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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⁻ RA 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.

Ever since the onset of the AIDS epidemic, it has been clear that HIV infection is associated with immunodeficiency (1, 2). In the intervening years, a number of mechanisms underlying this state of immunodeficiency have been described, ranging from quantitative loss of CD4⁺ T cells to qualitative changes in cell populations that persist (3, 4). Among the qualitative changes, one of the earliest to be discerned was the loss of recall response to Ag (5). Such recall, or memory, responses represent the bedrock upon which the adaptive immune system is based (6–10). In the T cell lineage, such recall is associated with specialized cell subpopulations that differentiate from naive T cells after exposure to Ag. Each memory T cell clone is endowed with an Ag-specific receptor, with the ability to persist for long periods of time, and with the propensity to rapidly proliferate and to differentiate into effector cells after secondary contact with cognate Ag. By this sequence of events, durable Ag-specific immunity is achieved.

Studies conducted in well-defined murine systems have illuminated some of the requirements for generation and maintenance of memory CD8⁺ T cell response during and after acute infection (10). Gene expression profiling and functional characterization of murine CD8⁺ T cell development have demonstrated that CD8⁺ T cell differentiation proceeds through a series of discrete steps (6, 11, 12). In mice, long-lived memory CD8⁺ T cells are thought to originate from effector CD8⁺ T cells that survive a death phase that follows the initial phase of T cell activation and expansion (13). Requirements for generation and maintenance of such memory CD8⁺ T cells include CD4⁺ T cell help (6, 14, 15) as well as acquisition of the cellular machinery that provides for either self-renewal (e.g., through the action of the transcriptional repressor, bcl6, which in turn may be important for asymmetrical differentiation of Ag-specific T cells upon secondary antigenic challenge) or for limited homeostatic proliferation (e.g., mediated by IL-7 through the IL-7Rα) (6, 16, 17). Homeostatic renewal itself appears to be dependent on rest from antigenic stimulation, an opportunity less frequent during the course of chronic viral infections that are associated with continuously high viral loads (VL). At the same time, low-level persistence of Ag and continued proliferation are likely necessary for the maintenance of virus-specific memory T cells in chronic infection, because these cells are not maintained by homeostatic cytokines (e.g., IL-7 and IL-15), inflammatory signals, and priming of recent thymic emigrants alone (18).

In humans, the long-lived memory CD8⁺ T cell compartment is poorly understood. Initial studies described three CD8⁺ T cell subpopulations (naive, memory, and effector), distinguished by their...
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 cell population that is endowed with high proliferative capacity, a broad TCR repertoire, and expression of IL-7Ra (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_{naive}) 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 subpopulations, which were thereafter sort 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 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 method 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 naive 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-uninfected 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 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.

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% H2O (Cambridge Isotope Laboratories) for a 7-wk labeling protocol. During the first week of labeling, subjects consumed 10 ml of 70% H2O per day followed by either 2 or 6 wk of two vials of 50 ml of 70% H2O per day. The first blood draw for cell sorting (Sort 1, or S1) was obtained in most cases 3 wk after the last dose of H2O was taken by the subject (i.e., wk 10 after the start of labeling). This blood draw was followed by two further blood draws (i.e., at weeks 14 and 18, termed S2 and S3, respectively). Some subjects came in for an additional blood draw during the week of labeling (data not shown). This time line is shown (see Fig. 1A). A pilot study in four healthy subjects labeled for only 3 wk with H2O revealed that H label incorporation into DNA of the T cells studied was insufficient to characterize die-away curves optimally (data not shown). The longer, 7-wk labeling protocol achieved higher initial levels of label incorporation and was therefore used for the data reported here.

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 5X concentrated hydrolysis mixture was added, containing sodium acetate buffer (pH 5), zinc sulfate, nuclease S1, and acid phosphatase (41), and the mixture was incubated at 37°C for 1–2 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 ml) was added, followed by 75 ml of glacial acetic acid, and the mixtures incubated at 100°C for 30 min. After cooling to room temperature, 1 ml of acetic anhydride was added to each sample, followed by 100 ml 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 ml 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 assign 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 with helium as the carrier gas, and selected ion monitoring was used to quantify the fractional abundance of deoxyribose. 2H enrichment was calculated as EM1, the excess fractional abundance of the M1 mass isotopomer above baseline (as determined by analysis of the unlabeled deoxyribose derivative, with correction for analyte abundance effects; Ref. 39). The EM1 value represents the abundance range that generated reliable EM1 values with the diluted, labeled 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.

As in previous studies (33, 39), 2H2O kinetic calculations during the 2H label incorporation phase were based on the precursor-product relationship. 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- netic model of cell survival, such as a single-pool, single-exponential 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. 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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 2H2O and two additional subjects who were not labeled). Long-term (7-wk) in vivo 2H2O 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...
This fact, coupled with refinements made to the FACS-MS method for measurements with low cell numbers enabled the analysis of more CD8\(^+\) 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\(^{hi}\)CCR7\(^{hi}\)CD28\(^{hi}\) (TN), CD45RA\(^{hi}\)CCR7\(^{lo}\)CD28\(^{lo}\) (TEMRA), CD45RA\(^{lo}\)CCR7\(^{hi}\)CD28\(^{lo}\) (TCM), CD45RA\(^{lo}\)CCR7\(^{lo}\)CD28\(^{lo}\) (TEM1) and CD45RA\(^{lo}\)CCR7\(^{lo}\)CD28\(^{hi}\) (TEM2) cells from 5 HIV-negative subjects and 20 HIV-infected subjects with a range of CD4 counts, sorted by decreasing CD4 counts. TEM cells in this study...
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% $T_{EM1}$ cells in HIV-infected subjects vs median 9.9%, IQR 5.6–19.3% $T_{EM1}$ cells in HIV-negative subjects, $p < 0.0001$). There was no significant correlation between $T_{EM1}$ cells per $\mu$L 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 $T_{EM2}$ cells in some HIV-infected subjects as compared with most HIV-negative subjects (median 6.5% IQR 3–11% $T_{EM2}$ cells in HIV-infected subjects vs median 1.6% IQR 0.6–3.2% $T_{EM2}$ cells in HIV-negative subjects, $p = 0.08$), there was no significant correlation between the absolute number of $T_{EM2}$ CD8$^+$ T cells and the CD4 count (Fig. 4C). Moreover, CD8$^+$ T cells with a $T_{EMRA}$ 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%) $T_{EMRA}$ cells in HIV-infected subjects vs median 22 (7.2%) IQR 19–39 (4–28%) $T_{EMRA}$ 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 $T_{CM}$ 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, $T_{EMRA}$ cells maintain a long lifespan but $T_{CM}$ cells do not.

The 7-wk $^2$H$_2$O labeling protocol resulted in higher $^2$H$_2$O enrichments in the different T cell subpopulations of HIV-negative subjects, including long-lived $T_N$ cells, than did three weeks of $^2$H$_2$O 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).

CD$^3$CD8$^+$ T cells (“CD8$^+$ T cells”), $T_{EM1}$, $T_{EM2}$, $T_{EMRA}$, $T_{CM}$, and $T_N$ cells from HIV-negative and HIV-infected subjects were sorted and analyzed for $^2$H$_2$O label enrichment after 7 wk of $^2$H$_2$O 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 $T_{EM1}$ 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 $T_{EM1}$ 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 $T_{EM2}$ 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 $T_{EM1}$ cells of HIV-infected subjects was very similar to that of
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 T N cells was low in both HIV-negative and HIV-infected subjects (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 T N 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,
CD45RA-CCR7- and TCM 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 TEMRA cells in HIV-infected subjects had a long half-life irrespective of the VL, the half-life of TCM 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 TEMRA 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 TEMRA 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 TEMRA cell compartment could not be estimated (see Table II footnotes), the percentage of labeled TEMRA cells remaining at the end of the delabeling period was significantly lower in HIV-infected subjects than in HIV-negative subjects (42.8% vs 175.5%, p < 0.01). The subject with the highest VL had the lowest number of TEMRA cells (range 23–66 TEMRA cells per μl at three different sort days), consistent with the trend observed (Fig. 4F and Ref. 24) between loss of TEMRA cells and progressive HIV disease.

More detailed correlations between HIV VL and the percentage of TEMRA, TEM2, TEMRA, or TCM cells remaining are shown in Fig. 6. No clear trend was observed for the correlation between VL and TEMRA cells remaining (Fig. 6A), likely due to their heterogeneous phenotypic composition. The more homogenous subpopulation of remaining TEM2 cells, however, correlated negatively with VL (Fig. 6B). There was no significant correlation of VL and the percentage of TEMRA cells remaining (Fig. 6C), but a negative correlation was found between VL and the percentage of TCM 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 TCM cells observed with decreasing CD4 counts documented in the larger cohort of HIV-infected subjects (Fig. 4E).

Expression of IL-7Ra, IL-18R1α, and CD57 on TCM and TEMRA 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 TCM cells or TEMRA cells express certain cell surface receptors (e.g., IL-7Ra 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 TCM cells from healthy HIV-negative subjects express IL-7Ra, whereas TCMRA cells express certain cell surface receptors (e.g., IL-7Ra 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 TCM cells from healthy HIV-negative subjects express IL-7Ra, whereas

Table II. Information and results for CD8+ T cell subpopulations from subjects labeled long-term (7 wk) with 2H2O

<table>
<thead>
<tr>
<th>T cell subset</th>
<th>Group</th>
<th>HIV copies/ml (log10)</th>
<th>k (d)</th>
<th>t1/2 (d)</th>
<th>% of Cells Remaining</th>
<th>Cells/μl</th>
<th>% of CD3+CD8+ T Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45RA-CCR7-</td>
<td>HIV negative</td>
<td>0.0117 ± 0.0217*</td>
<td>0.0146 ± 0.0022</td>
<td>0.0119</td>
<td>51.3</td>
<td>38.7</td>
<td>15.7</td>
</tr>
<tr>
<td>(TEM1) HIV infected</td>
<td>4.3 ± 1.0</td>
<td>35.9 ± 4.1</td>
<td>42.8 ± 20.4</td>
<td>391.7 ± 367.0</td>
<td>15.7 ± 9.1***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA-CCR7-</td>
<td>HIV negative</td>
<td>0.0119</td>
<td>51.3</td>
<td>8.3 ± 8.7</td>
<td>2.2 ± 1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD28+ (TEM1) HIV infected</td>
<td>4.3 ± 1.0</td>
<td>43.3 ± 12.2</td>
<td>241.4 ± 208.5</td>
<td>9.9 ± 5.6*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA-CCR7-</td>
<td>HIV negative</td>
<td>0.0007*</td>
<td>9762</td>
<td>99.6*</td>
<td>33.2 ± 27.3</td>
<td>8.9 ± 6.4</td>
<td></td>
</tr>
<tr>
<td>CD28+ (TEM1) HIV infected</td>
<td>4.3 ± 1.0</td>
<td>114.5 ± 47.2</td>
<td>68.5 ± 10.6</td>
<td>515.9 ± 162.3***</td>
<td>25.0 ± 5.3**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA-CCR7-</td>
<td>HIV negative</td>
<td>0.0079 ± 0.0029</td>
<td>977 ± 36.8</td>
<td>64.9 ± 10.4</td>
<td>30.5 ± 21.7</td>
<td>9.15 ± 7.5</td>
<td></td>
</tr>
<tr>
<td>CD28+ (TEM1) HIV infected</td>
<td>4.3 ± 1.0</td>
<td>51.0 ± 30.4d</td>
<td>45.5 ± 27.9d</td>
<td>96.7 ± 60.6</td>
<td>4.38 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45RA-CCR7-</td>
<td>HIV negative</td>
<td>0.0166 ± 0.0087d</td>
<td>175.5 ± 16.1</td>
<td>159.4 ± 82</td>
<td>44.0 ± 18.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD28+ (TEM1) HIV infected</td>
<td>4.3 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>249.7 ± 198.9</td>
<td>15.3 ± 11.6</td>
<td></td>
<td></td>
</tr>
</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 one 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 TCM cells could not be calculated, because label either increased during delabeling (all of the HIV-negative subjects and the two HIV-infected subjects with low VL) or label could no longer be detected at the last time point during delabeling (two of the HIV-infected subjects with high VL).
pression of CD57. These observations reveal kinetic differences accumulated in the face of progressive HIV disease, and lost expression of CD57 on TCM 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 $^{3}$H$_{2}$O, 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 variety of conclusions can be reasonably drawn.

First, the CD8+ TCM subpopulation that we show to be long-lived ($t_{1/2} = 97.7$ 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. Combinantly, 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 cell progenitors to these TCM subpopulation that we show to be long-lived T cells in HIV-infected subjects may be related to disease progression.

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down-regulation, in turn, would have predictably negative impact on T<sub>CM</sub> cell survival (59). In addition, T<sub>CM</sub> cells could transition more rapidly and/or in larger numbers to shorter-lived T<sub>EM2</sub> 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<sup>+</sup> T<sub>CM</sub> compartment is dependent on preservation of CD4<sup>+</sup> T<sub>CM</sub> 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<sup>+</sup> CD8<sup>+</sup> CD45RA<sup>−</sup>CCR<sup>+</sup> T<sub>CM</sub> cells (T<sub>EM1</sub>) contain CD28<sup>+</sup> cells, which may be cells transitioning from T<sub>EM2</sub> to T<sub>EM1</sub>, with the former being both CCR<sup>+</sup> and CD28<sup>+</sup> and the latter lacking expression of both of these receptors. Second, T<sub>EM1</sub> cells in this study do not refer to RA-expressing effector memory T (T<sub>EMRA</sub>) cells. T<sub>EMRA</sub> 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<sub>EMRA</sub> subpopulation. In HIV-negative subjects, these cells are low in number and slow in turnover. By contrast, circulating numbers of T<sub>EMRA</sub> cells are generally much higher in HIV-infected subjects, in whom they are also found to lose the expression of CD57, IL-7R<alpha>, and IL-18R<alpha>, but to remain long-lived. Collectively, these observations suggest that T<sub>EMRA</sub> 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<sub>EMRA</sub> 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<sup>+</sup> T<sub>EMRA</sub> 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<sub>EMRA</sub> cells in controllers vs progressors and, in the previous studies, the fraction of T<sub>EMRA</sub> 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<sup>+</sup> 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<sup>+</sup> T cells and the preservation of the T<sub>CM</sub> 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<sup>+</sup> 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-7R<alpha> and IL-18R<alpha> 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<sub>CM</sub> cells were found to express IL-7R<alpha> but only a small fraction (~20%) expressed IL-18R<alpha>. Thus, at this level of discrimination, we are unable to ascribe accepted traits of stem cells to these human CD8<sup>+</sup> 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<sup>+</sup> T cells, including T<sub>EM1</sub>, T<sub>EM2</sub>, T<sub>EMRA</sub>, T<sub>CM</sub>, and T<sub>N</sub> cells, in healthy subjects as well as in those infected with HIV. The data show that long-lived CD8<sup>+</sup> T cell subpopulations in healthy human subjects include T<sub>N</sub>, T<sub>CM</sub>, and T<sub>EMRA</sub> cells. In progressive HIV infection, the half-life of T<sub>CM</sub> cells becomes shorter while that of T<sub>EMRA</sub> cells remains the same. These changes in kinetics are reflected in the composition of the total CD8<sup>+</sup> T cell pool: progressive disease is associated with a loss of T<sub>CM</sub> cells while the fraction and absolute number of T<sub>EMRA</sub> 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<sup>+</sup> 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**

The authors have no financial conflict of interest.

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