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Reconstitution of EBV Latent but Not Lytic Antigen-Specific CD4+ and CD8+ T Cells after HIV Treatment with Highly Active Antiretroviral Therapy

Erwan Piriou,* Christine A. Jansen,* Karel van Dort,* Iris De Cuypers,* Nening M. Nanlohy,2* Joep M. A. Lange,†‡ Marinus H. J. van Oers,§ Frank Miedema,2*‡ and Debbie van Baarle3*§

The incidence of (EBV-related) malignancies in HIV-infected subjects has declined since the introduction of highly active antiretroviral therapy (HAART). To investigate the effect of HAART on EBV infection, we performed a longitudinal analysis of the T cell response to both a latent and a lytic Ag and EBV viral load in 10 subjects from early in HIV infection up to 5 years after HAART. All individuals responded to HAART by a decline in HIV viral load, a restoration of total CD4+ T cell numbers, and a decline in T cell immune activation. Despite this, EBV load remained unaltered, even after 5 years of therapy, although a decline in both CD4+ and CD8+ T cells specific for the lytic EBV protein BZLF1 suggested a decreased EBV reactivation rate. In contrast, latent EBV Ag EBNA1-specific CD4+ and CD8+ T cell responses were restored after 5 years of treatment to levels comparable to healthy individuals. In two individuals who were treated by HAART late during HIV progression, a lymphoma developed shortly after initiation of HAART, despite restoration of EBV-specific CD4+ and CD8+ T cells. In conclusion, long-term HAART does not alter the EBV DNA load, but does lead to a restoration of EBNA1-specific T cell responses, which might allow better control of EBV-infected cells when applied early during HIV infection. The Journal of Immunology, 2005, 175: 2010–2017.

The EBV is a widespread human gammaherpesvirus (1) that persists for life in a latent form in resting memory B cells after primary infection in the oropharynx (2, 3). EBV-infected B cells persist in a quiescent form in the peripheral blood, and can reemigrate to the tonsils or lymph nodes, where they can either be driven into proliferating lymphoblasts when the so-called growth program is induced (involving EBV latent genes), or differentiate into plasma cells, which induces the expression of lytic replication of EBV and ultimately virus release by a fraction of these reactivated cells (1). Control of EBV-infected B cells is achieved mainly by CD8+ T cells (4, 5). HIV infection is associated with an alteration of the EBV viral set point, reflected by an elevated EBV load early in infection which is followed by increased numbers of EBV lytic Ag-specific CD8+ T cells. This may be related to more frequent EBV reactivation (6, 7). After this increase early in HIV infection, EBV load increases only slowly during chronic infection (6). This results in the commonly observed elevated and fluctuating EBV load in untreated HIV infection (8), which may be the reason why the absolute level of EBV DNA in PBMC is not predictive of EBV-related non-Hodgkin lymphoma (NHL) (8, 9). Persistent high EBV burden might, however, lead to EBV-related NHL when EBV-specific CD8+ T cell function is lost. An association of this loss of CD8+ T cell function with a decrease in total CD4+ T cell numbers (10) suggested that it may be due to a lack of CD4+ T cell help, which is in accordance with many human (11–15) and animal studies (16–22).

In untreated HIV-infected individuals, the incidence of NHL is 60- to 250-fold increased compared with healthy individuals, a majority being systemic or primary CNS lymphomas, which in 70–80% of cases express latent EBV proteins (23–25). The incidence of both types of lymphomas has decreased since the introduction of highly active antiretroviral therapy (HAART) (26, 27–30), while their prognosis has greatly improved (27, 31–33).

The long-term effects of HAART on EBV viral load and EBV-specific immunity have not been studied. No consistent changes in EBV DNA load were found in either short-term longitudinal (34–36) or cross-sectional studies (9), while IFN-γ production by EBV-specific CD8+ T cells was increased shortly after initiation of HAART (34, 37). However, it remains unclear whether on the long term, HAART will lead to a restoration of the EBV load to levels measured in healthy EBV carriers, by a combination of decreased immune activation and possibly improved immune responses. Alternatively, it may be that HIV seroconversion irreversibly alters the individual EBV viral set point. The aim of this study was to investigate the long-term effects of HAART on EBV viral load and EBV-specific CD4+ and CD8+ T cell responses. To this end, we studied 10 HIV-infected subjects early (2 years postseroconversion) and late during untreated HIV infection (7 years) and at an early (7 mo) and later time point after HAART (5 years). All these individuals responded to HAART by both a decrease in HIV RNA load and a restoration of total CD4+ T cell numbers. Both CD4+ and CD8+ T

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cells to an EBV latent (EBNA1) and lytic protein (BZLF1) were studied, using an in vitro expansion method that was recently developed (38), next to the quantification of specific CD8+ T cells by direct staining with HLA-peptide tetramers, and in relation to EBV load.

Materials and Methods

Study population

All HIV seropositive subjects were 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. Ten patients were selected based on HLA class I typing and availability of cryopreserved PBMC at several time points before and after HAART (Table I). None of these 10 subjects was diagnosed with an NHL or other AIDS-defining event. Samples were studied early (73 mo prior to initiation of HAART, range 88–41) and later during HIV infection (11 mo pre-HAART, 16–1), and early (7 mo, 1–15) and late (56 mo, 43–81) after initiation of HAART. The median age of the patients at the first time point studied was 39.5 years (27.5–50). Start of HAART was defined by a drug regimen consisting of at least two nucleotide or nucleoside reverse transcriptase inhibitors and 1 protease inhibitor or 1 nucleoside reverse transcriptase inhibitor + 2 protease inhibitors. Time from HIV seroconversion, HLA class I and II typing, and medication of the HIV-infected individuals are indicated in Table I.

In addition, we studied two patients progressing to EBV-related AIDS NHL shortly after start of HAART, at the time points indicated in Fig. 4 (described in Results). Furthermore, a cross-sectional analysis of EBV-specific T cell responses was performed in PBMC from 14 healthy EBV-seropositive blood bank donors.

Flow cytometry and tetramer staining

MHC class I tetramers complexed to EBV peptides were produced as previously described (10, 39). The immunodominant epitopes were derived from both EBV lytic cycle proteins (A2-GLCTLVAML from BMLF1; B8-RAKFKQQLL from BZLF1), and EBV latent Ags (A11-AVFD KSDAK and A11-IVTDYSVIK from EBNA3B; B7-RPPIFIRRL from EBNA3A; B8-FLRGRAYGL from EBNA3A) (5). HIV-specific CD8+ T cells were studied using tetramers containing several epitopes, depending on the HLA type of the subject: A2-SLYNYATLV, B8-EIYKRWII and B57-KAFSPEVIPMF from Gag; and CD38 (Sanquin Reagents). A total of 200,000 events were acquired on a FACSCalibur flow cytometer (BD Biosciences). Responses were scored as positive when they were two times above the medium control value.

Measurement of HIV load

HIV RNA load was measured in plasma by several assays. The NASBA HIV-1 QT assay (Organon Teknika) and Amplicor HIV monitor (Roche Diagnostic) had a detection limit of 1000 and 400 copies/ml, respectively. After August 1999, load was determined by the more sensitive Quantiplex bDNA 3.0 assay (Bayer), with a detection limit of 50 copies/ml. Values of

Table I. Patient data: time from HIV seroconversion, HLA typing, treatment

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<th>t4a</th>
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a Time in months from HIV seroconversion.

b Ind = Indinavir; Saq = Saquinavir; Rit = Ritonavir.
1000, 400, and 50 (Table II) indicate that the load was undetectable by the method used, the cut-off values corresponding to the assay which was used.

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

EBV load was measured in duplicate in DNA from 2 × 10⁹ cells. Real-time PCR amplification was performed as previously described (8, 41), using PCR primers specific for the nonglycosylated membrane protein BNRFI p143 (42) and a fluorogenic probe (Applied Biosystems) to detect the 74-bp product. As a control for input DNA the amount of H9252-albumin DNA, a household gene present at 2 copies/cell, was also determined, using primers and probes as described before (43).

**Statistical analysis**

For calculation of longitudinal changes, the Wilcoxon signed rank test was used. Correlations were calculated using Spearman’s correlation test. Data from different groups were compared using Mann-Whitney U tests. All statistics were calculated using the software program SPSS 11.5 for Windows (SPSS).

**Results**

**Effects of HAART on EBV load and T cell immune activation**

All individuals studied responded to HAART by a reduction in HIV plasma RNA concentration, from a median of 57,000 RNA copies/ml plasma 1 year before treatment to 400 early after treatment (p = 0.007) and 50 at 5 years after treatment (p = 0.008) (Fig. 1A). Total CD4⁺ T cell numbers tended to increase from 400/μl to 460/μl late (p = 0.059) and 550/μl at 5 years after HAART (p = 0.107), while CD8⁺ T cell numbers were not altered by treatment. B cell numbers increased during untreated HIV infection (from 100 to 180 cells/μl, p = 0.012), but did not change after short-term (190/μl) or long-term (210/μl) HAART (Table II).

In accordance with earlier data, we found a high and fluctuating EBV DNA load in PBMC (6), and EBV load did not increase during untreated HIV infection (from 557 early to 393 copies/10⁶ PBMC late in untreated infection, p = 0.114). Initiation of HAART did not lead to a reduction in the number of EBV DNA copies measured in PBMC on the short term (421 copies/10⁶ PBMC at 7 mo after initiation of HAART, p = 0.878 compared with pretreatment value). Even long-term antiretroviral treatment did not alter the EBV load (759 copies/10⁶ PBMC at 56 mo post-HAART; p = 0.445 compared with pretreatment value, Table II). The contrast between EBV and HIV load became more pronounced when the load data were related to the first time point. HIV RNA load clearly decreased in each individual.
studied (Fig. 1A), whereas the median EBV load remained stable (Fig. 1B).

CD4 + T cell immune activation, as an indication of the general state of immune activation, which may induce an elevation of the EBV load, decreased significantly after HAART. The percentage of CD38 + HLA-DR + CD4 + T cells, which had increased from 4.39% at 73 mo to 11.21% at 11 mo before initiation of therapy \((p = 0.028)\), decreased to 3.76% at 7 mo \((p = 0.037)\) and 3.87% at 56 mo \((p = 0.037, \text{Fig. 1C})\). The percentage of CD38 + HLA-DR + CD8 + T cells changed from 19.50% at 11 mo before to 7.55% shortly after \((p = 0.013)\) and 2.93% at 56 mo after initiation of therapy \((p = 0.009, \text{Fig. 1D})\).

Restoration of the EBNA1-specific T cell response, and decrease in the BZLF1-specific T cell response

To study EBV-specific CD4 + T cell responses, we used a recently developed method, enabling specific and reproducible in vitro expansion and restimulation of specific T cells with EBNA1 or BZLF1 peptide pools \((38)\). Specific CD4 + central memory T cells capable of both proliferation and IFN-γ production in response to Ag are measured by this method, and were shown to correlate with protection against hepatitis C virus and malaria \((13, 44)\). For EBV we have shown that results from this assay correlated with EBV viral load and thus may be a good indication of an individuals’ ability to mount an effective EBV-specific CD4 + memory T cell response \((38)\).

As shown in representative FACS plots, EBNA1-specific CD4 + T cell responses tended to decline during untreated HIV infection, and were restored by antiretroviral treatment \((\text{Fig. 2A})\). In contrast, BZLF1-specific CD4 + T cell responses were maintained before treatment, but decreased after initiation of HAART \((\text{Fig. 2B})\). To better compare the changes within the whole group of individuals, we indexed the responses to the first time point measured for each subject. EBNA1-specific CD4 + T cell responses tended to decrease during untreated HIV infection in a majority (7 of 9) of the subjects studied \((\text{Fig. 2C, } p = 0.086)\), whereas no changes in BZLF1-specific CD4 + T cell responses were observed before initiation of HAART \((\text{Fig. 2D, } p = 0.374)\). Interestingly, we observed a significant restoration \((8 \text{ of 9 individuals})\) of EBNA1-specific CD4 + T cells after long-term HAART \((p = 0.021, \text{from 11 mo pre- to 56 mo post-HAART, Fig. 2C})\), whereas BZLF1-specific CD4 + T cells decreased significantly \((8 \text{ of 10 individuals})\) after initiation of HAART \((p = 0.038, \text{from 7 to 56 mo post-HAART, Fig. 2D})\).

To determine the level of restoration of EBNA1 and BZLF1-specific CD4 + T cells, we compared the responses after long-term HAART with those measured in healthy EBV carriers. In accordance with the restoration observed for EBNA1-specific T cells after initiation of therapy, numbers of CD4 + T cells specific for EBNA1 after long-term HAART were comparable to values measured in healthy EBV carriers \((3431 \text{ in healthy vs } 1319 \text{ after long-term HAART, } p = 0.109, \text{Fig. 2E})\). In contrast, numbers of BZLF1-specific T cells were lower than in healthy donors \((254 \text{ vs } 54, p = 0.03, \text{Fig. 2F})\).

Along with CD4 + T cells, CD8 + T cell responses were also measured after 12 days of expansion with EBV peptide pools \((\text{Fig. 3, A and B})\). Both EBNA1- and BZLF1-specific CD8 + T cells followed approximately the same kinetics as the respective CD4 + T cells \((\text{Fig. 3, C and E})\), although EBNA1-specific CD8 + T cell numbers were restored sooner after initiation of HAART than the CD4 + T cells, and the decrease in BZLF1-specific CD8 + T cells was not significant \((p = 0.953, 11 \text{ mo pre- vs } 7 \text{ mo post-HAART, } p = 0.314, 11 \text{ mo pre- vs 5 years post-HAART})\). Thus, antiretroviral treatment tended to lead to a restoration of latent Ag-(EBNA1, EBNA3A) specific T cells, and a decrease in lytic Ag-(BZLF1) specific T cells. Similar to the CD4 + T cell response after long-term HAART, EBNA1-specific CD8 + T cells were restored to levels observed in healthy individuals \((761 \text{ in healthy vs } 744 \text{ in HAART-treated, } p = 0.557, \text{Fig. 2D})\), and BZLF1-specific CD8 + T cells were lower than in healthy donors \((7754 \text{ vs } 254, p = 0.001, \text{Fig. 2F})\).

Interestingly, CD4 + and CD8 + T cell response to EBNA1 were positively correlated \((0.706, p < 0.001)\), which indicates a possible role for CD4 + T cells in helping the CD8 + T cell response. In contrast, the CD4 + and CD8 + T cell responses to BZLF1 were not correlated \((0.409, p = 0.769)\).

EBV- and HIV -specific CD8 + T cells were also enumerated directly by staining with HLA-peptide tetrameric complexes. HIV-specific CD8 + T cells declined in response to a reduction in HIV load \((\text{from a median of } 11.2/\mu \text{l at } 11 \text{ mo pre-HAART to } 4.6/\mu \text{l at } 7 \text{ and } 6.7/\mu \text{l at } 56 \text{ mo post-HAART, } p = 0.038 \text{ and } p = 0.017, \text{respectively, data not shown})\). In contrast, no consistent pattern could be distinguished in the kinetics of the sum of EBV-specific CD8 + T cells \((14.3/\mu \text{l at } 11 \text{ mo pre-HAART, to } 15.3/\mu \text{l at } 7 \text{ and } 15.2/\mu \text{l at } 56 \text{ mo post-HAART})\).

### FIGURE 2. Effects of HAART on EBV-specific CD4 + T cells. Representative FACS plots showing changes in CD4 + T cell responses to EBNA1 (A) and BZLF1 (B) during follow-up. C (EBNA1) and D (BZLF1), CD4 + T cell responses indexed to the first time point studied, showing a restoration of EBNA1-specific CD4 + T cell responses after 5 years of therapy, whereas BZLF1-specific CD4 + T cell responses declined. The dotted line represents the height of the first measurement. E (EBNA1) and F (BZLF1), Comparison of CD4 + T cell responses of the 10 subjects treated with HAART for 5 years (HIV +) with healthy EBV + donors (HIV -). The y-axes indicate the number of specific T cells measured after 12 days of in vitro expansion.
To study latent and lytic epitope EBV- and E with CD4 and CD8 between individuals, we indexed the numbers of tetramer-positive T cells measured after 12 days of in vitro expansion. Kinetics of FLR (FLR) and RAK (RAK), a wide array of patterns was observed, but for the group as a whole, no changes were observed over therapy.

Effects of HAART on EBV-specific CD8 T cells, CD8 T cell numbers had decreased already >5-fold, 54 (patient 68) and 37 (patient 343) before diagnosis (data not shown). In both patients restoration of the EBNA1-specific CD4 and CD8 T cell response was already observed before the start of HAART, which might have been driven by Ag from a developing malignancy, and continued to increase after HAART in subject 434 (Fig. 4, C and G). In subject 68, we were also able to enumerate IFN-γ-producing CD8 T cells specific for epitopes derived from EBNA3A (A30-AYSSWMSYS), EBNA3B (B44-VEITPYKPTW), and EBNA3C (B44-KEKHIQNAF), respectively. These responses were clearly restored rapidly after initiation of therapy (Fig. 4D). Thus, these data show that, despite a restoration of EBV-specific CD4 and CD8 T cell responses, the occurrence of an EBV-related NHL could not be prevented.

Discussion
In this study, we investigated whether long-term highly active antiretroviral therapy would lead to a lower EBV viral set point, which at least in part could explain the decreased incidence of AIDS NHL since the introduction of HAART. In 10 successful responders to antiretroviral therapy (both increase in CD4 T cell numbers and a decrease in HIV load), no alterations in EBV viral load were found, despite a clear decrease in immune activation, and a restoration of EBNA1-specific central memory CD4 and CD8 T cell responses. Interestingly, while a restoration of latent Ag-specific T cells occurred, lytic Ag-specific responses decreased, suggesting a reduction in the rate of reactivation of EBV after initiation of HAART.

Earlier studies did not report changes in EBV load shortly (up to 1 year) after initiation of HAART (34, 35), although higher numbers of IFN-γ-producing EBV-specific CD8 T cells (34, 37), and an increased concentration of EBV-specific Abs, were measured (36). We hypothesized that either 1) EBV viral load would decline after long-term antiretroviral treatment, due to a decline in immune activation and a restoration of EBV-specific T cell immunity or 2) EBV viral load would remain high as a consequence of a definite alteration of the equilibrium between EBV and immunity after HIV seroconversion. EBV load was not altered after 5 years of antiretroviral treatment, due to a decline in immune activation diminishes quickly after initiation of therapy, persistence of elevated IgG levels (46–48). In line with this, it may be that, although chronic activation of T cells is normalized in a few years, their ability to provide help to B cells is restored (49), which might help to maintain reactivation of EBV-carrying memory B cells (50). However, a reduction in lytic Ag-specific T cells does not support this explanation. Alternatively, while a decrease in EBV reactivation rate is suggested by the kinetics of EBV lytic Ag-specific T cells, it might still take a long time to reduce the pool of latently EBV-infected B cells. Furthermore, in untreated HIV infection, a slow increase in EBV load...
over years is usually observed (6, 51). Thus, it appears that long-term HAART does not influence the altered EBV viral set point initiated after HIV seroconversion (but may lead to a stabilization). Finally, one could argue that redistribution of EBV-infected B cells could explain our findings, but the relatively stable number of B cells after HAART in the individuals studied here argues this.

Interestingly, different patterns of recovery of EBNA1 and BZLF1-specific T cells were observed. Although no EBV reactivation is measurable by RT PCR in the blood of HIV-infected EBV carriers (9), the oropharynx is known as a major site of EBV replication (52), which is accompanied by an increase in the frequency of EBV lytic Ag-specific CD8+ T cells (7). Our data is compatible with a diminished rate of EBV reactivation, followed by a decreased lytic Ag-specific T cell response. At the same time, the general immune restoration associated with HAART (53) resulted in increased numbers of EBNA1-specific CD4+ T cells, possibly because of the restoration of the central memory CD4+ T cell pool specific for latent Ag EBNA1. The increase in CD8+ T cell function observed in earlier studies (34) might well be associated with the recovery of specific CD4+ Th cells (37). In addition, the CD8+ T cell response to EBNA1 was improved, possibly also through an improved CD4+ T cell helper function, as suggested by a correlation between the EBNA1-specific CD4+ and CD8+ T cell response. This is particularly interesting in light of recent papers showing that, in contrast to earlier reports (54–56), EBNA1-specific CD8+ T cells are able to recognize EBV-infected B cells (57–60), and, next to EBNA1-specific CD4+ T cells (61), might be an important factor in controlling outgrowth of EBV-positive tumors (60). It will be interesting to investigate whether the difference between EBNA1 and BZLF1-specific T cell responses is a general feature of latent vs lytic EBV Ags, or something specific for these two proteins.

Most recent data indicate a clear reduction in the incidence of NHL since the introduction of HAART (26, 27, 62), although it is still a matter of debate whether so-called “virological failers” will not be at higher risk on the longer term (29, 30). The 10 patients selected for our study had relatively preserved CD4+ T cell numbers, and responded to therapy by a clear reduction in HIV RNA load. They are thus likely to represent subjects who will have a decreased risk of developing NHL. A decrease in general immune activation and EBV reactivation, together with a restoration of EBV latent Ag-specific responses, may create a much “safer” equilibrium between EBV and its host. The development of lymphoma is known to be a multistep process, which can cover a period of several years, starting with alterations in immune control together with chronic antigenic stimulation and cytokine deregulation, followed by a phase of accumulation of genetic lesions, which can ultimately lead to the uncontrolled proliferation of a clonal B cell population (63, 64). EBV-specific CTL may be most efficient in controlling the early stages of EBV-associated polyclonal B cell proliferation. The two patients who developed NHL after the start of HAART likely represent individuals in which evolution toward an EBV-related malignancy was too advanced to be stopped by the immunological improvements of antiretroviral treatment. It may thus be important to start antiretroviral therapy before irreversible genetic alterations in EBV-infected B cells have occurred, although other reports show that the prognosis of NHL has also clearly improved since the introduction of HAART (27, 31, 32), even when antiretroviral treatment is initiated after diagnosis (33). In addition, it is known that central memory CD4+ T cell responses are better restored when HAART is initiated before total CD4+ T cell numbers drop below 350/μl (53).

In conclusion, the long-term follow-up of subjects who were successfully treated by HAART shows that despite an improved EBV-specific T cell response and a decrease in T cell immune activation, the EBV load remains high in these individuals. Interestingly, changes in the relative importance of latent and lytic
Ag-specific T cell responses suggest a decrease in EBV reactivation, but this does not alter the EBV load in the peripheral blood. The data are thus in accordance with the idea that an elevated EBV load in the HIV setting does not in itself correlate with the incidence of EBV-related malignancies (8). Thus, early initiation of HAART might result in a new equilibrium, much more favorable for the host, consisting of a still elevated EBV load, but in the presence of sufficient CD4 T help to preserve CD8 T cells.

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Disclosures

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