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Thomas J. Scriba, … , Michaela Lucas, Rodney E. Phillips


HIV-specific CD4+ T helper lymphocytes are preferred targets for infection. Although complete interruption of combination antiretroviral therapy (ART) can form part of therapeutic manipulations, there is grave concern that the resumption of viral replication might destroy, perhaps irreversibly, these T helper populations. High viremia blocks the proliferation capacity of HIV-specific helper cells. However, cytokine production assays imply that some antigen-specific effector function is retained. Despite this careful work, it remains unclear whether the return of HIV-1 replication physically destroys HIV-1–specific T helper cells in the peripheral blood. Difficulties in producing stable peptide-MHC class II complexes and the very low frequencies of antigen-specific CD4+ T cells have delayed the application of this powerful technique. Here we employ HLA class II tetramers and validate a sensitive, quantitative cell-enrichment technique to detect HIV-1 T helper cells. We studied patients with early-stage HIV infection who were given a short, fixed course of ART as part of a clinical study. We did not find significant deletion of these cells from the peripheral circulation when ART was stopped and unfettered HIV replication returned. The turnover of these virus-specific cells increased and they adopted an effector phenotype when viremia returned.

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HIV-1–specific CD4+ T lymphocyte turnover and activation increase upon viral rebound

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HIV-specific CD4+ T helper lymphocytes are preferred targets for infection. Although complete interruption of combination antiretroviral therapy (ART) can form part of therapeutic manipulations, there is grave concern that the resumption of viral replication might destroy, perhaps irreversibly, these T helper populations. High viremia blocks the proliferation capacity of HIV-specific helper cells. However, cytokine production assays imply that some antigen-specific effector function is retained. Despite this careful work, it remains unclear whether the return of HIV-1 replication physically destroys HIV-1–specific T helper cells in the peripheral blood. Difficulties in producing stable peptide-MHC class II complexes and the very low frequencies of antigen-specific CD4+ T cells have delayed the application of this powerful technique. Here we employ HLA class II tetramers and validate a sensitive, quantitative cell-enrichment technique to detect HIV-1 T helper cells. We studied patients with early-stage HIV infection who were given a short, fixed course of ART as part of a clinical study. We did not find significant deletion of these cells from the peripheral circulation when ART was stopped and unfettered HIV replication returned. The turnover of these virus-specific cells increased and they adopted an effector phenotype when viremia returned.

Introduction

CD4+ T lymphocytes are critical for immunological control of many viral infections, including HIV-1. Early studies showed that HIV-specific CD4+ responses, as measured by proliferation assays, are meager or absent in most chronically HIV-infected patients (1, 2). Since CD4+ T cells are the principal target of HIV, and HIV-specific CD4+ cells are preferentially infected (3), this T helper population may be particularly vulnerable to destruction. There is grave concern that high HIV replication, such as the rebound viremia seen when combination antiretroviral therapy (ART) is stopped, might destroy HIV-specific CD4+ T cell populations permanently (4, 5). Several reports have shown substantial HIV-specific proliferative responses in patients who receive effective ART (6–8) and in long-term nonprogressors (2, 9). Also, sensitive cytokine (IFN-γ) assays show that HIV-specific CD4+ cells persist in most chronically infected patients (2, 10–12). Although HIV-specific proliferative responses are associated with viral control (1, 13), there are reported discrepancies between the numbers of HIV-specific IFN-γ–producing cells and proliferative capacity (2, 11). Furthermore, loss of proliferative capacity was shown to be associated with failure of HIV-specific IL-2 production (14–16). It has been hypothesized that this loss in proliferative capacity and IL-2 production is due to CD4+ T cell dysfunction and not to the physical loss of these T cells.

Memory CD4+ T cells have been subdivided into central memory T cells (Tcm) and effector memory T cells (Tem) based on the expression of CCR7 and CD45RA. CD4+ Tem have been shown to produce predominantly IL-2 and have a high proliferative capacity, whereas Tcm produce mainly IFN-γ and have limited proliferative capacity (17). In agreement with this, a skewing of the HIV-specific CD4+ memory population from Tcm (CD45RA–CCR7+CD62L+) towards Tem (CD45RA+CCR7−CD62L−) in HIV-infected viremic individuals has been demonstrated (15, 16, 18, 19). These dysfunctional CD4+ cells may compromise CD8+ CTL activity and so erode immunity to HIV.

The study of antigen-specific CD4+ T cell function and phenotype is limited by requirements for in vitro stimulation before assaying cytokines or detecting proliferation. Although these methods have advanced our understanding of antigen-specific CD4+ T cells, it remains unknown whether or not there exist antigen-specific CD4+ T cell populations that do not produce the cytokines required for detection. These methods may bias analyses towards a limited subset of cytokine-producing cells. Also, in vitro stimulation is dependent on the activation of cells which results in altered expression of surface markers. For instance, upon antigenic stimulation, Tem transiently express CCR7 (20) and expression of CD62L on T cells in mice is rapidly lost (21). T cells can be directly visualized without antigen stimulation by staining them with fluorescently tagged MHC molecules folded around antigenic peptides. Peptide-MHC class II tetramers have greatly aided the study of antigen-specific CD8+ cells. Although the generation of MHC class II tetramers has been more difficult, more sensitive methods for detecting very low cell frequencies have identified virus-specific CD4+ cells directly ex vivo (22, 23).

In this study, we employed MHC class II tetramer complexes to enumerate and characterize Gag p24–specific CD4+ T cell populations ex vivo in HIV-infected individuals bearing HLA DRB1*0101.

Nonstandard abbreviations used: ART, antiretroviral therapy; Tcm, central memory T cells; Tem, effector memory T cells.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Results

Direct ex vivo detection of HIV Gag p24-specific CD4+ T cells by HLA class II tetramer staining. We stained HIV-specific CD4+ T cells directly ex vivo with HLA class II tetramers. For confident detection of HLA class I–restricted cell populations in PBMCs, the lower limit is 0.02% of CD8+ cells (24, 25). This limit, which has been reported to also apply to HLA class II tetramer staining (26), is not sensitive enough for direct detection of most tetramer-positive CD4+ T cells in peripheral blood. To improve detection sensitivity, we employed a magnetic bead enrichment step (22, 23), which revealed tetramer-positive cells in 13 of the 14 patients tested (Figure 1A).

To ensure that these tetramers were specific for T cells capable of recognizing HIV-1 p24 peptides bound to DRB1*0101, we stained fresh and cryopreserved PBMCs from healthy DRB1*0101 and HIV-infected non-DRB1*0101 individuals. No tetramer-positive cells were detected with either HIV-1-specific tetramer in 3 individuals who acted as controls (Figure 1B). The p24.17-DR1 and p24.14-DR1 tetramers showed no staining in HIV-negative, HCV-infected DRB1*0101 patients (A. Ulsenheimer et al., unpublished observations). A DRB1*0101 tetramer complexed to an irrelevant HCV peptide was also employed as a control. No HCV tetramer staining was found in 3 HIV-infected, HCV-negative individuals bearing DRB1*0101 (Figure 1B), confirming that tetramer specificity was derived from both the peptide antigen and the HLA class II molecule.

Validation of quantitative linear recovery of tetramer-positive cell enrichment. To validate the reproducibility and linear recovery efficiency of the magnetic bead enrichment technique employed in this study, we spiked PBMCs from an HIV-negative donor with a p24.17 epitope-specific CD4+ T cell clone as described in Methods. When magnetic bead enrichment of tetramer-positive cells was used to quantify the number of tetramer-positive cells in each sample, a highly significant linear recovery was obtained ($r^2 = 0.9887$, $P < 0.0001$; Figure 2 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI200523084DS1). Further quantitative validation was achieved by tetramer staining and enrichment of multiple replicates from individual patients in parallel (data not shown). This analysis confirmed that tetramer-positive CD4+ output frequencies were reproducible (SEM was consistently less than 11% of the mean response). Lucas et al. (23), who detected influenza virus hemagglutinin–specific CD4+ T cells directly ex vivo, had similar reproducibility.

In this study, we detected p24.17-DR1 and p24.14-DR1 tetramer-positive cells by direct ex vivo analysis at frequencies...
up to 0.14% and 0.008% of CD4+ T cells, respectively. To determine whether HIV-specific CD4+ T cells in PBMC samples from 6 patients with detectable p24.17-DR1 tetramer-positive cells were functional, we assayed for IFN-γ and IL-2 production using a magnetic bead enrichment–based cytokine-secretion technique. Upon p24.17 Gag peptide stimulation, IFN-γ production by CD4+ T cells was detected in all 6 patients tested, while IL-2 was detected in 3 patients (see Supplemental Figure 2). The number of cytokine-producing CD4+ T cells was found to be lower than that of tetramer-positive cells in all 6 patients.

Tetramer-positive CD4+ T cells survive when ART is stopped and viremia returns. HIV-specific CD4+ T cells lose their ability to proliferate and to produce IL-2 rapidly after primary HIV infection in most untreated viremic individuals (1, 2, 11, 14–16). The capacity for IFN-γ production persists, as judged by intracellular cytokine staining and ELISpot assay (1, 2, 10–12, 14–16). To determine whether cessation of ART and the resultant viral rebound destroys CD4+ T cells specific for the p24.17 epitope, we studied 8 DRB1*0101-positive patients who had a short fixed procedure was 188 weeks. All 8 patients had undetectable plasma viral loads during ART (Figure 3A). No significant difference in viral rebound that followed therapy cessation, and when the plateau after the initial viral rebound (viral setpoint) had been reached (Figure 3). The median expression of CD38 was found to be CD45RA–, CD27+, and CD28+, as well as almost exclusively CCR7+ and CD57+ (Figure 4B). An intermediate expression pattern was seen for L-selectin (CD62L). CCR7 expression on tetramer-positive cells in viremic patients was also compared to the expression levels in the naive (CD45RA+) and memory (CD45RA–) CD4+ T cell subsets (Figure 4C). This analysis reveals significantly lower surface expression of CCR7 on HIV-specific tetramer-positive CD4+ cells (median 12.5%) compared to the memory CD4+ T cell population (median 51.8%). Both HIV-specific tetramer-positive CD4+ cells and total memory CD4+ cells showed significantly less CCR7 expression than naive CD4+ T cells (median 95.1%).

The median expression of CD38 was found to be approximately 47% on tetramer-positive cells, suggesting a high level of T cell activation. When the level of CD38 expression was compared between HIV-specific tetramer-positive cells and the total memory (CD45RA–) CD4+ cell population, we found a significantly
The lower level of CD38 expression on the latter (median 23.7%; \(P = 0.011\); Figure 4D). This significance was upheld when the CD38 mean fluorescence index between the same populations was compared (data not shown).

**Elevated turnover of HIV-specific CD4+ T cells during viremia.** HIV-specific CD4+ T cells are preferentially infected (3), and they are probably destroyed as a result of the infection. However, we did not find a significant decrease in the numbers of HIV-specific tetramer-positive CD4+ T cell clones after viral rebound. A possible explanation for the maintenance of virus-specific helper cells is an increased turnover of these cells to compensate for the loss incurred during high viremia. Indeed, elevated CD4+ and CD8+ cell turnover, as measured by intracellular Ki67 staining, BrdU incorporation, and/or \(^{3}H\)glucose in viremic HIV infections, has been reported (28–33). To test whether HIV-specific CD4+ T cells display elevated turnover rates, we measured intracellular Ki67 expression on tetramer-positive CD4+ T cells. In 10 viremic patients, we found significantly higher Ki67 expression on tetramer-positive CD4+ T cells than on memory (CD45RA+) and naive (CD45RA–) CD4+ T cells (Figure 5, A and B). In addition, a significant direct correlation was found between the frequency of Ki67+ tetramer-positive CD4+ cells and plasma viral load (\(r^2 = 0.358, P = 0.024\); Figure 5C).

**Correlation between plasma viral load and CCR7 expression on p24-specific tetramer-positive CD4+ T cells.** HIV-specific CD4+ T cells have been shown to possess proliferative capacity in patients with very low viral replication and those who are ART suppressed (2, 6–9). During HIV viremia, antigen-specific proliferation (1, 2) and IL-2 production (14–16) are lost. To further determine the effects of HIV viremia, we quantified CCR7 on tetramer-positive cells. Tetramer-positive CD4+ T cells in patients receiving ART expressed significantly higher levels of CCR7 than those in viremic patients (\(P = 0.0021\); Figure 6, A and B). Furthermore, a significant inverse correlation was found between the percentage of CCR7-expressing tetramer-positive cells and plasma viral load (\(r^2 = 0.5033, P = 0.0021\); Figure 6C).

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**Figure 4**
Phenotypic analysis of HIV-specific memory CD4+ T cells using HLA class II tetramers. (A) Representative phenotypic staining with anti-CD27, anti-CD28, or anti-CD62L antibodies from subject Ox20. FACS plots are gated on CD4+ T lymphocytes, and the percentage of tetramer+ CD4+ T cells expressing the respective phenotypic marker is indicated in the upper right quadrant of each plot. (B) Phenotypic profile of HIV-specific CD4+ T cells in 9 viremic individuals. Each bar represents the mean percentage of tetramer+ CD4+ cells that express the indicated surface marker. Error bars represent SEM. (C) CCR7 expression on CD4+ T cell subsets in viremic HIV infection. CD45RA+ and CD45RA– CD4+ cells were gated on to distinguish between the naive and memory subsets, respectively. (D) The HIV–1-specific, tetramer+ CD4+ T cell subset was significantly more activated than the memory CD4+ T cell population. CD38 expression was measured in 9 viremic patients. Cells that fell into the CD45RA–CD4+ or tetramer+ CD4+ gates were analyzed. The means are represented by horizontal lines and the statistical differences between them were calculated with a paired Student’s \(t\) test.

**Figure 5**
Expression of the intracellular proliferation marker Ki67. (A) Representative costaining of CD4+ T cells with the p24.17-DR1 tetramer and Ki67 in 2 viremic patients. The percentage of tetramer+ cells expressing Ki67 is indicated in the upper-right quadrant. (B) Ki67 expression in CD4+ T cell subsets in viremic infection. CD45RA+ and CD45RA– CD4+ cells were gated on to distinguish between the naive and memory subsets, respectively. The means are represented by horizontal lines and the statistical differences between them were calculated with a paired Student’s \(t\) test. (C) Correlation between plasma viral load and Ki67 expression in HIV-specific tetramer+ CD4+ cells.
Tetramer-positive CD4+ cells in untreated chronically infected patients resemble those in patients treated acutely. To investigate whether these findings can be extended to chronically infected patients, we studied 2 ART naive DRB1*0101 patients (Figure 7). Longitudinal staining of PBMCs with p24.17-DR1 tetramer showed detectable frequencies of helper T cell populations up to 63 and 85 weeks following the onset of seroconversion symptoms (Figure 7A). In these treatment-naive patients, cell phenotypes were very similar to those found following viral rebound (Figure 7).

Discussion

While HLA class I tetramers have revolutionized the study of antigen-specific CD8+ T cell frequencies and phenotypes, application of the same technology to the study of CD4+ T cells has been more difficult. Initial efforts to make stable HLA class II peptide–MHC complexes were frustrating and the low frequencies of antigen-specific CD4+ cells in peripheral blood were not readily detectable by direct ex vivo staining (34, 35). In this study we used a magnetic bead enrichment technique to enhance the sensitivity of HLA class II tetramer staining. We consistently detected HIV Gag p24-specific CD4+ T cells in HIV-infected individuals at frequencies below the detection limit of direct flow cytometric analysis (0.02%) (24, 25). We demonstrated a linear recovery efficiency of the tetramer-enrichment technique. Quantitative analysis using HLA-class II tetramer staining and magnetic bead enrichment to detect influenza HA-specific CD4+ T cells was also highly reproducible (23).

Because HIV-specific CD4+ T cells are preferentially infected (3), there is concern that high levels of HIV replication, such as the rebound viremia seen when ART is stopped, might destroy HIV-specific helper T cells (4, 5). In resource-poor settings short-course ART has been recommended by the World Health Organization for the prevention of mother-to-child transmission. Unscheduled cessation of ART is also likely to occur where medical supervision is limited or absent. Using HLA class II tetramer staining we directly measured HIV-specific T helper cell numbers in patients while on ART, during and after ART cessation. Our analyses showed no significant destruction of HIV-specific CD4+ T cell clones when HIV viremia rebounded. This finding was confirmed by IFN-γ ELISpot analysis of CD8-depleted PBMCs. These data correspond with previous studies that quantified HIV-specific IFN-γ CD4+ T cell numbers and demonstrated increased responses in patients during viremia compared to ART treatment (14, 15, 36). Our analyses did not rule out the possibility that viral recrudescence could cause a redistribution of lymphocytes.

Figure 7

HIV-specific tetramer+ CD4+ cells in untreated chronic patients resemble those in viremic patients who received short-course ART at acute infection. (A) Longitudinal detection of tetramer+ CD4+ cells up to 63 weeks and 85 weeks after the first experience of seroconversion symptoms in patients Ox22 and Ox79, respectively. The frequency of tetramer+ CD4+ cells for each time point is indicated in the upper-right quadrant of each plot. (B) Phenotypic analysis of tetramer+ CD4+ cells from patient Ox79. These analyses were performed at time points 52 weeks or more after the first experience of seroconversion symptoms. The percentage of tetramer+ CD4+ cells expressing the relevant marker is indicated in parentheses in each plot.
HIV-specific proliferative responses significantly decrease in viremic patients (2, 11) along with virus-specific CD4+ responses as measured by IL-2 production (14–16). Our phenotypic analysis of HIV-specific tetramer-positive CD4+ cell clones reveals a dominant phenotype of CD45RA–CCR7+ representing effector cells and an inverse correlation between CCR7 expression and plasma viral load. Low CCR7 expression was also demonstrated on MHC class II tetramer-positive CD4+ cells during acute HCV infection (A. Ulsenheimer et al., manuscript submitted for publication).

These profiles contrast with those found on tetramer-positive CD4+ T cell specific for influenza or hepatitis C virus after infection has resolved; these CD4+ T helper cells display central memory phenotypes (CD45RA–CCR7+CD62L+) (22, 23). Our data suggest an “antigen dose”–dependent reduction in CCR7 expression on HIV-specific CD4+ T cells and are consistent with a recent study in which an inverse correlation was found between the percentage of CD45RA–CCR7+ Gag-specific CD4+ T cells as detected by intracellular cytokine staining (ICS) and plasma viral load (16). These data are consistent with analyses which relied on antigen-induced cytokine production in HIV-specific CD4+ T cells. In viremic patients only a small percentage of these cells were able to produce IL-2, while most produced IFN-γ (14–16, 18), a cytokine profile typical of effector cells (17). Harari et al. (19) and Younes et al. (15) also found low CCR7 expression on HIV-specific CD4+ T cells using an ICS assay. IFN-γ–specific HIV-specific CD4+ cells are increased in patients with viremia compared to treated HIV infection (14–16, 18). This may be the result of an increased capacity of effector cells to produce IFN-γ rather than an increase in overall cell numbers. These and our results therefore fit into the current paradigm of central and effector phenotypes and their respective functional capacities as described by Sallusto et al. (17). Assays based on IFN-γ detection may not be as useful for monitoring important immune responses as those measuring antigen-specific CD8+ and CD4+ lymphoproliferation and, in the case of CD4+ cells, IL-2 production.

Elevated turnover of total or memory CD4+ T cell populations in viremic HIV infection as measured by intracellular Ki67 staining, BrdU incorporation, and/or [3H]glucose has been described (29–33). In addition, a direct correlation between plasma viral load and Ki67 expression in the total CD4+ T cell pool was found (32). To determine whether high viral replication induces elevated turnover of HIV-specific CD4+ T cell populations, we measured intracellular Ki67 expression in the tetramer-positive cell population. HIV Gag p24-specific CD4+ cell clones had higher Ki67 expression than the memory CD4+ population. Ki67 expression in virus-specific tetramer-positive CD4+ cells also correlated directly with plasma viral load. This increased cell turnover may explain the maintenance of HIV-specific CD4+ cell numbers in the face of high viral replication such as rebound viremia.

This viral load-dependent effect on T cell differentiation and turnover is likely to be the result of antigenic stimulation when HIV replication returns. The high level of activation (Figure 4, B and D; CD38 expression) seen in the HIV-specific CD4+ T cell compartment supports this. These data are compatible with the finding that CD4+ and CD8+ activation as measured by CD38 expression are correlated directly with viral load and predictive of changes in total CD4 counts (37).

Although viral recrudescence does not significantly alter the quantity of HIV-1–specific helper T cell populations in peripheral blood, function may be compromised and may contribute to the impaired immunity found in untreated HIV infection. When HIV rebounds, HIV-specific CD4+ T cells adopt the same phenotype as those found in untreated chronic HIV infection. Control of viral replication is dependent on functional HIV-specific CD4+ T cells which can proliferate and produce IL-2 (1, 2), qualities attributed to CD45RA–CCR7+ cells. HIV-specific CD4+CCR7+ cells may be preserved by short-course ART for acute HIV infection. Postponement of the HIV-specific CD4+ T lymphocyte “functional switch” by a course of ART may translate as better long-term viral control. Our study supports evaluation of early treatment. A recent study showed that HIV-specific CD8+ proliferative responses returned in progressive infection when proliferative HIV-specific CD4+ helper cell responses were restored by therapeutic vaccination in patients receiving ART (38). Only controlled trials can determine if these interventions produce durable improvements in HIV-1 control.

In conclusion, we detected HIV-specific CD4+ T cells directly ex vivo with HLA class II tetramers in patients on ART and after these drugs were stopped. This avoided a reliance on detection systems based on cytokine production, which require antigen stimulation and therefore might alter the phenotype and/or proliferation marker expression of these cells artefactually (20, 21, 39). HIV-1–specific CD4+ T cell populations survive viral rebound physically intact, show increased activation and turnover, and acquire an effector phenotype wholly consistent with the return of antigen drive characteristic of untreated HIV-1 infection.

Methods

Study population. We recruited 105 patients with acute HIV infection at St. Mary’s Hospital, London. All but 16 patients received a short pulse of ART, after which therapy was stopped completely. These therapeutic interventions during acute infection have been demonstrated to preserve virus-specific CD4+ and CD8+ immune responses (13, 27, 38, 40, 41) and have been advocated for clinical use. Resumption of therapy constituted a study endpoint for that patient (27). From this open study we investigated 14 patients who expressed HLA-DRB1*0101 (Table 1). Two of these patients opted to remain untreated, while 12 received ART for a period of 2 to 6 months. Informed consent was obtained from all participants and the study was given appropriate ethical approval by the Local Research Ethics Committee at St. Mary’s Hospital Trust (99/IA/161E).

Table 1

<table>
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<th>ART duration (mo)</th>
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HLA typing and viral load determination. High-resolution HLA class I and II genotypes were determined for each patient by PCR using sequence-specific primers (42). Plasma viral load was quantified using the Ampli- cor RT-PCR kit (Roche Diagnostic Systems). The lower limit of detection was 50 HIV RNA copies/ml.

Cell lines and clones. Fresh PBMCs were depleted of CD8+ cells using anti- CD8 conjugated Dynabeads (Dynal UK Ltd.) and stimulated with 10 μg/ml peptide in medium containing 10% human AB+ serum, 0.4 mM Indinavir (Merck) and 0.5 μM Zidovudine (GlaxoSmithKline). The plates were incubated at 37°C in 5% CO₂ and after 3 days the medium was supplemented with 100 U/ml IL-2 (Proleukin) and 5% human T cell culture supplement (TSTIM; BD). Medium exchanges were done as necessary and after 12–14 days, cell lines were restimulated with 10 μg/ml peptide, IL-2, TSTIM, and irradiated autologous PBMCs. For generation of CD4+ T cell clones, cell lines were stimulated with peptide and cells producing IFN-γ and IL-2, which were selected using a cytotoxicity detection and enrichment method (Milenyi Biotech). IFN-γ- and IL-2–specific CD4+ cells were plated at limiting dilution in medium containing 10% human AB+ serum, 2 × 10⁵ irradiated mixed lymphocytes, 100 U/ml IL-2, 5% TSTIM, and 1 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich).

IFN-γ ELISpot assays for detection of CD4+ responses. Direct ex vivo IFN-γ ELISpot analysis was performed as previously described (27). Briefly, fresh PBMCs were depleted of CD8+ T cells using anti-CD8 conjugated Dynabeads. The efficiency of the CD8 depletion was tested routinely by FACS staining and less than 0.5% of CD8+ T cells were consistently detected (data not shown). Cells were stimulated in duplicate wells with recombinant proteins (p24, gp120, and p66 at 10 μg/ml; National Institute for Biological Standards and Controls [NIBSC]) and overlapping pooled peptides (20-mers overlapping by 10; Tat and Nef at 5 μg/ml). The HIV-unrelated recall antigens streptokinase and streptodornase (200 U/ml; Lederle), CMV lysate (10 μg/ml; Virusys), and PHA were used as positive controls. Nonrecombinant baculovirus and medium were used as negative controls. Spot quantification was automated and standardized with an ELISpot plate reader (Autoimmun Diagnostika).

Tetramer and antibody staining. The MHC class II tetramers used in this study were purchased from Beckman Coulter. The 2 HIV-specific tetramers were HLA-DRB1*0101 alleles complexed to the peptides p24.17 (AA 294–313, FRDYYDRFYKTLRAEQASQD; ref. 14) and p24.14 (AA 264–283, KRWILGLNKIVRMYSPTSI; ref. 1). A DRB1*0101 class II tetramer complexed to an HCV peptide was used as peptide control tetramer (kindly donated by A. Oxenius, University of Munich, Munich, Germany). Fresh or cryopreserved PBMCs were stained for 2 hours at room temperature with PE-conjugated MHC class II tetramer. Antibodies and Via-Probe (all from BD, CCR7-FITC from Rd&D Systems) were added during the last 30 minutes of incubation. The cells were washed and labeled with anti-PE microbeads (Milenyi Biotech) and 10% of the cells were reserved for FACS analysis, while 90% were subject to magnetic bead enrichment of PE-conjugated tetramer-positive cells using MACS columns (Milenyi Biotech).

For intracellular staining, cells were permeabilized using BD FixPerm (BD) following magnetic bead staining, stained with Ki67-FITC (Novo- castra) and then applied to the MACS columns. Negative CD25 staining on tetramer-positive cells confirmed that the 2-hour tetramer staining did not activate the CD4+ T cells. Cells were acquired on a FACScalibur flow cytometer (BD), gated on CD14+, CD19+, and Via-Probe-negative lymphocytes, and analyzed using CELLQuest software (BD).

Calculation of tetramer-positive T cell frequencies and validation of the method. Frequencies of tetramer-positive CD4+ T cells were determined as described previously (23). Briefly, the input number of CD4+ T cells in the enriched sample was calculated by multiplying the number of CD4+ cells in the pre-enrichment sample (10% of total) by a factor of 9. The frequency of tetramer-positive cells was thus calculated by dividing the post-enrichment output number of tetramer-positive cells by the calculated input number of CD4+ cells. To determine whether this technique gives a reproducibly linear enrichment recovery, we spiked HIV-negative PBMCs with a CD4+ T cell clone (Ox97 clone 10) specific for the DRB1*0101-restricted p24.17 epitope at frequencies ranging from 0.001% to 0.5%. These samples were then subjected to HLA class II tetramer staining and magnetic bead enrichment as done for ex vivo analysis.

IFN-γ and IL-2 secretion assays. IFN-γ and IL-2 production were determined with the IFN-γ and IL-2 Cell Enrichment and Detection Kit (Milenyi Bio- tech). We stimulated 2–10×10⁵ PBMCs with 5 μg/ml of peptide for 6 hours. Detection and enrichment of cytochrome-positive cells was performed according to the manufacturer’s protocol.

Statistical analysis. The paired Student’s t test and Mann-Whitney U test were performed using Prism version 3.0a for Macintosh (GraphPad). Differences were considered statistically significant when the P value was less than or equal to 0.05.

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