HLA-class II peptide tetramers vs. allergen-induced proliferation for identification of allergen-specific CD4 T cells.

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ABSTRACT

Background: Fluorescence-labeled MHC class II/peptide tetramer complexes are considered as optimal tools to characterize allergen-specific CD4⁺ T cells, but this technique is restricted to frequently expressed HLA-class II molecules and the knowledge of immunodominant epitopes. In contrast, allergen-stimulated proliferation assessed by CFSE-dilution is less sophisticated and widely applicable. The major mugwort allergen, Art v 1, contains only one single, immunodominant, HLA-DR1-restricted epitope (Art v 125-36). Thus, essentially all Art v 1-reactive cells should be identified by a HLA-DRB1*01:01/Art v 119-36 tetramer.

Methods: We compared specificity and sensitivity of tetramer⁺ and allergen-induced proliferating (CFSElo) CD4⁺ T cells by flow cytometry.

Results: The frequency of tetramer⁺CD4⁺ T cells determined ex vivo in PBMC of mugwort-allergic individuals ranged from 0 to 0.029%. After 2-3 weeks of in vitro expansion, sufficient tetramer⁺ T cells for phenotyping were detected in 83% of Art v 125-36-reactive T cell lines (TCL) from mugwort-allergic individuals, but not in TCL from healthy individuals. The tetramers defined bona fide Th2 cells. Notably, Art v 125-36-reactive TCL depleted of tetramer⁺ T cells still reacted to the peptide and only 44% Art v 125-36-specific T cell clones were detected by the tetramer. CFSElo CD4⁺ T cells contained only 0.3-10.7% of tetramer⁺ T cells and very low proportions of Th2 cells.

Conclusion: Allergen-specific T cells can be identified by HLA-class II tetramers with high specificity, but unexpected low sensitivity. In contrast, allergen-stimulated CFSElo CD4⁺ T cells contain extremely high fractions of bystander cells. Therefore, for T cell monitoring either method should be interpreted with caution.
INTRODUCTION

Allergen-specific CD4+ T cells play a major role in the initiation and cure of IgE-mediated allergy. Excessive Th2 cytokine responses (IL-4, IL-5, IL-13) to allergens are the hallmark of allergic responses, whereas the Th1 cytokine IFN-γ and IL-10 from regulatory T cells are associated with tolerance to allergens in non-allergic individuals or patients successfully treated with allergen-specific immunotherapy (AIT)(1,2). As the frequency of allergen-specific CD4+ T cells in peripheral blood is very low, their characterization has been based on T cell clones (TCC) derived from T cell lines (TCL) after in vitro expansion with allergen. Recently, functional reactivity in response to allergen-stimulation indicated by expression of activation markers, cytokine production or proliferation assessed by CFSE-dilution became frequently used to identify specific T cell responses (3-6).

For the characterization of antigen-specific CD8+ T cell responses, detection via peptide/major histocompatibility complex class I (pMHCI) tetramers (7) has become a “gold standard”. More recently available pMHCII tetramers allow direct detection of antigen-specific CD4+ T cells (8) and are currently praised as a tool to characterize and monitor CD4+ T cell responses at a single cell level, e.g. in AIT. However, their application has several limitations. The avidity of pMHCII tetramers and CD4+ T cells is lower than the avidity of pMHCI tetramers and CD8+ T cells (9,10). The knowledge of immunodominant T cell epitopes and MHC-restriction is a prerequisite to apply pMHC technology and a cumbersome pre-selection of patients is required. Thus, studies are usually restricted to the most frequent HLA-class II alleles and comprise low patient numbers.

pMHCII tetramers have been applied to investigate T cells specific for allergens from grass, birch, alder, cat, cow or peanut (11-17). Most allergens contain multiple T cell epitopes and allergic individuals differ markedly in epitope recognition patterns. However, the major allergen of mugwort pollen, Art v 1, contains only one single immunodominant T cell epitope (Art v 125-36) and its recognition is significantly associated with HLA-DR1 (18-20). We used a pMHCII tetramer consisting of HLA-DRB1*01:01 and the immunodominant peptide of Art v 1, which should ideally identify all Art v 1-reactive T cells, to address the specificity and sensitivity of tetramers. We also compared tetramer-staining with allergen-induced proliferation assessed by CFSE-dilution, a more commonly applicable and less costly method to identify and characterize antigen-specific CD4+ T cells.
MATERIALS AND METHODS

Patients

17 mugwort pollen-allergic patients were included who all had recurrent rhinitis/conjunctivitis during late summer, positive skin prick tests and specific IgE to mugwort pollen (ImmunoCAP, Thermo Fischer Scientific, Phadia, Uppsala, Sweden). All were sensitized to Art v 1 as determined by IgE-immunoblots and ELISA (19). Typing of the patients’ HLA-DRB1-alleles was performed by SSO typing kit (BAG Mr.Spot, Lich, Germany), and if necessary by All set SSP DR low resolution (Invitrogen, Bromborough, UK) and high resolution typing nucleotide sequencing (BigDye Terminator Cycle Sequencing Kit, ABI, Foster City, CA). 15 patients expressed at least one HLA-DRB1*01:01 allele and 2 patients at least 1 HLA-DRB1*15:01 allele. Five healthy donors with HLA-DRB1*01:01 alleles and no detectable serum IgE for Art v 1 were included for comparison. The study had been approved by the local ethics committee and all individuals had given informed consent.

Tetramer design

The peptide Art v 19-36 (NKKCDKKCIEWEKAQHGA) was chosen for the production of PE-labeled tetramers with HLA-DRA*01:01 and HLA-DRB1*01:01 (DR1/Art tetramers), because this 18-mer peptide covers more than 90% of T cell reactivity to Art v 1 in our population (19) and induces strong proliferation (21). The DR1/Art tetramer and DR1/CLIP control tetramer were purchased from Beckman Coulter Immunomics (Fullerton, CA).

Allergen-specific T cell cultures and cell sorting

Art v 25-36-specific TCL were established from PBMC of HLA-DR1+, Art v 1-sensitized mugwort-allergic patients by allergen-specific stimulation with recombinant Art v 1 (Biomay, Vienna, Austria; 95% purity; 0.012 EU/µg endotoxin) or Art v 1-peptides containing Art v 125-36 (Thermo, Ulm, Germany) in Ultraculture medium (Lonza, Walkersville, MD). Recombinant human IL-2 (10, 20 and 100 U/ml) was added at days 3 or 5 and cells were further expanded up to 5 weeks by addition of 10 U/ml IL-2 or IL-2 plus irradiated PBMC upon splitting. For 4 of 6 tested PBMC samples 20 U/ml IL-2 added at day 3 was optimal to obtain tetramer+ cells. TCC were obtained by limiting dilution of TCL (19).

For sorting of CD4+ tetramer+ and CD4+ tetramer- T cell populations, 5-10x10⁶ cells of TCL were stained with DR1/Art tetramer for 1h at 37°C and CD4-PE-Cy7 or CD4-PerCP-Cy5.5 for 30 min RT and sorted on a BD FACSARia (BD Biosciences, San Diego, CA). For proliferation assays, TCL or TCC (5x10⁶ cells) were stimulated in duplicates with
irradiated (60 Gy) APC and peptides at an optimum concentration of 3 µM, if not indicated otherwise. After 48 h, 0.5 µCi ³H–thymidine were added for 16 h, and proliferation assessed by β-counting. HLA-restriction of TCC was assessed by stimulation of 5x10⁴ cells with 2.5x10⁵ EBV-transformed B cells expressing defined HLA-DR molecules after pre-incubation for 2 hours with Art v 1 (5 µg/ml) or 18-mer Art v 1-peptide (2 µM) and subsequent irradiation (60 Gy). Proliferation was determined after 48h. Results are shown as stimulation indices (SI = mean cpm of T cell proliferation with stimulant / mean of T cell proliferation without stimulant).

**Cell staining and flow cytometry**

PBMC, TCL and TCC (up to 1x10⁶ cells) were incubated with 2 µl DR1/Art- or the DR1/CLIP control tetramer in 30 µl of HEPES-buffered RPMI1640 containing 10% human serum for 1 h at 37°C and 5% CO₂, and then stained for 30 min at RT with antibodies: CD3-Horizon V500, TCRαβ-FITC (both BD Biosciences), CRTh2-APC, CD27-PeCy7, CD28-APC, CD62L-PeCy7, CD45RO-APC, CXCR3-PerCP, CD14-APC-Cy7, CD19-APC-Cy7 (Biolegend, San Diego, USA), CD4-Brilliant violet 421, CXCR3-PerCP, CD14-APC-Cy7, CD19-APC-Cy7 (Biolegend, San Diego, USA). After washing with PBS, cells were stained with Fixable Viability Dye eFluor® 780 (eBioscience) for 30 min and analyzed by flow cytometry using a FACS Canto II (BD Biosciences) with Diva or FlowJo software. Within the lymphocyte gate, dead cells, B-cells and monocytes were excluded and a gate set on CD3⁺CD4⁺ T cells. For CFSE labeling, 1x10⁷ PBMC were stained in 500 µl PBS containing 0.5 µM CFSE (Molecular probes, Invitrogen, Camarillo, CA) for 15 min at 37°C and washed 3 times with RPMI1640 containing 10% FCS.

**Cytokine detection**

For cytokine detection in tetramer⁺ cells, TCL were stained with tetramer for 1 h at 37 °C, washed and stimulated with PMA (1 µg/ml) and ionomycin (100 µM) in the presence of brefeldin A (10 µg/ml; Sigma) for 5 h. TCL were subsequently labeled for live/dead, CD4, CD14 and CD19, and cytokine expression was determined. For intracellular cytokine staining of IFN-γ and IL-4, cells were fixed, permeabilized and stained with anti-IL-4-APC (BD), anti-IFN-γ-PECy7 (eBioscience). Cytokine capture assays for IL-10-detection was performed as described by the producer (Miltenyi, Bergisch-Gladbach, Germany) after stimulation with Art v 1₂₂₃₅ peptide and 2 µg/ml anti-CD28 for 5 h. Cytokines in TCC supernatants were determined using Luminex system 100 (Luminex, Austin,TX).

**Statistics**

Wilcoxon signed rank test was used to calculate significant differences by GraphPad Prism 5.0 (La Jolla, CA). P-values of <0.05 were considered significant.
RESULTS

The DR1/Art tetramer binds specifically to CD4+ T cells reactive to Art v 125-36

As reported previously, the PE-labeled DR1/Art tetramer containing the peptide Art v 119-36 (NKKCDKKCIEWEKAQHGA) specifically bound to several T cell cultures reactive with Art v 125-36 (KCIEWEKAQHGA) (22). A cloned TRAV17/TRBV18 TCR of a tetramer+ TCC (23) was transduced into Jurkat T cells (clone 41-19) by amphotropic retroviruses in the presence of polybrene as described (23) and 40-60% of these cells expressed the Art v 1-specific TCR and bound the DR1/Art tetramer but not a DR1/CLIP control tetramer (Fig. 1A). In a spike-recovery experiment, 0.1-25% of TCR-transgenic tetramer+ Jurkat cells were added to PBMC from a HLA-DR mismatched donor. The recovery of these cells after tetramer-staining correlated highly with the percentage of spiked cells and suggested a detection limit of about 0.1% (Fig. 1B). Art v 125-36-reactive TCL derived from two mugwort-allergic individuals with DRB1*15:01/*04:04 or DRB1*15:01/*15:02 were tetramer- indicating the exclusive identification of DR1-restricted T cells.

Ex vivo staining of PBMC with DR1/Art tetramer

Cryo-preserved PBMC from 4 different, DR1-positive, mugwort pollen-allergic patients and 2 DR1-positive, non-allergic donors were thawed and stained with DR1/Art or DR1/CLIP control tetramer. DR1/Art tetramer+ cells were not or only rarely detected corresponding to a maximum of 1:3.400 of CD3+CD4+ cells (0.0-0.029%) (Fig. 1C). Therefore, PE-labeled cells were enriched via magnetic anti-PE beads (n=4; 3 different allergic donors). The frequency of detectable tetramer+ T cells increased by about 25-fold to 1:303-1:143 CD3+CD4+ T cells (0.33%-0.70%). However, by this approach a large fraction of cells was lost and only a total number of 12-71 tetramer+CD4+ T cells were obtained from 3x10^7 PBMC of each patient (Fig. 1C). Repeated enrichment of aliquots from the same PBMC sample resulted in reproducible cell numbers, i.e. 38 and 40 tetramer+ cells. In 3x10^7 PBMC from the 2 non-allergic subjects 8 and 11 tetramer+ CD4+ T cells were detected after enrichment (data not shown).

Expansion of DR1/Art tetramer+ cells by in vitro stimulation with allergen

Since the number of tetramer+ T cells was not sufficient for further characterization ex vivo, in vitro enrichment by Art v 1-stimulation and expansion with IL-2 was performed. In time-course experiments of 8 Art v 1-specific TCL maximum percentages of tetramer+ cells were observed after 2 to 3 weeks of in vitro culture (Fig. 2A, B). In 24/29 (83%) TCL established from 12 allergic patients tetramer+ CD4+ T cells were obtained (median: 5.2%; range: 0-36%; Fig. 2C). The DR1/CLIP control staining ranged from 0.03%-0.23%
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Application of the same enrichment protocol for 5 non-allergic subjects did not result in detectable tetramer+ cells or Art v 1-specific proliferation.

Several approaches were tested to further improve our in vitro expansion procedure. First, we induced TCL with 12-mer Art v 1_{25-36} (KCIEWEKAQHGA), 14-mer Art v 1_{23-36} (NKKCIEWEKAQHGA) and 18-mer Art v 1_{19-36} (CDKNKKCIEWEKAQHGA) peptides, respectively. Several approaches were tested to further improve our in vitro expansion procedure. First, we induced TCL with 12-mer Art v 1_{25-36} (KCIEWEKAQHGA), 14-mer Art v 1_{23-36} (NKKCIEWEKAQHGA) and 18-mer Art v 1_{19-36} (CDKNKKCIEWEKAQHGA) peptides, respectively. The 14-mer was the most effective peptide to activate Art v 1-specific TCL and TCC (Fig. 3 A,B). Second, we included a short peptide sequence from the HLA

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**Figure 1:** Tetramer staining of Art v 125-36-specific T cells. A. Jurkat cells expressing an Art v 1-specific TCR. B. Spike-recovery experiment with TCRtg Jurkat cells. C. Ex vivo-enrichment of Art v 125-36-specific T cells. PBMC from mugwort-allergic donors were stained with DR1/Art tetramer-PE and then enriched by PE-labeled magnetic beads. Two representative experiments of 4 are shown. Tet, tetramer; CLIP, control tetramer.
invariant chain (LRMK) at the N-terminus of Art v 125-36, which had been described to facilitate direct loading of HLA-class II molecules (Fig. 3D) (24). Third, we added the dipeptide YR during stimulation with Art v 123-36, which should trigger peptide exchange in HLA class II molecules (Fig. 3E) (25). However, none of these approaches significantly improved the proliferation of established Art v 1-specific TCL and TCL or stimulation of PBMC. Art v 1 and the 14-mer were rather identical in stimulating proliferation of established TCL (n=23: 17.572 dpm (4.500-71.431 dpm) vs. 17.558 dpm (2.869-61.702 dpm) (Fig. 3C), but Art v 1 was slightly better in stimulating PBMC and yielded slightly higher numbers of tetramer+ T cells after expansion (n=6 donors; 0.76% (0-8%) vs. 0.38% (0-16%).

**Figure 2:** Kinetics of tetramer+ CD4+ T cells during expansion in vitro. PBMC from different mugwort-allergic donors were stimulated with rArt v 1 and cells expanded for up to 31 days by adding 20 U/ml IL-2 at day 3 and further cultivation with 10 U/ml and/or irradiated, allogeneic PBMC as feeder cells. A. Time course of one representative patient. B. The horizontal bar indicates median values of DR1/Art tetramer positive cells in TCL (n=8) at different time points of culture. C. Three examples for tetramer-staining of TCL after 3 weeks expansion.
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Figure 3: Optimisation of Art v 1,25-36-specific T cell stimulation. Art v 1-peptides were tested for stimulatory capacity with A. TCL (n=8) and TCC (n=7, 4 different patients). (Wilcoxon signed rank test; * p<0.05; **p< 0.01). C. Art v 1 was compared to 14-mer using TCL (n=23; 8 representative examples are shown). The Li-Key-Art v 1peptide (D.) or co-incubation with YR-dipeptide (E.) were not superior to stimulation with the 14-mer.

Phenotype of DR1/Art tetramer+ CD4+ T cells

The production of IL-4 and IFN-γ by tetramer+ T cells was analyzed by intracellular cytokine staining in 14 TCL from 11 patients after 2-3 weeks of expansion in vitro (Fig. 4A-C). The majority of tetramer+ cells (median: 68.3%; range: 41.1-90.9%) was IL-4+, while only 8.8% (0-83.6%) were IFN-γ+. The number of IL-4+IFN-γ+ tetramer+ cells was also low (8.2%; 0-44.8%). These results were in line with the high expression of CRTh2 (50.9%; 5.4-94.7%) and the low expression of CXCR3 (7.7%; 1.1-69.6%) on tetramer+ T cells. In 4 experiments, we combined Art/DR1 tetramer- and IL-10-staining. The few percent of IL-10+ CD4+ T cells detected were not co-stained with the tetramer.

In addition, various differentiation markers were assessed on tetramer+ CD4+ T cells (Fig. 4B). 98.8% (95.7-100%) expressed CD45RO, 9.1% (0.8-47.3%) CD62L and 1.6% (0-19.4%) CCR7. CD27 was expressed at low levels (3.3%; 0-34.3%) while CD28 was high (92.4%; 54.3-)
Together, in allergic individuals Art v 1-specific T cells identified by the tetramer predominantly were Th2 cells at an “intermediate” stage of differentiation (26).

**Detection of allergen-specific T cells by allergen-induced CFSE-dilution**

As an alternative method to identify Art v 1-specific CD4+ T cells, we assessed CFSE-dilution in short term cultures. Allergen-induced proliferation of PBMC had been proven optimal at day 8 for the analysis of CFSElo proliferating cells (3). In 14 cultures from 6 different patients we obtained a median of 2.8% (range: 0.3%–13.9%) CFSElo CD4+ T cells after stimulation with Art v 1. The median background proliferation was 0.7% (0.1-7.9%). The CFSElo fraction of CD4+ T cells contained significantly (p=0.002) lower percentages of IL-4-producing cells (4.7%; 0-48.3%) and less CRTh2+ cells (2.0%; 0.3-36.8%) compared to the tetramer+ cell fraction observed in IL-2-expanded TCL. In contrast, the number of IFN-γ-positive cells (21.6%; 2.7-48.3%) and the expression of CXCR3 (23.6%; 8.7-88.4%) was slightly higher (Fig. 4C). On the other hand, CFSElo cells expressed higher levels of CD27 (60.2%; 34.6-91.0%) and CD62L (78.3; 62.0-87.7%) than tetramer+ T cells in IL-2-expanded Art v 1-specific TCL.

Most notably, staining of CFSE-labeled short-time cultures with the DR1/Art tetramer revealed that merely 1.5% (0.3-10.7%; n=14) of the CFSElo cell fraction was tetramer+. The DR1/CLIP-tetramer resulted in a median reactivity of 0.02% (0-0.16%). One example is shown in Fig. 4D.

**Peptide-reactivity of DR1/Art tetramer-negative CD4+ T cells**

As only very small subpopulations of antigen-activated, proliferating CFSElo T cells were tetramer+, we also wondered whether Art v 125-36-reactive TCL contained peptide-reactive, tetramer+ T cells. Therefore, tetramer+ CD4+ T cells were removed from 3 Art v 125-36-reactive TCL (from 2 patients) by flow-cytometric cell sorting. Restimulation of the tetramer-CD4+ T cells resulted in a reduced, but still marked proliferation with Art v 1 or peptides in the range of 7%-89% (median: 39%) of the unsorted, total TCL (not shown). These experiments suggested that peptide-reactive T cells exist that were not detected by the tetramer.

**Limited DR1/Art tetramer reactivity with Art v 123-36-specific T cell clones**

To further investigate the discrepancy between peptide-reactivity and tetramer-binding, we investigated 27 DR1-restricted, Art v 123-36-reactive TCC derived from 5 different individuals (Table 1). Interestingly, binding of the DR1/Art tetramer was only detected in 12/27 TCC (44%), although they clearly reacted with Art v 123-36.
in the pMHCII tetramer. Notably, the proliferative response to Art v 1 23-36 did not differ between tetramer-positive and -negative TCC (Table 1, Fig. 5A). Moreover, the lack of tetramer-binding was apparently not due to reduced expression of the TCR on the respective TCC, as for 5 tetramer+ and 2 tetramer - TCC no difference in TCR\textsuperscript{ab}-staining was observed (MFI: 1,257-1,990 and 886 and 1,984 respectively). In addition, neither recent activation of the TCC (27, 28), nor application of the protein kinase inhibitor dasatinib (29), which both had been reported to improve staining with pMHCII tetramer, promoted binding of our DR1/Art-tetramer (data not shown). The tetramer staining varied in its intensity. Fig. 5B shows one tetramer and two tetramer+ TCC derived from

Figure 4: Phenotype comparison of tetramer+ CD4+ T cells in TCL after 3 weeks and CFSElo cells at day 8. PBMC were stimulated with Art v 1, IL-2 was added at day 3 and after 3 weeks of expansion with IL-2 cells stained with Art/DR1 tetramer. (A) Cytokine assessment after stimulation with PMA/ionomycin. One representative experiment of 14 is shown. (B) Staining of cytokines and markers of differentiation (n=14). In parallel, CFSE-stained PBMC were stimulated with Art v 1 and stained at day 8. (C) The Th-phenotype obtained in the two approaches was compared (**Wilcoxon signed rank test; p<0.01). (D) Tetramer-staining of CFSElo CD4+ T cells. One representative experiment out of 14 (patients n=6) is shown.
a single individual. Similarly, the staining intensities varied also in oligoclonal TCL (Fig. 3C) indicating that the affinity of the DR1/Art tetramer varies and might become limiting for certain TCR clonotypes.

**Table 1:** Characterization of Art v 1-specific T cell clones

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*HLA-restriction was tested in proliferation assays using APC homozygous for the respective HLA-DR allele and the 14-mer Art v 1 peptide except the TCC from patient 3 which were tested with the 18-mer Art v 1 peptide and TCC from patient 7 which were tested with Art v 1 (indicated in grey).

**TCC were assigned to Th subsets as follows: Th2: IL-4/IFN- >5, Th1: IFN- /IL-4 >5 and Th0: IFN- /IL-4 0.2–5.**
In order to address the possibility that tetramer-binding depends on differences in TCR epitope recognition, we analyzed critical amino acids involved in the activation of 6 tetramer+ and 5 tetramer TCC. The recognition patterns obtained for both types of TCC by stimulation with Art v 1-peptides containing single alanine-substitutions were not drastically different (Fig. 5C). The number of critical amino acid residues ranged from 3-11 amino acids for tetramer+ TCC and from 3-7 amino acids in tetramer TCC (Fig. 5C), possibly indicating a stronger interaction between TCR and pMHC in tetramer+ TCC. This difference was mostly localized at amino acids 26C and 28E.

**Figure 5:** The DR1/Art tetramer does not detect all Art v 1-peptide specific T cell clones. Of 27 DR1-restricted, Art v 123-36-reactive TCC derived from 5 different patients only 12 (=44%) were detected by the DR1/Art tetramer. (A) Similar proliferation of tetramer+ and tetramer- Art v 1-specific TCC in response to Art v 123-36 (Mann-Whitney; p=0.6782). (B) One tetramer- and 2 tetramer+ TCC from the same patient are shown. (C) Six tetramer+ and 5 tetramer-TCC derived from 5 different patients were mapped for critical amino acids within Art v 125-36 presented by HLA-DR1. T cell reactivity was tested with single mutant peptides. After 2 days proliferation was assessed by ³H-thymidine uptake. (-) denotes <30%, (+) >70% of the original proliferation value.
DISCUSSION

Monitoring of allergen-specific T cell responses during AIT could reveal its impact on T cells and their involvement in treatment efficacy. In this respect, pMHCII tetramers are ideal tools to monitor AIT-induced changes at the T cell level. The uniform T cell response in mugwort pollen allergy, involving one immunodominant T cell epitope restricted by HLA-DR1 (18,19) is a perfect model to compare pMHCII tetramers with the less costly allergen-induced CFSE-dilution for the characterization of allergen-specific CD4+ T cells. Application of the DR1/Art tetramer to determine the frequency of peptide-specific CD4+ T cells ex vivo revealed the expected low frequencies of Art v 1-specific CD4+ T cells (undetectable - 1:3400) in mugwort-allergic subjects. The frequency of tetramer+ CD4+ T cells in peripheral CD4+ T cells had been reported to range from 1:10^3-1:10^6 for pollen-allergens (14,30,31) and from 1:2000 to 1:10^4 for perennial allergens respectively (15,31,32). Similar to previous reports (12,14,30,31,33) we found even less tetramer+ CD4+ T cells in healthy individuals. Thus, the sensitivity of our tetramer matched the sensitivity of other pMHCII-reagents. However, the number of tetramer+ T cells was too low to characterize their phenotype. As in other studies on pollen allergens (13,16,31), after in vitro expansion, the DR1/Art tetramer identified predominantly IL-4+CRTh2+, i.e. a bona fide Th2-cells for allergic subjects (Fig. 4B,C). These data also matched our own data deduced from Art v 1-specific TCC (19) and indicate a high specificity of the tetramer.

Due to the uniform T cell response to Art v 1, with virtually all T cells recognizing the same epitope, we found that the DR1/Art tetramer failed to detect a major fraction of peptide-specific T cells. More than 60% of Art v 1-specific TCC which strongly responded to stimulation with the Art v 1-epitope were not identified by the DR1/Art tetramer (Table 1). Such a high prevalence of tetramer-negative effector T cells specific for autoimmune and viral antigens had previously been solely shown in mice in one report (34). To analyse whether TCR recognition of the epitope differed between tetramer-positive and -negative TCC, the aa critical for activation were analyzed with single alanine-mutant peptides of Art v 125-36. Slightly differential reactivity patterns for tetramer+ and tetramer- TCC (Fig. 5C) indicated subtle differences in TCR recognition possibly resulting in higher avidity for the tetramer. In fact, tetramer-staining in oligoclonal TCL (Fig. 3C) and different TCC (Fig. 5B) ranged from low to medium fluorescence intensities, suggesting varying avidities of this pMHCII tetramer for different TCR clonotypes. In summary, the DR1/Art tetramer shows high specificity, but a low sensitivity.

Notably, the specificity of the CSFE-dilution method proved to be strikingly poor. Only 1.5% (0.3-10.7%) of CFSEL0 cells were also tetramer+ positive. Even if one considers that the tetramer might not recognize 60% of peptide-reactive T cells, the fraction of estimated Art v 1-specific T cells is small within the CFSEL0 cell population. In addition,
only a minority of CFSElo cells were Th2 cells, i.e. produced IL-4 (4.7% vs. 68.3% in tetramer+ cells) or expressed CRTh2 (Fig. 4C), indicating a very high level of bystander activation. These apparently less polarized cells (as they were also only moderate IFN-γ producers) might represent freshly activated auto- or xeno-reactive T cells similar to the background of proliferating cells in the control cultures, which then additionally get expanded in allergen-stimulated cultures by cytokines released from allergen-reactive CD4+ T cells.

In this study, for the first time we compared allergen-induced proliferation and pMHCII tetramers directly for characterization of allergen-specific T cells. The application of an allergy model, in which a single tetramer should identify nearly all allergen-specific CD4+ T cells, revealed unexpected limitations in the use of tetramers because they failed to detect a relatively large fraction of peptide-reactive CD4+ T cells. If applied in monitoring of specific T cells, this deficit will result in the underestimation of their frequency and in a loss of T cell populations which may differ in their phenotype from the detected population. In contrast, the less sophisticated CFSE-dilution method showed low specificity and characterization of CFSElo T cells did not mirror the phenotype of allergen-specific T cells, but was strongly biased by an enormous fraction of bystander T cells. The shortcomings of each method to identify allergen-specific CD4+ T cells especially in evaluating their involvement in efficacy of AIT need to be taken into consideration in future AIT studies.
REFERENCES


