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A Steady State of CD4+ T Cell Memory Maturation and Activation Is Established during Primary Subtype C HIV-1 Infection

Pholo Maenetje,* Catherine Riou,* Joseph P. Casazza,† David Ambrozak,† Brenna Hill,‡ Glenda Gray,‡ Richard A. Koup,‡ Guy de Bruyn,‡ and Clive M. Gray*

The functional integrity of CD4+ T cells is crucial for well-orchestrated immunity and control of HIV-1 infection, but their selective depletion during infection creates a paradox for understanding a protective response. We used multiparameter flow cytometry to measure activation, memory maturation, and multiple functions of total and Ag-specific CD4+ T cells in 14 HIV-1– and CMV-coinfected individuals at 3 and 12 mo post-HIV-1 infection. Primary HIV-1 infection was characterized by elevated levels of CD38, HLA-DR, and Ki67 in total memory and Gag-specific CD4+ and CD8+ T cells. In both HIV-infected and 15 uninfected controls, the frequency of activated cells was uniformly distributed among early differentiated (ED; CD45RO+CD27−), late differentiated (CD45RO+CD27+), and fully differentiated (CD45RO−CD27−) memory CD4+ T cells. In HIV–1–infected individuals, activated CD4+ T cells significantly correlated with viremia at 3 mo postinfection (r = 0.79, p = 0.0007) and also harbored more gag provirus DNA copies than nonactivated cells (p = 0.04). Moreover, Gag-specific ED CD4+ T cells inversely associated with plasma viral load (r = −0.87, p < 0.0001). Overall, we show that low copy numbers of gag provirus and plasma RNA copies associated with low CD4 activation as well as accumulation of ED HIV-specific CD4+ memory. Significant positive correlations between 3 and 12 mo activation and memory events highlighted that a steady state of CD4+ T cell activation and memory maturation was established during primary infection and that these cells were unlikely to be involved in influencing the course of viremia in the first 12 mo of HIV-1 infection. The Journal of Immunology, 2010, 184: 4926–4935.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: ED, early differentiated; F, female; FD, fully differentiated; Int, intermediate; IQR, interquartile range; LD, late differentiated; M, male; NR, no response; NS, no sample; PID, participant identification number; pVL, plasma viral load.

Materials and Methods

Study participants

Primary HIV-infection cohort. HIV–1–infected individuals were recruited to a longitudinal cohort. All study participants were enrolled from an HIV-negative cohort and tested prospectively for HIV infection every 3 mo. The
time postinfection was estimated as the midpoint of the last Ab-negative and the first Ab-positive ELISA test prior to enrollment. None of the study participants received antiretroviral therapy during the first 12 mo of infection. All participants provided written informed consent for participation in this study. An additional cohort of 15 HIV-negative individuals were used as control subjects and have been described elsewhere (11). The clinical protocols were approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (M050832 and M070249, respectively; Johannesburg, South Africa).

**Plasma viral load and absolute CD4⁺ T cell counts**

Plasma HIV-1 RNA levels were quantified using the COBAS AMPLICOR HIV-1 monitor test version 1.5 (Roche Diagnostic Systems, Somerville, NJ). Absolute blood CD4⁺ and CD8⁺ T cell counts were measured using an FACScalibur flow cytometer (BD Biosciences, San Jose, CA) and expressed as cells/mm³.

**Synthetic subtype C peptides**

A panel of 66 overlapping peptides corresponding to the consensus subtype C sequence was made into a single pool covering the complete region of Gag and resuspended in DMSO (Sigma-Aldrich, St. Louis, MO) as previously described (25). A final peptide concentration of 2 µg/ml/peptide was used with <1% DMSO concentration. A set of 138 peptides (15-mers) overlapping by 11-aa residues corresponding to human CMV pp65 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Programs (Bethesda, MD). All prepared peptides were stored at −80°C prior to use.

**Cell preparation**

PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden), cryopreserved in 90% heat-inactivated FBS (Invitrogen, Paisley, U.K.) plus 10% DMSO, and stored in liquid nitrogen until needed. Thawed PBMCs were washed twice with RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 1.7 mM sodium glutamate (R10). The cells were then resuspended in R10 at 37°C and 5% CO₂ for 2 h in the presence of 10 U/ml DNase I (Roche Diagnostic Systems) prior to use in intracellular cytokine staining assays.

**Cell stimulation and intracellular staining**

Measurement of T cell activation. Thawed PBMCs were washed and resuspended at 2 × 10⁶ cells/ml with R10 and stimulated for 6 h at 37°C and 5% CO₂ with HIV-1 Gag and/or human CMV pp65 peptide pools (2 µg/ml) in the presence of 10 U/ml IL-2. CD28 and anti-CD49d, and 10 µg/ml brefeldin A. After washing, cells were stained with a panel consisting of CD14, CD3 APC-Cy7, CD38 APC, Ki67 FITC (BD Pharmingen, San Diego, CA) for 20 min in the dark at 4°C. The cells were then washed with PBS and surface stained with violet reactive dye (Vivid; Molecular Probes, Eugene, OR) and a mixture of CD27 PE-Cy5, and CD45RO Texas Red-PE (Beckman Coulter, Fullerton, CA) for 20 min in the dark at 4°C. The cells were then washed with PBS containing 1% FBS and 0.1% sodium azide and permeabilized according to the manufacturer’s instructions using a Cytofix/Cytoperm buffer kit (BD Biosciences) and stained intracellularly with IFN-γ and IL-2 PE (BD Biosciences). CD3, CD28, CD38 APC, CD107 Alexa 680 (BD Pharmingen, San Diego, CA), and CD4 PE-Cy5.5. After labeling, cells were washed and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich) and stored at 4°C prior to flow cytometry acquisition within 24 h.

Detection of T cell polyclonality. Under the same conditions as explained above, thawed PBMCs were stimulated for 6 h with or without HIV-Gag C peptides (2 µg/ml), but in the presence of CD107 Alexa 680 (conjugated under standard protocols) and 0.7 µg/ml monensin plus 1 µg/ml anti-CD28, anti-CD49d, and 10 µg/ml brefeldin A. After washing, cells were stained with a panel consisting of CD14 Pacific blue, CD19 Pacific blue, CD27 QD655, CD8 QD655 (all conjugated under standard protocols), CD27 PE-Cy5, and CD45RO Texas Red-PE (Beckman Coulter, Fullerton, CA) for 20 min in the dark at 4°C. The cells were then washed with PBS containing 1% FBS and 0.1% sodium azide and permeabilized according to the manufacturer’s instructions using a Cytofix/Cytoperm buffer kit (BD Biosciences) and stained intracellularly with IFN-γ and IL-2 PE (BD Biosciences). CD3, CD28, CD38 APC, CD107 Alexa 680 (BD Pharmingen, San Diego, CA), and CD4 PE-Cy5.5 (Caltag Laboratories, Burlingame, CA). After labeling, cells were washed and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich) and stored at 4°C prior to flow cytometry acquisition within 24 h.

**Flow cytometry analysis**

Approximately 500,000–1,000,000 events were collected per sample on an LSRII flow cytometer (BD Biosciences). Electronic compensation was conducted with Ab capture beads (BD Biosciences) stained separately with individual mAbs used in test samples. Data was analyzed with FlowJo version 8.8.6 (Tree Star, Ashland, OR). Dead cells (Vivid⁺), monocytes (CD14⁺), and B cells (CD19⁺) were removed from the analysis. Cells were then gated on singlets, live CD3⁺, CD4⁺, CD8⁺, and memory cells, and then on combinations of maturation and activation markers. A positive single cytokine response was defined as >0.06% of memory CD4⁺ T cell responses after background subtraction. This is consistent with other HIV-specific CD4⁺ T cell studies (26). For the Boolean gating analysis to detect multiple cytokine responses, values >0.01% and twice the background were considered as positive after background subtraction. A threshold of 0.01% has been previously applied for the analysis of CD4⁺ T cells producing multiple cytokines (21, 27).

**Cell sorting**

Cell sorting was performed using the method of the modification described by Douek et al. (28). HIV Gag-specific, activated, and nonactivated total memory CD4⁺ T cells were sorted using an FACSAria cell sorter (BD Biosciences) at 70 J/m². Activated cells were defined as cells expressing CD38, Ki67, or HLA-DR, whereas nonactivated cells did not express any of these markers. At least 40 million PBMCs were used for sorting in each experiment, and sorted populations were consistently >98% pure. The instrument setup was performed according to the manufacturer’s instructions. The level of HIV infection of these cells was then determined using real-time PCR to quantify the amount of HIV gag DNA per cell.

**Quantitative real-time PCR**

Immediately after cell sorting, cells were spun down in 1.5 ml polypropylene conical tubes, the supernatant was removed, and they were frozen at −20°C prior to use. Cells were then lysed in 25–100 µl 10 mM Tris buffer containing proteinase K (Qiagen, Valencia, CA). Supernatant (5 µl) was used as input DNA for the quantification of HIV gag DNA using the 5’ nuclelease (TaqMan) assay with an ABI 7500 system (Applied Biosystems, Foster City, CA) (28, 29). HIV gag-DNA degenerate primers and probes were designed in conserved regions of subtype C gag genes found in the Los Alamos HIV sequence database (www.hiv.lanl.gov). The gag C degenerate primers and probes were: gag-forward: 5’-GGGGAATGTGAGYAG-3’, gag-reverse: 5’-GGYCTCTGTTGTATTGCTCA-3’, and probe: 5’-Fam-CTACTGATVACTCTGARGACARATCAGTGA-BHQ1 (Inqaba Biotec, Pretoria, South Africa). For determining the cell number per reaction, quantitative real-time PCR was performed by sequencing nos for albumin copy numbers using primers and probes sequences previously described (28). Absolute quantitation of gag C and human albumin copy numbers were performed using DNA standards and standard curves generated from 10-fold serial dilutions starting at 10⁶ copies. Duplicate reactions were run and template copies calculated using ABI 7500 software (Applied Biosystems).

**Statistical analysis**

Statistical analysis and graphical presentation were performed using GraphPad Prism version 4.0 software (GraphPad, San Diego, CA). Data were expressed as median values and analyzed by the use of nonparametric statistics. Statistical significance was determined using the Mann-Whitney U test, Wilcoxon paired t test, or Kruskal-Wallis ANOVA using Dunn’s test for multiple comparisons. All tests were two-tailed, and a value of p < 0.05 was considered statistically significant. The relationship among the proportions of memory subpopulations, immune activation with absolute CD4 counts, and plasma viral loads were assessed by Spearman rank correlations.

**Results**

**Cohort characteristics and responses to Gag and CMV peptide pools**

All individuals were recruited within 3 mo of a first positive HIV Ab result (see Materials and Methods). Table I shows clinical characteristics of the participants, the majority of whom were women, stratified by change in viral load between 3 and 12 mo postinfection. Two participants (PHR006 and PHR009) were lost to follow-up, and there was no 12 mo viral load (Table I, D). The median reduction of plasma viremia in the group over the first 9 mo of follow-up was −0.27 log₁₀ RNA copies/ml, and median rate of absolute CD4 cell loss was −15 cells/mo (Table I). Viral loads at baseline ranged from 2.6–5.88 log₁₀ RNA copies/ml.
counts and percent of Ag-specific responses to CMV and Gag in
sponded to Gag peptides. Table I also shows the median CD4
PHR011), although one of these showed a positive response to
showed no CD4 response to Gag peptide pools (PHR009 and

providing a variance of 3.28 log_{10} to correlate with cell measure-
ments. One individual (PHR0012) showed an increase in vi-
rema in the first year (Table I,
urements. One individual (PHR0012) showed an increase in vi-

CD45RO+CD27 within CD8 + T cells, which we have previously shown to be
memory CD45RO

phases in memory subsets from HIV-infected and HIV-

CD27dim, late differentiated (LD) memory
2
CD27

CD27

and CD57 (11). We purposely employed a conservative gating
strategy (30–32) to avoid misclassifying cells bearing dim ex-
pression of CD27 or CD45RO in the ED or LD compartment.

Our first level of analysis assessed the proportions of total CD4
and CD8 memory populations. Fig. 1B compares the proportions
of ED-, LD-, and FD-memory CD4 and ED-, LD-, Int-, and FD-
memory CD8 populations between HIV-infected and HIV-
infected controls, in which no differences were identified. We
examined Ag-specific cells using a combined IL-2/IFN-γ readout
and identified distinct differences in maturation profiles between
HIV- and CMV-specific cells in both CD4+ and CD8+ T cells. For
the CD4 compartment (Fig. 1C), there was a significantly higher
proportion of ED-memory and lower proportions of LD-memory
Gag-specific cells relative to CMV-specific cells within the same
individuals (p = 0.0013 and p = 0.0006, respectively), which has
been shown previously (33). We noted from our parallel poly-
functional panel that the majority cytokine response was IFN-γ,
which was 5- and 16-fold greater than IL-2 expression at 3 and
12 mo postinfection, respectively, in all subsets (Supplemental
Fig. 1). The proportions of ED or LD CMV-specific memory pop-
ulations at 3 mo showed no difference between HIV-infected and
HIV-uninfected controls. For the CD8 compartment, the differences
between Gag- and CMV-specific cells were reflected in the Int-
memory cells. The maturation profile of CMV-specific CD8+ T cells
was comparable between HIV-infected and HIV-uninfected con-
trols. The differing numbers of absolute CD4 counts, which may
affect the difference in CMV- or Gag-specific CD4+ memory sub-
sets, were taken into account, and there were no significant differ-
ences in memory subsets between individuals with CD4 counts
below or above 500 cells/μl (not shown). Also taken into consid-
eration were those individuals who went on to control viremia
(Fig. 1B, 1C, Table I, open symbols) and those who had reached a
viral set point (Table I, closed symbols). Overall, these data show
that during primary HIV infection, regardless of the course of vi-
remia, Gag-specific CD4+ and CD8+ T cells possessed a pre-
dominantly ED-memory maturation status compared with CMV-
specific cells.

Defining memory maturation of HIV- and CMV-specific CD4+ T cells at 3 mo post HIV infection

We first wished to quantify the frequency of total and Ag-specific
memory subsets from HIV-infected and HIV-uninfected individuals
after short-term stimulation of isolated PBMCs with CMV and
subtype C-based Gag peptide pools. Using the differentiation
markers CD45RO and CD27, we were able to discriminate four and
five CD4+ and CD8+ T cell populations, respectively. Fig. 1A
shows representative plots of naive CD45RO–CD27−, early
differentiated (ED) memory CD45RO+CD27−, intermediate (Int)
memory CD45RO+CD27dim, late differentiated (LD) memory
CD45RO+CD27+, and fully differentiated (FD) effector memory
CD45RO–CD27− cells. Int memory was a unique population
distinct from naive and effector cells according to levels of CD127
and CD57 (11). We purposely employed a conservative gating
strategy (30–32) to avoid misclassifying cells bearing dim ex-
pression of CD27 or CD45RO in the ED or LD compartment.

Our first level of analysis assessed the proportions of total CD4
and CD8 memory populations. Fig. 1B compares the proportions
of ED-, LD-, and FD-memory CD4 and ED-, LD-, Int-, and FD-
memory CD8 populations between HIV-infected and HIV-
infected controls, in which no differences were identified. We

Table I. Clinical characteristics of the study subjects stratified by viral load differences between 3 and 12 mo postinfection

<table>
<thead>
<tr>
<th>HIV+ Participants</th>
<th>pVL (log_{10} copies/ml)</th>
<th>CD4 cells/mm3</th>
<th>Ag-Specific CD4 Responses (%)</th>
<th>Ag-Specific CD8 Responses (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PID</td>
<td>Age (y)</td>
<td>Sex</td>
<td>pVL 3 mo</td>
<td>pVL 12 mo</td>
</tr>
<tr>
<td>PHR009 24</td>
<td>F</td>
<td>2.60</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PHR008 27</td>
<td>F</td>
<td>3.67</td>
<td>3.88</td>
<td>0.25</td>
</tr>
<tr>
<td>PHR007 20</td>
<td>F</td>
<td>4.15</td>
<td>4.28</td>
<td>0.12</td>
</tr>
<tr>
<td>PHR006 40</td>
<td>F</td>
<td>4.90</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PHR010 32</td>
<td>M</td>
<td>4.15</td>
<td>4.28</td>
<td>0.12</td>
</tr>
<tr>
<td>PHR011 38</td>
<td>M</td>
<td>3.54</td>
<td>5.59</td>
<td>0.25</td>
</tr>
<tr>
<td>PHR004 46</td>
<td>F</td>
<td>5.52</td>
<td>5.26</td>
<td>–</td>
</tr>
<tr>
<td>PHR002 36</td>
<td>F</td>
<td>4.56</td>
<td>4.09</td>
<td>–</td>
</tr>
<tr>
<td>PHR013 26</td>
<td>F</td>
<td>3.43</td>
<td>2.60</td>
<td>–</td>
</tr>
<tr>
<td>PHR015 43</td>
<td>F</td>
<td>4.58</td>
<td>3.95</td>
<td>–</td>
</tr>
<tr>
<td>Median 30</td>
<td>73%</td>
<td>F</td>
<td>4.56</td>
<td>4.09</td>
</tr>
</tbody>
</table>

F, female; IQR, interquartile range; M, male; NR, no response; NS, no sample; PID, participant identification number; pVL, plasma viral load.

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stimulation did not result in increased expression of the activation markers used, our preliminary experiments showed that staphylococcal enterotoxin B stimulus had no effect on upregulating expression of CD38, HLA-DR, and Ki67 on Ag-specific memory CD4+ T cells when compared with no staphylococcal enterotoxin B stimulus. We also showed that the presence of brefeldin A during the 6-h stimulation did not limit the expression of HLA-DR and/or CD38 (data not shown). Fig. 2A shows representative expression plots of the three markers, in which distinct populations of activated cells could be discerned. Fig. 2B confirms that total memory CD4+ and CD8+ T cells from HIV-infected individuals were significantly more activated than total memory CD4+ T cells in HIV-infected individuals and HIV-uninfected controls for expression of CD38 and HLA-DR (p < 0.0005 and p < 0.0001, respectively; data not shown); and 2) Ki67 expression within Gag-specific CD4+ T cells was significantly (p < 0.0005) higher than in Gag-specific CD8+ T cells, indicating that HIV-specific CD4+ T cells have a higher turnover than CD8+ T cells during primary HIV-1 infection. The expression of activation markers on total and Gag-specific CD4+ T cells were unrelated to differences in the absolute number of CD4+ T cells (data not shown). Additionally, the differences in activation status were unrelated to whether HIV-infected individuals went onto control initial viral load or had already reached viral set point (Fig. 2B,2C, open and closed symbols). In summary, these data showed that Ag-specific CD8+ T cells during primary HIV infection were highly activated regardless of being HIV-specific or CMV-specific and that Gag-specific CD4+ T cells were characterized by high surface expression of CD38 and Ki67 expression levels as compared with CMV-specific CD4+ T cells.

Distribution of activation markers within CD4 memory subsets

To identify whether increased CD4 activation was preferentially distributed within a specific memory subset and to understand the relationship between activation and memory maturation, we employed Boolean gating to associate memory maturation...
phenotype with permutations of activation markers. Fig. 3A shows proportions of triple-, double-, and single-positive and triple-negative marker combinations of CD38, HLA-DR, and Ki67 in total and Ag-specific memory cells. A, Representative dot plots showing expression levels of CD38, HLA-DR, and Ki67 in total and Ag-specific CD4+ and CD8+ memory T cells. Comparing frequencies of activation markers in total memory CD4+ and CD8+ T cells from HIV-infected (n = 14) and HIV-uninfected controls (n = 15, 9) (B) and in Ag-specific CD4+ and CD8+ T cells (C). Open circles (○) represent individuals who showed an HIV viral load decline of >0.5 log_{10} RNA copies/ml between 3 and 12 mo post-infection; closed circles (●) represent individuals who showed a viral load change within ±0.5 log_{10} RNA copies/ml between 3 and 12 mo. The open triangle (△) represents the one participant who showed a viral load increase >0.5 log_{10} RNA copies/ml; open squares (□) represent two individuals whose viral evolution could not be determined due to missing viral load data at 12 mo. Statistical comparisons were determined by either Mann-Whitney U or Wilcoxon nonparametric t tests.

Reciprocal associations between CD4+ T cell activation and memory maturation with HIV-1 viral load

To examine the relationship between CD4+ T cell activation status and memory maturation with viral load, we correlated activation and memory profiles with viremia. For activation profiles, Fig. 4A shows a significant positive correlation between the frequency of CD38+HLA-DR+Ki67+ cells across each memory CD4 subset for either CMV or Gag specificities. However, Gag-specific CD4+ T cells were characterized by higher levels of activation relative to CMV-specific cells in HIV-infected individuals, as shown by the significant accumulation of activated cells that express two or three of the activation markers. The population of highly activated cells was equally expanded across ED-, LD-, and FD-memory populations (Fig. 3B), in which no significant differences were found in activation profiles among ED, LD, or FD CD4+ T cells. This is collectively shown in Fig. 3C for ED- or LD-memory cells, in which there was no significant correlation between memory maturation and triple-activated CD4+ T cells.
cells with viremia at 3 mo postinfection \((r = 0.79, p = 0.0007; \text{and} \ r = 0.58, p = 0.035, \text{respectively})\). As expected, when correlating the triple-negative CD4+ T cells (nonactivated cells), there were significant inverse correlations for both total and Gag-specific CD4+ T cells \((p = 0.0018, r = -0.75; \text{and} p = 0.012, r = -0.66, \text{respectively}; \text{data not shown})\). We also were able to show significant positive associations when using either double or single activation marker expression (Supplemental Fig. 2). We wished to understand if there was any grouping of individuals within the correlations who were able to control HIV within the first 12 mo of infection. It was evident (Fig. 4A) that there was a uniform spread of highly activated total and Gag-specific CD4+ T cells regardless of who was subsequently able to spontaneously reduce viremia. HIV, like other lentivirus, can infect both dividing and nondividing cells but requires T cell activation signals (34). To directly test the susceptibility of total memory-activated CD4+ T cells to in vivo HIV infection, we sorted populations of activated (defined by the expression of any of the three activation markers, CD38, HLA-DR, and Ki67 markers, orange corresponds to the frequency of cells expressing two of the three markers (i.e., CD38+HLADR+Ki67+, CD38−HLADR+Ki67+, and CD38−HLADR−Ki67+), yellow represents the frequency of cells expressing at least one of the activation markers (CD38+HLADR−Ki67+, CD38+HLADR+Ki67−, and CD38−HLADR−Ki67)), and green corresponds to the frequency of cells not expressing any of the markers (CD38−HLADR−Ki67−, triple negative). Statistical comparisons were performed in SPICE using permutation analysis of the pie distributions on proportions of single-, double-, triple-positive, and triple-negative cells. The \(p\) values are shown for comparisons between HIV− and HIV+ individuals (A) and Gag-specific and CMV-specific cells in HIV+ individuals (B). C, Correlations between the proportion of Gag-specific triple-positive cells (CD38+HLADR+Ki67+) and the proportion of Gag-specific ED (top panel) or Gag-specific LD (bottom panel).

**FIGURE 3.** Activation profiles of the different CD4+ T cells memory subsets at 3 mo postinfection. Boolean gating analysis was used to assess the activation profile of total (A) and Ag-specific cells (B) in ED-, LD-, and FD-memory CD4+ T cell subsets in HIV-infected \((n = 14)\) and HIV-uninfected \((n = 15)\) controls. The proportion of activated cells in each subset is represented as pie charts in which red corresponds to the frequency of cells expressing all three CD38, HLA-DR, and Ki67 markers, orange corresponds to the frequency of cells expressing two of the three markers (i.e., CD38+HLADR+Ki67+, CD38−HLADR+Ki67+, and CD38−HLADR−Ki67+), yellow represents the frequency of cells expressing at least one of the activation markers (CD38+HLADR−Ki67+, CD38+HLADR+Ki67−, and CD38−HLADR−Ki67), and green corresponds to the frequency of cells not expressing any of the markers (CD38−HLADR−Ki67−, triple negative). Statistical comparisons were performed in SPICE using permutation analysis of the pie distributions on proportions of single-, double-, triple-positive, and triple-negative cells. The \(p\) values are shown for comparisons between HIV− and HIV+ individuals (A) and Gag-specific and CMV-specific cells in HIV+ individuals (B). C, Correlations between the proportion of Gag-specific triple-positive cells (CD38+HLADR+Ki67+) and the proportion of Gag-specific ED (top panel) or Gag-specific LD (bottom panel).
3 mo postinfection for different combinations of CD107, IFN-γ, IL-2, MIP-1β, and TNF-α. It has been shown for CD8+ T cells that the same combination of multifunctionality is related to viral control (35, 36), and thus, we wished to explore whether such an association existed with multifunctional CD4+ T cells. Fig. 4D shows that at 3 mo postinfection there was an equal multifunctional profile (cells producing two or more cytokines simultaneously) between ED- and LD-memory Gag-specific CD4+ T cells. Taken together, these data show that ED- and LD-memory Gag-specific CD4+ T cells possess the same multifunctional profile, and the significant association of ED-memory with low viremia is independent and distinct from the activation and multifunctional nature of CD4+ T cell profiles.

Steady state of CD4+ T cell activation and memory maturation over time

To identify whether the status of CD4 activation or memory maturation at 3 mo postinfection may have reached a steady state or set point (2), we correlated the frequency of activated and ED-memory CD4+ T cells at 3 and 12 mo postinfection. We defined a steady state as the frequency of cells remaining within 20% variation between two time points post HIV infection. To identify whether activation and memory maturation had reached such a steady state or may have had a role in determining the course of viremia, we grouped participants into those having a viral load decline of >0.5 log10 RNA copies/ml and those who fell within a 60.5 log10 variation (Table I). Fig. 5A, 5B show significant positive correlations between 3 and 12 mo measurements of triple CD38+HLADR+Ki67+ (r=0.84, p=0.003) and ED-memory (r=0.94, p=0.0003) CD4+ T cells. These data suggest that the activation and ED-memory status of CD4+ T cells made at 3 mo postinfection had reached a steady state early during primary infection for the duration of the study period and that these cells are unlikely to determine the course of viremia. When we performed a similar analysis looking at the polyfunctional profile of Gag-specific CD4+ T cells (i.e., cells producing two or more cytokines simultaneously), we found that there was no significant association between measurements made at 3 and 12 mo, although there was a negative trend to less functionality at 12 mo. This trend disappeared when we made more stringent criteria of cells able to produce three to four cytokines per cell (data not shown). In Fig. 5C, when accounting for those individuals who were able to subsequently reduce viral loads (open symbols), there was a uniform spread of polyfunctional CD4+ T cells from these
**FIGURE 5.** Relationship between activation and proportions of ED total memory CD4+ T cells at 3 and 12 mo postinfection. A, Correlation between the proportions of triple-activated (CD38+HLADR+Ki67+) total memory CD4+ T cells at 3 and 12 mo postinfection. B, Correlation between the proportions of ED-memory CD4+ T cells at 3 and 12 mo postinfection. C, Correlation between the proportions of Gag-specific CD4+ T cells producing at least two cytokines at 3 and 12 mo postinfection; closed circles (●) represent individuals who showed a viral load change within ±0.5 log_{10} RNA copies/ml between 3 and 12 mo postinfection. The open triangle (▲) represents the one participant who showed a viral load increase >0.5 log_{10} RNA copies/ml. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation.

Discussion

The challenge of seeking what may constitute an anti-HIV protective function in the CD4+ T cell compartment is that these cells undergo early activation and depletion during infection, which is considered a clinical hallmark of immunopathogenesis and immunosuppression. We studied the behavior of CD4 cells during primary HIV-1 infection in antiretroviral-naive individuals to examine the balance between CD4+ memory maturation and activation and the association among activation, memory status, and viremia. Our central question was whether memory or activation status of HIV-specific CD4+ T cells had any impact on viral load and possibly infectivity. However, it remains to be determined if similar observations hold true for the CD8 compartment (48). Upon sorting activated CD4+ T cells, we were able to show that these cells were more susceptible to in vivo HIV infection, although we were unable to determine which memory subset was preferentially infected, due to lack of material. Prior studies have shown that memory CD4+ T cell subsets (49) and CD4+CD57+ cells (29) are more preferentially infected by HIV in vivo and support the notion that ED-activated cells may be susceptible targets. Collectively, these data confirm that CD4 activation events are directly proportional to viral load and possibly infectivity. However, it remains to be resolved whether activated CD4+ T cells are harbors of viral pools or whether higher levels of viral replication are causing CD4 activation, of which either or both scenarios would result in the significant correlations we observed.

Jointly, our observations represent an apparent paradox, assuming that memory maturation in the CD4 compartment is thought to be linked with cell activation. The conundrum is that low viremia, and possibly viral control, is associated with the maintenance of Gag-specific ED CD4+ memory T cells, of which
almost half are activated and likely to be susceptible to infection. There are four possible scenarios that could explain these observations: 1) there is preferential infection and depletion of activated ED- or LD-memory populations of CD4+ T cells, giving an apparent equal distribution of activation markers across cell subsets; 2) ED CD4 memory T cells, even in an activated state, are more resistant to HIV infection; 3) there is no causative link between ED CD4 memory T cells and control of viral replication; and 4) activation and memory differentiation are independent events. Although there is evidence to show that ED-memory CD4+ T cells have a higher survival potential (50, 51), it is also likely that these cells may be preferentially infected. Whatever the scenario, it is unlikely that activation events per se push CD4 memory maturation.

We propose, from our data, that the inverse association between HIV-specific ED CD4+ memory T cells and viral load is a reflection of Ag load and not a determining factor. This was supported when we found strong associations between 3 and 12 mo activation and memory maturation phenotypes, suggesting that levels of both activation and memory status were more a reflection of pre-existing and established events prior to the analysis and unlikely to be determining levels of viral replication. This appeared to be independent of the course of viral loads in which, in some individuals, there was spontaneous control of viremia despite possessing individuals of highly activated CD4+ T cells. The simplest interpretation from our data is that the dynamics of CD4+ T cell activation and memory maturation are determined by Ag load, and the course of viremia over time is unrelated to these events. Whatever the mechanisms, it was clear from our data that activation and memory status within the individuals studied had reached a steady state at some point during primary infection.

To address whether ED-memory are more polyfunctional than LD-memory cells and which may partly account for viral control, we assessed a five-functional profile that has been associated with viral control when applied to CD8+ T cells (35). As with the activation status of cells, we found that the polyfunctional nature of HIV-specific CD4+ T cells was equal between ED- and LD-memory cells, and the proportions of CD4+ T cells possessing polyfunctional characteristics did not strongly associate between 3 and 12 mo. Although the lack of temporal association was most likely due to loss of CD4 function over time, it was possible that the tools used to dissect differences between ED- and LD-memory were not sufficiently fine-tuned to discriminate functional differences and that the identity of CD4 function may not be as simple as translating those used to assess CD8 function. Casazza et al. (45) have shown, using a similar phenotype panel, that the multifunctional nature of CMV-specific CD4+ T cells increased with memory maturation, and that, in turn, was greater than HIV-specific CD4+ T cells. The latter study was performed in chronic infection, and it is possible, judging from our results, that the multifunctional nature of HIV-specific CD4+ T cells diminishes during primary infection, regardless of memory maturation phenotypes.

In conclusion, our data show that low viral load associated with both low activation levels and maintenance of ED HIV-specific CD4+ T cells. On closer examination, there was a steady state of CD4 activation and memory maturation profiles, regardless of viral load changes over time, suggesting that neither activation nor memory status was influencing the course of viremia in the first year of HIV infection in this cohort.

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Disclosures

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References
