

The DR4–DQ8 haplotype and a specific T cell receptor V β T cell subset are associated with absence of allergy to Can f 1

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Summary

Background The significance of specific T cell receptor (TCR) V β subtypes and human leucocyte antigen (HLA) class II alleles for the development of allergy to lipocalin allergens such as the major dog allergen Can f 1 is not clear at present.

Objective To characterize the TCR V β usage in the Can f 1-specific T cell lines and the HLA class II genotypes of Can f 1-allergic and non-allergic subjects.

Methods T cell lines were induced with recombinant Can f 1 from the peripheral blood mononuclear cells of 12 non-atopic dog owners and 26 dog-allergic patients. Thirteen of the dog-allergic subjects were sensitized to Can f 1. Expression of the TCR V β subtypes on CD4⁺ T cells in the T cell lines was measured by flow cytometry. The subjects were HLA genotyped for DRB1, DQB1 and DPB1 loci.

Results Can f 1-specific T cell lines were obtained from 18 subjects, with either positive ($n = 8$) or negative ($n = 10$) skin prick tests (SPTs) to recombinant Can f 1. The frequency of TCR V β 5.1⁺ T cells was significantly higher in the T cell lines of subjects with negative SPTs to the allergen. Moreover, DR4–DQ8 haplotype was over-represented among these subjects.

Conclusion The DR4–DQ8 haplotype and the TCR V β 5.1⁺ CD4⁺ T cells may be protective against allergy to Can f 1.

Keywords animal allergy, Can f 1, HLA, T cell receptors, T lymphocytes

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Introduction

Several major animal-derived respiratory allergens belong to the lipocalin family of proteins [1]. Although the allergenicity of lipocalins is well established, its basis remains unresolved [2]. For some other allergens, the biological function, especially an enzyme activity, contributes to their allergenicity [3]. Two lipocalins, Bos d 5 (β -lactoglobulin) and tear lipocalin, have been reported to display non-specific endonuclease activity [4], but the effect of this on the allergenicity of these proteins is not known. Taking into account the heterogeneity of biological functions of known allergens, it seems clear, however, that the allergenicity of a protein cannot be assigned simply to its biological function [5]. Therefore, the immunological properties of an allergen, especially how it is recognized by the immune system, are likely to be important. In this context, it is of interest to note that lipocalins can exhibit considerable sequential homology between species. For example, the major dog allergen Can f 1 has a close to 60% amino acid identity with the human tear

lipocalin (the BLAST network service at the Swiss Institute of Bioinformatics, April 12, 2004 [6]). We have previously proposed that the presence of endogenous lipocalins might be a factor favouring the development of T helper type 2 (Th2)-type responses against exogenous lipocalin allergens [7].

The interaction between a peptide associated with the major histocompatibility complex (MHC) class II molecule on an antigen-presenting cell (APC) and the T cell receptor (TCR) on a CD4⁺ T cell is a crucial event for initiating an antigen-specific immune response. Although several factors, such as the genetic background [8], costimulation [9] or the cytokine milieu [10], all influence the outcome of immune response, the TCR–MHC interaction has been shown to be involved in determining the quality of the response. For example, high doses of antigen tended to induce Th1-type responses, whereas low doses tended to induce Th2-type responses [11]. Accordingly, the weak affinity of the TCR–MHC interaction has been observed to favour Th2-type responses [12–14]. Stimulation through TCR can also be important, in that it can lead to the restricted usage of particular TCR V β subtypes. T cell responses against some allergens have been observed to exhibit this feature [15–19].

In this study, we sought to establish whether the human T cell response against the dog major allergen Can f 1 shows a predilection for the usage of particular TCR V β subtypes

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and/or associates with particular human leucocyte antigen (HLA) class II antigens. We also wanted to discover whether these factors distinguish subjects sensitized to Can f 1 from non-sensitized subjects. We found that the DR4-DQ8 haplotype was preferentially expressed in subjects with negative skin prick tests (SPTs) to Can f 1. This phenomenon was associated with the expansion of TCR V β 5.1⁺ CD4⁺ T cells in the Can f 1-specific T cell lines of these subjects. These findings suggest that a certain MHC-TCR combination may protect from the development of allergy to Can f 1.

Materials and methods

Subjects

Twenty-six clinically diagnosed dog-allergic subjects with positive SPTs (≥ 3 mm) to a commercial dog dander preparation (ALK-Abellø, Hørsholm, Denmark) participated in this study. Thirteen of the dog-allergic subjects also had a positive SPT to recombinant (r)Can f 1 [20]. Twelve non-atopic dog owners were recruited as control subjects: their SPTs to both the commercial dog dander preparation and rCan f 1 were negative. The study was approved by the Ethics Committee of Kuopio University Hospital, and written informed consent was given by all the subjects.

T cell lines

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized peripheral blood by Ficoll Paque Plus (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. For the generation of allergen-specific T cell lines, PBMCs were stimulated with rCan f 1 at a concentration of 100 μ g/mL in 12-well plates (Corning Inc., Corning, NY, USA) at a density of 6×10^6 cells/well. The culture medium was RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 20 μ M 2-mercaptoethanol, sodium pyruvate (BioWhittaker), non-essential amino acids (BioWhittaker), 100 IU/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES (BioWhittaker) and 5% inactivated human AB serum (Finnish Red Cross, Helsinki, Finland). Phytohaemagglutinin (PHA; Murex Biotech, Dartford, UK) at a concentration of 100 ng/mL was used to establish control T cell lines.

On day 6, recombinant IL-2 (Strathmann Biotech, Hannover, Germany) was added to the cultures at a final concentration of 5 IU/mL. On day 9, the rIL-2 concentration was raised to 20 IU/mL. On day 14, the cells were harvested, washed and counted. Then, the cells were restimulated on 12-well plates at a density of 3×10^6 cells/well, together with 6×10^6 γ -irradiated (3000 rad) autologous PBMCs as feeder cells and with rCan f 1 or PHA as previously. The restimulation was completed as described above.

T cell proliferation assays

T cell proliferation assays were set up in triplicates in 96-well round-bottomed micro-titre plates (Corning Inc.) with 2×10^5 PBMCs or 5×10^4 T cells and 10^5 γ -irradiated (3000 rad) autologous PBMCs per well in complete RPMI 1640. rCan f 1 was added at a concentration of 100 μ g/mL to

the cultures. Recombinant (r) Bos d 2 [21] at a concentration of 100 μ g/mL was used as an irrelevant control. After an incubation of 5 days (PBMCs) or 72 h (T cell lines), [³H]thymidine was added (1 μ Ci/well; Amersham Pharmacia Biotech, Little Chalfont, UK), and after an additional 16 h, the cells were harvested on to glass fibre filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1450), and the results were expressed as counts per minute (c.p.m.). T cell lines were considered antigen specific when the stimulation index (SI: ratio between the mean c.p.m. of wells stimulated with APCs plus rCan f 1, and the mean c.p.m. of wells without the allergen) was greater than 1.5 and the difference of the proliferative response between the stimulated and unstimulated cultures was greater than 1000 c.p.m.

Analysis of T cell receptor V β expression by flow cytometry

TCR V β subtypes expressed on CD4⁺ T cells were assessed by flow cytometry using the IOTest Beta Mark kit (Immunotech, Marseille, France) reagents. The kit contains PE, FITC or PE-FITC double-labelled antibodies against the following V β subtypes: V β 1, V β 2, V β 3, V β 4, V β 5.1, V β 5.2, V β 5.3, V β 7.1, V β 7.2, V β 8, V β 9, V β 10, V β 11, V β 12, V β 13.1, V β 13.2, V β 14, V β 16, V β 17, V β 18, V β 20, V β 21.3, V β 22 and V β 23. For the gating of CD4⁺ cells, anti-CD4-PE-Cy5 (Immunotech) was used. Isotype control IgG₁-PE-Cy5 (Immunotech) was included in the analyses to determine the level of background staining (data not shown). Freshly isolated PBMCs or Can f 1-specific T cell lines were incubated with the anti-V β and anti-CD4 antibodies for 30 min at room temperature in the dark. After washing twice with phosphate-buffered saline (PBS), the cells were analysed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Five thousand CD4⁺ gated events were collected for the analysis. V β distribution was calculated and expressed as a percentage of CD4⁺ cells. The expansions of TCR V β subtypes in the T cell lines were regarded as significant when the level of expression was more than 5% and at least two times greater than the expression in PBMCs.

Human leucocyte antigen class II genotyping

HLA DRB1, DQB1 and DPB1 typings were performed using the standard Inno-LIPA reversed dot blot assays (Innogenetics, Ghent, Belgium).

Statistical analysis

Statistical differences between groups were determined with the Kruskal-Wallis test, and *post hoc* comparisons with Dunn's test (data in Figs 1 and 2) or the Mann-Whitney U-test (data in Fig. 3). Fisher's exact test was used to analyse the association between the occurrence of DR4-DQ8 haplotype and the expansion of V β 5.1⁺ T cells in the Can f 1-specific T cell lines. *P*-values < 0.05 were considered significant.

Results

Proliferative responses of peripheral blood mononuclear cells and T cell lines upon stimulation with rCan f 1

The proliferative responses of PBMCs of dog-allergic subjects with positive SPTs to rCan f 1 ($n = 13$), dog-allergic subjects with negative SPTs to rCan f 1 ($n = 13$) and non-atopic dog owners ($n = 12$) upon stimulation with rCan f 1 proved to be weak (Fig. 1a). After two cycles of propagation *in vitro* with rCan f 1, the proliferative responses of the T cell lines upon stimulation with the allergen were enhanced (Fig. 1b). Although both the PBMCs and T cell lines of dog-allergic

subjects with positive SPTs to rCan f 1 exhibited a tendency for slightly stronger proliferative responses in comparison with those of subjects from other groups, the differences were not statistically significant. According to the criteria adopted for a positive proliferative response, eight of the 13 T cell lines from dog-allergic subjects with positive SPTs to rCan f 1 were specific to the allergen. Four of the 13 T cell lines from dog-allergic subjects with negative SPTs to rCan f 1 and six of the 12 T cell lines from non-atopic control subjects were antigen specific. None of the T cell lines had a positive proliferative response to the irrelevant control allergen rBos d 2 (data not shown).

T cell receptor V β subtype distribution of peripheral blood CD4⁺ T cells

The TCR V β distribution of peripheral blood CD4⁺ T cells is shown in Fig. 2. Significantly higher frequencies of V β 21.3⁺ and V β 22⁺ T cells were observed in the peripheral blood of dog-allergic subjects with positive SPTs to rCan f 1 compared with non-atopic control subjects ($P < 0.05$ and $P < 0.01$, respectively). Other V β subtypes did not show statistically significant differences between the three groups.

Expression of T cell receptor V β subtypes in the T cell lines and its association with human leucocyte antigen class II genotypes

The expansions of TCR V β subtypes in the Can f 1-specific T cell lines and the HLA-DRB1, DQB1 and DPB1 genotypes of the subjects in the study are shown in Table 1. rCan f 1 was able to induce the expansion of 0–4 V β subtypes in each of the 18 Can f 1-specific T cell lines studied. Out of the 24 V β subtypes examined, 17 were significantly expanded in at least one of the T cell lines. When the subjects in the study were arranged into two groups according to their SPT reaction to rCan f 1, an expansion of V β 5.1⁺ T cells was observed in the Can f 1-specific T cell lines of five of the 10 subjects with negative SPTs to rCan f 1, but in none of the T cell lines of the eight subjects with positive SPTs to rCan f 1 and in none of the 18 PHA-induced control T cell lines (Table 1 and Fig. 3). In contrast, three T cell lines of the subjects with positive SPTs to rCan f 1 showed a strong expansion of V β 7⁺ T cells (Table 1). This TCR V β subtype was not expanded in any of

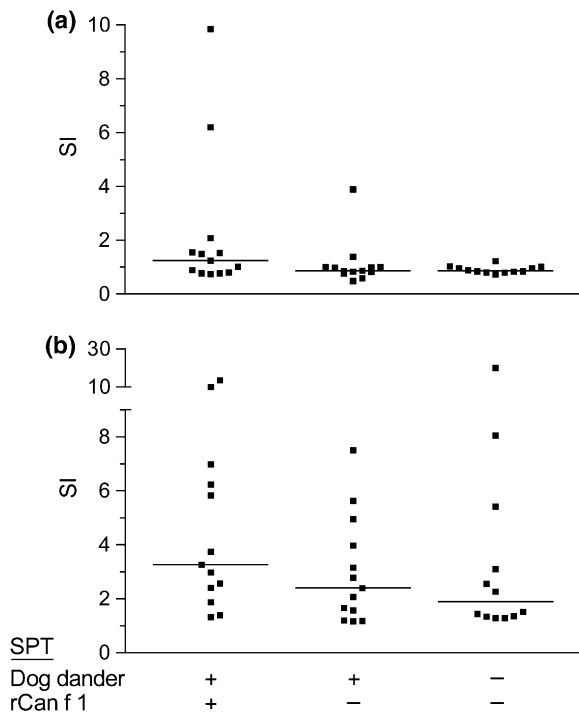


Fig. 1. Proliferative responses of peripheral blood mononuclear cells (PBMCs) (a) and rCan f 1-induced T cell lines (b) upon stimulation with rCan f 1. Results are expressed as stimulation indices (SI). There were no statistically significant differences between the groups ($P > 0.05$). Background proliferation varied between 474 and 4600 c.p.m. for PBMCs and between 20 and 4600 c.p.m. for the T cell lines.

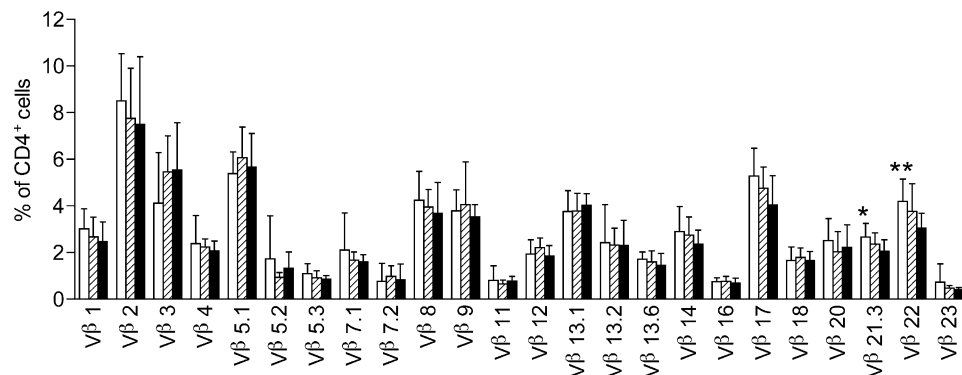


Fig. 2. Expression of T cell receptor V β subtypes on the peripheral blood CD4⁺ T cells of dog-allergic subjects with positive skin prick tests (SPTs) to rCan f 1 (open columns), dog-allergic subjects with negative SPTs to rCan f 1 (hatched columns) and non-atopic control subjects (filled columns). * $P < 0.05$ and ** $P < 0.01$ in comparison with non-atopic control subjects.

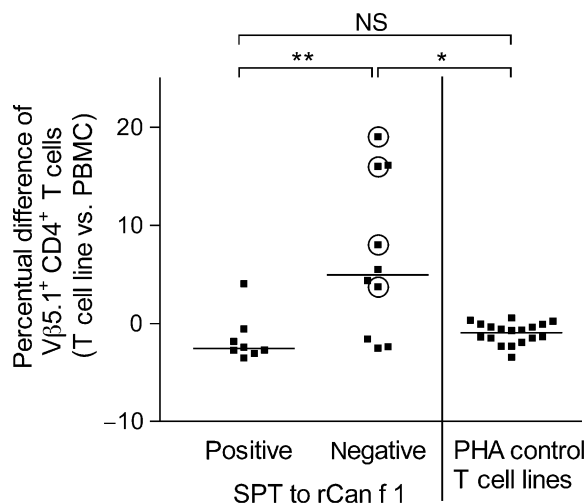


Fig. 3. Expansion of T cell receptor Vβ 5.1⁺ CD4⁺ T cells in the Can f 1-specific T cell lines of subjects with positive ($n = 8$) or negative ($n = 10$) SPTs to rCan f 1. Phytohaemagglutinin-induced T cell lines of the same subjects ($n = 18$) served as controls. Circled dots represent dog-allergic subjects with negative SPTs to rCan f 1. * $P < 0.05$ and ** $P < 0.01$, NS, non-significant.

the T cell lines of the subjects with negative SPTs to rCan f 1 (Table 1) or in any of the PHA-induced control T cell lines (data not shown).

The subjects with positive SPTs to rCan f 1 did not show accumulation of any HLA class II haplotype (Table 1). The subjects with negative SPTs to rCan f 1 had, however, a higher frequency of the haplotype HLA-DRB1*04-DQB1*0302 (DR4-DQ8). This was found in six out of the 10 subjects in this group, whereas only one of the eight subjects with positive SPTs to rCan f 1 had the same haplotype. Four of the subjects with the haplotype expressed the DRB1*0401 allele and three expressed the DRB1*0404 allele. Interestingly, the occurrence of the DR4-DQ8 haplotype was associated with the expansion of Vβ5.1⁺ T cells in the cell lines. Four of the five subjects with negative SPTs to rCan f 1 and a significant expansion of Vβ5.1⁺ T cells had the DR4-DQ8 haplotype ($P < 0.05$).

Discussion

Our results suggest that a certain TCR-MHC combination may be responsible for either an allergenic or non-allergenic T cell response against Can f 1. Three of the eight T cell lines from the subjects with positive SPTs to the allergen exhibited a strong expansion of Vβ7⁺ T cells (Table 1). Two of the three subjects had the same HLA-DRB1*0101-DQB1*0501 (DR1-DQ5) haplotype. Importantly, five of the 10 subjects with negative SPTs to rCan f 1 had a significant expansion of Vβ5.1⁺ T cells in their Can f 1-specific T cell lines. Four of these subjects had the DR4-DQ8 haplotype. This haplotype was also found in two additional subjects with negative SPTs to rCan f 1, whereas only one subject with a positive SPT to the allergen had this haplotype. To our knowledge, such a TCR Vβ-MHC association has not been observed with respect to lipocalin allergens, although such associations have been observed in responses against a

ragweed allergen, Amb a 5 [15], and against other antigens [22, 23]. It seems that we may have identified subjects with non-allergenic or protective T cell responses to Can f 1 among those exposed to the allergen. Our results are in accordance with those studies showing that in a subgroup of subjects, immune response to an allergen can provide protection against allergic sensitization [24, 25].

HLA associations with immune responsiveness to lipocalin allergens remain largely unexplored. However, the studies of Sakaguchi et al. [26] suggest that the HLA-DRB1*0405 allele is important for the T cell response to milk β-lactoglobulin, a lipocalin allergen. In another study, both positive (DR7) and negative (DR3) HLA associations were observed with sensitization to rat urine [27], the main allergen of which is Rat n 1, a lipocalin protein [28]. In this study, we did not find an association between a specific HLA class II allele and allergy to Can f 1, which is in accordance with findings in previous studies [29, 30]. In contrast, we observed that protection against sensitization to Can f 1 was associated with the HLA haplotype DR4-DQ8. This haplotype was clearly over-represented in subjects with negative SPTs to rCan f 1 (60%) but under-represented in subjects with positive SPTs to the allergen (13%), since the frequency of the HLA-DR4-DQ8 haplotype is approximately 20% in the general Finnish population (J. Partanen, unpublished data). DR4 is an intriguing allele since it has been shown to be positively associated with autoimmune diseases such as autoimmune diabetes [31] and rheumatoid arthritis [32] in Finnish patients. In contrast to autoimmune disorders, DR4 has been reported to have a protective effect against sensitization to various allergens, such as house dust mite (HDM) [33], mellitin and phospholipase A2 [34], mountain-cedar [35] and citrus red mite [36]. It is of interest to note that in a recent study, a protective response (modified Th2 response) to cat Fel d 1 was associated with DR7 [25]. Although not examined in detail, it has been suggested that the protective effect of an MHC allele might be mediated by allergen-specific regulatory T cells [34, 35]. The increased production of IL-10 by the T cells of DR7-positive subjects with a modified Th2 response upon *in vitro* stimulation with Fel d 1 peptides supports this view [25]. Nevertheless, our and other investigators' studies suggest that protection against allergic sensitization to an allergen can be provided by different HLA alleles.

The proliferative responses of PBMCs of dog-allergic patients and non-atopic control subjects upon stimulation with rCan f 1 were weak (Fig. 1a). In parallel with this, the induction of specific T cell lines with rCan f 1 turned out to be laborious, resulting in Can f 1-specific T cell lines with only a moderate proliferative capacity upon stimulation with the allergen (Fig. 1b). Both of these findings are in accordance with our previous results with the cow dander allergen Bos d 2, another lipocalin allergen [37]. Recently, the capacity of Rat n 1 to induce the proliferation of PBMCs from rat-allergic individuals was also observed to be weak [38]. Interestingly, the weak stimulatory capacity appears not to be restricted to lipocalins, as another mammalian allergen, cat Fel d 1, is also weakly stimulatory [39, 40]. To explain the previously mentioned observations concerning Bos d 2, we have suggested that the poor stimulatory capacity of Bos d 2 may be a characteristic associated with its allergenicity [7]. Our finding that human T cell clones specific for Bos d 2

Table 1. Significant TCR Vβ subtype expansions in the Can f 1-specific T cell lines indicated as percentages (expression in T cell line/expression in PBMC) and the HLA class II genotypes of the subjects with positive or negative SPTs to rCan f 1

HLA Class II genotype																															
TCR Vβ subtype																															
Subject	1	2	3	4	5.1	5.2	5.3	7.1	7.2	8	9	11	12	13.1	13.2	13.6	14	16	17	18	20	21.3	22	23	DRB1	DQB1	DPB1				
SPT ⁺	S. R.						7/1																		0701	0901	02	0301	0401	0402	
	T. R.						5/1		33/2																0101	1301	0501	0603	0402	1101	
	A. V.		19/9														9/2								0801	0901	0303	0402	0401	0402	
	I. N.																								0701	1101	02	0301	0401	0301	
	R. K.							28/2																	0101	1501	0501	0602	0401	0201	
	J. T.	7/3																							1101	1501	0301	0602	0401	0201	
	P. K.								10/2																0401	–	0302	–	0401	3301	
	M. S.																9/2		11/4				10/3		0101	1501	0501	0602	0301	–	
	M. V.*	5/2				15/7																			0404	0801	0302	0402	0201	0301	
SPT [–]	R. V.*					26/7						23/2	14/3												0101	1501	0501	0602	0402	0501	
	T. P.*			7/3																		7/2		9/3		1501	–	0602	–	0401	0201
	S. H.*	9/3				22/6											6/3								0401	–	0302	–	0401	3301	
	H. K.					23/6																			0401	0101	0302	0501	0401	0402	
	T. H.																					10/3			0404	15	0302	0602	0201	2301	
	H. V.																						24/2		0401	1501	0302	0602	0401	0201	
	M. T.																	7/2		9/4			8/1		1101	13	0301	0603	0401	–	
	S. S.					10/5				23/4															0404	0801	0302	0402	0201	0402	
	J. V.	6/3																							0101	0801	0501	0402	0301	0402	

*Dog-allergic subjects with negative SPTs to rCan f 1.
TCR, T cell receptor; PBMC, peripheral blood mononuclear cells; HLA, human leucocyte antigen; SPT, skin prick test.

recognized its immunodominant epitope in a suboptimal way points to the possibility that the weak recognition of the allergen contributes to its poor stimulatory capacity [41]. Whether the weak stimulatory capacity of Can f 1 is associated with a similar property remains to be elucidated.

The mean proliferative responses of the rCan f 1-induced T cell lines upon stimulation with the allergen were at similar levels in the three study groups, although there was a tendency for stronger responses in the T cell lines from subjects with positive SPTs to rCan f 1 (Fig. 1b). Several studies have shown that the proliferative responses of T cells of allergic and non-allergic individuals stimulated with allergens *in vitro* are similar [19, 42, 43]. However, we observed that half of the T cell lines from non-atopic controls did not proliferate at all when stimulated with rCan f 1, whereas almost all T cell lines from dog-allergic subjects proliferated at least weakly. This is in line with findings in a previous study in which a significant proportion of non-atopic control subjects showed no proliferative T cell response to Der p 1, whereas all atopic subjects showed a response against the allergen whether or not they were allergic to HDM [19].

A significantly higher frequency of V β 21.3⁺ and V β 22⁺ CD4⁺ T cells was observed in the peripheral blood of dog-allergic subjects with positive SPTs to rCan f 1 compared with non-atopic controls (Fig. 2). As the frequency of the V β subtypes was not further increased in the Can f 1-specific T cell lines of these subjects (Table 1), the greater frequency of the V β subtypes in the peripheral blood was probably not associated with allergy to Can f 1. It is conceivable that other factors, such as *in vivo* expansion of T cells reactive against other dog allergens or against other allergen sources or the atopic status of the subjects, explain the finding. This view is supported by the observation that dog-allergic subjects with negative SPTs to rCan f 1 also had a greater frequency of these V β subtypes in their peripheral blood than the non-atopic controls. Some studies have suggested that allergic subjects can have increased frequencies of allergen-specific T cells with particular V β subtypes in the peripheral blood [17, 19]. Beyer et al. [17] were able to show that this phenomenon was most probably associated with strong allergen exposure to birch, since birch pollen-sensitized individuals had higher frequencies of T cells with specific V β subtypes in their peripheral blood only during the pollen season.

In conclusion, the subjects with negative SPTs to rCan f 1 exhibited an increased frequency of TCR V β 5.1⁺ CD4⁺ T cells in their Can f 1-specific T cell lines, and this was associated with the HLA-DR4-DQ8 haplotype. This observation points to the possibility that a specific TCR-MHC combination may protect against sensitization to an allergen.

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