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Suboptimal recognition of a T cell epitope of the major dog allergen Can f 1 by human T cells

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ABSTRACT

We have previously proposed that mammalian lipocalin allergens are recognized suboptimally by the human immune system due to their homology with endogenous lipocalins. Here, we have characterized in detail the human T cell recognition of one of the previously identified T cell epitopes of the major dog allergen Can f 1, contained in peptide p105–120. A panel of peptide analogues (altered peptide ligands, APLs) of p105–120 was tested on two specific T cell clones restricted by different human leukocyte antigen (HLA) alleles. Interestingly, we identified for both of the clones several heteroclitic APLs that were capable of stimulating them at 10–30-fold lower concentrations than the natural peptide. Moreover, one of the heteroclitic APLs identified with the T cell clones, L115F, was observed to induce a stronger polyclonal T cell response than the natural allergen peptide from the peripheral blood mononuclear cells (PBMCs) of six Can f 1-allergic subjects studied. The heteroclitic APLs bound with the same affinity as p105–120 to common HLA-DR- and HLA-DP-alleles, suggesting that their improved stimulatory capacity is attributable to a more efficient T cell receptor (TCR) recognition rather than increased HLA binding. Collectively, our data suggest that p105–120 is recognized suboptimally by human T cells. This may contribute to the allergenicity of Can f 1.

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1. Introduction

By definition, allergens are proteins capable of inducing a Th2-deviated CD4+ T cell response leading to IgE synthesis in susceptible individuals. Despite the ever-growing information on allergens and their structure, the basis for their allergenicity has remained elusive. It seems clear, however, that only a limited subset of proteins is allergenic (Radauer et al., 2008). For some allergens, proteolytic activity appears to play a role in their allergenicity (Ghaemmaghami et al., 2001; Lamhamedi-Cherradi et al., 2008) whereas for some other allergens, glycan or lipid structures can mediate a Th2 adjuvant effect through dendritic cells (DCs) (Traidl-Hoffmann et al., 2005; Shreffler et al., 2006). Nevertheless, no structural or functional property has been identified to explain the allergenic capacity of the vast majority of allergens.

Mammalian-derived proteins are important respiratory allergens. With the exception of the cat allergen Fel d 1, all currently identified significant mammalian allergens belong to the lipocalin family of proteins (Virtanen and Kinnunen, 2008). Lipocalins also exist as endogenous proteins in humans and several of them have a considerable degree of sequence identity (40–60%) with exogenous lipocalin allergens. Based on this observation, we have proposed that the presence of endogenous lipocalins could be a factor favoring the Th2 dominance of the immune response against exogenous lipocalin allergens (Virtanen et al., 1999).

The proliferative responses of peripheral blood mononuclear cells (PBMCs) of animal-allergic subjects to different mammalian lipocalin allergens, such as cow Bos d 2, dog Can f 1, horse Equ c 1 and rat Rat n 1, have consistently been observed to be weak (Zeiler et al., 1999; Kinnunen et al., 2005b; Immonen et al., 2007a; Jeal et al., 2004). One possible explanation for this finding is that the lipocalin allergen-specific T cell repertoire does not contain T

Abbreviations: APC, antigen-presenting cell; APL, altered peptide ligand; cpm, counts per minute; DC, dendritic cell; EC_{50}, concentration needed to obtain a half-maximal response; HLA, human leukocyte antigen; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; TCL, T cell line; TCR, T cell receptor.

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cells with a high T cell receptor (TCR) avidity, as a result of negative selection in the thymus by homologous self-peptides. Since low-avidity stimulation through the TCR has been observed to favor the Th2 differentiation of naïve T cells (Rogers and Croft, 1999; Brogdon et al., 2002), a T cell repertoire lacking high-avidity T cells could predispose the immune response against lipocalin allergens to be Th2-dominated. To support this hypothesis, we have previously observed that the natural immunodominant T cell epitope of the cow allergen Bos d 2 is suboptimally recognized by human T cells. Altered peptide ligands (APLs) of the epitope were observed to induce the proliferation and cytokine production of Bos d 2-specific T cell clones at 10–100-fold lower concentrations than the natural peptide (Kinnunen et al., 2003, 2005a). Moreover, one of these heteroclitic APLs has also been shown to induce a Th1-/Th0-deviated T cell response instead of a Th2-deviated response from the PBMCs of allergic subjects *in vitro* (Kinnunen et al., 2007) demonstrating that TCR recognition may play an important role in the development of an allergic immune response.

We have previously observed that the human T cell response against the major dog allergen, Can f 1, is directed against several epitopes (Immonen et al., 2005, 2007b). In this study, we examined in detail the human T cell recognition of one of the dominant epitopes of Can f 1 which is contained in the peptide 105–120 (p105–120). We found this epitope to be suboptimally recognized, as several heteroclitic APLs capable of stimulating specific human T cell clones at 10–30-fold lower concentrations than p105–120 were identified. Moreover, one of the heteroclitic APLs identified with the T cell clones, L115F, was found to have an increased capacity to induce a polyclonal T cell response and to expand p105–120-reactive T cells from the PBMCs of six dog-allergic subjects with different human leukocyte antigen (HLA) backgrounds *in vitro*. Collectively, our current data further support the hypothesis that the suboptimal T cell recognition of lipocalin allergens may contribute to the weak and Th2-deviated immune response against them.

2. Materials and methods

2.1. Subjects and antigens

Six dog-allergic subjects (A–F) with positive skin prick tests to Can f 1 (Kinnunen et al., 2005b) participated in this study. The study was approved by the Ethics Committee of Kuopio University Hospital, and written informed consent was given by all the subjects. Recombinant Can f 1 was produced in *Pichia pastoris* as previously described (Saarelainen et al., 2004). The Can f 1 peptide p105–120 (HGRQIRMAKLLGRDPE) and its single amino acid variants were synthesized and purified as previously described (Immonen et al., 2005). All peptides were readily soluble in water.

2.2. The generation of T cell clones and lines

The clones 2C9 and 5G2 were isolated from two p105–120-specific T cell lines from subject A by the limiting dilution method as previously described (Immonen et al., 2005). The clonality of the clones was proven by PCR using a panel of TCR Vβ subfamily-specific primers (Kinnunen et al., 2003). The TCR Vβ usage of the clones was Vβ8 for 2C9 and Vβ4 for 5G2.

For the generation of peptide-specific T cell lines (TCLs), PBMCs were separated by Ficoll Paque Plus density gradient centrifugation (GE Healthcare Bio-Sciences, Uppsala, Sweden) and seeded in 96-well round-bottomed plates (20 wells per peptide, 2×10^5 PBMCs per well) together with 5 μg/ml of the peptides. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, 20 μM 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (all from Lonza, Verviers, Belgium) and 5% inactivated human AB serum (Sigma–Aldrich, St. Louis, MO). At day 5, half of the culture medium was replaced and 10 IU/ml of IL-2 (Strathmann Biotec, Hannover, Germany) was added to the cultures. At day 10, the cultures were restimulated with 1.5×10^5 autologous γ-irradiated (3000 RAD) PBMCs together with 5 μg/ml of the peptides and IL-2 (10 IU/ml). The restimulation cycle was completed as described above. At day 20, half of the cells in each well were split into two new daughter plates and stimulated with autologous γ-irradiated PBMCs with or without the peptides. After 3 days, proliferation was measured as described below and positive cultures with a stimulation index (cpm in the presence of a peptide divided by cpm in the absence of a peptide) >2 were restimulated with autologous γ-irradiated PBMCs, 5 μg/ml of the peptides and 25 IU/ml of IL-2 in 48-well plates. Thereafter, half of the culture medium was replaced with fresh medium and IL-2 every 2–3 days.

2.3. Proliferation assay

T cell proliferation assays were set up in triplicates (T cell clones) or duplicates (T cell lines) in 96-well round-bottomed plates with 2.5×10^4 T cells, 5×10^4 autologous γ-irradiated PBMCs and antigens at different concentrations. After 3 days, [^{3}H]thymidine was added (1 μCi per well; GE Healthcare, Little Chalfont, UK), and after an additional 16 h, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1540) and the results expressed as counts per minute (cpm). The functional TCR avidity of the T cell clones and T cell lines was assessed by determining the concentration needed to obtain a half-maximal response (EC_{50}) from the dose response curves. The HLA restriction of the T cell clones was determined by inhibiting the response to p105–120 with monoclonal antibodies (mAbs) to HLA-DR (clone L243) or HLA-DQ (clone SPVL3) at a final concentration of 1 μg/ml. BLS-DQ0602, an EBV-transformed B lymphoblastoid cell line (B-LCL) transfected with HLA-DQA1*0102/DQB1*0602 (Ettinger et al., 1998), was kindly provided by Dr. William W. Kwok (Benaroya Research Institute at Virginia Mason, Seattle, WA).

2.4. Cytokine assay

Supernatants (100 μl per well) were collected from the proliferation assay plates at day 3 and were stored at −70 °C until analyzed. For the T cell clones, the concentrations of IL-5, IFN-γ and IL-10 were measured in duplicate using commercial ELISA kits (DuoSet; R&D Systems, Minneapolis, MN). For the T cell lines, the concentrations of IL-4, IL-5, IFN-γ, IL-10 and IL-17 were measured by the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

2.5. Flow-cytometric analyses

The different Vβ subtypes expressed by the T cell lines were analyzed with the IOTest Beta Mark kit (Immunotech, Marseille, France), as previously described (Kinnunen et al., 2005b). TCR Vβ expansions were considered significant when more than 10% of the cells expressed a particular Vβ subtype and the level of expression was at least two times greater than the expression in PBMC.

2.6. HLA class II peptide-binding assays

HLA-DR and HLA-DP4 molecules were immunopurified from homologous EBV cell lines by affinity chromatography using the monomorphic mAbs L243 and B7/21, respectively (Texier et al., 2000, 2001; Castelli et al., 2002). The binding to HLA-DR and HLA-DP4 molecules was assessed by competitive ELISA as previously

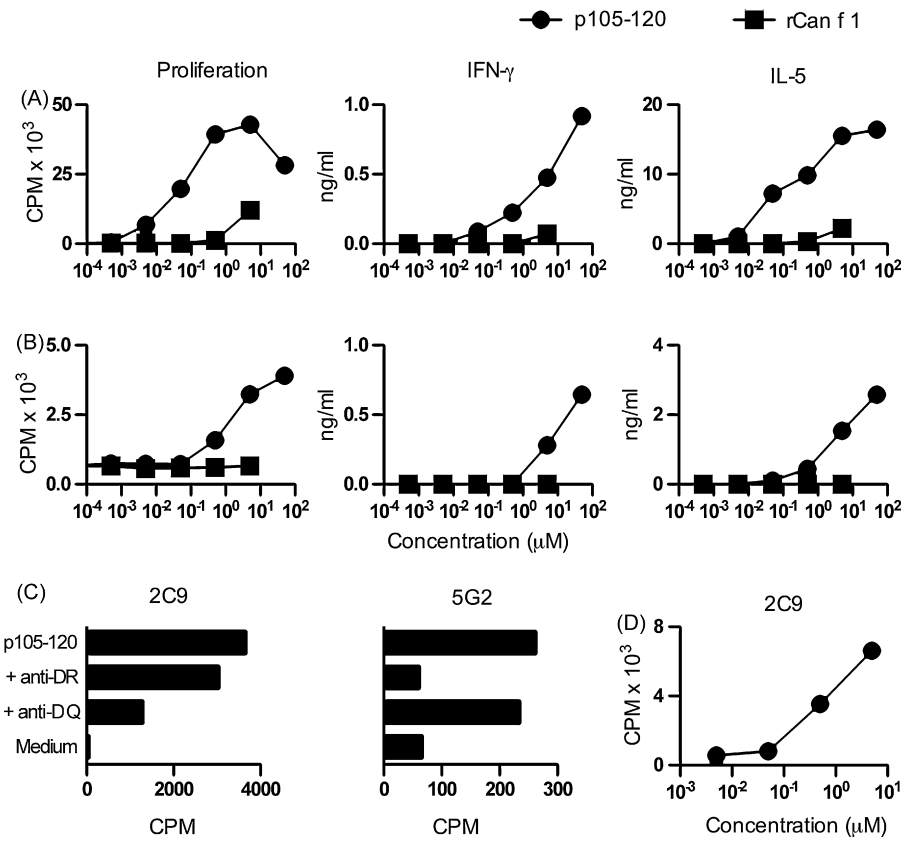


Fig. 1. Proliferative and cytokine responses of the clones 2C9 (A) and 5G2 (B) upon stimulation with p105–120 and rCan f 1 at different concentrations. The proliferative responses are presented as mean cpm of triplicate wells and the cytokine responses as mean concentrations of duplicate measurements. (C) DR/DQ restriction of the clones 2C9 and 5G2. p105–120 was used at 5 μM and the HLA-DR- and -DQ-antibodies at 1 μg/ml. (D) Proliferative response of clone 2C9 upon stimulation with different concentrations of p105–120 with BLS-DQ0602 cells as APCs. The results are representative of two independent experiments.

reported (Texier et al., 2000, 2001; Castelli et al., 2002). Binding specificity for each HLA class II allele was ensured by the choice of the biotinylated peptides as previously described (Texier et al., 2001; Castelli et al., 2002). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment.

2.7. Statistical analyses

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA). Fisher's exact test was used to analyze the difference between the numbers of TCLs induced with p105–120 or APLs and the Mann–Whitney *U*-test to analyze the difference in the cytokine production between the TCLs. *p* values of 0.05 or less were considered significant.

3. Results

3.1. Characteristics of the p105–120-specific T cell clones 2C9 and 5G2

In general, lipocalin allergen-specific T cell clones exhibit limited capacity to survive and expand *in vitro* (Kinnunen et al., 2003; Immonen et al., 2005). Two T cell clones with sustained expansion capacity, 2C9 and 5G2, specific for the Can f 1 peptide p105–120 were isolated from the peripheral blood of Can f 1-allergic subject A (HLA-DRB1*1101, *1501 and DQB1*0301, *0602). The dose–response curves of the clones for proliferation and for IFN-γ and IL-5 production are shown in Fig. 1A and B. Clone 2C9 appeared to recognize p105–120 with a high functional avidity (EC_{50} 0.05 μM, corresponding to 0.1 μg/ml) whereas the avidity of clone 5G2 was considerably lower (EC_{50} 1.3 μM, corresponding to 2.4 μg/ml). Clone 2C9 also recognized the whole Can f 1 protein, if only at high concentrations (Fig. 1A). Clone 5G2, in contrast, was not able to recognize Can f 1 at the concentrations tested, possibly because of its low functional avidity for p105–120 (Fig. 1B).

Both clones produced higher levels of IL-5 than IFN-γ (Fig. 1A and B) and neither of them produced notable amounts of IL-10 (above 0.2 ng/ml, data not shown). Based on their cytokine production pattern, clone 2C9 can be regarded as Th2-like (IL-5/IFN-γ ratio >10) and clone 5G2 as Th0-/Th2-like (IL-5/IFN-γ ratio ~5). The proliferative response of clone 2C9 to p105–120 could be inhibited by anti-HLA-DQ mAb but not with HLA-DR mAb, and the response of clone 5G2 by anti-HLA-DR mAb but not with HLA-DQ mAb (Fig. 1C). The genotyping of subject A's DQ-locus revealed two alleles, DQB1*0301 and DQB1*0602. Subsequent proliferation tests using allogeneic PBMCs expressing either DQB1*0301 or DQB1*0602 suggested that the response of clone 2C9 against p105–120 was restricted by DQB1*0602 (data not shown). The finding was confirmed by using the DQB1*0602-transfected cell line BLS-DQ0602 as antigen-presenting cells (APCs) (Fig. 1D). The exact HLA-DR-restricting element of clone 5G2 could not be reliably confirmed due to its weak proliferative capacity (data not shown).

3.2. Determination of the core residues recognized by p105–120-specific T cell clones

To determine the core residues of the T cell epitope recognized by the p105–120-specific T cell clones 2C9 and 5G2,

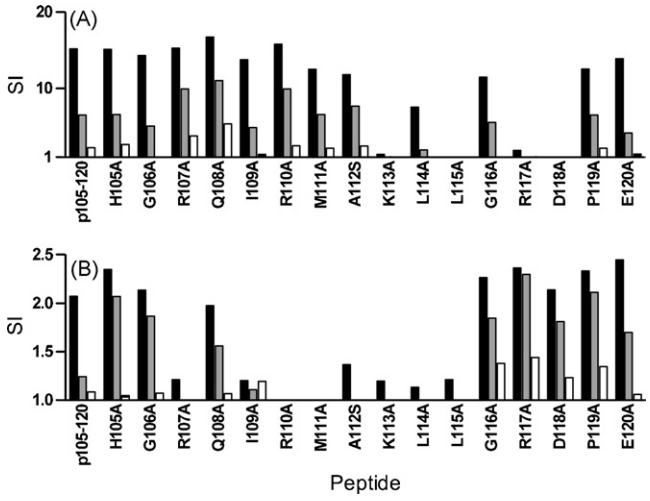


Fig. 2. Proliferative responses of the clones 2C9 (A) and 5G2 (B) to alanine- (or serine-) substituted APLs of p105–120. The peptide concentrations from left to right are 0.1, 0.01 and 0.001 μg/ml for 2C9 and 10, 1 and 0.1 μg/ml for 5G2, respectively. The results are expressed as stimulation indices (SI) and are representative of two independent experiments.

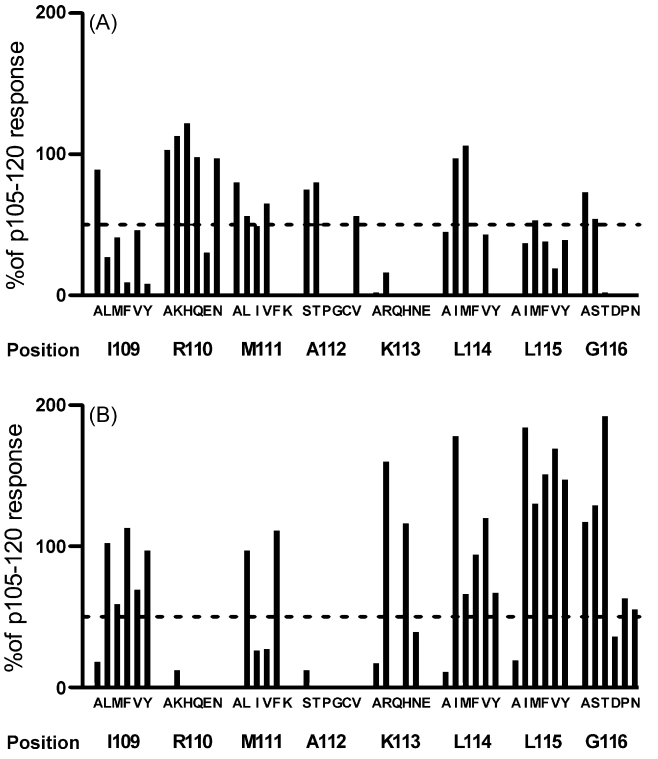


Fig. 3. Proliferative responses of the clones 2C9 (A) and 5G2 (B) to APLs of p105–120 containing conservative or semiconservative amino acid substitutions were tested at several peptide concentrations. The results are presented as percentages of the proliferative response against p105–120 at 0.1 μg/ml for 2C9 and at 10 μg/ml for 5G2, respectively. Responses lower than 50% of that to p105–120 correspond to at least a 10-fold increase in EC_{50}. The results are representative of three independent experiments.

their proliferative responses to a panel of alanine- (or serine-) substituted peptides were analyzed. The clones were stimulated with several concentrations of the peptides (0.001–0.1 μg/ml for 2C9 and 0.1–10 μg/ml for 5G2) to determine tolerated or non-tolerated amino acid substitutions (Fig. 2). Clone 2C9 appeared not to tolerate substitutions at the residues 113, 115, 117 and 118 whereas the response of clone 5G2 was abrogated by substitutions at the residues 107 and 109 to 115 (Fig. 2). The results with the DR-restricted clone 5G2 are in accordance with our previous results (Immonen et al., 2005) and the prediction by ProPred (Singh and Raghava, 2001), which suggest that the HLA-DR-binding motif of p105–120 is between the residues 109 and 117 (IRMAKLLGR). Based on the results obtained with clone 2C9 it seems that the DQB1*0602-binding motif of p105–120 is different, but due to the limited information on DQB1*0602-binding (Database Syfpeithi; http://www.syfpeithi.de/) it cannot be predicted more accurately.

3.3. p105–120-specific T cell clones recognize the Can f 1 T cell epitope suboptimally

The proliferative responses of the clones 2C9 and 5G2 were further analyzed with additional peptide analogues of p105–120 containing conservative or semiconservative substitutions between the residues 109 and 116 (Fig. 3). Clone 2C9 tolerated no amino acid substitutions for K113 and clone 5G2 for R110 or A112, whereas at least one conservative substitution was tolerated for the other core residues. Overall, the pattern of reactivity to the analogues suggests that the T cell receptors of the clones 2C9 and 5G2 recognize different residues within p105–120.

Interestingly, a total of 4 and 10 heteroclitic APLs were identified for the clones 2C9 and 5G2, respectively. Substitution of K, A, H, or N for R110 resulted in at least 10-fold decrease in EC_{50} for clone 2C9. A similar phenomenon was observed for clone 5G2 with substitutions of R for K113, I for L114, F, I, M, V, or Y for L115 and T, A or S for G116. The proliferative responses of the clones to the most potent heteroclitic APLs for each residue are shown in Fig. 4. Stimulation with the heteroclitic APLs also lead to the production of IFN-γ and IL-5 at lower peptide concentrations than with p105–120, but no qualitative change in the cytokine profile of the clones could be observed (data not shown).

3.4. Stimulatory capacity of the heteroclitic APLs of p105–120 on polyclonal T cells

Next, we wanted to examine whether the heteroclitic APLs identified with the two T cell clones also had an increased stimulatory capacity on T cells from dog-allergic subjects in general. For this, specific long-term T cell lines (TCLs) were generated from the PBMCs of six Can f 1-allergic subjects with different HLA genotypes using p105–120 and the heteroclitic APLs R110K, K113R, L114I, L115F and G116T (Table 1). The allergen peptide p105–120 was able to induce specific TCLs from only two out of six subjects (Table 1; subjects A and B). This is not surprising since we have previously shown that all Can f 1-allergic subjects do not mount a T cell response to the epitope contained within p105–120 (Immonen et al., 2005). Interestingly, the heteroclitic APLs were more potent than p105–120 in inducing specific TCLs. The most stimulatory APL, L115F, was able to induce specific TCLs in all six subjects (Table 1) and the total number of TCLs induced with it was significantly greater than that induced by p105–120 (19 and 4, respectively, $p = 0.002$). The APLs R110K, K113R and G116T also appeared to be slightly more stimulatory than p105–120, each inducing specific T cell lines from five out of six allergic subjects. However, the number of TCLs induced with each of these peptides was not statistically significantly greater than that induced by p105–120 ($p > 0.05$; Table 1). In contrast, L114I appeared to be weaker than p105–120, as it induced specific T cell lines from only one of the subjects.

The capacity of the APL-induced TCLs to recognize the natural allergen epitope was assessed by stimulating them with different concentrations of p105–120. Interestingly, the majority of the APL-induced TCLs appeared to be highly cross-reactive, as 31 of

Table 1
Number of peptide-induced TCLs obtained from each Can f 1-allergic subject (per 20 replicate wells seeded) and the subjects' HLA class II genotypes.

Subject	T cell line induced with						HLA class II genotype	
	p105–120	R110K	K113R	L114I	L115F	G116T	DRB1	DQB1
A	3	1	3	–	8	1	1101,1501	0301,0602
B	1	2	3	–	2	1	0101,1301	0501,0603
C	–	2	2	–	2	–	0101,1501	0501,0602
D	–	1	1	–	1	3	0101,1301	0501,0603
E	–	1	–	2	4	1	1302,1501	0602,0604
F	–	–	2	–	2	1	0801,0901	0303,0402
Total	4	7	11	2	19[a]	7		

[a] $p = 0.002$ as compared with p105–120.

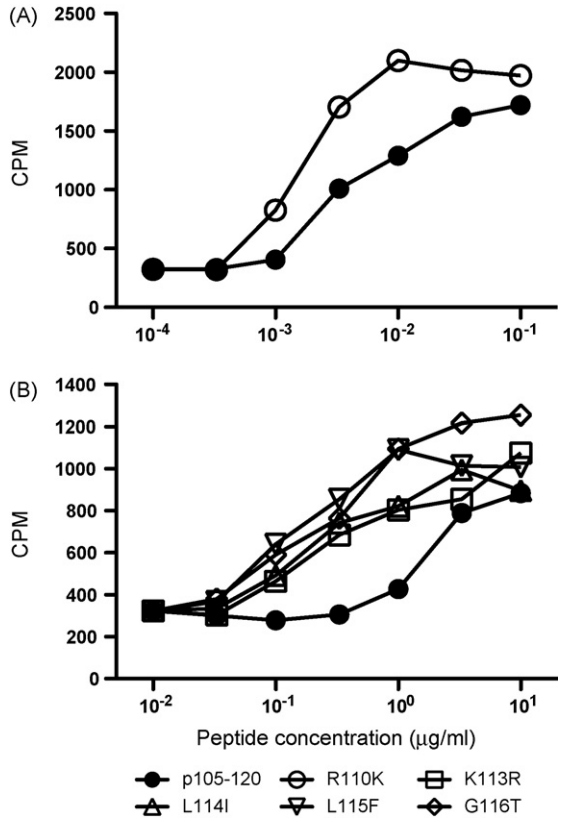


Fig. 4. Proliferative response of clone 2C9 to p105–120 and R110K (A) and clone 5G2 to p105–120, K113R, L114I, L115F and G116T (B) at different peptide concentrations. The results are representative of three independent experiments.

the 46 TCLs showed reactivity to p105–120 (Table 2). Most of the cross-reactive responses were of similar or lower functional avidity and only 2 TCLs recognized p105–120 with a higher avidity than the APL they were induced with. Importantly, 13 of the 19 TCLs induced with L115F were cross-reactive and more than half of these TCLs (7 out of 13) recognized p105–120 with a lower avidity (Table 2). These results demonstrate that L115F could expand p105–120-reactive T cells more efficiently than the natural peptide *in vitro* (13 TCLs (Table 2) vs. 4 TCLs (Table 1), $p = 0.04$) and that the majority of the induced TCLs recognized p105–120 suboptimally.

Table 2
Functional avidity of the APL-induced T cell lines for p105–120.

Avidity for p105–120[a]	T cell line induced with					
	R110K	K113R	L114I	L115F	G116T	Total
Higher	–	1	–	1	–	2
Similar	3	3	2	5	2	15
Lower	2	4	–	7	1	14
None	2	3	–	6	4	15

[a] Functional avidity was evaluated by EC_{50} ratios (EC_{50} of p105–120/EC_{50} of the APL used to induce the TCL). Lower avidity, EC_{50} ratio > 10; similar avidity, $0.1 < EC_{50}$ ratio < 10; higher avidity, EC_{50} ratio < 0.1.

3.5. Characteristics of the APL-induced T cell lines

The APL-induced TCLs displayed variability in their TCR Vβ usage with no predilection for any Vβ subtypes. Thirteen of the 24 Vβ subtypes analyzed were expanded in at least one of the TCLs (data not shown). Moreover, most of these Vβ subtype expansions were observed in TCLs induced with different APLs. These findings suggest that the APL-induced T cells are polyclonal and use a broad repertoire of different TCR structures.

The cytokine response of the TCLs was generally very weak with IL-5 and IFN-γ predominating (Table 3). Detectable levels of IL-17 were not observed with any of the TCLs (data not shown). There was a tendency for slightly stronger cytokine responses, in particular with IL-5, in the APL-induced TCLs than in those induced with p105–120, although this did not reach a statistical significance ($p > 0.05$). Moreover, there appeared to be slight variability in the cytokine profiles of TCLs induced with different APLs. The TCLs induced with K113R and L114I produced predominantly IL-5 whereas the TCLs induced with R110K, L115F and G12T appeared to produce both IL-5 and IFN-γ at similar levels.

3.6. Binding of the APLs of p105–120 to HLA class II alleles

To determine whether the enhanced T cell responses induced with the heteroclitic APLs were due to increased HLA binding, the binding capacities of the APLs to a panel of common DR and DP alleles were measured (Table 4). As previously reported (Immonen et al., 2005), p105–120 binds to several HLA alleles with the strongest binding (IC_{50} ratio <10) observed with DRB1*0301, DRB1*1101, DRB1*1301, DRB4 and DRB5. The APLs appeared to bind to the HLA alleles examined with a similar affinity as the natural peptide. Only minor alterations (less than 6-fold) in the IC_{50} ratios were observed as compared with p105–120, with two exceptions: L115F was observed to bind to DRB4 with a slightly improved affinity (a 12-fold difference) and G116T to DRB1*1101 with a decreased affinity (a 36-fold difference). Collectively, these data strongly suggest that the heteroclitic stimulatory capacity of the APLs on the T cell clones is due to a more efficient TCR recognition rather than increased HLA binding. Moreover, alterations in the HLA binding are not sufficient to explain the increased frequency of TCLs induced with the APL L115F, since only one of the subjects (subject F) had the DRB4 gene.

Table 3
Cytokine production by the peptide-induced TCLs[a].

	T cell line induced with					
	p105–120	R110K	K113R	L114I	L115F	G116T
IL-4	1.1 ± 0.3	6.3 ± 5.0	9.1 ± 3.5	17.6 ± 7.9	11.1 ± 6.4	3.8 ± 1.1
IL-5	6.0 ± 2.6	198.8 ± 145.0	314.5 ± 121.9	785.0 ± 277.5	122.1 ± 67.0	128.1 ± 111.5
IFN-γ	63.9 ± 45.6	157.1 ± 67.4	47.1 ± 18.9	14.3 ± 8.2	206.1 ± 139.9	152.2 ± 93.2
IL-10	24.4 ± 20.1	22.5 ± 14.3	12.3 ± 5.8	70.4 ± 17.4	36.5 ± 18.7	71.2 ± 29.2

[a] Results are shown as mean concentrations (pg/ml) ± SEM.

Table 4
Binding capacities of p105–120 and its heteroclitic APLs to HLA class II alleles[a].

Peptides	DRB1 alleles							DRB3	DRB4	DRB5	DP0401	DP0402
	0101	0301	0401	0701	1101	1301	1501					
p105–120	46	2	316	467	0.5	1	123	>447	6	1	1,000	456
R110K	126	2	>1291	>816	2	2	257	>447	16	2	>1581	>2184
K113R	30	4	61	267	0.2	0.5	62	>447	2	0.5	397	85
L114I	37	2	90	400	1	1	86	>447	5	1	>1581	1080
L115F	8	4	155	300	1	0.5	58	>447	0.5	0.5	887	>2184
G116T	45	3	548	600	18	0.5	102	>447	4	0.5	1125	>2184

[a] The binding capacity of the peptides to HLA class II alleles was determined as described in Section 2. The results are expressed as IC_{50} ratios (ratio between the IC_{50} value and the IC_{50} of the reference peptide) to take into account the differences in sensitivity of the binding assays. The IC_{50} values for the reference peptides were: DRB1*0101, 2 nM; DRB1*0301, 200 nM; DRB1*0401, 8 nM; DRB1*0701, 12 nM; DRB1*1101, 5 nM; DRB1*1301, 73 nM; DRB1*1501, 46 nM; DRB3, 22 nM; DRB4, 17 nM; DRB5, 3 nM; DPB1*0401, 6 nM; DPB1*0402, 5 nM.

4. Discussion

In the current study, one of the natural allergen epitopes of Can f 1, contained in peptide p105–120, was found to be suboptimal for human T cells. Altogether 14 APLs able to induce proliferation and cytokine production at 10–30-fold lower concentrations than the natural allergen peptide were identified for two p105–120-specific T cell clones restricted by different HLA alleles. Importantly, one of these heteroclitic APLs, L115F, was shown to induce a stronger T cell response and to expand p105–120-reactive T cells more efficiently than the natural peptide from the PBMCs of six dog-allergic subjects with different HLA backgrounds *in vitro*. The heteroclitic APLs bound with the same affinity as p105–120 to a panel of common HLA-DR- and HLA-DP-alleles suggesting that their improved stimulatory capacity is attributable to a more efficient TCR recognition rather than increased HLA binding.

We have previously identified heteroclitic APLs for two DR4-restricted T cell clones specific to the immunodominant epitope of Bos d 2, a lipocalin allergen from cow dander (Kinnunen et al., 2003). Here, we extend these findings to two additional T cell clones, restricted by two separate HLA alleles, specific to another mammalian lipocalin allergen, Can f 1. Together, these studies suggest that the occurrence of heteroclitic APLs for lipocalin allergen epitopes is not an isolated phenomenon. It is of interest to note that heteroclitic APLs have readily been identified for the epitopes of self-antigens, such as myelin basic protein (Hemmer et al., 1997, 1998) and proteolipid protein (Nicholson et al., 1998). In contrast, little improvement over the natural ligand has been observed for the epitopes of true foreign antigens, such as influenza virus hemagglutinin (Zhao et al., 2001). Interestingly, influenza virus hemagglutinin-specific T cells have been demonstrated to be generally of higher avidity than those specific to myelin basic protein (Bielekova et al., 2004). Therefore, it is possible that heteroclitic APLs are more common for T cells that recognize their ligand with a low avidity than for those T cells that recognize their ligand with a high avidity. In this study, more heteroclitic APLs were identified for the lower avidity clone 5G2 than for the higher avidity clone 2C9 (10 and 4, respectively) supporting the idea that there is an inverse correlation between T cell avidity and the probability of identifying heteroclitic APLs.

Five of the most potent heteroclitic APLs identified with the two T cell clones were tested for their capacity to induce a polyclonal T cell response from the PBMCs of six Can f 1-allergic subjects with a heterogeneous HLA background. Four of these APLs were more potent than p105–120 in inducing specific TCLs. Each of the APLs R110K, K113R, L115F and G116T induced specific TCLs from five to six subjects whereas specific TCLs were induced from only two subjects with p105–120 (Table 1). However, one of the APLs, L114I, induced a T cell response in only one of the subjects demonstrating that not all amino acid substitutions lead to an enhanced T cell response at the polyclonal level. Importantly, the total number of specific TCLs induced with one of the APLs, L115F, was statistically significantly higher than that induced with p105–120 (19 TCLs vs. 4 TCLs, $p = 0.002$). Moreover, 13 of the 19 TCLs induced with L115F were cross-reactive with p105–120 demonstrating that L115F could expand p105–120-reactive T cells more efficiently than the natural peptide *in vitro* (13 TCLs vs. 4 TCLs, $p = 0.04$). Since most of the L115F-induced TCLs recognized p105–120 with a lower avidity, the increased frequency of p105–120-reactive TCLs may be explained by the enhanced capacity of L115F to activate low-avidity p105–120-specific T cells.

In previous studies, the proliferative responses of PBMCs of allergic subjects to mammalian lipocalin allergens, such as cow Bos d 2, dog Can f 1, horse Equ c 1 and rat Rat n 1, have been observed to be weak (Zeiler et al., 1999; Kinnunen et al., 2005b; Immonen et al., 2007a; Jeal et al., 2004). One possible explanation for this phenomenon is that the lipocalin allergen-specific T cells recognize allergen peptide–HLA complexes on APCs with a weak avidity. Our current results with Can f 1-specific T cell clones and those obtained previously with Bos d 2-specific clones (Kinnunen et al., 2003) directly support the idea of suboptimal recognition of lipocalin allergen epitopes by specific T cells. Here, we extend these findings to polyclonal T cells. The increased frequency of p105–120-reactive TCLs induced by the APLs, especially by L115F, suggests that the natural allergen peptide is a suboptimal ligand for a large number of peripheral blood T cells from several dog-allergic subjects. Our current results leave exact mechanisms for this to be elucidated but they suggest that weak T cell recognition rather than weak HLA binding of p105–120 is implicated in the phenomenon. To address this question in more detail, the frequency and avidity

of p105–120- and APL-specific T cells in the peripheral blood needs to be analyzed more directly in future experiments, for example, with HLA class II tetramers (Kinnunen et al., 2005a, 2007).

In addition to weak cellular proliferation, suboptimal antigen recognition can also be a factor affecting the quality of a T cell response. Weak stimulation through the TCR has been observed to favor the Th2 differentiation of naïve T cells whereas stronger stimulation leads to Th1 dominance (Rogers and Croft, 1999; Brogdon et al., 2002). Activation of DCs by TLR signaling can compensate for a weak TCR signal to induce Th1 deviation (Nembrini et al., 2006), but no such effect on DCs by lipocalin allergens has been observed (unpublished observations). Therefore, a T cell repertoire lacking high-avidity T cells for lipocalin allergens could predispose the immune response to be Th2-dominated. An interesting link between lipocalin allergen-specific low-avidity T cells and the allergenic capacity of the proteins could be offered by the evolutionary relatedness of humans and other mammals: it can be speculated that T cells recognizing lipocalin allergens with a high avidity would have been negatively selected in the human thymus by peptides homologous with those of exogenous lipocalin allergens (Virtanen et al., 1999; Platts-Mills, 2007).

In a recent study, an APL of the immunodominant epitope of Bos d 2 was shown to induce a Th1-deviated T-cell response cross-reactive with the natural allergen epitope *in vitro* (Kinnunen et al., 2007). Hypothetically, such APLs could be therapeutic *in vivo* through the induction of immune deviation or bystander suppression. In the current study, the majority of the TCLs, 31 out of 46, induced with the APLs of p105–120 were also shown to be cross-reactive with the natural epitope, an important prerequisite for therapeutic potential. Moreover, there appeared to be variability in the quality of the T cell response induced by different APLs. Two of the APLs induced a clearly Th2-deviated response with IL-5 production predominating, whereas the other three induced a more balanced Th0-type response (Table 3). Interestingly, different natural epitopes of Can f 1 and the cat allergen Fel d 1 have also been observed to induce qualitatively different T cell responses *in vitro*, with either IFN-γ or IL-10 production predominating (Immonen et al., 2007b; Reefer et al., 2004). Since deviation from Th2 to Th1 and the induction of IL-10 are considered to be important mechanisms in allergen immunotherapy (Jutel et al., 1995, 2003; Bellinghausen et al., 1997; Bohle et al., 2007), the identification of allergen epitopes or APLs with Th1- or Tr1-deviating properties may enable the development of more efficient modes of peptide-based immunotherapy.

In conclusion, we have demonstrated in this study that one of the natural T cell epitopes of the dog allergen Can f 1 is suboptimally recognized by human T cells. We identified heteroclitic APLs that were able to stimulate specific T cell clones at lower concentrations and to induce a stronger polyclonal T cell response *in vitro* than the natural allergen peptide. These data provide further evidence that suboptimal recognition of the T cell epitopes of lipocalin allergens may account for the weak and Th2-deviated T cell responses against them.

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