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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*  

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IV-1 infection is characterized by generalized immune activation (1, 2), and the hyperactivation of T cells may accelerate HIV disease progression (3, 4). Immune activation in HIV-1 infection may be either a direct consequence of HIV Ag load or a consequence of exposure to other pathogens, such as bacteria translocating from the gut (4–6), or from endemic coinfections (7). Whether this occurs to the same degree in acute and primary infection is unclear. Nevertheless, the resulting persistent activation of the immune system is accompanied by loss of peripheral CD4+ T cells (2, 4, 8, 9) and a skewing of CD8+ T cell differentiation to a more mature memory phenotype that would lead to accumulation of effector cells and premature terminal differentiation (10, 11). For example, persistent exposure to HIV-1 drives the differentiation of central memory HIV-specific IL-2–producing CD4+ T cells to an IFN-γ–producing effector memory phenotype, and this latter phenotype has been associated with higher levels of HIV viremia (12–14). It is thus important to understand the effect of immune activation on the maturation and functionality of T cell memory and specifically within the CD4 compartment.

Until recently, there has been little focus on unraveling the relationship between activation and maturation of HIV-specific CD4+ T cell memory with viral control. HIV-specific CD4+ T cells have been shown to play an important role in maintaining functional HIV-specific CD8+ T cell responses (15, 16) and control of viremia during chronic HIV infection (17–19). The maintenance and preservation of HIV-specific CD4+ T cells endowed with the ability to produce multiple cytokines in individuals also coincides with apparent protective immunity against HIV (20–22). However, individuals with progressive HIV disease still exhibit significant numbers of cytokine-producing HIV-specific CD4+ T cells (23, 24), implying that the causative link between HIV-specific CD4+ T cell responses and viral control remain to be resolved.

To explore the association of CD4+ T cells with in vivo viral replication, we examined the association among markers of memory maturation, activation, and polyfunction in total and HIV-specific CD4+ T cells in a prospective cohort of recently HIV-infected individuals in South Africa. We show that profiles of CD4+ T cell memory maturation and activation reach an established steady state early after HIV-1 infection and are unlikely to be related to control of viremia.

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

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The online version of this article contains supplemental material.
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 cells were measured using an FACS Calibur 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 were made into a single pool covering the complete region of the gag gene (11). Synthetic subtype C peptides were used as control subjects and have been described elsewhere (11). The gag nucleotide sequence was determined for each individual and used for the calculation of the number of peptide copies using primers and probe sequences previously described (28).

**Cell preparation**

PBMCs were isolated by standard ficoll-hypaque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden), cryopreserved in 90% heat-inactivated FBS (Invitrogen, Paisely, U.K.) plus 10% DMSO, and stored at −80°C prior to use.

**Cell sorting**

PBMCs were isolated by standard ficoll-hypaque density gradient centrifugation (Amersham Pharmacia, Uppsala, Sweden), cryopreserved in 90% heat-inactivated FBS (Invitrogen, Paisely, U.K.) plus 10% DMSO, and stored at −80°C prior to use. thawed PBMCs were washed twice with RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 1.7 mM sodium glutamate (R10). The cells were then resuspended at 2 × 106 cells/ml with R10 and stimulated for 6 h at 37°C and 5% CO2 with HIV-1 C Gag and/or human CMV pp65 peptide pools (2 μg/ml) in the presence of 1 μg/ml oC28 and oC49d costimulatory Abs (BC Biosciences) and 10 μg/ml brefeldin A (Sigma-Aldrich, St. Louis, MO). A negative control containing PBMCs and costimulatory Abs from the same subject, but without the peptide mix, was also included for each assay. Following stimulation, cells were washed with PBS and surface stained with violet reactive dye (Vivid; Molecular Probes, Eugene, OR) and a mixture of mAbs containing HLA-DR Alexa 680, CD14 Pacific blue, CD19 Pacific blue, anti-CD28, anti-CD49d, and 10 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, CD57 QD565, CD28 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). CD28 APC-Cy7, anti-CD4 APC, PE-CD3 (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.

**Detection of T cell polygonal function.** 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, CD57 QD565, CD28 QD655, CD27 PE-Cy5, and CD45RO Texas Red, and the viability violet reactive dye. Following incubation, cells were washed and permeabilized using the Cytofix/Cytoperm buffer kit (BD Biosciences) and then stained intracellularly with CD3 APC-Cy7, IFN-γ FITC, IL-2 APC, MIP-1α PE, TNF-α PE-Cy7 (BD Pharmingen), 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.

**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 (Vivid4), 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**

Immediately after cell sorting, cells were spun down in 1.5 ml polystyrene 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 protease K (Qiagen, Valencia, CA). Supernatant (5 μl) was used as input DNA for the quantification of HIV gag-DNA using the 5′ nuclease (TaqMan) assay with an ABI 7500 system (Applied Biosystems, Foster City, CA). A single Ct value on each sample was used as the measure for 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 sequence were: gag-forward: 5′-GGGGAAGTGAYA-GACGTATCAGGAG-3′, gag-reverse: 5′-GGYCCCTGTYTTAATGTCCAA-3′, and probe: 5′-Fam-CTACTGATVCCTTCATGACARATCATGATGAG-BHQ1 (Inqaba Biotec, Pretoria, South Africa). For determining the cell burdens in the reaction, quantitated real-time PCR was performed on combinations of maturation and activation 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.

**Quantitative real-time PCR**

Immediately after cell sorting, cells were spun down in 1.5 ml polystyrene 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 protease K (Qiagen, Valencia, CA). Supernatant (5 μl) was used as input DNA for the quantification of HIV gag-DNA using the 5′ nuclease (TaqMan) assay with an ABI 7500 system (Applied Biosystems, Foster City, CA). A single Ct value on each sample was used as the measure for 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 sequence were: gag-forward: 5′-GGGGAAGTGAYA-GACGTATCAGGAG-3′, gag-reverse: 5′-GGYCCCTGTYTTAATGTCCAA-3′, and probe: 5′-Fam-CTACTGATVCCTTCATGACARATCATGATGATGAG-BHQ1 (Inqaba Biotec, Pretoria, South Africa). For determining the cell burdens in the reaction, quantitated real-time PCR was performed on combinations of maturation and activation 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.

**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, □). 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/ml (Table I). Viral loads at baseline ranged from 2.6–5.88 log10 RNA copies/ml.
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/mm^3</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>3 mo</td>
<td>12 mo</td>
</tr>
<tr>
<td>PHR009 24 F</td>
<td>2.60</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PHR006 40 F</td>
<td>4.90</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PHR012 34 M</td>
<td>4.09</td>
<td>4.87</td>
<td>0.78</td>
<td>△</td>
</tr>
<tr>
<td>PHR014 38 M</td>
<td>5.34</td>
<td>5.59</td>
<td>0.25</td>
<td>●</td>
</tr>
<tr>
<td>PHR008 27 F</td>
<td>3.67</td>
<td>3.88</td>
<td>0.21</td>
<td>●</td>
</tr>
<tr>
<td>PHR010 32 M</td>
<td>4.15</td>
<td>4.28</td>
<td>0.12</td>
<td>●</td>
</tr>
<tr>
<td>PHR007 20 F</td>
<td>2.60</td>
<td>2.60</td>
<td>0.00</td>
<td>●</td>
</tr>
<tr>
<td>PHR005 32 F</td>
<td>4.75</td>
<td>4.55</td>
<td>–0.20</td>
<td>●</td>
</tr>
<tr>
<td>PHR004 46 F</td>
<td>5.52</td>
<td>5.26</td>
<td>–0.27</td>
<td>●</td>
</tr>
<tr>
<td>PHR001 21 F</td>
<td>4.56</td>
<td>3.99</td>
<td>–0.57</td>
<td>○</td>
</tr>
<tr>
<td>PHR011 38 M</td>
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<td>4.09</td>
<td>–0.67</td>
<td>●</td>
</tr>
<tr>
<td>PHR003 21 F</td>
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<td>4.42</td>
<td>–0.81</td>
<td>○</td>
</tr>
<tr>
<td>PHR014 38 M</td>
<td>3.43</td>
<td>2.60</td>
<td>–0.82</td>
<td>○</td>
</tr>
<tr>
<td>PHR002 34 F</td>
<td>3.48</td>
<td>2.60</td>
<td>–0.88</td>
<td>○</td>
</tr>
<tr>
<td>PHR015 43 F</td>
<td>5.88</td>
<td>3.95</td>
<td>–1.93</td>
<td>△</td>
</tr>
<tr>
<td>Median 30 F</td>
<td>5.61</td>
<td>4.09</td>
<td>–0.27</td>
<td>○</td>
</tr>
</tbody>
</table>

IQR – 3.58–5.07 3.88–4.55 –0.81–0.12 457–772 391–614 –19–5 0.13–0.26 0.14–0.21 0.22–0.37 0.1–0.72

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

Table I

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Providing a variance of 3.28 log_{10} to correlate with cell measurements. One individual (PHR0012) showed an increase in viremia in the first year (Table I, △); six individuals showed a change of ±0.5 log_{10} RNA copies/ml and were considered as having reached a set point (Table I, ○), and six individuals showed reduced RNA copies/ml below 0.5 log_{10} change between 3 and 12 mo (Table I, ○). In terms of cellular responses, two patients showed no CD4 response to Gag peptide pools (PHR009 and PHR011), although one of these showed a positive response to CMV. Fifteen HIV-uninfected individuals were used as control subjects, and they all responded to CMV peptide pools; none responded to Gag peptides. Table I also shows the median CD4 counts and percent of Ag-specific responses to CMV and Gag in both CD4^+ and CD8^+ T 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^+CD27^dim, late differentiated (LD) memory CD45RO^+CD27^- , and fully differentiated (FD) effector memory CD45RO^-CD27^- cells. Int memory was a unique population within CD8^+ T cells, which we have previously shown to be 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 expression 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-uninfected 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 polyfunctional 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 populations 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- and LD-memory populations (p = 0.00014 and p = 0.0008, respectively), in which there was a higher proportion of the Int-memory cells. The maturation profile of CMV-specific CD8^+ T cells was comparable between HIV-infected and HIV-uninfected controls. The differing numbers of absolute CD4 counts, which may affect the difference in CMV- or Gag-specific CD4^+ memory subsets, were taken into account, and there were no significant differences in memory subsets between individuals with CD4 counts below or above 500 cells/μl (not shown). Also taken into consideration 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 viremia, Gag-specific CD4^+ and CD8^+ T cells possessed a predominantly ED-memory maturation status compared with CMV-specific cells.

Activation profiles of HIV- and CMV-specific CD4^+ T cells at 3 mo post HIV infection

To understand the profile of activated T cells during primary HIV infection, we first assessed the activation status of total memory and Ag-specific CD4^+ and CD8^+ T cells using a combination of Ki67, HLA-DR, and CD38 markers. To ensure that short-term
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 Ki67 on Ag-specific memory CD4+ T cells. Additionally, the differences in 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).

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 was significantly 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).

**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 and HLA-DR and intracytoplasmic expression of 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, • (B) and in Ag-specific CD4+ and CD8+ T cells (○). Open circles (○) represent individuals who showed a viral load decline of >0.5 log10 RNA copies/ml between 3 and 12 mo post-infection; closed circles (•) represent individuals who showed a viral load change within ±0.5 log10 RNA copies/ml between 3 and 12 mo. The open triangle (△) represents the one participant who showed a viral load increase >0.5 log10 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.
cells with viremia at 3 mo postinfection (\( r = 0.79, p = 0.0007; \) and \( r = 0.58, p = 0.035, \) 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 (\( p = 0.0018, r = -0.75; \) and \( p = 0.012, r = -0.66, \) respectively; 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, Ki67, or HLA-DR, and CD45RO+) and nonactivated (CD38-HLA-DR-Ki67- and CD38-HLA-DR+Ki67-) memory CD4+ T cells and quantified the number of gag proviral DNA copies/cell in the sorted populations. Fig. 4B shows that activated cells possessed significantly higher quantities of gag proviral copies when compared with sorted nonactivated cell fractions. Although found at very low frequency of CD4+ T cells (0.15 gag copies/cell maximum), these data directly show that activated total memory CD4+ T cells are preferred targets for in vivo HIV infection and that activated memory CD4+ T cells support ongoing viral replication.

For memory maturation, Fig. 4C shows a significant negative correlation between the frequency of Gag-specific ED-memory CD4+ T cells with viremia at 3 mo postinfection (Fig. 4C; \( r = -0.87, p < 0.0001 \)) and a positive correlation with LD-memory cells (\( r = 0.85, p = 0.0002; \) not shown). Similarly, accounting for those individuals who were able to subsequently reduce viral loads in the first 12 mo of infection (Fig. 4A, 4C, open symbols), there was a uniform spread of total and Gag-specific ED- and LD-memory CD4+ T cells, suggesting that frequencies of these cell populations were not determining the trajectory of viremia. Of note, the proportion of total memory CD4+ ED or LD T cells did not associate with viral load.

**Multifunctional profile of Gag-specific ED- and LD-memory CD4+ T cells at 3 mo postinfection**

To explore the possibility that CD4+ T cell multifunctionality may have played a role in viral control rather than memory maturation, we compared ED- and LD-memory Gag-specific CD4+ T cells at
3 mo postinfection for different combinations of CD107, IFN-γ, IL-2, MIP-1β, TNF-α, CD3, CD4, CD8, CD45RO, and CD27. Using Boolean gating, the distribution of cells presenting any combination of functional profiles was determined for ED- and LD-memory cells. Cells expressing two or more cytokines simultaneously were considered as multifunctional. Open circles (○) represent individuals who showed an HIV viral load decline of >0.5 log_{10} RNA copies/ml between 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. The open triangle (△) represents the one participant who showed a viral load increase >0.5 log_{10} RNA copies/ml, and open squares (□) represent two individuals whose viral evolution could not be determined due to missing viral load data at 12 mo. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation.

FIGURE 4. Correlations between memory CD4+ T cell activation profiles and memory subsets with viral load at 3 mo postinfection. A, Correlations between the proportion of CD38+HLADR+Ki67+ in total memory and Gag-specific CD4+ T cells with viral load at 3 mo. B, Differences in gag DNA copies/10^3 CD4+ T cells between nonactivated (CD38+HLADR−Ki67−) and activated (cells expressing at least one of the activation Ags) total memory CD4+ T cells at 3 mo postinfection (n = 8). Each symbol represents the average of three measurements performed independently. Statistical significance was determined by Wilcoxon signed-rank t test. C, Correlations between the proportion of ED-memory cells in total memory and Gag-specific CD4+ T cells with viral load at 3 mo. D, Comparison of proportion of multifunctional Gag-specific CD4+ T cells between ED- and LD-memory cells. PBMCs from HIV-infected individuals (n = 14) at 3 mo postinfection were stimulated with Gag peptide pools for 6 h and labeled with Abs against CD107, IFN-γ, IL-2, MIP-1β, TNF-α, CD3, CD4, CD8, CD45RO, and CD27. Using Boolean gating, the distribution of cells presenting any combination of functional profiles was determined for ED- and LD-memory cells. Cells expressing two or more cytokines simultaneously were considered as multifunctional. Open circles (○) represent individuals who showed an HIV viral load decline of >0.5 log_{10} RNA copies/ml between 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. The open triangle (△) represents the one participant who showed a viral load increase >0.5 log_{10} RNA copies/ml, and open squares (□) represent two individuals whose viral evolution could not be determined due to missing viral load data at 12 mo. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation.

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 log_{10} RNA copies/ml and those who fell within a ±0.5 log_{10} 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
provide evidence that ED HIV-specific memory CD4+ T cells are 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 maturation in the CD4 compartment is likely related to time of HIV versus CMV exposure (45). In fact, the wide range of HIV loads at 3 mo postinfection allowed us to associate ED- and LD-memory CD4+ T cells with viral replication. By so doing, we identified an imbalance toward ED HIV-specific memory T cells with low viremia, with LD CD4+ memory T cells associating with high viremia. These data would appear consistent with previous reports suggesting that high numbers of less differentiated HIV-specific CD4+ T cells would favor a better clinical outcome (46, 47). When we looked at memory, our data showed that HIV-specific CD4 memory had a predominantly ED phenotype during primary infection relative to CMV-specific CD4+ T cell subsets. Memory phenotypes of Ag-experienced CD4+ T cells is related to Ag exposure and persistence (44), and the relative ED Gag-specific CD4+ T cells is likely related to time of HIV versus CMV exposure. This notion may be supported by recent data showing that Gag-specific CD4+ T cells were more mature than CMV-specific cells in chronic infection (45). 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 stage of CD8 memory maturation. In our current work, we wished to ask whether a population of ED CD4+ T cells would also associate with viral control and whether activation events may be the driving force behind memory differentiation in the CD4 compartment and with the inability to control viral load. It is clear from studies in Sooty Mangabeys that attenuated immune activation most probably protects the natural host of SIV from progression to AIDS (37) and is probably related to no or little microbial translocation that would lead to systemic hyperimmunity activation (6, 38) and increased viral replication (39). In humans, the extensive immune activation during chronic infection is thought to be the driver of pathogenesis, in which there is either microbial translocation of bacterial DNA and LPS into the lymphatic circulation (40, 41) or coinfections with multiple endemic pathogens (7), resulting in nonspecific hyperactivation of T cells (42, 43). Whether this occurs during primary or acute infection and whether high levels of viral replication during the initial stages of HIV infection can result in Ag-specific immune activation is not clear. One fundamental question is whether immune activation during primary HIV infection causes an imbalance in CD4+ T cell memory lineage.

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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 ED 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 decline of >0.5 log10 RNA copies/ml between 3 and 12 mo postinfection; open circles (○) represent individuals who showed a viral load increase >0.5 log10 RNA copies/ml. Statistical associations were performed by a two-tailed nonparametric Spearman rank correlation.
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 populations 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 polyfunctional 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 polyfunctional 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

The authors have no financial interests of interest.

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