA-DR molecules were diluted (1/20 to 1/100) in 10 mM phosphate, 150 mM NaCl, 1 mM n-dodecyl β-D-maltoside, 10 mM citrate and 0.003% thimerosal buffer. Serial dilutions of Bet v 1 peptides were incubated with HLA-DR molecules in presence of a biotinylated reporter peptide. The latter included the influenza hemagglutinin $HA_{306–318}$ peptide, used at a concentration of 1 and 30 nM as a reporter for binding to HLA-DRB1*0101 and HLA-DRB1*0401, respectively. The HLA-$A3_{152–166}$ peptide (10 nM) was used as a reporter peptide for binding to the HLA-DRB1*1501 molecule [25]. After a 24-hour incubation at 37°C (except for HLA-DRB1*1501 binding, evaluated after a 72-hour incubation) with competing peptides, HLA-DR-peptide complexes were added to plates previously coated with 10 μg/ml of the L243 antibody, and further incubated for 2 h at room temperature. Bound biotinylated peptides were detected using a streptavidin-alkaline phosphatase conjugate (Amersham, Little Chalfont, UK) and 4-methylumbelliferyl phosphate as a substrate (Sigma, St. Quentin-Fallavier, France). Fluorescence was measured at 450 nm following excitation at 365 nm on a Victor II spectrofluorimeter (Perkin Elmer Instruments, Les Ulis, France).

Table 1. Binding capacities of Bet v 1 peptides to immunopurified MHC class II molecules

Peptides	Amino acid sequences	HLA-DRB1 alleles		
		0101*	0401*	1501*
Bet v $1_{141–155}$	ETLLRAVESYLLAHS	1	2	0.1
AEB-1	Ac-LRMK-ETLLRAVESYLLA-NH2	4	38	0.4
AEB-4	Ac-LRMK-LRAVESYLLA-NH2	7	108	0.1
AEB-6	Ac-LRMK-AVESYLLA-NH2	>4,722	>1,748	85
AEB-8	Ac-ETLLRAVESYLLA-NH2	3	23	0.4
AEB-11	Ac-LRMK-ETLLRAVESYLLAHS-NH2	4	30	1
AEB-12	Ac-ETLLRAVESYLLAHS-NH2	4	23	2

Peptide-binding capacities for common HLA class II molecules were evaluated in a competitive ELISA as described in Material and Methods. Results are expressed as a ratio between IC_{50} (inhibitory concentration) values obtained with either tested peptides or corresponding reference peptides. Results have been confirmed in at least 2 independent experiments. Reference peptides were $HA_{306–318}$ for both HLA-DRB1*0101 (IC_{50} = 2 nM) and DRB1*0401 alleles (IC_{50} = 6 nM), and HLA-$A3_{152–166}$ for the HLA-DRB1*1501 allele (IC_{50} = 11 nM). The AEB series of peptides consists of serially truncated versions of the Bet v $1_{141–155}$ peptide coupled with a polymethylene linker to the Ii-Key peptide. Collectively, these peptides are referred to as the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. Frequencies of HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*1501 in the Caucasian population are approximately 17, 11 and 15%, respectively.

In vitro Stimulation of Bet v 1-Specific CD4+ T Cells

Serially truncated versions of the Bet v $1_{141–155}$ peptide were chemically conjugated with the Ii-Key peptide from the MHC invariant chain to facilitate peptide exchange and binding to MHC class II molecules (Antigen Express, Worcester, Mass., USA) [26, 27]. A Bet v $1_{141–155}$ Ii-Key conjugate peptide mix consisting of a pool of such acetylated and amidated peptides (combining AEB-1, AEB-4, AEB-6, AEB-8, AEB-11 and AEB-12; table 1) was selected based on both its high-affinity binding to MHC class II molecules and a proven capacity to stimulate PBMCs from allergic patients. Recombinant Bet v 1 was produced in *Escherichia coli*, and purified as described elsewhere [28]. Natural Der p 1, used as an irrelevant stimulus, was obtained from Indoor Biotechnologies (Deeside, UK).

Allergen-specific T cells were expanded as previously described [29–31] after several rounds of in vitro stimulation. Briefly, freshly isolated PBMCs were seeded in 12-well plates (TPP, Trasadingen, Switzerland) at 5×10^6 cells/well in 2 ml culture medium and stimulated with either the Bet v $1_{141–155}$ peptide (10 μg/ml), the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (10 μg/ml) or purified recombinant Bet v 1 (50 μg/ml). Cells were subsequently fed with fresh medium containing 10 IU/ml IL-2 (Roche, Meylan, France) every 3 days. For long-term cultures beyond 12–14 days, cells were restimulated in presence of irradiated autologous PBMCs pulsed with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix.

Proliferation Assays

Proliferation was assessed using a standard 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oreg., USA) dye dilution assay. Adherent cells were obtained after plating 1×10^6 PBMCs per well in 48-well plates (TPP) for 2 h. Nonadherent cells were removed after washing twice with PBS. Adherent cells were incubated for 3 h with either 10 μg/ml Bet v $1_{141–155}$ Ii-Key conjugate peptide mix, 10 μg/ml Bet v $1_{141–155}$, or for 16 h with 50 μg/ml recombinant Bet v 1 or medium alone. Nonadherent cells were washed twice and stained with 0.8 μg/ml CFSE in PBS for 10 min at 37°C, as described elsewhere [32]. Staining was stopped after adding pure human AB serum (v/v) and washing twice. CFSE-stained cells (1.0×10^6 cells/ml) were added to adherent cells in 48-well culture plates. After 3 days, 10 IU/ml IL-2 was added to cultures. Expanded cells were then harvested and stained with both phycoerythrin (PE)-Cy5-labeled anti-CD4 and PE-Cy7-labeled anti-CD3 antibodies (Beckman Coulter/Immunotech, Marseille, France) for 30 min at 4°C. Cells were washed and analyzed by flow cytometry using an FC500 cytometer (Beckman Coulter, Miami, Fla., USA).

MHC Class II Bet v $1_{141–155}$ Tetramer Staining

MHC class II DRB1*0101, DRB1*0401 and DRB1*1501 tetramers were produced by Beckman Coulter/Immunotech using recombinant α- and β-chains from human MHC class II molecules produced in a baculovirus expression system. Two sets of tetramers complexed with either the Bet v $1_{141–155}$ peptide (ETLLRAVESYLLAHS) or with the control CLIP peptide (LPKPPKPVSKMRMATPLLMQALPM) were produced as described elsewhere [33].

MHC class II peptide tetramer staining was performed on PBMCs after 5–30 days of in vitro stimulation as described above. To this aim, 0.2×10^6 cells were washed and incubated with 0.5 μg PE-labeled MHC class II peptide tetramers in 50 μl culture medium for 2 h at 37°C. FITC-labeled anti-CD4, PE-Cy7-labeled anti-CD3 antibodies (Beckman Coulter/Immunotech) and a combination of ECD-labeled anti-CD14 antibody and 7-aminoactinomycin D (Viaprobe reagent; BD Biosciences Pharmingen, San Diego, Calif., USA) were used to detect T cells, while exclud-

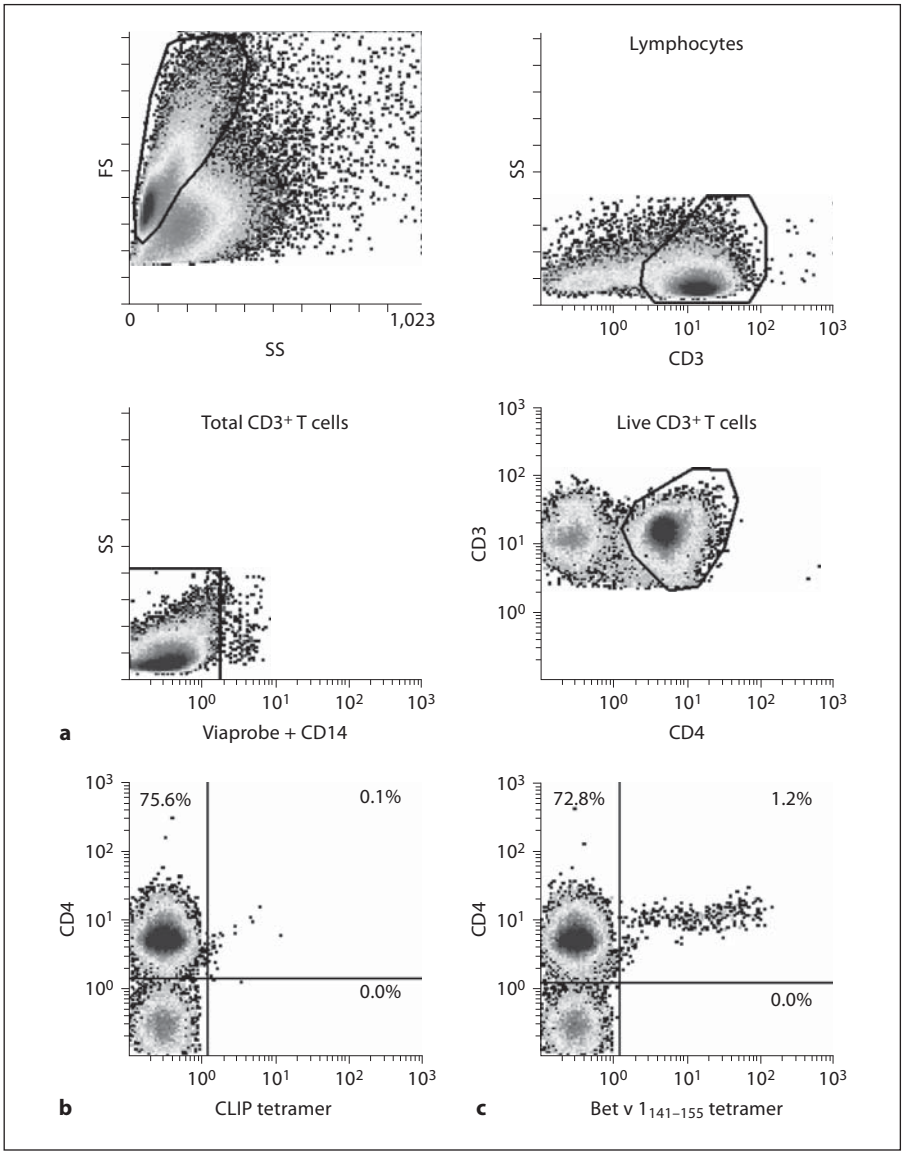


Fig. 1. Flow cytometry analysis of Bet v $1_{141–155}$-specific CD4+ T cells. **a** The gating strategy was based on the identification of lymphocytes, based on forward scatter (FS) and side scatter (SS) patterns. Viable CD4+ T cells were selected based on both coexpression of CD3 and CD4, absence of CD14 and negative Viaprobe staining. **b** Measurement of background staining using an irrelevant CLIP tetramer within the CD3+ T cell population. **c** Measurement of Bet v $1_{141–155}$-specific T cells within the CD3+ T cell population. Staining was performed in PBMCs from an HLA-DRB1*0401 birch pollen-allergic donor after 17 days of in vitro stimulation with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix.

ing monocytes and dead cells. After 20 min at 4°C, cells were washed twice in PBS and analyzed by flow cytometry. Appropriate fluorochrome-conjugated isotype (IgG1)-matched antibodies were used as controls (all from Beckman Coulter/Immunotech).

Cytokine Surface Capture Assay in Bet v $1_{141–155}$-Specific T Lymphocytes

Measurement of IFN-γ, IL-5 or IL-10 production by Bet v $1_{141–155}$-specific CD4+ T cells was performed after allergen stimulation, combining MHC class II peptide tetramer staining and a cytokine surface capture assay using a Cytokine Secretion Assay kit (Miltenyi Biotec, Bergisch Gladbach, Germany). All experiments were performed on cell cultures containing at least 0.8% Bet v $1_{141–155}$ tetramer-positive CD4+ T cells. Before activation, cells were stained with MHC class II peptide tetramers (5 μg/ml) for 30 min at 37°C (suboptimal conditions) in order to reduce the potential downregulation of T cell receptors and allow subsequent allergen stimulation [34]. To induce cytokine secretion, cells were restimulated for 3 h with irradiated autologous PBMCs pulsed with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix or with an irrelevant stimulus as a control. Cells were subsequently washed and labeled for 5 min at 4°C with a cytokine-specific high-affinity bivalent capture antibody directed against both CD45 and a given cytokine (IL-5, IL-10 or IFN-γ). After 45 min at 37°C to allow cytokine secretion, cells were washed and incubated for 30 min at 4°C with a PC7-labeled anti-CD4 antibody, ECD-labeled anti-CD14 antibody, Viaprobe and either FITC-labeled anti-IFN-γ, allophyco-cyanin-labeled anti-IL-5 or anti-IL-10 monoclonal antibodies (Miltenyi Biotec) [35, 36]. Cells were washed twice and immediately analyzed by flow cytometry.

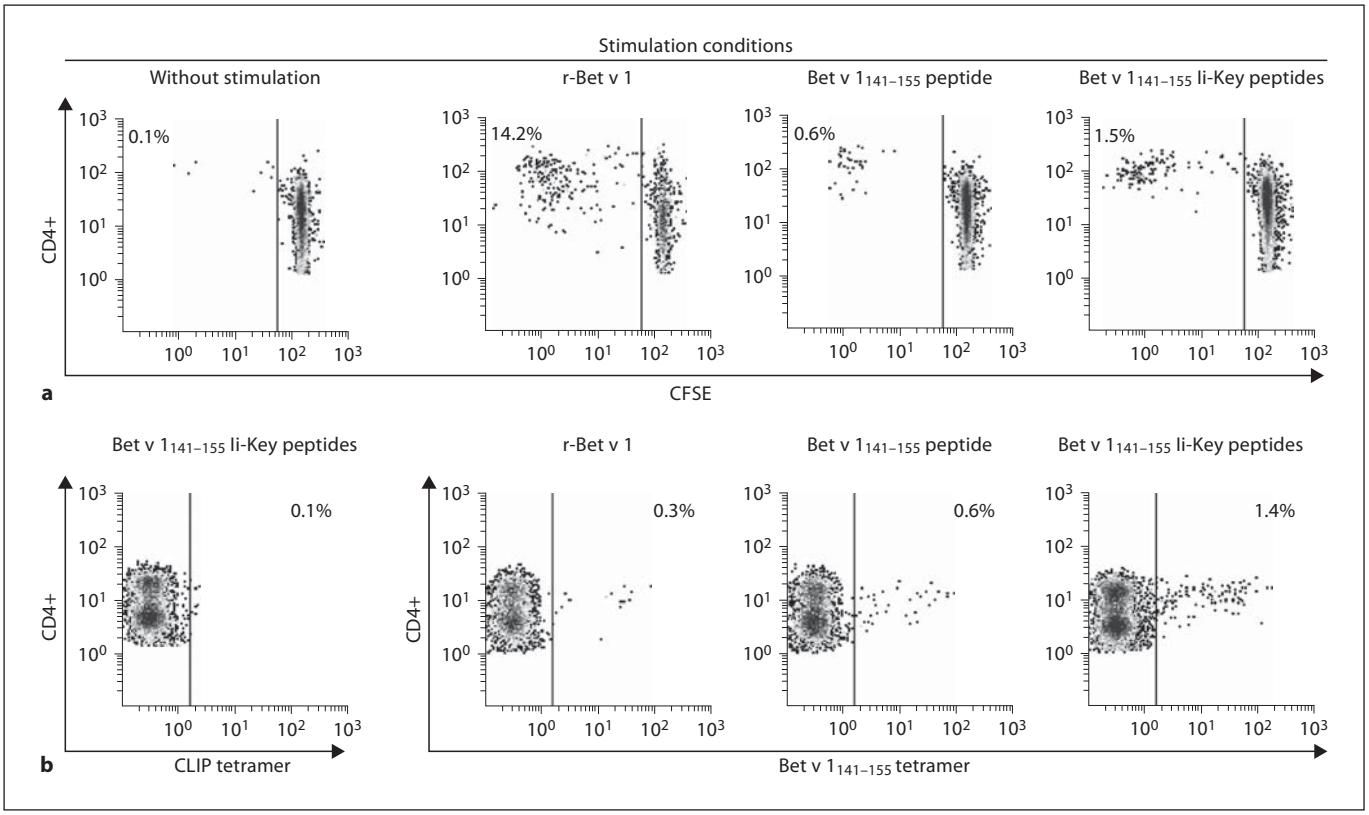


Fig. 2. In vitro expansion of Bet v $1_{141–155}$-specific T cells. Freshly isolated PBMCs from an HLA-DRB1*1501 birch pollen-allergic individual were stimulated with either purified Bet v 1 protein (50 μg/ml), Bet v $1_{141–155}$ peptide or Bet v $1_{141–155}$ Ii-Key conjugate peptide mix at 10 μg/ml for 14 days. Cells were fed with 10 IU/ml IL-2 at day 5 and every 3–4 days afterwards and restimulated on day 14 with irradiated autologous PBMCs pulsed with cognate peptide. In the CFSE dye dilution experiment, cells were stained with CFSE at day 14 and analyzed 7 days later (**a**); tetramer staining was performed at day 21 after first stimulation (**b**). Percentages of dividing cells or tetramer-positive cells are indicated within the live CD4+ T cell population. In this experiment, background staining with corresponding CLIP tetramers was always less or equal to 0.1% CD4+ T cells.

Results

Design and Development of MHC Class II Bet v $1_{141–155}$ Tetramers

Predictive algorithms [37, 38] were used to identify potential MHC class II binding motifs within the Bet v 1 protein. Peptides containing putative T cell epitopes were synthesized and used to perform in vitro binding assays with purified common Caucasian HLA-DRB1 molecules. Using such assays, we confirmed that Bet v $1_{141–155}$ exhibited a high affinity (in the nanomolar range) for HLA-DRB1*0101, DRB1*0401 and DRB1*1501 molecules (table 1). Corresponding tetramers were produced as complexes with either the Bet v $1_{141–155}$ peptide or with the irrelevant $CLIP_{81–104}$ peptide as a control, and used to detect Bet v $1_{141–155}$-specific CD4+ T cells. In order to avoid nonspecific MHC class II peptide tetramer binding, we excluded monocytes based on CD14 staining. Dead cells were removed using Viaprobe staining (fig. 1).

In vitro Expansion of Bet v $1_{141–155}$-Specific T Cells

The frequency of allergen-specific CD4+ T cells in peripheral blood is too low in most circumstances to allow detection without any in vitro expansion of antigen-reactive cells [19]. Thus, we tested various stimulation schemes to expand those T cells efficiently. To this aim, PBMCs from an allergic donor were stimulated with either the recombinant Bet v 1 protein, Bet v $1_{141–155}$ peptide alone or a pool of 6 peptides chemically conjugated to the Ii-Key peptide (Bet v $1_{141–155}$ Ii-Key conjugate peptide mix). The latter comprises entire or truncated versions of the Bet v $1_{141–155}$ epitope coupled with a polymethylene linker to the Ii-Key peptide, known to facilitate peptide exchange and binding to MHC class II molecules [17, 26, 27]. Those con-

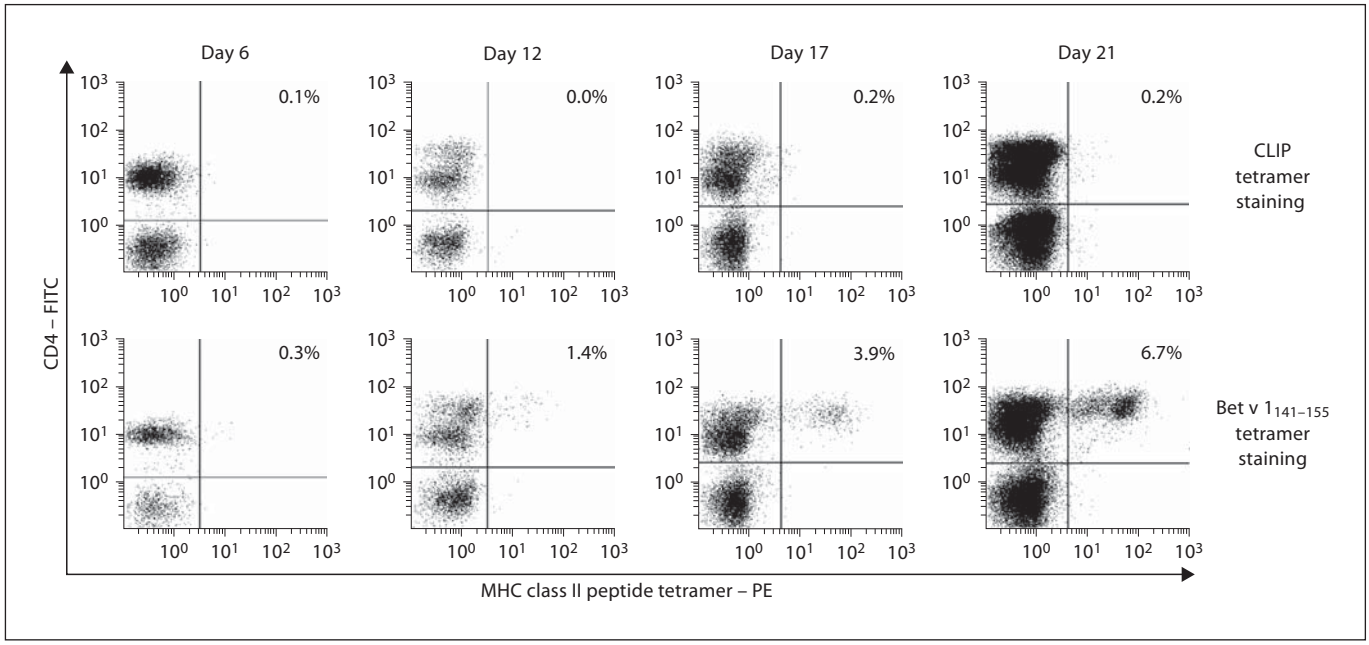


Fig. 3. MHC class II Bet v $1_{141–155}$ tetramer staining in the course of in vitro expansion. PBMCs from an HLA-DRB1*0101 birch pollen-allergic individual were stimulated with 10 μg/ml Bet v $1_{141–155}$ Ii-Key conjugate peptide mix and subsequently stained with HLA-DRB1*0101 Bet v $1_{141–155}$ tetramer or HLA-DRB1*0101 CLIP tetramer 6, 12, 17 or 21 days after stimulation. Percentages of CD4+ tetramer-positive cells within the live CD3+ cell population are indicated in the upper right quadrant. Data are representative of 5 independent experiments.

jugates were confirmed to bind to HLA-DRB1-purified molecules with a high affinity (table 1). To compare those various stimuli, we performed parallel in vitro stimulations of CFSE-labeled PBMCs. In presence of the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix, a 2.5-fold increase in the T cell response was observed when compared with the Bet v $1_{141–155}$ peptide alone, with 1.5 and 0.6% proliferating CD4+ T cells, respectively (fig. 2a). A high proliferative response was observed in presence of the recombinant Bet v 1 protein as expected, since Bet v 1 contains multiple T cell epitopes beyond Bet v $1_{141–155}$ [13]. However, when comparing percentages of MHC class II Bet v $1_{141–155}$ tetramer-positive T cells obtained after a 21-day stimulation with those various reagents, specific staining was significantly higher after stimulation with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix when compared with the purified Bet v 1 protein or Bet v $1_{141–155}$ peptide alone (fig. 2b). Importantly, patterns of cytokine production in T cells stimulated under those various conditions were similar, suggesting that there was no bias in expanding a specific cellular subset (see below). Thus, all subsequent experiments were conducted using the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix as a stimulus.

We further investigated the kinetics of allergen-specific CD4+ T cell expansion under those in vitro stimulation conditions. Figure 3 shows a representative example of MHC class II Bet v $1_{141–155}$ tetramer staining in PBMCs obtained from a birch pollen-allergic individual at various time points during cell cultures. For samples obtained outside the pollen season, only minor tetramer staining (<1% CD4+ T cells) is generally observed before 1–2 weeks of in vitro stimulation. Generally, the peak of MHC class II Bet v $1_{141–155}$ tetramer detection (with 1–13% tetramer-positive T cells detectable in the cultures) is reached after 15–21 days of PBMC stimulation for allergic individuals and within 21–28-days for healthy donors.

Specificity and Sensitivity of MHC Class II Bet v $1_{141–155}$ Tetramer Staining

Several lines of evidence confirm that MHC class II Bet v $1_{141–155}$ tetramers detect specifically Bet v 1-reactive CD4+ T cells. Firstly, this cell population does not react over background level (≤0.1% in this experiment) with a control MHC class II peptide tetramer made with the irrelevant CLIP peptide (fig. 4a) nor with a mismatched

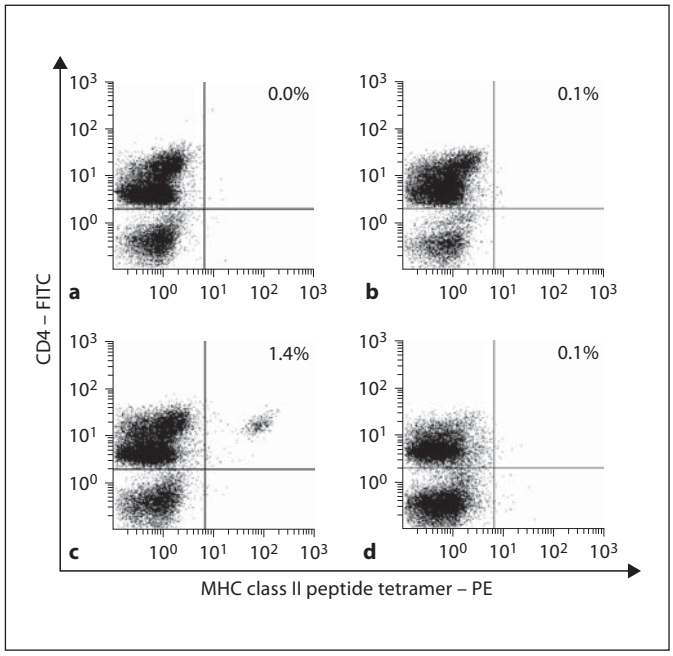


Fig. 4. Specificity of MHC class II Bet v $1_{141–155}$ tetramer staining. HLA-DRB1*1501 CLIP tetramer staining (**a**), HLA-DRB1*0101 Bet v $1_{141–155}$ tetramer staining (**b**) and HLA-DRB1*1501 Bet v $1_{141–155}$ tetramer staining (**c**) of a Bet v $1_{141–155}$-specific cell line generated from an HLA-DRB1*1501 birch pollen-allergic individual. **d** HLA-DRB1*1501 Bet v $1_{141–155}$ tetramer staining of Der p 1-specific T cells generated from the same HLA-DRB1*1501 birch pollen-allergic individual as in **c**. Percentage of CD4+ tetramer-positive cells within the live CD3+ cell population are indicated in the upper right quadrant.

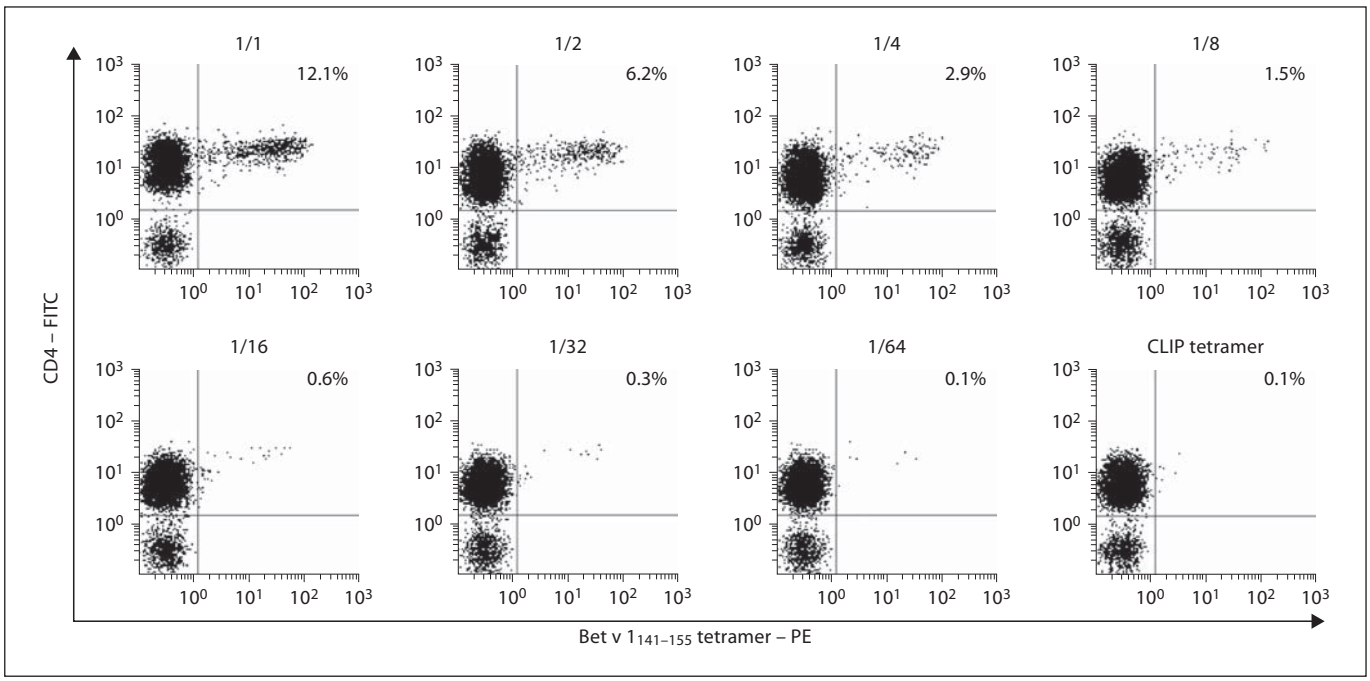


Fig. 5. Sensitivity of MHC class II Bet v $1_{141–155}$ tetramer staining. PBMCs from an HLA-DRB1*0101 patient allergic to birch pollen were stimulated with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (10 μg/ml) for 14 days. Cells were fed with 10 IU/ml IL-2 starting at day 5 and every 3–4 days afterwards. At day 14, cells were mixed with PBMCs from an HLA-DRB1*1501 donor at various ratios. Cell mixtures were stained with an HLA-DRB1*0101 Bet v $1_{141–155}$ tetramer. Percentages of CD4+ tetramer-positive cells within the live CD3+ cell population are indicated in the upper right quadrant.

MHC class II Bet v $1_{141–155}$ tetramer (fig. 4b). Further, Bet v $1_{141–155}$-specific T cells are detected with the MHC class II Bet v $1_{141–155}$ tetramer based on a relevant MHC class II allotype (fig. 4c). Lastly, no detectable staining is observed with MHC class II Bet v $1_{141–155}$ tetramer in Der p 1-specific T cells generated from the same individual (fig. 4d). Thus, the Bet v $1_{141–155}$ tetramer specificity is confirmed both in terms of Bet v $1_{141–155}$ epitope and HLA-DRB1 molecule recognition.

In order to assess the sensitivity of detection with Bet v $1_{141–155}$ tetramers, Bet v $1_{141–155}$-specific T cells expanded from a birch pollen-allergic individual were mixed in decreasing amounts with unstimulated PBMCs from a healthy HLA-DRB1-compatible donor. As expected, the percentage of Bet v 1 tetramer-positive cells in cell mixtures was reduced during serial dilution with tetramer-negative cells (fig. 5). We consider that we can reliably detect Bet v $1_{141–155}$-specific T cells at a level of 0.2% CD4+ cells over background staining with the control CLIP tetramer. We estimated from multiple proliferation experiments that 1-month in vitro stimulation (that is, the longest stimulation time we use) results in approximately a 10,000-fold expansion of allergen-specific cells. From this, we calculate that this assay allows to detect a single Bet v $1_{141–155}$-specific T cell within 5×10^6 CD4+ T cells.

Precursor Frequencies of Bet v $1_{141–155}$-Specific CD4+ T Cells in Peripheral Blood

To estimate the frequency of Bet v $1_{141–155}$-specific CD4+ T cells circulating in peripheral blood, we combined tetramer staining with CFSE labeling to assess cell divisions following allergen stimulation. Stimulation of PBMCs with phytohemagglutinin and IL-2 results in cell divisions, characterized by distinct CFSE fluorescence levels. The latter were used to calculate the average number of cell divisions in cells stimulated with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. As shown in figure 6a, after 10 days of culture, all tetramer-positive cells are predominantly in the CFSE-low cell population. The mean CFSE fluorescence of these cells indicates that they underwent approximately 10 cell divisions in response to the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. The detection of 0.7% tetramer-positive cells after 10 cell divisions leads to an estimate of the initial precursor frequency of 0.0007% CD4+ T cells. Using this approach, we confirmed that CD4+ T cells from both allergic and nonallergic individuals undergo an average of 1 cell division each day during the first 10 days of in vitro stimulation with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (fig. 6b). Thus, precursor frequencies were determined by dividing the percentage of tetramer-positive cells detected by 2^n [39], where n is the number of days of in vitro stimulation needed to detect specific tetramer staining (applicable within $n \leq 10$). From such experiments, we estimate that Bet v $1_{141–155}$-specific T cells are present outside the pollen season in peripheral blood at a level of 1 in 10^4–10^6 CD4+ T cells. During the pollen season, Bet v $1_{141–155}$-specific T cell frequencies in the blood raise to up to 2–4 in 10^3 CD4+ T cells in nonallergic and allergic individuals, demonstrating that a significant expansion of specific T cells occurs during exposure to seasonal allergens (fig. 6c). No differences were observed depending upon the HLA-DR background of the donors.

Single Cell Assessment of Cytokine Secretion in MHC Class II Bet v $1_{141–155}$ Tetramer-Positive Cells

We combined tetramer staining with a cytokine surface capture assay in order to characterize at a single cell level patterns of cytokine released from tetramer-positive CD4+ cells following allergen stimulation [40]. In such studies, cells were stained under suboptimal conditions with tetramers (5 μg/ml) at 37°C for 30 min to allow further stimulation with antigen [34, 41]. To this aim, cells were incubated for 3 h with irradiated autologous PBMCs pulsed with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. Secreted cytokines were captured at the cell surface using bispecific antibodies directed to both the CD45 molecule and either IFN-γ, IL-5 or IL-10. The percentage of Bet v $1_{141–155}$-specific T cells producing any given cytokine was subsequently detected after staining with FITC or allophyco-cyanin-labeled cytokine-specific monoclonal antibodies [42] and flow cytometry analysis (fig. 7). Although not shown, T cell receptor engagement with MHC class II Bet v $1_{141–155}$ tetramers alone does not stimulate any detectable cytokine release. In contrast, we consistently observed that approximately 30–70% of Bet v $1_{141–155}$ tetramer-positive CD4+ T cells produce cytokines following allergen restimulation. Using such a functional assay combined with tetramer staining in 3 birch pollen-allergic versus 3 nonallergic individuals, we confirmed, as expected, that most Bet v $1_{141–155}$-specific CD4+ T cells from birch pollen-allergic donors produce high levels of IL-5 (mean 34 ± 2.9% versus 10.1 ± 2.7%, for allergic versus nonallergic subjects). In contrast, tetramer-positive cells from nonallergic individuals rather produce IFN-γ (mean 4.9 ± 1.6% versus 36 ± 2.6%, for allergic versus nonallergic subjects) and to a lesser extent IL-10 (mean 2.3 ± 0.8% versus 8.5 ± 2%, for allergic versus nonallergic subjects; fig. 8a, b) [43].

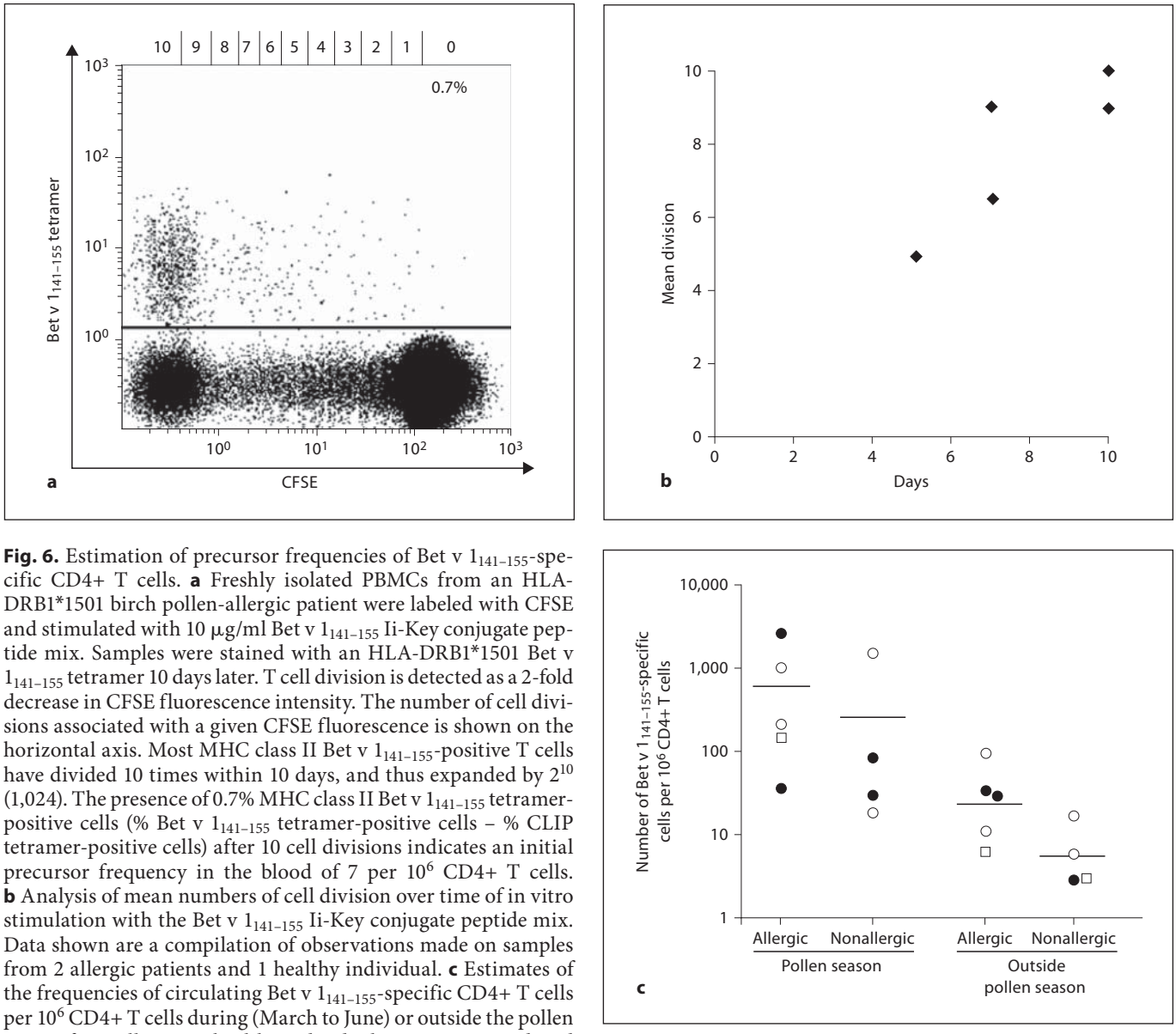


Fig. 6. Estimation of precursor frequencies of Bet v $1_{141–155}$-specific CD4+ T cells. **a** Freshly isolated PBMCs from an HLA-DRB1*1501 birch pollen-allergic patient were labeled with CFSE and stimulated with 10 μg/ml Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. Samples were stained with an HLA-DRB1*1501 Bet v $1_{141–155}$ tetramer 10 days later. T cell division is detected as a 2-fold decrease in CFSE fluorescence intensity. The number of cell divisions associated with a given CFSE fluorescence is shown on the horizontal axis. Most MHC class II Bet v $1_{141–155}$-positive T cells have divided 10 times within 10 days, and thus expanded by 2^{10} (1,024). The presence of 0.7% MHC class II Bet v $1_{141–155}$ tetramer-positive cells (% Bet v $1_{141–155}$ tetramer-positive cells – % CLIP tetramer-positive cells) after 10 cell divisions indicates an initial precursor frequency in the blood of 7 per 10^6 CD4+ T cells. **b** Analysis of mean numbers of cell division over time of in vitro stimulation with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. Data shown are a compilation of observations made on samples from 2 allergic patients and 1 healthy individual. **c** Estimates of the frequencies of circulating Bet v $1_{141–155}$-specific CD4+ T cells per 10^6 CD4+ T cells during (March to June) or outside the pollen season from allergic or healthy individuals. Data were produced with blood samples obtained from individuals with either an HLA-DRB1*0101 (filled circles), HLA-DRB1*1501 (open circles) or HLA-DRB1*0401 (open squares) background.

Using this cytokine surface capture assay, we also confirmed that stimulation conditions used in this assay do not alter patterns of cytokine production. As shown in figure 9, percentages of tetramer-positive cells producing either IL-5 (27.5%) or IFN-γ (9.2%) after stimulation of Bet v 1-specific T cells with the recombinant Bet v 1 protein were similar to those observed when using the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (31.7% and 5.2%, respectively) as a stimulus. Furthermore, as shown in fig. 10, a comparable pattern of cytokine production was observed at various time points (days 14, 21 and 28) during in vitro expansion of Bet v 1-specific CD4+ T cells in response to allergen. Collectively, these data demonstrate that the in vitro stimulation protocol used in this study does not introduce any bias in terms of the population of T cells expanded.

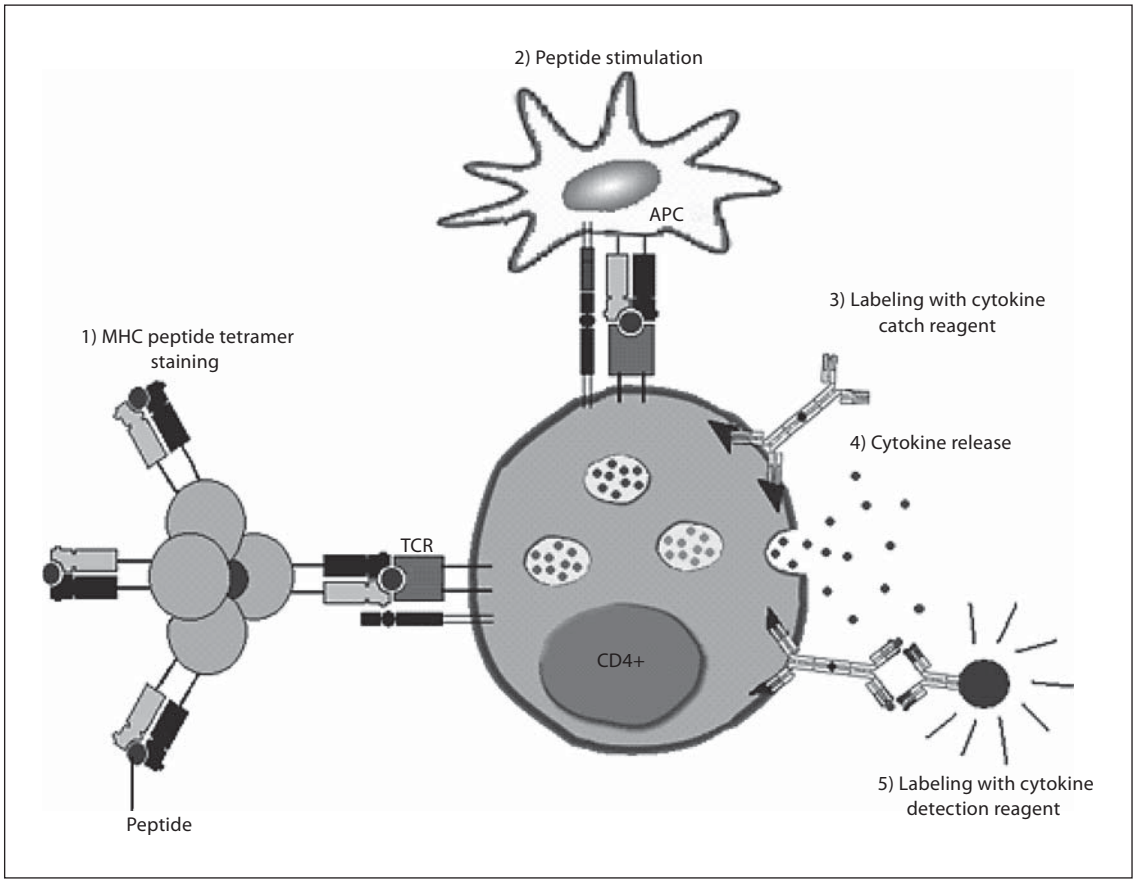
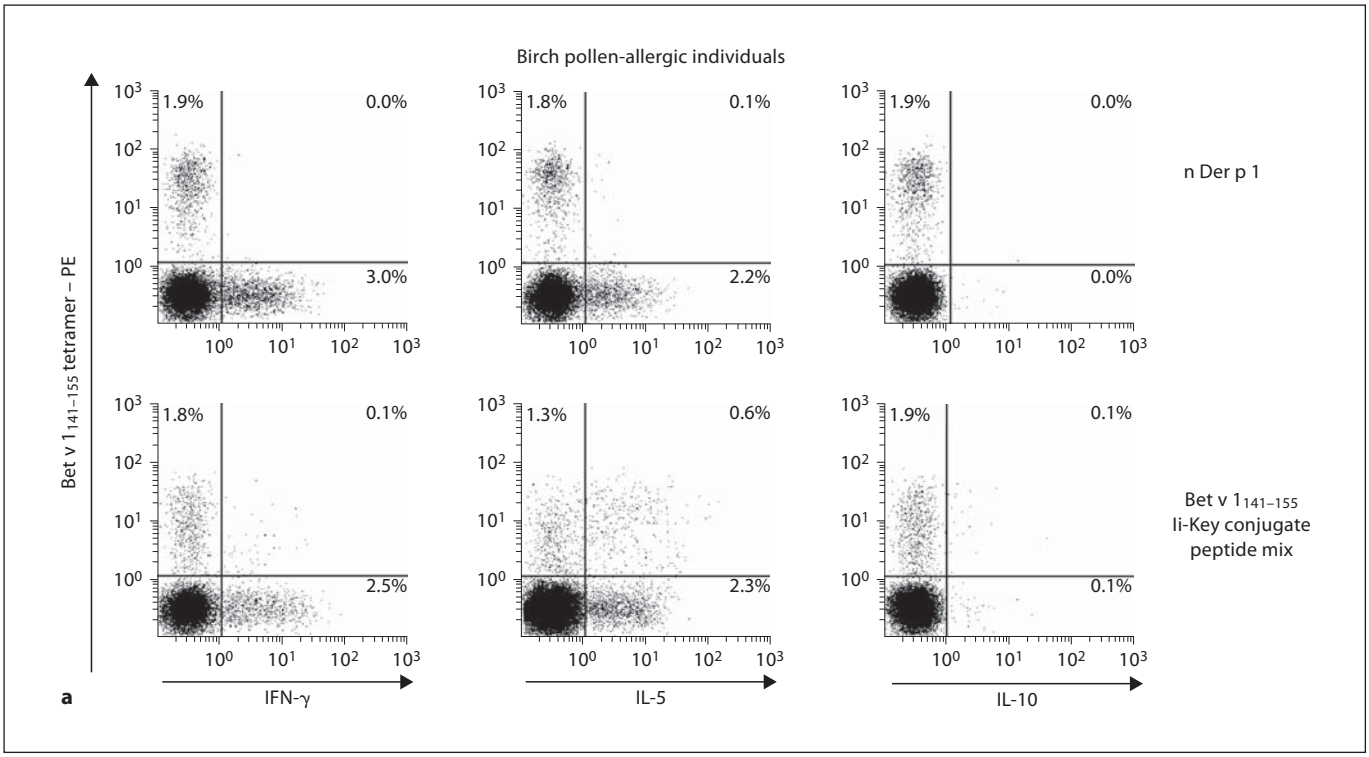
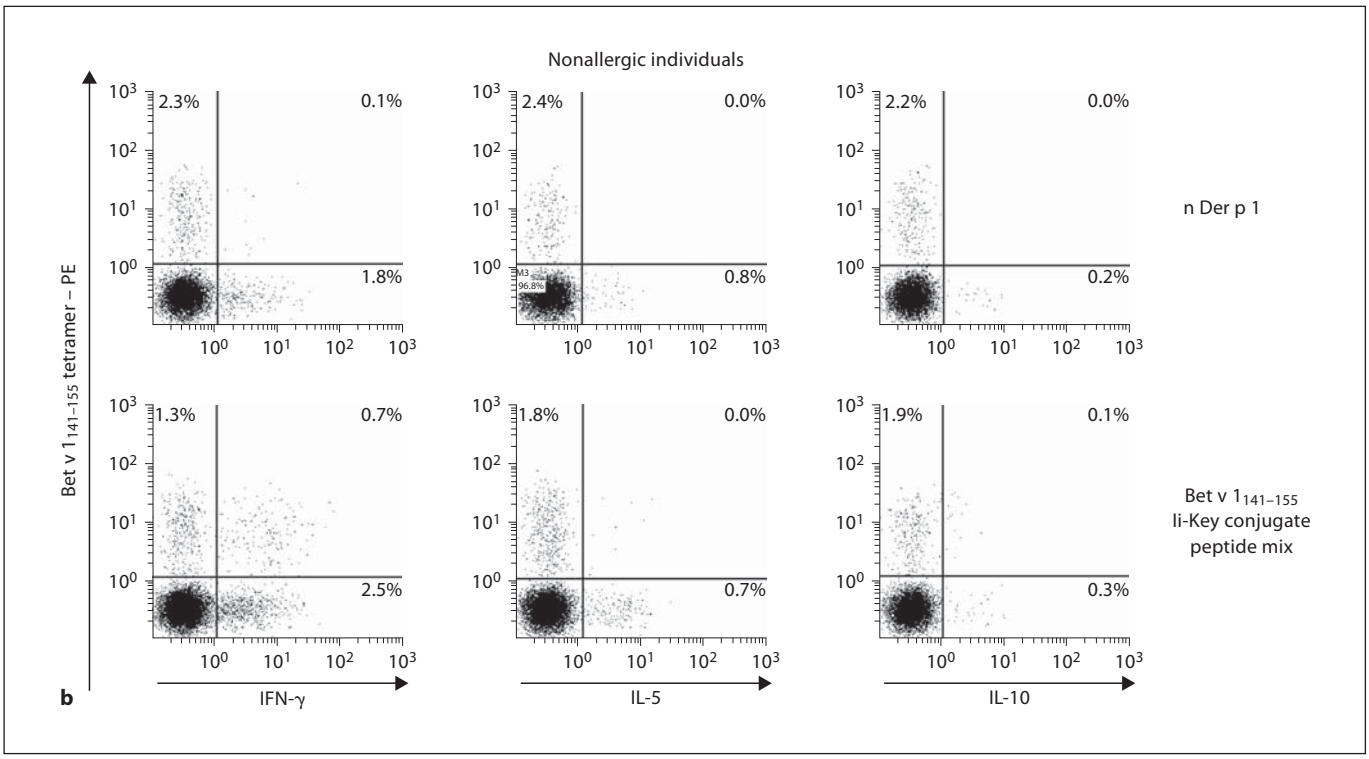


Fig. 7. Single cell analysis of T cell polarization. Bet v 1-specific CD4+ T cells were stained with MHC class II Bet v $1_{141–155}$ tetramers for 30 min at 37°C (1). To induce cytokine secretion before activation, cells were stimulated in vitro for 3 h with irradiated autologous PBMC pulsed with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (2). Cells were subsequently labeled with a bispecific antibody-antibody conjugate directed against both the CD45 molecule and a specific cytokine (3), and then transferred into a 37°C warm medium for 45 min to allow cytokine secretion and capture (4). Samples were stained for 30 min at 4°C with fluorochrome-labeled anti-cell surface markers antibodies and anti-cytokine antibodies specific for either IL-5, IL-10 or IFN-γ (5). Cells were washed and analyzed by flow cytometry. APC = Antigen-presenting cell; TCR = T cell receptor.

Fig. 8. Characterization of Bet v $1_{141–155}$-specific CD4+ T cells in birch pollen-allergic and healthy individuals. **a** PBMCs from an HLA-DRB1*1501 birch pollen-allergic patient were cultured for 2 weeks in presence of Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (10 μg/ml). Cells were fed with 10 IU/ml IL-2 at day 5 and every 3–4 days subsequently. Cells were stained with the MHC class II Bet v $1_{141–155}$ tetramer or the MHC class II CLIP control tetramer for 30 min. Cells were further stimulated for 3 h with irradiated autologous PBMCs pulsed with purified Der p 1 as a control (upper panel) or the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (lower panel). Detection of cells secreting IFN-γ, IL-5 and IL-10 was performed as described in figure 7, using an FITC-labeled anti-IFN-γ and an allophyco-cyanin-labeled anti-IL-5 or anti-IL-10 antibody. **b** Similar experiments were conducted as above using PBMCs from an HLA-DRB1*1501 healthy individual. Results are representative of experiments conducted in 3 birch pollen-allergic patients and 3 nonallergic individuals. In these representative experiments, percentages within Bet v $1_{141–155}$ tetramer-positive CD4+ T lymphocytes of cells secreting either IFN-γ, IL-5 or IL-10 were 3.5, 42.6 and 4.2%, respectively, for birch pollen-allergic patients and 31.9, 8.4 and 6.1%, respectively, for healthy individuals.


Birch pollen-allergic individuals
Bet v 1141–155 tetramer – PE
1.9%
0.0%
3.0%
1.8%
0.1%
2.2%
1.9%
0.0%
0.0%
n Der p 1
1.8%
0.1%
2.5%
1.3%
0.6%
2.3%
1.9%
0.1%
0.1%
Bet v 1141–155 Ii-Key conjugate peptide mix
IFN-γ
IL-5
IL-10
a


Nonallergic individuals
Bet v 1141–155 tetramer – PE
2.3%
0.1%
1.8%
2.4%
0.0%
M3
96.8%
0.8%
2.2%
0.0%
0.2%
n Der p 1
1.3%
0.7%
2.5%
1.8%
0.0%
0.7%
1.9%
0.1%
0.3%
Bet v 1141–155 Ii-Key conjugate peptide mix
IFN-γ
IL-5
IL-10
b

8

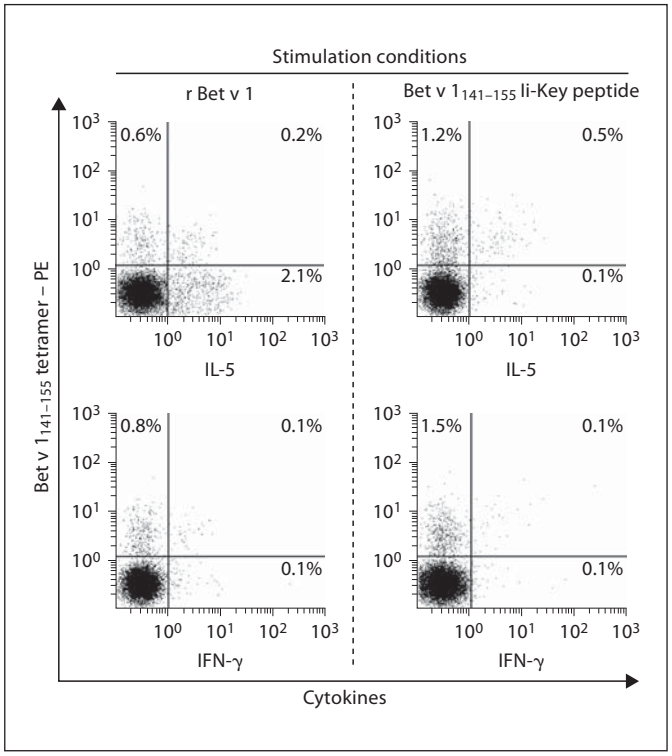


Fig. 9. Comparison of CD4+ T cells expanded with either the recombinant Bet v 1 protein or the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. PBMCs from an HLA-DRB1*0101 birch pollen-allergic individual were stimulated with purified recombinant Bet v 1 protein (50 μg/ml) or Bet v $1_{141–155}$ Ii-Key conjugate peptide mix (10 μg/ml). Cells were fed with 10 IU/ml IL-2 at day 5 and every 3–4 days afterwards. At day 14, cells were stained with the HLA-DRB1*0101 Bet v $1_{141–155}$ tetramer for 30 min and stimulated with irradiated autologous PBMCs pulsed with the cognate antigen for 3 h. The cytokine secretion profile of CD4+ Bet v $1_{141–155}$ tetramer-positive cells was subsequently determined as described in figure 7. In these representative experiments, percentages within Bet v $1_{141–155}$ tetramer-positive CD4+ T lymphocytes of cells secreting IL-5 or IFN-γ were 27.5 and 9.2%, respectively, for the r-Bet v 1 stimulation condition, and 31.7 and 5.2%, respectively, following stimulation with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix. Negative controls with irrelevant peptide restimulation were all negative (data not shown).

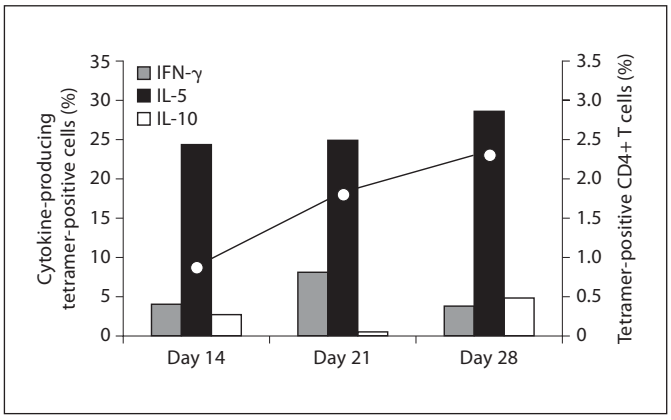


Fig. 10. Patterns of cytokine production among MHC class II Bet v $1_{141–155}$ CD4+ T cells during in vitro expansion. PBMCs from a birch pollen-allergic individual were stimulated with the Bet v $1_{141–155}$ Ii-Key conjugate peptide mix for 28 days. MHC class II Bet v $1_{141–155}$ tetramer staining combined with the cytokine surface capture assay were performed at 3 consecutive time points (days 14, 21 and 28) during the culture. Percentages of cells secreting IFN-γ, IL-5 and IL-10 within the CD4+ tetramer-positive population (closed bars) are indicated in parallel with numbers of MHC class II Bet v $1_{141–155}$ tetramer-positive CD4+ T cells (open circles), referring to the left or right ordinate scale, respectively.

Discussion

The analysis of antigen-specific T cells has been largely relying upon functional assays such as cell proliferation or cytokine production following antigen stimulation. These methods, however, tend to underestimate the number of antigen-reactive T cells, since some of them have a limited capacity to proliferate or produce little amounts of cytokines [44]. In contrast, MHC peptide tetramer staining allows to identify antigen-specific T cells independently of their functional capacity. MHC class I peptide tetramers have been successfully used to investigate CD8+ T cell responses, but studies with MHC class II peptide tetramers are much less frequent, due to the difficulty to develop high-avidity tetramer complexes to detect rare circulating CD4+ T cell progenitors [45, 46].

In this study we demonstrate the value of MHC class II peptide tetramers to assess, at a single cell level, allergen-specific T lymphocytes. Using the birch pollen allergen Bet v 1 as a model, we observed that the Bet v $1_{141–155}$ peptide exhibits a high affinity in the nanomolar range for HLA-DRB1*0101, DRB1*0401 and DRB1*1501 allotypes, which collectively are found in 40% of the Caucasian population. Importantly, the identification of a high-affinity binder epitope for such HLA molecules is critical to produce stable high-avidity tetramers. In most circumstances, the frequency of allergen-specific CD4+ T cells in peripheral blood is below the limit of detection to perform direct staining of blood samples, and, thus, appropriate strategies for in vitro expansion of those cells are required [18–22]. In this study, we stimulated PBMCs using truncated versions of the Bet v $1_{141–155}$ immunodominant epitope chemically coupled to the Ii-Key peptide from the MHC invariant chain. The latter is known

to bind to an allosteric site on the MHC molecule, thereby facilitating peptide exchange [47]. This Bet v $1_{141–155}$ Ii-Key conjugate peptide mix is significantly more potent than either the Bet v $1_{141–155}$ peptide alone or the recombinant Bet v 1 protein to expand Bet v $1_{141–155}$-specific CD4+ T cells, whereas under all those stimulatory conditions, proliferating T cells exhibit similar functional properties (in terms of cytokine production). MHC class II peptide tetramer staining is highly specific, reproducible and sensitive, allowing to detect a single allergen-specific T cell within 5×10^6 CD4+ T cells. Although in vitro stimulation with allergen could lead to the preferential expansion of selected T cell subsets, we noticed in our studies that the cytokine secretion profile of tetramer-positive T cells obtained after either short- or long-term cultures is similar. This suggests that stimulation conditions used in the present study do not skew allergen-specific T cell populations. Allergen-specific CD4+ T cells labeled with MHC class II peptide tetramers can be further characterized functionally, combining a cytokine surface capture assay with tetramer staining. This assay allows to detect cytokine produced after short-term antigen stimulation in CD4+ T cells stained with tetramers, under conditions precluding T cell receptor internalization after tetramer binding. Using this method, we observed that allergen-specific CD4+ T cells from allergic patients produce high levels of IL-5, but limited amounts of IFN-γ after allergen stimulation. In contrast, healthy individuals have circulating Bet v $1_{141–155}$-specific T cells in their blood, which rather secrete IFN-γ, and to a lesser extent IL-10, in response to allergen. Tetramer staining also provides insights into the frequencies in peripheral blood of such allergen-specific CD4+ T cell progenitors, suggesting a 2–3 log expansion during the peak of pollen exposure.

Altogether, we report here methodological conditions to ensure the successful use of MHC class II peptide tetramers to assess allergen-specific T cell responses. The development of both stable tetramers based on a high-affinity T cell epitope presented by common MHC class II molecules as well as optimized in vitro stimulation conditions are critical success factors to ensure the specificity and sensitivity of this technique. MHC class II peptide tetramer staining conjugated with a cytokine surface capture or surface phenotyping can provide a detailed picture of T cell responses at a single cell level. MHC class II peptide tetramer staining will allow to further investigate allergen-specific T cell responses in patients and healthy individuals. It will also be extremely useful to monitor the efficacy of various immunotherapeutic strategies in humans, using an immunological readout, possibly helping to identify surrogate biological markers of clinical efficacy.

Acknowledgments

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