

T-cell receptor contact and MHC binding residues of a major rye grass pollen allergen T-cell epitope

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Background: T cells are pivotal in the elicitation of allergic diseases. Analogues of T-cell epitope peptides with a modification at a T-cell receptor (TCR) contact site can alter selected T-cell effector functions. Thus the ability to modulate allergen-specific T-cell responses towards T_{H1}-like by stimulation with peptide analogues may downregulate allergic inflammation. **Objectives:** The purpose of this study was to characterize the minimal epitope recognized by cloned T cells of a dominant Lol p 5 epitope, p105-116, and identify the critical residues involved in TCR and MHC contact.

Methods: Using peptides with progressive truncation of N- and C-terminal residues in T-cell proliferation assays, we identified the core epitope recognized by cloned CD4⁺ T cells. An additional series of peptides with single amino acid substitutions were used in T-cell proliferation and live-cell MHC binding assays. Taken together, these results allowed identification of MHC binding and TCR contact residues of p105-116.

Results: The core epitope of p105-116 was identified as residues 107-114. Within this core epitope, 3 residues were found to be important for MHC binding, positions 107, 110, and 112, whereas those at positions 108, 109, 110, 111, and 113 were putative TCR contact residues.

Conclusions: The identification of the TCR and MHC contact residues of a dominant Lol p 5 T-cell epitope and analogues of this peptide capable of modulating T-cell responses will allow the evaluation of these peptides' potential as immunotherapeutic agents for rye grass pollen allergic disease. (*J Allergy Clin Immunol* 1999;103:255-61.)

Key words: Lol p 5, T-cell epitopes, peptide analogues, rye grass pollen allergen, MHC class II

Characterization of allergen-specific T-cell responses in individuals with atopic allergic reaction based on the analysis of peripheral T cells, short-term T-cell lines and T-cell clones, and clinical biopsy specimens suggests that both T_{H2}-type (producing IL-4, IL-5, IL-10, IL-13) and

Abbreviations used

APC:	Antigen-presenting cell
LCB-HA:	Long-chain biotinylated hemagglutinin
RT-EBV:	Irradiated autologous EBV-transformed B cells
TCR:	T-cell receptor

T_{H0}-type cells (producing IL-2, TGF- β , and IFN- γ and T_{H2}-type cytokines) contribute to the functional phenotype of the CD4⁺ T-cell repertoire.¹⁻⁴ In contrast allergen-specific T cells from individuals with no atopic allergic reaction express a T_{H1/0}-type phenotype.⁵⁻⁷ Thus the ability to drive naive T_{H0}-type allergen-specific T cells in individuals with atopic allergic reactions to a more T_{H1}-type phenotype should be advantageous in the alleviation of allergic disease symptoms. Indeed studies have implicated such an immune mechanism to be one involved in current desensitization regimens where a shift in the allergen-specific T-cell cytokine response from a dominant T_{H2}-type to that of T_{H1}-type has been demonstrated.⁸⁻¹³ However, the exact mechanism involved in inducing this change is yet to be elucidated. The associated risk of anaphylaxis as the result of the use of whole allergen molecules has limited the efficacy of current immunotherapy regimens. Thus the use of dominant T-cell epitope peptides of allergen molecules for immunotherapy offers the advantages of more selective targeting of the allergen-specific T cell but being too small to cross-link mast cell bound IgE, eliminating the risk of anaphylaxis.

In recent years it has been demonstrated that analogues of immunogenic peptides modified at T-cell receptor (TCR) contact sites (ie, peptide analogues or altered peptide ligands) are capable of inducing a spectrum of T-cell responses.^{14,15} Peptide analogues capable of dissociating or altering selected T-cell functions, such as proliferation, cytokine production, and effector functions, have been described.¹⁶⁻²¹ Such peptide analogues that are capable of modulating the cytokine profile of allergen-specific T cells may be useful in the treatment of allergic disease by directly increasing the production of T_{H1}-type cytokines, such as IFN- γ , and/or the abrogation of IL-4 production. The promotion of IFN- γ production and the resulting alteration in the local cytokine milieu may be sufficient to drive naive allergen-specific T_{H0} cells to a T_{H1} phenotype and may aid in the reduction of

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TABLE I. Characteristics of T-cell clone RT9.1

Surface phenotype	CD4 ⁺ /8 ⁻	
	TCR $\alpha\beta$	
HLA restriction	DR4	
Cytokine production	IL-4	(1.1 ng/mL)
	IL-5	(2.2 ng/mL)
	IFN- γ	(5.9 ng/mL)
	IL-2	(0.2 U/mL)

clinical disease symptoms by decreasing allergen-specific IgE production and inflammatory cell infiltrate.

In attempting to design peptides for use in immunotherapeutic strategies, the problems associated with the ability of a single peptide to bind a range of different MHC haplotypes as seen in outbred populations are raised. However, HLA binding motifs, involving only a few peptide residues,²² and promiscuous peptides (containing supermotifs), which bind to several different class II MHC molecules, have been described.²³ Furthermore, HLA molecules may be grouped according to peptide anchor sites (HLA supertypes). Thus the identification of TCR contact residues of dominant T-cell epitopes and of those residues involved in binding to the MHC allows for a rational approach in designing peptide analogues that induce selected T-cell functions and bind to a wide range of MHC haplotypes.

Rye grass pollen (*Lolium perenne*) is a major aeroallergen in cool temperate climates during the grass flowering season. One of the major allergens of rye grass pollen is Lol p 5, which is recognized by more than 90% of sera from donors allergic to rye grass.²⁴ Lol p 5 has a molecular mass ranging from 28 to 32 kd and is present in the starch granules of pollen grains released as respirable particles into the atmosphere after rain.²⁵ Previous studies by us have identified T-cell epitopes of Lol p 5, with peptide 105-116 stimulating most Lol p 5-specific T-cell clones generated from a donor with atopic allergic reaction.²⁶

We present the identification of the core epitope and the TCR contact and MHC binding residues of Lol p 5 (105-116) by characterizing T-cell proliferation of cloned p105-116-specific T cells isolated from an individual with atopic allergic reaction in response to analogue p105-116 peptides and the MHC binding capacity of these analogue peptides. Analogue peptides modified at TCR contacts were tested for their potential to modulate T-cell responses with a view to identifying p105-116 analogues that could be used as immunotherapeutic agents against rye grass pollen allergic disease.

METHODS

Antigens

Lol p 5 peptides, p105-116 (AKYDAFVTALTE), analogues thereof, and N- and C-terminal truncated peptides of Lol p 5 residues 98-116 were synthesized on an automated multiple peptide synthesizer (ACT 350; Advanced ChemTech, Louisville, Ky) as previously described.²⁷ Synthesis of the hemagglutinin 307-319

peptide and N-terminal long-chain biotinylated hemagglutinin (LCB-HA) 307-319 peptide has been described previously.²⁸

Generation of cloned Lol p 5 (105-116)-specific T lymphocytes

T-cell clone RT9.1 was isolated by limit dilution cloning from a Lol p 5-specific long-term T-cell line generated from the peripheral blood of an individual who was allergic to rye grass pollen (DRB1*0401/0408, DPB1*0401, DQB1*0301). The characteristics of this T_H0-like clone are detailed in Table I (supernatants for cytokine analysis by ELISA were harvested at 48 hours after stimulation with 10 μ g/mL of p105-116). T cells were maintained in RPMI 1640 medium supplemented with 2 mmol/L L-glutamine (Gibco BRL, Melbourne, Australia), 0.05 mg/mL gentamicin (David Bull Laboratories, Victoria, Australia), and 5% heat-inactivated human AB⁺ serum. The antigen-reactive T cells were maintained by stimulation every 7 days with Lol p 5 (p105-116) (1 μ g/mL) and irradiated autologous EBV-transformed B cells (RT-EBV; 1×10^6 /mL, 6500 rad) as a source of antigen-presenting cells (APCs) together with 10 U/mL rIL-2 (Cetus Therapeutics, Emeryville, Calif). In all experiments T cells were rested for 6 to 7 days after the last addition of peptide and APCs.

T-cell proliferation assays

Cloned T cells (5×10^4 /well) were cultured in 96-well U-bottom plates (ICN, Aurora, Ohio) with p105-116 or analogues (10 μ g/mL) in the presence of irradiated RT-EBV cells (5×10^4 /well). Cultures were incubated for 4 days, pulsed for the last 8 hours with tritiated thymidine (1 μ Ci/well), and harvested onto glass fiber filters. Tritiated thymidine incorporation was measured by liquid scintillation spectroscopy. Results are expressed as the mean counts per minute plus standard deviation for triplicate cultures.

Cell-surface MHC class II binding assays

The relative MHC class II binding affinities of Lol p 5 peptide analogues compared with p105-116 were tested by their ability to compete with LCB-HA p307-319 for binding to RT-EBV cells with a modification of a live-cell binding assay previously described.^{29,30} Briefly RT-EBV cells were coincubated with LCB-HA p307-319 (5 μ g/mL) and a 100-fold excess of p105-116 or analogues in 96-well microtiter plates overnight at 37°C, 5% carbon dioxide in sterile PBS/0.1% BSA and RPMI 1640/10% FBS. Cells were washed in 1% FBS/PBS containing sodium azide (wash buffer) and incubated with fluorescein avidin D (Vector Laboratories, Burlingame, Calif) for 20 minutes on ice, washed and incubated with biotinylated anti-avidin D (Vector Laboratories) for 20 minutes on ice, and washed and incubated again with fluorescein avidin D for 20 minutes on ice. After a final wash, the mean fluorescence intensity of stained cells was determined from 10,000 events with a flow cytometer (FAC-Scan; Becton Dickinson, San Jose, Calif) and "Cell Quest" software (Becton Dickinson). A live gate (propidium iodide exclusion) was used in all flow cytometric analyses.

RESULTS

Determination of the core epitope of Lol p 5 (105-116) recognized by p105-116-reactive cloned T cells

To identify the core epitope of p105-116 recognized by the Lol p 5 (105-116)-specific T-cell clone RT9.1, N- and C-terminal truncated peptides were tested for their ability to stimulate RT9.1 in proliferation assays (Fig 1). Truncation of residues from the N-terminus was tolerat-

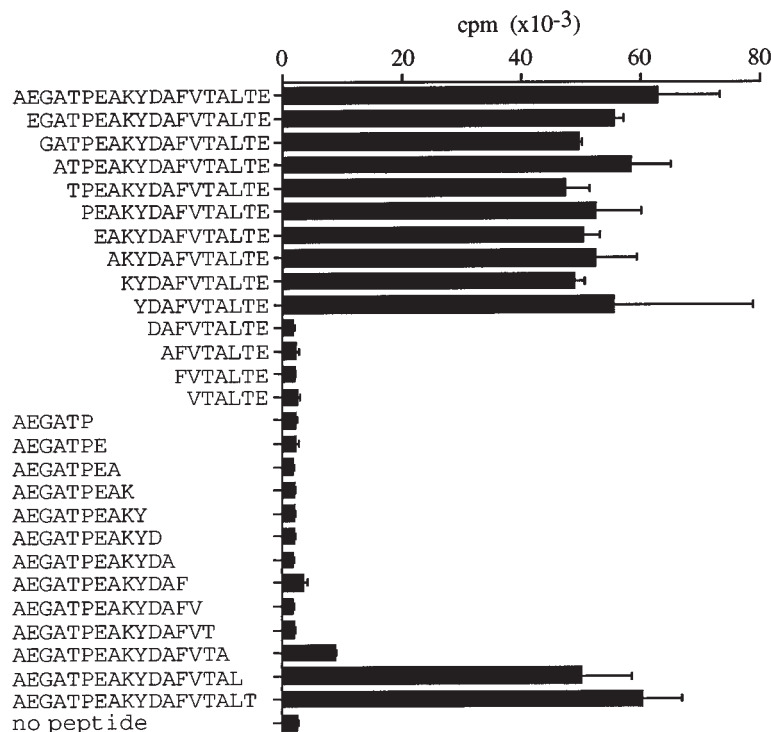


FIG 1. The proliferative response of cloned p105-116-specific T cells (RT9.1) to N- and C-terminal truncated peptides of the 98-116 Lol p 5 sequence. T-cell clone RT9.1 was stimulated with N- and C-terminal truncated peptides (10 μ g/mL) in the presence of RT-EBV cells as APCs. Proliferation was determined by tritiated thymidine incorporation. Data is expressed as the mean counts per minute (cpm) plus SD of triplicate cultures. Background responses of T cells cultured with irradiated APCs in the absence of antigen were <2000 cpm.

ed to residue Y107. Peptides truncated beyond this residue failed to activate RT9.1, indicating that this residue was highly important within the p105-116 sequence. Marked reduction in the activation of RT9.1 by C-terminal truncated peptides was observed on truncation of residue L114. Total unresponsiveness was achieved with removal of residue A113. The core epitope recognized by T-cell clone RT9.1 was therefore concluded to be Lol p 5 107-114 (YDAFVTAL). This experiment was repeated 3 times with similar results.

Mapping critical residues of p105-116

The first peptide analogue series tested comprised single amino acid alanine-substituted peptides of the Lol p 5 (105-116) sequence. Native p105-116 and analogues were tested for their ability to activate RT9.1 at doses ranging from 0.1 to 100 μ g/mL. Maximal proliferative responses were generally induced at a concentration of 10 μ g/mL. Substitution at positions 107, 108, 110, and 111 all markedly affected proliferation compared with that induced by the native peptide (Fig 2, A). Peptides D108A and F110A were nonstimulatory, and Y107A was capable of minimal activation generally only at the highest peptide concentration tested. Weak stimulation was achieved with V111A.

To further investigate the importance of these residues and others within the p105-116 sequence, an additional

series of peptides with single conservative or nonconservative amino acid substitutions were synthesized, and all were tested for their ability to stimulate T-cell clone RT9.1 in proliferation assays (Fig 2, B). Substitutions at positions 105, 106, 114, 115, and 116 had no effect on the proliferation of RT9.1. In addition to the critical residues identified by the alanine-substituted analogues, positions 109, 112, and 113 were also identified as important in p105-116 recognition by RT9.1. Substitutions at residues 109 and 113 were generally well tolerated, with only the substitution with a charged residue (glutamic acid) significantly affecting activation of RT9.1. This charge substitution at residue A113 resulted in the peptide being unable to activate the clone, whereas suboptimal activation was observed with this substitution at residue A109. The substitutions with charged residues at position T112 were not well tolerated, with the substitution of a lysine failing to activate the clone and only minimal activation observed with a glutamic acid substitution. These experiments were repeated 3 times with similar results.

Identification of p105-116 MHC binding residues

The reduction or loss of stimulation of T-cell clone RT9.1 observed with a number of peptide analogues could have occurred as a result of the reduction of the

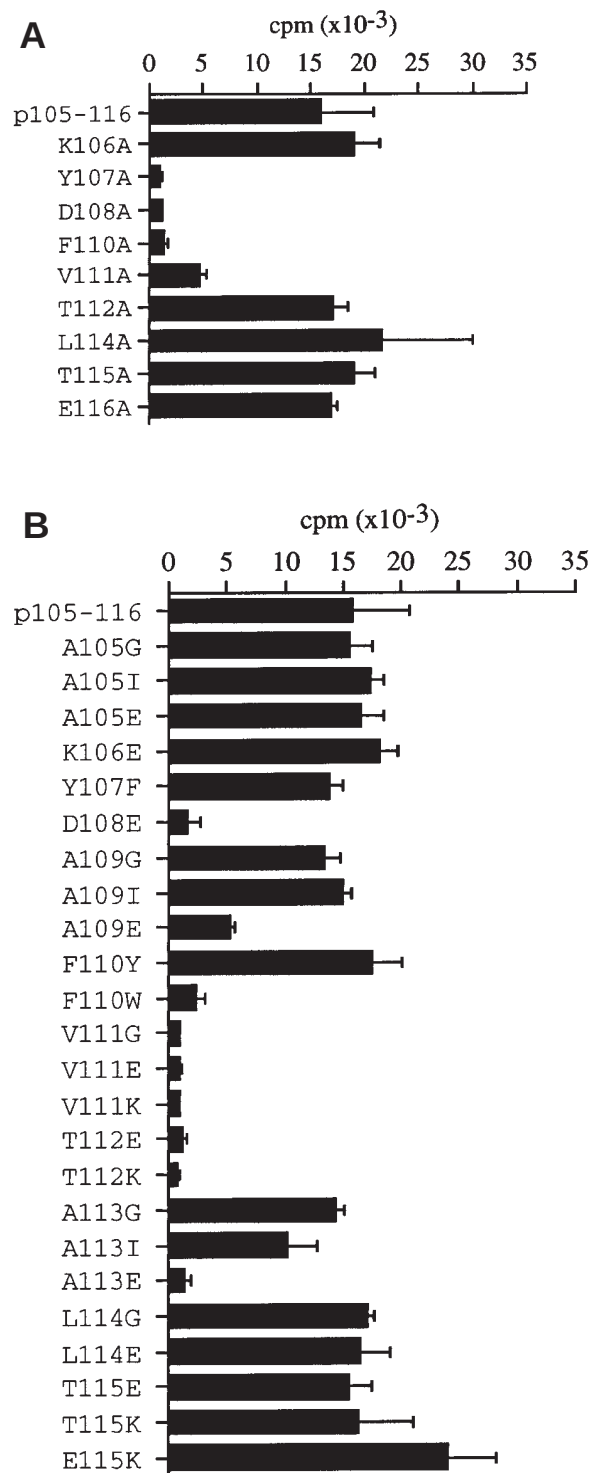


FIG 2. Proliferative response of cloned p105-116-specific T cells (RT9.1) to p105-116 or analogue peptides. T-cell clone RT9.1 was stimulated with peptide (10 μ g/mL) in the presence of RT-EBV cells as APCs. Proliferation was determined by tritiated thymidine incorporation. Data are expressed as the mean counts per minute (cpm) plus SD of triplicate cultures. **A**, Substitutions with alanine residues; **B**, substitutions with conservative or nonconservative residues. Background responses of T cells cultured with irradiated APCs in the absence of antigen were <1200 cpm.

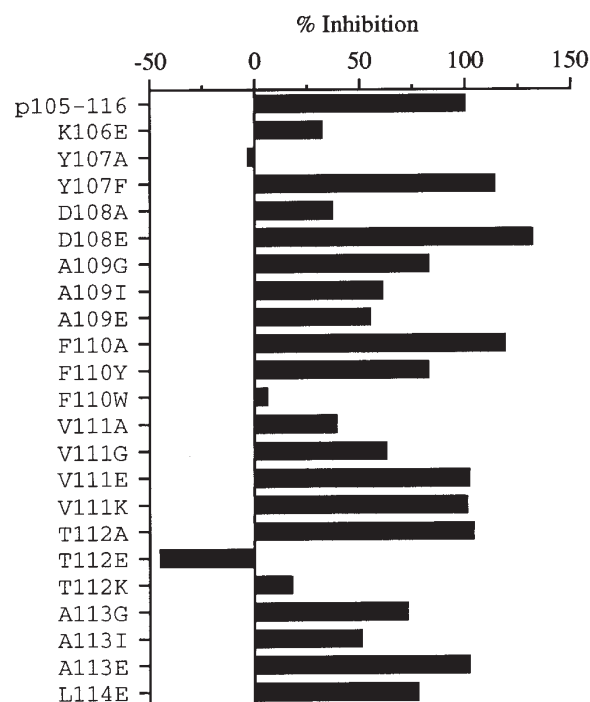


FIG 3. Relative binding affinity of peptide analogues to MHC expressed on RT-EBV cells. Bars indicate the percent inhibition of LCB-HA p307-319 binding relative to that induced by p105-116. Native p105-116 and analogues (500 μ g/mL) were added with LCB-HA p307-319 (5 μ g/mL) to RT-EBV cells and incubated overnight at 37°C. Cells were stained with avidin-FITC followed by a biotinylated anti-avidin antibody and a further layer of avidin-FITC. Mean fluorescence intensity was determined from 10,000 events by flow cytometry. A live gate (propidium iodide exclusion) was used in all analyses. Values correspond to the mean of 1 to 6 experiments.

binding affinity of the peptide to the MHC or a reduction in the affinity of the peptide-TCR interaction. To discriminate between these 2 possibilities, MHC binding studies were performed. Peptide 105-116 and analogues were investigated for their ability to compete with LCB-HA p307-319 for binding to RT-EBV cells (Fig 3). Single amino acid substitutions at positions 106, 108, 109, 111, 113, and 114 did not prevent the peptide's ability to compete with LCB-HA p307-319 for binding to the MHC in comparison to p105-116. However, introduction of alanine at position 107 or a charged residue (glutamic acid or lysine) at position 112 resulted in the abrogation of binding. Substitution of phenylalanine at position 110 with an alanine or tyrosine did not alter the ability of the peptide to bind to MHC; however, substitution with a tryptophan resulted in virtually no binding to the MHC.

Thus from the combined results of proliferation and competitive MHC binding assays (Table II), it can be concluded that amino acids at positions 108, 109, 111, and 113 are TCR contact residues for the T-cell clone RT9.1 and that positions 107 and 112 are important MHC binding residues. Additionally, because of the differing capacities of the position 110 analogues to modu-

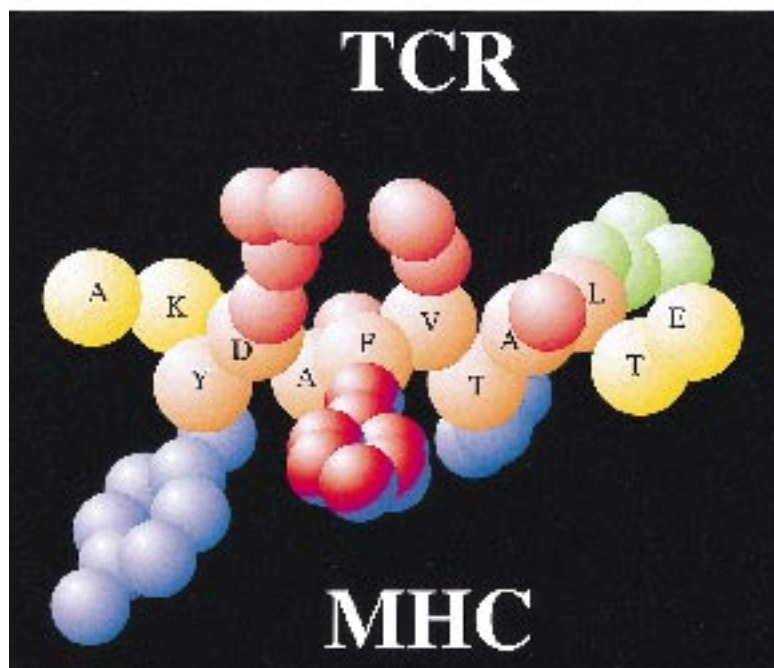


FIG 4. Schematic model of the p105-116 determinant for T-cell clone RT9.1. The view is side on with MHC to the bottom and TCR to the top. MHC-contacting side chains are depicted *blue*; TCR contacts are depicted *red*, and the side chain for the potential TCR and MHC contact residue F110 is depicted as a *mixture of these 2 colors*. The peptide backbone for residues that are within the core epitope are colored *orange*, whereas those residues that are outside this core epitope are colored *yellow*. The side chain for L114, which is within the core epitope but for which no role has been identified, is depicted *green*. This schematic is based on the crystal structure of HLA-DR1 complexed with the hemagglutinin peptide residues 306-318.³²

late RT9.1 proliferative responses and their respective affinities for MHC binding, phenylalanine at position 110 is proposed to play a dual role in both TCR contact and anchoring of the peptide to the MHC. These conclusions are summarized in Fig 4.

DISCUSSION

In this study we have determined the core epitope of Lol p 5 (105-116) recognized by cloned p105-116-specific T cells (RT9.1) and identified the residues that contact the TCR and the residues that are involved in anchoring this peptide to the MHC.

The stimulation of T-cell clone RT9.1 with N- and C-terminal truncated peptides encompassing residues 98-116 of the Lol p 5 sequence revealed the core epitope to be 8 amino acids in length, comprising residues 107-114. Single amino acid-substituted peptides of p105-116 revealed 7 residues (Y107, D108, A109, F110, V111, T112, and A113) where a substitution abrogated or significantly reduced the proliferative response of RT9.1, suggesting that these residues are involved in interactions with the TCR, MHC, or both. All 7 residues are within the defined core epitope. It is interesting to note that none of the amino acid substitutions at position 114 affected activation of RT9.1. Even the insertion of a charged residue that was poorly tolerated at any of the other posi-

tions within the core epitope region had no effect on the stimulation of RT9.1 or MHC binding. Further amino acid substitutions at this position may give insight into its potential function. However, it is possible that residue 114 contributes main chain binding to the MHC as identified in HLA class II-peptide crystal structures,^{31,32} with its side chain having little or no functional role.

Analysis of the relative MHC class II binding capabilities of peptides substituted at each of the 7 critical positions revealed 3 residues (Y107, F110, and T112) to be involved in anchoring the peptide to the MHC. Tyrosine at position 107 appeared to be the primary MHC anchor, and T112 appeared to be a secondary anchor residue because it was not as sensitive as Y107 to amino acid substitution. This is demonstrated by the fact that the conservative substitution Y107F was tolerated, but Y107A was not, yet in contrast the analogue T112A still bound well to the MHC. Phenylalanine at position 110 appears to play a dual role in MHC and in TCR recognition by RT9.1. Presumably the orientation of the residue within the peptide allows interaction with residues of both the MHC and TCR molecules. Indeed crystal structures of peptide bound to HLA-DR molecules indicate that residues occupying the MHC binding pocket corresponding to that potentially occupied by F110 are still partially solvent accessible and thus could be recognized by a TCR.^{32,33} Alternatively, F110 may not contact the

TABLE II. Relative MHC binding affinity of p105-116 analogue peptides and proliferative response of T-cell clone RT9.1

	Binding*	T-cell clone R9.1 proliferation† peptide concentration (μg/mL)			
		0.1	1	10	100
p105-116	++++	+	+++	++++	+++
A105G	NT	—	++++	++++	++
A105I	NT	+	++++	++++	++
A105E	NT	+	++++	++++	+++
K106A	NT	—	+++	++++	++++
K106E	++	—	++	++++	+++
Y107A	—	—	—	—	+
Y107F	++++	+	++++	++++	++
D108A	++	—	—	—	—
D108E	++++	—	—	—	—
A109G	++++	+	+++	++++	+++
A109I	+++	—	++++	++++	+++
A109E	+++	—	—	+	++
F110A	++++	—	—	—	—
F110W	+	—	—	+	+++
F110Y	+++	—	++	++++	+++
V111A	++	—	—	+	+
V111G	+++	—	—	—	—
V111E	++++	—	—	—	—
V111K	++++	—	—	—	—
T112A	++++	—	+	++++	+++
T112E	—	—	—	—	+
T112K	+	—	—	—	—
A113G	+++	—	—	++++	++
A113I	+++	—	—	++	++
A113E	++++	—	—	—	—
L114A	NT	—	+++	++++	+++
L114G	NT	—	+++	++++	+++
L114E	++++	—	++	++++	++++
T115A	NT	+	++++	++++	+++
T115E	NT	—	++	++++	++++
T115K	NT	+	+++	++++	+++
E116A	NT	—	+++	++++	++
E116K	NT	—	++++	++++	++++

NT, Not tested.

*Relative binding affinity was measured in a competition assay with LCB-HA p307-319. Data are expressed as percentage of binding with respect to native peptide: —, no binding; +, <25%; ++, <50%; +++, <75%; +++++, >75%.

†T-cell clone RT9.1 proliferative response relative to p105-116 is indicated as follows: —, no response; +, <25%; ++, <50%; +++, <75%; +++++, >75%.

TCR, and the recognition pattern observed with the different F110 analogues by RT9.1 may be due to an alteration in the surface of the peptide that directly contacts the TCR. The ability of buried peptide residue substitution to alter T-cell recognition while minimally affecting peptide binding has been described.³⁴

The binding of p105-116 to the MHC expressed by RT-EBV cells appears to be DR restricted. This is indicated by antibody inhibition experiments whereby only an anti-HLA-DR monoclonal antibody and neither an anti-HLA-DP nor anti-HLA-DQ monoclonal antibody could reduce the proliferative response of the T-cell clone

RT9.1. Similarly, the binding of p105-116 to the MHC expressed on the RT-EBV cell line was only reduced by the anti-HLA-DR antibody (data not shown). If residue Y107 is assigned position 1, the presence of a large aromatic side-chained residue at positions 1 and 4 and a polar residue at position 6 as seen in the p105-116 sequence is consistent with several HLA-DR motifs described previously.²² The HLA-DRB 1 haplotype of the donor from which the T-cell clone RT9.1 and RT-EBV cell line were derived is DRB1*0401/0408. The MHC binding motif that we have identified for p105-116 fits that described for DRB1*0401,^{22,33} but no motifs for DRB1*0408 have yet been described.

The 5 TCR contact residues of p105-116 identified for T-cell clone RT9.1 (D108, A109, F110, V111, and A113) vary in their importance in TCR signaling, demonstrated by their respective sensitivities to amino acid substitution. Two critical TCR contacts were identified, D108 and V111. Aspartic acid at position 108 appeared to be totally intolerant to substitution and is thus designated the primary TCR contact. We have designated V111 a secondary TCR contact because of its greater (yet still poor) tolerance to amino acid substitution. The remaining TCR contact residues A109, F110, and A113 are deemed minor TCR contacts because substitutions at these positions were generally well tolerated except for the introduction of a charge at position A113. The identification of these MHC and TCR contact residues will aid in the design of peptide analogues of p105-116 modified at TCR contacts that preferentially induce a more T_{H1}-type pattern of cytokine expression in p105-116 specific T cells; this may aid in the reduction of rye grass pollen-induced allergic disease symptoms.

The substitution of the primary TCR contact site within a peptide often results in a failure to elicit any measurable T-cell response, whereas the generation of analogues capable of altering or dissociating selected T-cell functions might be expected when secondary or minor TCR contacts are altered.¹⁵ The proliferative responses of RT9.1 to peptides substituted at secondary or minor TCR contact positions concurs with these observations. Thus substitutions at positions 109, 110, 111, and 113 in p105-116 are the most likely candidate positions for the generation of analogues capable of modulating the cytokine response of T-cell clone RT9.1 and other p105-116-specific T cells with a similar TCR footprint, favoring production of T_{H1}-type cytokines such as IFN-γ. However, the degree of inflexibility observed at the designated secondary TCR contact site V111 may indicate that such peptides may be difficult to generate when a substitution is made at this position and that the best possibilities are at the remaining 3 positions. We are currently investigating the ability of these and other peptide analogues to modulate the proliferative and cytokine profile generated relative to p105-116 in oligoclonal T-cell lines from this T-cell clone donor and other donors with atopic allergic reaction.

In summary we have identified the core epitope of an immunodominant Lol p 5 T-cell peptide (105-116) and

delineated the critical residues involved in MHC binding and TCR contact. This will provide useful information for the investigation of peptide analogues that can induce altered signals in this T-cell clone and in other p105-116-specific T cells with a similar TCR footprint.

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