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Altered EBV Viral Load Setpoint after HIV Seroconversion Is in Accordance with Lack of Predictive Value of EBV Load for the Occurrence of AIDS-Related Non-Hodgkin Lymphoma

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In contrast to the situation in the post-transplant setting, in HIV-infected individuals an elevated EBV load is not predictive of EBV-related malignancies. To study whether a high EBV load is already a normal situation early in HIV infection and is not related to a decrease in immune function over time, we investigated EBV load and EBV-specific CD8 T cells ~1 year before and 1 year after HIV seroconversion. EBV load significantly increased after HIV seroconversion from 205 to 1002 copies/10⁶ PBMC (p < 0.001), whereas no further increase in EBV load was observed between 1 and 5 years after HIV seroconversion (median, 1827–2478 copies/10⁶ PBMC; p = 0.530). Interestingly, the absolute number of EBV lytic epitope, RAKFKQLL-specific CD8⁺ T cells increased over HIV seroconversion (4.78 to 9.54/µl; p = 0.011). Furthermore, the fraction of CD27-negative effector, RAK-specific CD8⁺ T cells tended to increase (from 12.2 to 17.31% CD27⁻; p = 0.051), in accordance with Ag-driven differentiation. In conclusion, both virological and immunological data support the idea that a new EBV viral setpoint is reached early in HIV infection, probably by EBV reactivation, as suggested by the preferential increase in EBV lytic epitope-specific CD8⁺ T cells. These data may thus help to explain the lack of predictive value of EBV load for the occurrence of AIDS-related lymphoma.


The EBV is a widespread human γ-herpesvirus. Primary infection with EBV is usually asymptomatic, but can cause infectious mononucleosis when occurring in adolescence (1). After primary infection the virus persists for life in a latent form in resting memory B cells (2, 3). As in many viral infections, CD8⁺ T cells are thought to play a major role in the control of both primary infection as well as subsequent reactivation of the virus from the latently infected B cell pool (4, 5). In immunosuppressed individuals, a combination of factors, including inappropriate immune control of the virus, may lead to lymphoproliferative disorders. In HIV-infected individuals, the incidence of non-Hodgkin lymphomas (NHL) is considerably increased compared with that in healthy individuals, and ~75% of these lymphoma are EBV positive (6). Most EBV-positive AIDS-NHL are either systemic or primary CNS lymphoma (7). During the acute phase of primary EBV infection, which is accompanied by an elevated EBV load in both infectious mononucleosis and asymptomatic EBV seroconversion (8, 9), most CD8⁺ T cells are directed against EBV early lytic proteins, whereas during the transition to latent infection, relatively more CD8⁺ T cells are directed toward latent Ags (10, 11).

Latent EBV infection is characterized by a tightly regulated number of infected B cells in the peripheral blood, with <10% variation over a period of years in healthy individuals (12), and generally a viral load of <0.5–1×10⁶ copies/10⁶ PBMC (13). Cross-sectional studies have shown that in transplant recipients and AIDS patients with lymphoproliferative disorders, EBV load at diagnosis was much higher than that in healthy individuals (4×10⁶ to >1.5×10⁶ and >2×10⁶ EBV copies/10⁶ PBMC, respectively) (13, 14). In the post-transplantation setting, an increased EBV load is highly predictive of EBV-related malignancies (13, 15). In contrast, retrospective studies have shown that in HIV-infected individuals, EBV load is high and fluctuates regardless of the subsequent development of EBV-positive lymphoproliferative disorders (16, 17). Although the number of EBV copies increases over time in patients progressing to AIDS-related NHL, there is no correlation between absolute EBV load and the occurrence of NHL, in contrast to the situation in transplant patients. Individuals progressing to EBV-positive NHL were not found among those with the highest EBV load (16).

In HIV-infected individuals who do not progress to EBV-related malignancies, EBV-specific CD8⁺ T cells are relatively well preserved (18, 19). CD8⁺ T cell function, as measured by the ability to secrete IFN-γ in short term Ag-specific stimulation assays, is lower than that in healthy individuals (20) and collapses completely in individuals who subsequently develop malignancies, suggesting that EBV is still under control during HIV infection in most individuals. Recent data suggest that also in the transplantation setting in both solid organ as well as stem cell transplantation, a high EBV load is not necessarily associated with progressive viral disease as long as a sufficient EBV-specific CD8⁺ T cell response is present (21, 22). To explain why EBV load is increased in HIV-infected individuals despite sufficient immune control to

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3 Abbreviations used in this paper: NHL, non-Hodgkin’s lymphoma; LTNP, long term nonprogressor; FLR, FLRGRAYGL; RAK, RAKFKQLL.
avoid malignancies, other factors could be involved. HIV infection is associated with a state of high immune activation (23, 24), in which both acute and chronic activation of B cells (25) may lead to an increase in EBV replication and an elevated EBV load.

We therefore hypothesized that after HIV seroconversion the EBV viral setpoint, defined as an equilibrium between virus and immune response, may be altered, resulting in an elevated EBV load in combination with an apparently adequate CD8\(^+\) T cell response. To investigate whether such a new balance is reached after HIV seroconversion, we studied both EBV load and EBV-specific CD8\(^+\) T cells before and after seroconversion for HIV.

Materials and Methods

Study population

This study was performed on participants of the Amsterdam Cohort studies on AIDS and HIV-1 infection. Blood samples from these homosexual men at risk for HIV-1 infection were collected every 3 mo for HIV-1 serology and immunological studies. In addition, at all time points PBMC were cryopreserved.

In a cross-sectional analysis, we studied EBV load in 61 HIV-1-seronegative homosexual men and 26 homosexual men who remained HIV-1 seronegative over time. The HIV-1-positive homosexual men and 26 homosexual men who remained HIV-1 negative had too short a follow-up time to enable classification as an equilibrium between virus and host immune response (longitudinal analysis).

In a longitudinal analysis we measured EBV load in 36 cohort participants who seroconverted for HIV during follow-up. Among the 36 seroconverters for HIV, 26 were progressors to AIDS, five were LTNP, and five had too short a follow-up time to enable classification in either of these two categories. In all 36 individuals, EBV load was measured 1–3 years before and 1 year after seroconversion for HIV. In 13 of these individuals we could also measure EBV load at 5 years post-HIV seroconversion. Based on HLA type and availability of PBMC, we were able to measure EBV-specific CD8\(^+\) T cells in 16 of these individuals.

The characteristics of the individuals under (longitudinal) study are summarized in Table I. None of the individuals studied received highly active antiretroviral therapy before or at the time points studied.

Lymphocyte isolation and DNA extraction

PBMC were isolated from heparinized blood by Ficoll-Hypaque density centrifugation, and 1\(\times\)10\(^5\) cells were lysed by addition of L6 lysis buffer. Genomic DNA was extracted by precipitation with isopropanol, washed twice with 70% ethanol, and dissolved in distilled H\(_2\)O.

Real-time quantitative PCR assay for measurement of EBV load in PBMC

DNA from 2\(\times\)10\(^5\) cells was amplified in duplicate using PCR primers specific for the nonglycosylated membrane protein BNRFL p143 (27). Real-time PCR amplification was performed as previously described (16, 16, 28), using a fluorogenic probe (PE Biosystems, Nieuwekerk aan de IJssel, The Netherlands) to detect the 74-bp product. Amplification and detection were performed using an ABI PRISM 7700 Sequence detector (PE Biosystems). Real-time measurements were taken, and a threshold cycle value was calculated for each sample by determining the point at which the fluorescence exceeded a threshold limit of 0.10. The detection limit of this assay was initially reported as 50 copies/10\(^6\) PBMC; however, values >10 copies/10\(^6\) PBMC were included when <10% variation was observed between duplicates and otherwise scored as 0. Values below the detection limit were included in the statistics.

As a control for the input DNA and to compensate for differences between samples, for each sample the amount of \(\beta\)-albumin DNA, a house-hold gene present at two copies per cell, was also determined. The forward primer was F-ALB (5'-TGA.AAC.ATA.CGT.TCC.CAA.AGA.GTT.T-3') and the reverse primer was R-ALB (5'-CTC.TCT.TCC.TCA.GAA.AGT.GTT.CAT-AT-3'). The fluorogenic probe was 5'-TGC.TGA.AAC.ATT.CAC.CTT.CCA.TGC.AGA-3', with a FAM-reported molecule attached to the 5' end and a TAMRA quencher linked at the 3' end (29).

Flow cytometry and tetramer staining

MHC class I tetramers complexed with EBV peptides were produced as previously described (20, 30). Two HLA B8-restricted immunodominant peptides were used, derived from the EBV lytic cycle protein BZLF1 (RAKFQKLL) and from the latent Ag EBNA-3A (FLRGRAYGL), respectively (5). Biotinylated class I peptide complexes were tetramerized by addition of aliphosphycocyanin- or PE-conjugated streptavidin.

Four-color fluorescence analysis was performed. Briefly, PBMC were thawed and 1–1.5\(\times\)10\(^6\) cells were stained with PerCP-conjugated mAb CD8, CD27-PE, CD45RO-FITC (BD Biosciences), San Jose, CA), and one of both HLA peptide tetramers conjugated with aliphosphycocyanin to study tetramer numbers and phenotype. To study the fraction of EBV-specific CD8\(^+\) T cells in culture, cells were stained for CD8 and both tetramers; subsequently, cells were permeabilized (FACS Permeabilizing Solution; BD Biosciences) and stained intracellularly with an FITC-conjugated mAb for Ki67 (NovoCastra, Newcastle, U.K.). At least 200,000 events were acquired using a FACS Calibur flow cytometer (BD Biosciences). Lymphocytes were gated by forward and side scatter, and data were analyzed using the software program CellQuest (BD Biosciences).

Detection of IFN-\(\gamma\)-producing CD8\(^+\) T cells

After ex vivo stimulation with EBV peptides, IFN-\(\gamma\)-producing cells were enumerated by IFN-\(\gamma\)-specific ELISPOT and/or intracellular cytokine staining assays as previously described (20, 30). For the ELISPOT, we used 96-well, nylon-backed plates (Nunc, Roskilde, Denmark) and mAbs from MAABTECH (Stockholm, Sweden). PBMC were added in triplicate wells at 1\(\times\)10\(^5\) cells/well in the absence or the presence of 10 \(\mu\)g/ml peptide and incubated overnight at 37°C in 5% CO\(_2\).

As a positive control to test the capacity of PBMC to produce IFN-\(\gamma\) in general, PHA (Murex Diagnostics, Dartford, U.K.) was added. Individual cytokine-producing cells were detected as dark purple spots, which were counted using an automated spot reader (Automated ELISA-Spot Assay, AELVIS, software version 3.2; Video Analysis Systems, Hannover, Germany). The number of responsive EBV-specific CD8\(^+\) T cells was calculated by subtracting negative control values and compensated for the fraction of viable lymphocytes within the PBMC, as measured by FACS analysis in the same samples.

For measurement of EBV-specific, IFN-\(\gamma\)-producing CD8\(^+\) T cells by intracellular cytokine staining, 10\(^5\) PBMC were stimulated in 500 \(\mu\)l of medium containing 10% FCS for 6 h with 10 \(\mu\)g/ml peptide in the presence of 3 \(\mu\)M monensin for the last 5 h to allow accumulation of cytokines. Unstimulated cells were used as a negative control, and as a positive control cells were stimulated with 10 ng/ml PMA and 2 \(\mu\)g/ml ionomycin. After stimulation, the cells were stained for extracellular CD8 and intracellular IFN-\(\gamma\), as described previously (30).

Table I. Characteristics of 36 individuals studied before and after HIV seroconversion

<table>
<thead>
<tr>
<th>Group</th>
<th>N(^0)</th>
<th>N(^3)</th>
<th>Age (years)</th>
<th>t1</th>
<th>t2</th>
<th>t3</th>
<th>CD4(^+)</th>
<th>CD4(^+)</th>
<th>CD4(^+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>36</td>
<td>3</td>
<td>35 (23–51)</td>
<td>–30</td>
<td>14</td>
<td>64</td>
<td>900 (310–1740)</td>
<td>400 (160–1690)</td>
<td>410 (190–990)</td>
</tr>
<tr>
<td>LTNP</td>
<td>5</td>
<td>3</td>
<td>42 (29–51)</td>
<td>–34</td>
<td>15</td>
<td>64</td>
<td>810 (760–1740)</td>
<td>490 (360–1690)</td>
<td>800 (600–990)</td>
</tr>
<tr>
<td>PROG</td>
<td>26</td>
<td>10</td>
<td>35.5 (23–50)</td>
<td>–29</td>
<td>14</td>
<td>64</td>
<td>900 (310–1700)</td>
<td>510 (160–1190)</td>
<td>350 (190–480)</td>
</tr>
<tr>
<td>UND</td>
<td>5</td>
<td>0</td>
<td>37 (29–43)</td>
<td>–117</td>
<td>11</td>
<td>ND</td>
<td>760 (420–960)</td>
<td>460 (350–1350)</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) Data are expressed as median values (range). Age is given in years.

\(^b\) Number of individuals studied at time point 1 and 2 (N) and time point 3 (N3), respectively.

\(^c\) Time to seroconversion in months.

\(^d\) CD4 numbers per microliter at different time points; t1, before HIV seroconversion; t2, 1 year after HIV seroconversion; t3, 5 years after HIV seroconversion.
Table II. Comparison of EBV load between different groups of HIV-infected individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>EBV Load/10^6 PBMC (median)</th>
<th>Range</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonprogressors</td>
<td>22</td>
<td>388</td>
<td>0–88.750</td>
<td>0.417</td>
</tr>
<tr>
<td>Progressors</td>
<td>39</td>
<td>597</td>
<td>0–35.313</td>
<td></td>
</tr>
<tr>
<td>CD4 ≥ 400</td>
<td>28</td>
<td>846</td>
<td>0–27.460</td>
<td>0.685</td>
</tr>
<tr>
<td>CD4 &gt; 400</td>
<td>33</td>
<td>425</td>
<td>0–88.750</td>
<td></td>
</tr>
<tr>
<td>CD4 ≤ 200</td>
<td>9</td>
<td>198</td>
<td>0–9.802</td>
<td>0.290</td>
</tr>
<tr>
<td>CD4 &gt; 200</td>
<td>52</td>
<td>583</td>
<td>0–88.750</td>
<td></td>
</tr>
</tbody>
</table>

* n, number of individuals tested; nonprogressors, HIV-1-infected individuals who were LTNP or slow progressors to AIDS; progressors, progressors to AIDS; CD4, CD4^+ T cell numbers/per microliter. EBV load was measured between 1 and 3 years after study entry or seroconversion.

Results

High EBV load in HIV-infected individuals is not related to immunodeficiency

To investigate EBV load in HIV-infected compared with noninfected individuals, we studied EBV load in 25 HIV− and 61 HIV+ homosexual men. As shown in Fig. 1A, EBV load in HIV+ (homosexual) men (median, 565/10^6 PBMC; range, 0–88.750) was much higher than that in HIV− (homosexual) men (median, 12/10^6 PBMC; range, 0–132; p < 0.001, by Mann-Whitney U test).

To study whether the degree of HIV-related immune deficiency is associated with the observed elevated EBV load, the 61 HIV+ homosexual men were subdivided into 22 long term non- or slow-progressing individuals and 39 rapid progressors to AIDS. No difference in EBV load was found between these groups (median, 388 and 597, respectively; p = 0.417; Table II). In addition, no difference in EBV load was found between 28 HIV+ homosexual men with CD4^+ T cell numbers <400/μl and 33 with CD4^+ T cell numbers >400/μl (median, 846 and 425, respectively; p = 0.685; Table II). Likewise, a similar EBV load was observed in HIV+ homosexual men with CD4^+ T cell numbers <200/μl (n = 9) and >200/μl (n = 52; median, 198 and 583, respectively; p = 0.290; Table II). Moreover, there was no correlation between EBV load and CD4^+ T cell numbers in HIV+ homosexual men (r = 0.027; p = 0.838, by Spearman’s correlation test; Fig. 1B).

Increase in EBV load after HIV seroconversion

The higher EBV load observed in our cross-sectional analysis may be caused by different factors. One possibility is that there is a difference between the homosexual men who remained HIV seronegative over time and those who seroconverted for HIV. The most obvious explanation, however, is that EBV load increases over HIV seroconversion. To confirm that the higher EBV load observed in HIV-infected individuals compared with HIV-seronegative individuals is due to HIV infection, we determined EBV load in 36 HIV seroconverters before (median, 30 mo; range, 4–130 mo) and after (median, 14 mo; range, 5–30) HIV seroconversion. As shown in Fig. 2A, a significant increase from 205 EBV copies/10^6 PBMC (range, 0–4,603) before HIV seroconversion to 1,002 copies/10^6 PBMC (0–50,416) after HIV seroconversion was observed (p < 0.001, by Wilcoxon signed rank test).

To investigate whether EBV load further increased later during chronic HIV infection, we measured EBV load 5 years after seroconversion for HIV in 13 of the 36 individuals. Also within this smaller group we found an increase in EBV load early after HIV seroconversion (from 276 to 1827 copies; p = 0.01). In contrast, the increase in EBV load from 1 to 5 years after HIV seroconversion was not significant (1827 and 2478 copies, respectively; p = 0.530; Fig. 2B). Thus, it seems that the high EBV load often observed in HIV-infected individuals is already reached early in HIV infection.

Statistical analysis

To compare EBV DNA copy numbers, numbers of EBV-specific CD8^+ T cells and numbers of IFN-γ-producing cells in paired samples before and after seroconversion for HIV, the Wilcoxon signed rank test was used. For comparison of EBV load between different groups, Mann-Whitney tests were used. Correlations between CD4^+ T cell numbers and EBV load were calculated with Spearman’s correlation test, using the software program SPSS 10.0 for Windows (SPSS, Chicago, IL).

FIGURE 1. Cross-sectional analysis of EBV load in HIV− and HIV+ homosexual men. A, EBV load (copies per 10^6 PBMC) was measured by real-time TaqMan PCR in 26 HIV-negative homosexual men (left) and 61 HIV-positive homosexual men at 1–3 years after HIV seroconversion (right). EBV load was significantly higher in the HIV-positive group (median, 565 copies/10^6 PBMC) than in the HIV-negative group (12 copies/10^6 PBMC; p < 0.001, by Mann-Whitney test). B, No correlation was found between EBV load (copies per 10^6 PBMC) and CD4^+ T cell counts (cells per microliter of blood; r = 0.027; p = 0.838, by Spearman’s correlation test).

FIGURE 2. Longitudinal analysis of EBV load in PBMC before and after HIV seroconversion. A, EBV load was measured in 36 homosexual men at a median of 30 mo before (Pre SC; left) and 14 mo after (Post SC; right) HIV seroconversion. A significant increase from 205 to 1002 copies/10^6 PBMC was observed (p < 0.001, by Wilcoxon signed rank test). B, In 13 homosexual men from the group above, EBV load (copies per 10^6 PBMC) was measured 11 mo before (Pre SC) and at two time points after HIV seroconversion (1 and 5 years, respectively). A significant increase (p = 0.01) was found over HIV seroconversion, whereas EBV load did not increase between 1 and 5 years after HIV seroconversion (p = 0.530).
Increase in EBV load is paralleled by increase in number of EBV lytic Ag-specific CD8\(^+\) T cells after HIV seroconversion

To study whether the increase in EBV load after HIV seroconversion was accompanied by changes in EBV-specific CD8\(^+\) T cells, we used HLA-EBV peptide tetrameric complexes to measure the number of EBV-specific CD8\(^+\) T cells in 16 HLA-B\(^*\) individuals before and after HIV seroconversion. CD8\(^+\) T cells recognizing immunodominant epitopes from latent EBV protein EBNA3A (FLRGRAYGL (FLR)) and early lytic EBV protein BZLF1 (RAKFKQLL (RAK)) were studied.

Fig. 3A shows staining representing the most frequent response, an increase in RAK-specific CD8\(^+\) T cells after HIV seroconversion and no change in FLR-specific CD8\(^+\) T cells. Overall, the percentage of CD8\(^+\) T cells recognizing RAK tended to increase after HIV seroconversion (median, 0.67 to 1.06% of CD8\(^+\) T cells; \(p = 0.163\); data not shown). As both the absolute number (median, 570 to 840/\(\upmu\)l blood; \(p = 0.055\)) and the percentage (27.82 to 40.85; \(p = 0.001\)) of CD8\(^+\) T cells had increased in a majority of individuals, we calculated the absolute numbers of EBV-specific CD8\(^+\) T cells in the peripheral blood for 13 of 16 individuals for whom these data were available. As shown in Fig. 3B (left panel), the absolute number of RAK-specific CD8\(^+\) T cells increased significantly (median, 4.78 to 9.54/\(\upmu\)l; \(p = 0.011\)). In contrast, the frequencies and numbers of CD8\(^+\) T cells specific for FLR did not change significantly (median, 0.14 to 0.16% (\(p = 0.73\)) and 0.83 to 1.60/\(\upmu\)l (\(p = 0.53\)), respectively; data not shown and Fig. 3B, right panel).

Evidence for Ag-driven differentiation of EBV lytic Ag-specific CD8\(^+\) T cells

The differentiation state of CD8\(^+\) T cells can be defined by several markers. Using CD27 and CD45RO, it has been shown that Ag-driven phenotypical changes occur when CD8\(^+\) T cells differentiate from naive (CD27\(^-\)CD45RO\(^+\)) to memory (CD27\(^+\)CD45RO\(^-\)) and effector (CD27\(^-\)CD45RO\(^++\)) CD8\(^+\) T cells (31). We have recently shown that in asymptomatic HIV-infected individuals the fraction of CD27-negative, EBV-specific T cells increases over time, suggesting an adequate response to a high EBV burden (32). To obtain more insight into possible phenotypic changes in EBV-specific T cells over HIV seroconversion, we analyzed CD27 and CD45RO expression on EBV-specific CD8\(^+\) T cells. Fig. 4A shows an example of the (phenotypic) distribution of EBV-specific T cells over seroconversion. Interestingly, RAK-specific T cells showed a trend to an increased percentage of CD27\(^-\) T cells over HIV seroconversion (from 12.2 to 17.3%; \(p = 0.051\); Fig. 4B, middle panel). In accordance with this, a correlation between the increase in the percentage of CD27-negative RAK-specific T cells and the increase in the number of RAK-specific T cells was found (\(r = 0.750\); \(p = 0.020\), by Spearman’s correlation test; Fig. 4C). We did not observe significant phenotypical changes in FLR-specific CD8\(^+\) T cells or the whole CD8\(^+\) T cell population (Fig. 4B, lower and upper panels, respectively). These data thus corroborate an Ag-induced expansion of RAK-specific CD8\(^+\) T cells.

No increase in IFN-\(\gamma\) production by EBV-specific CD8\(^+\) T cells after HIV seroconversion

We have shown previously that in HIV-infected individuals, CD8\(^+\) T cell function, defined as the fraction of IFN-\(\gamma\)-producing cells within the tetramer-positive cells, is diminished compared with that in healthy EBV carriers (20). To assess the functional capacity of the EBV-specific CD8\(^+\) T cells over HIV seroconversion, we stimulated PBMC from 16 donors with RAK and FLR peptides. IFN-\(\gamma\)-production was measured by either ELISPOT (after 18 h) or intracellular cytokine staining (after 6 h). As these two techniques do not lead to different percentages of IFN-\(\gamma\)-producing CD8\(^+\) T cells in our hands (data not shown) (20), we used both, depending on the number of cells available. An example of intracellular cytokine staining is given in Fig. 5A, showing an increased percentage of IFN-\(\gamma\)-producing RAK-specific CD8\(^+\) T cells after HIV seroconversion. In general, however, both the frequency (data not shown) and the absolute numbers (Fig. 5B) of cells specific for the lytic epitope RAK (median, 0.27 to 0.21% of CD8\(^+\) T cells (\(p = 0.91\)) and 1.5 to 2.01 cells/\(\upmu\)l (\(p = 0.239\)) and latent epitope FLR (0.07 to 0.06% of CD8\(^+\) T cells (\(p = 0.215\)) and 0.45 to 0.58 cells/\(\upmu\)l; \(p = 0.65\)) did not change significantly.

To estimate the changes in the functional capacity of EBV-specific CD8\(^+\) T cells on a per cell basis, we calculated the ratio between the number of cells producing IFN-\(\gamma\) and the number of cells binding for HLA peptide tetramers from the same EBV epitope. We did not find significant changes for either RAK (median, 0.29 to 0.25; \(p = 0.639\)) or FLR (0.63 to 0.44; \(p = 0.177\)), indicating that 1 year after HIV infection the capacity of EBV-specific CD8\(^+\) T cells to produce IFN-\(\gamma\) was not yet significantly reduced.

Discussion

EBV-related NHL are a frequent event in immunocompromised patients. Unlike in the post-transplant setting, an elevated EBV load is not a useful marker for prediction of EBV-related malignancy in HIV-infected patients (13, 15–17). In healthy individuals
a very constant number of EBV copies is maintained over time. As for other chronic viral infections, the exact combination of factors determining a so-called viral setpoint, which can include viral strain(s), numbers, and function of CD4+ and CD8+ T cells; HLA type; Ab response; and coinfection with other pathogens, is unknown. We hypothesized that after HIV infection, the tightly regulated equilibrium between EBV and host could be altered by a very constant number of EBV copies maintained over time. As for other chronic viral infections, the exact combination of factors determining a so-called viral setpoint, which can include viral strain(s), numbers, and function of CD4+ and CD8+ T cells; HLA type; Ab response; and coinfection with other pathogens, is unknown. We hypothesized that after HIV infection, the tightly regulated equilibrium between EBV and host could be altered by a number of factors, including activation of B cells and possibly declining CD8+ T cell function, well before control over EBV is lost. Therefore, we studied both EBV load and EBV-specific CD8+ T cell responses in individuals seroconverting for HIV. EBV load was investigated in HIV-infected and noninfected individuals in both a cross-section of HIV− and HIV+ homosexual men and longitudinally in individuals seroconverting for HIV. Our results show a higher EBV load in HIV-infected individuals compared with HIV-seronegative individuals. In longitudinal measurements of individuals seroconverting for HIV, the increase in EBV load occurs during HIV seroconversion, whereas EBV load does not further increase later in HIV infection. Furthermore, numbers of CD8+ T cells directed toward the early lytic protein BZLF1 increased after HIV seroconversion. These data suggest that the EBV viral setpoint is altered after seroconversion for HIV.

The most obvious explanation for a higher EBV load in HIV-infected compared with noninfected homosexual men would be the HIV-related immunodeficiency. However, we found no difference in EBV load between nonprogressors and progressors to AIDS, and no relation to CD4+ T cell counts, suggesting that the degree of immunodeficiency has no influence on EBV load. A higher EBV load in HIV+ individuals compared with HIV− individuals has been reported (16, 17). In addition, we observed a higher EBV load in HIV− homosexual men compared with healthy heterosexual individuals from earlier reports (13). This suggests that factors other than immune deficiency determine EBV load, and that a high EBV load is not necessarily a reflection of loss of EBV immune control. This is also in accordance with a previous study (16) in which we observed that EBV load was not higher in patients progressing to EBV-related NHL compared with other HIV+ individuals. Longitudinal analysis of EBV load in individuals seroconverting for HIV confirms that indeed most of the increase occurs early in HIV infection, whereas the increase later during chronic...
HIV infection is more gradual. It might well be that substantial B cell activation during acute HIV infection, which is associated with lymphadenopathies, mainly due to germinal center formation, causes the major increase in EBV load. After acute HIV infection, chronic activation of B cells might maintain a high EBV load. High levels of Abs to the VCA-p18 in HIV carriers with elevated EBV loads suggest that EBV reactivation indeed occurs frequently (17).

With respect to EBV-specific CD8\(^+\) T cells, we observed an increase in the absolute numbers of EBV-lytic Ag-specific CD8\(^-\) T cells after HIV seroconversion, whereas the pattern for latent Ag-specific CD8\(^+\) T cells seems less consistent. This might indicate a response to EBV reactivation during HIV seroconversion, which would resemble the situation in primary EBV, where mostly lytic Ags are targeted (11, 33). Another drive for expansion of lytic Ag-specific CD8\(^-\) T cells could be EBV replication in epithelial tissue, which is frequently reported in HIV (34, 35). In support of an Ag-driven expansion and differentiation of lytic Ag-specific CD8\(^-\) T cells, we found an increase in the percentage of CD27-negative RAK-specific cells after HIV seroconversion. This may be comparable to the expansion observed in post-transplant patients for CMV-specific CD8\(^+\) T cells (36). Furthermore, there was a correlation between the increase in the number of RAK-specific CD8\(^-\) T cells and the increase in the fraction of CD27-negative cells within this subset. We could not find evidence for an increased proliferation of RAK-specific CD8\(^+\) T cells by Ki67 measurement (data not shown). However, it is likely that the peak of this expansion occurred at the time of HIV seroconversion.

Regarding the capacity of EBV-specific CD8\(^+\) T cells to produce IFN-\(\gamma\), both the percentage and the total numbers of these cells were maintained after HIV seroconversion. In accordance with this, the ratio between IFN-\(\gamma\)-producing CD8\(^+\) T cells and tetramer-positive CD8\(^+\) T cells did not significantly decrease. This seems in contrast with our previous study, where we found a significantly lower fraction of IFN-\(\gamma\)-producing cells within the tetramer-positive cells in HIV-infected homosexual men compared with healthy heterosexual individuals (20). An explanation could be that we now made observations earlier in HIV infection, and that the decline in CD8\(^-\) T cell function was not yet prominent. Functional defects may not occur immediately (1 year) after HIV seroconversion, but occur over time, possibly related to chronic high antigenic load (37, 38). These data together suggest that, at least early in HIV infection, CD8\(^-\) T cell-mediated control of EBV is sufficient. Furthermore, whereas in our earlier report we compared HIV-infected men with healthy blood bank donors, we now studied HIV seroconverters longitudinally. As most of these individuals already seemed to have an elevated EBV load, compared with healthy blood bank donors (with an undetectable load), before HIV seroconversion, it may be that their CD8\(^+\) T cells were already less functional.

Our results suggest the following sequence of events. First, primary HIV infection leads to a high degree of B cell stimulation and a cytokine environment favorable for proliferating B cells (39) and reactivation of EBV from these cells, leading to a higher EBV load. Consequently, in response to EBV reactivation, EBV-specific CD8\(^+\) T cells expand. It may seem contradictory that EBV load does not return to basal levels if the CD8\(^+\) T cell response reacts adequately. However, as chronic HIV infection is also associated with increased immune activation, it might be that an altered equilibrium is reached with higher background replication of EBV and a sufficient CD8\(^+\) T cell response to avoid uncontrolled proliferation of infected B cells.

In support of an altered EBV setpoint after HIV seroconversion, recent reports on the effects of highly active antiretroviral therapy do not show spectacular changes in EBV load, thus demonstrating that a relatively stable new equilibrium is attained (40, 41).

In conclusion, our study shows that the high EBV load observed in HIV-infected individuals can be assigned to an increase in EBV load during HIV seroconversion and not to a gradual increase later during HIV infection due to immune deficiency. Furthermore, confirming previous data (16), we found no link between the absolute EBV load and the degree of progression to AIDS or loss of CD4\(^+\) T cells. The fact that EBV load can be very high despite the presence of a functional CD8\(^+\) T cell response explains earlier studies, which showed that measurement of EBV load in itself is not enough to predict malignancy. Recent reports in both the solid organ as well as the bone marrow transplantation field also demonstrate that a high EBV load is not necessarily associated with lymphoproliferative disease as long as an adequate CD8\(^+\) T cell response is also present (21, 22). However, iatrogenic and HIV-induced immune suppression are very different settings, which probably explains why in the first case EBV load has a much better predictive value for EBV-related malignancies. In transplanted patients, a high EBV load results mainly from a lack of EBV-specific CD8\(^+\) T cell immunity. In contrast, chronic HIV leads to gradual degradation of the immune system. The combination of HIV-associated chronic immune activation, CD4\(^+\) T cell depletion, exhaustion of CD8\(^+\) T cells, and diverse B cell dysfunctions can ultimately lead to uncontrolled proliferation of EBV-infected B cells and lymphoma. Our data show that in HIV infection, an elevated EBV load together with a sufficient CD8\(^+\) T cell response is a normal situation and thus explains why absolute EBV load in itself is not a good predictive marker for EBV-related malignancies.

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References
