

Potential of an altered peptide ligand of lipocalin allergen Bos d 2 for peptide immunotherapy

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Background: Peptide immunotherapy is a promising alternative for treating allergic diseases. One way to enhance the efficacy of peptide immunotherapy is to use altered peptide ligands (APLs) that contain amino acid substitutions compared with the natural peptide.

Objective: To evaluate the potential of an APL of the immunodominant epitope of lipocalin allergen Bos d 2 for peptide immunotherapy.

Methods: Peripheral blood CD4⁺ T-cell responses of 8 HLA-DR4-positive subjects to the natural ligand of Bos d 2 (p127-142) or to an APL (pN135D) were analyzed by MHC class II tetramer staining after *in vitro* expansion with the peptides. Long-term T-cell lines (TCLs) were induced with the peptides, and the cytokine production, cross-reactivity, and T-cell receptor V β subtype expression of the TCLs were analyzed. **Results:** CD4⁺ T cells specific for both p127-142 and pN135D were readily detected in peripheral blood after a single *in vitro* stimulation. Whereas the TCLs induced with p127-142 were T_H2/T_H0-deviated, those induced with pN135D were T_H1/T_H0-deviated and highly cross-reactive with p127-142. Moreover, the pN135D-induced TCLs appeared to use a broader repertoire of T-cell receptor V β subtypes than those induced with p127-142.

Conclusion: An APL of an immunodominant allergen epitope was able to induce a novel T_H1-deviated T-cell population cross-reactive with the natural epitope *in vitro*. This cell

population could have a therapeutic immunomodulatory function *in vivo* through bystander suppression.

Clinical implications: These results support the idea that altered peptide ligands may be used to enhance the efficacy of peptide immunotherapy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergy, altered peptide ligand, Bos d 2, lipocalin allergen, peptide immunotherapy, T cells, T-cell receptors, T_H1/T_H2

CD4⁺ T cells play a key role in both allergic and autoimmune diseases. For these diseases, antigen-specific modulation of T-cell response is a promising therapeutic alternative. Peptide-based immunotherapy involves the administration of short synthetic peptides containing the T-cell epitopes of allergens or self-antigens. Several clinical trials have demonstrated the potential of peptide immunotherapy in treating allergy.¹⁻⁵ Recently, 2 clinical trials have also provided encouraging results for the peptide treatment of type 1 diabetes⁶ and rheumatoid arthritis.⁷

One way to enhance the efficacy of peptide immunotherapy is to use altered peptide ligands (APLs). These peptides contain amino acid substitutions compared with the natural peptide. They have been shown to induce both quantitatively and qualitatively distinct signals in the responding T cell.⁸ Initial experiments in murine models have demonstrated the potential of APL immunotherapy for autoimmune diseases⁹⁻¹¹ and allergic asthma.¹² Two clinical trials have assessed the feasibility of APL immunotherapy in human beings.^{13,14} In these trials, an APL of the immunodominant epitope of myelin basic protein was administered to patients with multiple sclerosis (MS). However, the response to the treatment was heterogeneous, with no clear improvement in clinical outcomes, and both studies were suspended as a result of adverse reactions, such as disease exacerbations¹³ or hypersensitivity reactions.¹⁴ The prospect of using APLs in allergen immunotherapy has remained largely unexplored.

We have previously identified heteroclitic APLs for human T-cell clones specific for the immunodominant epitope of Bos d 2, a lipocalin allergen from cow dander.^{15,16} These APLs were able to stimulate the T-cell clones at 10-fold to 100-fold lower peptide concentrations than the natural ligand. Moreover, they exhibited immunomodulatory

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Supported by Kuopio University Hospital (project #5021605), the Academy of Finland (contract #205871), the Ida Montin Foundation, the Finnish-Norwegian Medical Foundation, and the Research and Science Foundation of Farnos.

Disclosure of potential conflict of interest: A. Immonen has patent licensing arrangements on the use of the Can f 1 sequences. T. Virtanen has patent licensing arrangements on the use of the Can f 1 sequences. The rest of the authors have declared that they have no conflict of interest.

Received for publication August 25, 2006; revised January 4, 2007; accepted for publication January 11, 2007.

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0091-6749/\$32.00

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doi:10.1016/j.jaci.2007.01.011

Abbreviations used

APL:	Altered peptide ligand
CDR3:	Complementarity-determining region 3
CFSE:	5-(And-6)-carboxyfluorescein diacetate succinimidyl ester
DC:	Dendritic cell
EC:	Effective concentration
FITC:	Fluorescein isothiocyanate
HA:	Hemagglutinin
MS:	Multiple sclerosis
PE:	Phycoerythrin
TCL:	T-cell line
TCR:	T-cell receptor
SSCP:	Single-strand conformational polymorphism

potential on the clones.¹⁷ In the current study, we analyzed *in vitro* the CD4⁺ T-cell responses of HLA-DR4-positive subjects to the natural ligand of Bos d 2 (p127-142) and to one of its heteroclitic peptide analogs, pN135D, to evaluate the potential of pN135D for peptide-based immunotherapy.

METHODS

Cells and peptides

PBMCs from 6 patients with cow dust-induced asthma (A-1 to A-6) with positive skin prick tests to Bos d 2¹⁵ and from 2 nonatopic individuals (NA-1 and NA-2) were separated by Ficoll Paque Plus density gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). All subjects were genotyped to be positive for HLA-DRB1 *0401. The study was approved by the Ethics Committee of the Kuopio University Hospital, and written informed consent was given by all subjects. The T2.DR4 cell line expressing the DRB1 *0401 allele¹⁸ was kindly provided by Dr H. Zarour (Pittsburgh, Pa). The Bos d 2 peptide p127-142 (ELEKYQQLNSERGVPN) and its single amino acid variant pN135D (ELEKYQLDSEKRGVPN) were synthesized as described previously.¹⁶

MHC class II tetramers

The generation of soluble DRA1 *0101/DRB1 *0401 molecules and the procedure used for peptide loading have been described earlier.¹⁹ The peptides described here were used to load DRB1*0401 molecules to generate DR0401/peptide tetramers. The hemagglutinin (HA) 306-318 peptide PRYVKQNTLKLAT was used to generate DR0401/HA306-318 as a control tetramer for staining. The staining was performed by incubating T cells with 0.5 µg of the phycoerythrin (PE)-labeled tetramers in 50 µL culture medium for 2 hours at 37°C. Anti-CD4-PE-Cy5 (Immunotech, Marseille, France) and, in some experiments, anti-Vβ13.1-fluorescein isothiocyanate (FITC; Immunotech) were added, and the cells were incubated for an additional 20 minutes at 4°C. The cells were washed twice and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif).

Dendritic cell culture

Mature dendritic cells (DCs) were prepared according to the procedure of Ponsaerts et al.²⁰ Briefly, PBMCs were incubated for 2 hours in serum-free AIM-V medium (Life Technologies, Paisley, United Kingdom) in 6-well plates (Corning Inc, Corning, NY).

Nonadherent cells were gently removed by repeated washing, and the adherent cells were cultured for 2 days in AIM-V supplemented with 100 ng/mL GM-CSF (Strathmann Biotech, Hannover, Germany). PolyI:C (Sigma, St Louis, Mo) was added at a concentration of 25 µg/mL 24 hours after starting the culture. The typical purity of the DC cultures was 60% to 70% according to staining with FITC-labeled anti-CD14, anti-CD86, and anti-CD83 mAbs (BD Biosciences, San Jose, Calif). Before use, DCs were pulsed with the peptides (10 µmol/L) in AIM-V for 4 hours, washed, and resuspended in RPMI 1640 supplemented with 2 mmol/L L-glutamine, 20 µmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, nonessential amino acids, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10 mmol/L HEPES (BioWhittaker, Verviers, Belgium), and 5% inactivated human AB serum (Finnish Red Cross, Helsinki, Finland).

CFSE/tetramer analysis of the CD4⁺ T-cell response

CD4⁺ T cells were positively selected from PBMCs with immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cells assessed by staining with anti-CD4-FITC (BD Biosciences) was routinely > 98%. Purified CD4⁺ T cells were stained with 1 µmol/L 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Leiden, The Netherlands) in PBS for 10 minutes at 37°C. Staining was stopped by adding 100% human AB serum and subsequent washing. T cells (1 × 10⁶ per well) were stimulated with 2 × 10⁵ autologous peptide-pulsed DCs in 48-well plates (Corning Inc.) in 1 mL complete RPMI 1640 supplemented with 5 ng/mL IL-7 (Strathmann Biotech). On day 8, fresh medium supplemented with 10 IU/mL IL-2 (Strathmann Biotech) and 5 ng/mL IL-7 was added to the cultures. The T cells were stained with tetramers and anti-CD4-PE-Cy5 and analyzed by flow cytometry on days 8 and 15.

Long-term CD4⁺ T-cell lines

For the generation of long-term CD4⁺ T-cell lines (TCLs), 1 × 10⁴ peptide-pulsed DCs and 5 × 10⁴ CD4⁺ T cells were seeded in 96-well U-bottomed plates (30 wells per peptide) in complete RPMI 1640. After 4 days, IL-2 (10 IU/mL) and IL-7 (5 ng/mL) were added to the cultures. The cultures were restimulated with fresh peptide-pulsed DCs every 10 to 11 days. After the second and third stimulation, half the T cells in each well were split into 2 new daughter plates and stimulated with γ-irradiated (6000 rad) T2.DR4 cells (1 × 10⁴ per well) pulsed for 4 hours with or without the peptides (10 µmol/L). After 3 days, [³H]thymidine was added (1 µCi per well; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), and after an additional 16 hours, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1450) and the results expressed as cpm. The stimulation index (cpm in the presence of a peptide divided by cpm in the absence of a peptide) was determined. Positive wells (stimulation index > 2 and a Δcpm of at least 5000) were transferred to a 24-well plate and expanded with 2 × 10⁶ autologous PBMCs, 10 µmol/L of the peptide, 10 IU/mL IL-2, and 5 ng/mL IL-7. The established TCLs were stimulated in quintuplicates in 96-well U-bottomed wells with 1 × 10⁴ irradiated T2.DR4 cells and different concentrations of the peptides for 3 days, and the proliferation was measured as described. The functional T-cell receptor (TCR) avidity, assessed by effective concentration (EC)₅₀ values—the peptide concentration needed to induce half-maximal proliferation—was calculated from the titration curves of individual TCLs. Supernatants (100 µL per well) were collected from the cultures stimulated with 50 µmol/L of the peptides after 3 days, and the production of IL-4, IFN-γ and IL-10 was measured by using commercial ELISA kits (DuoSet; R&D Systems, Minneapolis, Minn). The detection limits for IL-4, IFN-γ, and IL-10 were 20, 30, and 30 pg/mL,

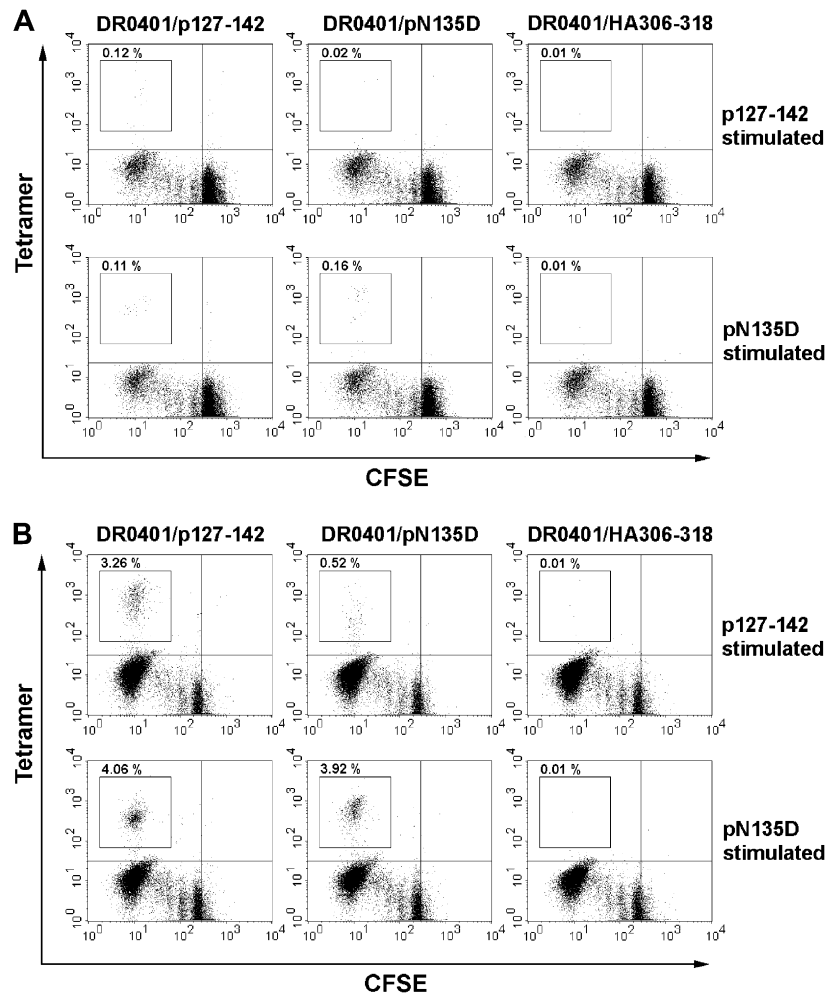


FIG 1. Detection of peptide-specific T cells with MHC class II tetramers and CFSE dye dilution. Tetramer staining was performed on day 8 (**A**) and on day 15 (**B**) after stimulation with peptide-pulsed DCs. Representative results are shown for subject A-3. The percentages of divided tetramer-positive cells are indicated. The DR0401/HA306-318 tetramer was used as a control for staining.

respectively. IFN- γ /IL-4 ratios were used for the classification of the TCLs: T_H1 (IFN- γ /IL-4 ratio > 10 and IFN- $\gamma > 100$ pg/mL), T_H2 (IFN- γ /IL-4 ratio < 0.1 and IL-4 > 100 pg/mL), T_H0 (ratios in between). The different V β subtypes expressed by the TCLs were analyzed with the IOTest Beta Mark kit (Immunotech, Marseille, France) as previously described.²¹ TCR V β expansions were considered significant when more than 10% of the cells expressed the particular V β subtype and the level of expression was at least 2 times greater than the expression in PBMC. The TCLs were also stained with the MHC class II tetramers and anti-CD4-PE-Cy5 together with anti-V β 13.1-FITC.

TCR RT-PCR/SSCP analysis of V β 13.1⁺ T cells

Total RNA was isolated from the TCLs with the RNeasy total RNA isolation system (Promega, Madison, Wis). The first-strand cDNA was synthesized using TCR C β -specific primer after amplification with a V β 13-specific primer as described previously.²² The amplification product was subjected to single-strand conformational polymorphism (SSCP) analysis.²³ Briefly, the sample was diluted in a denaturing solution containing 95% formamide and 20 mmol/L EDTA and boiled for 5 minutes. The sample was loaded onto 6%

polyacrylamide Tris-boric acid-EDTA (TBE) gel containing 10% glycerol. The gel was run at 8 Vcm⁻¹ constant voltage for 2.5 hours in 0.5 $\times$ TBE buffer at room temperature. After electrophoresis, the gel was silver-stained, and the individual DNA bands of interest were reamplified with V β 13 and C β primers using the bandstab technique.²⁴ The V β 13-C β fragments were cloned into pCR2.1-TOPO vectors, and the constructs were transformed into *Escherichia coli* by using the TOPO TA Cloning kit (Invitrogen, Carlsbad, Calif). The nucleotide sequences of the V β 13-C β clones were confirmed by DNA sequencing. In some cases, identical sequences were obtained from 2 independent SSCP-derived clones of a specific TCL.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, Calif). The Mann-Whitney *U* test was used to analyze the differences between the specific proliferative and cytokine responses of long-term TCLs. The χ^2 test for trend was used to analyze the differences between the p127-142-induced and pN135D-induced TCLs in the distribution of cytokine phenotypes and the distribution of avidities for the homologous peptide. *P* values of .05 or less were considered significant.

TABLE I. The percentage of divided tetramer-positive CD4⁺ T cells in peptide-induced cultures on day 15*

Subject	Induction with			
	p127-142		pN135D	
	Tetramer analysis with DR0401/			
	p127-132	pN135D	p127-132	pN135D
A-1	0.64	0.08	0.52	4.62
A-2	3.01	0.03	0.55	5.11
A-3	3.26	0.52	4.06	3.92
A-4	0.08	0.05	0.07	0.22
A-5	6.69	0.36	1.11	0.49
A-6	0.36	0.01	0.02	0.01
NA-1	0.05	0.03	0.05	0.07
NA-2	0.38	0.05	0.08	0.04

*Background staining with the DR0401/HA306-318 control tetramer ranged from 0.01% to 0.09% in individual experiments. Positive staining (>0.10% of CD4⁺ T cells) is indicated in boldface.

RESULTS

Expansion of specific CD4⁺ T cells on stimulation with p127-142 and pN135D

We have previously demonstrated that an allergen peptide with a single amino acid substitution (pN135D) was capable of activating a human Bos d 2–specific T-cell clone F1-9 restricted by DRB1 *0401 at about a 100-fold lower concentration than the natural peptide p127-142.¹⁶ In this study, we wanted to evaluate the potential of pN135D for peptide immunotherapy by analyzing the *in vitro* T-cell responses of 8 DR4-positive subjects to p127-142 and pN135D. For the analysis, CD4⁺ T cells isolated by immunomagnetic sorting were stained with CFSE and stimulated with DCs pulsed with the peptides. The cells were stained with peptide-loaded DRB1 *0401 tetramers on days 8 and 15.

On day 8, the percentage of divided tetramer-positive CD4⁺ T cells in the cultures was very low. Distinct populations of DR0401/p127-142-positive and DR0401/pN135D-positive T cells could be observed in the peptide-induced cultures of the subject A-3 only (0.12% and 0.16%, respectively; Fig 1, A). However, after culturing the cells for additional 7 days together with IL-2 and IL-7, DR0401/p127-142–positive and DR0401/pN135D-positive CD4⁺ T cells were observed in the p127-142-induced and pN135D-induced cultures from 6 and 5 subjects, respectively. A representative example is shown for subject A-3 in Fig 1, B. The percentage of DR0401/p127-142–positive T cells ranged from 0.36% to 6.69% in the p127-142–induced cultures and that of DR0401/pN135D–positive T cells from 0.22% to 5.11% in the pN135D-induced cultures (Table I). The peptide-induced responses of the nonatopic subjects NA-1 and NA-2 appeared to be weaker than the responses of most of the atopic subjects.

Several of the tetramer-positive cultures exhibited cross-reactivity for the homologous peptide, as shown for

TABLE II. The number of specific TCLs obtained from the subjects (30 replicate wells per subject were seeded)

Subject	p127-142	pN135D
A-1	1	3
A-2	2	2
A-3	4	1
A-4	2	3
A-5	9	5
A-6	—	2
NA-1	—	1
NA-2	—	1
Total	18	18

subject A-3 (Fig 1, A and B). However, only 2 out of the 6 p127-142-induced cultures that were positively stained with DR0401/p127-142 were stained with DR0401/pN135D. In contrast, positive staining with DR0401/p127-142 was observed in 4 out of the 5 pN135D-induced T-cell cultures that were positively stained with DR0401/pN135D (Table I).

Generation of long-term TCLs with p127-142 and pN135D

Because pN135D was observed to induce T cells cross-reactive with p127-142, we wanted to analyze this phenomenon in more detail. We generated long-term CD4⁺ TCLs from all subjects with p127-142 and pN135D (Table II). Eighteen TCLs were induced with each of the peptides. Interestingly, at least 1 pN135D-specific TCL was obtained from all 8 subjects, whereas p127-142–specific TCLs were obtained from only 5 subjects.

The proliferative responses of the p127-142–induced and pN135D-induced TCLs were of the same magnitude (Fig 2, A). Moreover, the functional TCR avidity of the TCLs, estimated by EC₅₀ values (ie, the peptide concentration needed to induce a half-maximal response), was similar (median EC₅₀ of p127-142–induced TCL, 1.0 μmol/L; pN135D-induced TCL, 1.9 μmol/L; *P* = .38; Mann-Whitney *U* test). Of the peptide-specific TCLs, 1 p127-142–specific and 5 pN135D-specific TCLs failed to stain with the DR0401/p127-142 and DR0401/pN135D tetramers, respectively (less than 1% tetramer positive; data not shown), probably because of a low TCR affinity.¹⁷

Cytokine phenotype of the p127-142–induced and pN135D-induced TCLs

Interestingly, there was a difference in cytokine production by the TCLs. The pN135D-induced TCLs produced significantly higher levels of IFN-γ than the p127-142–induced TCLs (Fig 2, B). In contrast, there was no statistically significant difference in the production of IL-4 (Fig 2, C) or IL-10 (Fig 2, D). Consequently, the p127-142–induced TCLs were predominantly of the T_H2/T_H0 phenotype, whereas the pN135D-induced TCLs were of the T_H1/T_H0 phenotype (Table III; *P* = .004).

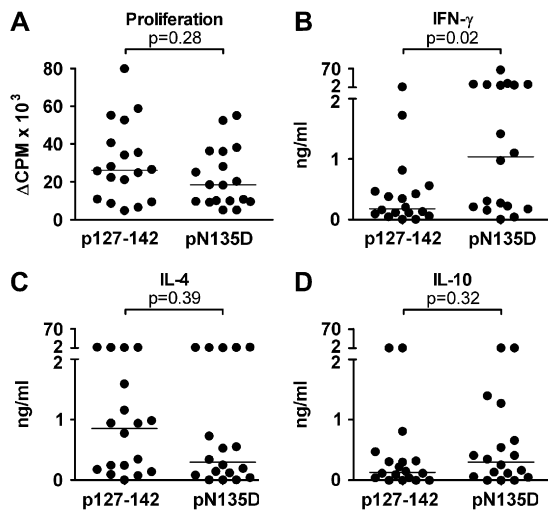


FIG 2. Proliferative and cytokine responses of p127-142-induced and pN135D-induced long-term TCLs. **A**, The proliferative responses of the TCLs are expressed as Δcpm (mean of stimulated wells – mean of unstimulated wells). The amounts of IFN- γ (**B**), IL-4 (**C**), and IL-10 (**D**) produced were detected in the supernatants. The peptide concentration used for stimulation was 50 $\mu\text{mol/L}$.

TABLE III. T_H cytokine phenotype of the long-term TCLs*

	p127-142-induced TCL	pN135D-induced TCL
T_H2	5	1
T_H0	12	9
T_H1	1	8

*The phenotypes were based on the IFN- γ /IL-4 ratio (T_H1 , IFN- γ /IL-4 ratio > 10 and IFN- γ > 100 pg/mL; T_H2 , IFN- γ /IL-4 ratio < 0.1 and IL-4 > 100 pg/mL; T_H0 , ratios in between). The χ^2 test for trend was used to analyze the differences in the distribution of cytokine phenotypes between the p127-142-induced and pN135D-induced TCLs ($P = .004$).

Cross-reactivity of the p127-142-induced and pN135D-induced TCLs

Both the p127-142-induced and pN135D-induced TCLs frequently showed reactivity to the homologous peptide. Nine of the 18 p127-142-induced TCLs exhibited a cross-reactive response to pN135D. Importantly, 14 of the 18 pN135D-induced TCLs recognized p127-142. Moreover, at least 1 cross-reactive pN135D-induced TCL was obtained from every subject in the study. Both p127-142-induced and pN135D-induced TCLs displayed a spectrum of functional avidities for the homologous peptide (Table IV), as demonstrated with 3 pN135D-induced TCLs from subject A-5 (Fig 3). Although most of the cross-reactive T-cell responses were of lower or similar avidity than the responses to the peptide used for inducing the TCLs, 5 pN135D-induced TCLs (from 4 different subjects) recognized p127-142 with a higher avidity than pN135D (Table IV). In contrast, only 1 p127-142-induced TCL recognized pN135D with a higher avidity. In general, the avidity of the pN135D-induced TCL for p127-142 tended to be higher than that of the

TABLE IV. Functional avidity of the long-term TCLs for the homologous peptide*

	Avidity of p127-142-induced TCL for pN135D	Avidity of pN135D-induced TCL for p127-142
Higher	1	5
Similar	3	2
Lower	5	7
None	9	4

*Functional avidity was evaluated by EC_{50} ratios (EC_{50} of the homologous peptide/ EC_{50} of the peptide used in the induction of the TCL). Lower avidity, EC_{50} ratio > 10; similar avidity, $0.1 < EC_{50}$ ratio < 10; higher avidity, EC_{50} ratio < 0.1. The χ^2 test for trend was used to analyze the differences in the distribution of functional TCR avidities of the p127-142-induced and pN135D-induced TCLs for the homologous peptide ($P = .06$).

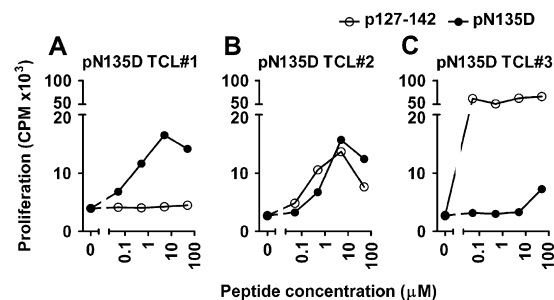


FIG 3. Different patterns of T-cell cross-reactivity: the homologous peptide is not recognized (**A**); the homologous peptide is recognized with a similar avidity (**B**) or with higher avidity than the peptide used for induction (**C**). All 3 TCLs were obtained from subject A-5 on stimulation with the peptide analog pN135D.

p127-142-induced TCL for pN135D (Table IV; $P = .06$). On stimulation with the homologous peptide, the cytokine phenotype of the TCLs (Table III) remained unaltered (data not shown).

TCR V β subtypes used by the p127-142-induced and pN135D-induced TCLs

The expansion of TCR V β subtypes in the p127-142-induced and pN135D-induced long-term TCLs was analyzed by flow cytometry (see this article's Fig E1 in the Online Repository at www.jacionline.org). Interestingly, an expansion of V β 13.1⁺ T cells was observed in 14 of the 18 p127-142-induced TCLs but only in 4 of the 18 pN135D-induced TCLs. All the pN135D-induced TCLs with an expansion of V β 13.1⁺ T cells were cross-reactive with p127-142 (data not shown). Expansion of a specific V β subtype other than V β 13.1 was observed in only 1 to 3 of the p127-142-induced or pN135D-induced TCLs. The antigen-specificity of the expanded V β 13.1⁺ T-cell populations was confirmed by staining the TCLs with both the V β 13.1-specific antibody and the DR0401/peptide tetramers (a representative example is shown in this article's Fig E1 in the Online Repository at www.jacionline.org).

TABLE V. TCR β -chain CDR3 amino acid sequences used by peptide-specific V β 13.1⁺ T cells from p127-142-induced and pN135D-induced TCLs*

Subject	TCL	Amino acid sequence			CDR3 length
		V β 13.1	N-D β -N-J β	J β	
A-2	p127-142 TCL 1	CASS	RTGD GQAFF	J β 1.1	9
	pN135D TCL 1	CAS	RFRAQVCPNEWLHF	J β 1.2	14
A-3	p127-142 TCL 1	CASS	RTSG GLETQYF	J β 2.5	11
	p127-142 TCL 2	CAS	TGRVTMATTF	J β 1.2	10
	p127-142 TCL 3	CAS	RRSTSTDTQYF	J β 2.3	11
A-4	p127-142 TCL 1	CASS	LQGSRFTLHF	J β 1.6	10
	p127-142 TCL 2	CASS	PRGADTQYF	J β 2.3	9
		CASS	RTGG YGYTF	J β 1.2	9
A-5	p127-142 TCL 1	CA	TH RGG AAGMNTAEFF	J β 1.1	15
	p127-142 TCL 2	CA	AGGGGAQEKLF	J β 1.4	12
	p127-142 TCL 3	CASS	PQGVNTGELFF	J β 2.2	11
	p127-142 TCL 4	CASS	RAGF LDTEAFF	J β 1.1	11
	p127-142 TCL 5	CASS	RAGF LDTEAFF	J β 1.1	11
	p127-142 TCL 6	CASS	P RTEK QYF	J β 2.7	8
	p127-142 TCL 7	CASS	RVAG VGNEQFF	J β 2.1	11
	p127-142 TCL 8	CASS	RVAG VGNEQFF	J β 2.1	11
	pN135D TCL 3	CASS	QILAGGPDEQFF	J β 2.1	12
	pN135D TCL 1	CASS	YST RGGP NTIYF	J β 1.3	12
NA-1		CASS	VSKYPDRFRQETQYF	J β 2.5	15
		CASS	RTGF GLETQYF	J β 2.5	11

*The RTGG motif, or minor variations of it, are indicated by boxing.

TCR β -chain CDR3 amino acid sequences of peptide-specific V β 13.1⁺ T cells

The TCLs showing an expansion of peptide-specific V β 13.1⁺ T cells were subjected to SSCP analysis. In 16 of the 18 TCLs, a monoclonal SSCP pattern²⁵ was detected, whereas in 2 TCLs, the SSCP pattern was oligoclonal (data not shown). The SSCP bands were reamplified directly from the gels, and their nucleotide sequences were determined after subcloning. The deduced TCR β -chain complementarity-determining region 3 (CDR3) amino acid sequences are shown in Table V. Diverse J β segments were used by the TCLs, even intraindividually, and the CDR3 length varied between 8 and 15 amino acids. Interestingly, a common amino acid motif, RTGG, or minor variations of it, was observed in 11 TCLs from 6 different subjects (Table V). Moreover, a CDR3 sequence was shared by p127-142-induced TCLs 4 and 5 from subject A-5, whereas the TCLs 7 and 8 from the same subject shared a different sequence (Table V). This finding suggests that at least 2 different p127-142-specific T-cell clones had expanded in subject A-5 *in vivo*.

DISCUSSION

In this study, we evaluated the potential of an APL of the dominant T-cell epitope of lipocalin allergen Bos d 2¹⁶ for human peptide-based immunotherapy. This APL, pN135D, was found to be stimulatory for CD4⁺ T cells *in vitro*. Interestingly, 14 of the 18 pN135D-induced TCLs recognized p127-142, 7 of these with a similar or

higher avidity than pN135D. Moreover, the pN135D-induced TCLs were predominantly of the T_H1/T_H0 phenotype, in contrast with the T_H2/T_H0 phenotype of p127-142-induced TCLs. Furthermore, the pN135D-induced TCLs appeared to use a broader repertoire of TCR V β subtypes than the p127-142-induced TCLs.

Altered TCR-mediated activation by APLs has been shown to have a potential therapeutic effect—for example, by promoting immune deviation, antagonism, anergy, or deletion of specific T cells.^{17,26-30} Some studies also suggest that APLs can induce a new cross-reactive T-cell population able to modulate the immunologic microenvironment containing the pathogenic T cells—for example, by bystander suppression.^{9,11,31} This phenomenon has also been demonstrated in one of the clinical trials of MS.¹³ In that study, an *in vivo* expansion of APL-specific T cells, a large fraction of which were cross-reactive with the natural myelin basic protein peptide, was observed in every patient treated with the APL. Unfortunately, some of these T cells were not of the desired T_H2 phenotype but of the T_H1 phenotype, which presumably led to disease exacerbations in 2 of the 8 subjects in the study.

Our data support the view that bystander suppression can be a potential mechanism in APL immunotherapy. Almost all the pN135D-induced TCLs cross-recognized the natural peptide p127-142. Importantly, the T_H1/T_H0 cytokine phenotype of these TCLs remained unaltered on stimulation with p127-142. Furthermore, the difference in the TCR V β use by the p127-142-induced and pN135D-induced TCLs supports the notion that most of the TCLs induced with the APL were derived from a different pool of T-cell precursors.

Hypothetically, the induction of bystander suppression with an APL in allergen immunotherapy requires 2 steps *in vivo*. First, immunization with the APL should create a novel T_H1-biased T-cell population cross-reactive with the natural allergen epitope. Subsequently, on allergen exposure, these T cells would become activated with the natural peptide and be able to modulate the T_H2-deviated immune response. Another possibility is that immunization with the APL could result in the induction of regulatory T cells, a phenomenon previously observed in both conventional allergen immunotherapy^{32,33} and immunotherapy with natural allergen peptides.³⁴ In the future, immunomodulatory treatments that promote the induction of regulatory T cells³⁵ might be used in combination with APL immunotherapy to enhance its efficacy.

One of the major risks associated with APL immunotherapy is the cross-activation of pathogenic memory T cells.³⁶ In this study, we observed that the peptide analog pN135D was capable of activating a substantial proportion of the T_H2/T_H0-deviated p127-142-induced TCLs. This could lead to disease exacerbation *in vivo*, as observed in the clinical trial of MS.¹³ On the other hand, transient activation of allergen-specific T cells has been shown to precede the development of tolerance *in vivo* in immunotherapy with natural allergen peptides.^{37,38}

Most of the previous research on APL immunotherapy has been directed against autoimmune diseases, in particular MS. Allergic disorders may be a safer alternative for the clinical trials of APL immunotherapy.³⁹ The efficacy and safety of immunotherapy with natural peptides have been evaluated in several clinical trials of allergic diseases,¹⁻⁵ whereas experience with autoimmune diseases is more limited.^{6,7} It is also conceivable that potential disease exacerbations associated with high APL doses¹³ are more easily controllable in allergic subjects than in patients with autoimmune diseases.⁴

The current study demonstrated that the TCR β -chains of T cells specific for p127-142 were highly conserved in DR4-positive subjects. An expansion of p127-142-specific V β 13.1⁺ T cells was observed in 14 of the 18 p127-142-induced TCLs but in only 4 of the 18 pN135D-induced TCLs. Moreover, a common amino acid motif, RTGG, or minor variations of it, was observed in the TCR β -chain CDR3 amino acid sequences of 11 different TCLs. These findings suggest a common structural requirement for the recognition of p127-142 in the context of DRB1 *0401. The significance of the highly homogenous TCR repertoire for the allergenicity of Bos d 2 in DR4-positive subjects needs to be clarified in further studies. Potentially, the identification of common TCR structures in the pathogenic T-cell population could allow the development of TCR-based therapies, such as TCR-based vaccination.⁴⁰

The difference in TCR V β usage by the p127-142-induced and pN135D-induced TCLs suggests that most of the pN135D-induced T cells were derived from a different pool of T-cell precursors than the p127-142-induced T cells. It is possible that most of the p127-142-induced T cells originated from the memory T-cell pool, whereas the

pN135D-induced T cells were mainly of naive origin. The finding that 2 CDR3 sequences were shared by distinct p127-142-induced TCLs suggests that the p127-142-specific T cells had previously been expanded in allergic subject A-5 *in vivo*.

In conclusion, we have demonstrated in this study that the CD4⁺ T-cell response of DR4-positive allergic subjects against the immunodominant epitope of Bos d 2 (p127-142) was predominantly T_H2/T_H0-deviated and remarkably uniform in TCR V β usage. In contrast, the CD4⁺ T-cell response against the peptide analog pN135D was mostly T_H1/T_H0-deviated and appeared to use a broader repertoire of TCR V β subtypes. Importantly, pN135D-induced TCLs cross-reactive with the natural peptide were obtained from all 8 subjects, demonstrating that it can be possible to induce a novel cross-reactive T-cell population with an APL in a group of subjects sharing a similar MHC background. Through bystander suppression, this cell population can have a therapeutic immunomodulatory effect *in vivo*. Our results support the notion that, with a careful selection of patients, peptide analogs may be used to enhance the efficacy of peptide-based allergen immunotherapy.

The skillful technical assistance of Virpi Fisk is gratefully acknowledged. We thank Professor Emeritus Rauno Mäntyjärvi, MD, PhD, for critical review of the manuscript.

REFERENCES

1. Norman PS, Ohman JL Jr, Long AA, Creticos PS, Gefter MA, Shaked Z, et al. Treatment of cat allergy with T-cell reactive peptides. *Am J Respir Crit Care Med* 1996;154:1623-8.
2. Muller U, Akdis CA, Fricker M, Akdis M, Blesken T, Bettens F, et al. Successful immunotherapy with T-cell epitope peptides of bee venom phospholipase A2 induces specific T-cell anergy in patients allergic to bee venom. *J Allergy Clin Immunol* 1998;101:747-54.
3. Pene J, Desroches A, Paradis L, Lebel B, Farce M, Nicodemus CF, et al. Immunotherapy with Fel d 1 peptides decreases IL-4 release by peripheral blood T cells of patients allergic to cats. *J Allergy Clin Immunol* 1998;102:571-8.
4. Oldfield WL, Larche M, Kay AB. Effect of T-cell peptides derived from Fel d 1 on allergic reactions and cytokine production in patients sensitive to cats: a randomised controlled trial. *Lancet* 2002;360:47-53.
5. Fellrath JM, Kettner A, Dufour N, Frigerio C, Schneeberger D, Leimgruber A, et al. Allergen-specific T-cell tolerance induction with allergen-derived long synthetic peptides: results of a phase I trial. *J Allergy Clin Immunol* 2003;111:854-61.
6. Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. *Lancet* 2001;358:1749-53.
7. Prakken BJ, Samodal R, Le TD, Giannoni F, Yung GP, Scavulli J, et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. *Proc Natl Acad Sci U S A* 2004;101:4228-33.
8. Sloan-Lancaster J, Allen PM. Altered peptide ligand-induced partial T cell activation: molecular mechanisms and role in T cell biology. *Annu Rev Immunol* 1996;14:1-27.
9. Nicholson LB, Greer JM, Sobel RA, Lees MB, Kuchroo VK. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity* 1995;3:397-405.
10. Brocke S, Gijbels K, Allegretta M, Ferber I, Piercy C, Blankenstein T, et al. Treatment of experimental encephalomyelitis with a peptide analogue of myelin basic protein. *Nature* 1996;379:343-6.

11. Alleva DG, Gaur A, Jin L, Wegmann D, Gottlieb PA, Pahuja A, et al. Immunological characterization and therapeutic activity of an altered-peptide ligand, NBI-6024, based on the immunodominant type 1 diabetes autoantigen insulin B-chain (9-23) peptide. *Diabetes* 2002;51:2126-34.
12. Janssen EM, van Oosterhout AJ, van Rensen AJ, van Eden W, Nijkamp FP, Wauben MH. Modulation of Th2 responses by peptide analogues in a murine model of allergic asthma: amelioration or deterioration of the disease process depends on the Th1 or Th2 skewing characteristics of the therapeutic peptide. *J Immunol* 2000;164:580-8.
13. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* 2000;6:1167-75.
14. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med* 2000;6:1176-82.
15. Zeiler T, Mantyjarvi R, Rautiainen J, Rytönen-Nissinen M, Vilja P, Taivainen A, et al. T cell epitopes of a lipocalin allergen colocalize with the conserved regions of the molecule. *J Immunol* 1999;162:1415-22.
16. Kinnunen T, Buhot C, Narvanen A, Rytönen-Nissinen M, Saarelainen S, Pouvelle-Moratille S, et al. The immunodominant epitope of lipocalin allergen Bos d 2 is suboptimal for human T cells. *Eur J Immunol* 2003;33:1717-26.
17. Kinnunen T, Kwok WW, Narvanen A, Rytönen-Nissinen M, Immonen A, Saarelainen S, et al. Immunomodulatory potential of heteroclitic analogs of the dominant T-cell epitope of lipocalin allergen Bos d 2 on specific T cells. *Int Immunol* 2005;17:1573-81.
18. Turvy DN, Blum JS. Detection of biotinylated cell surface receptors and MHC molecules in a capture ELISA: a rapid assay to measure endocytosis. *J Immunol Methods* 1998;212:9-18.
19. Novak EJ, Liu AW, Nepom GT, Kwok WW. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J Clin Invest* 1999;104:R63-7.
20. Ponsaerts P, Van den Bosch G, Cools N, Van Driessche A, Nijs G, Lenjou M, et al. Messenger RNA electroporation of human monocytes, followed by rapid in vitro differentiation, leads to highly stimulatory antigen-loaded mature dendritic cells. *J Immunol* 2002;169:1669-75.
21. Kinnunen T, Taivainen A, Partanen J, Immonen A, Saarelainen S, Rytönen-Nissinen M, et al. The DR4-DQ8 haplotype and a specific T cell receptor Vbeta T cell subset are associated with absence of allergy to Can f 1. *Clin Exp Allergy* 2005;35:797-803.
22. Genevee C, Diu A, Nierat J, Caignard A, Dietrich PY, Ferradini L, et al. An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. *Eur J Immunol* 1992;22:1261-9.
23. Yoshida K, Arai T, Kaburaki J, Ikeda Y, Kawakami Y, Kuwana M. Restricted T-cell receptor beta-chain usage by T cells autoreactive to beta(2)-glycoprotein I in patients with antiphospholipid syndrome. *Blood* 2002;99:2499-504.
24. Wilton SD, Lim L, Dye D, Laing N, Bandstab: a PCR-based alternative to cloning PCR products. *BioTechniques* 1997;22:642-5.
25. Yamamoto K, Sakoda H, Nakajima T, Kato T, Okubo M, Dohi M, et al. Accumulation of multiple T cell clonotypes in the synovial lesions of patients with rheumatoid arthritis revealed by a novel clonality analysis. *Int Immunol* 1992;4:1219-23.
26. Sloan-Lancaster J, Evavold BD, Allen PM. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 1993;363:156-9.
27. Franco A, Southwood S, Arrhenius T, Kuchroo VK, Grey HM, Sette A, et al. T cell receptor antagonist peptides are highly effective inhibitors of experimental allergic encephalomyelitis. *Eur J Immunol* 1994;24:940-6.
28. Windhagen A, Scholz C, Hollsberg P, Fukaura H, Sette A, Hafler DA. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity* 1995;2:373-80.
29. Vergelli M, Hemmer B, Utz U, Vogt A, Kalbus M, Tranquill L, et al. Differential activation of human autoreactive T cell clones by altered peptide ligands derived from myelin basic protein peptide (87-99). *Eur J Immunol* 1996;26:2624-34.
30. Verhoef A, Lamb JR. Threshold signaling of human Th0 cells in activation and anergy: modulation of effector function by altered TCR ligand. *J Immunol* 2000;164:6034-40.
31. Santambrogio L, Lees MB, Sobel RA. Altered peptide ligand modulation of experimental allergic encephalomyelitis: immune responses within the CNS. *J Neuroimmunol* 1998;81:1-13.
32. Francis JN, Till SJ, Durham SR. Induction of IL-10+CD4+CD25+ T cells by grass pollen immunotherapy. *J Allergy Clin Immunol* 2003;111:1255-61.
33. Jutel M, Akdis M, Budak F, Aebischer-Casaulta C, Wrzyszc M, Blaser K, et al. IL-10 and TGF-beta cooperate in the regulatory T cell response to mucosal allergens in normal immunity and specific immunotherapy. *Eur J Immunol* 2003;33:1205-14.
34. Verhoef A, Alexander C, Kay AB, Larche M. T cell epitope immunotherapy induces a CD4+ T cell population with regulatory activity. *PLoS Med* 2005;2:e78.
35. Larche M, Akdis CA, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* 2006;6:761-71.
36. Ausubel LJ, Bieganowska KD, Hafler DA. Cross-reactivity of T-cell clones specific for altered peptide ligands of myelin basic protein. *Cell Immunol* 1999;193:99-107.
37. Haselden BM, Kay AB, Larche M. Immunoglobulin E-independent major histocompatibility complex-restricted T cell peptide epitope-induced late asthmatic reactions. *J Exp Med* 1999;189:1885-94.
38. Oldfield WL, Kay AB, Larche M. Allergen-derived T cell peptide-induced late asthmatic reactions precede the induction of antigen-specific hyporesponsiveness in atopic allergic asthmatic subjects. *J Immunol* 2001;167:1734-9.
39. Larche M. Inhibition of human T-cell responses by allergen peptides. *Immunology* 2001;104:377-82.
40. Ria F, van den Elzen P, Madakamuttil LT, Miller JE, Maverakis E, Sercarz EE. Molecular characterization of the T cell repertoire using immunoscope analysis and its possible implementation in clinical practice. *Curr Mol Med* 2001;1:297-304.