

## Persistent central memory phenotype of circulating Fel d 1 peptide/DRB1\*0101 tetramer-binding CD4<sup>+</sup> T cells

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**Background:** Although substantial evidence suggests that T cells are important in the pathogenesis of atopic dermatitis (AD), little is known of the differentiation status of CD4<sup>+</sup> T cells specific for common environmental allergens.

**Objective:** To determine the frequency, differentiation phenotype, and function of circulating allergen-specific CD4<sup>+</sup> T cells in adult individuals with severe persistent AD and controls.

**Methods:** Using tetrameric complexes of an HLA DRB1\*0101 restricted epitope from Fel d 1, the major IgE-reactive component of cat dander, we studied *ex vivo* and cultured T-cell frequency and phenotype in individuals with AD and healthy controls. Cytokine secretion was measured by *ex vivo* and cultured IFN- $\gamma$ , IL-4, and IL-10 enzyme linked immuno-spot analysis.

**Results:** *Ex vivo* Fel d 1-specific DRB1\*0101-restricted CD4<sup>+</sup> T cells express high levels of CCR7, CD62L, CD27, and CD28 and proportionately low levels of tissue-specific homing receptors and T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production, placing the cells largely within the central memory subgroup.

**Conclusion:** Circulating Fel d 1-specific DRB1\*0101-restricted CD4<sup>+</sup> T cells maintain central memory capacity, consistent with a potential to contribute to persisting clinical atopic disease.

**Clinical implications:** Persisting central memory characteristics of allergen-specific CD4<sup>+</sup> T cells in individuals with AD may contribute to chronic disease. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

**Key words:** T cells, atopic dermatitis, memory T cells, Fel d 1

### Abbreviations used

AD: Atopic dermatitis

CLA: Cutaneous lymphocyte-associated antigen

DR1 LPV: HLA DRB1\*0101 LPVVLENARILKNCVDAK tetramer

ELISpot: Enzyme Linked Immuno-Spot

R0: RPMI supplemented with penicillin, streptomycin, and L-glutamine

R10: RPMI with penicillin, streptomycin, L-glutamine, and 10% FCS

T<sub>CM</sub>: Central memory T cell

T<sub>EM</sub>: Effector memory T cell

UK: United Kingdom

Atopic dermatitis (AD) is a chronic inflammatory skin condition associated with adaptive immune responses to harmless environmental allergens.<sup>1</sup> A recent United Kingdom (UK) prospective birth cohort study established the prevalence of AD at age 6 to 18 months as 25.6%,<sup>2</sup> and adult prevalence is believed to be approximately 2% to 3%.<sup>3,4</sup> The prevalence of AD and other atopic diseases has risen substantially over the last 30 years.<sup>5</sup>

Cat allergy is a significant association of atopic disease, with 16.9% of adults found to be sensitized in 1 UK study<sup>6</sup> and 5.8% rising to 19.4% in children with AD.<sup>7</sup> A modified response to cat allergens has been described in which there is little cat specific IgE but detectable cat specific IgG<sub>4</sub>.<sup>8</sup> It is proposed that exposure to high levels of cat dander and/or immunotherapy induces such a modified T<sub>H</sub>2 response and associates with reduced disease.<sup>8</sup> The major IgE-reactive component of cat dander is Fel d 1, a glycoprotein dimer of 36 kd found on the skin, fur, and saliva of the cat. Numerous studies have demonstrated proliferative T-cell responses to Fel d 1 protein and peptides in both cat atopic and nonatopic subjects by means of thymidine incorporation proliferation assays,<sup>8-10</sup> accompanied in 1 study by increased IL-5 and decreased IFN- $\gamma$  response to Fel d 1 in cat-atopic subjects compared with controls, measured by ELISA.<sup>8</sup> IL-4 response measured by Enzyme Linked Immuno-Spot (ELISpot) has been found by a number of groups to be raised in cat-atopic subjects compared with controls.<sup>11-13</sup> Furthermore, IL-10 secretion in response to Fel d 1 protein has been

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demonstrated in cat-atopic subjects and healthy controls, and Fel d 1 peptides elicited an enhanced HLA DR7 restricted IL-10 response within the modified cat allergy group.<sup>8</sup> Numerous peptide regions of Fel d 1 have been proposed as epitopes that have been shown to induce proliferation and cytokine production by T cells derived from atopic individuals.<sup>8,10,14-16</sup> Although these studies have been vital to characterize the T-cell response to Fel d 1, it is possible that not all allergen-specific T cells have proliferative and/or cytokine-producing capacity. Thus far, there are no studies that have capitalized on the advantages of using HLA peptide tetrameric complexes to characterize the overall *ex vivo* CD4<sup>+</sup> T-cell response to a common persistent allergen and to define phenotypic and functional subgroups.

Naive CD4<sup>+</sup> T cells undergo unique developmental programs after antigen encounter, resulting in the generation of central (T<sub>CM</sub>) and effector (T<sub>EM</sub>) memory T cells. Although considerable controversy exists regarding the precise phenotypic characteristics of such subpopulations, there is general acceptance that T<sub>CM</sub> express CD62L and CCR7 and have a high replicative capacity and proportionately low effector function. Coupled with relatively low expression of tissue-specific homing receptors (such as the skin homing receptor cutaneous lymphocyte-associated antigen [CLA]), it is believed that T<sub>CM</sub> are able to maintain lymph node homing and long-lived responses to antigen. In contrast, T<sub>EM</sub> express low levels of CD62L and CCR7 and high levels of tissue-specific homing receptors and have good effector function and a lower replicative capacity. The importance of both T<sub>CM</sub> and T<sub>EM</sub> subsets for the control of infectious disease and the effectiveness of vaccines has been shown in several studies.<sup>17-19</sup>

We used HLA peptide tetrameric complexes based on a defined DRB1\*0101-restricted Fel d 1 epitope to identify and characterize the *ex vivo* differentiation phenotype of allergen-specific CD4<sup>+</sup> T cells in adult individuals with severe persistent AD.<sup>16</sup>

## METHODS

### Subjects

Under the ethical approval of Oxford Clinical Research Committee, 26 subjects with moderate to severe AD were recruited through the Department of Dermatology, Churchill Hospital, Oxford, UK. The UK AD diagnostic criteria were used to define all patients with AD.<sup>20,21</sup> All donors were HLA-typed by polymerase chain reaction-sequence specific primer phototyping.<sup>22</sup> From these people, 4 subjects with AD and cat specific IgE were found to be DRB1\*01 and studied further (15%). In addition, we screened 94 asymptomatic individuals and identified 5 who were positive for DRB1\*01 (5%). We chose this allele because HLA-DRB1\*0101 is 1 of only limited HLA class II alleles that have been successfully refolded to form tetrameric complexes and subsequently shown to be useful in identifying antigen-specific CD4<sup>+</sup> T cells.

### Specific IgE measurement

Specific IgEs to cat dander, house dust mite, and German cockroach were measured at the Department of Immunology, Churchill Hospital, Oxford, using the Pharmacia Unicap system (Uppsala, Sweden). Results are expressed in classes with 1 to 6 positive.

### PBMC separation

PBMCs were separated from heparinized peripheral blood using Lymphoprep (1114547, Nycomed, Oslo, Norway) density gradient. PBMCs were then washed in RPMI supplemented with penicillin, streptomycin, and L-glutamine (R0) and resuspended in RPMI with penicillin, streptomycin, L-glutamine, and 10% FCS (R10).

### IFN- $\gamma$ and IL-4 ELISpots

Ninety-six-well multiscreen Immobilon-P plates (IP-plates) (MAIPS4510, Millipore, Watford, UK) were coated with 50  $\mu$ L per well at 15  $\mu$ g/mL with IFN- $\gamma$  catcher antibody (3420-2A, Mabtech, Nacka Strand, Sweden) in sterile PBS, pH 7.4, or IL-4 catcher antibody (3410-2A, Mabtech) in sterile carbonate buffer, pH 9.6, and incubated 16 hours at 4°C (adapted from Pala et al<sup>13</sup> and Gabrielson et al<sup>23</sup>). Plates were washed 6 times with R0 and then blocked with R10 for 1 hour at 37°C. Cells were added at  $1 \times 10^5$ /well (PBMCs on IFN- $\gamma$  ELISpot),  $3 \times 10^5$ /well (PBMCs on IL-4 ELISpot), or  $4 \times 10^4$ /well (T-cell line). Cells were stimulated with 10  $\mu$ mol/L peptide or 10  $\mu$ g/mL recombinant Fel d 1 or Bla g 1 protein (Indoor Biotechnologies, Cardiff, UK). Phytohemagglutinin was used as the positive control at 2  $\mu$ g/mL, dengue fever (NS5) peptide as a negative control, and R0 as a blank. The plate was incubated 40 hours at 37°C before being developed as per the manufacturer's instructions using Biorad (170-643, Biorad, Hemel Hempstead, UK) developing buffer.

### Flow cytometry

Cells were stained with DRB1\*0101 iTAG MHC Tetramer (Beckman Coulter, San Diego, Calif) for 2 hours at 37°C 5% CO<sub>2</sub>. Then directly conjugated antibodies to surface markers were added as per the manufacturer's instructions and incubated 20 minutes in the dark at room temperature. Cells were then washed in cold PBS and fixed in PBS with 0.5% formaldehyde. Four-color flow cytometry was performed within 24 hours of cell staining on a FACScalibur with CellQuest software (Becton Dickinson, Immunocytometry Systems, San Jose, Calif). To maximize tetramer positive cell numbers,  $3 \times 10^6$  PBMCs were used per stain.

### Cultured ELISpots

Fresh PBMCs at  $2$  to  $4 \times 10^6$ /mL in R0 with 10% human AB serum (Rh10) were incubated on 24-well flat bottom culture plate with 3.75  $\mu$ mol/L peptide for 10 days at 37°C 5% CO<sub>2</sub>. On days 3 and 7, the cells were supplemented with Rh10 plus 200 IU/mL human IL-2. On day 13, cells were removed from the plate, washed twice in sterile PBS, and returned to a clean well overnight in Rh10 to rest before ELISpot or tetramer analysis on day 14.

### Statistics

Student *t* test and Pearson correlation were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, Calif).

## RESULTS

### Confirmation and optimization of DRB1\*0101Fel d 1 epitope

Before generation of the class II tetrameric complex (tetramer), we first refined the Fel d 1 restricted DRB1\*0101 peptide sequence to help ensure tetramer stability.<sup>16</sup> Using short-term cultured peptide-stimulated T-cell lines derived from HLA DRB1\*0101 (DR1)-positive individuals, we confirmed that significant responses were detectable to both the peptide and to recombinant Fel d 1 protein

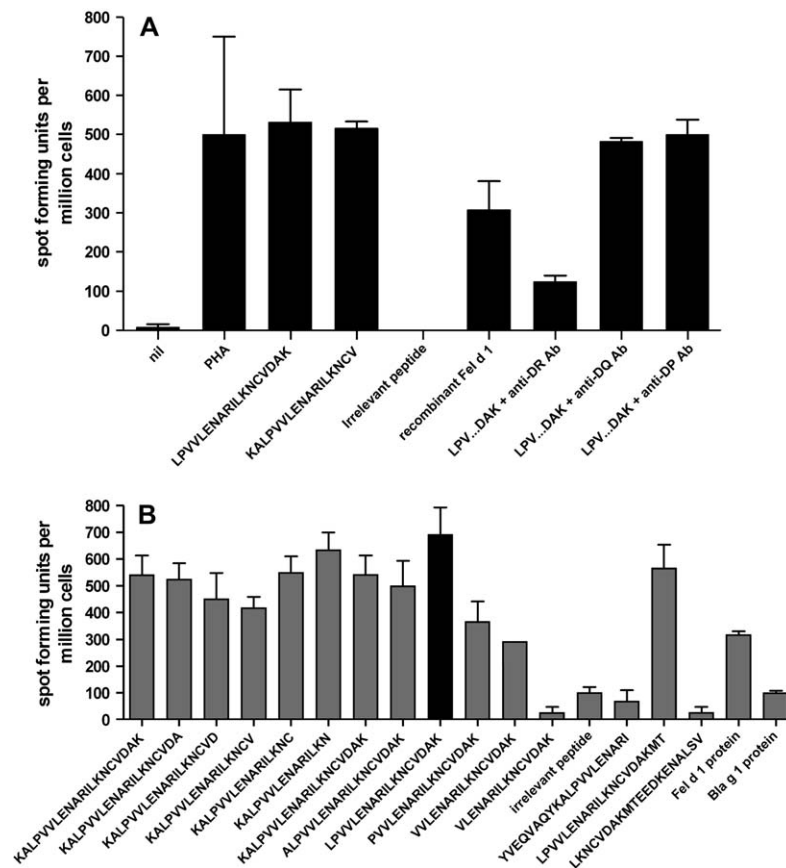


**FIG 1.** ELISpot analysis of short-term cultured T-cell lines showing (A) recognition of both Fel d 1 peptides and recombinant protein and inhibition by anti-HLA-DR antibodies; (B) full analysis of truncated peptides confirming that overall the shifted LPV...DAK sequence yielded an enhanced response. Data shown from 1 patient and undertaken in triplicate.

(Fig 1). We then proceeded to test short-term cultured T-cell lines using truncated peptides and found that LPVVLENARILKNCVDAK, a peptide shifted 2 amino acids toward the C-terminus compared with the published sequence, frequently yielded an enhanced response (Fig 1). We showed that anti-HLA-DR antibodies significantly blocked the response, consistent with HLA-DRB1\*0101 restriction (Fig 1). These data confirmed that DR1-expressing atopic individuals have circulating HLA DRB1\*0101-restricted Fel d 1-specific CD4<sup>+</sup> T cells, and having refined the epitope, we were in a position to use HLA class II tetrameric complexes with potentially greater stability.

### Ex vivo and cultured T-cell line tetramer frequency

We proceeded to investigate the *ex vivo* frequency of epitope-specific CD4 T cells in the peripheral blood of subjects with cat allergy with AD and healthy individuals using the DRB1\*0101 LPVVLENARILKNCVDAK (DR1 LPV) tetramer. DRB1\*0101 is not a common allele, and we had to undertake HLA typing of 26 individuals with severe AD and 94 controls before identifying our DRB1\*0101-positive cohort. Fig 2, A and C, shows an

example of *ex vivo* tetramer staining from individuals with and without AD. The frequency of tetramer-positive CD4 T cells was observed to be significantly ( $P < .05$ ) higher in the individuals with AD compared with those without (Fig 2, E). We also investigated the replicative potential of the allergen-specific T cells using peptide stimulation to expand specific T-cell lines and showed that the cells are able to proliferate efficiently *in vitro* (Fig 2). Furthermore, we found that individuals without AD have DR1-restricted Fel d 1-specific CD4<sup>+</sup> T cells that can be expanded *in vitro*, compatible with studies suggesting that subjects without atopy do have circulating allergen-specific T cells.<sup>8</sup> Overall, these data show that although individuals with AD or without AD have circulating Fel d 1-specific DR1-restricted CD4<sup>+</sup> T cells that can proliferate *in vitro*, the frequencies in those with disease are significantly higher than those in disease-free controls.

### Phenotypic analysis of Fel d 1-specific T cells

Phenotypic analysis was performed on tetramer-positive CD4 T cells derived *ex vivo* from all DR1 individuals to establish the activation and differentiation status of the allergen specific cells. Fig 3 demonstrates the expression

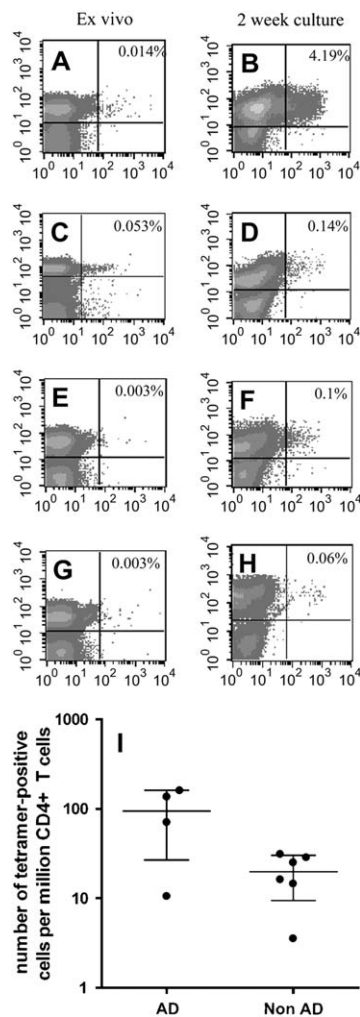


**FIG 2.** Examples of DR1 LPV Fel d 1 tetramer FACS analyses from HLA-DRB1\*0101-positive individuals. Y-axis anti-CD4; X-axis tetramer. Left column shows examples of *ex vivo* responses, and right column shows examples of staining after 2 weeks of culture. **A-F**, Atopic individuals; **G and H**, nonatopic healthy controls; **I**, proportion of tetramer-binding cells in the peripheral blood of individuals with AD and controls.

level of the markers on tetramer-positive CD4 T cells. The skin homing marker CLA was found to be expressed on  $12.7\% \pm 2.4\%$  of the tetramer-binding CD4 T cells, which is higher than the levels observed on the *ex vivo* CD4 T-cell population as a whole (mean,  $1.1\% \pm 0.4\%$ ). The proportion of tetramer-positive CD4 T cells expressing CCR4 and CCR8 was comparatively higher (mean,  $60.5\% \pm 3.7\%$  and  $34.5\% \pm 2.9\%$ , respectively) than CLA. The proportion of DR1 LPV-specific cells expressing CCR4 or CCR8 was also substantially higher than the expression of the CD4<sup>+</sup> T-cell population as a whole (mean,  $17.8\% \pm 1.9\%$  and  $0.13\% \pm 0.04\%$ , respectively). The activation marker CD38 was observed on  $35.3\% \pm 6.5\%$  of DR1 LPV-positive CD4 T cells, which was also higher than seen in the CD4<sup>+</sup> T-cell population (mean,  $14.5\% \pm 6.5\%$ ).

The Fel d 1-specific tetramer-positive T cells were found to express predominantly CCR7 and CD62L (mean,

$77.7\% \pm 3.6\%$  and  $77.4\% \pm 4.8\%$ ), although coexpression was lower at  $63.0\% \pm 6.3\%$ . These levels were similar to those observed on the whole CD4 T-cell population. Expression of the costimulatory markers CD27 and CD28 was also high on tetramer-positive CD4<sup>+</sup> T cells, with  $84.2\% \pm 2.7\%$  and  $75.1\% \pm 3.7\%$ , respectively, expressing the individual markers and  $70.8\% \pm 4.1\%$  with coexpression. Levels of expressing cells were, however, lower compared with the whole CD4 T-cell population (CD27<sup>+</sup> mean,  $93.8\% \pm 0.8\%$ ; CD28<sup>+</sup> mean,  $94.7\% \pm 1.8\%$ ; CD27<sup>+</sup>CD28<sup>+</sup> mean,  $90.3\% \pm 1.9\%$ ). The use of 4-color analyses limits the detailed subset characterization of the tetramer-binding cells, but it is hoped that this will be improved with future access to multilaser flow cytometry. Overall, these data showed even in individuals with severe active AD that the Fel d 1-specific DR1-restricted CD4<sup>+</sup> T cells largely maintained expression markers associated with early differentiation and a central memory phenotype. We therefore proceeded to investigate the functional capacity of the Fel d 1-specific CD4<sup>+</sup> T cells *ex vivo*.

### Functional analysis of Fel d 1-specific CD4 T cells

To investigate the functional ability of CD4 T cells specific to the Fel d 1 epitope, IFN- $\gamma$ , IL-4, and IL-10 ELISpots were performed both *ex vivo* and using short-term cultured T-cell lines. We observed that when compared with overlapping Fel d 1-derived peptides, the DR1-restricted peptide was typically one of the highest responses (data not shown). Fig 4, A, shows that the frequency of peptide-specific cytokine responses was very low *ex vivo*, but consistent with previous reports, subjects with and without atopy had detectable circulating Fel d 1-specific CD4<sup>+</sup> T cells.<sup>8</sup> We also confirmed the cytokine-producing capacity of cultured allergen-specific CD4<sup>+</sup> T cells and confirmed that there were no significant differences in cytokine production between those with disease and the controls (see this article's Fig E1 in the Online Repository at [www.jacionline.org](http://www.jacionline.org)). When comparing frequency of tetramer-positive cells and cytokine responses by ELISpot of cultured T cells, it was found that IL-4 responses and combined IL-4 and IFN- $\gamma$  responses correlated significantly with tetramer frequency ( $r = 0.75$ ,  $P < .0005$ ; and  $r = 0.48$ ,  $P < .05$ , respectively; Fig 4, B). We attempted to undertake costaining with class II tetramer and anticytokine antibodies but were limited by the T-cell receptor downregulation that occurred after antigen stimulation. Such an approach has been published using class I tetrameric complexes,<sup>24</sup> but despite extensive experiments, we were unable to replicate this in the class II system. Overall, these data showed using IFN- $\gamma$ , IL-4, and IL-10 ELISpots that approximately 20% of the tetramer-positive cells from individuals with AD express IL-4, but that there is variable expression of IFN- $\gamma$  and IL-10. Thus, the cell surface central memory phenotypic characteristics associate with proportionately low cytokine production.

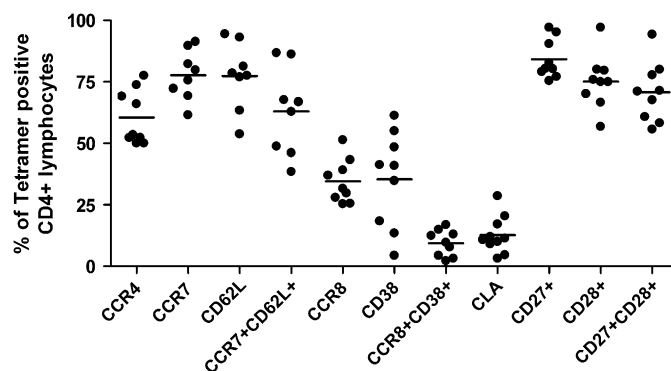


FIG 3. *Ex vivo* phenotypic analysis of tetramer-binding cells from the peripheral blood of subjects with AD.

## DISCUSSION

By using HLA class II tetrameric complexes, we have been able to provide the first data documenting the phenotype of allergen-specific CD4<sup>+</sup> T cells *ex vivo* in individuals with atopic disease and controls and show that the majority of the Fel d 1-specific HLA-DRB1\*0101-restricted CD4<sup>+</sup> T cells express a central memory phenotype characterized by high levels of CCR7, CD62L, CD27, and CD28 expression coupled with high replicative potential and relatively low levels of T<sub>H</sub>1 and T<sub>H</sub>2 cytokine production. However, approximately 20% of the allergen-specific CD4<sup>+</sup> T cells showed an effector memory phenotype with low levels of CCR7 and CD62L and high expression of the skin homing receptor CLA and the activation marker CD38.

T<sub>CM</sub>s are associated with persisting long-lived responses to antigen and in persistent viral infections typically make up the minority of the circulating antigen-specific T cells. In contrast, for persistent antigens, the peripheral blood antigen-specific CD4<sup>+</sup> T cells are believed to be usually dominated by T<sub>EM</sub> characterized by potent effector function and poor proliferative potential. CD4<sup>+</sup> T<sub>EM</sub> show a rapid turnover with a proliferation rate of 4.7% per day in comparison with the slower proliferation rate of 1.5% per day for T<sub>CM</sub>,<sup>25</sup> which is thought to equate to a half-life of months to years.<sup>26</sup> Persistence of T<sub>CM</sub> would be predicted to provide a long-term supply of effector antigen-specific CD4<sup>+</sup> T cells that may contribute to chronic inflammatory responses and clinical disease. In our study, approximately 20% of the circulating Fel d 1-specific T cells showed a skin homing T<sub>EM</sub> phenotype that may have made up the dominant source for the rapid production of effector T<sub>H</sub>1 and T<sub>H</sub>2 cytokines in response to peptide.

The majority of the Fel d 1-specific CD4<sup>+</sup> T cells expressed CCR4 and thus would be predicted to be largely pre-T<sub>H</sub>2 cells with an associated susceptibility for subsequent full T<sub>H</sub>2 differentiation.<sup>27</sup> Furthermore, the receptors CCR4 and CCR8 are known for their roles in recruiting T<sub>H</sub>2 T cells to sites of allergic inflammation, both the skin and the lungs.<sup>28</sup> CCR4 is the receptor for CCL17 (thymus and activation-regulated chemokine

[TARC]) and CCL22 (macrophage-derived chemokine [MDC]), both important chemokines in the recruitment of activated T<sub>H</sub>2 cells.<sup>29</sup> TARC is produced by basal keratinocytes and is upregulated in AD skin lesions,<sup>30,31</sup> and therefore, CCR4 expression on T cells is thought to be important in their recruitment to the skin.<sup>32</sup> CCR8 is the receptor for the chemokine CCL1, which is constitutively expressed by epidermal antigen-presenting cells. High expression on T cells derived from healthy skin has been described, along with a proinflammatory cytokine profile and activated phenotype of these cells.<sup>33</sup>

It will be important to investigate the mechanism for such persistent maintenance of a dominant population carrying the T<sub>CM</sub> phenotype because this may provide novel preventive or therapeutic approaches. It has been shown that IL-4 promotes CLA downregulation<sup>34</sup> and CCR7 upregulation by viral-specific CD8<sup>+</sup> T cells<sup>35</sup> and thus may also play a role in the sustained expression of CCR7 by allergen-specific CD4<sup>+</sup> T cells. Furthermore, recently Wang et al<sup>36</sup> have shown that keratinocyte production of thymic stromal lymphopoietin may also contribute to the maintenance of a central memory phenotype of T<sub>H</sub>2 cells. Although IL-4 and thymic stromal lymphopoietin may alter the balance of CCR7/CLA expression, such effects are clearly not absolute, because CLA<sup>+</sup> T cells are readily detectable in inflamed atopic skin. Nevertheless, interference of the possible effects of IL-4 on T-cell differentiation may provide novel preventive or therapeutic strategies.

The presence or absence of allergen-specific CD4<sup>+</sup> T cells in the peripheral blood of nonatopic subjects has been the source of much debate in the literature, but by using HLA class II tetrameric complexes, we have shown that Fel d 1-specific CD4<sup>+</sup> T cells are present within the peripheral blood of nonatopic individuals, albeit at significantly lower frequencies than levels in individuals with severe atopic disease. Thus we would favor a model in which the majority of individuals have circulating allergen-specific CD4<sup>+</sup> T cells, but the frequency and function differs between atopic and nonatopic subjects.<sup>8</sup>

Having identified and characterized the *ex vivo* Fel d 1-specific CD4<sup>+</sup> T cell response, there are clearly a number of further investigations that are required, such as

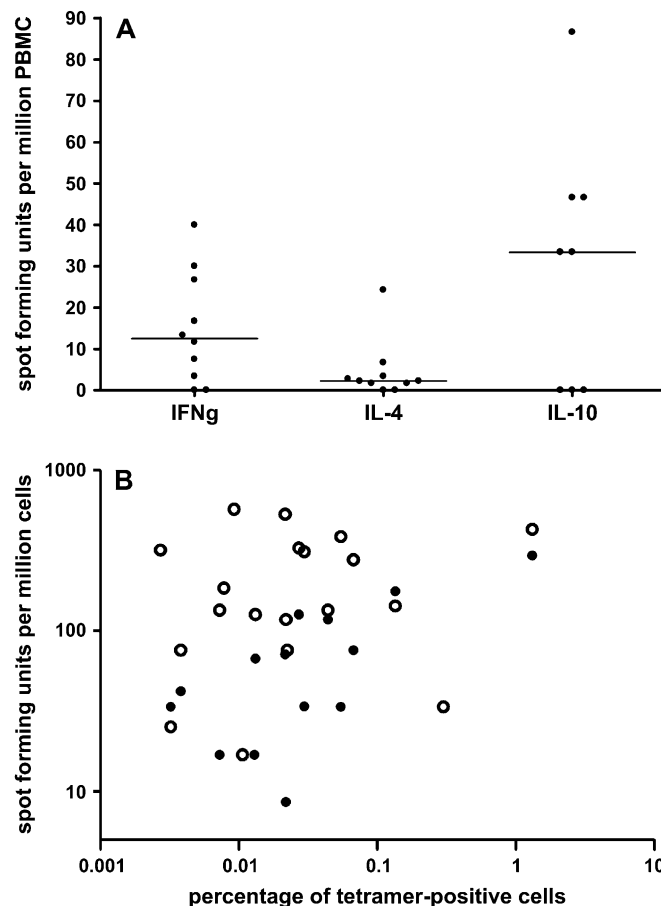


**FIG 4. A,** *Ex vivo* ELISpot responses to the DR1 LPV peptide in individuals with AD and controls. Mean negative control values: IFN- $\gamma$ ,  $9.0 \pm 4.76$ ; IL-4,  $1.0 \pm 0.50$ ; and IL-10,  $35.9 \pm 13.3$  spot forming units/million. **B,** Correlation between tetramer staining and ELISpot for short-term T-cell lines using AD and control subjects. *Solid circles* represent IL-4, which shows significant correlation between tetramer staining and ELISpot data ( $P < .0005$ ). *Open circles* represent IFN- $\gamma$  (nonsignificant).

longitudinal studies during relapse and remission in both children and adults. It will be important to document changes during treatment and immunotherapy and to characterize the cells within lesional tissue. Last, we are limited by the number of fully characterized allergen epitopes that are restricted through HLA class II molecules for which we can generate HLA class II tetrameric complexes, but as these become available, in due course it will be crucial to compare the phenotypes between different allergen-specific CD4 $^{+}$  T cells.

In summary, we have shown that the majority of Fel d 1-specific HLA-DR1-restricted CD4 $^{+}$  T cells in the peripheral blood of severely affected adult atopic subjects and controls show a T<sub>CM</sub> phenotype characterized by high expression of CCR7, CD62L, CD27, and CD28 and low expression of the tissue-specific homing receptor CLA. The cells had good replicative potential and relatively poor effector function. In contrast, approximately 20% of the circulating allergen-specific CD4 $^{+}$  T cells from individuals with AD showed rapid effector function through production of IFN- $\gamma$ , IL-4, and IL-10. Such

maintenance of cells with a T<sub>CM</sub> phenotype in adults with severe chronic AD may contribute to long-lived recognition of antigen and persistent disease.

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