

Characterization of human memory CD4⁺ T-cell responses to the dog allergen Can f 4

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Background: The recently identified dog lipocalin allergen Can f 4 is an important respiratory allergen.

Objective: We sought to comprehensively characterize the memory CD4⁺ T-cell responses of allergic and nonallergic subjects to Can f 4.

Methods: Can f 4-specific CD4⁺CD45RO⁺ T-cell lines (TCLs) from allergic and healthy subjects were established and characterized by their functional and phenotypic properties. The epitope specificity of the TCLs was tested with 48 overlapping 16-mer peptides spanning the sequence of Can f 4. HLA restriction of the specific TCLs and the binding capacity of the epitope-containing peptides to common HLA class II molecules were studied.

Results: Can f 4-specific memory CD4⁺ TCLs were obtained at an 8-fold higher frequency from allergic than from nonallergic subjects. Functionally, the TCLs of allergic subjects exhibited a higher T-cell receptor avidity and expression of CD25 and predominantly produced IL-4 and IL-5. The TCLs of nonallergic subjects mostly secreted IFN- γ and IL-10, with high CXCR3 expression. Several distinct T-cell epitope regions along the allergen were identified. Importantly, the peptides from the region between amino acids 43 and 67 showed promiscuous HLA-binding capacity and induced memory CD4⁺ T-cell responses in 90% of the allergic donors.

Conclusion: Productive T_H2-deviated memory T-cell responses to Can f 4 are observed in allergic but not nonallergic subjects. A 19-mer peptide sequence covering the core of the immunodominant region of the allergen is a potential target for the development of peptide-based allergen immunotherapy. (J Allergy Clin Immunol 2015;■■■■:■■■-■■■.)

Key words: Memory T cells, T_H1/T_H2, T-cell epitope, HLA class II, Can f 4, lipocalin allergen, peptide, allergy, immunotherapy

Abbreviations used

APC: Antigen-presenting cell
EC₅₀: Half-maximal effective concentration
IC₅₀: Half-maximal inhibitory concentration
PIT: Peptide-based immunotherapy
SI: Stimulation index
TCL: T-cell line
TCR: T-cell receptor

Sensitization to dog dander is highly prevalent in patients with atopic conditions, such as rhinitis, eczema, or asthma.¹⁻³ To date, the IgE reactivity of 4 dog allergens of the lipocalin protein family, Can f 1,^{4,5} Can f 2,^{4,5} Can f 4,⁶ and Can f 6,⁷ as well as the dog serum albumin Can f 3⁸ and the dog prostatic kallikrein Can f 5,⁹ has been characterized in detail. However, despite the central role of CD4⁺ T cells in allergic inflammation, T-cell reactivity to only one of them, Can f 1, has been systematically studied.^{10,11}

In susceptible subjects allergen exposure leads to induction of specific CD4⁺ T_H2 cells that provide critical signals promoting the production of IgE and eosinophilic inflammation through direct cellular interactions and production of the cytokines IL-4, IL-5, and IL-13.¹² Instead, immune tolerance, possibly associated with a T_H1/T_R1 phenotype of allergen-specific CD4⁺ T cells in nonatopic subjects, is proposed to be maintained through several nonexclusive mechanisms, such as immune ignorance, deletion of allergen-reactive T cells, and induction of regulatory T cells.¹³ Similar mechanisms have also been linked to tolerance induced by allergen-specific immunotherapy,^{14,15} the only treatment targeting the immune pathology of allergy.¹⁶ An alternative to conventional allergen immunotherapy is administration of short peptides containing the T-cell epitopes of an allergen (peptide-based immunotherapy [PIT]). PIT specifically targets allergen-specific CD4⁺ T cells with the advantage of a reduced risk for adverse systemic reactions.^{17,18} Understanding the allergen-specific T-cell responses in allergic and healthy subjects at the T-cell epitope level is pivotal for the development of PIT. For population-wide therapeutic coverage, identification of peptides containing allergen epitopes with a capacity to bind to a variety of commonly expressed HLA class II molecules is of special interest.

Here we extend the knowledge of dog allergen-specific human T-cell responses to Can f 4, a clinically relevant dog lipocalin, which has been shown to exhibit an IgE reactivity of 35% to 60% within patients with dog allergy.^{5,6} We established and analyzed Can f 4-specific CD4⁺ memory T-cell lines (TCLs) from both allergic and nonallergic subjects and found that the responses differed considerably between the subject groups in terms of

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the memory CD4⁺ T-cell frequency and phenotype. Importantly, we discovered a highly stimulatory epitope region with promiscuous HLA class II-binding capacity. The core of the region, a 19-mer sequence between amino acids 46 and 64 could be used in the development of peptide-based allergen immunotherapy.

METHODS

Subjects and antigens

Peripheral blood samples were collected from 13 patients with clinically diagnosed dog allergy and rCan f 4-specific IgE reactivity and from 12 dog dander-exposed nonatopic subjects for this study. For detailed demographic data, see Table E1 and the Methods section in this article's Online Repository at www.jacionline.org. The study was approved by the Ethics Committee of Kuopio University Hospital, and written informed consent was obtained from all the participants in the study. rCan f 4 was produced in *Pichia pastoris*, as previously described.¹⁹ The 48 16-mer peptides overlapping by 13 amino acids and covering the entire sequence of Can f 4, as well as the truncated versions of the peptide p46-61, were synthesized at greater than 80% purity by GL Biochem (Shanghai, China; see Table E2 in this article's Online Repository at www.jacionline.org).

Generation and functional analyses of Can f 4-specific CD4⁺ T cells

Can f 4-specific memory CD4⁺ T cells were generated with the split-well method, and their proliferation was assessed, as previously described,^{20,21} with minor modifications (see the Methods section in this article's Online Repository). Details of cytokine measurements, surface marker analyses, HLA restrictions, and HLA class II peptide binding assays are outlined in the Methods section in this article's Online Repository.

HLA class II peptide-binding predictions

The HLA class II-binding predictions for the sequence of Can f 4 were made on July 10, 2014, by using the Immune Epitope Database analysis resource Consensus tool.^{22,23} The alleles expressed by the study population were included. The top 3% of each loci were selected.

Statistical analyses

Statistical analyses were conducted with the commercial software GraphPad Prism (GraphPad Software, San Diego, Calif). *P* values of less than .05 were considered statistically significant. For detailed information, see the Methods section in this article's Online Repository.

RESULTS

Can f 4-specific memory CD4⁺ T cells exist at a higher frequency in peripheral blood of allergic than that of nonallergic subjects

Specific T cells were established from freshly isolated and highly purified CD4⁺CD45RA⁺CD45RO⁺ memory T cells by using the split-well method to characterize the human *in vivo*-generated CD4⁺ T-cell responses to the dog lipocalin allergen Can f 4. With this technique, we obtained Can f 4-specific memory CD4⁺ T cells from 12 (92%) of 13 allergic subjects (86 T cells) but only 3 (25%) of 12 nonallergic subjects (11 T cells; *P* = .001, Fisher exact test). In general, the estimated frequency of memory CD4⁺ T cells in peripheral blood was low, less than 1 per 100,000 CD4⁺ memory T cells in all subjects enrolled in the study (Fig 1, A). However, when the subject groups were compared, the mean frequency of Can f 4-specific CD4⁺

memory T cells was approximately 8-fold higher in allergic than nonallergic subjects (2.46 ± 2.45 vs 0.31 ± 0.59 per 1 million memory CD4⁺ T cells, respectively).

Because the interaction between T and B cells plays a central role in allergic sensitization, it is conceivable that there would be a link between the responses of these subsets. To assess this, we compared the Can f 4-specific serum IgE levels and the number of memory T cells obtained per allergic donor. In line with previous studies using different approaches to measure T-cell reactivity,^{24,25} the correlation turned out to be low (*r* = 0.32, *P* = .28; see Fig E1 in this article's Online Repository at www.jacionline.org). However, it is noteworthy that we obtained the highest numbers of T cells from the 2 subjects with the highest specific IgE levels.

Can f 4-specific CD4⁺ T-cell responses differ functionally between allergic and nonallergic subjects

To compare the functional characteristics of Can f 4-specific T cells obtained from subjects with and without Can f 4 allergy, we first assessed the functional avidity of the T cells by calculating the half-maximal effective concentration (EC₅₀) values for each T cell. It turned out that the average EC₅₀ values of the T cells of allergic subjects were significantly lower than those of nonallergic subjects, indicating higher functional avidity (*P* = .007; Fig 1, B). By using an arbitrary EC₅₀ cutoff of 2 μg/mL, 25% of the allergic subjects' T cells but none of those of nonallergic subjects could be classified as higher-avidity T cells (*P* = .03, Fisher exact test). These T cells were obtained from 6 allergic subjects, and they recognized several Can f 4 epitope regions (see Table E3 in this article's Online Repository at www.jacionline.org).

Next, we measured production of the cytokines IL-4, IL-5, IFN-γ, IL-10, and IL-17 by the Can f 4-specific T cells on stimulation with allergen. The results are presented in Fig 2, A, with the exception of IL-17, which was not detected in the supernatant of any T cell. We observed that the T cells of allergic subjects produced predominantly the T_H2-associated cytokines IL-4 and IL-5, whereas within the T cells of nonallergic subjects, the production of IL-4 was absent and that of IL-5 was lower in comparison with that seen in allergic subjects. In contrast, concentrations of the T_H1/T_R1-linked cytokines IFN-γ and IL-10 were significantly greater in culture supernatants of T cells from nonallergic subjects. Next, we categorized phenotypically each T cell by calculating an IFN-γ/IL-5 ratio (Fig 2, B). We found that 64% of T cells from allergic subjects and 27% of those from healthy subjects were of the T_H2 phenotype (*P* = .03). In contrast, only 4% of T cells from allergic subjects but 36% from healthy subjects were T_H1 biased (*P* = .003).

To complement the functional analyses, we determined the expression of selected T-cell surface markers. The activation status of the T cells, as assessed based on expression of CD25, the IL-2 receptor alpha-chain, was observed to be greater in the T cells from allergic subjects than in those from nonallergic subjects (Fig 2, C). Moreover, in line with the cytokine data, expression of the chemokine receptor CXCR3, a marker of T_H1 deviation,²⁶ was found to be less in T cells from allergic than nonallergic subjects (Fig 2, C). Expression of the other surface markers studied (CD45RO, CD45RA, CD62 ligand (CD62L), CCR7, cytotoxic T lymphocyte-associated protein 4 (CTLA-4), CCR4, and CCR6) did not differ between the groups (data not shown).

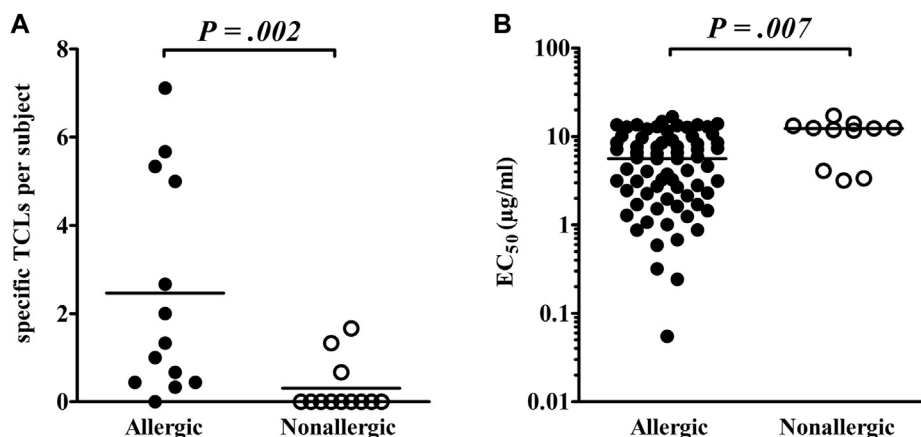


FIG 1. Frequencies and functional avidities of the Can f 4-specific T cells of memory origin from allergic (solid circles) and nonallergic (open circles) subjects. **A**, Frequencies of specific T cells per 10^6 $CD4^+$ $CD45RO^+$ memory T cells. **B**, Functional avidities of specific T cells assessed as EC_{50} values calculated from the titrated response curves of each T cell.

T-cell epitope mapping of Can f 4 reveals a dominant epitope region

The T-cell epitopes of Can f 4 were identified by testing the reactivity of 80 Can f 4-specific T cells obtained from 11 allergic and 3 nonallergic subjects to 48 overlapping 16-mer peptides covering the allergen sequence. The epitopes were found to scatter throughout the allergen sequence (Fig 3 and see Fig E2 in this article's Online Repository at www.jacionline.org), with an average of 3.3 ± 0.62 and 2.3 ± 0.88 epitopes recognized per allergic and nonallergic subject, respectively. Four Can f 4 regions, amino acids 13 to 31, 43 to 67, 91 to 127, and 124 to 148 (highlighted in Fig 3), were observed to be the most prevalently recognized because they were covered by overlapping peptides that activated the Can f 4-specific T cells of at least 3 subjects. Moreover, the mean stimulatory capacity of the peptides covering these regions, as assessed by the stimulation indices (SIs) of the specific T cells, was found to be higher than that of the peptides covering other epitope regions (46.4 ± 66.5 vs 29.6 ± 27.6 ; $P = .007$, Mann-Whitney U test). The most frequently recognized region was amino acids 43 to 67, the peptides of which stimulated the T cells from 10 (90%) of 11 allergic and 1 (33%) of 3 nonallergic subjects. Three allergic subjects and 1 nonallergic subject harbored T cells specific to this region only. The core sequences (ie, the amino acids shared by 2-3 consecutive peptides able to stimulate a T cell) of the epitope-specific T cells are presented in Fig 4. The core of the most stimulatory region, amino acids 43 to 67, was found to reside within the 19-mer sequence PRLKMLFNFYVKVDGECVE (amino acids 46-64, Fig 4). To further dissect the T-cell reactivity against this epitope region, we stimulated 12 p46-61-reactive T cells from 6 patients with truncated versions of the peptide. We observed 5 distinct minimal core epitopes within the sequence (see Fig E3 in this article's Online Repository at www.jacionline.org).

Variable HLA class II restriction of the T-cell response to Can f 4

To determine the HLA class II restriction of the T-cell response to Can f 4 epitopes, we tested the capacity of antibodies specific to the HLA-DR, HLA-DP, and HLA-DQ molecules to inhibit the proliferation of the Can f 4-specific T cells on stimulation with

peptides. In total, 34 T cells from 8 allergic subjects and 1 nonallergic subject were analyzed (see Table E4 in this article's Online Repository at www.jacionline.org). It was discovered that 74% of the T-cell responses were restricted by HLA-DR, 21% by HLA-DQ, and 4% by HLA-DP. Importantly, T-cell responses to none of the 4 most stimulatory regions of Can f 4 were observed to be exclusively restricted by HLA-DR, HLA-DP, or HLA-DQ molecules. Similar variability in the HLA class II restriction was also observed in the analysis of specific restricting alleles with partially HLA-matched allogeneic PBMCs or homozygous EBV-transformed B-cell lines (see Table E4). Collectively, our results suggest that Can f 4 epitopes, including those covered by the immunodominant region of amino acids 43 to 67, are presented by a wide array of HLA class II molecules.

Bioinformatic predictions and immunochemical measurements of HLA binding of Can f 4 peptides

We used bioinformatic algorithms (see the Methods section in this article's Online Repository) to predict HLA class II peptide-binding motifs in Can f 4. Altogether, 38 HLA-DR-binding motifs, 6 HLA-DP-binding motifs, and 7 HLA-DQ-binding motifs in Can f 4 were predicted. Twenty-five percent (13/51) of the predicted motifs were not recognized by the Can f 4-specific T cells in our epitope mapping assay (overlapping by 9 amino acids was required, Fig 4). Instead, we discovered 8 additional T-cell epitope core sequences outside the HLA-binding predictions. Most (37/51 [73%]) of the predicted HLA-binding sites were found to be contained in the 4 most stimulatory epitope regions of Can f 4, each of them containing at least 4 distinct motifs predicted (Fig 4), suggesting that prevalent HLA-binding capacity is linked to the immunogenicity of Can f 4 epitopes. Of note, 4 of the predicted HLA-binding motifs within the core of the immunodominant epitope region (amino acids 46-64, Fig 4) correspond to the experimentally verified minimal core epitopes (see Fig E3). To further experimentally validate the HLA-binding predictions, we measured the binding of 15 peptides covering these regions to a panel of prevalent HLA-DR1 molecules (Fig 5). In line with the predictions, promiscuous HLA binding was observed

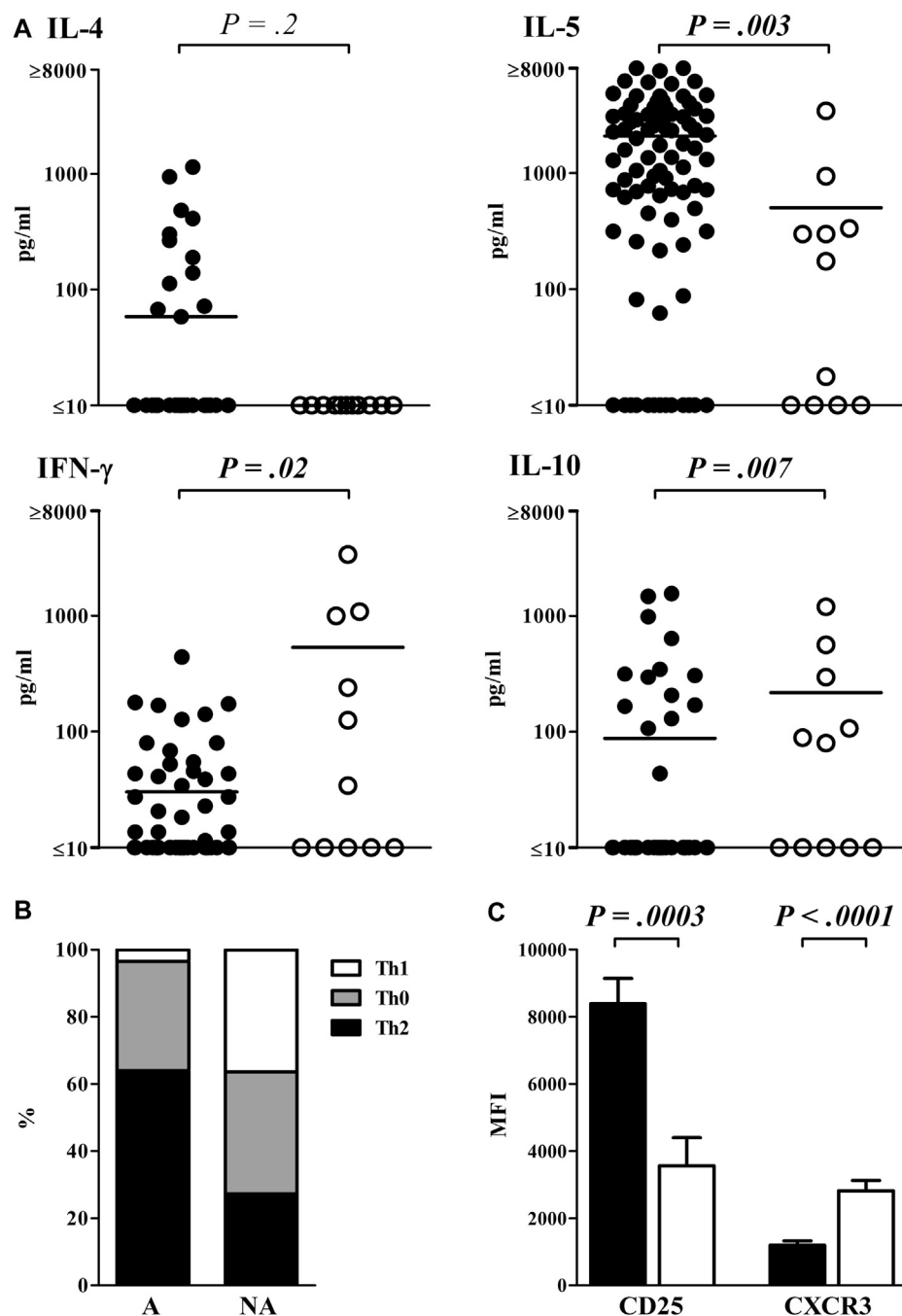


FIG 2. Phenotypic characterization of the Can f 4-specific memory T cells. **A**, Production of the cytokines IL-4, IL-5, IFN- γ , and IL-10 by the Can f 4-specific T cells of allergic (solid circles) and nonallergic (open circles) subjects on stimulation with Can f 4 at 100 μ g/mL. **B**, Cytokine phenotypes of the T cells from allergic (A) and nonallergic (NA) subjects based on IFN- γ /IL-5 ratio (T_H1 , >5 ; T_H2 , <0.01 ; T_H0 , 0.01-5). **C**, Mean fluorescence intensities (MFI; $\pm$ SEMs) of the cell-surface markers CD25 and CXCR3 on Can f 4-specific CD4 $^+$ T cells.

because 3 of the 4 regions contained peptides that were capable of binding to a minimum of 3 different HLA class II molecules with a moderate-to-high affinity. Notably, the peptides of amino acids 43 to 58 and 46 to 61 from the most antigenic epitope region of Can f 4 displayed the most prevalent binding capacity to the HLA class II molecules (strong binding affinity to 5/6 and 4/6 of the HLA alleles tested, respectively; Fig 5).

DISCUSSION

Understanding the mechanisms of allergen-specific T-cell reactivity in subjects with and without allergy is essential in elucidating the pathogenesis of allergy. This study provides a comprehensive analysis of the human memory CD4 $^+$ T-cell responses of allergic and nonatopic subjects to Can f 4, an important dog allergen,^{5,6} including the systematic characterization of its previously undefined T-cell epitopes. We demonstrate

	Peptides (aa)	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	A-11	A-12	A-13	NA-5	NA-6	NA-8	mean SI (±SEM)
region I	1-16															2.9±0.0 23.2±15.8
	4-19															
	7-22															
	10-25															
	13-28															46.2±45.9
	16-31															61.4±70.7
	19-34															77.5±53.5 36.7±33.2 10.0±8.8 7.9±5.5
	22-37															
	25-40															
	28-43															
region II	31-46															5.9±0.0
	34-49															4.5±0.0
	37-52															73.0±100.1 57.1±116.1 17.3±27.1 10.8±9.0
	40-55															
	43-58															
	46-61															
	49-64															22.8±0.0 26.6±5.8 18.3±20.5 4.9±0.0
	52-67															
	55-70															
	58-73															2.4±0.0 7.9±6.8 10.1±0.0 27.6±9.7
region III	61-76															
	64-79															
	67-82															
	70-85															47.6±30.8 21.2±16.1 61.4±79.4 80.4±82.7
	73-88															
	76-91															
	79-94															
	82-97															55.6±56.7 49.5±37.9 43.0±39.0 41.8±0.0
	85-100															
	88-103															
region IV	91-106															15.0±9.5 53.7±66.9 20.9±28.5 13.8±8.7
	94-109															
	97-112															
	100-115															
	103-118															25.3±32.1
	106-121															
	109-124															
	112-127															
	115-130															15.0±9.5 53.7±66.9 20.9±28.5 13.8±8.7
	118-133															
region V	121-136															
	124-139															
	128-142															25.3±32.1
	131-145															
	134-148															
	137-151															
	140-154															25.3±32.1
	143-158															

FIG 3. T-cell epitope regions (gray boxes) recognized by the Can f 4-specific TCLs of allergic (A) and nonallergic (NA) subjects. A proliferative response with an SI of greater than 2 by a specific TCL to at least 2 consecutive 16-mer peptides (exception being the terminal p143-158) was regarded as epitope specific. The most stimulatory epitope regions (I-IV) are highlighted in red. The most prevalently recognized region (ie, amino acids 43-67) is marked with a red box.

that Can f 4-specific memory CD4⁺ T cells can be isolated at a distinctly higher frequency from the peripheral blood of allergic than nonallergic subjects. Moreover, the cells of the former group exhibit a stronger T-cell receptor (TCR) avidity and T_H2 bias. Peptides covering the Can f 4 sequence amino acids 43 to 67 were found to elicit the most widespread T-cell reactivity in our study population comprising subjects with diverse HLA class II backgrounds. Therefore we consider this region an important mediator of allergenicity to Can f 4.

In the current study we isolated Can f 4-specific memory CD4⁺ TCLs from the majority (92%) of the allergic subjects but only from 25% of the nonallergic subjects. Although specific TCLs were obtained at a relatively low frequency in both subject groups, ranging from 1 to 10 per 10⁶ CD4⁺CD45RO⁺ T cells per subject (Fig 1, A), we observed an 8-fold higher frequency of allergen-specific memory CD4⁺ T cells in the allergic subjects in comparison with those without allergy. A number of other studies exploiting the same methodology,^{11,27} an *ex vivo*

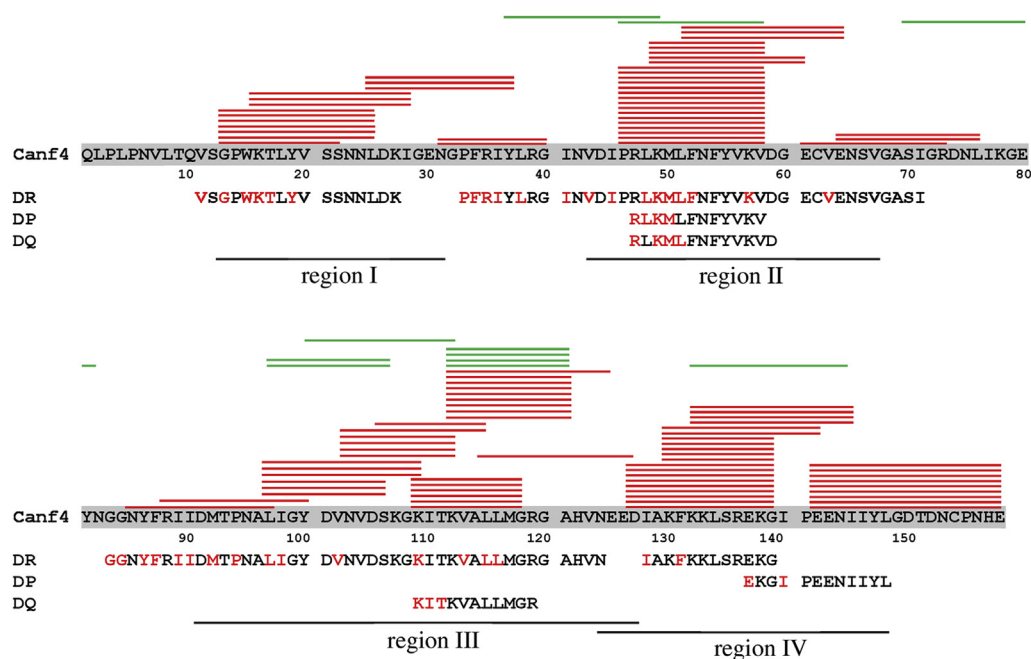


FIG 4. Alignment of the T-cell epitope core sequences and predicted HLA-binding motifs on the Can f 4 sequence. Lines above the Can f 4 sequence represent the core sequences recognized by each TCL obtained from allergic (red) and nonallergic (green) subjects. Below the sequence are presented the predicted HLA-binding motifs (the length of 9 amino acids, with P1 anchor residues colored in red). Black lines indicate the most frequently recognized regions (I-IV) of Can f 4.

region	aa	DR1	DR3	DR4	DR7	DR11	DR15
I	13-28	293	424	71	4	1768	6
	43-58	0.2	>3 333	11	4	6	0.3
II	46-61	2	>3 333	5 882	4	8	1
	49-64	>13 353	>3 333	>6 215	>6 873	>9210	>1 167
	52-67	24	132	1 363	205	2236	116
III	91-106	41	>3 333	756	1 168	685	8
	94-109	>13 353	>3 333	43	600	>9210	20
	97-112	5 143	1	682	3 500	1531	1 500
	100-115	22 857	1	>6 215	>6 873	>9210	>1 167
	103-118	2 309	>3 333	>6 215	83	5000	232
	106-121	48	94	323	39	23	31
	109-124	1	>3 333	>6 215	18	17	3
IV	112-127	5	>3 333	>6 215	21	200	13
	131-145	3 237	>3 333	>6 215	>6 873	24	>1 167

FIG 5. Binding affinities of the Can f 4 peptides to HLA-DR molecules. The binding affinities of 15 peptides covering the 4 most prevalently recognized epitope regions (I-IV) of Can f 4 to a panel of 6 common HLA-DR molecules are indicated as relative binding ratios. Lower numbers correspond to higher binding affinities. Green, Moderate binding (IC_{50} ratio, 20-100); red, strong binding (IC_{50} ratio, <20). aa, Amino acids.

tetramer-based approach,^{15,27-30} or both have similarly demonstrated increased levels of allergen-specific memory CD4⁺ T cells in allergic subjects.

Interestingly, we found both here (Fig 1, B) and previously with another dog allergen, Can f 1,¹¹ that the memory CD4⁺ T cells of allergic subjects are of higher functional avidity. The observation is likely attributed to the selective expansion of allergen-specific T cells *in vivo*, a phenomenon known to favor clonotypes with strong antigen-binding capacity.³¹⁻³³ Of note, we also observed a higher expression of CD25, the IL-2 receptor α chain, on the T cells of allergic subjects (Fig 2, C). On the basis of these findings,

the Can f 4-specific T cells of allergic and healthy subjects exhibit differential capacities to become activated and expand in response to allergen stimulation *in vivo*, which might partially account for the observed difference in specific memory T-cell frequencies between the groups. In support of this interpretation, we found that in all allergic subjects with 3 or more TCLs obtained, at least 2 individual TCLs from a subject recognized exactly the same epitope regions of Can f 4 (see Fig E2). Although not analyzed here at the TCR sequence level, these TCLs likely represent clonal expansions of the same allergen-specific precursors. The reason for the restricted capacity of nonallergic subjects to

generate allergen-specific memory CD4⁺ T-cell responses of higher avidity remains currently unclear. The suggested mechanisms at least include factors associated with differences in TCR recognition of an allergen^{27,34} and peripheral regulation of the immune response.¹³

The phenotypic characterization of the TCLs showed a T_H2-biased response with a predominant IL-4 and IL-5 production and a low expression of the surface marker CXCR3 in allergic subjects (Fig 2). The findings are most likely attributed to the atopic status of the subjects because no phenotypic polarization was observed in relation to the functional avidity or the epitope specificity of the TCLs (data not shown). Among the nonallergic subjects, the few memory T cells, which were of low avidity, were observed to respond to the Can f 4 stimulation with IFN- γ - and IL-10-biased production of the cytokines. This seems to be a predominant finding also in other recent studies,^{15,30,35} suggesting that the weak allergen-specific memory CD4⁺ T-cell response induced in nonallergic subjects is predominantly T_H1 or T_R1 deviated.

A potential caveat in the interpretation of the current data is that the adopted long-term *in vitro* stimulation protocol of T cells could selectively modify the T-cell frequencies or phenotypes discovered. However, these outcomes are likely minimal because a similar bias in the frequency and phenotype of memory CD4⁺ T cells between the allergic and nonallergic subjects has also been demonstrated *ex vivo* by using HLA class II tetramers.^{27,28,36} In line with this notion, in a study by Van Overtvelt et al,³⁵ the phenotypic characteristics of birch Bet v 1-specific TCLs were not skewed by a long-term *in vitro* culture. Importantly, compared with the HLA class II tetramer approach, our *in vitro* stimulation protocol can identify allergen-specific CD4⁺ T cells irrespective of their HLA restriction and TCR avidity, allowing a more comprehensive analysis of the responding T-cell repertoire.

The use of vaccines composed of allergen peptides is an attractive alternative for crude allergen extracts in allergen immunotherapy because of the lower risk of adverse systemic reactions caused by IgE cross-linking on mast cells.^{17,18} In this context allergen peptides with a capacity to bind to a wide range of HLA class II molecules can open prospects for the development of population-wide peptide immunotherapy. Therefore it is of interest that our epitope mapping revealed 4 Can f 4 regions capable of stimulating T cells of multiple subjects with diverse HLA backgrounds (see Fig 3 and Table E5 in this article's Online Repository at www.jacionline.org). Moreover, the peptides covering these regions induced a stronger proliferation in the TCLs of allergic subjects than the rest of the Can f 4 peptides. Importantly, the most promiscuous region, amino acids 43 to 67, was recognized by 90% of the allergic subjects tested. The significance of this region for the allergenicity of Can f 4 was further emphasized by the observation that 3 of the allergic subjects reacted exclusively to it. The core of this region, covered by the 19-mer sequence between amino acids 46 and 64 (PRLKMLFNFYVKVDGECVE), contains multiple predicted HLA-binding motifs (Fig 4), 4 of which we could also experimentally verify as minimal core epitopes with truncated peptides (see Fig E3). A single 19-mer peptide or a few shorter peptides covering this immunodominant region could be attractive candidates for the development of PIT of dog allergy. Because not all allergic patients are sensitized to Can f 4, peptide preparations considered for immunotherapeutic trials should

probably also include dominant T-cell epitopes from other important dog allergens, such as those previously identified in Can f 1.¹⁰

When the epitope-specific TCLs were studied in terms of their HLA class II restriction, the majority of the responses were observed to be restricted by HLA-DR (see Table E4). Previously, similar results have been obtained with at least German cockroach,²⁵ timothy grass,³⁷ ragweed,³⁸ and horse²⁰ allergens. Importantly, restrictions by at least 2 different HLA loci/alleles were observed with TCLs specific to each of the 4 most prevalently recognized Can f 4 epitope regions. In line with these results, the bioinformatically predicted HLA-binding motifs were found to cluster in these regions (Fig 4), and peptides covering them were commonly observed to be capable of binding to several HLA molecules (Fig 5). Of special interest, the core of the most stimulatory Can f 4 region, amino acids 43 to 67, was predicted to contain a total of 13 HLA-DR-, HLA-DP-, and HLA-DQ-binding motifs (Fig 4). It also exhibited a high binding affinity to most of the HLA molecules tested (Fig 5). These data collectively suggest that the immunogenicity of the Can f 4 epitopes is linked with their promiscuous binding capacity to different HLA class II molecules.³⁹⁻⁴¹

In summary, we have provided here an in-depth analysis of human T-cell reactivity to Can f 4, an important dog allergen. Both allergic and nonallergic subjects were found to harbor allergen-specific memory CD4⁺ T cells that differed significantly in both frequency and phenotype. The binding capacity of Can f 4 epitope-containing peptides to a diversity of HLA class II molecules, as demonstrated both bioinformatically and experimentally, turned out to play a major role in their capacity to induce T-cell responses *in vivo*. Therapeutically, a 19-mer sequence between amino acids 46 to 64 containing the core sequence of the promiscuous epitope region of Can f 4 appears to be a useful target for further investigation.

The skillful technical assistance of Virpi Fisk and Merja Esselström is gratefully acknowledged.

Key messages

- The frequency of Can f 4-specific memory CD4⁺ T cells is 8-fold higher in allergic than nonallergic subjects.
- The immunodominance of Can f 4 epitopes is linked with their HLA class II-binding capacity.
- A 19-mer peptide sequence containing the core of the immunodominant epitope region of Can f 4 demonstrates potential for immunotherapy.

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METHODS

Subjects

Peripheral blood samples were collected from 13 patients with clinical dog allergy and from 12 dog dander-exposed nonatopic subjects for this study. The 13 allergic subjects were characterized at the Pulmonary and Dermatology Clinics of Kuopio University Hospital. They exhibited rCan f 4-specific IgE reactivity and reported exacerbation of allergic symptoms on dog dander exposure (Table E1). IgE antibodies to rCan f 4 were measured with an indirect ELISA, as described previously.^{E1} The cutoff for a positive reaction was defined as the mean OD of the control subjects + 3 SDs. The 12 nonallergic subjects reported no medical history of atopy or allergic symptoms of any kind. All of the subjects enrolled in the study reported previous dog dander exposure, and the exposure frequency did not differ between the subject groups ($P > .05$, Fisher exact test). HLA class II genotyping for the DQ and DR alleles of the subjects was performed in the Clinical Laboratory of Finnish Red Cross Blood Service (Helsinki, Finland)^{E2} or the Immunogenetics Laboratory of the University of Turku (Turku, Finland)^{E3} with PCR-SSO and PCR-SSP methods (Table E5). The distribution of the HLA class II alleles did not differ between allergic and healthy subjects ($P > .05$, Fisher exact test).

Generation of Can f 4-specific CD45RO⁺CD4⁺ TCLs with the split-well method

After purifying the PBMCs by using Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden), memory (CD4⁺CD45RO⁺CD45RA⁻) T cells were isolated with the memory CD4⁺ isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The mean purity of the memory T cells of allergic and nonallergic subjects was $99.7\% \pm 0.3\%$ and $99.2\% \pm 0.6\%$, respectively, as assessed by staining with anti-CD4–fluorescein isothiocyanate, anti-CD45RA–allophycocyanin, and anti-CD45RO–phycoerythrin-Cy7 (all from BD Biosciences, San Jose, Calif). The TCLs were generated and analyzed, as previously described,^{E4,E5} with minor modifications. The isolated memory T cells were seeded in 96-well U-bottom plates (Corning Incorporated, Corning, NY; 5×10^4 cells per well; 60–90 wells per subject) in the presence of $10 \mu\text{g/mL}$ rCan f 4 in a $150\text{-}\mu\text{L}$ volume of RPMI 1640 culture medium supplemented with 2 mmol/L L-glutamine, $20 \mu\text{mol/L}$ 2-mercaptoethanol, 1 mmol/L sodium pyruvate, nonessential amino acids, 100 IU/mL penicillin, $100 \mu\text{g/mL}$ streptomycin, 10 mmol/L HEPES (all from Lonza, Verviers, Belgium), and 5% inactivated human AB serum (Sigma-Aldrich, St Louis, Mo). Cells retained in the MACS separation columns after memory T-cell isolation were flushed out and γ -irradiated (3000 rad) and used as antigen-presenting cells (APCs) at 1.5×10^5 cells per well. Ten wells with $1 \mu\text{mol/L}$ purified protein derivative (PPD) served as a positive control. On day 5, $50 \mu\text{L}$ of fresh medium supplemented with 20 IU/mL IL-2 (Strathmann Biotech, Hannover, Germany) was added to the cultures. On day 10, half of the culture medium was removed, and the cultures were restimulated with 1.5×10^5 γ -irradiated (3000 rad) PBMCs, $10 \mu\text{g/mL}$ rCan f 4, and 20 IU/mL IL-2 in a final medium volume of $150 \mu\text{L}$. On day 15, $50 \mu\text{L}$ of fresh medium supplemented with 20 IU/mL IL-2 was added to the cultures. On day 20, after replacing the supernatants with fresh medium, half of the cultures were split into 2 daughter plates stimulated with or without rCan f 4 ($10 \mu\text{g/mL}$) in the presence of autologous γ -irradiated (3000 rad) PBMCs (5×10^4) as APCs. After an incubation of 72 hours, tritiated thymidine was added ($1 \mu\text{Ci}$ per well; GE Healthcare, Little Chalfont, United Kingdom), and after an additional 16 hours, cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by means of scintillation counting (Wallac Micro Beta 1540), and the results were expressed as counts per minute. The counts per minute differences between stimulated and unstimulated wells (ΔCPM) and SIs (counts per minute in the presence of antigen divided by counts per minute in the absence of antigen) were determined. Positive cultures ($\Delta\text{CPM} > 1000$ and $\text{SI} > 2$) were next restimulated in 48-well plates with autologous γ -irradiated PBMCs (10^6 per well), $10 \mu\text{g/mL}$ rCan f 4, and 25 to 50 IU/mL IL-2. Half of the culture medium was replaced with fresh medium supplemented with IL-2 (25 IU/mL) every 2 to 3 days during the 14-day culture before proliferation assays.

Proliferation assays

Proliferation assays were set in 96-well U-bottom plates. Cultured T cells (2.5×10^4) were stimulated in duplicates with rCan f 4 (five 10-fold dilutions from $100 \mu\text{g/mL}$) and 16-mer peptides ($5 \mu\text{g/mL}$). Wells containing PHA ($1 \mu\text{g/mL}$) and those without stimulant were included as positive and negative controls, respectively. Autologous γ -irradiated (3000 rad) PBMCs (5×10^4) served as APCs. Proliferation was measured based on radionuclide uptake (see above). Responses of SIs of greater than 2 to rCan f 4 at $100 \mu\text{g/mL}$ and to at least 2 adjacent peptides were regarded as specific. The epitope reactivity of 9 TCLs could not be tested because of low cell numbers. The frequencies of Can f 4-specific memory CD4⁺ T cells were estimated by the number of specific cultures per seeded memory CD4⁺ T cell ($3\text{--}4.5 \times 10^6$) per subject, assuming that each of them represents monoclonal T-cell growth. EC_{50} values (ie, the rCan f 4 concentration needed to induce a half-maximal proliferative response) were determined from the dose-response curves of individual TCLs.

Cytokine assays

Supernatants ($100 \mu\text{L}$ per well in duplicates) were collected from proliferation assay plates after a 72-hour stimulation with $100 \mu\text{g/mL}$ rCan f 4 and stored at -70°C until analyzed. The production of IL-4, IL-5, IFN- γ , IL-10, and IL-17 was measured by using commercial ELISA kits (DuoSet; R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions.

Flow cytometric analyses

Expression of cell-surface markers was analyzed with a BD FACSCanto II flow cytometer (BD, San Jose, Calif) after staining T cells with anti-CD4–fluorescein isothiocyanate and a panel of phycoerythrin-, allophycocyanin-, or phycoerythrin-Cy7–labeled mAbs (CD45RO, CD45RA, CD62 ligand, CCR7, CD25, cytotoxic T lymphocyte-associated protein 4, CXCR3, CCR4, and CCR6; all from BD Biosciences) or corresponding isotype control mAbs on day 40.

HLA restriction analyses

HLA restriction of Can f 4 epitope-recognizing TCLs was assessed by inhibiting the proliferative responses to epitope-containing peptides with HLA antigen-specific antibodies *in vitro*, as previously described.^{E4,E6} In brief, after a 1-hour incubation of 5×10^4 APCs in duplicates with $10 \mu\text{g/mL}$ mAbs to HLA-DR (L243), HLA-DQ (SPVL3), and HLA-DP (B7/21) in 96-well U-bottom plates, 2.5×10^4 T cells together with $10 \mu\text{g/mL}$ of the specific peptide were added to the wells. Proliferation was measured based on radionuclide uptake (see above), and an inhibition of the response by at least 50% was considered significant. Moreover, individual restricting alleles were determined, as previously described,^{E4,E6} by using allogeneic, partially HLA-matched PBMCs and/or EBV-transformed B-cell lines with known HLA phenotypes as APCs.

HLA class II peptide-binding assays

HLA-DR molecules were purified from homozygous EBV cell lines by means of affinity chromatography with the monomorphic mAb L243.^{E7} The binding to HLA-DR molecules was assessed by means of competitive ELISA, as previously reported.^{E7} Each peptide-MHC combination was evaluated in 2 independent experiments. A peptide concentration that prevented binding of 50% of the labeled peptide (half-maximal inhibitory concentration [IC_{50}]) was evaluated. Data were expressed as a relative affinity (ie, ratio of the IC_{50} of the peptide to the IC_{50} of the reference peptide, which is a high binder to the HLA-DR molecule). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their sequences and mean IC_{50} values were the following: HA 306-318 (PKYVKQNTLKLAT) for DRB1*0101 (3 nmol/L), DRB1*0401 (70 nmol/L), DRB1*1101 (5 nmol/L), and DRB1*0701 (7 nmol/L); A3 152-166

(EAEQLRAYLDGTGVE) for DRB1*1501 (40 nmol/L); and MT 2-16 (AKTIAYDEEARRGLE) for DRB1*0301 (30 nmol/L).

Statistical analyses

The Mann-Whitney *U* test was used for group analyses of TCL frequencies (Fig 1, A), EC₅₀ values (Fig 1, B), cytokine production (Fig 2, A), and cell-surface phenotyping (Fig 2, C). The Fisher exact test was used for analysis of categorical phenotyping of cytokine data (Fig 2, B), the Wilcoxon signed rank test was used for analysis of HLA peptide binding between the peptides (Fig 5), and the Spearman test was used for correlation analysis of IgE production and T-cell frequencies of the subjects (Fig E1). In other cases statistical tests were used as indicated.

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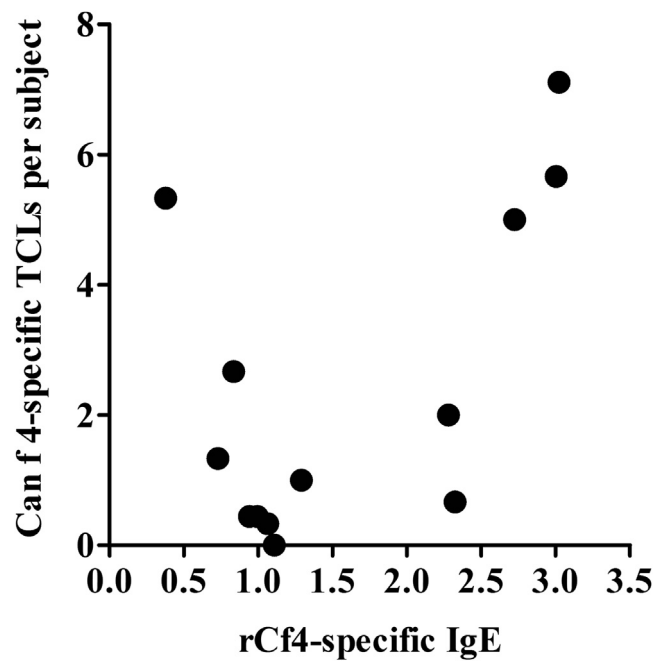


FIG E1. Correlation between serum IgE reactivity to rCan f 4 (OD: 450 nm, *x-axis*) and the number of Can f 4-specific T cells per 10^6 CD4⁺CD45RO⁺ T cells obtained from 13 allergic subjects (*y-axis*). Spearman $r = 0.3219$, $P = .28$.

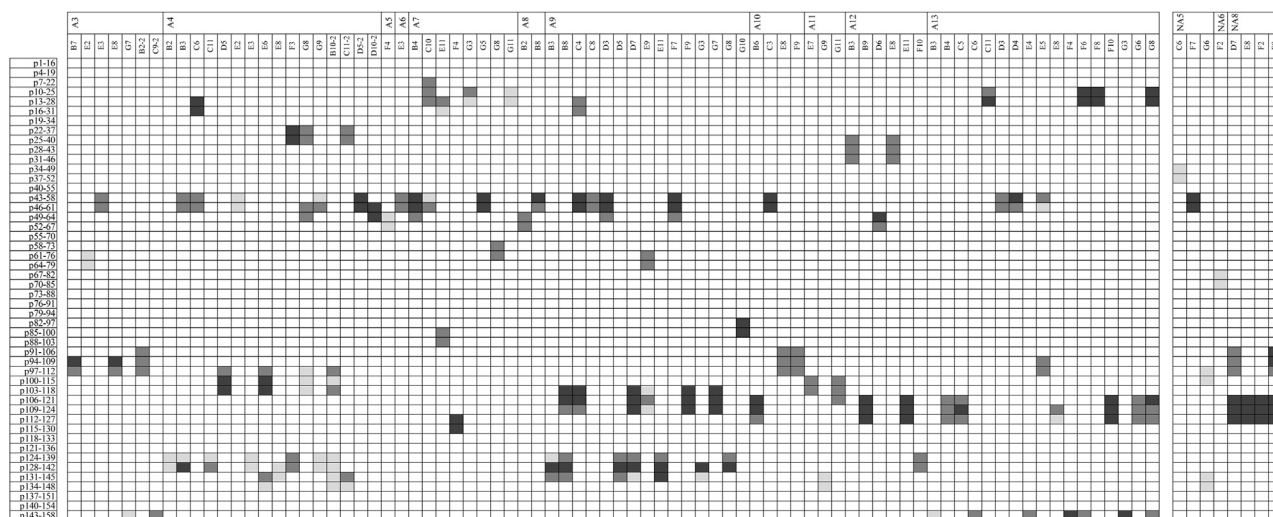


FIG E2. Full map of the epitope regions recognized by each Can f 4-specific TCL obtained. A T-cell response with SI of greater than 2 to at least 2 consecutive peptides was considered epitope specific. *Black boxes*, Proliferation of Δ CPM of greater than 5000; *dark gray boxes*, Δ CPM of 1000 to 5000; *light gray boxes*, Δ CPM of less than 1000.



FIG E3. Fine epitope mapping. Five minimal T-cell core epitopes within the core of the immunodominant epitope region of amino acids 46 to 64 were discovered by stimulating 12 TCLs from 6 different subjects specific to the region with a panel of 16 peptides of 9 to 16 amino acids truncated at the N- or C-terminus. An SI of greater than 2 and a Δ CPM of greater than 1000 (dotted line) was considered a positive response. The minimal core epitopes are indicated with vertical lines.

TABLE E1. Demographic data

Study group	ID	Sex	Age (y)	Dog dander exposure	Asthma	Allergic symptoms	Exacerbation*	Specific IgE ELISA (OD: 450 nm)		
								Can f 4	Can f 1	Can f 2
Allergic	A-1	M	21	Occasional	No	R, U, C, W	Yes	1.108	ND	ND
	A-2	M	62	Daily	Yes	R, U, C, W	Yes	0.942	0.166	0.000
	A-3	F	55	Daily	Yes	W	Yes	0.375	0.544	0.000
	A-4	F	49	Occasional	No	R	Yes	3.023	ND	ND
	A-5	M	61	Occasional	Yes	R, U, C, W	Yes	2.171	2.819	0.000
	A-6	F	44	Daily	Yes	R, U	Yes	1.108	0.456	0.000
	A-7	F	48	Occasional	Yes	W	Yes	1.579	2.504	1.850
	A-8	F	55	Daily	Yes	R, U, W	Yes	2.850	0.858	0.774
	A-9	M	40	Childhood	Yes	W	Yes	3.071	2.608	2.707
	A-10	F	56	Daily	Yes	R	Yes	1.382	0.224	0.061
	A-11	M	65	Occasional	Yes	W	Yes	1.290	0.000	ND
	A-12	M	36	Occasional	Yes	R, U, W	Yes	2.280	ND	ND
	A-13	M	36	Occasional	Yes	R, W	Yes	3.003	3.131	ND
Nonallergic	NA-1	F	48	Occasional	No	No	—	—	—	—
	NA-2	F	51	Occasional	No	No	—	—	—	—
	NA-3	M	22	Childhood	No	No	—	—	—	—
	NA-4	F	34	Occasional	No	No	—	—	—	—
	NA-5	M	37	Occasional	No	No	—	—	—	—
	NA-6	M	32	Occasional	No	No	—	—	—	—
	NA-7	F	26	Occasional	No	No	—	—	—	—
	NA-8	M	34	Occasional	No	No	—	—	—	—
	NA-9	M	26	Daily	No	No	—	—	—	—
	NA-10	F	27	Daily	No	No	—	—	—	—
	NA-11	F	27	Occasional	No	No	—	—	—	—
	NA-12	F	57	Occasional	No	No	—	—	—	—

C, Conjunctivitis; ND, not done; R, rhinitis; U, urticaria; W, wheezing.

*Self-reported exacerbation of symptoms under dog dander exposure.

TABLE E2. Peptides used in the study

1	QLPLPNVLTQVSGPWK (1-16)	25	RDNLIKGEYNGGNYFR (73-88)
2	LPNVLTQVSGPWKTLY (4-19)	26	LIKGEYNGGNYFRIID (76-91)
3	VLTQVSGPWKTLYVSS (7-22)	27	GEYNGGNYFRIIDMTP (79-94)
4	QVSGPWKTLYVSSNNL (10-25)	28	NGGNYFRIIDMTPNAL (82-97)
5	GPWKTLYVSSNNLDKI (13-28)	29	NYFRIIDMTPNALIGY (85-100)
6	KTLYVSSNNLDKIGEN (16-31)	30	RIIDMTPNALIGYDVN (88-103)
7	YVSSNNLDKIGENGPF (19-34)	31	DMTPNALIGYDVNVDS (91-106)
8	SNNLDKIGENGPFRIY (22-37)	32	PNALIGYDVNVDSKGG (94-109)
9	LDKIGENGPFRIYLRG (25-40)	33	LIGYDVNVDSKGGKITK (97-112)
10	IGENGPFRIYLRGINV (28-43)	34	YDVNVDSKGGKITKVAL (100-115)
11	NGPFRIYLRGINVDIP (31-46)	35	NVDSKGGKITKVALLMG (103-118)
12	FRIYLRGINVDIPRLK (34-49)	36	SKGGKITKVALLMGRGA (106-121)
13	YLRGINVDIPRLKMLF (37-52)	37	KITKVALLMGRGAHVN (109-124)
14	GINVDIPRLKMLFNFY (40-55)	38	KVALLMGRGAHVNEED (112-127)
15	VDIPRLKMLFNFYVKV (43-58)	39	LLMGRGAHVNEEDIAK (115-130)
16	PRLKMLFNFYVKVDGE (46-61)	40	GRGAHVNEEDIAKFVK (118-133)
17	KMLFNFYVKVDGECVE (49-64)	41	AHVNEEDIAKFVKLSR (121-136)
18	FNFYVKVDGECVENS (52-67)	42	NEEDIAKFVKLSREKG (124-139)
19	YVKVDGECVENSVGAS (55-70)	43	DIKFVKLSREKGIP (127-142)
20	VDGECVENSVGASIGR (58-73)	44	KFKLSREKGIP (130-145)
21	ECVENSVGASIGRDNL (61-76)	45	KLSREKGIP (133-148)
22	ENSVGASIGRDNLIG (64-79)	46	REKGIP (136-151)
23	VGASIGRDNLIGGEYN (67-82)	47	GIP (139-154)
24	SIGRDNLIGGEYNGGN (70-85)	48	EENIYLGDNDNCPNHE (142-158)

TABLE E3. Can f 4 peptide specificity of the higher-avidity TCLs

Peptide specificity (amino acids)	Subject/TCL	EC ₅₀
13-31	A-7/E11*	1.1
13-28	A-4/C6*	0.9
22-40	A-4/F3*	1.2
25-46	A-12/E8	1.6
43-58	A-9/C8	1.5
43-58	A-10/C3*	1.1
43-64	A-9/D3	1.0
43-64	A-9/F7	1.3
49-67	A-12/D6	0.6
82-100	A-9/G10	0.9
94-112	A-3/E8	1.7
97-112	A-4/D5	1.1
97-118	A-4/B10-2*	0.1
103-124	A-9/C4*	1.4
106-121	A-12/B9	0.2
106-121	A-12/E11	0.3
124-142	A-4/B2	1.2
124-142	A-12/F10	0.1
143-158	A-3/C9-2	1.9

*Specificity to 2 to 3 epitope regions; the result from the most stimulatory region (highest SI) is presented.

TABLE E4. HLA class II restrictions of the Can f 4 epitope-specific TCLs

Region	Peptide (amino acids)	Subject/TCL	HLA locus	Restricting allele
I	10-25	A-7/C10	DQ	DQB1*02/DQB1*0303
	13-28	A-9/C4	DR	DRB1*1501, DRB5*0101
	13-28	A-13/F6	DR	DRB1*0101/DRB1*0401
II	31-46	A-12/E8	DR/DQ	DRB1*0801/DRB1*0101, DQB1*0202/0603
	43-58	A-13/D3	DR	DRB1*0101/DRB1*0401
	43-58	A-7/G5	DR/DP	DRB1*0701, DRB4*0101
	43-58	A-8/B8	DR/DP	DRB1*1501/DRB1*0101
	43-58	A-9/D3	DR/DP	DRB1*1501, DRB5*0101
	43-58	A-10/C3	DR	DRB1*04/DRB1*0101, DRB4*0103
	46-61	A-13/G11	DR	DRB1*0101
	46-61	A-6/E3	DR	DRB1*0101/DRB1*1301
	46-61	A-9/F7	DR/DP	DRB1*1101/DRB1*1501, DRB5*0101
	49-64	A-8/B2	DR	DRB1*1501
	61-76	A-7/G8	DQ	DQB1*02/DQB1*0303
	64-79	A-9/E9	DQ	DQB1*0301/DQB1*0602
	85-100	A-9/G10	DR	DRB1*1101/DRB1*1501, DRB5*0101
	85-100	A-7/E11	DQ	DQB1*02
	94-109	NA-8/D7	DP/DQ	DQB1*0501
III	94-109	A-10/F9	DR	DRB1*04/DRB1*0101, DRB4*0103
	100-115	A-11/G11	DR	DRB1*0701/DRB1*1301, DRB4*0101
	106-121	A-13/G8	DR	DRB1*0101/DRB1*0401
	106-121	A-9/G7	DQ	DQB1*0301/DQB1*0602
	109-124	A-13/C5	DR	DRB1*0101/DRB1*0401
	109-124	A-12/B9	DR/DQ	DRB1*0801/DRB1*0101, DQB1*0202/0603
	109-124	A-10/B6	DR	DRB1*04/DRB1*0101, DRB4*0103
	109-124	A-13/F10	DR	DRB1*0101/DRB1*0401
	112-127	NA-8/D7	DR	DRB1*0101
	112-127	A-7/F4	DQ	DQB1*02/DQB1*0303
IV	124-139	A-12/F10	DR	N/A
	128-142	A-9/E11	DR	DRB1*1101/DRB1*1501, DRB5*0101
	134-148	A-11/G9	DP	N/A
	143-158	A-13/E4	DR	DRB1*0101/DRB1*0401
	143-158	A-13/F4	DQ	DQB1*0302
	143-158	A-13/G3	DR	DRB1*0101/DRB1*0401

TABLE E5. HLA class II genotypes of the subjects

Study group	ID	HLA genotype			
		DRB1_1	DRB1_2	DQB1_1	DQB1_2
Allergic	A-1	0801	1501	04	0602
	A-2	0101	1501	501	0602
	A-3	0401	0404	0302	03/04
	A-4	1501	1302	0602	0604
	A-5	0101	1501	0501	0602
	A-6	0101	1301	0501	0603
	A-7	0701	0901	02	0303
	A-8	0101	1501	0501	0602
	A-9	1101	1501	0301	0602
	A-10	04	0101	0301	0501
	A-11	0701	1301	0202	0603
	A-12	0801	0101	04	0501
	A-13	0401	0101	0302	0501
Nonallergic	NA-1	11/12/1303	—	0301	0502/3
	NA-2	0403	1501	0302	0602
	NA-3	1501	1302	0602	0604
	NA-4	NA	NA	NA	NA
	NA-5	0801	1501	04	0602
	NA-6	11/12/1303	1501	0301	0602
	NA-7	04	11/12/1303	0301	0301
	NA-8	0801	0101	04	0501
	NA-9	0801	1301	04	0603
	NA-10	0403	1501	0302	0602
	NA-11	0301	01/10	02	0501
	NA-12	01/10	1501	0501	0602