

Allergen-specific naïve and memory CD4⁺ T cells exhibit functional and phenotypic differences between individuals with or without allergy

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Although allergen-specific CD4⁺ T cells are detectable in the peripheral blood of both individuals with or without allergy, their frequencies and phenotypes within the memory as well as naïve repertoires are incompletely known. Here, we analyzed the DRB1*0401-restricted responses of peripheral blood-derived memory (CD4⁺CD45RO⁺) and naïve (CD4⁺CD45RA⁺) T cells from subjects with or without allergy against the immunodominant epitope of the major cow dander allergen Bos d 2 by HLA class II tetramers *in vitro*. The frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood-derived cultures appeared to be higher in subjects with allergy than those without, whereas naïve Bos d 2_{127–142}-specific T cells were detectable in the cultures of both groups at nearly the same frequency. Surprisingly, the TCR avidity of Bos d 2_{127–142}-specific T cells of naïve origin, as assessed by the intensity of HLA class II tetramer staining, was found to be higher in individuals with allergy. Upon restimulation, long-term Bos d 2_{127–142}-specific T-cell lines generated from both memory and naïve T-cell pools from individuals with allergy proliferated more strongly, produced more IL-4 and IL-10, and expressed higher levels of CD25 but lower levels of CXCR3 than the T-cell lines from individuals without allergy, demonstrating differences also at the functional level. Collectively, our current results suggest that not only the memory but also the naïve allergen-specific T-cell repertoires differ between individuals with or without allergy.

Key words: Allergology · CD4⁺ T cells · Memory cells · Naïve cells · TCR

Introduction

CD4⁺ Th2 cells are key players in the pathogenesis of type I allergy. Through mechanisms including the production of IL-4,

IL-5 and IL-13, they regulate the synthesis of specific IgE and the effector function of mast cells and eosinophils, which in turn are responsible for the allergic phenotype [1]. Although it is well established that pathogenic Th2-type CD4⁺ T-cell responses dominate in individuals with allergy, the specific nature of allergen-specific T-cell responses in individuals without allergy is less well understood. A number of mechanisms have been proposed to explain the tolerance to allergens in these

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individuals, including immunological ignorance, immune deviation to Th0/Th1 phenotype and the induction of regulatory T cells, especially IL-10-producing type 1 regulatory (Tr1) cells [2].

Allergen-specific CD4⁺ T cells are present at very low frequencies in the peripheral blood of both subjects with or without allergy. This phenomenon has hampered detailed studies of human allergen-specific T-cell responses, since commonly used techniques, such as the measurement of proliferation or cytokine production, are relatively insensitive and usually require extensive *in vitro* culture to expand T cells for analyses. Moreover, these techniques do not allow a clear discrimination between antigen-specific T cells and nonspecific T cells induced by bystander activation. Therefore, it is not surprising that previous studies measuring the proliferative responses or cytokine production of PBMC or T-cell lines (TCL) upon stimulation with allergens or allergen peptides have failed to demonstrate a consistent difference between subjects with allergy and those without [3–7]. The current situation has been significantly improved through the development of HLA class II tetramer technology, which allows the direct visualization of rare antigen-specific T cells by flow cytometry [8]. In recent years, HLA class II tetramers have successfully been used to detect CD4⁺ T cells specific to allergen epitopes in both healthy subjects and those with allergy [9–13].

Most of the previous research on human antigen-specific CD4⁺ T-cell responses has been performed using PBMC or purified peripheral blood CD4⁺ T cells. It is important to note, however, that the peripheral blood CD4⁺ T-cell repertoire in humans consists of both memory and naïve T cells that can be distinguished by their mutually exclusive expression of the CD45RO and CD45RA molecules, respectively. The importance of studying human antigen-specific T-cell responses arising from these separate subsets has only recently been appreciated. For example, it has been demonstrated that a difference in T-cell reactivity to autoantigens between subjects with autoimmune diseases and healthy individuals can only be observed when analyzing T-cell responses derived from the memory compartment [14–16].

Lipocalin allergen Bos d 2, the major respiratory allergen in cow dander, contains a single immunodominant epitope that is recognized by T cells from patients with cow dust-induced asthma [17]. The Bos d 2_{127–142} peptide containing this epitope has been shown to bind to DRB1*0401 [18]. It has been observed that both subjects with or without allergy mount DRB1*0401-restricted T-cell responses to the peptide [11]. Moreover, DRB1*0401 tetramers loaded with Bos d 2_{127–142} have successfully been used to stain specific T-cell clones, TCL, as well as peripheral blood CD4⁺ T cells after a short *ex vivo* expansion [11, 19]. Because of these properties, Bos d 2_{127–142} is ideally suited for detailed studies of HLA-restricted allergen-specific T-cell responses.

In the current study, we have separately examined DRB1*0401-restricted T-cell responses arising from memory (CD45RA[−]CD45RO⁺) and naïve (CD45RA⁺CD45RO[−]) CD4⁺ T-cell subsets against the Bos d 2_{127–142} peptide in subjects with

or without allergy. We observed several functional and phenotypic differences in the specific T-cell repertoires between these subject groups. Our current results suggest that not only the memory but also the naïve allergen-specific T-cell repertoires differ between subjects with or without allergy.

Results

Higher frequency of Bos d 2_{127–142}-specific memory CD4⁺ T cells in subjects with allergy

We have previously demonstrated that DR0401/p127-142 HLA class II tetramers can be used to detect Bos d 2_{127–142}-specific CD4⁺ T cells in the peripheral blood of subjects with allergy after a short *ex vivo* expansion [11]. Using this approach, we sought to determine the frequency of circulating Bos d 2-specific memory and naïve CD4⁺ T cells in subjects with or without allergy.

Highly purified memory (CD45RA[−]CD45RO⁺) and naïve (CD45RA⁺CD45RO[−]) CD4⁺ T cells isolated from the PBMC of five cow-asthmatic and five subjects without atopy were stimulated with DC pulsed with the Bos d 2_{127–142} peptide *in vitro*. After 14 days, the cultures were stained with tetramers to detect Bos d 2_{127–142}-specific T cells. Representative results are shown for T-cell cultures derived from memory (Fig. 1A and B) and naïve (Fig. 1C and D) CD4⁺ T cells for one subject with or without allergy, respectively.

Bos d 2_{127–142} tetramer-positive cells were detected in the memory T-cell-derived cultures of four out of five subjects with allergy, but only in two out of five cultures of subjects without allergy (Table 1). The mean ($\pm$ SEM) frequency of tetramer-positive cells was $0.38 \pm 0.19\%$ and $0.18 \pm 0.16\%$ for subjects with and without allergy, respectively. Previous experiments suggest that allergen-specific CD4⁺ T cells undergo at least 10–12 cell divisions during a 14-day *in vitro* culture [11, 12]. Based on this assumption, it can be calculated that the frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood of subjects with allergy is low, in the range of 10^{-5} and 10^{-6} circulating CD4⁺ T cells. Notably, since tetramer-positive responses were undetectable in the cultures of most of the subjects without allergy, the frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood of subjects with allergy appears to be even lower than that of subjects without allergy.

Ex vivo-expanded Bos d 2_{127–142}-specific naïve CD4⁺ T cells are of higher avidity in subjects with allergy

In previous studies, antigen concentration has been demonstrated to be a factor affecting the TCR avidity of the induced T-cell population *in vitro*: high antigen concentrations favor the expansion of low-avidity T cells, whereas low concentrations favor the expansion of high-avidity T cells [20–22]. To enable the expansion of both high- and low-avidity naïve Bos d 2_{127–142}-specific T cells,

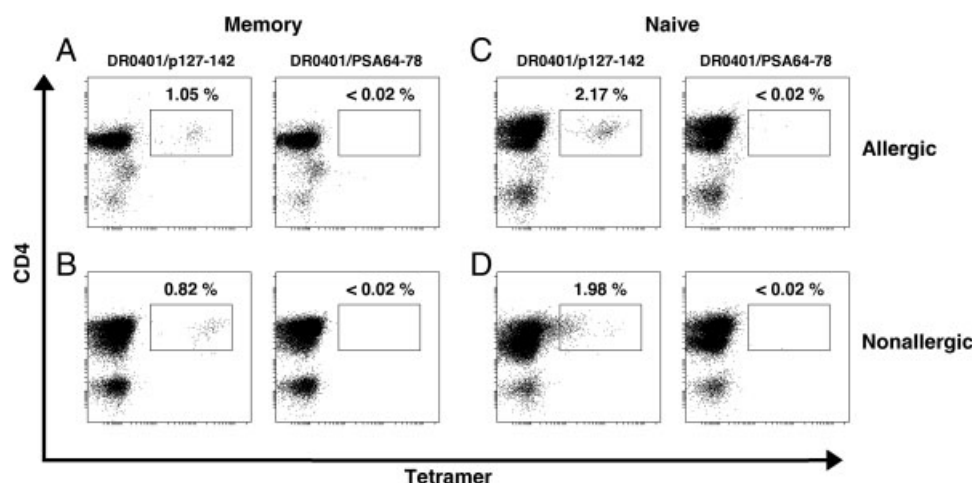


Figure 1. Detection of Bos d 2_{127–142} tetramer-positive CD4⁺ T cells of memory (A and B) or naïve (C and D) origin after *ex vivo* expansion. Representative results are shown for T-cell cultures from one subject with allergy (A and C) and one subject without allergy (B and D). Staining with DR0401/p127–142 or the control tetramer DR0401/PSA64–78 was performed on day 14 after *in vitro* stimulation with Bos d 2_{127–142} peptide-pulsed DC. The percentages of tetramer-positive CD4⁺ T cells are indicated. Background staining with the control tetramer was constantly <0.02%.

Table 1. The percentage of Bos d 2_{127–142} tetramer-positive CD4⁺ T cells in memory and naïve cell-derived cultures after *ex vivo* expansion at a peptide concentration of 10 or 0.1 μ M^{a)}

Subject	Memory T cells (%)	Naïve T cells (%)	
	10 μ M	10 μ M	0.1 μ M
A-1	–	0.12	ND
A-2	0.04	0.82	1.08
A-3	0.31	0.37	–
A-4	0.51	–	0.32
A-5	1.05	2.17	0.05
Mean $\pm$ SEM	0.38 $\pm$ 0.19	0.70 $\pm$ 0.39	0.36 $\pm$ 0.22
NA-1	–	0.31	–
NA-2	–	0.13	0.09
NA-3	–	0.52	0.38
NA-4	0.09	0.09	0.05
NA-5	0.82	1.98	–
Mean $\pm$ SEM	0.18 $\pm$ 0.16	0.61 $\pm$ 0.35	0.10 $\pm$ 0.07

^{a)} –, tetramer staining below 0.02 %; ND, not determined.

we stimulated naïve CD4⁺ T cells at two peptide concentrations, *i.e.* 10 and 0.1 μ M. By this approach, Bos d 2_{127–142} tetramer-positive cells were detected in the naïve T-cell-derived cultures of all the subjects studied (Table 1).

Although the frequency of the tetramer-positive cells in general appeared to be lower after expansion of naïve T cells at the lower (0.1 μ M) peptide concentration (Table 1), the phenomenon was less in subjects with allergy, as two of them (A-2 and A-4) showed a higher frequency of tetramer-positive T cells in their cultures after expansion at the lower concentration. However, when the highest frequencies of the tetramer-positive cells from each individual, regardless of the peptide concentration used in the expansion (0.1 μ M for A-2 and A-4, 10 μ M for the other subjects), were included in the analysis, the mean frequency of Bos d 2_{127–142}

tetramer-positive cells in the naïve T-cell-derived cultures from subjects with or without allergy was largely the same, $0.81 \pm 0.38\%$ and $0.61 \pm 0.35\%$, respectively (Fig. 2A). Moreover, based on these results it can be calculated that the frequency of Bos d 2_{127–142}-specific naïve T cells in the peripheral blood appears to be within the same range as that of memory T cells, between 10^{-5} and 10^{-6} circulating CD4⁺ T cells.

The intensity of tetramer staining of the *ex vivo*-expanded Bos d 2_{127–142}-specific CD4⁺ T cells appeared to be higher in the naïve T-cell-derived cultures from subjects with allergy than those without (a representative example shown in Fig. 1C and D). A statistically significant difference in the MFI of tetramer staining was observed between the cultures from the two groups (Fig. 2B; $p = 0.02$, Mann–Whitney *U* test). Of particular interest, previous studies have demonstrated that the capacity of T cells to bind HLA class II tetramers directly correlates with their TCR avidity [19, 23, 24]. Therefore, our current results suggest that despite largely similar precursor frequencies (Fig. 2A) the naïve Bos d 2_{127–142}-specific CD4⁺ T cells in subjects with allergy are of higher avidity than those in subjects without allergy (Fig. 2B).

Ex vivo-expanded Bos d 2_{127–142}-specific CD4⁺ T cells recognize the naturally processed epitope

To confirm that the *ex vivo*-expanded Bos d 2_{127–142} tetramer-positive T cells can specifically recognize the Bos d 2 allergen and its naturally processed T-cell epitope, T-cell clones were generated by single cell sorting tetramer-positive cells after *ex vivo* expansion of memory and naïve CD4⁺ T cells. The results of two memory cell and three naïve cell-derived clones established from three different allergic subjects and one naïve cell-derived clone from a control subject are shown in Fig. 3. All clones stained positively with the Bos d 2_{127–142} tetramer (data not shown) and proliferated in response to both the

Bos d 2_{127–142} peptide and recombinant Bos d 2, confirming their capacity to recognize the naturally processed epitope of Bos d 2 (Fig. 3A). Moreover, upon stimulation with the peptide the memory cell-derived clones from subjects with allergy produced IL-4 but no IFN- γ or IL-10, suggesting a Th2 phenotype. In contrast, the naïve cell-derived clones produced low levels of both IL-4 and IFN- γ , suggesting an undifferentiated Th0 phenotype.

Poor proliferation and tetramer staining of Bos d 2_{127–142}-specific TCL from subjects without allergy

To further examine the functional and phenotypic characteristics of Bos d 2_{127–142}-specific T cells in the peripheral blood, a complementary approach, the split-well method, was used to generate mono-/oligoclonal DRB1*0401-restricted long-term TCL from peripheral-blood memory and naïve CD4⁺ T cells [11].

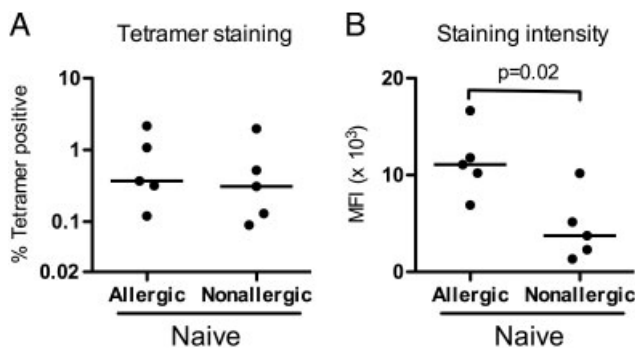


Figure 2. The percentage (A) and the MFI (B) of Bos d 2_{127–142} tetramer-positive CD4⁺ T cells in the naïve cell-derived cultures from subjects with or without allergy after *ex vivo* expansion. The T cells were expanded at the peptide concentration of 0.1 μ M (subjects A-2 and A-4) or 10 μ M (other subjects). Statistical analysis was done by the Mann–Whitney *U* test.

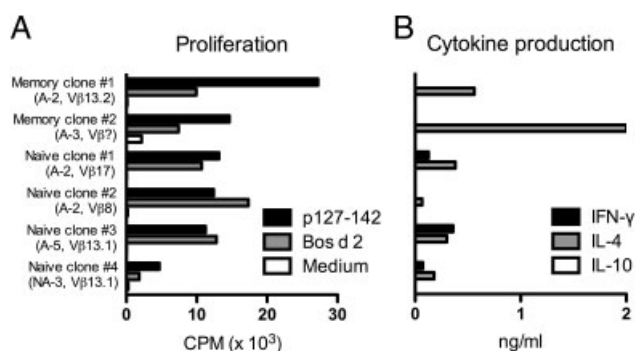


Figure 3. Proliferative responses (A) of two memory cell and three naïve cell-derived Bos d 2_{127–142} tetramer-positive CD4⁺ T-cell clones from subjects with allergy and one naïve cell-derived clone from a subject without allergy to the Bos d 2_{127–142} peptide (10 μ M) or recombinant Bos d 2 (5 μ M). IFN- γ , IL-4 and IL-10 production (B) of the clones upon stimulation with the Bos d 2_{127–142} peptide (10 μ M). The subject from whom the clone was derived and the TCR V β subset expressed by the clone is indicated in parentheses.

With this technique, we were able to obtain specific TCL of memory origin from four out of five allergic subjects, but from only two out of five subjects without allergy (Table 2). Moreover, the frequency of the memory cell-derived TCL generated from the subjects with allergy was significantly higher than that from the subjects without allergy (7.1 and 2.0%, respectively, $p < 0.05$, Fisher's exact test). This finding further supports the notion that the frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood of subjects with allergy is higher than that of control subjects. Furthermore, assuming that each TCL represents a monoclonal expansion of Bos d 2_{127–142}-specific T cells, these results corroborate the estimated frequency of Bos d 2_{127–142}-specific memory T cells to be in the range of 10^{-5} and 10^{-6} circulating CD4⁺ T cells.

Bos d 2_{127–142}-specific TCL of naïve origin could be generated using the split-well method from all but one of the subjects (NA-3). There appeared to be a tendency for a higher frequency of naïve cell-derived TCL generated from the allergic subjects than from the nonallergic subjects (8.5 versus 4.0%), though this did not reach statistical significance ($p = 0.14$). Moreover, upon stimulation with the Bos d 2_{127–142} peptide, the naïve cell-derived TCL of subjects with allergy tended to proliferate more strongly than those of subjects without allergy (Fig. 4A; Mann–Whitney *U* test: $p = 0.06$). A similar tendency was observed between the memory cell-derived TCL from both groups (Fig. 4A), though the small number of memory cell-derived TCL established from subjects without allergy precludes a definite conclusion.

To clarify this phenomenon, we stained the TCL with DR0401/Bos d 2_{127–142} tetramers (Fig. 4B). We observed that 14 of the 17 TCL of memory or naïve origin (82%) analyzed from subjects with allergy stained with the specific tetramers, whereas only one of the five TCL (20%) from control subjects showed a positive staining ($p = 0.02$, Fisher's exact test). Therefore, the weaker proliferative capacity of the Bos d 2_{127–142}-specific TCL

Table 2. The number of long-term Bos d 2_{127–142}-specific TCL generated from the memory and naïve T-cell pools from subjects with or without allergy (per 20–30 wells seeded)^{a)}

Subject	Memory T cells	Naïve T cells
A-1	1/30	2/30
A-2	–/30	3/30
A-3	4/20	3/20
A-4	2/30	2/30
A-5	3/30	1/20
Total	10/140 (7.1%)	11/130 (8.5%)
NA-1	2/30	2/30
NA-2	1/30	1/30
NA-3	–/30	–/30
NA-4	–/30	2/30
NA-5	–/30	1/30
Total	3/150 (2.0%) ^{a)}	6/150 (4.0%)

^{a)} $p < 0.05$ as compared with memory T cells from allergic subjects using Fisher's exact test.

from control group seems to be associated with their diminished capacity to bind specific tetramers, which in turn is known to reflect a weaker TCR avidity, as mentioned above.

Th2-bias of Bos d 2_{127–142}-specific TCL derived from subjects with allergy

The cytokine production of the long-term Bos d 2_{127–142}-specific TCL was assessed by measuring IFN- γ , IL-4 and IL-10 in the culture supernatants (Fig. 5A–C). Memory cell-derived TCL from subjects with allergy produced predominantly IL-4 and to a lesser extent IL-10, whereas the cytokine production of memory cell-derived TCL from control group and naïve cell-derived TCL from both groups were generally weak. Consequently, the memory cell-derived TCL from subjects with allergy were predominantly of a Th2 phenotype, whereas almost all memory cell-derived TCL from the control group and naïve cell-derived TCL from both groups were of an undifferentiated Th0 phenotype (Fig. 5D). It was, however, interesting that a low level of IL-4 production was detected in five out of 11 TCL of naïve origin from subjects with allergy (Fig. 5B). In contrast, IL-4 production was detected in none of the six TCL of naïve origin from subjects without allergy. This finding points to a subtle Th2 bias in TCL of naïve origin from subjects with allergy compared with those without allergy.

Higher CD25 and lower CXCR3 expression in Bos d 2_{127–142}-specific TCL from subjects with allergy

Finally, the expression of phenotypic cell-surface markers on long-term Bos d 2_{127–142}-specific TCL was analyzed by flow cytometry (Fig. 6). After a long *in vitro* culture, both memory and naïve cell-derived TCL from subjects with or without allergy exhibited an effector memory phenotype (CD45RO⁺CD45RA[−]CD62L[−]CCR7[−]). However, consistent with previous studies [22, 25], a low residual level of CD45RA expression was observed on naïve cell-derived TCL

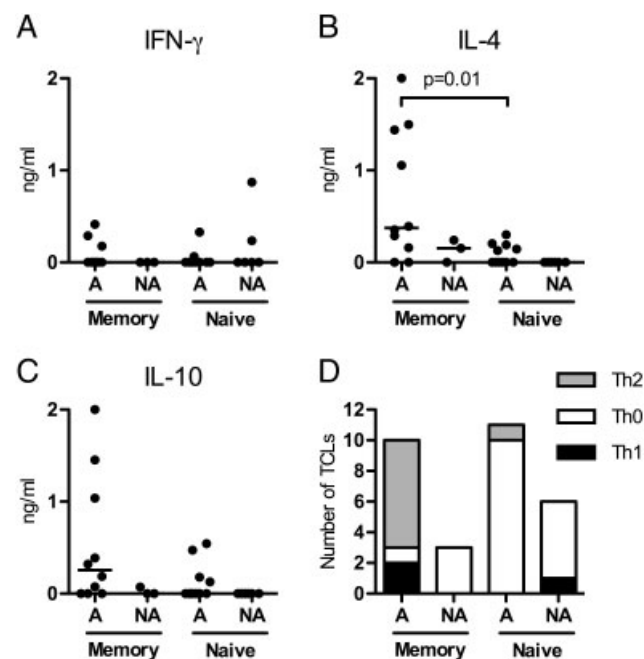


Figure 5. Cytokine production by the long-term Bos d 2_{127–142}-specific TCL derived from memory and naïve T-cell pool of subjects with (allergic, “A”) or without allergy (nonallergic, “NA”). The amounts of IFN- γ (A), IL-4 (B) and IL-10 (C) produced were detected in the supernatants. The peptide concentration used for stimulation was 10 μ M. Th cytokine phenotypes of the TCL (D). The phenotypes were based on the IFN- γ /IL-4 ratio (Th1: IFN- γ /IL-4 ratio > 5; Th2: IFN- γ /IL-4 ratio < 0.2; Th0: IFN- γ /IL-4 ratios in between). Statistical analysis was done by the Mann-Whitney U test.

(CD45RA expression 4.4 ± 1.7 versus $0.7 \pm 0.4\%$ for TCL of naïve and memory origin, respectively; $p = 0.004$, Mann-Whitney U test). No expression of CTLA-4, CCR4 or CCR6 was observed in the TCL, whereas most of the TCL expressed CD25 and CXCR3 at variable levels. The expression of these markers was comparable between memory and naïve cell-derived TCL within the allergic (Fig. 6A) and nonallergic (Fig. 6B) subject groups. However, both memory and naïve cell-derived TCL from subjects with allergy expressed significantly higher levels of CD25 (Fig. 6C; $p = 0.001$ Mann-Whitney U test) but lower levels of CXCR3 (Fig. 6D; $p = 0.006$) than the TCL from subjects without allergy. The higher expression of the T-cell activation marker CD25, the IL-2 receptor α -chain, by TCL from subjects with allergy is probably associated with their greater capacity to proliferate *in vitro* (Fig. 4A). The lower expression of the Th1-associated chemokine receptor CXCR3 [26], on the other hand, may be associated with the observed Th2-bias in TCL of both memory and naïve origin from subjects with allergy (Fig. 5).

Discussion

In the current study, we have dissected the responses of peripheral blood T cells of memory (CD4⁺CD45RO⁺) and naïve (CD4⁺CD45RA⁺) origin against the immunodominant epitope of the major cow dander allergen Bos d 2 by HLA class II tetramers *in vitro*. The frequency of Bos d 2_{127–142}-specific memory T cells

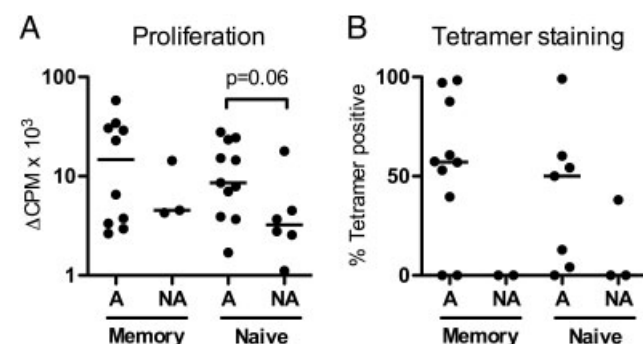


Figure 4. (A) Proliferative responses of Bos d 2_{127–142}-specific long-term TCL derived from memory and naïve pools from subjects with (allergic, “A”) or without allergy (nonallergic, “NA”). The proliferative responses are expressed as ΔCPM (mean of wells stimulated with the Bos d 2_{127–142} peptide (10 μ M) - mean of unstimulated wells). (B) The percentage of Bos d 2_{127–142} tetramer-positive CD4⁺ T cells in the TCL. Statistical analysis was done by the Mann-Whitney U test.

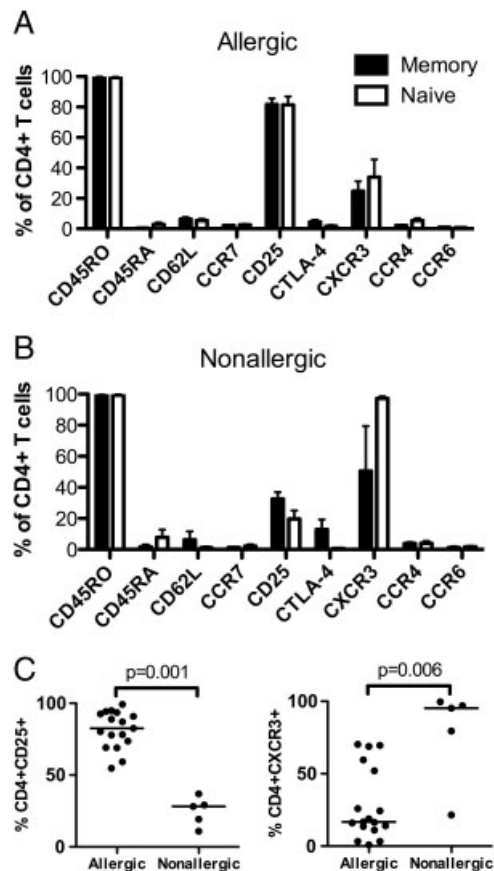


Figure 6. The expression of phenotypic cell-surface markers on Bos d 2_{127–142}-specific long-term TCL derived from memory and naïve T-cell pools of subjects with (A) or without (B) allergy. Results are expressed as mean % ± SEM of CD4⁺ T cells expressing the markers. The expression of CD25 (C) and CXCR3 (D) by all TCL (of memory and naïve origin) from subjects with or without allergy. Ten memory cell-derived and seven naïve-cell derived TCL from allergic subjects and two memory-cell derived and three naïve-cell derived TCL from nonallergic subjects were analyzed. Statistical analysis was done by the Mann-Whitney U test.

in the peripheral blood-derived cultures appeared to be higher in subjects with allergy than those without, whereas naïve Bos d 2_{127–142}-specific T cells were detectable in the cultures of both groups at nearly the same frequency. Despite this largely similar frequency, *ex vivo*-expanded Bos d 2_{127–142}-specific naïve CD4⁺ T cells from subjects with allergy exhibited a higher TCR avidity, as assessed by their capacity to bind HLA tetramers, than those from control group. A complementary approach, generation of long-term TCL by the split well technique, produced results in line with those obtained upon short-term *ex vivo* culture.

To our knowledge, this is the first study in which the responses of human CD4⁺ T cells of naïve origin against an allergen epitope have been analyzed in detail. In fact, information on naïve cell-derived antigen-specific CD4⁺ T-cell responses in other pathological conditions is limited as well. Although several previous studies have demonstrated that naïve cell-derived autoantigen-specific CD4⁺ T cells are detectable in the

peripheral blood cultures of both healthy subjects and those with autoimmune disease [15, 16, 22, 25], their avidity and functional capacity have not been studied in detail. Interestingly, a recent study employing HLA class II tetramers demonstrated that highly purified naïve CD4⁺ T cells from subjects with diabetes were more sensitive to stimulation with a proinsulin peptide than those from healthy individuals, suggesting a possible difference in the avidity of the naïve autoantigen-specific T-cell repertoires between these subject groups [27].

It has recently been demonstrated that the composition of the naïve T-cell repertoire may directly influence the diversity, quality and magnitude of a primary immune response [28, 29]. Therefore, it is possible that subtle differences in the TCR avidity of naïve antigen-specific T-cell repertoires, such as those observed in this study, may have an effect on the strength and nature of the CD4⁺ T-cell response generated. In line with this view, we were able to demonstrate functional differences between Bos d 2_{127–142}-specific CD4⁺ TCL of naïve origin from subjects with allergy and the control group. First, despite a comparable precursor frequency between the two groups (Fig. 2A), after repeated *in vitro* stimulations, a lower number of naïve-cell derived Bos d 2_{127–142}-specific long-term TCL were generated from the control group than from subjects with allergy (Table 2). Second, the few naïve cell-derived TCL established from the control group tended to proliferate weakly and failed to bind specific tetramers (Fig. 4). Finally, these TCL expressed lower levels of the T-cell activation marker CD25 than those from subjects with allergy (Fig. 6C).

In the current study, the frequency of Bos d 2_{127–142}-specific memory T cells was observed to be low in the peripheral blood of subjects with allergy. Estimates based both on tetramer staining after a short-term *ex vivo* culture (Table 1) and on the induction of specific long-term TCL (Table 2) suggest a frequency in the range of 10^{–5} to 10^{–6} circulating memory CD4⁺ T cells. Using HLA class II tetramers, the frequencies of CD4⁺ T cells in the peripheral blood of allergic subjects specific to the immunodominant epitopes of other allergens, such as cat Fel d 1 and birch Bet v 1, have been estimated to be in the same range or slightly higher [10, 12].

The existence of allergen-specific T cells in individuals without allergy has been a source of much discussion in the literature. Earlier studies measuring the proliferative responses of PBMC or TCL to allergens or allergen peptides have failed to demonstrate a clear difference between allergic and nonallergic subjects, though some of the studies have reported a tendency for stronger responses in allergic subjects [3–7]. In the current study, the frequency of Bos d 2_{127–142}-specific memory T cells in the peripheral blood of subjects without allergy was estimated to be approximately two- to four-fold lower than that of subjects with allergy (Tables 1 and 2). One possible explanation for the lower frequency of memory T cells in the control group is that they have not been exposed to the cow allergen at a similar intensity as the subjects with allergy with occupational cow dust-induced asthma. However, this explanation is controversial, since our recent studies comparing the frequencies of memory CD4⁺

T cells responsive to other mammalian lipocalin allergens, dog Can f 1 [30] and horse Equ c 1 (A. Nieminen, manuscript in preparation), demonstrate a similar difference between allergy sufferers and healthy animal owners.

Importantly, in a study using HLA class II tetramer staining directly *ex vivo*, Bateman *et al.* demonstrated that there is a clearly higher frequency of Fel d 1-specific T cells in the peripheral blood of subjects with allergy than in the peripheral blood of subjects without allergy [10]. Other studies employing HLA class II tetramers have not examined the frequencies of allergen-specific T cells in subjects without allergy in detail. However, Macaubas *et al.* showed that allergen-specific T cells were detectable after *ex vivo* expansion in subjects with allergy but not in the control group [9]. Moreover, Van Overtvelt *et al.* observed that a longer *in vitro* time period was needed for detecting allergen-specific T cells in cultures from subjects without allergy than those with allergy [12]. Both of these findings are therefore consistent with the view that the frequency of allergen-specific memory T cells in subjects without allergy is lower than in allergic subjects. The exact mechanisms leading to a higher frequency of allergen-specific memory CD4⁺ T cells in individuals with or without allergy remain to be elucidated. Differences in Treg function may, at least in part, account for this phenomenon [31].

Another interesting observation in the current study is that the frequencies of Bos d 2_{127–142}-specific memory and naïve T cells in the peripheral blood appeared to be largely similar (Tables 1 and 2). This finding has important implications, since analysis of peripheral blood allergen-specific T-cell responses often requires an *in vitro* expansion step, thus introducing the possibility that a sizable portion of the responding T cells may be derived from naïve, *in vitro*-activated T cells. This may confound the detection of subtle differences in the frequency, avidity and phenotype of allergen-specific T cells in subjects with or without allergy. To support this view, several studies have recently demonstrated that a difference in T-cell reactivity to autoantigens between subjects with autoimmunity and healthy individuals can only be demonstrated when analyzing T-cell responses derived from the memory compartment [14–16].

In agreement with the current paradigm for the importance of Th2 differentiation in the allergic immune response, the cytokine phenotype of Bos d 2_{127–142}-specific T cells derived from the memory pool of allergic subjects was observed to be clearly Th2-biased. Both memory cell-derived tetramer-positive T-cell clones sorted directly after *ex vivo* expansion and memory cell-derived long-term TCL produced predominantly IL-4 upon stimulation (Figs. 2 and 5). In contrast, the cytokine response of T cells of memory origin from nonallergic subjects and T cells of naïve origin from both allergic and nonallergic subjects was weak and characteristic of an undifferentiated Th0 phenotype (Figs. 2 and 5). Interestingly, however, a low level of IL-4 production was observed in almost half of the naïve cell-derived TCL from allergic subjects but in none of those from nonallergic subjects (Fig 5B and C).

Both memory and naïve cell-derived Bos d 2_{127–142}-specific TCL from allergic and nonallergic subjects differed in their

expression of the chemokine receptor CXCR3. While most of the TCL from allergic subjects expressed CXCR3 only at low levels, the expression levels on TCL from nonallergic subjects were considerably higher (Fig. 6D). Interestingly, a higher expression of CXCR3 on allergen-specific T cells from nonallergic subjects was also observed in the study of Van Overtvelt *et al.* [12]. CXCR3 is considered to be a Th1-associated chemokine receptor, since it is expressed at higher levels on Th0- or Th1-polarized cells than on Th2-polarized cells [26]. Therefore, the lower expression of CXCR3 may be related to the Th2-polarized phenotype of the TCL from allergic subjects (Fig. 5). Interestingly, CXCR3-expressing T cells from individuals with atopy have been shown to be more susceptible to activation-induced cell death than those from subjects without atopy [32]. This mechanism may lead to the selective depletion of these cells and can contribute to the Th2-dominant allergen-specific T-cell responses in atopic subjects. Intriguingly, the low CXCR3 expression on Bos d 2_{127–142}-specific TCL of naïve origin from allergic subjects and their tendency to produce IL-4 support the notion that atopy may have an effect on the phenotype of naïve allergen-specific T cells from allergic subjects, at least *in vitro*.

In conclusion, our results suggest that both memory and naïve Bos d 2_{127–142}-specific T-cell repertoires exhibit several functional and phenotypic differences between allergic and nonallergic subjects. Importantly, these results propose that not only the memory but also the naïve Bos d 2_{127–142}-specific T-cell repertoires differ between allergic and nonallergic subjects. Additional studies, however, are needed to confirm whether these findings apply to allergen-specific T-cell responses against other mammalian allergens or allergens in general. Moreover, it is important to note that our results are based on analyses of *in vitro* expanded cells since direct *ex vivo* analysis of rare antigen-specific human CD4⁺ T cells is not feasible for the time being. Nevertheless, it is conceivable that the use of HLA class II tetramers and the careful dissection of human T-cell responses arising from different CD4⁺ T-cell subsets will ultimately lead to a better understanding of the allergen-specific immune response in individuals with or without allergy.

Materials and methods

Subjects and antigens

PBMC from five cow-asthmatic patients (A-1 to A-5) with positive skin prick tests to Bos d 2 [17] and from five individuals without atopy (NA-1 to NA-5) were separated by Ficoll-Paque Plus density gradient centrifugation (GE Healthcare Biosciences, Uppsala, Sweden). All subjects were genotyped to be positive for HLA-DRB1*0401. The study was approved by the Ethics Committee of the Kuopio University Hospital, and written informed consent was given by all subjects. Recombinant Bos d 2 and the Bos d 2_{127–142} peptide (ELEKYQLNSERGVPN) were synthesized as previously described [17, 18].

Tetramer staining

The DR0401/p127–142 and DR0401/PSA64–78 tetramers were generated as previously described [19]. Tetramer staining was performed by incubating T cells with 0.5 μ g of the PE-labeled tetramers in 50 μ L of culture medium for 2 h at +37°C. Anti-CD4-FITC, anti-CD19-PerCP-Cy5.5 and anti-CD14-PerCP-Cy5.5 (all from BD Biosciences, San Jose, CA) were added, and the cells were incubated for an additional 20 min at +4°C. The cells were then washed twice and further incubated for 10 min with the Via-Probe reagent (BD Biosciences) at +4°C, and analyzed with a BD FACSCanto II flow cytometer. Dead cells, CD19⁺ B cells and CD14⁺ monocytes were excluded from the analyses.

Isolation and ex vivo expansion of memory and naïve CD4⁺ T cells

Memory (CD45RA[−]CD45RO⁺) and naïve (CD45RA⁺CD45RO[−]) CD4⁺ T cells were isolated from freshly purified PBMC with the “no touch” memory or naïve CD4⁺ T-cell isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany). The mean purity of the memory cells was 97% (range 90–99%) and that of the naïve cells 94% (range 88–99%), as assessed by staining with anti-CD4-FITC, anti-CD45RA-APC and anti-CD45RO-PE-Cy7 (all from BD Biosciences). Non-CD4⁺ cells that were retained in the MACS separation columns were flushed out and used to generate monocyte-derived mature DC, as previously described [11].

Purified memory or naïve CD4⁺ T cells (10⁶ per well) were stimulated with 2 $\times$ 10⁵ autologous DC pulsed with 0.1 or 10 μ M of the Bos d 2_{127–142} peptide in 48-well plates in a volume of 1 mL (Corning, Corning, NY). The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, 20 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, 100 IU/mL penicillin, 100 μ g/mL streptomycin, 10 mM HEPES (all from Lonza, Verviers, Belgium) and 5% inactivated human AB serum (Sigma-Aldrich, St. Louis, MO). On days 7 and 10, fresh medium supplemented with 25 IU/mL of IL-2 and 10 ng/mL of IL-15 (both from Strathmann Biotech, Hannover, Germany) was added to the cultures. On day 14, the T cells were stained with tetramers and analyzed. For cloning, tetramer-positive CD4⁺ T cells were single cell sorted using an EPICS Elite ESP flow cytometer (Beckman Coulter, Fullerton, CA) into 96-well round-bottomed plates and expanded in the presence of 10⁵ allogeneic γ -irradiated (6000 rad) PBMC, 5 $\times$ 10³ irradiated EBV-transformed B cells, 1 μ g/mL of PHA and 25 IU/mL of IL-2.

Generation of long-term CD4⁺ TCL from memory and naïve pools

The split-well method was used to generate highly mono-/oligoclonal long-term TCL, as previously described [11]. Briefly,

2.5 $\times$ 10⁴ memory or naïve CD4⁺ T cells were stimulated with 5 $\times$ 10³ peptide-pulsed (10 μ M) DC in 96-well round-bottomed plates (20–30 replicate wells). After 4 days, fresh medium and 10 IU/mL of IL-2 were added to the cultures. After 10 days, the cultures were restimulated with autologous peptide-pulsed γ -irradiated (3000 rad) PBMC. On day 20, half of the T cells in each well were split into two new daughter plates, and stimulated with γ -irradiated (6000 rad) T2.DR4 cells (1 $\times$ 10⁴ per well) pulsed for 4 h with or without the peptide. After 3 days, [³H]thymidine was added (1 μ Ci per well; GE Healthcare, Little Chalfont, UK), and following an additional 16 h, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1450) and the results expressed as cpm. Positive cultures were transferred to a 48-well plate and expanded with 1 $\times$ 10⁶ irradiated autologous PBMC, 10 μ M of the peptide and 25 IU/mL of IL-2 for 2 wk before analyses.

Functional T-cell assays

T-cell proliferation assays were set up in triplicates (TCL) or duplicates (T-cell clones) in 96-well round-bottomed plates with 2.5 $\times$ 10⁴ (TCL) or 1 $\times$ 10⁴ (T-cell clones) T cells together with 5 $\times$ 10³ irradiated T2.DR4 cells (TCL) or 2.5 $\times$ 10⁴ irradiated autologous PBMC (T-cell clones) as APC. After 3 days, proliferation was measured, as described above. Supernatants (100 μ L per well) were collected from the cultures stimulated with 10 μ M of the Bos d 2_{127–142} peptide after 3 days. The production of IFN- γ , IL-4 and IL-10 was measured using commercial ELISA kits (DuoSet, R&D Systems, Minneapolis, MN). IFN- γ /IL-4 ratios were used for the classification of the TCL (Th1: IFN- γ /IL-4 ratio > 5; Th2: IFN- γ /IL-4 ratio < 0.2; Th0: IFN- γ /IL-4 ratios in between). The expression of cell-surface markers by the TCL was assessed by staining with anti-CD4-FITC and a panel of PE-, APC- or PE-Cy7-labeled mAb (CD45RO, CD45RA, CD62L, CCR7, CD25, CTLA-4, CXCR3, CCR4 and CCR6, all from BD Biosciences) or corresponding isotype control mAb before flow-cytometric analyses.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism (Graphpad Software, San Diego, CA). The Mann–Whitney *U* test and Fisher's exact test were used as indicated. *p*-Values of 0.05 or less were considered significant.

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Abbreviation: TCL: T-cell line

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