

Characterization of HLA Class II/Peptide-TCR Interactions of the Immunodominant T Cell Epitope in Art v 1, the Major Mugwort Pollen Allergen¹

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More than 95% of mugwort pollen-allergic individuals are sensitized to Art v 1, the major allergen in mugwort pollen. Interestingly, the CD4 T cell response to Art v 1 involves only one single immunodominant peptide, Art v 1_{25–36} (KCIEWEKAQHGA), and is highly associated with the expression of HLA-DR1. Therefore, we investigated the molecular basis of this unusual immunodominance among allergens. Using artificial APC expressing exclusively HLA-DRB1*0101 and HLA-DRA*0101, we formally showed that DR1 acts as restriction element for Art v 1_{25–36}-specific T cell responses. Further assessment of binding of Art v 1_{25–36} to artificial HLA-DR molecules revealed that its affinity was high for HLA-DR1. Amino acid I27 was identified as anchor residue interacting with DR molecules in pocket P1. Additionally, Art v 1_{25–36} bound with high affinity to HLA-DRB1*0301 and *0401, moderately to HLA-DRB1*1301 and HLA-DRB5*0101, and weakly to HLA-DRB1*1101 and *1501. T cell activation was also inducible by Art v 1_{25–36}-loaded, APC-expressing HLA molecules other than DR1, indicating degeneracy of peptide binding and promiscuity of TCR recognition. Specific binding of HLA-DRB1*0101 tetramers containing Art v 1_{19–36} allowed the identification of Art v 1_{25–36}-specific T cells by flow cytometry. In summary, the immunodominance of Art v 1_{25–36} relies on its affinity to DR1, but is not dictated by it. Future investigations at the molecular HLA/peptide/TCR and cellular level using mugwort pollen allergy as a disease model may allow new insights into tolerance and pathomechanisms operative in type I allergy, which may instigate new, T cell-directed strategies in specific immunotherapy. *The Journal of Immunology*, 2008, 181: 3636–3642.

Mugwort (*Artemisia vulgaris*) is a common weed widely spread in temperate and humid zones of the Northern hemisphere and along the Mediterranean basin (1). In Europe, mugwort is the major cause of pollen allergy in late summer, and, with a prevalence of 15–20% of pollen allergies, it is the third most common cause of sensitization to pollen. Type I allergy to mugwort pollen is associated with allergic symptoms induced by ragweed pollen and certain food allergens due to IgE cross-reactivity (2, 3). Mugwort pollen contains several minor allergens in the range of 9 to >60 kDa (4), but only one major allergen, Art v 1, which is recognized by >95% of mugwort-allergic patients (5).

This 28-kDa glycoprotein has an unusual modular structure, being composed of a cysteine-rich globular (defensin-like) domain and a hydroxy-proline-rich tail containing uncommon carbohydrate structures (5, 6).

CD4⁺ T cells are key players in the pathogenesis and maintenance of type I allergy. IL-4 and IL-13 produced by Th2 cells is directly responsible for the Ig isotype switch to IgE in B cells. A balance of Th1, Th2, and regulatory CD4⁺ T cell subsets are involved in the homeostasis of a nonpathological immune response to allergens and in successful specific immunotherapy (7, 8). Mugwort-allergic patients show a typical Th2-dominated T cell response to Art v 1 (9, 10). However, in striking contrast to other allergens that contain multiple T cell epitopes (11–13), Art v 1 harbors only one single immunodominant epitope, Art v 1_{25–36} (KCIEWEKAQHGA), located in the defensin domain and recognized by 85% of Art v 1-reactive mugwort pollen-allergic patients. The minimal epitope in Art v 1_{25–36} consists of 5–10 aa, and 3–5 aa within this core region have been shown to be relevant for T cell proliferation depending on the clonotypic TCR (14). The presentation of Art v 1_{25–36} has been shown to be HLA-DR-restricted (10, 14). Strikingly, recognition of Art v 1_{25–36} is highly associated with the HLA-DR1 phenotype ($p < 10^{-6}$), which is expressed by ~70% of the patients. Only few and by far less strong associations of HLA class II Ags with responses to major allergens have been reported (15–18). Due to its uniform T cell response, mugwort pollen allergy appears to be an ideal model for the investigation of HLA/peptide-TCR interactions, especially with respect to peptide immunotherapy. The concept of using natural or altered peptide ligands as vaccines has been revived recently as a promising therapeutic approach in allergy and other T cell-mediated diseases (19, 20).

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So far, the almost exclusive T cell recognition of Art v 1_{25–36} could neither be attributed to an exclusive presentation of this epitope after Ag processing nor to a restricted TCR repertoire (10, 14). Therefore, a preferential binding of Art v 1_{25–36} to HLA-DR1 might be crucial for the particular immunodominance of this peptide. In the present study, we defined the fine specificity of the interaction of Art v 1_{25–36} with HLA-DR1 and other frequently expressed HLA-DR molecules. To elucidate HLA-binding sites within Art v 1_{25–36}, binding assays with single amino acid mutant peptides were performed. In functional assays, the HLA restriction of Art v 1_{25–36}-specific T cells was investigated using APC with diverse HLA types. Additionally, HLA class II/peptide tetramers containing the immunodominant peptide were tested for applicability to identify Art v 1_{25–36}-specific T cells.

Materials and Methods

Allergens and peptides

Natural (n)³ Art v 1 was purified from mugwort extract by cation exchange and size exclusion chromatography and characterized as previously described (5). Recombinant (r)Art v 1 was obtained from Biomay. For T cell epitope mapping, synthetic overlapping 12-mer peptides spanning the Art v 1 sequence were obtained from Biotrend. Art v 1 peptides for HLA-binding studies were purchased from Thermo Electronics and were 87–97% pure. Biotinylated peptides were synthesized using standard Fmoc chemistry on an Apex synthesizer (Advanced ChemTech Europe) and were purified by RP-HPLC on a C₁₈ Vydac column (Interchim).

Allergen-specific T cells

Allergen-specific T cells were derived from mugwort pollen-allergic patients with typical clinical history, that is, recurrent rhinitis/conjunctivitis during late summer and positive skin prick tests to mugwort pollen extract (ALK; wheal diameter >5 mm). The CAP-FEIA test for mugwort pollen (w6, Pharmacia Diagnostics) was ≥3, and all individuals were sensitized to Art v 1. The study was approved by the ethics committee of the Medical University of Vienna (EK No. 497/2005).

T cell lines (TCL) and T cell clones (TCC) were established as described previously from PBMC of mugwort-allergic individuals using purified nArt v 1 or rArt v 1 as initial stimulus (10, 14). TCL or TCC (2 × 10⁴) were stimulated with allergen (10 μg/ml) or peptides (5 μg/ml, unless noted otherwise) presented by autologous PBMC or HLA-defined EBV-transformed B cell lines or HEK293 cells (human embryonic kidney epithelial cells) transfected with Ag-presenting molecules. T cell proliferation was measured by [³H]thymidine uptake as described (10).

HLA class II typing

Typing of HLA-DRB1 alleles was performed using a commercial sequence-specific oligonucleotide (SSO) typing kit (Dynal Reli SSO HLA-DRB typing kit, Dynal Biotech). Samples with only a single detectable DRB1 allele were typed in addition by sequence-specific primer (SSP; Dynal AllSet SSP HLA-DR low resolution, Dynal Biotech). High-resolution typing was performed by nucleotide sequencing (BigDye terminator cycle sequencing kit, Applied Biosystems).

Transfection of HEK293 cells

Transfection of HEK293 cells with expression plasmids was performed as described elsewhere (21). Briefly, 3 × 10⁶ HEK293 cells were transfected with plasmid DNA (30 μg) by Ca₃PO₄ transfection (22). Where indicated, 2.5 μg of the following pEAK12 expression plasmids was transfected: HLA-DRA*0101, HLA-DRB1*0101, CD54, and CD80. Additionally, 2.5 μg of the invariant chain (Ii) constructs harboring Ii::Art v 1_{25–34} or alternatively Ii::CLIP in pcDNA1.1amp were cotransfected. The construction of Ii fusion proteins has been described elsewhere (23). DNA amounts were brought to 30 μg with empty pEAK12 vector. Eighteen hours after transfection, medium was changed, and cells were harvested 48 h later and washed once in medium.

Peptide-binding assays specific for HLA-DR molecules

HLA-DR molecules were purified from homozygous EBV B cell lines by affinity chromatography using mAb L243, and peptide binding to HLA-DR

molecules was assessed by competitive ELISA as previously reported (24, 25). Peptide concentrations that prevented binding of 50% of the labeled peptide (IC₅₀) were evaluated. Unlabelled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their amino acid sequences were: HA_{306–318} (PKYVKQNTLKLAT) for DRB1*0101, DRB1*0401, DRB1*1101, and DRB5*0101, YKL (AAYAAAKAAALAA) for DRB1*0701, A3_{152–166} (EAEQLRAYLDGTGVE) for DRB1*1501, MT_{2–16} (AKT IAYDEEARRGLE) for DRB1*0301, B1_{21–36} (TERVRLVTRHIYNREE) for DRB1*1301, LOL_{191–210} (ESWGAVWRIDTPDKLTGPFT) for DRB3*0101, and E₂/E₁₆₈ (AGDLLAIETDKATI) for DRB4*0101.

HLA class II/peptide tetramer staining

Custom-made HLA class II/peptide tetramers (class II iTag MHC tetramers) containing HLA-DRB1*0101, DRA*0101, and Art v 1_{19–36} (NKKCKKKCIEWEKAQHGA) were obtained from Beckman Coulter/Immunomics. A control HLA class II tetramer containing the same HLA-DR1 molecules and the immunodominant peptide from the major birch pollen allergen, Bet v 1_{141–156} (ETLLRAVESYLLAHS) (13), was kindly provided by Philip Moingeon (Stallergenes, Antony, France; Ref. 26). Optimum conditions for tetramer staining were established in pilot experiments. Briefly, 1 × 10⁵ T cells from Art v 1_{25–36}-specific TCL or TCC were incubated with 0.6, 1.2, 2.5, 5, or 10 μg PE-labeled tetramers in 50 μl HEPES-buffered RPMI 1640 plus 10% human AB serum for 2 h at room temperature or 30 min at 37°C in the dark. During the last 20 min, cells were additionally stained with anti-CD4-FITC and anti-CD3-PerCP or isotype control Abs (BD Biosciences). Cells were then washed with PBS and fixed with 0.5% formaldehyde. Fluorescence analyses were performed with FACSCanto using DiVa software (BD Biosciences). The optimum staining (i.e., incubation of cells with 5 μg tetramers in 50 μl medium for 2 h at room temperature) was used for all experiments shown in this study.

HLA-DR crosspresentation experiments

Molecules involved in HLA restriction were revealed by proliferation assays performed in the presence of blocking anti-HLA-DP, -DQ, or -DR Abs as described before (10, 14). To study the capacity of different HLA-DR molecules to present Art v 1_{25–36}, peptide-specific TCC were stimulated with different, irradiated EBV-transformed B cells expressing defined HLA-DR-molecules (as listed in Fig. 2). Experiments were performed in duplicates, using 2.5 × 10⁴ irradiated autologous or allogeneic APC and 5 μg/ml (~3 μM; unless stated otherwise) Art v 1_{25–36} peptide or medium alone as control. In some experiments, nArt v 1 or rArt v 1 (10 μg/ml) was also used as stimuli. A stimulation index (SI = ratio between mean of T cell proliferation with peptide and mean of T cell proliferation without peptide) of ≥3 was considered positive.

Results

Art v 1_{25–36} is presented by HLA-DR1

In a previous study, using EBV-transformed B cells as APC, we provided strong evidence that Art v 1_{25–36} is presented by HLA-DR1 (14). To unequivocally confirm HLA-DR1 restriction, artificial APCs were established. HLA class II-negative HEK293 cells (23) were transfected with the costimulatory molecules CD54 and CD80 together with HLA-DRB1*0101, DRA*0101, and Ii::Art v 1_{25–34}. Ii::CLIP served as control peptide. These artificially created APC-presenting Art v 1_{25–34} in an exclusive HLA-DR1 context induced proliferative responses in nine Art v 1_{25–34}-specific TCC derived from four different patients with comparable efficiency as autologous EBV-transformed B cells. One representative experiment is depicted in Fig. 1. Control cultures containing T cells and peptide alone showed ~85% lower proliferative responses.

Binding of Art v 1_{25–36} to HLA-DR1

The binding capacity of Art v 1_{25–36} to the HLA-DR1 molecule was assessed in competition studies by using HA_{306–318} as competitor, a peptide known to bind to DR1 molecules with high affinity (IC₅₀ 2 nM; Table I). Art v 1_{25–36} potently inhibited HA_{306–318} binding to DR1, that is, only ~10-fold less than the reference peptide HA_{306–318} itself (Table II). Additionally, Art v 1 peptides extruding more to the N terminus such as Art v 1_{19–30} and Art v 1_{22–33}, which were recognized by T cells of some patients (10), were

³ Abbreviations used in this paper: n, natural; Ii, invariant chain; r, recombinant; SI, stimulation index; TCC, T cell clone; TCL, T cell line.

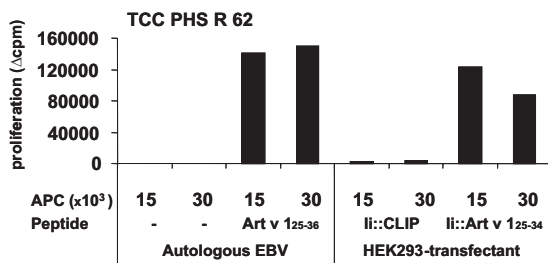


FIGURE 1. Presentation of Art v I_{25-36} by HLA-DR1. Art v I_{25-36} -specific T cell clones were stimulated with autologous EBV-transformed B cells (HLA-DRB1*0101/HLA-DRB1*1601) plus Art v I_{25-26} peptide, respectively, and medium alone as negative control, or with HEK293 cells transfected with costimulatory molecules (CD54 and CD80), HLA-DRB1*0101, and HLA-DRA*0101, Ii::Art v I_{25-34} , respectively, and Ii::CLIP as negative control. One representative TCC of nine reactive TCC derived from four different individuals is shown.

tested. Art v I_{22-33} showed weak binding and Art v I_{19-30} did not show any detectable binding to DR1 (Table II).

To allocate the amino acid residues crucial for anchoring the peptide within the groove of the HLA molecule, peptides with single alanine/glycine substitutions at each amino acid position of Art v I_{25-36} were used to compete with HA₃₀₆₋₃₁₈. A marked loss of binding was obtained with the alanine mutant replacing isoleucine at position 27 (I_{27}), while substitution of other positions had minor effects. Weak binding losses were induced by the glycine substitutions of original alanine residues (A_{32} , A_{36}) and might result from enhancement of the peptide flexibility. A slight effect was also provoked by substituting tryptophan at position 29 (W_{29}). As binding to HLA-DR1 is mainly governed by the anchorage of aromatic or aliphatic residues in the P1 pocket, I_{27} is located in P1, where it mainly contributes to the binding to DR1. E_{30} is therefore located in P4, A_{32} in P6, Q_{33} in P7, and G_{35} in P9 (Table II).

Binding of Art v I_{25-36} to HLA-DR molecules other than HLA-DR1

Previously we found that ~30% of mugwort pollen-allergic individuals who recognize the T cell epitope Art v I_{25-36} do not express HLA-DR1 (14). These HLA-DR1-negative patients expressed one or two of the following phenotypes: HLA-DR7, DR15, DR16, DR4, DR3, DR11, DR13, and the associated, less polymorphic products of the second HLA-DR β -chain (HLA-DR52, HLA-DR53, or HLA-DR51) as possible restriction elements. The respective phenotype frequencies are noted in Table I.

Table II. Binding of Art v I_{19-30} , Art v I_{22-33} , Art v I_{25-36} , and monosubstituted peptides of Art v I_{25-36} to HLA-DRB1*0101^a

Peptide	Amino Acid Sequence	Binding Loss (IC ₅₀ in nM)	Relative Binding Loss Compared to Art v I_{25-36}
HA ₃₀₆₋₃₁₈	PKYVKQNTLKLAT	1.6	
Art v I_{19-30}	NKKCDKKKCIWE	>10,000	>476.00
Art v I_{22-33}	CDKKKCIWEKAQ	3,158	150.00
Art v I_{25-36}	KCIWEKAQHGA	21	1.00
Ala ¹	ACIWEKAQHGA	17	0.81
Ala ²	KAIWEKAQHGA	29	1.38
Ala ³	KCAWEKAQHGA	4,718	225.00
Ala ⁴	KCIAWEKAQHGA	10	0.48
Ala ⁵	KCIEAEKAQHGA	126	6.00
Ala ⁶	KCIEWAKAQHGA	11	0.52
Ala ⁷	KCIEWEAAQHGA	31	1.48
Gly ⁸	KCIEWEKGQHGA	114	5.42
Ala ⁹	KCIEWEKAAGHGA	20	0.95
Ala ¹⁰	KCIEWEKAQAGA	14	0.67
Ala ¹¹	KCIEWEKAQHAA	8	0.38
Gly ¹²	KCIEWEKAQHGG	134	6.38
Suggested anchor positions	KCIEWEKAQHGA P1P4 P6P7 P9		

^a Relative binding affinity of peptides to HLA-DR1 molecules was assessed by competitive inhibition of the high-affinity interaction of biotinylated HA₃₀₆₋₃₁₈ with HLA-DR1 as described in *Materials and Methods*. P1–9 indicate pockets in the peptide binding groove of HLA-DR1 molecules.

This observation suggested a certain degeneracy of peptide binding, that is, additional binding to other HLA-DR molecules. To define additional restriction elements for Art v I_{25-36} , frequently expressed HLA-DR molecules other than DR1 (Table I) were tested in inhibition studies. As shown in Table III, Art v I_{25-36} also bound with high affinity to HLA-DR3 and HLA-DR4, moderately to HLA-DR13 and HLA-DR51, and weakly to HLA-DR11 and HLA-DR15. No binding to HLA-DR7, HLA-DR52, or HLA-DR53 was observed. The neighboring peptides Art v I_{22-33} and Art v I_{19-30} did not bind to any of these additional HLA-DR molecules tested (Table III).

Art v I_{25-36} presentation by HLA-DR molecules on APC

Inhibition experiments using anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP Abs and 10 TCL and 5 TCC derived from 10 different patients confirmed that naturally processed peptides derived from the entire recombinant or natural allergen were also exclusively presented by HLA-DR molecules on autologous APC (data not shown), as had been previously observed for the synthetic Art v 1 peptide (10, 14).

Since synthetic Art v I_{25-36} exhibited promiscuity in binding to different isolated HLA class II molecules, the T cell stimulatory

Table I. HLA-DR phenotype, HLA-DR molecules, and biotinylated peptides used for competitive binding assays

Specificities	Phenotype ^a (%) in Art v 1-Reactive Allergic Individuals	Phenotype ^b (%) in Healthy Individuals	DRB Molecules Used in Binding Assay	Biotinylated Peptides ^c	IC ₅₀ (nM)
DR1	68.6	20.6	DRB1*0101	HA ₃₀₆₋₃₁₈	2
DR3	15.7	20.6	DRB1*0301	MT ₂₋₁₆	365
DR4	9.8	20.3	DRB1*0401	HA ₃₀₆₋₃₁₈	38
DR7	15.7	23.7	DRB1*0701	YKL	6
DR11	17.6	24.7	DRB1*1101	HA ₃₀₆₋₃₁₈	5
DR13	5.1	26.2	DRB1*1301	B1 ₂₁₋₃₆	322
DR15	21.5	24.8	DRB1*1501	A3 ₁₅₂₋₁₆₆	26
DR52	49.0	68.0	DRB3*0101	LOL ₁₉₁₋₂₁₀	15
DR53	25.0	44.0	DRB4*0101	E ₂ /E ₁₆₈	10
DR51	33.0	29.0	DRB5*0101	HA ₃₀₆₋₃₁₈	6

^a HLA-DR phenotype of the local Austrian population ($n = 355$) representing also other Caucasian populations (41).

^b HLA-DR phenotype of Austrian mugwort pollen-allergic individuals (13).

^c For amino acid sequences, see *Materials and Methods*.

Table III. Binding of Art v 1 peptides to frequently expressed HLA-DR alleles

HLA Specificity (Allele)	Peptides		
	Art v I ₁₉₋₃₀	Art v I ₂₂₋₃₃	Art v I ₂₅₋₃₆
DR1 (DRB1*0101)	>13,813 ^a	2,025	12
DR3 (DRB1*0301)	>27	>27	14
DR4 (DRB1*0401)	>261	>261	6
DR7 (DRB1*0701)	>1,547	>1,547	>1,547
DR11 (DRB1*1101)	>1,833	396	249
DR13 (DRB1*1301)	>31	>31	20
DR15 (DRB1*1501)	>377	>377	137
DR52 (DRB3*0101)	>656	>656	>656
DR53 (DRB4*0101)	>1,032	>1,032	>1,032
DR51 (DRB5*0101)	>1,805	1,414	98

^aRelative activity. Binding of Art v 1 peptides was assessed by inhibition of HLA-DR binding of the appropriate reference peptides (Table I). IC₅₀ values were converted into relative activities (ratio between IC₅₀ value and IC₅₀ of the respective reference peptide) to take into account the differences of sensitivity of the binding assays.

capacity of native HLA class II molecules on APC with different DR types was investigated in 15 TCC. In three of these experiments, the response of Art v I₂₅₋₃₆-specific T cells to naturally processed peptides and synthetic peptides was compared. Representative TCC are shown in Fig. 2. TCC PHS R9 (Fig. 2A) reacted to Art v 1 peptides presented by autologous APC expressing DR1 and DR16, to APC expressing exclusively DR1, but not to APC homozygous for DR16. These data indicate a distinct restriction by DR1 molecules; DR51 could be excluded as restriction element by crosspresentation. In addition to HLA-DR1, heterologous HLA-DR4-positive APC were also able to present the peptide. TCC PHS R62 (Fig. 2B) recognized the cognate peptides in an even more degenerate fashion on autologous HLA-DR1 and DR16, as well as presented by heterologous HLA-DR4, HLA-DR7, and HLA-DR8, but not by HLA-DR53 or HLA-DR51. A comparable presentation of synthetic and naturally processed peptides derived from rArt v 1 or nArt v 1 by the same HLA class II molecules was observed with these two TCC and with four other TCC derived from the same patient.

Two TCC from individuals lacking HLA-DR1 are shown in Fig. 2, C and D. One TCC from a donor expressing HLA-DR15, HLA-DR7, HLA-DR53, and HLA-DR51 reacted to Art v I₂₅₋₃₆ presented by HLA-DR15 (Fig. 2C). For another TCC derived from a donor expressing HLA-DR3, HLA-DR16, HLA-DR52, and HLA-DR51, we identified HLA-DR16 as restricting molecule (Fig. 2D).

Regarding heterologous HLA molecules capable of presenting Art v I₂₅₋₃₆ to TCC from HLA-DR1-positive donors, HLA-DR4 was the second most frequently used HLA class II molecule (10 of 13 (77%) TCC and in 4 of 5 (80%) TCL); additionally, DR7 (3 of 9 (33%) TCC) and DR8 (8 of 13 (61%) TCC) also acted as Ag-presenting molecules. Neither HLA-DR52, HLA-DR53, nor HLA-DR51 were relevant for presentation of Art v I₂₅₋₃₆ in most experiments (data not shown).

Although the HLA-DR4-expressing EBV B cells induced similar or even better T cell proliferations to superantigens (data not shown), HLA-DR1-expressing EBV B cells were more effective in Art v I₂₅₋₃₆ presentation at limiting concentrations in three of three different TCC. One representative experiment is shown in Fig. 3. For one TCC (PHS R9) the minimal T cell epitope was assessed by truncated peptide mapping for DR1- and DR4-expressing APC respectively (not shown). The minimal epitope for DR4⁺ APC was 1 aa longer (IEWEKAQ) than that determined for DR1⁺ APC (EWEKAQ).

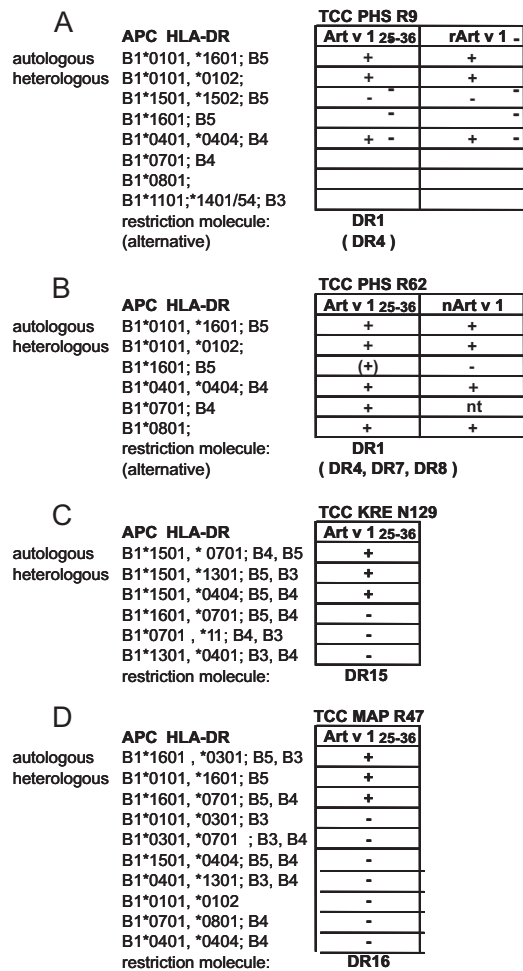


FIGURE 2. Cross-stimulation experiments using EBV-transformed B cells with defined expression of HLA-DR molecules. T cells (4×10^4) were stimulated with 2.5×10^5 irradiated APC and nArt v 1 or rArt v 1 and Art v I₂₅₋₃₆ ($5 \mu\text{g/ml}$). Proliferation was measured by [³H]thymidine uptake. SI of TCC stimulated with autologous APC ranged from 5.6 to 56.2, with background counts without peptide varying from 663 to 3068 cpm. + indicates proliferation of TCC (SI ≥ 3), and - indicates no proliferation of TCC (SI < 3). The TCC used expressed the following TCRV α /V β families: PHS R9 (A), V α 9, V β 5.2; PHS R62 (B), V α 3, V β 18; KRE N129 (C), V α nd, V β 20; MAP R47 (D), V α nd, V β 6; nd indicates not determined.

Cytokine production measured in parallel was quantitatively congruent to proliferation for this Th2 TCC and also did not change qualitatively (data not shown).

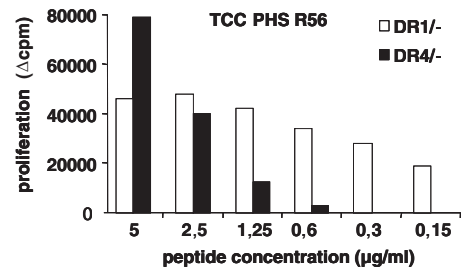
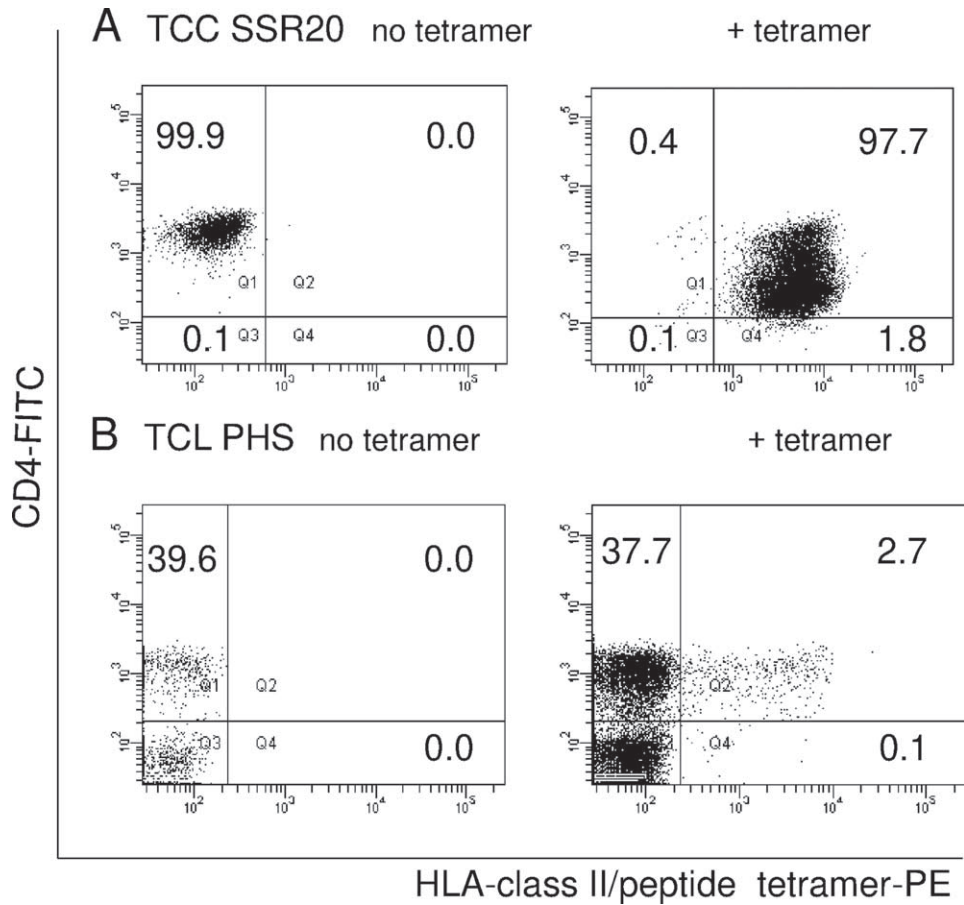


FIGURE 3. Comparison of HLA-DR1 and HLA-DR4 as Ag-presenting molecules for Art v I₂₅₋₃₆. EBV cell lines expressing HLA-DRB1*01 or HLA-DRB1*04 were used as APC. One representative TCC of five TCC tested is shown.

FIGURE 4. Identification of Art v 1_{25–36}-specific T cells using HLA class II/peptide tetramers. Custom-made PE-labeled tetramers containing HLA-DRA*0101, HLA-DRB1*0101, and the peptide Art v 1_{19–36} were used to demonstrate allergen-specific T cells by flow cytometry. *A*, An Art v 1_{25–36}-specific TCC and (*B*) a representative TCL (*n* = 8) are shown.



Detection of Art v 1_{25–36}-specific T cells by HLA-DR1/peptide tetramers

Both, the immunodominance of one single T cell epitope in Art v 1 and the highly significant association of this response with HLA-DR1 expression make mugwort pollen allergy an ideal disease model for the application of MHC class II/peptide tetramers. Fluorescence-labeled tetramers consisting of HLA-DRA*0101, DRB1*0101, and Art v 1_{19–36} recognized the TCR of an Art v 1_{25–36}-specific TCC (Fig. 4A). These tetramers were used to identify Art v 1_{25–36}-specific CD4⁺ T cells in various short-term TCL (*n* = 8; six different patients) by flow cytometry. In these TCL, 0.5–15.9% (median of 6.0%) of CD4⁺tetramer⁺ cells were detected. Two representative examples are shown in Figs. 4B and 5A. In two Art v 1-reactive TCL, control tetramers containing the same DR molecules, but the unrelated peptide Bet v 1_{141–155}, were incubated in parallel with Art v 1_{19–36}-containing tetramers. A population positive with the original tetramer, but not with the control

tetramer, was clearly discernable (Fig. 5). Otherwise, no staining was observed in two control TCL obtained from different HLA-DRB1*01-expressing individuals and reactive with Amb a 1, the major ragweed pollen allergen, but not with Art v 1 (Fig. 5B). Furthermore, the tetramer generally did not bind to CD3⁺CD4[−] T cells (Figs. 4B and 5B).

Discussion

Using allergen-specific T cell lines and clones, we have defined Art v 1_{25–36} as the single immunodominant T cell epitope in Art v 1, the major allergen in mugwort pollen, and found its recognition highly associated with HLA-DR1 expression (10, 14). Interestingly, in silico analysis for potential T cell epitopes using algorithms predicting HLA class II/peptide binding such as “syfpeithy” (27) or “tepitope” (28, 29) revealed no peptide containing Art v 1_{25–36} with top scores for binding to HLA-DRB1*0101 or other common HLA haplotypes. In contrast, even a negative scoring was

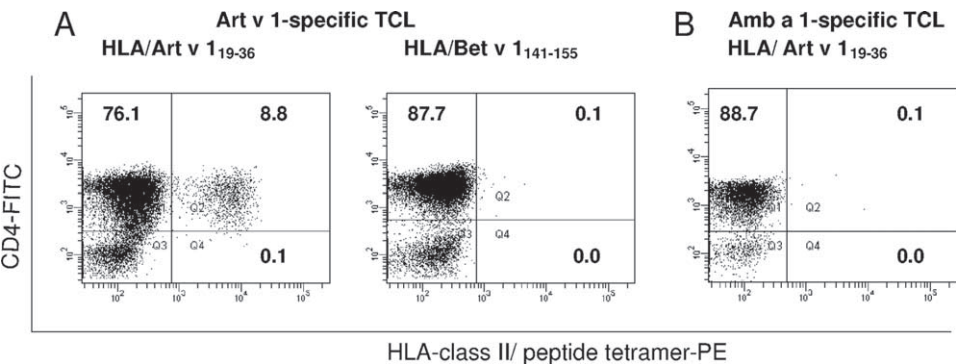


FIGURE 5. Specific binding of HLA class II/peptide tetramers. *A*, An Art v 1_{25–36}-specific TCL is recognized by the DR1 tetramers containing Art v 1_{19–36}, but not by DR1 tetramers containing Bet v 1_{142–156} as negative control peptide. *B*, A control TCL specific for Amb a 1 (major allergen in ragweed pollen) did not bind the DR1 tetramer containing Art v 1_{19–36}. One of two different TCL each is shown.

obtained for HLA-DRB1*0401. To elucidate the reason for the discrepancy between T cell approach and computer algorithms, we focused on HLA class II molecule/peptide interactions in this study.

Using HEK293 cells transfected with HLA-DR1 and an Ii::Art v 1 peptide fusion protein, we first confirmed that Art v 1_{25–36} is presented by HLA-DR1 (Fig. 1). Employing purified HLA-DR molecules as well as variant peptides, the binding capacity of HLA-DR1 for Art v 1_{25–36} (KCIEWEKAQHGA) was evaluated. We observed strong binding of Art v 1_{25–36} to HLA-DR1 and identified I27 as the most probable amino acid anchor in pocket P1 of the peptide-binding groove (Table II) (30, 31). This information permits differentiation of HLA- vs TCR-binding sites within the minimal epitope of the Art v 1 peptide (14). However, in vitro binding of Art v 1_{25–36} was not confined to HLA-DR1, but was also high for HLA-DR3 and HLA-DR4, moderate for HLA-DR13 and HLA-DR51, and low for HLA-DR11 and HLA-DR15 (Table III). Such degenerate binding characteristics of antigenic peptides to HLA class II “supertypes” have been reported for other antigenic peptides with high-affinity interactions (30, 32–34) and could explain the Art v 1_{25–36}-specific T cell responses of a minority of patients being negative for DR1(14). Interestingly, although Art v 1_{25–36} bound strongly to HLA-DR3 and HLA-DR4 molecules in vitro, no increase in the frequency of these alleles had been found in mugwort-allergic patients at the population level (14). Hence, the profound association of the allergic response to Art v 1 with HLA-DR1 could not fully be explained by in vitro HLA-binding studies.

To address the degenerate HLA binding of Art v 1_{25–36} at the T cell activation level, peptide-specific TCC were stimulated with APC expressing defined HLA class II molecules. Likewise, the TCC showed a broad scope of promiscuity in HLA restriction ranging from a single to multiple alleles. However, at limiting concentrations of the Art v 1 peptide the T cell response seemed to favor HLA-DR1 over HLA-DR4 (Fig. 3). Since the T cell-stimulating capacity of the DR4-expressing APC for superantigens was slightly better than the DR1-expressing APC (data not shown), a difference in intrinsic Ag-presenting capacity between both cell lines can be excluded. Limiting concentrations of the allergen in vivo could, therefore, explain a bias for DR1 as Ag-presenting molecule. Despite the lack of peptide binding to purified HLA-DR7 molecules, in this test system DR7 was able to present Art v 1_{25–36}. The discrepancy of T cell response and HLA peptide binding data may indicate a different sensitivity of the assays used. Additionally, rare HLA alleles such as HLA-DR16 and HLA-DR8, which had not been included in the binding studies, presented Art v 1_{25–36} (Fig. 2). Since the TCR gene usage in Art v 1-specific TCC is extremely diverse (9, 10, 14), the promiscuity of T cell recognition appears to be determined rather by TCR than by HLA binding of the peptide. Although Art v 1 has an unusual structure (5, 6), naturally processed Art v 1 peptides were recognized in an equal manner by Art v 1_{25–36}-specific T cells (Fig. 3), indicating that the degenerate recognition in our system was not an artifact due to the synthetic nature of the peptides. Thus, partly divergent data were obtained in different test systems and neither of them could fully explain the epidemiological finding of a selective DR1 association. Therefore, sensitization in vivo with natural Ag via locally specialized APC may underlie different selective conditions that are not achievable in vitro.

Customized HLA class II/peptide tetramers represent powerful tools to identify and characterize allergen-specific CD4⁺ T cells. Their application has been reported for T cell epitopes from Lol p 1 (35), Fel d 1 (36), Der p 1 (37), Bos d 2 (20), and Bet v 1 (26, 38). Herein, we demonstrate that stable, high-affinity MHC class II

tetramers containing HLA-DR1 molecules and Art v 1_{19–36}, a peptide containing the immunodominant epitope, can also be used to identify Art v 1-specific T cells (Figs. 4 and 5). Mugwort pollen allergy represents an ideal model for studying allergen-specific T cell responses using tetramers. The fact that most mugwort-allergic patients are sensitized to Art v 1 and possess T cells that recognize primarily only one T cell epitope provides the possibility to address all representative allergen-specific T cells of an individual by using HLA-DRB1*0101/Art v 1_{19–36} tetramers. This may be of advantage regarding the very low frequency of peptide-specific CD4⁺ T cells in peripheral blood (36, 35). Preliminary data indicate that ex vivo detection of Art v 1-specific T cells in PBMC by HLA class II tetramers is possible. We will proceed to address differences between mugwort pollen-allergic and nonallergic individuals, the plasticity of the Art v 1-specific T cell repertoire, and to investigate peripheral tolerance mechanisms at the T cell level operative during specific immunotherapy (39), including regulation and immune deviation.

In conclusion, our in vitro studies of HLA class II/peptide interactions further specified our knowledge on the trimolecular interaction of TCR/peptide/MHC underlying the T cell response to Art v 1. We possess a well-defined experimental test system consisting of a clinically relevant allergen that is mainly dependent on HLA-DR1 presentation and a cloned, functionally expressible Art v 1-specific TCR (40). These experimental tools will allow us to establish new in vitro and in vivo models (e.g., artificial APC expressing different costimulatory molecules or TCR/HLA-DR1 transgenic mice) to further investigate immune mechanisms at the level of allergen-specific T cells and TCR-MHC/peptide interactions using mugwort pollen allergy as disease model. The insights gained can be expanded to other allergens and may ultimately lead to new strategies in specific immunotherapy of allergic diseases.

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Disclosures

The authors have no financial conflicts of interest.

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