

Differential CD4⁺ T-cell responses of allergic and non-allergic subjects to the immunodominant epitope region of the horse major allergen *Equ c 1*

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Introduction

Recent studies suggest that allergen-specific T-cell repertoires between allergic and non-allergic individuals differ. It has been discovered, for example, that the frequency of allergen-specific CD4⁺ memory T cells, despite being low in general, is considerably higher in allergic individuals sensitized to mammalian or plant allergens than in healthy individuals.^{1–7} Accordingly, one recent study reported that the terminally differentiated CD27-negative allergen-specific CD4⁺ T cells, producing the T helper type 2 (Th2) cytokines and expressing chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2), were only found in allergic subjects; in non-allergic individuals, these cells were absent.⁶ In our

Summary

The responses of allergen-specific CD4⁺ T cells of allergic and healthy individuals are still incompletely understood. Our objective was to investigate the functional and phenotypic properties of CD4⁺ T cells of horse-allergic and healthy subjects specific to the immunodominant epitope region of the major horse allergen *Equ c 1*. Specific T-cell lines (TCLs) and clones were generated from peripheral blood mononuclear cells with *Equ c 1*_{143–160}, the peptide containing the immunodominant epitope region of *Equ c 1*. The frequency, proliferative response, cytokine production and HLA restriction of the cells were examined. The frequency of *Equ c 1*-specific CD4⁺ T cells was low (approximately 1 per 10⁶ CD4⁺ T cells) in both allergic and non-allergic subjects. The cells of allergic subjects had a stronger proliferative capacity than those of non-allergic subjects, and they predominantly emerged from the memory T-cell pool and expressed the T helper type 2 cytokine profile, whereas the cells of non-allergic subjects emerged from the naive T-cell pool and produced low levels of interferon- γ and interleukin-10. T-cell response to *Equ c 1*_{143–160} was restricted by several common HLA class II molecules from both DQ and DR loci. As the phenotypic and functional properties of *Equ c 1*-specific CD4⁺ T cells differ between allergic and non-allergic subjects, allergen-specific T cells appear to be tightly implicated in the development of diseased or healthy outcome. Restriction of the specific CD4⁺ T-cell response by multiple HLA alleles suggests that *Equ c 1*_{143–160} is a promising candidate for peptide-based immunotherapy.

Keywords: CD4⁺ T-cell response; *Equ c 1*; frequency; horse; lipocalin allergen.

studies with the mammalian lipocalin allergens for cow *Bos d 2* and dog *Can f 1*, allergen-specific CD4⁺ T cells of allergic individuals were predominantly of the Th2 phenotype and they showed higher functional or structural T-cell receptor (TCR) avidity than did the cells from non-allergic individuals.^{1,2} Moreover, the allergen-specific CD4⁺ T cells of non-allergic subjects were mostly either unpolarized or produced low levels of interferon- γ (IFN- γ) and interleukin-10 (IL-10).^{1,2}

In the current study, we sought to confirm these findings by examining the CD4⁺ T-cell response to the major horse allergen *Equ c 1*, an important lipocalin allergen⁸ with the prevalence of IgE reactivity close to 80% among horse dust-allergic subjects.^{9,10} For this purpose, we analysed the CD4⁺ T-cell responses of horse dust-exposed

Equ c 1-sensitized and healthy subjects focusing on the dominant epitope region of the allergen. This region is strongly recognized by the T cells of almost all *Equ c 1*-sensitized subjects examined.¹¹ As with the major allergen of dog, *Can f 1*, and the major allergen of cow, *Bos d 2*, the frequency of *Equ c 1*-specific CD4⁺ T cells in the peripheral blood is very low. In allergic subjects, it is mostly higher than in non-allergic ones. Moreover, the function and phenotype of *Equ c 1*-specific CD4⁺ T cells differ between these two subject groups.

Materials and methods

Antigens

p143–160 (GIVKENIIDLTIDRCFQ), an 18-mer peptide containing the immunodominant epitope region of *Equ c 1*, was synthesized and purified by GL Biochem (Shanghai, China). Recombinant (r) *Equ c 1* was produced in *Pichia pastoris*, as described previously.¹¹

Subjects and HLA genotyping

Fourteen clinically diagnosed horse-allergic subjects (subjects A–N) with positive (≥ 3 mm) skin prick tests with r*Equ c 1* and nine horse dust-exposed non-atopic control subjects (subjects O–W) with negative skin prick tests were recruited to the study. The subjects were characterized at the Pulmonary Clinic of Kuopio University Hospital, as described in detail previously.¹¹ In brief, the allergic subjects exhibited a positive horse UniCAP result (FEIA; Pharmacia, Uppsala, Sweden; > 0.7 kU/l) and a positive skin prick test (≥ 3 mm) with a commercial horse epithelial extract (ALK Abellø, Hørsholm, Denmark), whereas the control subjects were negative in these tests. The non-atopic control subjects had horse riding as a hobby, and were therefore constantly exposed to horse allergens. Human leucocyte antigen (HLA) class II genotyping for the DQ and DR alleles of the subjects was performed in the Clinical Laboratory of the Finnish Red Cross Blood Service (Helsinki, Finland¹²) or in the Immunogenetics Laboratory of the University of Turku (Turku, Finland¹³) with PCR-based lanthanide-labelled sequence-specific oligonucleotide hybridization (Supplementary material, Table S1). Signed informed consent was provided by all subjects participating in the study and the study was approved by the Ethics Committee of Kuopio University Hospital, permission # 182/99.

Induction of p143–160-specific T-cell lines by the split-well method

To obtain TCLs specific to the immunodominant epitope region of *Equ c 1*, the peripheral blood mononuclear cells (PBMCs) of each donor were first separated by Ficoll-

Table 1. HLA class II restriction of *Equ c 1*-specific T-cell lines and clones

	HLA locus	Restricting allele
T-cell lines		
A11	DR	DRB1*0404/DRB4*0101
A3	DR	DRB1*0404/DRB4*0101
A9	DR	DRB1*0404/DRB4*0101
C3	DR	DRB1*0404/DRB4*0101
C4	DR	DRB1*0404/DRB4*0101
I3	DQ	DQB1*0602
J2	DQ	DQB1*0501
J4	DQ	DQB1*0501
K7	DR	ND
T-cell clones		
A1	DR	DRB1*0404/DRB4*0101
C1	DR	DRB1*0404/DRB4*0101
C2	DR	DRB1*0404/DRB4*0101
J1	DQ	DQB1*0603
K1	DQ	DQB1*0603
Q1	DR	DRB1*0404/DRB4*0101
Q2	DR	DRB1*0404/DRB4*0101

ND, not determined.

Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). Then, the cells were cultured in 30 wells of 96-well round-bottomed plates (Corning Inc., Corning, NY) at a density of 2×10^5 cells per well in 150 μ l of complete RPMI-1640 medium supplemented with 2 mM L-glutamine, 20 μ M 2-mercaptoethanol, sodium pyruvate, non-essential amino acids, 100 IU/ml penicillin and 100 μ g/ml streptomycin, 10 mM HEPES (all from Lonza, Verviers, Belgium), and 5% inactivated human AB serum (Sigma-Aldrich, St Louis, MO) together with p143–160 of *Equ c 1* (10 μ g/ml) at + 37°C. On day 5, 50 μ l of fresh medium was added together with recombinant human IL-2 (rIL-2, final concentration 10 IU/ml; Miltenyi Biotec, Bergisch Gladbach, Germany). On day 10, the cells were restimulated with p143–160 along with 1.5×10^5 γ -irradiated (3000 rads) autologous PBMCs as antigen-presenting cells (APCs) and rIL-2 (10 IU/ml) in a total volume of 150 μ l. On day 15, 50 μ l of fresh medium was added together with rIL-2 (final concentration 10 IU/ml). Finally, on day 20, the wells were split to create two replicate plates by transferring 50 μ l of cell suspension per well to new 96-well daughter plates. Cultures in one of the daughter plates were stimulated with *Equ c 1*_{143–160} (10 μ g/ml) and the other served as a control plate. Proliferation was measured, as described below. Positive cultures (stimulation index > 2) were transferred onto a 48-well plate and restimulated with *Equ c 1*_{143–160} (10 μ g/ml) and rIL-2 (25 IU/ml) in the presence of 10^6 γ -irradiated autologous PBMCs as APCs. The cell lines were incubated for 14 days and supplemented with fresh medium and rIL-2 (25 IU/ml) every 2–3 days before analyses.

T-cell proliferation assay

The T-cell proliferation assays were set up in triplicates on 96-well round-bottomed plates with 2.5×10^4 T cells and 5×10^4 autologous PBMCs together with the *Equ c 1* peptide p143–160 (10 µg/ml) and r*Equ c 1* (100 µg/ml). The plates were then incubated for 3 days at +37°C, after which the cells were pulsed for 16 hr with 1.0 µCi of [³H]thymidine (GE Healthcare, Little Chalfont, UK) per well and harvested onto glass-fibre filters (Wallac, Turku, Finland). Thymidine incorporation was then measured by scintillation counting (MicroBeta TriLux 1450, Wallac), and the results were displayed as mean counts per minute (CPM) or as stimulation indices (SI; CPM of a stimulated culture divided by CPM of an unstimulated culture).

HLA restriction

The HLA restriction of the p143–160-specific TCLs was studied by inhibiting the proliferative response to p143–160 with monoclonal antibodies (1 µg/ml) to HLA-DR (clone L243) and HLA-DQ (clone SPVL3), as described previously.¹⁴ A response with at least a 50% inhibition to p143–160 was considered significant. In addition, allogeneic, partially HLA-matched PBMCs from a person expressing only one shared allele with the subject from whom the TCL was derived were used in proliferation assays.

Cytokine assays

The concentrations of IL-4, IL-5, IL-10, and IFN-γ produced by TCLs specific to both p143–160 and *Equ c 1* were determined by the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Supernatants for the assays (100 µl per well) were collected from the proliferation assay plates on day 3 and they were stored at –70°C until analysed.

CFSE dilution assay and cloning

Memory (CD45RA[–] CD45RO⁺) or naive (CD45RA⁺ CD45RO[–]) CD4⁺ T cells were isolated from freshly purified PBMCs with the no-touch memory or naive CD4⁺ T-cell isolation kits (Miltenyi Biotec). The purity of the cells was 91–99%, as assessed by staining with anti-CD4 FITC, anti-CD45RA allophycocyanin, and anti-CD45RO phycoerythrin-Cy7 antibodies (all from BD Biosciences, San Jose, CA). Non-CD4⁺ cells retained in the separation column were eluted out and used as APCs after irradiation (3000 rads). One million memory or naive T cells were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE; CellTrace CFSE Cell Proliferation Kit, Invitrogen, Eugene, OR) according to the manufacturer's instructions and expanded in a 24-well plate along with

3×10^6 APCs and p143–160 (10 µg/ml) at +37°C. On day 7, half of the cells were analysed with the FACSCanto II flow cytometer (BD Biosciences) for CFSE intensity. Cell division index (CDI) was calculated by dividing the number of CFSE_{low} cells in the stimulated sample by the number of CFSE_{low} cells in the unstimulated sample, and CDI > 2 was considered a positive proliferative response. For the rest of the cells, half of the volume was replaced with fresh medium supplemented with rIL-2 (25 IU/ml). On day 14, the CFSE-labelled TCLs were analysed again for CFSE intensity. Dividing cells were then single-cell sorted into U-bottomed 96-well plates containing 5×10^4 γ-irradiated PBMCs, 2.5×10^3 γ-irradiated Epstein–Barr virus-transformed B cells (both 6000 rads), 1 µg/ml of phytohaemagglutinin (Remel Europe Ltd., Dartford, UK) and 25 IU/ml of rIL-2 using the EPICS Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA). The clonality of the sorted T cells was verified by flow cytometric TCR Vβ-chain analysis, as previously described.¹⁵

HLA class II tetramers

The DRB4*0101:*Equ c 1*_{143–160} tetramer and the control tetramer DRB4*0101:GAD65_{555–567} were generated as described elsewhere.¹⁶ Tetramer staining was performed by incubating T cells with 0.5 µg of the phycoerythrin-labelled tetramers in 50 µl of culture medium for 2 hr at +37°C. After incubation, anti-CD4 FITC was added and the cells were incubated for a further 20 min at +4°C. Finally, the cells were washed twice and analysed with the flow cytometer.

Statistical analyses

Statistical analyses were performed using GRAPHPAD PRISM (GraphPad Software, San Diego, CA). The Mann–Whitney *U*-test, Fisher's exact test and Grubb's test were used as indicated. *P*-values of 0.05 or less were regarded as significant.

Results

CD4⁺ T-cell lines specific to the horse allergen *Equ c 1*_{143–160} peptide are obtained in similar numbers from both allergic and non-allergic individuals

Recent studies have shown that the frequency and proliferative capacity of effector CD4⁺ T helper (Th) cells differ between allergic and non-allergic subjects.^{1–7} In order to evaluate the frequency of peripheral blood CD4⁺ T cells of horse-allergic and healthy subjects specific to the immunodominant epitope region of the horse major allergen *Equ c 1*¹¹, 30 T-cell cultures per person were

initiated from PBMCs with *Equ c 1*_{143–160} employing the split-well method. In total, we obtained 52 and 30 *Equ c 1*_{143–160}-specific TCLs from allergic and non-allergic subjects, respectively (Fig. 1). When the number of *Equ c 1*_{143–160}-specific TCLs was analysed per person, it was found to be similar between the subject groups [3.7 ± 0.6 (mean $\pm$ SEM) and 3.3 ± 1.1 TCLs, respectively; $P > 0.05$, Fisher's exact test]. However, when the *Equ c 1*_{143–160}-specific TCLs that were also specific to the *Equ c 1* protein (protein-specific TCLs) were analysed (30 lines from allergic and 12 from non-allergic subjects) the number of TCLs showed some tendency for difference between the groups (2.1 ± 0.6 and 1.3 ± 0.9 TCLs per person, $P = 0.19$; Fig. 1; black columns). When one non-allergic subject out of nine (subject Q, Fig. 1; Grubb's test for outliers $P < 0.01$ = significant outlier) with an exceptionally high number of protein-specific TCLs (eight; the next largest number for a non-allergic individual was two, Fig. 1) was excluded from the analysis, the difference was statistically highly significant (0.5 ± 0.3 TCLs per non-allergic person, $P < 0.001$). Therefore, this finding suggests that the recognition of the naturally processed epitope from *Equ c 1* by CD4⁺ T cells may be a distinguishing factor between the allergic patients and most of the healthy subjects.

Frequency of *Equ c 1*-specific CD4⁺ T cells in the peripheral blood is low

The frequency of *Equ c 1*_{143–160}-specific CD4⁺ T cells was estimated by the number of positive wells in the split-well cultures on 96-well plates. When a total of six million PBMCs were seeded per person (30 wells, 2×10^5 PBMCs per well), assuming that each positive well represents a monoclonal T-cell growth, the mean frequency of *Equ c 1*_{143–160}-specific T cells of allergic subjects was 0.63 per 10^6 and that of non-allergic subjects was 0.56 per 10^6 PBMCs. Presuming that a person's PBMCs contain 30% of CD4⁺ T cells it can be estimated that there are approx-

imately 2.10 per 10^6 and 1.85 per 10^6 *Equ c 1*_{143–160}-specific CD4⁺ cells in the circulating CD4⁺ T-cell pool of allergic and non-allergic subjects, respectively. Extending the estimation to the CD4⁺ cells that were *Equ c 1* protein-specific as well, the frequencies of specific cells were even lower, around 1.18 per 10^6 CD4⁺ cells for an allergic and 0.74 per 10^6 for a non-allergic subject. Again, if the eight protein-specific lines obtained from the non-allergic subject Q were excluded, the protein-specific CD4⁺ T cells were detected extremely rarely in most non-allergic subjects (0.28 per 10^6).

Equ c 1 protein-specific CD4⁺ T-cell lines from allergic subjects have a stronger proliferative capacity than those from non-allergic subjects

We have previously observed that although T-cell responses to lipocalin allergens are weak in general,^{11,15,17} allergen-specific TCLs from allergic subjects have stronger proliferative capacity than TCLs from non-allergic subjects.^{1,2} Our present results corroborate these findings, as the *Equ c 1* protein-specific TCLs from sensitized subjects proliferated significantly more strongly upon stimulation with the *Equ c 1*_{143–160} peptide than those from non-sensitized subjects ($P < 0.01$, Mann-Whitney *U*-test; Fig. 2). Moreover, the protein-specific TCLs derived from allergic subjects mounted significantly stronger proliferative responses than the TCLs, which only recognized the *Equ c 1*_{143–160} peptide ($P < 0.01$, Fig. 2). This finding may reflect the higher TCR avidity of the *Equ c 1* protein-specific TCLs and further implies that the T cells reactive to the naturally processed epitope are the allergy-associated cells.

Equ c 1 protein-specific T-cell lines from allergic subjects are Th2-biased

We assessed the cytokine profiles of the *Equ c 1* protein-specific TCLs by measuring the concentrations of IL-4,

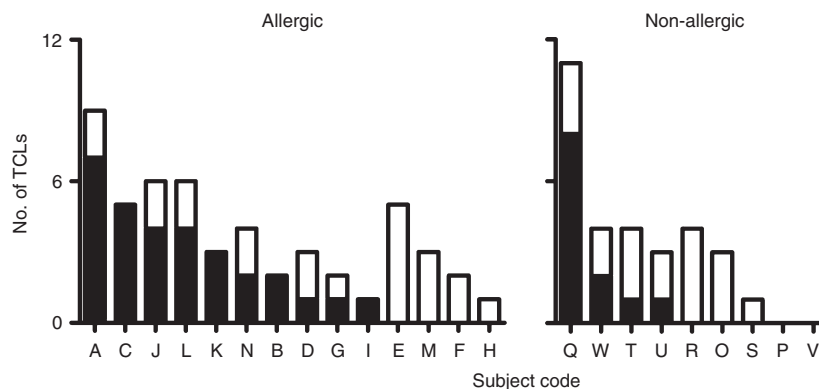


Figure 1. Number of *Equ c 1*_{143–160}-specific TCLs per 30 peripheral blood mononuclear cell cultures obtained with the split-well method from allergic subjects (left panel) and non-allergic subjects (right panel). The number of *Equ c 1* protein-specific T-cell lines is indicated by black.

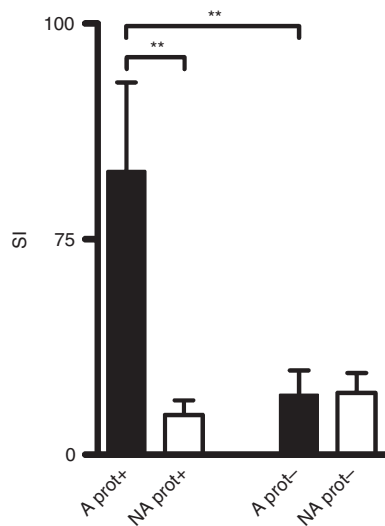


Figure 2. Proliferative responses of the *Equ c 1* protein-specific (prot⁺) T-cell lines and those specific only to the peptide (prot⁻) from allergic (A) and non-allergic (NA) subjects upon stimulation with *Equ c 1*_{143–160} (10 µg/ml). The results are expressed as stimulation indexes (SI). ***P* < 0.01.

IL-5, IL-10 and IFN- γ in the cell culture supernatants (Fig. 3). The TCLs from allergic subjects produced significantly higher levels of the Th2 cytokines IL-4 and IL-5 than TCLs from non-allergic subjects (*P* < 0.01 and *P* < 0.05, respectively, Mann–Whitney *U*-test; Fig. 3). There was no statistically significant difference in the IL-10 and IFN- γ production (*P* > 0.05; Fig. 3). These findings corroborate previous observations,^{2,5,18–20} demonstrating that allergen-specific CD4⁺ T-cell responses in allergic subjects are Th2-biased compared with those in non-allergic subjects.

Equ c 1-specific T-cell responses are derived from the memory CD4⁺ T-cell pool in allergic but not in non-allergic subjects

In order to assess whether the *Equ c 1*-specific responses emerge from the memory or naive T-cell pool, additional short-term T-cell cultures were generated from memory

(CD4⁺ CD45RO⁺) and naive (CD4⁺ CD45RA⁺) T cells purified from PBMCs of eight allergic and six non-allergic subjects. First, the purified cells were stained with the CFSE dye and stimulated with the *Equ c 1*_{143–160} peptide. After *ex vivo* expansion for 7 days, the dividing cells were visualized by flow cytometry (representative examples shown in Fig. 4a). Specific proliferative responses (CDI > 2) were detected in the memory T-cell-derived cultures of five allergic subjects out of eight (63%), whereas no responses were observed in the memory T-cell-derived cultures of the six non-allergic subjects studied (*P* < 0.05, Fisher's exact test; Fig. 4b). All the peptide-specific proliferative responses of the non-allergic subjects were detected in the naive T-cell-derived cultures (Fig. 4b), including the response of the non-allergic subject Q (CFSE analysis shown in Fig. 4a) that had an abnormally high frequency of *Equ c 1*-specific T cells (Fig. 1). To confirm that the *ex vivo*-expanded CFSE_{low} T cells were specific to the *Equ c 1*_{143–160} and the *Equ c 1* protein, T-cell clones generated by single-cell sorting of the expanded T cells were stimulated with the peptide and the protein. The positive results of five memory T-cell-derived clones from allergic subjects and two naive T-cell-derived clones from a non-allergic subject are shown in Fig. 5(a). Taken together, our results demonstrate that the *Equ c 1*-specific CD4⁺ T-cell responses of allergic subjects derive from memory cells while those of non-allergic subjects derive from naive cells.

Equ c 1-specific CD4⁺ T-cell responses are restricted by both HLA-DQ and -DR alleles

The HLA class II restriction of *Equ c 1* protein-specific TCLs and clones from allergic subjects was assessed by inhibiting the responses with anti-HLA-DQ and -DR antibodies (representative examples shown in Fig. 5b) and by using partially HLA-matched PBMCs for antigen presentation. As shown in Table 1, restriction by HLA-DQ was seen in three and by HLA-DR in six out of the nine TCLs investigated. In line with the findings with the TCLs, both HLA-DQ and -DR restrictions were detected with the seven *Equ c 1* protein-reactive T-cell clones from

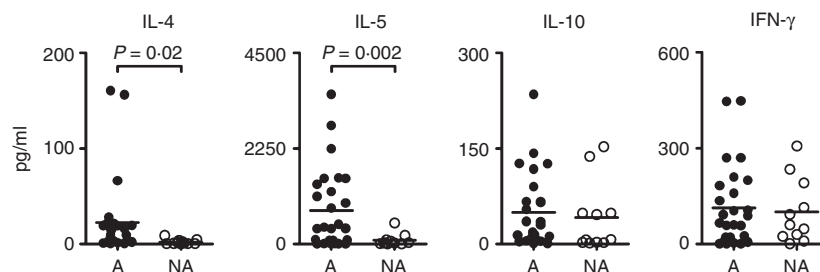


Figure 3. Production of interleukin-4 (IL-4), IL-5, IL-10 and interferon- γ (IFN- γ) by the *Equ c 1*_{143–160}-stimulated (10 µg/ml) *Equ c 1* protein-specific T-cell lines (TCLs). The production of IL-4 and IL-5 by TCLs from allergic subjects (filled symbols) is significantly higher than that by TCLs from non-allergic subjects (open symbols). The production of IL-10 and IFN- γ does not differ between the subject groups (*P* > 0.05).

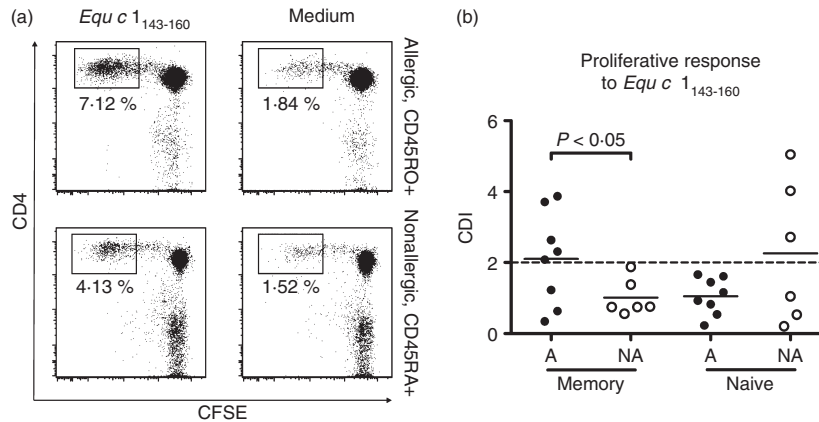


Figure 4. (a) Detection of *Equ c 1*_{143–160}-specific proliferation of peripheral blood memory and naive CD4⁺ T cells by the CFSE dilution assay. CD4⁺ CD45RO⁺ memory cells from the allergic subject B (top panels) and CD4⁺ CD45RA⁺ naive cells from the non-allergic subject Q (bottom panels) were either stimulated with *Equ c 1*_{143–160} (10 µg/ml) or left unstimulated for 7 days. The percentage of divided, CFSE_{low} cells in the samples is indicated. (b) Proliferative responses of the short-term cultures of allergic and non-allergic subjects upon stimulation with the *Equ c 1*_{143–160} peptide (10 µg/ml), analysed by CFSE staining. CDI, cell division index; A, allergic and NA, non-allergic subject.

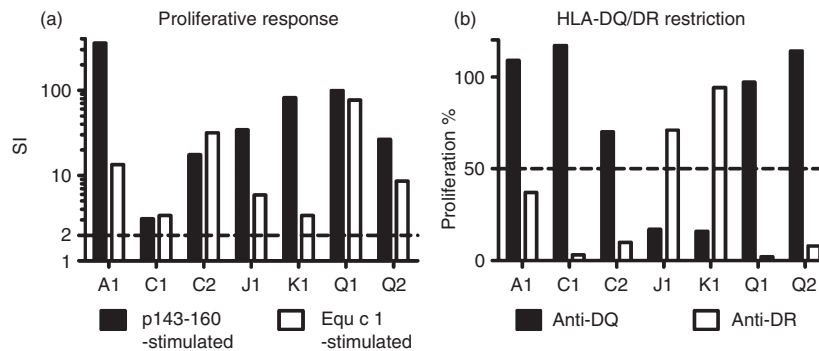


Figure 5. Proliferative responses (a) and HLA restriction (b) of the *Equ c 1*-specific T-cell clones. The T-cell clones derived from memory cells of allergic subjects (A, C, J and K) and those derived from naive cells of a non-allergic subject (Q), generated by cloning the *ex vivo*-expanded CFSE_{low} T cells of the short-term cultures, were stimulated with both the *Equ c 1*_{143–160} peptide (10 µg/ml; filled bars) and the *Equ c 1* protein (100 µg/ml; open bars) (a). The clones were also stimulated with the *Equ c 1*_{143–160} peptide (10 µg/ml) in the presence of anti-HLA-DQ (filled bars) or anti-HLA-DR (open bars) antibodies. Results are expressed as percentages of proliferation (³H]thymidine incorporation) in comparison with the uninhibited *Equ c 1*_{143–160} responses (b). Inhibition of more than 50% of the proliferative response with the anti-HLA antibodies was considered significant.

five different subjects (Fig. 5b and Table 1). More detailed investigations using partially HLA-matched allogeneic PBMCs as APCs revealed that two of the DQ-restricted TCLs were restricted by DQB1*0501 and one by DQB1*0602 and both of the DQ-restricted T-cell clones were restricted by DQB1*0603 (Table 1). Interestingly, we observed that five of the six DR-restricted TCLs and all of the five DR-restricted T-cell clones were restricted by either DRB1*0404 or DRB4*0101 (one TCL was not determined). As the DRB1*0404 and DRB4*0101 restrictions could not be distinguished with partially HLA-matched PBMCs in this experimental setting because of the linkage disequilibrium between these two alleles, we stained one monoclonal and one oligoclonal TCL from a DRB1*0404/DRB4*0101 positive horse-allergic subject with a DRB4*0101:*Equ c 1*_{143–160} HLA

class II tetramer (Fig. 6). Positive staining with the tetramer confirmed that the DRB4*0101 allele is involved in restricting the CD4⁺ T-cell response to *Equ c 1*_{143–160}. Taken together, our findings suggest that a wide array of HLA class II alleles, including DRB4*0101, is able to bind and present the immunodominant epitope region of *Equ c 1*.

Discussion

In the present study, we have examined allergen-specific peripheral blood CD4⁺ T-cell responses of subjects sensitized to the major allergen of horse, *Equ c 1*, and compared them with those of non-allergic horse dust-exposed individuals. As we have previously found that *Equ c 1* contains one immunodominant epitope region between

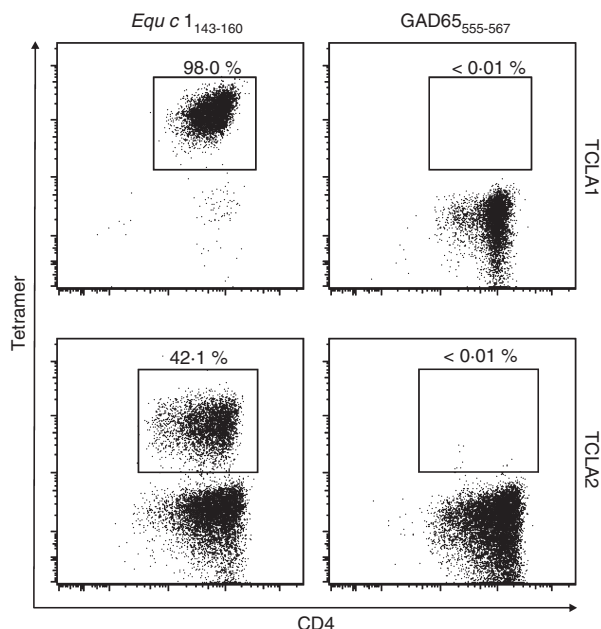


Figure 6. DRB4*0101:Equ *c* 1_{143–160} tetramer staining of a monoclonal (TCL A1; top) and oligoclonal (TCL A2; bottom) CD4⁺ T-cell line derived from the allergic subject A. DRB4*0101 tetramer loaded with the irrelevant GAD65_{555–567} peptide was used as a control.

the amino acids 143 and 160 against which almost all Equ *c* 1-sensitized individuals mount a strong T-cell response,¹¹ we chose to analyse the CD4⁺ T-cell responses to this particular region.

Recent studies with lipocalin and non-lipocalin allergens have suggested that there is a difference in the frequency of allergen-specific CD4⁺ T cells between allergic and non-allergic subjects.^{1–7} In line with these findings we observed here that the number of Equ *c* 1 protein-specific TCLs, but not the number of Equ *c* 1_{143–160} peptide-specific TCLs, from allergic subjects tended to be higher than that from non-allergic subjects (Fig. 1). However, the difference in the number of Equ *c* 1 protein-specific TCLs between the groups did not reach a statistical significance because the number of protein-specific TCLs derived from one non-allergic individual was exceptionally high, as described in Results. Importantly, our detailed analysis demonstrates that the Equ *c* 1_{143–160}-specific CD4⁺ T-cell responses from this, as well as other non-allergic individuals examined, appeared to derive solely from the naive CD4⁺ T-cell subset (Fig. 4a, b). In contrast, all the Equ *c* 1_{143–160}-specific CD4⁺ T-cell responses from allergic subjects derived from the memory CD4⁺ T-cell subset (Fig. 4a, b). Consequently, the situation with the Equ *c* 1 allergen appears to be similar to our previous observations with the Bos *d* 2 and Can *f* 1 allergens in that allergic subjects have elevated frequencies of CD4⁺ memory T cells in their peripheral blood.^{1,2} This notion is also in line with the available data on CD4⁺ T-cell responses to other allergens, such as cat Fel *d* 1³ and

peanut Ara *h* 1.⁴ Taken together, our current results further support the concept that the frequency of allergen-specific CD4⁺ T cells, especially those of the memory phenotype, is higher in allergic subjects.^{1–7}

As reported above, one non-allergic subject had strong cellular reactivity to Equ *c* 1, which was derived from the naive CD4⁺ T-cell subset (Fig. 4a). Although reasons for the reactivity are not known, it can be speculated that this individual has a predisposition for sensitization to Equ *c* 1. Nevertheless, the finding points to a possibility that healthy subjects are not a homogeneous group with low or non-existent levels of allergen-specific T cells. Therefore, further investigations are clearly necessary to explore the complete repertoire of T-cell reactivity to allergenic proteins among healthy subjects.

The estimated frequency of Equ *c* 1 protein-specific CD4⁺ T cells was very low, in the range of 1 per 10⁶ CD4⁺ T cells, in the peripheral blood of sensitized and healthy subjects. Although methodological and other differences between studies may complicate direct comparison, the frequency corresponds well with our previous estimates with the Bos *d* 2 and Can *f* 1 allergens.^{1,2} In line with our observations, the frequency of birch pollen Bet *v* 1-specific CD4⁺ T cells was reported to be in the same range in the peripheral blood of sensitized subjects outside the birch pollen season. At the peak of the season, however, this frequency was strongly increased.¹⁹ It is of interest that a tetramer-based enrichment method showed high frequencies (up to 1 in 7000 cells), and considerable variation, of specific CD4⁺ T cells to an important animal-derived allergen, cat Fel *d* 1, in allergic subjects.⁷ Elevated frequencies of allergen-specific CD4⁺ T cells compared with healthy donors have also been found in allergy to the peanut Ara *h* 1, rye grass Lol *p* 1, and alder Aln *g* 1 allergens.^{4–6} In the current study, the frequency of Equ *c* 1-specific CD4⁺ T cells in most healthy subjects was also lower than that in allergic subjects. Together, these studies emphasize that the overall frequencies of allergen-specific CD4⁺ T cells in the peripheral blood are very low, although they can be allergen-dependent and vary upon exposure.

In line with our and others' reports with other allergens,^{1,2,5,6,18,19,21} the Equ *c* 1-specific TCLs of allergic subjects were found to produce higher levels of IL-4 and IL-5 than those of non-allergic subjects, whereas the TCLs of non-allergic subjects produced only IFN- γ and IL-10, the levels of which, however, did not differ between the subject groups (Fig. 3). These results indicate that Equ *c* 1-specific T cells in allergic subjects are Th2-deviated whereas those in non-allergic subjects are unpolarized or weakly regulatory T cell 1 (Tr1)- or Th1-deviated, probably through their predominant origin from the naive CD4⁺ T-cell subset. In our previous study with the Can *f* 1 allergen, we noted that only allergic subjects had TCLs with a 'higher' functional avidity and these higher-avidity TCLs produced the highest levels of IL-4

and IL-5, suggesting that TCR avidity may be associated with Th2 polarization, possibly through the preferential selection of higher-avidity T-cell clones *in vivo*.¹ Therefore, it is of interest that the *Equ c 1*-specific TCLs from allergic subjects, examined here, exhibited a significantly stronger proliferative capacity than those from non-allergic subjects (Fig. 2). This points to a possibility that elevated TCR avidity, although not directly examined here, may be associated with Th2-polarized memory CD4⁺ T-cell responses in allergic subjects.

We did not find a difference in the IL-10 and IFN- γ production by the TCLs of allergic and non-allergic subjects (Fig. 3), so it appears unlikely that these cytokines would have affected the proliferative capacity of the TCLs examined. Therefore, these results are in line with those of several other studies in that the activity of regulatory T cells does not explicitly explain the missing CD4⁺ T-cell responses of healthy subjects to allergens. Although one early study suggested that CD4⁺ CD25⁺ cells can suppress allergen-specific T-cell responses in non-allergic subjects,²² a later study found no increase in the allergen-specific responses after the depletion of regulatory CD4⁺ CD25⁺ T cells *in vitro*.²³ Similarly in our previous study, when we depleted CD4⁺ CD25⁺ cells or blocked IL-10 production with antibodies *in vitro*, no significant effect on the allergen-induced T-cell proliferation was observed in either allergic or non-allergic subjects.¹ It is of interest, however, that if the allergen-specific CD4⁺ T cells of non-allergic subjects are activated, they do produce IFN- γ and IL-10 (Fig. 3). Wambre *et al.*⁶ observed a population of allergen-specific, IFN- γ - and IL-10-producing cells that in non-allergic subjects could contribute to a protective effect against allergy. They did not discover, however, significant differences in the number of CD4⁺ CD25⁺ regulatory T cells among peripheral blood allergen-specific CD4⁺ T cells between subjects who were allergic or not to alder pollen.

Better understanding of the differing T-cell responsiveness between allergic and healthy subjects is essential for developing functional immunotherapeutic tools, such as peptide-based immunotherapy (PIT). Peptides for PIT should derive from major allergens and be ideally presented by HLA class II molecules that are prevalent in a population to maximize the efficacy of PIT.²⁴ We have previously shown that the *Equ c 1*_{143–160} peptide, covering the immunodominant epitope region of *Equ c 1*, contains two distinct T-cell epitopes.¹¹ Our current analyses reveal that the CD4⁺ T-cell response to *Equ c 1*_{143–160} is restricted by multiple HLA alleles (Table 1 and Fig. 5). Specifically, we demonstrate that the HLA-DQ alleles DQB1*0501, DQB1*0602 and DQB1*0603 are involved in presenting the *Equ c 1* peptide to T cells. As to the DR-restricted responses, they were found to be restricted by either DRB1*0404 or DRB4*0101 alleles, but because of the linkage disequilibrium between these two alleles the exact restricting element

could not be determined by using the PBMCs at our disposal as APCs. However, tetramer staining of two TCLs from a DRB1*0404/DRB4*0101-positive subject revealed that they were restricted with DRB4*0101 (Fig. 6). Taken together, these findings indicate that the *Equ c 1* peptide is presented by several different HLA class II molecules and that one of these is DRB4, which is encoded by a gene carried and expressed by all DR4-, DR7- and DR9-positive individuals, so covering around 25–30% of the Caucasian population.^{12,25}

Our current results parallel those previously obtained by Van Overtvelt *et al.*¹⁹ and Jahn-Schmid *et al.*²⁶ with the birch and ragweed major allergens *Bet v 1* and *Amb a 1*, respectively, in that the T-cell epitopes from these allergens were also presented by several HLA class II loci. Similarly, Oseroff *et al.*¹⁸ demonstrated that the major immunodominant regions of the timothy grass allergens were restricted by multiple HLA class II molecules and loci. Taken together, the aforementioned features suggest that the peptide 143–160 is a promising candidate for PIT of *Equ c 1* allergy. Moreover, because DRB4 is a common allele the DRB4:*Equ c 1*_{143–160} tetramer may prove to be a useful tool to monitor *Equ c 1*-specific CD4⁺ T-cell responses.

In conclusion, our current results demonstrate that the frequency of *Equ c 1*-specific CD4⁺ T cells in most allergic subjects is higher than in non-allergic subjects. The responses of allergic subjects were found to arise from memory cells, suggesting expansion *in vivo*. Moreover, the allergen-specific CD4⁺ T cells from allergic subjects were confirmed to be of the Th2 phenotype whereas those from non-allergic subjects were either unpolarized or produced low levels of IFN- γ and IL-10. Taken together, these findings consolidate our understanding of the atopic and healthy CD4⁺ T-cell response against allergens of the lipocalin family.

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Disclosures

The authors declare that they have no competing interests.

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Supporting Information

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

Table S1. The DQ-HLA and DR-HLA alleles of the test subjects.

SUPPLEMENTAL TABLE 1. The DQ- and DR-HLA alleles of the test subjects.

ID	DQB1*	DQB1*	DRB1*	DRB1*	DRB4*
A	0302	0301	0404	1201	0101
B	0501	0602	0101	1501	
C	0302	0602	0404	1501	0101
D	0402	0301	0801	1201	
E	0402	0602	0801	1501	
F	0302	N/A	0401	N/A	
G	0602	0604	1302	1501	
H	0201	0301	0301	1201	
I	0501	0602	0101	1501	
J	0501	0603	0101	1301	
K	0501	0603	0101	1301	
L	0301	0602	1101	1501	
M	0501	0201	0101	0301	
N	0302	0302	0401	0404	0101
O	0303	0501	0101	0901	0101
P	04	0501	1/10	0801	
Q	02	0501	0101	0701	0101
R	0301	0501	1/10	11/12/13	
S	0301	0603	11/12/13	1301	
T	0501	0603	1/10	1301	
U	02	04	0701	0801	0101
V	0303	0501	1/10	0901	
W	0303	0501	1/10	N/A	