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Central Memory CD8+ T Cells Appear to Have a Shorter Lifespan and Reduced Abundance as a Function of HIV Disease Progression

Kristin Ladell,² Marc K. Hellerstein,‡ Denise Cesar,‡ Robert Busch,§ Drina Boban,† and Joseph M. McCune⁴*

Progressive HIV disease has been associated with loss of memory T cell responses to Ag. To better characterize and quantify long-lived memory T cells in vivo, we have refined an in vivo labeling technique to study the kinetics of phenotypically distinct, low-frequency CD8+ T cell subpopulations in humans. HIV-negative subjects and antiretroviral-untreated HIV-infected subjects in varying stages of HIV disease were studied. After labeling the DNA of dividing cells with deuterated water (²H₂O), ²H-label incorporation and die-away kinetics were quantified using a highly sensitive FACS/mass spectrometric method. Two different populations of long-lived memory CD8+ T cells were identified in HIV-negative subjects: CD8⁺CD45RA⁻CCR7⁺CD28⁺ central memory (T_CM) cells expressing IL-7Rα and CD8⁺CD45RA⁺CCR7⁻CD28⁺ effector memory (T_EMRA) cells expressing CD57. In pilot studies in HIV-infected subjects, T_CM cells appeared to have a shorter half-life and reduced abundance, particularly in those with high viral loads; T_EMRA cells, by contrast, retained a long half-life and accumulated in the face of progressive HIV disease. These data are consistent with the hypothesis that IL-7Rα⁺ T_CM cells represent true memory CD8+ T cells, the loss of which may be responsible in part for the progressive loss of T cell memory function during progressive HIV infection. The Journal of Immunology, 2008, 180: 7907–7918.
cell surface phenotype (19). Memory T cells were found to exhibit high proliferative capacity, whereas effector T cells demonstrated strong cytotoxic potential. More recently, based on the presence or absence of expression of the chemokine receptor, CCR7, the memory compartment has been subdivided into central memory and effector memory subpopulations (20). Since then, additional cell surface markers have been used to distinguish various T cell subpopulations, but different studies have been nonuniform in the use of these markers (21, 22). The general consensus is that long-lived memory CD8 T cells reside within a CD3+ CD8+ CD45RA– CD28+ CCR7+ central memory T (T CM)3 population that is endowed with high proliferative capacity, a broad TCR repertoire, and expression of IL-7Rα (19, 23). Another subpopulation of CD8+ T cells that may be long-lived in vivo (24) is the CD3+ CD8+ CD45RA– CD28+ CCR7+ RA effector memory (T EMRA) subpopulation. A very high fraction of these cells expresses CD57, a marker associated with senescent T cells (25, 26), and it is not clear whether they possess substantial memory function in vivo.

The fate of these long-lived CD8+ T cell subpopulations is even less well studied in the context of HIV-infected individuals. Usually, HIV disease progression is associated with a decrease in the absolute number of circulating naive CD8+ T cells (T N) and an increase in the number of circulating memory/effector CD8+ T cells. Labeling studies with deuterium-labeled glucose or water (2H2O) have shown that the latter compartment can be subdivided into two kinetically distinct subpopulations, one of which is short-lived (presumably containing effector memory, or T EM, CD8+ T cells) and the other longer lived (presumably containing the T CM and/or T EMRA cells). In HIV-infected individuals classified as long-term nonprogressors, cells with a T CM-like phenotype are maintained in the circulation (27). Ag-specific CD8+ T EMRA cells are also more frequent in those who control HIV replication after acute infection than in those who develop progressive disease (28). Analytical limitations (e.g., cell numbers below detection limits and low isotope enrichments), however, have made it difficult to directly measure the lifespan of these two cell subpopulations. Accordingly, it is not clear whether their lifespan or relative abundance changes as a function of HIV disease progression.

In this study, we have refined the stable isotope-FACS-mass spectrometric (MS) method for the analysis of T cell turnover in vivo so that it can be used to simultaneously measure the in vivo kinetics and lifespan of multiple subpopulations of CD8+ T cells, including, for the first time, those that are relatively rare. A long-term (7-wk) 2H2O labeling protocol was used, allowing sufficient time for 2H to be incorporated into the deoxyribose moiety of DNA in slowly dividing cells, including various CD8+ T cell populations. These were thereafter sorted purified according to their expression of CD45RA, CCR7, and CD28 (21, 29–32). Label incorporation and die-away kinetics into each of these subpopulations were then quantified by MS analysis (33–37), taking advantage of recent advances in the sensitivity of these techniques for measurement of label in low cell numbers. In addition to evaluating the applicability of the refined stable isotope/FACS-MS to the kinetic analysis of low-abundance T cell subpopulations in vivo, our aim was to provide information about the following questions: 1) what is the phenotype and the lifespan of long-lived memory CD8+ T cells in healthy HIV-negative subjects; and 2) does this phenotype and/or lifespan change in the context of progressive HIV disease? Our results demonstrate that the turnover of low-abundance T cell subpopulations can be quantified using the refined stable isotope-FACS-MS method. Moreover, data obtained by this method suggest that both CD8+ T CM and T EMRA are long-lived in vivo in humans, but that T CM cells are lost and T EMRA cells accumulate as HIV disease progresses.

Materials and Methods

Human subjects

Subjects were recruited by advertisement. Written informed consent was obtained from all subjects, and protocols were approved by the University of California Committee on Human Research. Eight HIV-negative and five antiretroviral-uninfected HIV-infected subjects were included in the in vivo stable isotope labeling study. Except for one subject, the labeled HIV-infected subjects were naïve to antiretroviral treatment. All of the labeled subjects were male. Additional immunophenotyping was conducted on 4 of the labeled and 2 additional HIV-negative subjects, all 5 of the labeled HIV-infected subjects, 2 more HIV-negative subjects (1 female and 1 male), and 15 more chronically infected, antiretroviral-treated HIV-infected subjects (13 male and 2 transgender, one of the latter known to be treated with feminizing sex hormones) from the SCOPE cohort (an ongoing prospective cohort study aimed at investigating the long-term clinical and immunological consequences associated with HIV infection and treatment of HIV infection; Ref. 38). Subjects were excluded if, at any time within the 3-mo period before enrollment, they had either used a medication (e.g., glucocorticoids or other immunosuppressive drugs) or been diagnosed with a disease (e.g., lymphoproliferative diseases or cancer) that might affect T cell turnover. All the labeled subjects came in for a screening visit at which time they filled out a questionnaire and blood was drawn to determine their eligibility to participate in the study. The characteristics of those subjects who were studied are shown in Table I. Changes in the relative proportion of CD8+ T cell subpopulations were determined by obtaining a complete blood count and a T cell phenotyping flow cytometric panel (see Materials and Methods, “Preparation of whole blood samples for sorting” and Fig. 1B) at each sort date. Both CD8+ T cell counts and the relative composition of CD8+ T cell subpopulations were stable for individual subjects during the time period of study.

Labeling protocol

All subjects enrolled for labeling were seen at the Clinical and Translational Science Institute Clinical Research Center at San Francisco General Hospital. After enrollment, they were provided with a sufficient number of 50-ml vials of 70% 2H2O (Cambridge Isotope Laboratories) for a 7-wk labeling protocol. During the first week of labeling, subjects ingested prospective cohort study aimed at investigating the long-term clinical and immunological consequences associated with HIV infection and treatment of HIV infection; Ref. 38). Subjects were excluded if, at any time within the 3-mo period before enrollment, they had either used a medication (e.g., glucocorticoids or other immunosuppressive drugs) or been diagnosed with a disease (e.g., lymphoproliferative diseases or cancer) that might affect T cell turnover. All the labeled subjects came in for a screening visit at which time they filled out a questionnaire and blood was drawn to determine their eligibility to participate in the study. The characteristics of those subjects who were studied are shown in Table I. Changes in the relative proportion of CD8+ T cell subpopulations were discerned by obtaining a complete blood count and a T cell phenotyping flow cytometric panel (see Materials and Methods, “Preparation of whole blood samples for sorting” and Fig. 1B) at each sort date. Both CD8+ T cell counts and the relative composition of CD8+ T cell subpopulations were stable for individual subjects during the time period of study.

Measurement of T cell DNA labeling

The stable isotope/FACS-MS method for measuring T cell turnover has been described in detail previously (33, 36, 39). This method was refined so that smaller numbers of cells could be analyzed while maintaining accuracy for isotope enrichment (40), enabling the simultaneous kinetic characterization of multiple subpopulations of CD8+ T cells, including rare phenotypic subsets. Sorted cell pellets were resuspended in 200 ml of PBS, boiled for 10 min to release DNA from chromatin, and rapidly chilled. For DNA hydrolysis, 50 ml of 5× concentrated hydrolysis mixture was added, containing sodium acetate buffer (pH 5), zine sulfate, nuclease S1, and acid phosphatase (41), and the mixture was incubated at 37°C for 1–16 h. Samples were transferred to screw-cap glass hydrolysis tubes. Aqueous O-(2,3,4,5,6-pentafluorobenzoyl)hydroxylamine hydrochloride solution (1 mg/ml, 100 μl) was added, followed by 75 μl of glacial acetic acid, and the mixtures incubated at 100°C for 30 min. After cooling to room temperature, 50 μl of acetic anhydride was added to each sample, followed by 50 μl of N-methylimidazole with rapid mixing. The reaction was allowed to proceed for 15–20 min. After cooling, 2 ml of water were added, and the reactions were extracted twice with 750 μl of dichloromethane. The
organic layers were pooled, dried over sodium sulfate, evaporated to dryness, reconstituted in 50 ml of ethyl acetate, and transferred to gas chromatography (GC) vials for analysis.

In addition to controls used routinely in the stable isotope-FACS-MS method (33, 36, 39), additional sets of blanks and standards were included in each run. DNA standards were appropriately matched to the range of cell counts in the experimental samples, assuming −6 pg of DNA per human diploid nucleus. Labeled cell samples and DNA standards of known 2H enrichment were diluted and run with each preparation to verify the stability of measured enrichments at low sample abundance, and reagent blanks were used to assess contamination by extraneous deoxyribose or DNA. Before analyzing samples with low cell counts, the entire procedure was checked several times, using only DNA standards, blanks, and titrated amounts of cells from an abundant source with known 2H enrichment.

GC-MS analysis was performed using an Agilent model 5973 MS with a 6890 GC and an autosampler in negative chemical ionization mode with methane as reagent gas. Samples were resolved on a 30-m DB-17 column in each run. DNA standards were approximately matched to the range of cell counts in the experimental samples, assuming 100% 2H2O with natural abundance H2O in known proportions.

In this pulse-chase experiment, the loss of label enrichment in cellular DNA at S1 (wk 10, or 3 wk after the last dose of 2H2O), representing the baseline value (or pulse) after washout of 2H2O from body water pools, was used as the time zero value. This time point was chosen because body water 2H2O enrichments fall to low levels (i.e., with a half-life of ~7 days, data not shown), allowing subsequent die-away kinetics to be assessed without the confounding effects of continued label incorporation. The subsequent loss of label from cellular DNA of each subset was quantified between S1 and S3 (wk 18). The decay constant (k) was calculated using the equation for exponential decay: k = [ln(S3/S1)]/Dt, where S3 and S1 represent 2H enrichments measured at wk 18 and 10, respectively, and Dt is the time between measurements (8 wk). The half-life was calculated as t1/2 = ln(2)/k. For some T cell subpopulations in some individuals (e.g., TCM cells in subjects who were HIV-negative), exponential label decay was not observed between S1 and S3 or S2 and S3 (e.g., TCM cells in three of four HIV-negative subjects). Therefore, we also calculated the percentage of initially incorporated label (i.e., the 2He n-2H enrichment in cellular DNA at S1 (wk 10, or 3 wk after the last dose of 2H2O), representing the baseline value (or pulse) after washout of 2H2O from body water pools, was used as the time zero value. This time point was chosen because body water 2H2O enrichments fall to low levels (i.e., with a half-life of ~7 days, data not shown), allowing subsequent die-away kinetics to be assessed without the confounding effects of continued label incorporation. The subsequent loss of label from cellular DNA of each subset was quantified between S1 and S3 (wk 18). The decay constant (k) was calculated using the equation for exponential decay: k = [ln(S3/S1)]/Dt, where S3 and S1 represent 2H enrichments measured at wk 18 and 10, respectively, and Dt is the time between measurements (8 wk). The half-life was calculated as t1/2 = ln(2)/k. For some T cell subpopulations in some individuals (e.g., TCM cells in subjects who were HIV-negative), exponential label decay was not observed between S1 and S3 or S2 and S3 (e.g., TCM cells in three of four HIV-negative subjects). Therefore, we also calculated the percentage of initially incorporated label (i.e., the 2He n-2H enrichment measured at S1) that was retained at S3 (i.e., percent labeled cells remaining = [S3/S1]). This parameter does not assume any particular kinetic model of cell survival, such as a single-pool, single-exponential kinetics of label die-away, and was calculated for all cell populations.

### Statistical analyses

Statistically significant differences between groups were assessed using the parametric Student’s t test. The parametric Pearson correlation (calculated with 95% confidence intervals and two-tailed p values) was used to determine the strength of linear relationships between two variables. Parametric
statistical tests were applied due to their greater sensitivity with the low sample sizes found in some of the analyses of this study. All data sets were tested for normality using the Kolmogorov-Smirnov normality test and all that showed significant differences were found to be normal using this test.

Results

Characteristics of study populations

To investigate the impact of HIV infection on long-lived CD8⁺ T cell subpopulations, we first phenotypically characterized the CD8⁺ T cell compartment in antiretroviral-untreated HIV-infected subjects at varying stages of disease (n = 20, including five labeled HIV-infected subjects) and compared the results to those obtained in healthy HIV-negative subjects (n = 6, comprising four subjects labeled with ³H₂O and two additional subjects who were not labeled). Long-term (7-wk) in vivo ³H₂O labeling was also conducted in five of the HIV-infected subjects and four of the HIV-negative subjects. The median CD8 count of the HIV-infected subjects (1175 CD8 T cells/µl; range, 578–3388) was significantly higher than that of the HIV-negative subjects (334 CD8 T cells/µl; range, 246–563) (p < 0.002 HIV-infected vs

FIGURE 1. In vivo stable isotope labeling protocol and gating strategy for T cell phenotyping. A, Long-term ³H₂O label administration. ³H₂O was administered over a period of 7 wk (3 × 50 ml of 70% ³H₂O per day during the first week and 2 × 50 ml of 70% ³H₂O per day during the next 6 wk). T cell subpopulations were sorted at three time points during the delabeling phase: weeks 10 (S1), 14 (S2), and 18 (S3). (B) Flow cytometric gating strategy: Upper row from left to right: Lymphocytes were gated using forward scatter-area (FSC-A) vs side scatter-area (SSC-A). Doublets were excluded using FSC-A vs FSC-height (FSC-H). Live cells were negative for live/dead fixable aqua (Amine). CD3⁺CD8⁺ T cells were gated. CD8low T cells were included in the gate. The fraction of CD8low T cells of total CD8⁺ T cells did not significantly differ between groups and also did not change significantly with lower CD4 counts. Middle row from left to right shows the FMOs (explained in Materials and Methods) for CD45RA, CD28, and CCR7, as well as the all stain (including all Abs used for staining) for CD3⁺CD8⁺CD45RA⁺ cells (farthest left gate in the lower left plot). The bottom row from left to right shows CD45RAbright (farthest right gate in the lower left plot), then the FMOs for CD28 and CCR7 as well as the “all stain” for CD3⁺CD8⁺CD45RAbright cells.
HIV-negative). This fact, coupled with refinements made to the FACS-MS method for measurements with low cell numbers enabled the analysis of more CD8\(^+\)/H11001 T cell subpopulations from HIV-infected than from HIV-negative subjects. Further characteristics of the study subjects are shown in Table I and the time line of the labeling protocol is shown in Fig. 1A.

With progressive HIV disease, the number of circulating \(T_N\) and \(T_{CM}\) cells decreases while the number of \(T_{EMRA}\) cells remains high. The cell surface markers used to differentiate effector memory \(1\) (\(T_{EM1}\)), \(T_{EM2}\) (a subset of \(T_{EM1}\)), RA effector memory \(T\) (\(T_{EMRA}\)), central memory \(T\) (\(T_{CM}\)), or naive \(T\) (\(T_N\)) cell subpopulations within the CD3\(^+\)CD8\(^+\) T cell population were chosen based on findings from previous human studies (21, 29–32). The gating strategy used for cell sorting and analysis is shown in Fig. 1B. The cytometric flow profiles in Figs. 2 and 3 show the different CD3\(^+\)CD8\(^+\) T cell subpopulations studied here, including CD45RA\(^{bright}\)CCR7\(^{+}\)CD28\(^{-}\) (\(T_N\)), CD45RA\(^{bright}\)CCR7\(^{+}\)CD28\(^{-}\) (\(T_{EMRA}\)), CD45RA\(^{bright}\)CCR7\(^{-}\)CD28\(^{+}\) (\(T_{EM1}\)), CD45RA\(^{bright}\)CCR7\(^{-}\)CD28\(^{+}\) CD28\(^{-}\) (\(T_{EM2}\)) cells from 5 HIV-negative subjects and 20 HIV-infected subjects with a range of CD4 counts, sorted by decreasing CD4 counts.

FIGURE 2. Flow plots of CD3\(^+\)CD8\(^+\)CD45RA\(^{bright}\) T cell subpopulations from the HIV-negative (A) and HIV-infected (B) subjects. The same gating strategy as in Fig. 1, bottom row, was used with CCR7 on the \(x\)-axis and CD28 on the \(y\)-axis. The flow plots are shown with the location of the CD3\(^+\)CD8\(^+\)CD45RA\(^{bright}\)CD28\(^{-}\)CCR7\(^{+}\) naive T (\(T_N\)) cells and the CD3\(^+\)CD8\(^+\)CD45RA\(^{bright}\)CD28\(^{-}\)CCR7\(^{+}\) RA effector T cells (\(T_{EMRA}\)) cells (labeled in the farthest right plot in A). Labels for \(T_N\) and \(T_{EMRA}\) cell subpopulations are located on the right next to the respective cell subpopulation or gate. Gates for individual subjects were set based on FMO stains. The capital letters in the lower right corner of each plot correspond to the subject IDs listed in Table I and are displayed by decreasing CD4 count (from top left to bottom right).
were defined as RA−CCR7− based on the definition of Sallusto and coworkers (30). It is evident from the flow cytometric profiles shown in Figs. 1 and 3 that the CD8+CD45RA−CCR7−TEM1 cells (red box) are heterogeneous and consist of two distinct subpopulations when analyzed for expression of CD45RA, CCR7, and CD28, namely, CD45RA−CCR7−CD28+ and CD45RA−CCR7−CD28−TEM2 cells (red box around TEM1 cells in Figs. 1 and 3A, right flow plot in the upper row).

The relationship of each of these CD3+CD8+ T cell subpopulations to the CD4 count was assessed in the cohort of HIV-infected subjects (Fig. 4). As shown in Fig. 4A, the absolute number of CD3+CD8+ T cells remained more or less constant across the range of CD4 T cell counts studied in this cohort. It has been previously reported that the transition of HIV-specific cells from the TEM1 phenotype to the TEMRA phenotype does not occur (43, 44). In line with this finding, the fraction of total TEM1 cells is increased in most HIV-infected subjects as compared with

FIGURE 3. Flow plots of CD3+CD8+CD45RA− T cell subpopulations from the HIV-negative (A) and HIV-infected (B) subjects. The same gating strategy as in Fig. 1, middle row, was used with CCR7 on the x-axis and CD28 on the y-axis. The flow plots are shown with the location of CD3+CD8+CD45RA−CD28+CCR7+ central memory T (TCM) cells and CD3+CD8+CD45RA−CD28−CCR7− effector T (TEM2) cells (labeled in the farthest right plot in A). Labels for TCM, TEM1, and TEM2 cell subpopulations are located either above or on the right next to the respective gates. Gates for individual subjects were set based on FMO stains. The capital letters in the lower right corner of each plot correspond to the subject IDs listed in Table I. CD8+CD45RA−CCR7− T cells, which are here referred to as TEM1 cells, are heterogeneous and consist of two distinct subpopulations when analyzed for expression of CD45RA, CCR7, and CD28, namely CD45RA−CCR7−CD28+ as well as CD45RA−CCR7−CD28−TEM2 cells (red box around TEM1 cells in A, right flow plot in the upper row).
HIV-negative subjects (median 69.6%, interquartile range (IQR) 44.5–82.6% TEM1 cells in HIV-infected subjects vs median 9.9%, IQR 5.6–19.3% TEM1 cells in HIV-negative subjects, p < 0.0001). There was no significant correlation between TEM1 cells per μl of blood and the CD4 count in HIV-infected subjects (Fig. 4B). Similarly, although the flow cytometric profiles reveal an increase in the fraction of TEM1 cells in some HIV-infected subjects as compared with most HIV-negative subjects (median 6.5% IQR 3–11% TEM1 cells in HIV-infected subjects vs median 1.6% IQR 0.6–3.2% TEM1 cells in HIV-negative subjects, p = 0.08), there was no significant correlation between the absolute number of TEM1 cells and the CD4 count (Fig. 4C). Moreover, CD8⁺ T cells with a TEMRA phenotype were rare in healthy HIV-negative subjects (see Fig. 2A), but their fraction and absolute number was elevated in HIV-infected subjects, irrespective of the CD4 count (median 273 (1.4%) IQR 182–406 (6–28%) TEMRA cells in HIV-infected subjects vs median 22 (7.2%) IQR 19–39 (4–2%) TEMRA cells in HIV-negative subjects, p < 0.01), even though there was no relationship between their absolute count and the CD4 count (Fig. 4D). In contrast, the number of TCM cells was observed to fall as the CD4 count declined (p = 0.02; Fig. 4E). Finally, and consistent with the previously reported decrease of T N cell numbers found in adults with progressive HIV disease (45), T N cells significantly decreased with decreasing CD4 counts (p = 0.02; Fig. 4F).

In HIV infection, TEMRA cells maintain a long lifespan but TCM cells do not

The 7-wk ²H₂O labeling protocol resulted in higher ²H₂O enrichments in the different T cell subpopulations of HIV-negative subjects, including long-lived T N cells, than did three weeks of ²H₂O labeling (data not shown). This finding is consistent with a meta-analysis of previous stable isotope labeling studies with different periods of labeling, which showed that longer labeling protocols resulted in progressive incorporation of label into longer-lived cells (46).

CD³⁺CD8⁺ T cells (“CD8⁺ T cells”), TEM₁, TEM₂, TEMRA, TCM, and T N cells from HIV-negative and HIV-infected subjects were sorted and analyzed for ²H₂O label enrichment after 7 wk of ²H₂O labeling and during the subsequent delabeling period. Fig. 5 shows the decay curves of label enrichment in each of these subpopulations. A few points are apparent. First, the observation of consistent and distinct patterns of label enrichment in the different T cell subpopulations confirms that use of the refined stable isotope/FACS-MS method allowed measurement of label incorporation into as few as 5,000–20,000 cells (the data shown here were generally derived from 20,000 cells). Secondly, it is evident from the labeling patterns that HIV-infected subjects had higher levels of label enrichment in several T cell subpopulations than HIV-negative subjects, as would have been expected from previous studies (34, 39). Also, label incorporation into TEM₁ cells from HIV-negative subjects was relatively high, but varied between subjects at the different time points (Fig. 5C), possibly reflecting their heterogeneous phenotypic composition in these HIV-negative subjects (Fig. 3A). In HIV-infected subjects, label incorporation into the TEM₁ subpopulation was higher, especially in the HIV-infected subject with the highest VL (Fig. 5D).

Additional insights into the turnover of different CD8⁺ T cell subpopulations were obtained on inspection of the die-away curves. In the case of the TEM₂ subpopulation, a high fraction of these cells was labeled in four HIV-infected subjects, and a rapid delabeling occurred during the period of observation (Fig. 5E). Given the small numbers of such cells in HIV-negative subjects, the kinetics of this subpopulation could be studied in only a single individual (Fig. 5E). Label incorporation and rapid loss of label in TEM₁ cells of HIV-infected subjects was very similar to that of

**FIGURE 4.** Circulating CD8⁺ T cell subpopulations in HIV-infected subjects. Linear regressions and Pearson correlations (calculated with 95% confidence intervals and two-tailed p values) between the circulating CD4⁺ T cell count in HIV-infected patients (n = 20) and the absolute number of circulating CD8⁺ T cells (A), TEM₁ (B), TEM₂ (C), TEMRA (D), TCM (E), and T N (F) cells/μl. Pearson correlations are shown, as data sets were found to be normal as determined using the Kolmogorov-Smirnov normality test (see Materials and Methods).
TEM2 cells, notwithstanding the heterogeneous composition of the former subpopulation. In HIV-negative subjects, the TEMRA (Fig. 5F) and TCM (Fig. 5G) subpopulations both revealed low uptake of label and slow loss after labeling was discontinued. In all HIV-infected subjects, TEMRA cells also showed a very slow loss of label (Fig. 5F). In contrast, however, some but not all HIV-infected subjects showed a high uptake of label and a very rapid loss of label in the TCM subpopulation (Fig. 5H). Label incorporation into TN cells was low in both HIV-negative and HIV-infected sub-
jects (on average 0.18% in HIV-negative subjects vs 0.4% in HIV-infected subjects) and its loss after cessation of labeling was slow, if evident at all in HIV-negative subjects, consistent with the expected long half-life of this subpopulation of cells (Refs. 9 and 34 and Fig. 5, I and J). In HIV-infected subjects with high VL, however, label decreased or was lost completely by the last time point in TN cells.

The decay curves shown in Fig. 5 were used to calculate the decay constant (k) and the half-life as well as the percentage of cells remaining at the end of the protocol. These data are summarized in Table II, along with the number of cells per microliter and the frequency of a given subpopulation among total CD8 T cells. TEM1 cells had a very variable half-life (mean 105.8 ± 88.4 days) in HIV-negative subjects, likely due to the fact that the fraction of CD28 and CD28 cells within this CD8 T cell subpopulation was variable in these subjects. This variability was also reflected in the variable percentage of TEM1 cells remaining at wk 18. In HIV-infected subjects, in contrast, the half-life of TEM1 cells was shorter and less variable (35.9 ± 4.1 days) and the percentage of cells remaining at wk 18 was similar to that found for TEM2 cells. TEM2 cells, meanwhile, showed a short half-life in both HIV-negative and HIV-infected subjects (58.1 and 49.3 ± 19.3 days, respectively). Of the memory/effector CD8 T cell subpopulations,
Table II. Information and results for CD8+ T cell subpopulations from subjects labeled long-term (7 wk) with ²H₂O

<table>
<thead>
<tr>
<th>CD3+CD8+ T cell subset</th>
<th>Group</th>
<th>HIV copies/ml (log₁₀)</th>
<th>k</th>
<th>t₁/₂ (d)</th>
<th>% of Cells Remaining</th>
<th>Cells/µl</th>
<th>% of CD3+CD8+ T Cells</th>
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<tr>
<td>CD45RA-CCR7 (TEM₁)</td>
<td>HIV negative</td>
<td>-</td>
<td>0.0117 ± 0.0217</td>
<td>105.8 ± 88.4</td>
<td>53.7 ± 34.7</td>
<td>31.3 ± 20.6</td>
<td>9.7 ± 8.3</td>
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<td>CD45RA-CCR7 CD28</td>
<td>HIV negative</td>
<td>-</td>
<td>0.0119 ± 0.0047</td>
<td>49.3 ± 19.3</td>
<td>43.3 ± 12.2</td>
<td>241.4 ± 208.5</td>
<td>9.9 ± 5.6</td>
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<td>CD45RA-CCR7 CD28</td>
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<td>99.6 ± 27.3</td>
<td>8.9 ± 6.4</td>
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<td>45.5 ± 27.9</td>
<td>96.7 ± 60.6</td>
<td>4.38 ± 1.2</td>
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</tbody>
</table>

* Means ± SD.
* * p < 0.05, ** p < 0.01, *** p < 0.001 HIV negative vs HIV infected.
* To prevent underestimation, the ²H₂O enrichment from the subject at S1 and the mean ²H₂O enrichment of three subjects at S3 were used to calculate k, the half-life, and the percentage of cells remaining.
* p < 0.05 HIV-negative versus HIV-infected high VL.
* Decay constants and half-lives for T₇ cells could not be calculated, because label either increased during delabeling (all of the HIV-negative subjects and the two HIV-infected subjects with high VL) or label could no longer be detected at the last time point during delabeling (two of the HIV-infected subjects with high VL).

T_EMRA and T_CCM cells had the longest half-lives (9762 and 97.7 days, respectively) in the HIV-negative subjects and also the highest fraction of cells (99.6 and 64.9%, respectively) remaining at the last time point evaluated during delabeling. However, whereas T_EMRA cells in HIV-infected subjects had a long half-life irrespective of the VL, the half-life of T_CCM cells was shorter (51 days) and was particularly short (28.8 days) in those with a high VL (p < 0.05). In those subjects with a high VL, the fraction of remaining labeled T_CCM cells was significantly reduced at the end of the protocol (26.0% vs 74.7% in those with a low VL and vs 64.9% in HIV-negative subjects; p < 0.01 for HIV-negative subjects vs HIV-infected subjects with high VL). By contrast, the fraction of T_EMRA cells remaining at the end of the protocol was the same (68.5% ± 10.6%), irrespective of the VL. Although the half-life of cells in the T₇ compartment could not be estimated (see Table II footnotes), the percentage of labeled T₇ cells remaining at the end of the delabeling period was significantly lower in HIV-infected subjects than in HIV-negative subjects (42.8% vs 75.5%, p < 0.01). The subject with the highest VL had the lowest number of T₇ cells (range 23–66 T₇ cells per µl at three different sort days), consistent with the trend observed (Fig. 4F and Ref. 24) between loss of T₇ cells and progressive HIV disease.

More detailed correlations between HIV VL and the percentage of T_EM₁, T_EM₂, T_EMRA, or T_CCM cells remaining are shown in Fig. 6. No clear trend was observed for the correlation between VL and T_EM₁ cells remaining (Fig. 6A), likely due to their heterogeneous phenotypic composition. The more homogenous subpopulation of remaining T_EM₂ cells, however, correlated negatively with VL (Fig. 6B). There was no significant correlation of VL and the percentage of T_EMRA cells remaining (Fig. 6C), but a negative correlation was found between VL and the percentage of T_CCM cells remaining at the last time point during de-labeling (Fig. 6D).

Keeping in mind the caveat of the small numbers of samples, the correlations shown in Fig. 6, B and D, were significant (p < 0.01 and p < 0.05, respectively). These observations are consistent with the decline in T_CM cells observed with decreasing CD4 counts documented in the larger cohort of HIV-infected subjects (Fig. 4E).

Expression of IL-7Rα, IL-18R1α, and CD57 on T_CM and T_EMRA cells in the different CD8+ T cell subpopulations

Because long-lived memory T cells may behave like self-renewing stem cells (16, 17, 47), we asked whether long-lived T_CM cells or T_EMRA cells express certain cell surface receptors (e.g., IL-7Rα and IL-18R1α) that have been previously shown (16) to be expressed on murine hematopoietic stem cells as well as on memory B and T cells. We also evaluated changes in the expression patterns of these surface receptors between HIV-negative and HIV-infected subjects in varying stages of disease progression as well as the fraction expressing CD57, a marker of more terminally differentiated cells. As shown in Fig. 7A (left), almost 100% of T_CM cells from healthy HIV-negative subjects express IL-7Rα, whereas T_EMRA cells express certain cell surface receptors (e.g., IL-7Rα and IL-18R1α) that have been previously shown (16) to be expressed on murine hematopoietic stem cells as well as on memory B and T cells. We also evaluated changes in the expression patterns of these surface receptors between HIV-negative and HIV-infected subjects in varying stages of disease progression as well as the fraction expressing CD57, a marker of more terminally differentiated cells. As shown in Fig. 7A (left), almost 100% of T_CM cells from healthy HIV-negative subjects express IL-7Rα, whereas
the percentage of IL-7Rα+ TCM cells was significantly lower in HIV-infected subjects (p < 0.05). Expression of IL-18R1α or CD57 on TCM cells was much lower and did not differ between groups. The fraction of TemRA cells expressing IL-7Rα was significantly lower in HIV-infected subjects than in HIV-negative subjects (p < 0.01; Fig. 7B, left) as was the fraction expressing IL-18R1α (p < 0.05; Fig. 7B, middle) and CD57 (p < 0.05; Fig. 7B, right). When these subpopulations were examined in the context of CD4 T cell counts, the percentage of TCM cells positive for IL-7Rα trended to fall as the CD4 count fell (Fig. 7C, left). There was a significant decrease in the MFI of CD57 on CD8+ T cells; this decrease was specifically observed on CD8high TemRA cells (p = 0.003; Fig. 7C, middle) and a decrease in the percentage of CD8high TemRA cells that were CD57+ (p = 0.06; Fig. 7C, right).

Discussion

It is clear that the memory/effector CD8+ T cell population is phenotypically and functionally heterogeneous, including multiple subpopulations of short-lived effector cells and long-lived memory cells (39, 48). In a previous study using an in vivo labeling technique (34), we found that long-lived CD4+ and CD8+ T cell subpopulations are lost as a function of HIV disease progression. Here, we have used a refinement of this labeling technique that permitted kinetic characterization of the lifespan of small numbers of cells to discern two subpopulations of long-lived CD8+ T memory/effector cells: CD8+ CD45RA−CCR7+CD28+ central memory (TCM) cells expressing IL-7Rα and CD8+ CD45RA−CCR7−CD28− RA effector memory (TemRA) cells, of which a high fraction expresses CD57. TCM cells were found to have a significantly shorter half-life in HIV-infected subjects with high VL, compared with healthy HIV-negative subjects, and to decrease in number and in their expression of IL-7Rα as a function of disease progression. TemRA cells, by contrast, retained a long half-life, accumulated in the face of progressive HIV disease, and lost expression of CD57. These observations reveal kinetic differences between certain subpopulations of CD8+ T cells in HIV-infected subjects that may be related to disease progression.

An important, and we believe generally applicable, feature of this study is its demonstration that the kinetics of thousands, as opposed to hundreds of thousands, of cells can be studied in vivo. Prior methodology could only be used to estimate the half-life of larger numbers of cells that were assumed to contain varying fractions of phenotypically distinct subpopulations. By using the newly refined techniques described here for accurate measurement of isotope enrichments in small numbers of cells, combined with the use of long-term “pulse” labeling with H2O, it is now possible to take full advantage of the power of state-of-the-art multiparameter, multidirectional FACS, and to simultaneously measure the kinetics of multiple, even rare, subpopulations of cells. A caveat to any interpretation that might be drawn from our kinetic analysis is that the number of subjects studied (four HIV-negative and five HIV-infected) is small. Nonetheless, and especially when coupled with phenotypic data obtained from an additional 17 subjects, a number of conclusions can be reasonably drawn.

First, the CD8+ TCM subpopulation that we show to be long-lived (t1/2 = 97.7 days) in HIV-negative subjects has die-away kinetics that are markedly faster in HIV-infected subjects, with a half-life reaching 28.8 days in those with the highest VL. Concomitantly, this subpopulation appears to be lost from the circulation as the CD4 count drops with disease progression, although this should be verified in a longitudinal study using PBMC directly ex vivo from untreated HIV-infected patients. Possibly, TCM cells are lost because they are less efficiently produced: circulating naive CD8+ T cells; alternatively or in addition, TCM cells may be lost because they survive less well. Thus, although IL-7 is necessary for the survival of long-lived memory T cells (49), the high levels of IL-7 (50, 51) and of chronic immune activation (38, 52) found in late-stage HIV disease may result in down-regulation of the IL-7Rα on TCM cells, as reported previously (53–58) and as also observed in these studies. Such
down-regulation, in turn, would have predictably negative impact on T_{CM} cell survival (59). In addition, T_{CM} cells could transition more rapidly and/or in larger numbers to shorter-lived T_{EM2} cells in HIV-infected individuals, similar to the observation made recently in SIV-infected macaques (60). It is also possible that maintenance of the CD8^+ T_{CM} compartment is dependent on preservation of CD4^+ T_{CM} cells, which have been shown to be lost as a function of progressive SIV disease (60) and are likely also lost in progressive HIV disease (34). Two points must be added for clarification. First, CD3^+ CD8^+ CD45RA^-CCR7^- T cells (T_{EM1}) contain CD28^+ cells, which may be cells transitioning from T_{EM1} to T_{EM2}, with the former being both CCR7^- and CD28^- and the latter lacking expression of both of these receptors. Second, T_{EM1} cells in this study do not refer to RAexpressing effector memory T (T_{EMRA}) cells. T_{EMRA} cells are sometimes called terminally differentiated effector memory T cells (28, 44).

A second tentative conclusion is underscored by the markedly different behavior of the long-lived T_{EMRA} subpopulation. In HIV-negative subjects, these cells are low in number and slow in turnover. By contrast, circulating numbers of T_{EMRA} cells are generally much higher in HIV-infected subjects, in whom they are also found to lose the expression of CD57, IL-7Rα, and IL-18Rαβ, but to remain long-lived. Collectively, these observations suggest that T_{EMRA} cells maintain a long lifespan in HIV-infected subjects but that they change phenotypically, and perhaps also functionally, as HIV disease progresses. Thus, it has recently been shown that HIV-specific T_{EMRA} cells are more frequently detectable in controlled than in progressive HIV disease (28) and that they may predict the VL set point after acute infection (61). In these stages of disease and with this phenotype, such cells may accordingly be protective. The CD57^+/IL-7Rα^- T_{EMRA} cells studied here, by contrast, accumulate in late stages of disease and may not share the same beneficial properties. It should also be pointed out that, unlike the previous studies (28, 61), we did not compare the fractions of T_{EMRA} cells in controllers vs progressors and, in the previous studies, the fraction of T_{EMRA} cells was not compared in HIV-negative vs HIV-infected subjects.

Until now, it has not been possible to study the turnover of Ag-specific T cells in vivo. However, a few in vitro studies suggest that the maintenance of the proliferative capacity of epitope-specific CD8 T cell responses is an important functional correlate of viral control in HIV infection (27, 62, 63). It is likely that both the maintenance of the proliferative capacity of HIV-specific CD8^+ T cells and the preservation of the T_{CM} compartment contribute to the control of HIV as well as to the control of other infections, thereby preventing immunodeficiency.

In this study, we also addressed the question of whether cells in either of the long-lived memory CD8^+ T cell subpopulations have properties similar to those of stem cells. In previous studies, for example, it has been proposed that bcl6b is responsible for maintaining "stem cell-like" memory T cells in a quiescent state (17, 29) and, by microarray analysis, transcripts for IL-7Rα and IL-18Rα have been detected within murine hematopoietic stem cells, memory B cells, and memory T cells (16). In our hands, the expression of bcl6b was not higher in memory than in naive T cells (data not shown), and T_{CM} cells were found to express IL-7Rα but only a small fraction (~20%) expressed IL-18Rα. Thus, at this level of discrimination, we are unable to ascribe accepted traits of stem cells to these human CD8^+ memory T cell subpopulations.

In summary, a highly sensitive in vivo labeling technique has been used here to define the kinetic properties of low abundance subpopulations of CD8^+ T cells, including T_{EM1}, T_{EM2}, T_{EMRA}, T_{CM}, and T_{N} cells, in healthy subjects as well as in those infected with HIV. The data show that long-lived CD8^+ T cell subpopulations in healthy subjects include T_{N}, T_{CM}, and T_{EMRA} cells. In progressive HIV infection, the half-life of T_{CM} cells becomes shorter while that of T_{EMRA} cells remains the same. These changes in kinetics are reflected in the composition of the total CD8^+ T cell pool: progressive disease is associated with a loss of T_{CM} cells while the fraction and absolute number of T_{EMRA} cells is increased, irrespective of disease stage. Further exploration of these observations using the refined stable isotope/FACS/MS method may provide a more complete understanding of the manner in which the CD8^+ T cell compartment is eroded, both numerically and functionally, as HIV disease advances, and may also provide insight into the recovery of this population after the institution of highly active antiretroviral therapy.

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Disclosures

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