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

Kristin Ladell, Marc K. Hellerstein, Denise Cesar, Robert Busch, Drina Boban and Joseph M. McCune


http://www.jimmunol.org/content/180/12/7907

**References**

This article *cites 63 articles*, 23 of which you can access for free at: http://www.jimmunol.org/content/180/12/7907.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
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-7Ra and CD8⁺CD45RA⁺CCR7⁻CD28⁻ RA effector memory (T_EMRA) cells expressing CD87. 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-7Ra⁺ 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, memory (TCM) cells expressing IL-7R α and CD8+CD45RA⁻CCR7⁺CD28⁺ central memory (T_CM) cells expressing IL-7Ra and CD8+CD45RA⁺CCR7⁻CD28⁻ RA effector memory (T_EMRA) cells expressing CD87. 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-7Ra⁺ 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.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

*Division of Experimental Medicine and ¹Department of Medicine, University of California, San Francisco, CA 94110; ²Department of Nutritional Sciences, University of California, Berkeley, CA 94720; and ³KineMed, Inc., Emeryville, CA 94608

Received for publication January 7, 2008. Accepted for publication April 14, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by National Institutes of Health Grant RO1 AI43866 (to M.K.H.) and National Institutes of Health Awards U38 AI43641 and R37 AI03132 (to J.M.M.), who is the recipient of the Burroughs Wellcome Fund Clinical Scientist Award in Translational Research and the National Institutes of Health Director’s Pioneer Award Program, part of the National Institutes of Health Roadmap for Medical Research, through Grant DIPDOD00329. Funding for the optimization of the low-count techniques was provided by KineMed. The Clinical and Translational Science Institute Clinical Research Center was supported by Grant UL1 RR024131-01 from the National Center for Research Resources, a component of the National Institutes of Health, and National Institutes of Health Roadmap for Medical Research.

2 Current address: Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, U.K.

3 Current address: Department of Medicine, University of Cambridge, Cambridge, U.K.

4 Address correspondence and reprint requests to Dr. Joseph M. McCune, Division of Experimental Medicine, Department of Medicine, University of California, 1001 Potrero Avenue, Building 3, Room 601, San Francisco, CA 94110. E-mail address: mike.mccune@ucsf.edu

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

www.jimmunol.org
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 of 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<sub>CM</sub>) 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<sub>EMRA</sub>) 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<sub>NA</sub>) and an increase in the number of circulating memory/effector CD8+ T cells. Labeling studies with deuterium-labeled glucose or water (H<sub>2</sub>O) 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<sub>EM</sub>; CD8+ T cells) and the other longer lived (presumably containing the T<sub>CM</sub> or T<sub>EMRA</sub> cells). In HIV-infected individuals classified as long-term nonprogressors, cells with a T<sub>CM</sub>-like phenotype are maintained 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.

**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-untreated 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-untreated 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 completed 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.

**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% H<sub>2</sub>O (Cambridge Isotope Laboratories) for a 7-wk labeling protocol. During the first week of labeling, subjects drank three vials of 50 ml of 70% H<sub>2</sub>O per day followed by either 2 or 6 wk of two vials of 50 ml of 70% H<sub>2</sub>O 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 H<sub>2</sub>O 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 last 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 H<sub>2</sub>O revealed that H<sub>2</sub>O 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 5% 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 1–16 h. 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 
\(^2\text{H}\) 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 assay 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 
\(^2\text{H}\) 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 molar abundances of the parent ion [M – HF, = M0] (m/z 435) and the M1 mass isotope (m/z 436) of the pentafluorobenzyl triacetyl derivative of deoxyribose. 
\(^2\text{H}\) enrichment was calculated as EM1, the excess fractional abundance of the M1 mass isotope above baseline (as determined by analysis of the unlabeled deoxyribose derivative, with correction for analyte abundance effects; Ref. 39). The EM1 value represents isotope enrichment from \(^2\text{H}\) above natural abundance and is analogous to specific activity values with radioisotopes (33, 42). Data were rejected if the signal to background ratio fell below 10, or if the M0 abundance fell below the abundance range that generated reliable EM1 values with the diluted, labeled standards. 
\(^2\text{H}\) enrichments in body water were calculated as described previously (34, 39) by comparison with standard curves generated by mixing \(^2\text{H}\) with natural abundance \(^2\text{H}\)2O in known proportions.

Calculations of decay constants, half-lives, and percentage of CD8\(^+\) T cells remaining after 7 wk of \(^2\text{H}\)2O labeling

As in previous studies (33, 39), \(^2\text{H}\)2O kinetic calculations during the \(^2\text{H}\) label incorporation phase were based on the precursor-product relationship. In this pulse-chase experiment, the loss of label enrichment in cellular DNA during the delabeling phase was determined for each T cell subset. \(^2\text{H}\) enrichment in cellular DNA at S1 (wk 10, or 3 wk after the last dose of \(^2\text{H}\)2O), representing the baseline value (or pulse) after washout of \(^2\text{H}\)2O from body water pools, was used as the time zero value. This time point was chosen because body water \(^2\text{H}\)2O 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 
\(^2\text{H}\) enrichments measured at wk 18 and wk 10, respectively, and Dt is the time between measurements (8 wk). The half-life was calculated as \(t_{1/2} = \ln(2)/k\). For some T cell subpopulations in some individuals (e.g., \(T_C\) cells in subjects who were HIV-negative), exponential label decay was not observed between S1 and S3 or S2 and S3 (e.g., \(T_C\) cells in three of four HIV-negative subjects). Therefore, we also calculated the percentage of initially incorporated label (i.e., the \(^2\text{H}\) 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.

Table 1. Characteristics of study subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject ID</th>
<th>Age (yr)</th>
<th>VL (copies/ml)</th>
<th>Yr HIV Infected</th>
<th>CD4 Count/µl of Blood</th>
<th>CD8 Count/µl of Blood</th>
<th>Wk of (^2\text{H})2O Labeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV negative</td>
<td>A</td>
<td>27</td>
<td>Negative</td>
<td>–</td>
<td>586</td>
<td>376</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>56</td>
<td>Negative</td>
<td>–</td>
<td>513</td>
<td>292</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>59</td>
<td>Negative</td>
<td>–</td>
<td>555</td>
<td>488</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>64</td>
<td>Negative</td>
<td>–</td>
<td>903</td>
<td>258</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>35</td>
<td>Negative</td>
<td>–</td>
<td>1289</td>
<td>563</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>35</td>
<td>Negative</td>
<td>–</td>
<td>832</td>
<td>246</td>
<td>7</td>
</tr>
<tr>
<td>HIV infected (untreated)</td>
<td>L</td>
<td>41</td>
<td>&lt;75</td>
<td>5</td>
<td>1028</td>
<td>998</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>40</td>
<td>8,278</td>
<td>9</td>
<td>973</td>
<td>1,500</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>40</td>
<td>2,059</td>
<td>NA(^a)</td>
<td>852</td>
<td>3,388</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>52</td>
<td>713</td>
<td>5</td>
<td>784</td>
<td>1,052</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>O</td>
<td>52</td>
<td>&lt;75</td>
<td>17</td>
<td>730</td>
<td>929</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>52</td>
<td>8,469</td>
<td>16</td>
<td>1,657</td>
<td>1,969</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>59</td>
<td>&lt;75</td>
<td>11</td>
<td>642</td>
<td>1,067</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>38</td>
<td>392</td>
<td>13</td>
<td>594</td>
<td>2,079</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>H</td>
<td>41</td>
<td>2,220</td>
<td>NA</td>
<td>532</td>
<td>1,764</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>42</td>
<td>6,897</td>
<td>11</td>
<td>493</td>
<td>884</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>38</td>
<td>30,851</td>
<td>NA</td>
<td>431</td>
<td>1,646</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>U</td>
<td>37</td>
<td>16,212</td>
<td>6</td>
<td>360</td>
<td>578</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>36</td>
<td>448,343</td>
<td>NA</td>
<td>349</td>
<td>2,445</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>41</td>
<td>17,090</td>
<td>11</td>
<td>331</td>
<td>1,067</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>J</td>
<td>53</td>
<td>74,199</td>
<td>NA</td>
<td>330</td>
<td>1,397</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>W</td>
<td>46</td>
<td>32,236</td>
<td>11</td>
<td>277</td>
<td>1,207</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>X</td>
<td>40</td>
<td>15,534</td>
<td>15</td>
<td>170</td>
<td>1,133</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>60</td>
<td>222,000</td>
<td>17</td>
<td>78</td>
<td>586</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Z</td>
<td>47</td>
<td>467,160</td>
<td>21</td>
<td>66</td>
<td>1,281</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) NA. Not available; ND, not determined.
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

FIGURE 1. In vivo stable isotope labeling protocol and gating strategy for T cell phenotyping. A, Long-term ²H₂O label administration. ²H₂O was administered over a period of 7 wk (3 × 50 ml of 70% ²H₂O per day during the first week and 2 × 50 ml of 70% ²H₂O 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$^+$ 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$^+$ (TN), CD45RA$^{bright}$CCR7$^{-}$CD28$^+$ (TEMRA), CD45RA$^{-}$CCR7$^{-}$CD28$^+$ (TCM), CD45RA$^{-}$CCR7$^{-}$CD28$^{-}$ (TEM1) and CD45RA$^{-}$CCR7$^{-}$CD28$^{-}$ (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 Sallustio 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...
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, \( p < 0.00001 \)). There was no significant correlation between T_{EM1} 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 T_{EM1} cells in some HIV-infected subjects as compared with most HIV-negative subjects (median 6.5% IQR 3–11% T_{EM1} cells in HIV-infected subjects vs median 1.6% IQR 0.6–3.2% T_{EM1} 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 (4–2%) T_{EMRA} cells in HIV-negative subjects, \( p < 0.01 \)). 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).

CD3\(^+\)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 studied 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 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 ex-pected 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, how-ever, 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 summa-rized in Table II, along with the number of cells per microliter and the frequency of a given subpopulation among total CD8+ T cells. TEM2 cells, meanwhile, showed a short half-life in both HIV-neg-ative and HIV-infected subjects (58.1 and 49.3 (±19.3) days, re-spectively). 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>
</tr>
</thead>
<tbody>
<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>
</tr>
<tr>
<td>CD45RA+CCR7+CD28+</td>
<td>HIV infected</td>
<td>4.3 ± 1.0</td>
<td>0.0164 ± 0.0022</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>
</tr>
<tr>
<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>
</tr>
<tr>
<td>CD45RA+CCR7+CD28-</td>
<td>HIV infected</td>
<td>–</td>
<td>0.00076</td>
<td>976.2</td>
<td>99.6*</td>
<td>33.2 ± 27.3</td>
<td>8.9 ± 6.4</td>
</tr>
<tr>
<td>CD45RA+CCR7+CD28-</td>
<td>HIV negative</td>
<td>–</td>
<td>0.0069 ± 0.0028</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>
</tr>
<tr>
<td>CD45RA+CCR7+CD28-</td>
<td>HIV infected</td>
<td>4.3 ± 1.0</td>
<td>0.0166 ± 0.0087</td>
<td>51.0 ± 30.4</td>
<td>45.5 ± 27.9</td>
<td>96.7 ± 60.6</td>
<td>4.38 ± 1.2</td>
</tr>
<tr>
<td>CD45RA-CCR7+CD28-</td>
<td>HIV negative</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CD45RA-CCR7+CD28-</td>
<td>HIV infected</td>
<td>4.3 ± 1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

*Means ± SD.
**p < 0.05, ***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.

TEMRA cells express certain cell surface receptors (e.g., IL-7Ra and IL-18Rα) 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 TCM cells express certain cell surface receptors (e.g., IL-7Ra and IL-18Rα) 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

Expression of IL-7Ra, IL-18Rα, 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-18Rα) 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...
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 \((p < 0.01)\) as was the fraction expressing IL-18R1α \((p < 0.05)\) and CD57 \((p < 0.05)\). When these subpopulations were examined in the context of CD4 T cell counts, the percentage of TCM cells positive for IL-7Rα tended to fall as the CD4 count fell. There was a significant decrease in the MFI of CD57 on CD8+ T cells; this decrease was specifically observed on CD8high TEMRA cells \((p = 0.003)\) and a decrease in the percentage of CD8high TEMRA cells that were CD57+ \((p = 0.06)\).

**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. In a previous study using an in vivo labeling technique, 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 H\(^2\)O, it is now possible to take full advantage of the power of state-of-the-art multivariate, 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 \((t_{1/2} = 97.7\) 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. 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 naïve CD8+ T cell progenitors to these cells decline in number as a function of HIV disease progression. 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, 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 TCM cell survival (59). In addition, TCM cells could transition more rapidly and/or in larger numbers to shorter-lived TEM2 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+ TCM compartment is dependent on preservation of CD4+ TCM 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 (TEM1) cells contain CD28+ cells, which may be cells transitioning from TEM to TEM2 with the former being both CCR7+ and CD28+ and the latter lacking expression of both of these receptors. Second, TEM cells in this study do not refer to RA-expressing effector memory T (TEMRA) cells. TEMRA 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 TEMRA subpopulation. In HIV-negative subjects, these cells are low in number and slow in turnover. By contrast, circulating numbers of TEMRA 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-18R1α, but to remain long-lived. Collectively, these observations suggest that TEMRA 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 TEMRA 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 CD57low TEMRA 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 TEMRA cells in controllers vs progressors and, in the previous studies, the fraction of TEMRA 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 TCM 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 bcl6 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-18R1α have been detected within murine hematopoietic stem cells, memory B cells, and memory T cells (16). In our hands, the expression of bcl6 was not higher in memory than in naive T cells (data not shown), and TCM cells were found to express IL-7Rα but only a small fraction (~20%) expressed IL-18R1α. 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 TEM1, TEM2, TEMRA, TCM, and TN cells, in healthy subjects as well as in those infected with HIV. The data show that long-lived CD8+ T cell subpopulations in healthy human subjects include TCM, TEM, and TEMRA cells. In progressive HIV infection, the half-life of TCM cells becomes shorter while that of TEMRA 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 TCM cells while the fraction and absolute number of TEMRA 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.

Acknowledgments

We thank the participants in the study; the nurses at the Clinical and Translational Science Institute Clinical Research Center at San Francisco General Hospital; and Rebecca Hoh, Marcia Smith, Joy Madamba, and Regan Gage at the Positive Health Program, San Francisco General Hospital (San Francisco, CA), for subject selection and recruitment from the SCOPE cohort. We acknowledge Dr. Steven Deeks from the University of California Positive Health Program for advice on the selection criteria of the subjects from the SCOPE cohort and Dr. Jason Barbour from the Positive Health Program for assistance with the statistical analysis. We appreciate the help with cell sorting by Dr. Marty Bigos, Tomasz Polonoski, and Valerie Stepps from the Flow Cytometry Core at the J. David Gladstone Institutes (San Francisco) and by CK Poon from the Flow Cytometry Core at the University of California Division of Experimental Medicine. Lastly, we thank Dr. Claire Emson, Ben Hunrichs, and Bridget McEvoy-Hein of the Department of Nutritional Sciences at University of California for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References


