

# Activated human epitope-specific T cells identified by class II tetramers reside within a CD4<sup>high</sup>, proliferating subset

Erik J. Novak<sup>1,2</sup>, Susan A. Masewicz<sup>1</sup>, Andrew W. Liu<sup>1</sup>, Åke Lernmark<sup>2</sup>, William W. Kwok<sup>1</sup> and Gerald T. Nepom<sup>1</sup>

<sup>1</sup>Virginia Mason Research Center, Benaroya Research Institute, 1201 Ninth Avenue, Seattle, WA 98101-2795, USA

<sup>2</sup>R. H. Williams Laboratory, Molecular and Cellular Biology Program and Department of Medicine, University of Washington, Seattle, WA 98195, USA

**Keywords:** cellular activation, FACS, HLA, MHC, T Lymphocytes

## Abstract

Antigen-specific T cells acquire a distinctive phenotype during activation, with characteristic acquisition of surface markers and patterns of gene expression. Early after antigen stimulation, CD4<sup>+</sup> T lymphocytes increase their surface density of the CD4 marker, a trait which has been used to identify antigen-activated cells. The recent development of MHC tetramer technologies has greatly improved the ability to detect HLA class I-restricted T cells specific for known antigen epitopes. We have recently extended these studies to human class II-restricted CD4<sup>+</sup> T cell responses and now describe antigen-specific T cell responses from human peripheral blood in which elevated CD4 expression levels in human T cells following antigen stimulation identify the activated and proliferating subset of cells. The CD4<sup>high</sup> population is substantially enriched in epitope-specific cells identified by class II tetramer staining and almost all tetramer-positive cells are contained within the CD4<sup>high</sup> population. T cell clones derived from the tetramer-positive, CD4<sup>high</sup> population demonstrate antigen specificity and maintain tetramer staining, while the substantial number of CD4<sup>high</sup> cells which fail to stain with tetramer appear to proliferate as a result of bystander activation. Epitope-specific components of a polyclonal immune response are directly visualized and quantitated by tetramer detection, providing a direct measure of the heterogeneity of the human immune response.

## Introduction

The acquired immune response depends on activation and proliferation of antigen-specific CD4<sup>+</sup> T cells, a process which represents a sequential acquisition of new cell markers and gene expression (1,2). In spite of the presence of these activation markers, however, detailed study of the antigen-specific T<sub>h</sub> cell response in humans has been hampered by low precursor frequencies of epitope-specific T cells and an inability to identify these cells from the vast excess of T cells with other specificities present in the peripheral blood. In many clinical applications, ranging from synthetic vaccine design to antigen-specific therapies for autoimmunity, it is essential to identify immunodominant components of the immune response, in which specific T cells respond to individual peptide epitopes. In experimental studies, the use of TCR transgenic mice provides a means to increase the

precursor frequency of antigen-specific cells allowing study of the development of the antigen-specific response (3–7). Likewise, use of V<sub>β</sub> clonotypic antibodies has allowed study of the response to particular antigens that elicit a very restricted TCR usage (8). Nonetheless, these approaches are limited to selected situations and are generally not applicable for the study of antigen-specific responses in humans.

The recent description in mice of elevated CD4 levels on the surface of T cells responding *in vivo* suggests a means to identify and enrich these antigen-specific populations (9–11). In these studies, autoreactive, alloreactive or foreign antigen-responsive T cells were recovered from a subset of CD4<sup>+</sup> T cells based on high levels of CD4 expression. These CD4<sup>high</sup> cells represented ~5–20% of the CD4 T cell population depending on the antigen and time-point analyzed. Included

within this activated cohort were T cells specific for recent antigen exposure, demonstrated by cloning and functional assays.

The recent development of class I and class II MHC tetramers to detect antigen-specific T cells has greatly improved the ability to track and analyze antigen-specific T cell responses where the reactive antigen epitope and MHC restriction are known (12–14). In a number of studies, the use of class I tetramers has provided a wealth of information on the behavior and phenotypes of T cells specific for a variety of pathogens and tumor antigens (12, 15–17). We have recently described the use of class II tetramers to detect peripheral blood T cells specific to immunodominant epitopes of influenza A hemagglutinin protein and tegument protein from herpes simplex-2 virus (14, 18). In this paper we demonstrate that up-regulated surface expression of CD4 identifies human T<sub>H</sub> cells activated and proliferating to specific antigen stimulation. Using class II tetramer staining we show that the CD4<sup>high</sup> population contains almost all of the antigen-specific cells and that the tetramer-positive cells account for ~15% of the CD4<sup>high</sup> population. These cells continue to demonstrate antigen specificity when sorted and cloned, validating the use of a specific marker phenotype to directly quantitate antigen-specific T cells in humans.

## Methods

### *Isolation, antigen stimulation and staining of cells*

Peripheral blood mononuclear cells (PBMC) from healthy donors previously vaccinated for tetanus and influenza were separated from heparinized venous blood by gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway). Cells were cultured in RPMI 1640 (Gibco/BRL, Rockville, MD), supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml penicillin/streptomycin and 15% v/v pooled human serum. Adherent cells were prepared by plating PBMC at  $5 \times 10^6$  cells/well in 24-well plates for 1 h. Non-adherent cells were removed using a transfer pipette and washing once with PBS. Adherent cells were incubated for 3 h with 10 µg/ml influenza hemagglutinin peptide, residues 307–319 (HA<sub>307–319</sub>), a maximally stimulating dose of whole tetanus toxoid or no antigen. The non-adherent fraction was passed through a nylon wool column, washed twice with serum-free PBS and stained with either 0.8 µM 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) in PBS for 10 min at 37°C or 2 µM PKH-26 in Diluent C (Sigma, St Louis, MO) for 1 min at 37°C. Staining was stopped by adding 100% FBS and subsequently washing the cells twice in RPMI 1640 culture media. The stained nylon wool-purified T cells were then added back to the adherent cells at a density of  $2.5 \times 10^6$  cells/well.

### *Identification of antigen-specific cells using class II tetramers*

The construction of the expression vectors for generation of soluble DRA1\*0101/DRB1\*0401 has been described previously (14). Briefly, a chimeric cassette containing the extracellular coding region for the DRB1\*0401 chain appended to the acidic leucine zipper motif was generated from DRB1\*0401 and leucine zipper cDNA using the PCR-mediated

splicing overlap technique (19,20). A site-specific biotinylation sequence was then added to the 3' end of the DRB1\*0404/leucine zipper cassette. The chimeric cDNA was subcloned into the Cu-inducible pRmHa-3 *Drosophila* expression vector. The DRα expression vector was generated in a similar fashion using a basic leucine zipper motif and the coding region for the DRA1\*0101 chain. For class II production, DRα and DRβ expression vectors were co-transfected into Schneider S-2 cells. Soluble class II molecules produced following addition of CuSO<sub>4</sub> were purified by affinity chromatography, concentrated and subsequently biotinylated using the BirA enzyme (Avidity, Denver, CO) (21). Specific peptide was loaded at a concentration of 5-fold molar excess over the class II concentration in 100 mM sodium phosphate, pH 6.0 and 0.2% *n*-octyl-*D*-glucopyranoside. Tetramers were formed by incubating class II molecules with phycoerythrin (PE)-labeled streptavidin (Biosource International, Camarillo, CA) for 6 h at room temperature at a molar ratio of 8:1.

For staining, cells were incubated with 1 µg PE-labeled tetramer in 50 µl media for 1–3 h at 37°C, and subsequently with combinations of fluorochrome-labeled anti-CD3, CD4, CD8, CD25 and CD69 for 20 min on ice (PharMingen, San Jose, CA/Becton Dickinson, San Jose, CA). Cells were then washed in PBS containing 1% FBS and 0.1% NaN<sub>3</sub>, and analyzed using a Becton Dickinson FACSCalibur flow cytometer. The flow cytometer was calibrated using cells stained with single fluorochromes. Data analysis was performed using CellQuest software (Becton Dickinson) and WinMdi software (Stanford University).

### *Calculation of cell divisions and precursor frequency*

A portion of CFSE-stained cells was stimulated with 2.5 µg/ml phytohemagglutinin (PHA) and 10 U IL-2, and examined by FACS on day 7. Polyclonal stimulation of T cells with PHA and IL-2 results in cell division with distinct CFSE fluorescence peaks allowing determination of the mean CFSE fluorescence for each generation. These values were used to calibrate the CFSE fluorescence scale for each experiment. Precursor frequencies were determined by following the general approach described by Wells *et al.* (22). Briefly, to determine the absolute number of precursors of the divided cells, the cells in each generation were divided by 2<sup>*x*</sup>, where *x* is the number of cell divisions. This value was then divided by the total number of cells (undivided cells + precursor number of divided cells) to provide an estimate of precursor frequency. Precursor frequencies are expressed as the fraction of CD4<sup>+</sup> T cells out of the total CD3<sup>+</sup> population.

### *Cloning of antigen-specific cells*

HA<sub>307–319</sub> tetramer-positive and -negative T cells stained with CFSE and CD4 were single-cell sorted into 96-well plates using a FACS Vantage cell sorter (Becton Dickinson). Clones were expanded for 14 days with irradiated PBMC ( $10^5$ /well) plus 2.5 µg/ml PHA and 10 U IL-2. To confirm specificity of the clones, clonal cells were stimulated with 10 µg/ml HA<sub>307–319</sub> using either a transfected bare lymphocyte syndrome cell line (BLS-1) which expresses the sole class II molecule DRA1\*0101/DRB1\*0401 (23) or an autologous Epstein-Barr-transformed B cell line. [<sup>3</sup>H]Thymidine incorporation was measured at

72 h. Clonal cells were also stained with PE-labeled tetramer and analyzed by FACS as described above.

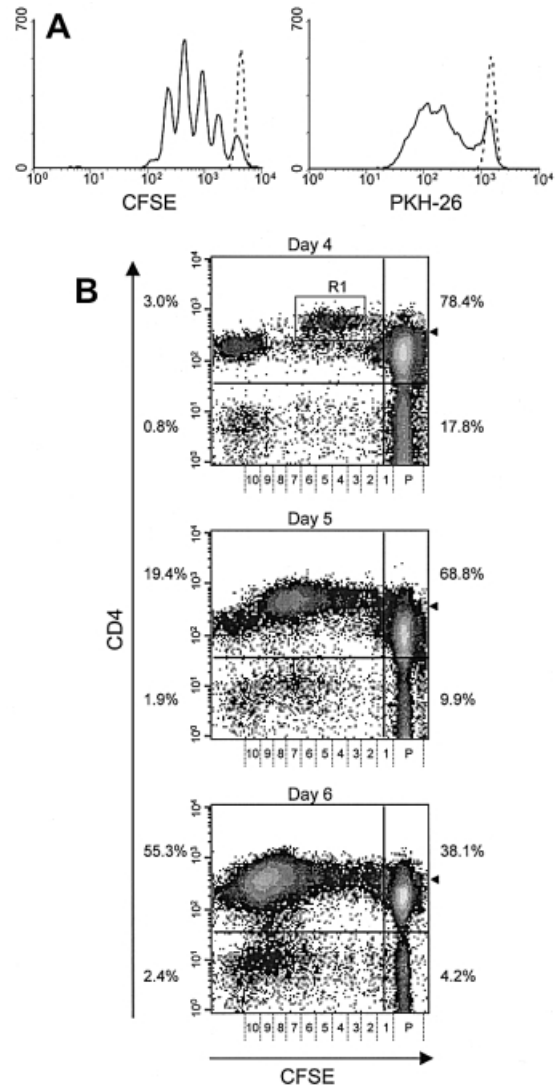
For studies of T cell activation in the absence of antigen-presenting cells (APC), selected clones were cultured with 10 µg/ml of DR0401 tetramers loaded with either HA<sub>307-319</sub> or VP16<sub>465-484</sub> (VP16 is a tegument protein of herpes simplex-2 virus). [<sup>3</sup>H]Thymidine incorporation was again measured at 72 h, while CD69 expression was measured at 18 h. These responses were both tetramer- and peptide-dependent as tetramer lacking specific peptide or peptides in the absence of tetramer showed no significant proliferative response (stimulation index < 2).

## Results

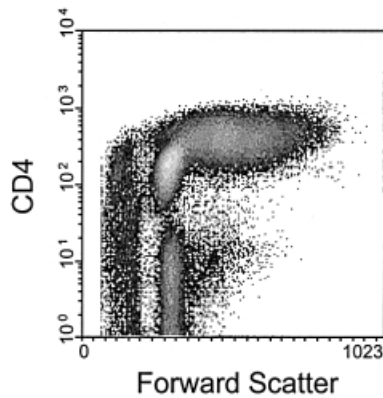
### Cytofluorometric profiling of antigen-specific T cell subsets

Recent FACS-based studies of multiple rounds of proliferation in human cells have utilized primarily two types of tracking dyes, PKH-26 and CFSE (24–26). PKH-26 is a lipophilic dye which incorporates into the cell membrane, while CFSE stably binds cytoskeletal actin following activation by cellular esterases. Each time a cell divides, both PKH-26 and CFSE are apportioned equally between daughter cells, resulting in a halving of fluorescence. We stained nylon wool-purified T cells with either CFSE or PKH-26, and polyclonally stimulated the cells with PHA and IL-2. The cells were cultured for 4 days and analyzed by flow cytometry (Fig. 1A). In the CFSE-stained sample there was an approximate halving of median fluorescence intensity as dye was apportioned equally among daughter cells with each successive generation. Division of CFSE-stained cells produced distinct peaks in contrast to PKH-26-stained cells, where peaks were much less resolved, making it difficult to determine generation boundaries.

We therefore used CFSE staining to identify T cells dividing in response to stimulation with tetanus toxoid antigen from an individual previously vaccinated for tetanus. We examined nylon wool-purified T cells on days 4, 5 and 6 following stimulation using autologous adherent APC pulsed with tetanus toxoid. CFSE proliferation profiles on CD3<sup>+</sup> gated cells, with CFSE fluorescence over a four-decade logarithmic scale along the horizontal axis and CD4 levels along the vertical axis, are shown in Fig. 1(B). The horizontal axis indicates the corresponding number of cell divisions with 'P' denoting the undivided parent population. Cells at the far left of the CFSE plot represent the small fraction of unstained, CD3<sup>+</sup> T cells that remained in the adherent population. As early as day 4 following stimulation, there is a small population of dividing CD4<sup>+</sup> T cells, indicated in gate R1, comprising 0.9% of the total cells. With time, the population of divided cells increases and shifts further to the left on the plot of CFSE fluorescence. By day 6, 55% of the total cells are present in the CD4<sup>+</sup> divided population with the majority having divided  $\geq 7$  times, demonstrating marked accumulation of divided cells over a 3- to 4-day period in response to tetanus toxoid stimulation. A small number of proliferating, non-CD4<sup>+</sup> cells are also evident (2.4% on day 6), representing cells of other subsets, mainly CD8<sup>+</sup> T cells. By using the shift in CFSE-stained cells to calculate the average number of cell divisions, the precursor frequency of CD4<sup>+</sup> T cells proliferating to tetanus toxoid is



**Fig. 1.** Dye dilution analysis of stimulated peripheral T cells. (A) CFSE- or PKH-26-stained, nylon wool-purified T cells from a single donor were added to autologous adherent cells and a portion were stimulated with 2.5 µg/ml PHA and 10 U IL-2. Cells were harvested on day 4, stained with anti-CD3 and -CD4 surface markers, and analyzed by flow cytometry. Histograms of CFSE fluorescence (left panel) and PKH-26 fluorescence (right panel) of CD3<sup>+</sup>, CD4<sup>+</sup> cells are shown. PHA/IL-2-stimulated cells are shown with a solid line, while unstimulated controls are shown with a dotted line. Equal numbers of events are shown in both panels. (B) Nylon wool-purified T cells, labeled with CFSE before culture with autologous adherent cells and tetanus toxoid antigen, were harvested on day 4, 5 and 6 of culture and stained with anti-CD3 and -CD4 surface markers, and analyzed by flow cytometry. In all panels, cells are gated on forward and side scatter and CD3<sup>+</sup> cells, the vertical axis shows CD4 fluorescence and the horizontal axis shows CFSE fluorescence over a four-decade logarithmic scale, indicating the corresponding number of cell divisions, with 'P' depicting the undivided parent population. This scale was calculated from the distinct CFSE fluorescence peaks produced by polyclonal stimulation with PHA and IL-2. Arrowheads indicate the boundary between CD4<sup>normal</sup> and CD4<sup>high</sup> cells selected for Fig. 3, corresponding to the top 1% of undivided, CD4<sup>+</sup> cells. 'R1' indicates the early proliferating CD4<sup>high</sup> population.



**Fig. 2.** Cells with CD4<sup>high</sup> phenotype span a range of cell sizes. Nylon wool-purified T cells stimulated with tetanus toxoid as described were harvested on day 6. Panel indicates CD4 fluorescence versus forward scatter on all events collected.

**Table 1.** Elevated CD4 expression levels in divided cells following tetanus toxoid stimulation

	Geometric mean CD4 fluorescence (four-decade logarithmic scale) <sup>a</sup>			
	Day 4	Day 5	Day 6	Day 7
Divided	440	478	436	393
Undivided	156	115	195	114

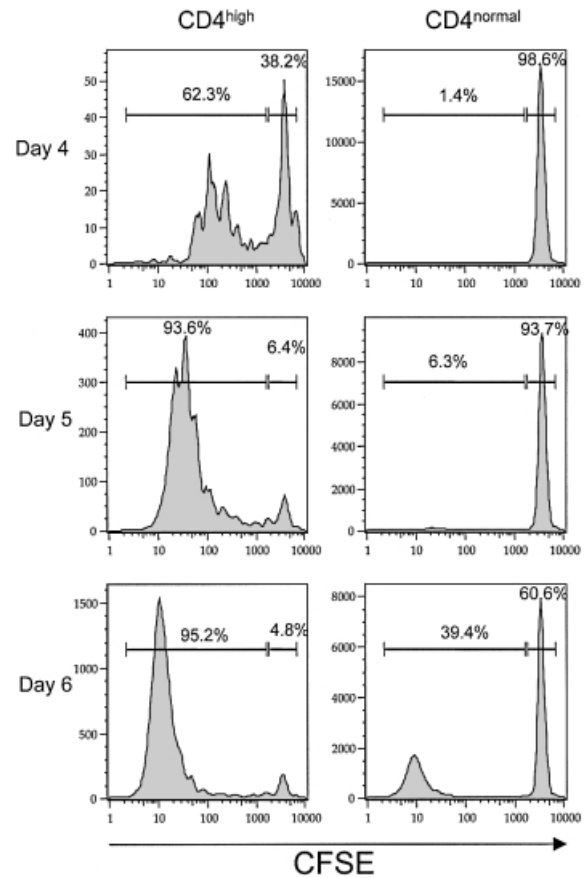
<sup>a</sup>Cells gated on the CD3<sup>+</sup>, CD4<sup>+</sup> population.

estimated to be 0.1%, within the range identified by earlier studies of tetanus-immunized individuals using limiting dilution analysis (27,28).

#### Activated, proliferating human cells show CD4<sup>high</sup> phenotype

Comparison of CD4 levels in the dividing and non-dividing fractions of tetanus toxoid-stimulated T cells revealed that the proliferating cells showed significantly elevated CD4 levels compared to the non-proliferating cells (Table 1). Levels in the non-proliferating population were generally unchanged from levels in unstimulated peripheral blood lymphocytes samples, and there were no observed differences in CD3 levels between the proliferating and non-proliferating fractions (data not shown). The CD4<sup>high</sup> population generally showed increased forward scatter, reflective of blasting cells undergoing cell division, as shown in Fig. 2 for day 6 following tetanus toxoid stimulation. Nonetheless, the broad range of cell sizes for the CD4<sup>high</sup> population on day 6 indicates that the cell size and CD4 fluorescence are not completely correlated. This increase in CD4 expression upon antigen-specific activation has been shown previously in murine T cells stimulated with specific antigen and alloantigen (9–11). Of note, CD8<sup>+</sup> expression levels in the small 'bystander' population of non-CD4<sup>+</sup> cells which divide did not increase relative to non-proliferating CD8<sup>+</sup> cells.

To determine whether the CD4<sup>high</sup> marker was specific for the proliferating cells we examined CFSE profiles for cells



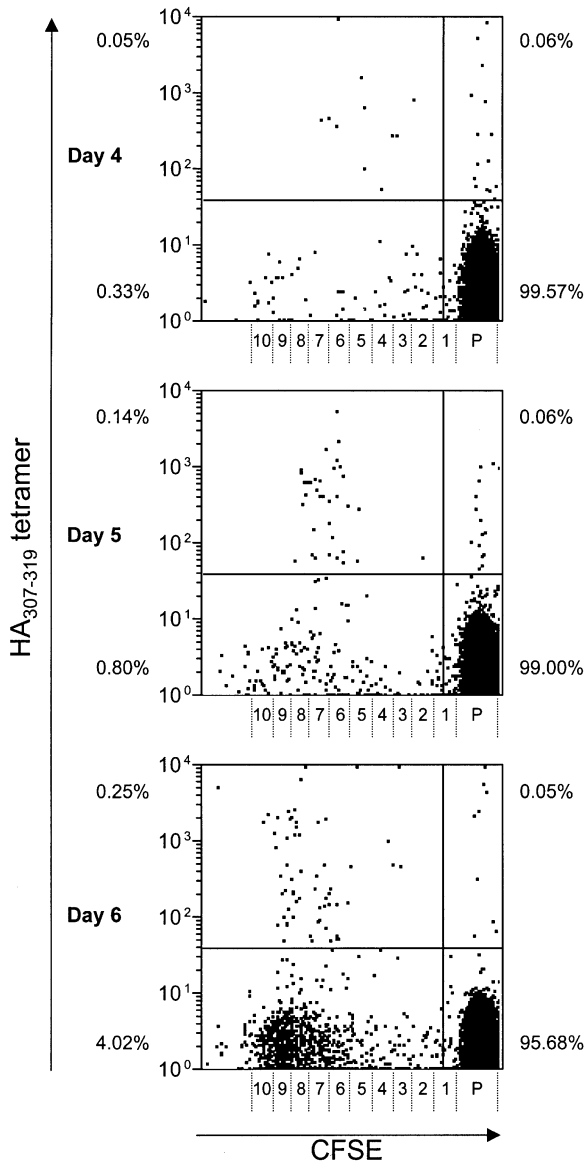
**Fig. 3.** Dividing cells are in the CD4<sup>high</sup> subset. Nylon wool-purified T cells were labeled with CFSE before culture with autologous adherent cells and tetanus toxoid antigen. Each panel indicates the percent of divided and undivided cells on days 4, 5 and 6 for CD4<sup>high</sup> and CD4<sup>normal</sup> cells, with the CD4<sup>high</sup> population containing the ~1% of the undivided CD4<sup>+</sup> population, as described in the legend of Fig. 1.

gated as CD4<sup>high</sup> and CD4<sup>normal</sup>. As shown in Fig. 3, the CD4<sup>high</sup> gate is substantially enriched in the proliferating cells starting at day 4, and consists almost entirely of proliferating cells by days 5 and 6. In the CD4<sup>normal</sup> population there are initially very few cells which have undergone proliferation. However, by day 6, increasing numbers of divided cells exhibit lower levels of CD4 expression. This may reflect cells returning to a resting state at the later time points following antigen stimulation.

#### Antigen-specific cells identified by class II tetramer staining show CD4<sup>high</sup> phenotype

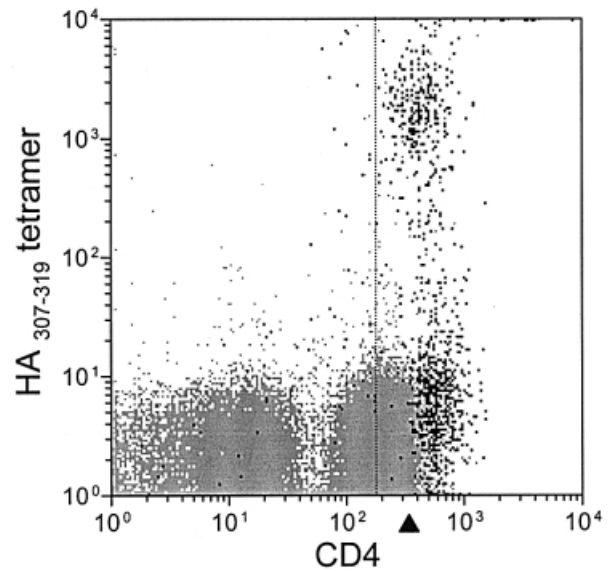
In order to identify T cells specific for a particular antigen, we stimulated cells from a DRB1\*0401 donor using an immunodominant epitope of the hemagglutinin protein (HA<sub>307–319</sub>) and subsequently identified these cells using class II tetramers. Tetramer-positive cells were first detectable at day 4 following peptide stimulation and continued to expand in number on subsequent days in accordance with continued division as reflected by decreased CFSE staining, shown in Fig. 4.

The small percentage of undivided cells which are tetramer-



**Fig. 4.** Temporal expansion of HA<sub>307-319</sub>-specific cells following antigen stimulation. Nylon wool-purified T cells were labeled with CFSE before culture with autologous adherent cells and 10 µg/ml HA<sub>307-319</sub> peptide. Panels are gated on CD3<sup>+</sup> cells. Percentages indicate the percent of CD3<sup>+</sup> cells in the upper left quadrant reflecting the tetramer-positive, divided population. The same numbers of total events are shown in each panel.

positive likely reflect non-specific background staining of the tetramer since we have observed similar levels of staining using both tetramers loaded with irrelevant control peptide and tetramers not loaded with any peptide (data not shown). Combining tetramer staining with CFSE staining to separate out dividing cells allows us to improve the detection resolution of the antigen-specific population. Among the different individuals tested, the tetramer-negative proliferating cells comprised 80–96% of the total number of proliferating cells on day 6 with the majority of these cells (>80%) staining positive for CD4.



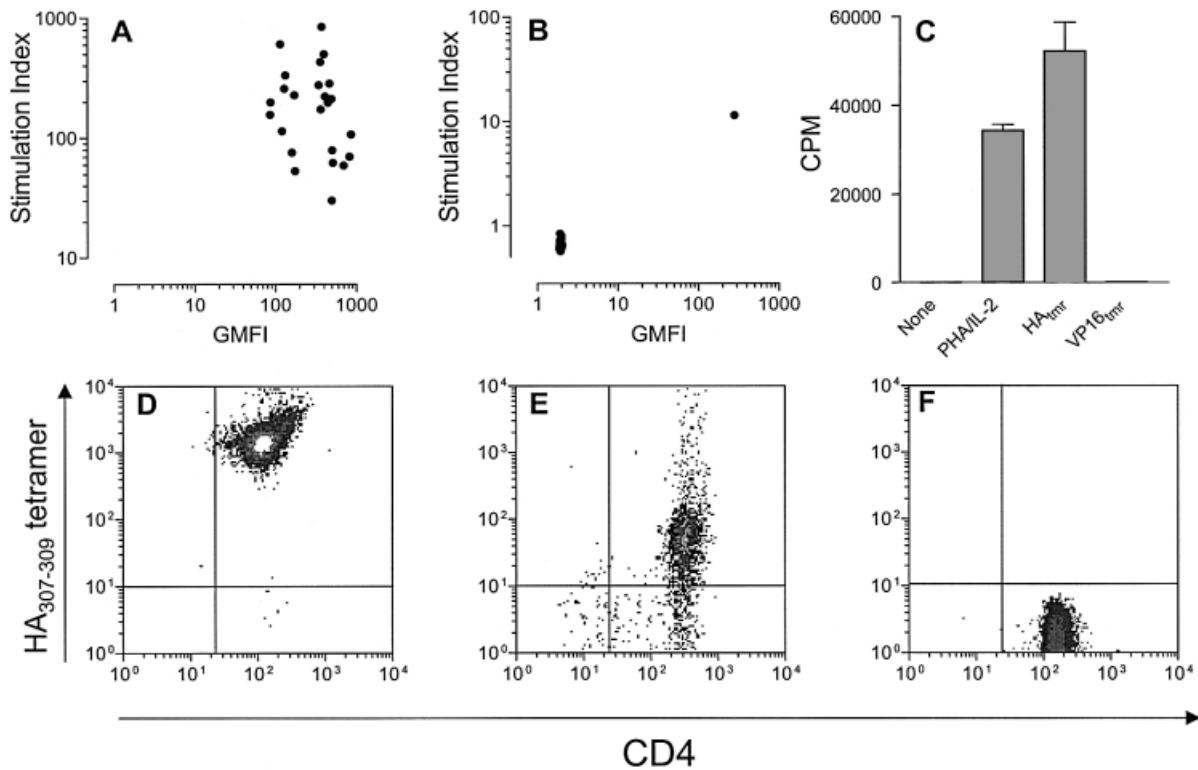
**Fig. 5.** CD4<sup>high</sup> phenotype of tetramer-positive cells. Nylon wool-purified T cells were labeled with CFSE before culture with autologous adherent cells and 10 µg/ml HA<sub>307-319</sub> peptide. On day 6 of culture, cells were harvested and stained with HA<sub>307-319</sub> tetramer and anti-CD4 antibody. Black dots identify divided cells as indicated by decreased CFSE fluorescence, while grey dots identify undivided cells remaining in the parent population. The dotted line indicates the geometric mean fluorescence of the CD4<sup>+</sup> population. The arrowhead indicates the boundary for the top 5% of CD4<sup>+</sup> cells.

We then examined the CD4 surface expression levels of tetramer-positive cells on day 6 (Fig. 5). CD4 levels on the horizontal axis are plotted against tetramer staining on the vertical axis. The dotted line indicates the geometric mean fluorescence of the CD4<sup>+</sup> population. The data shows that the population of proliferating cells, identified by black dots, is CD4<sup>high</sup>, consistent with the findings using tetanus toxoid. Eighty-two percent of the tetramer-positive cells are within the top 5% of CD4<sup>+</sup> T cells and 95% are within the top 20%. Conversely, in the CD4<sup>high</sup> population consisting of the top 5% of CD4<sup>+</sup> T cells, 16% of the cells are tetramer-positive.

*Tetramer sorted cells maintain antigen-specific phenotype*

In our studies using peptide-specific stimulation, we have consistently observed a subset of proliferating, CD4<sup>high</sup> cells that fail to stain with tetramer. We hypothesized that these cells could either be peptide specific but restricted by an HLA molecule other than DRB1\*0401 or expanding non-specifically as a result of bystander activation. To more fully characterize the proliferating, CD4<sup>high</sup> population we performed single-cell sorting of both tetramer-positive and -negative cells of the CD4<sup>high</sup>, divided population on day 6 following peptide stimulation.

For cells sorted on the tetramer-positive gate, all 24 clones analyzed demonstrated peptide-specific, DRB1\*0401-restricted proliferation when stimulated with a DRB1\*0401-specific BLS-1 cell line (Fig. 6A). In addition, all 24 clones stained positive with tetramer with staining intensities for the individual clones ranging from very strong to more moderate and broad (Fig. 6D and E). Interestingly, strength of staining



**Fig. 6.** Characterization of CD4<sup>high</sup> T cell clones. (A) Twenty-four clones identified through sorting of tetramer-positive/CD4<sup>high</sup>/CFSE<sup>low</sup> cells were stimulated using a DRB1\*0401-transfected BLS-1 cell line pulsed with 10  $\mu$ g/ml HA<sub>307-319</sub> peptide. [<sup>3</sup>H]Thymidine was added for the last 18 h of a 72 h incubation. (B) Thirty clones identified through sorting of tetramer-negative/CD4<sup>high</sup>/CFSE<sup>low</sup> cells were similarly stimulated using an autologous Epstein-Barr virus-transformed B cell line pulsed with 10  $\mu$ g/ml HA<sub>307-319</sub> peptide. The stimulation index is the ratio of c.p.m. of cultures with and without HA<sub>307-319</sub> peptide. The geometric mean fluorescence intensity (GMFI) of tetramer staining is plotted for each clone on the horizontal axis. (C) Comparison of [<sup>3</sup>H]thymidine incorporation at 72 h between a representative tetramer-positive T cell clone cultured with no stimulus, 2.5  $\mu$ g/ml PHA/10U IL-2, 10  $\mu$ g/ml HA<sub>307-319</sub> tetramer or 10  $\mu$ g/ml VP16<sub>465-484</sub> tetramer. Error bars indicate SEM of triplicates. The lower panels show tetramer staining for representative clones demonstrating the variety of staining intensities. Staining was performed 37°C for 1 h using 10  $\mu$ g/ml of PE-labeled tetramer. The clone in (D) shows strong tetramer staining, while the clone in (E) shows weaker and more heterogeneous staining. (F) Tetramer staining of a clone representative of the proliferation-negative clones.

did not correlate with stimulation index (Fig. 6A). Moreover, there was no correlation between TCR levels measured by FACS before or after tetramer staining and the intensity of tetramer staining (data not shown). As shown with a representative clone in Fig. 6(C), re-stimulation of the T cells with 10  $\mu$ g/ml of the peptide-specific tetramer, but not an irrelevant tetramer loaded with VP16<sub>465-484</sub>, induced cell proliferation indicated by [<sup>3</sup>H]thymidine incorporation; this stimulation by soluble tetramer occurred with both tetramer high- and moderate-staining T cell clones. Among 10 clones tested, representing a range of tetramer staining, all responded to stimulation by tetramer and maximal proliferative responses were generally 10–30% higher than those seen using conventional APC (data not shown).

Thirty clones sorted on the tetramer-negative gate were also studied. To test for antigen specificity restricted to any of the self class II molecules, we stimulated cells using an autologous B cell line pulsed with the HA<sub>307-319</sub> peptide. Only one clone showed peptide-specific proliferation, while the remaining 29 were negative (Fig. 6B). Upon staining with tetramer, the clone which demonstrated antigen-specific proliferation also showed tetramer staining, while the other 29 clones did not stain with tetramer (Fig. 6F).

## Discussion

Identification of antigen-specific T cells in human peripheral blood is increasingly important with the development of epitope-specific immune monitoring and therapeutic strategies. A number of recent studies have employed class I tetramers to identify and harvest cytotoxic T cells as a means to engineer immune-based therapies for tumors and viral infections (15,17,29,30). Similar strategies identifying antigen-specific T helper cells will be needed as CD4<sup>+</sup> T cell responses are essential in mediating sustained cytotoxic T cell responses and in the pathogenesis of a number of autoimmune diseases (31–33). As early as 4 days following tetanus toxoid or HA peptide stimulation, proliferating CD4<sup>high</sup> cells are readily identified by FACS analysis. Using class II tetramer staining we have shown that the CD4<sup>high</sup> population contains almost all of the proliferating, epitope-specific T cells following stimulation with the HA<sub>307-319</sub> peptide.

An earlier study using DBA/2 mice identified the CD4<sup>high</sup> population following sperm whale myoglobin stimulation as enriched in antigen-specific cells as shown by antigen-induced proliferation and limiting dilution analysis (11). A follow-up study in the NOD mouse model for type 1 diabetes

showed that the CD4<sup>high</sup> subset in prediabetic mice was significantly more effective at transferring disease than the CD4<sup>normal</sup> subset (10). The present study identifies a similar phenomenon in human lymphocytes responding to antigen, and confirms that the CD4<sup>high</sup> cells are indeed proliferating and enriched for antigen-reactive cells.

Our comparison of tetramer staining with CD4 expression levels revealed that while almost all tetramer-positive cells are CD4<sup>high</sup>, there exists a considerable number of proliferating, activated CD4<sup>high</sup> cells which do not stain with tetramer. Single-cell sorting of tetramer-positive and -negative populations, and subsequent characterization of these clones demonstrated that tetramer staining correlates very well with antigen specificity. All clones identified as tetramer-positive showed DRB1\*0401-restricted proliferation on stimulation with HA<sub>307-319</sub> peptide. While all clones restained with tetramer, we observed a variety of staining intensities despite comparable TCR expression. We interpret this as a reflection of the range of different TCR MHC-peptide complex avidities for the different T cell clones. The intensity of tetramer staining did not correlate with the extent of proliferation, suggesting that proliferative capacity depends on other factors in addition to TCR avidity. Nonetheless, the tetramers themselves provide sufficient stimulus to induce specific proliferation in both strong and weak staining clones. Analysis of the tetramer-negative T cell population suggests that the majority of CD4<sup>high</sup>, proliferating cells which fail to stain with tetramer are indeed not antigen specific and appear to proliferate as a result of bystander activation. We have also noted that addition of IL-2 to cultures increases the fraction of tetramer-negative, proliferating cells, consistent with the mechanism of bystander activation.

Our study demonstrates that there exists variation in the levels of CD4 expression in human lymphocytes upon activation, distinct from the effects of cell size and TCR expression. In our antigen stimulated conditions, levels begin to slowly return towards normal after ~5 days and therefore elevated CD4 expression can be viewed as an intermediate-phase marker of a CD4<sup>+</sup> T cell response profile. The biological significance of elevated CD4 surface expression upon activation remains unclear. A number of studies in different contexts have shown that interaction of the CD4 molecule with the TCR-peptide-MHC complex enhances TCR signaling, possibly by prolonging the TCR-ligand contact time (34). In light of this role for CD4 as a co-receptor for T cell signaling, increased levels of CD4 during activation could serve to maintain T cell sensitivity to continued specific antigen stimulation, ensuring a strong proliferative response. The present study demonstrates that elevated CD4 expression is a distinct phenotype for the activated subset of human T cells proliferating in response to antigen stimulation. CD4<sup>high</sup>, antigen-specific cells identified by class II tetramer staining are activated, proliferating cells capable of expansion, properties which are hallmarks of the helper-inducer subset of lymphocytes important in protective immunity and autoimmune disease.

### Acknowledgements

The authors would like to thank Lori Moriarity and Sharon Kochik for expert technical assistance. This study supported in part by the

National Institutes of Health (DK53004 and DK53345). E. J. N. is supported by Poncin and Achievement Awards for College Scientists predoctoral fellowships.

### Abbreviations

APC	antigen-presenting cell
CFSE	5-(and -6)-carboxyfluorescein diacetate succinimidyl ester
HA <sub>307-319</sub>	influenza hemagglutinin peptide, residues 307-319
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PHA	phytohemagglutinin

### References

- Marrack, P., Mitchell, T., Hildeman, D., Kiedl, R., Teague, T. K., Bender, J., Rees, W., Schaefer, B. C. and Kappler, J. 2000. Genomic-scale analysis of gene expression in resting and activated T cells. *Curr. Opin. Immunol.* 12:206.
- Wulfig, C., Rabinowitz, J. D., Beeson, C., Sjaastad, M. D., McConnell, H. M. and Davis, M. M. 1997. Kinetics and extent of T cell activation as measured with the calcium signal. *J. Exp. Med.* 185:1815.
- Bot, A., Casares, S., Bot, S., von Boehmer, H. and Bona, C. 1998. Cellular mechanisms involved in protection against influenza virus infection in transgenic mice expressing a TCR receptor specific for class II hemagglutinin peptide in CD4<sup>+</sup> and CD8<sup>+</sup> T cells. *J. Immunol.* 160:4500.
- Goverman, J., Woods, A., Larson, L., Weiner, L. P., Hood, L. and Zaller, D. M. 1993. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell* 72:551.
- Kaye, J., Hsu, M. L., Sauron, M. E., Jameson, S. C., Gascoigne, N. R. and Hedrick, S. M. 1989. Selective development of CD4<sup>+</sup> T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. *Nature* 341:746.
- Sagerstrom, C. G., Kerr, E. M., Allison, J. P. and Davis, M. M. 1993. Activation and differentiation requirements of primary T cells *in vitro*. *Proc. Natl Acad. Sci. USA* 90:8987.
- Scott, B., Bluthmann, H., Teh, H. S. and von Boehmer, H. 1989. The generation of mature T cells requires interaction of the alpha beta T-cell receptor with major histocompatibility antigens. *Nature* 338:591.
- Davis, M. M., McHeyzer-Williams, M. and Chien, Y. H. 1995. T-cell receptor V-region usage and antigen specificity. The cytochrome c model system. *Ann. NY Acad. Sci.* 756:1.
- Krieger, N. R., Fathman, C. G., Shaw, M. K. and Ridgway, W. M. 2000. Identification and characterization of the antigen-specific subpopulation of alloreactive CD4<sup>+</sup> T cells *in vitro* and *in vivo*. *Transplantation* 69:605.
- Lejon, K. and Fathman, C. G. 1999. Isolation of self antigen-reactive cells from inflamed islets of nonobese diabetic mice using CD4<sup>high</sup> expression as a marker. *J. Immunol.* 163:5708.
- Ridgway, W., Fasso, M. and Fathman, C. G. 1998. Following antigen challenge, T cells up-regulate cell surface expression of CD4 *in vitro* and *in vivo*. *J. Immunol.* 161:714.
- Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J. and Davis, M. M. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94.
- Crawford, F., Kozono, H., White, J., Marrack, P. and Kappler, J. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity* 8:675.
- Novak, E. J., Liu, A. W., Nepom, G. T. and Kwok, W. W. 1999. MHC class II tetramers identify peptide-specific human CD4(+) T cells proliferating in response to influenza A antigen. *J. Clin. Invest.* 104:R63.
- Lee, P. P., Yee, C., Savage, P. A., Fong, L., Brockstedt, D., Weber, J. S., Johnson, D., Swetter, S., Thompson, J., Greenberg, P. D., Roederer, M. and Davis, M. M. 1999. Characterization of

- circulating T cells specific for tumor-associated antigens in melanoma patients. *Nat. Med.* 5:677.
- 16 Romero, P., Dunbar, P. R., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., Chen, J. L., Lienard, D., Cerottini, J. C. and Cerundolo, V. 1998. *Ex vivo* staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med.* 188:1641.
  - 17 Yee, C., Savage, P. A., Lee, P. P., Davis, M. M. and Greenberg, P. D. 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J. Immunol.* 162:2227.
  - 18 Kwok, W. W., Liu, A. W., Novak, E. J., Gebe, J. A., Ettinger, R. A., Nepom, G. T., Reymond, S. N. and Koelle, D. M. 2000. HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2-infected individuals: direct detection of immunodominant antigen-responsive cells. *J. Immunol.* 164:4244.
  - 19 Horton, R. M., Cai, Z. L., Ho, S. N. and Pease, L. R. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques* 8:528.
  - 20 Chang, H. C., Bao, Z., Yao, Y., Tse, A. G., Goyarts, E. C., Madsen, M., Kawasaki, E., Brauer, P. P., Sacchettini, J. C. and Nathenson, S. G. 1994. A general method for facilitating heterodimeric pairing between two proteins: application to expression of alpha and beta T-cell receptor extracellular segments. *Proc. Natl Acad. Sci. USA* 91:11408.
  - 21 Schatz, P. J. 1993. Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in *Escherichia coli*. *Biotechnology* 11:1138.
  - 22 Wells, A. D., Gudmundsdottir, H. and Turka, L. A. 1997. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J. Clin. Invest.* 100:3173.
  - 23 Kovats, S., Nepom, G. T., Coleman, M., Nepom, B., Kwok, W. W. and Blum, J. S. 1995. Deficient antigen-presenting cell function in multiple genetic complementation groups of type II bare lymphocyte syndrome. *J. Clin. Invest.* 96:217.
  - 24 Allsopp, C. E., Nicholls, S. J. and Langhorne, J. 1998. A flow cytometric method to assess antigen-specific proliferative responses of different subpopulations of fresh and cryopreserved human peripheral blood mononuclear cells. *J. Immunol. Methods* 214:175.
  - 25 Givan, A. L., Fisher, J. L., Waugh, M., Ernstoff, M. S. and Wallace, P. K. 1999. A flow cytometric method to estimate the precursor frequencies of cells proliferating in response to specific antigens. *J. Immunol. Methods* 230:99.
  - 26 Lyons, A. B. and Parish, C. R. 1994. Determination of lymphocyte division by flow cytometry. *J. Immunol. Methods* 171:131.
  - 27 Adams, P. W., Opremcak, E. M. and Orosz, C. G. 1991. Limiting dilution analysis of human, tetanus-reactive helper T lymphocytes. A rapid method for the enumeration of helper T lymphocytes with specificity for soluble antigens. *J. Immunol. Methods* 142:231.
  - 28 van Oers, M. H., Pinkster, J. and Zeijlemaker, W. P. 1978. Quantification of antigen-reactive cells among human T lymphocytes. *Eur. J. Immunol.* 8:477.
  - 29 Dunbar, P. R., Chen, J. L., Chao, D., Rust, N., Teisserenc, H., Ogg, G. S., Romero, P., Weynants, P. and Cerundolo, V. 1999. Cutting edge: rapid cloning of tumor-specific CTL suitable for adoptive immunotherapy of melanoma. *J. Immunol.* 162:6959.
  - 30 Gray, C. M., Lawrence, J., Schapiro, J. M., Altman, J. D., Winters, M. A., Crompton, M., Loi, M., Kundu, S. K., Davis, M. M. and Merigan, T. C. 1999. Frequency of class I HLA-restricted anti-HIV CD8<sup>+</sup> T cells in individuals receiving highly active antiretroviral therapy (HAART). *J. Immunol.* 162:1780.
  - 31 Baxevanis, C. N., Voutsas, I. F., Tsitsilonis, O. E., Gritzapis, A. D., Sotiriadou, R. and Papamichail, M. 2000. Tumor-specific CD4<sup>+</sup> T lymphocytes from cancer patients are required for optimal induction of cytotoxic T cells against the autologous tumor. *J. Immunol.* 164:3902.
  - 32 Pardoll, D. M. and Topalian, S. L. 1998. The role of CD4<sup>+</sup> T cell responses in antitumor immunity. *Curr. Opin. Immunol.* 10:588.
  - 33 Romagnani, S. 1998. T-cell subsets (T<sub>H</sub>1, T<sub>H</sub>2) and cytokines in autoimmunity. In Rose, N. R. and Mackay, I. R., eds, *The Autoimmune Diseases*, 3rd edn, p. 163. Academic Press, San Diego, CA.
  - 34 Janeway, C. A. and Bottomly, K. 1994. Signals and signs for lymphocyte responses. *Cell* 76: 275.