

Human memory CD4⁺ T cell response to the major dog allergen Can f 5, prostatic kallikrein

A. Kailaanmäki¹, T. Kinnunen¹, A. Rönkä¹, M. Rytönen-Nissinen^{1,2}, J. Lidholm³, L. Mattsson³, J. Randell⁴ and T. Virtanen¹

¹Department of Clinical Microbiology, Institute of Clinical Medicine and Biocenter Kuopio, University of Eastern Finland, Kuopio, Finland, ²Institute of Dentistry, School of Medicine, University of Eastern Finland, Kuopio, Finland, ³Thermo Fisher Scientific, Uppsala, Sweden and ⁴Department of Pulmonary Diseases, Kuopio University Hospital, Kuopio, Finland

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Summary

Background Human CD4⁺ T cell responses to important animal allergens are still insufficiently understood.

Objective To comprehensively characterize *in vitro* and *ex vivo* the peripheral blood memory CD4⁺ T cell responses of subjects with and without allergy to the major dog allergen Can f 5, the only known animal allergen in the kallikrein family of proteins.

Methods Can f 5-specific memory CD4⁺ T cell lines (TCLs) were established from the peripheral blood of 12 subjects with and 12 subjects without allergy to Can f 5 and characterized for their functional and phenotypic properties. The results were evaluated with those obtained *ex vivo* with a novel CD154 enrichment method. The epitopes recognized by the Can f 5-specific TCLs were determined with 72 overlapping 16-mer peptides covering the sequence of the allergen.

Results Can f 5-specific TCLs were obtained at about tenfold higher frequency from allergic than from non-allergic subjects. Functionally, the TCLs of allergic subjects displayed a Th2-biased cytokine phenotype and increased T cell receptor avidity, whereas the TCLs of non-allergic subjects displayed a Th1-/Th0-biased cytokine phenotype and lower TCR avidity. The higher frequency and the Th2 phenotype of Can f 5-specific memory CD4⁺ T cells in allergic subjects were confirmed by the CD154 enrichment method *ex vivo*. Six distinct T cell epitope regions of Can f 5 were predominantly recognized by the TCLs from allergic subjects.

Conclusions and Clinical Relevance Can f 5-specific memory CD4⁺ T cell responses differ considerably between subjects with and without allergy, as assessed by both *in vitro* and *ex vivo* approaches. Peptides containing the dominant T cell epitopes of Can f 5 can be employed for developing peptide-based immunotherapy for dog allergy.

Keywords Can f 5, Memory T cells, Th1/Th2, T cell epitope, allergy, immunotherapy, peptide
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Correspondence:

Anssi Kailaanmäki, Department of Clinical Microbiology, Institute of Clinical Medicine and Biocenter Kuopio, University of Eastern Finland, PO Box 1627, FI 70211 Kuopio, Finland.

E-mail: anssi.kailaanmaki@uef.fi

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Introduction

Allergen-specific CD4⁺ T cell responses have a critical role in the development of IgE-mediated allergy. Determination of the characteristics of allergen-specific T cells is therefore of utmost importance for both understanding the pathogenesis of the disease and developing antigen-specific therapies. Recent investigations suggest that individuals with allergy have increased frequencies of Th2-deviated memory CD4⁺ T cells in peripheral blood whereas memory CD4⁺ T cells in non-allergic individuals are generally very

rare and mostly of the Th1/Th0 or type 1 regulatory (Tr1) phenotype [1–6].

Dog-derived proteins are a common cause of respiratory hypersensitivity [7]. To date, six of them have been designated as allergens [8]. Nevertheless, human CD4⁺ T cell responses have only been characterized in-depth to two of these, the lipocalin proteins Can f 1 [9, 10] and Can f 4 [4]. One of the designated dog allergens, Can f 5, is prostatic kallikrein. It is reported to be a major allergen, as it sensitizes up to 70% of dog-allergic patients [11]. Interestingly, Can f 5 shares a high sequence identity, around 60%, with human pros-

tate-specific antigen (PSA). It has been suggested that IgE cross-reactivity between Can f 5 and PSA can play a role in allergy to human semen [11, 12]. To date, Can f 5 and human PSA are the only mammalian kallikreins found to be allergenic.

Earlier studies examining CD4⁺ T cell responses to allergens have largely employed methods requiring prolonged *in vitro* culture, which potentially can affect both the frequency and the phenotype of allergen-specific T cells. To overcome this caveat, several approaches have been developed to directly analyse allergen-specific CD4⁺ T cells *ex vivo*. Especially HLA class II tetramer-based methods have been widely employed to this end with considerable success in recent years [1, 13–15]. However, the major limitation of the tetramer approach is that it requires prior knowledge of the CD4⁺ T cell epitopes within the allergen of interest as well as their HLA class II restriction and therefore is typically limited to the analysis of single epitope specificities.

In the current study, we set out to investigate in detail the human CD4⁺ memory T cell responses to Can f 5, using two parallel approaches, a conventional split-well approach to generate long-term allergen-specific TCLs and a novel *ex vivo* analysis based on the CD154 enrichment. Our current results demonstrate that memory CD4⁺ T cells specific to Can f 5, a non-lipocalin allergen from dog, exist at a distinctively higher frequency in the peripheral blood of dog-allergic than non-allergic subjects and that these T cells are clearly Th2-biased. These results are in accordance with our previous observations on human memory CD4⁺ T cell responses to the lipocalin dog allergens Can f 1 [10] and Can f 4 [4]. Importantly, the results obtained *ex vivo* with the CD154 enrichment method correlated accurately with those obtained with the split-well method. In this work, we also characterized the T cell epitopes of the major dog allergen Can f 5. At least one of the six identified epitope regions was recognized by every Can f 5-allergic subject studied. The T cell epitopes identified here can be employed in the development of peptide-based immunotherapy for dog allergy.

Materials and methods

Antigens

Recombinant (r) Can f 5 protein was produced, as previously described [11], and tuberculin purified protein derivative (PPD) was provided by Statens Serum Institut (Copenhagen, Denmark). *Staphylococcus enterotoxin B* (SEB) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Seventy-four 16-mer peptides covering the whole Can f 5 sequence with an overlap of 13 amino acids

were synthesized at a minimum of 80% purity by GL Biochem (Shanghai, China; Table S1).

For the CD154 enrichment assay (see below), we chose 27 Can f 5 peptides that were recognized by at least one of the Can f 5-specific TCLs generated from 12 allergic subjects (Fig. 5). These peptides (Table S1) were pooled and used at a final concentration of 1 µg/mL per peptide in the stimulation. The peptides were dissolved in dimethyl sulfoxide (DMSO, Fisher Scientific, Waltham, MA, USA) with a final concentration of < 0.3% in the cultures.

Subjects

Peripheral blood samples were collected from twelve clinically diagnosed dog-allergic patients and twelve dog dander-exposed non-allergic donors (Tables S2 and S3). The allergic subjects were characterized at the Pulmonary Clinic of Kuopio University Hospital. They exhibited rCan f 5-specific IgE reactivity in ELISA (Table S2) and reported an exacerbation of their allergic symptoms upon exposure to dog dander. The Can f 5-specific IgE antibodies were measured by ELISA, as described previously [16]. Total serum IgE was measured at the Eastern Finland Laboratory Centre Joint Authority Enterprise (ISLAB, Kuopio, Finland) by electrochemiluminescence immunoassay (cobas e 601 analyser, Hitachi High Technology Co., Tokyo, Japan). The non-allergic control subjects reported no allergic symptoms when exposed to dogs or dog dander. Signed informed consent was provided by all subjects participating in this study, and the study was approved by the Ethics Committee of Kuopio University Hospital, permission #182/99.

Induction of Can f 5-specific T cell lines by the split-well method

Can f 5-specific T cell lines were generated with the split-well method, essentially as described previously [4, 17]. Peripheral blood mononuclear cells (PBMCs) of each donor were separated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Memory T cells (CD4⁺CD45RO⁺) were isolated with the no-touch memory CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs retained in the separation column were flushed out and used as antigen-presenting cells after γ -irradiation (3000 rad). 5×10^4 memory CD4⁺ T cells together with 1.5×10^5 irradiated PBMCs were seeded in 50 replicate wells on a 96-well plate (Corning Inc., Corning, NY, USA) and stimulated with rCan f 5 (10 µg/mL). The cells were cultured in 100 µL of complete RPMI 1640 medium supplemented with 10 mM HEPES, 100 µg/mL streptomycin, 100 IU/mL penicillin,

20 μ M 2-mercaptoethanol, 2 mM L-glutamine, 1 mmol/l sodium pyruvate, non-essential amino acids (all from Lonza, Verviers, Belgium), and 5% inactivated human AB serum (Sigma-Aldrich). On day 5, 50 μ L of fresh culture medium and 25 IU/mL of recombinant human interleukin-2 (rIL-2) were added in the wells. On day 10, the cells were restimulated with rCan f 5 together with 1.5×10^5 irradiated PBMCs in a total volume of 200 μ L per well. Finally, on day 20, half of the cells were split into two replicate 96-well daughter plates together with irradiated PBMCs with or without rCan f 5. After 3 days, proliferation was measured by the thymidine incorporation assay by pulsing the plates for 16 h with [^{3} H]thymidine (0.5 μ Ci; PerkinElmer, Waltham, MA, USA) and harvesting them on glass fibre filters (PerkinElmer). The uptake of [^{3} H]thymidine was subsequently measured by liquid scintillation counting (MicroBeta Trilux 1450; Wallac, Turku, Finland), and the results were displayed as counts per minute (CPM) or as stimulation indices (SI; CPM of a stimulated culture divided by CPM of the corresponding unstimulated culture). Positive wells (SI > 2 and Δ CPM > 1000) were transferred from the original plate to a 48-well plate (Corning) and restimulated with rCan f 5 along with 10^6 irradiated PBMCs. The T cell lines were cultivated for 14 days and supplemented with fresh medium and rIL-2 (50 IU/mL) every 2 to 3 days before further analyses.

T cell proliferation assays

Proliferation assays were performed in 96-well plates. For the Can f 5 dose-response assays, 2.5×10^4 T cells were stimulated in duplicate with five tenfold dilutions of rCan f 5 starting at 10 μ g/mL together with 5×10^4 irradiated PBMCs. EC₅₀ values (i.e. the rCan f 5 concentration needed to induce a half-maximal proliferative response) were determined from the dose-response curves of individual TCLs. For the T cell epitope mapping, 10^4 T cells were stimulated with each of the 16-mer peptides (10 μ g/mL) in duplicate together with 2.5×10^4 irradiated PBMCs. Proliferation was measured by the thymidine incorporation assay, as described above. In peptide screening, responses of SI > 2 to two or three adjacent peptides were regarded as specific, and the sequence shared with these peptides was defined as the core epitope recognized by the TCL.

Cytokine ELISA

Supernatants from duplicate Can f 5-stimulated (10 μ g/mL) wells and non-stimulated controls were collected from the proliferation assay plates after 72 h. The production of IL-5 and IFN- γ was measured by the DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA).

CD154 enrichment assay

The *ex vivo* analysis of allergen-specific CD4⁺ T cells was performed by stimulating PBMCs from seven allergic patients and four non-allergic controls with a pool of Can f 5 peptides (1 μ g/mL of each; see above) in 2–3 wells (2.5×10^7 PBMCs in 2.5 mL of medium per well) on 6-well plates (Corning). An equal number of PBMCs were seeded on identical plates with no antigen to serve as negative controls. Moreover, 5×10^6 PBMCs were seeded on 48-well plates in 1 mL of medium with PPD (10 μ g/mL) or SEB (1 μ g/mL) as memory T cell-specific and nonspecific positive controls, respectively. Then, 10 μ g/mL of anti-CD40 (BioLegend, clone HB14) was added to each well, and the cells were incubated for 16 h at +37°C before the magnetic bead enrichment. Antigen-specific T cells were enriched with the CD154 Microbead kit (Miltenyi) according to the manufacturer's instructions. In brief, the cells were washed and then incubated with biotinylated anti-CD154 antibodies and anti-biotin microbeads. After the staining, 1/100 fractions of Can f 5-stimulated and negative control samples were collected as pre-enrichment samples. The rest of the cells were applied into an MS column and washed once. Next, surface staining of the cells was performed in a total volume of 100 μ L within the column for 15 min at room temperature with the following antibodies (all from BioLegend, with the exception of CD4-APC/H7 from BD Biosciences, San Jose, CA, USA): anti-CD14-BV510, -CD19-BV510, -CD69-PerCp/Cy5.5, -CD4-APC/H7, -CD45RA-FITC, -CRTh2-BV421, -CXCR3-Alexa647, -CCR6-PE/Cy7, and anti-biotin-PE (for CD154). The column was washed twice, and the column-bound cells were eluted to collect the CD154-enriched fraction of cells. Pre-enrichment samples, as well as PPD- and SEB-stimulated controls, were stained with the same antibodies with the exception of anti-CD154-PE (Miltenyi Biotec) instead of anti-biotin-PE for PPD and SEB. Counting beads (Sphero AccuCount Fluorescent Particles 7.7 μ m; Spherotech Inc., Lake Forest, IL, USA) were added to each sample for absolute cell counts. Data were analysed by gating on forward scatter/side scatter and excluding doublets, CD14⁺ and CD19⁺ events. Frequencies were calculated using the following formula: $n/(N \times 100)$, where n is the total number of CD4⁺CD45RA⁺CD69⁺CD154⁺ events in the enriched fraction and N is the total number of CD4⁺CD45RA⁺ memory T cells in the pre-enrichment sample.

HLA class II restriction analyses

The HLA restriction of TCLs was examined by inhibiting the proliferative response to a given peptide with specific antibodies, as described previously [4]. In brief, 5×10^4 irradiated PBMCs in duplicate were incubated

for 1 h at +37°C with 1 µg/mL of antibodies to HLA-DP (B7/21), HLA-DQ (SPVL3), and HLA-DR (L243) in 96-well plates. Then, 2.5×10^4 T cells and 10 µg/mL of the relevant peptide were added to the wells, and proliferation was measured as described above.

Statistical analyses

The Fischer's exact test, the Mann–Whitney *U*-test, the Wilcoxon signed rank test, and the Spearman correlation test were used as indicated.

Results

The frequency of Can f 5-specific memory CD4⁺ T cells is markedly higher in allergic than in non-allergic subjects

As a first approach, we employed the split-well method [4, 10] to generate long-term memory TCLs specific to Can f 5 from highly purified peripheral blood CD4⁺CD45RO⁺ T cells. Can f 5-specific TCLs were obtained from all (100%) of the 12 Can f 5-sensitized dog-allergic subjects but only from 7 (58%) of the 12 non-allergic subjects studied ($P = 0.04$; Fischer's exact test). Furthermore, the calculated frequency of memory CD4⁺ T cells (assuming that a TCL originated from a single T cell) was approximately tenfold higher in allergic than in non-allergic subjects (7.9 ± 7.4 [mean $\pm$ SD] vs. 0.8 ± 0.8 per 10^6 CD4⁺ T cells, respectively; $P < 0.001$, Fig. 1a).

Can f 5-specific memory T cell lines from allergic subjects exhibit higher functional avidity and a Th2-biased cytokine phenotype

The functional T cell receptor avidity of Can f 5-specific TCLs was assessed by calculating the half-maximal

effective concentration (EC_{50}) for each TCL. The functional avidity of TCLs of allergic subjects was significantly higher (i.e. the concentration of Can f 5 needed to induce proliferation was lower) than the avidity of TCLs obtained from non-allergic subjects (EC_{50} 3.1 ± 3.1 vs. 6.4 ± 4.1 µg/mL for allergic and non-allergic subjects, respectively; $P < 0.05$, Fig. 1b). We also assessed the cytokine production of the Can f 5-specific TCLs by measuring the concentrations of the prototypical Th2 and Th1 cytokines, and IL-5 and IFN- γ , in the cell culture supernatants. The production of IL-5 was significantly higher in TCLs obtained from allergic subjects than in those from non-allergic subjects (613.4 ± 897.7 vs. 120.0 ± 95.8 pg/mL; $P = 0.02$, Fig. 2a). Conversely, the production of IFN- γ was significantly higher in the TCLs of non-allergic subjects (19.8 ± 38.9 vs. 91.7 ± 87.8 pg/mL; $P < 0.01$). Overall, 44% of the TCLs of allergic subjects exhibited a Th2-biased cytokine secretion profile as determined by the IFN- γ /IL-5 ratio (Th1: > 5 ; Th0: 0.01 – 5 ; Th2: < 0.01). For non-allergic subjects, the corresponding fraction of Th2-deviated TCLs was 14% (Fig. 2b).

The CD154 enrichment assay confirms the increased frequency and the Th2 phenotype of Can f 5-specific CD4⁺ T cells in allergic subjects ex vivo

To further validate the results obtained with long-term TCLs, the recently described CD154 enrichment method [18] was employed to detect Can f 5-specific memory CD4⁺ T cells directly *ex vivo* by flow cytometry. One allergic subject (A-5) had an exceptionally high (469 per 10^6) frequency of Can f 5-reactive memory CD4⁺ T cells in peripheral blood, permitting us to directly ascertain that the frequency of Can f 5-specific T cells was similar before and after the CD154 enrichment (Fig. S1). Using the *ex vivo* method, Can f 5-specific memory CD4⁺ T cell frequencies varied between 1 and 469 per 10^6 cells and were significantly higher in allergic than in non-allergic subjects (Fig. 3a and b). Notably, the frequencies of Can f 5-specific T cells extrapolated from the split-well data (Fig. 1a) correlated well (Fig. 3c; $r = 0.87$, $P < 0.001$) with the frequencies detected *ex vivo* with the CD154 enrichment method (Fig. 3b), although the frequencies obtained with the latter approach were systematically slightly higher (Fig. 3c). The *ex vivo* frequencies of memory CD4⁺ T cells specific to the microbial control antigen PPD as well as SEB were similar between allergic and non-allergic subjects (Figs. 3b and S2).

To determine the phenotype of Can f 5-specific memory T cells in allergic subjects directly *ex vivo*, the expression of the chemokine receptors CCR6, CXCR3, and CRTh2 was analysed after the CD154 enrichment (Figs. 4 and S3). Can f 5-specific CD4⁺ T cells were

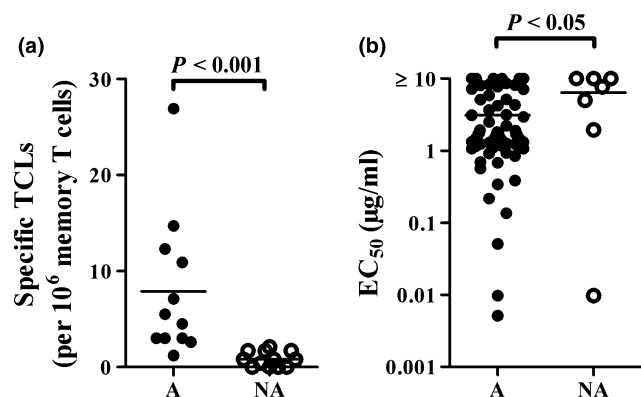


Fig. 1. (a) The frequencies of Can f 5-specific TCLs generated per 10^6 memory CD4⁺ T cells from allergic (A) and non-allergic (NA) subjects. (b) Functional avidities of specific TCLs assessed as EC_{50} values calculated from the titrated dose–response curves of each TCL. The data were analysed by the Mann–Whitney *U*-test.

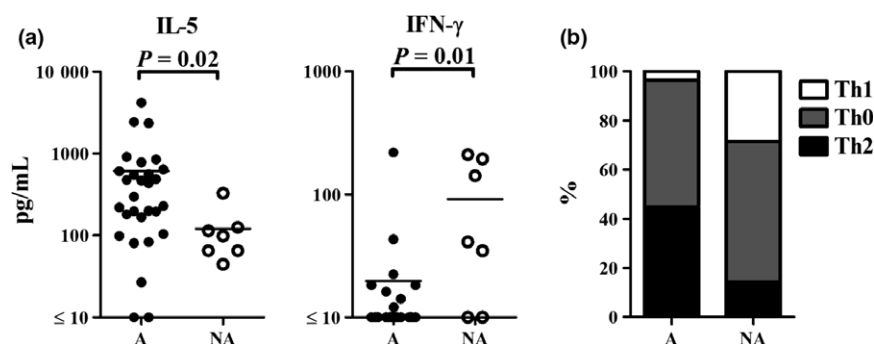


Fig. 2. (a) Production of IL-5 and IFN- γ by the Can f 5-specific T cells from allergic (A) and non-allergic (NA) subjects. (b) The cytokine phenotypes of Can f 5-specific T cells based on the IFN- γ /IL-5 ratio (Th1: > 5 ; Th0: 0.01–5; Th2: < 0.01). The data were analysed by the Mann–Whitney *U*-test.

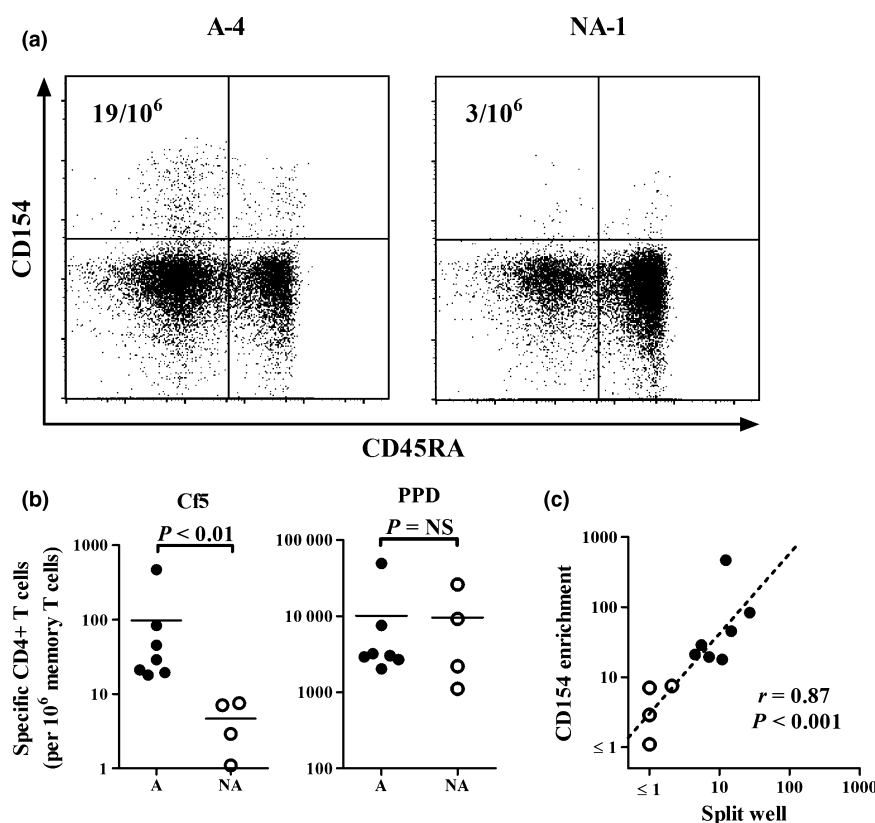


Fig. 3. (a) Detection of Can f 5-specific CD4⁺ T cells *ex vivo* using the CD154 enrichment assay for a representative allergic (left) and non-allergic individual (right). The calculated frequency of Can f 5-specific memory CD4⁺ T cells is indicated. (b) The *ex vivo* frequencies of Can f 5 (Cf5) and PPD-specific memory CD4⁺ T cells from 7 allergic and 4 non-allergic subjects, as determined with the CD154 enrichment assay. *NS* = not significant. The data were analysed by the Mann–Whitney *U*-test. (c) Correlation of the frequencies of Can f 5-specific memory CD4⁺ T cells obtained by the split-well and the CD154 enrichment methods (allergic subjects, closed dots; non-allergic subjects, open dots). The X and Y axes indicate the number of specific cells per 10^6 memory CD4⁺ T cells obtained with each of the two methods. The data were analysed by the Spearman correlation test.

clearly concentrated in the CCR6–CXCR3[–] subset, which is reported to contain mostly Th2-type memory T cells [19]. Additionally, Can f 5-specific T cells displayed elevated expression of CRTh2, a more specific marker of Th2-polarized cells [20]. Contrary to Can f 5, PPD-specific T cells were distinctively concentrated in the CCR6+CXCR3[–] and CCR6+CXCR3⁺ subsets that contain

memory T cells polarized towards Th17 and Th1/17 phenotypes, respectively [19].

The Can f 5 protein contains six T cell epitope regions

The T cell epitopes of Can f 5 were identified by testing the reactivity of 52 distinct Can f 5-specific T cells

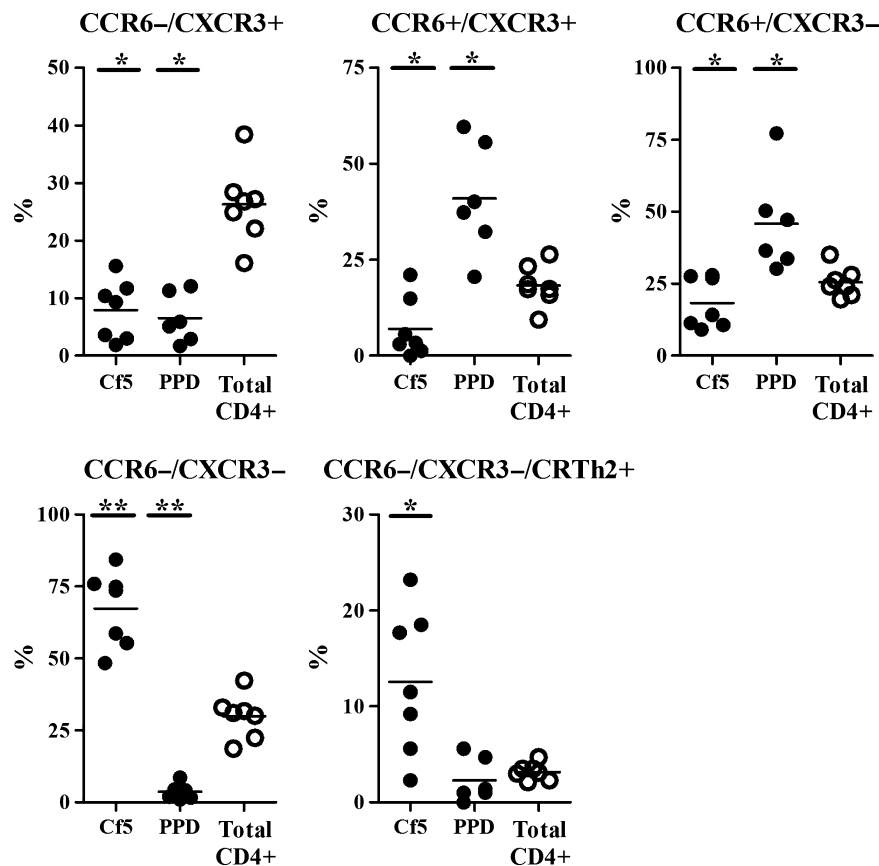


Fig. 4. Expression of the phenotypic markers CCR6, CXCR3, and CCR6+CXCR3+CXCR3- by Can f 5 (Cf5) and PPD-specific T cells from allergic subjects as compared to unstimulated total memory CD4+ T cells. Percentages of CD154+ events positive for the combination of chemokine receptors are indicated. ** = $P < 0.01$, * = $P < 0.05$ as compared to total memory CD4+ T cells. The data were analysed by the Mann–Whitney *U*-test.

generated from the 12 allergic subjects to overlapping 16-mer peptides that covered the amino acid sequence of the allergen. With this approach, we detected several distinct T cell epitopes throughout the molecule, with a median of 4 epitopes (range 1–6) recognized by each allergic subject. Importantly, the identified epitopes comprised six evident epitope regions, each of which was recognized by at least 4 of the 12 subjects (Figs. 5 and S4). All the 12 allergic subjects studied recognized at least one of these six regions. The most prominent epitope region, located between the amino acid residues 163–184, was recognized by 7 of the 12 subjects (58%).

The HLA restriction of 38 Can f 5-specific TCLs was determined by blocking their proliferative responses to Can f 5 peptides by HLA class II-specific antibodies [4]. As 47% of the Can f 5-specific T cell responses were restricted by HLA-DR, 40% by HLA-DQ, and 13% by HLA-DP (Fig. S5), the responses to Can f 5 were observed to be restricted by a wide array of HLA class II molecules. Of interest, bioinformatic predictions of HLA class II peptide-binding motifs in Can f 5 (Fig. S4) correlated only modestly with the experimentally identified T cell epitopes: 55% of *in silico*-predicted epitopes

were confirmed as T cell epitopes by the experimental approach used in this study, whereas 50% of the confirmed epitopes were not included in the predicted ones.

Discussion

Investigations of the T cell reactivity to environmental allergens in atopic and healthy individuals are important both for understanding the pathogenesis of allergy and for developing allergen-specific therapies. Among animal allergens, dog-derived proteins are a common cause of respiratory hypersensitivity [11, 21, 22]. In the current study, we sought to examine in detail the memory CD4+ T cell responses of allergic and non-allergic subjects to the recently identified major dog allergen Can f 5, prostatic kallikrein.

Based on the split-well data, the estimated frequency of Can f 5-specific memory CD4+ T cells was approximately tenfold higher in allergic than in non-allergic subjects (Fig. 1a). Interestingly, the difference in the frequency of Can f 5-specific T cells observed here was surprisingly similar to that previously observed with

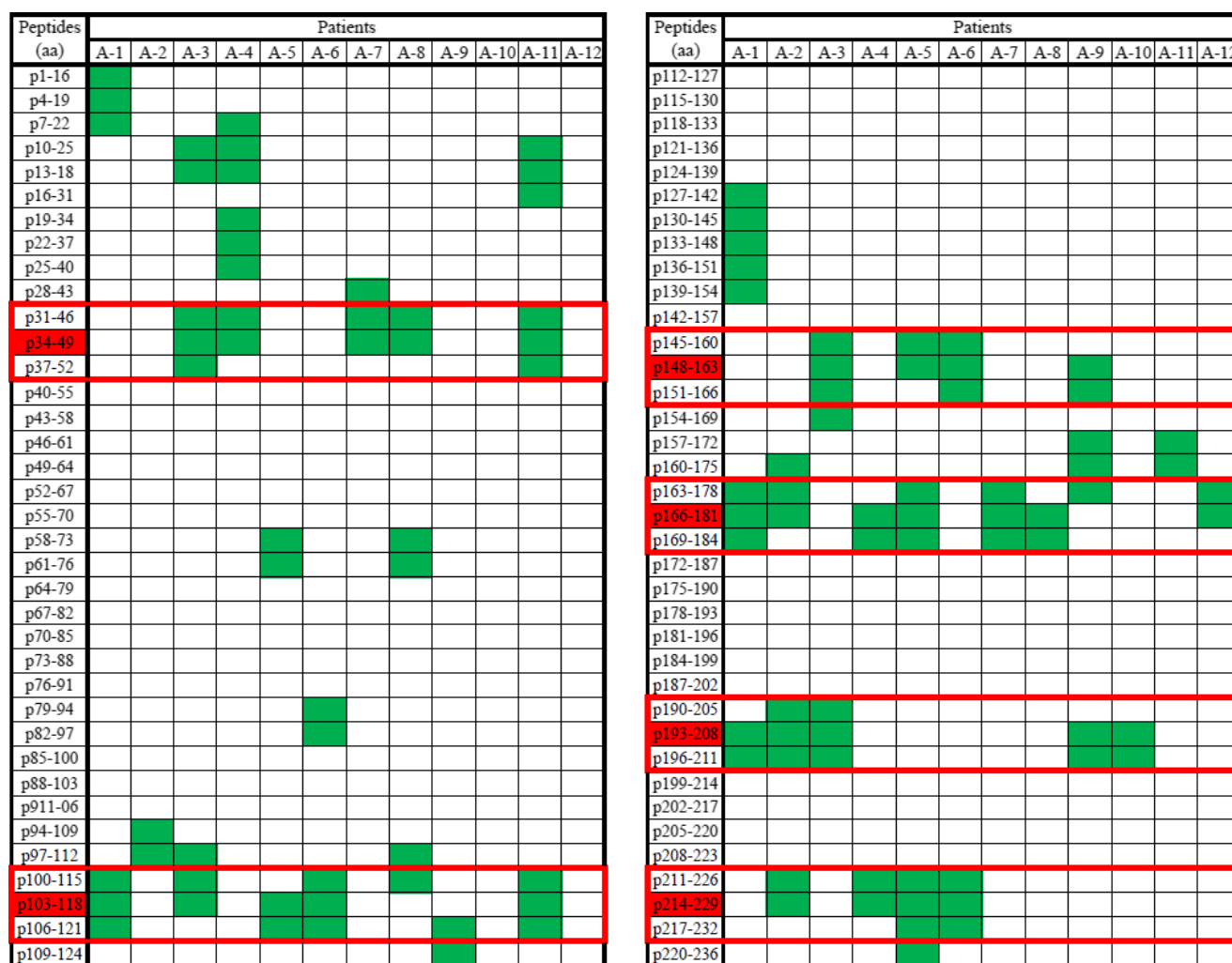


Fig. 5. T cell epitope regions of Can f 5 recognized by the TCLs of 12 dog-allergic patients. A proliferative response of SI > 2 to two or three adjacent peptides was considered epitope specific. Red boxes indicate the epitope regions that were capable of stimulating the TCLs of at least 4 different patients. Peptides indicated by a red background contain the sequence common to the 3 peptides within each of the immunodominant regions.

Can f 1 and Can f 4, two other major dog allergens [4, 10]. Other studies with various allergens employing *in vitro* or tetramer-based *ex vivo* methods have similarly found elevated numbers of memory T cells in the blood of allergic subjects [1–5, 23]. In line with studies with the other dog allergens Can f 1 [10] and Can f 4 [4], the Can f 5-specific TCLs also displayed a higher functional TCR avidity in allergic than in non-allergic subjects (Fig. 1b). This phenomenon is likely the result of a selective expansion of allergen-specific T cell clones with high-affinity TCRs *in vivo* [24–26]. The consistent lack of expanded high-avidity allergen-specific memory T cells in non-allergic subjects remains enigmatic. Possible explanations for this phenomenon include increased peripheral regulation or differential T cell recognition of the allergen in these individuals [23, 27, 28].

Further phenotypic characterization of Can f 5-specific TCLs revealed a Th2-biased cytokine secretion profile in the TCLs of allergic patients, whereas TCLs from non-allergic subjects were skewed towards the Th1 phenotype (Fig. 2b). These results are also consistent with those from several other studies with other allergens [2, 5, 13, 14, 23].

As prolonged *in vitro* stimulation may have an effect on both the phenotype and the observed frequency of antigen-specific cells [29], we utilized a novel *ex vivo* enrichment method based on the upregulation of the activation marker CD154 to further validate our data [18]. Importantly, one of the allergic subjects (A-5) had an extremely high frequency of Can f 5-specific cells in peripheral blood, which permitted us to directly calculate the frequency of the allergen-specific memory cells *ex vivo* and hence confirm that both the frequency and

phenotype of Can f 5-specific cells remained unaffected by the magnetic enrichment step (Fig. S1). As with the split-well method, the frequency of Can f 5-specific memory CD4+ T cells was significantly higher in allergic subjects than in non-allergic subjects (Fig. 3b). Moreover, there was a consistent tendency of the CD154 enrichment method to report slightly higher frequencies of allergen-specific cells than the split-well method (Fig. 3c). This discrepancy may be a result of the inability of some of the Can f 5-specific memory CD4+ T cells to undergo long-term expansion *in vitro* due to their low T cell receptor avidity or terminally differentiated phenotype. Importantly, the CD154 enrichment method also confirmed the Th2-deviated phenotype of Can f 5-specific memory CD4+ T cells in allergic subjects: Can f 5-specific cells were highly concentrated in the CCR6-CXCR3- subset, which mostly contains memory Th2 cells [19]. In addition, Can f 5-specific cells exhibited elevated expression of CRTh2, which is a more specific marker for Th2-deviated cells [20].

Based on our current results, the split-well method and the *ex vivo* CD154 enrichment method appear to produce comparable results. This observation confirms the validity of our earlier results obtained with the former method on the nature of human CD4+ T cell responses to various animal allergens [2, 4, 10, 23]. Importantly, both of these methods allow the analysis of antigen-specific CD4+ T cells regardless of previous knowledge of the epitopes or the HLA restriction elements, which is an important caveat with the widely used HLA class II tetramer approach [18, 29]. Obviously, the potential of monitoring allergen-specific T cells directly *ex vivo* in a short timescale is an additional advantage of the CD154 enrichment method.

The use of allergen preparations containing complete allergen molecules in allergen immunotherapy poses a risk of adverse reactions caused by the cross-linking of allergen-specific IgE on mast cells. In the most severe cases, systemic or even life-threatening reactions, such as anaphylaxis, may occur [30, 31]. Immunotherapeutic vaccines composed of selected peptides offer a promising alternative for whole allergen preparations, as short peptides circumvent the issue of IgE binding while still retaining the T cell epitopes required for the desired immunomodulation [31]. For the development of pep-

tide-based immunotherapy, it is important that candidate peptides are shown to exert allergen-specific T cell responses *in vivo* and that they exhibit a wide range of HLA class II binding, enabling a maximal coverage within the allergic population. Our epitope mapping of Can f 5 revealed that the protein contains six distinct T cell epitope regions that were capable of stimulating T cells from 100% (12/12) of the studied allergic subjects with diverse HLA phenotypes (Fig. 5 and Table S3). Of the six identified immunodominant regions, the epitope region located between the amino acid residues 163–184 emerged as the most prominent one, as more than half (7/12) of the allergic subjects reacted to it.

In summary, we have analysed here in detail the human memory CD4+ T cell reactivity to the major dog allergen Can f 5. Allergen-specific memory T cell responses were identified in both allergic and non-allergic subjects, but they were markedly more frequent and substantially more Th2-deviated in allergic subjects. The results obtained here with the novel *ex vivo* CD154 enrichment method were largely concordant with those generated with a more conventional split-well approach. Finally, we identified six distinct epitope regions within the Can f 5 molecule. Peptides covering these epitope regions can be considered as candidates for future peptide-based immunotherapeutic trials against dog allergy, especially if combined with previously identified dominant epitopes from other major dog allergens, such as Can f 1 [9] and Can f 4 [4].

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Conflict of interest

The authors declare that they have no relevant conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Ex vivo* detection of Can f 5-specific memory CD4⁺ T cells from subject A-5 before and after the CD154 enrichment.

Figure S2. The *ex vivo* frequencies of SEB-reactive memory CD4⁺ T cells from 7 allergic and 4 nonallergic subjects, as determined with the CD154 enrichment assay.

Figure S3. A representative figure of the expression of the phenotypic markers CCR6, CXCR3 and CCR2 on total unstimulated memory CD4⁺ T cells (left) and Can

f 5-specific memory CD4+ T cells after the CD154 enrichment (right) from subject A-5.

Figure S4. Alignment of the experimentally verified T cell epitopes and the computer algorithm-predicted HLA-binding motifs on the Can f 5 sequence.

Figure S5. (a) Representative figures of the HLA class II restriction of Can f 5-specific TCLs by HLA-DP, -DQ and -DR as determined using blocking anti-DP, -DQ

and -DR monoclonal antibodies. Results are expressed as stimulation indices upon stimulation with Can f 5 in the presence or absence of the antibodies. **(b)** Percentage of Can f 5-specific TCLs restricted by HLA class II -DP, -DQ and -DR alleles.

Table S1. Peptides used in the study.

Table S2. Demographic data.

Table S3. HLA genotypes of the test subjects.