

Novel method for detection of virus-specific CD4⁺ T cells indicates a decreased EBV-specific CD4⁺ T cell response in untreated HIV-infected subjects

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A lower function of EBV-specific CD8⁺ T cells in HIV-infected subjects could be related to a lack of specific CD4⁺ T cell help. Therefore, we studied EBV-specific CD4⁺ T cells in both healthy donors and untreated or highly active antiretroviral therapy (HAART)-treated HIV-seropositive homosexual men. To this end, PBMC were stimulated with overlapping peptide pools from a latent and a lytic EBV protein, EBV nuclear antigen (EBNA) 1 and EBV lytic-switch protein ZEBRA (BZLF1), respectively. EBV-specific CD4⁺ T cell frequencies measured directly *ex vivo* were low. To measure EBV-specific memory CD4⁺ T cells, capable of both expansion and IFN- γ production upon antigenic challenge, we developed a specific and reproducible assay, combining *ex vivo* expansion of specific T cells with flow cytometric analysis of IFN- γ production. Untreated HIV-infected individuals had a lower CD4⁺ T cell response to both EBNA1 and BZLF1 as compared to healthy EBV carriers and HAART-treated HIV-positive subjects. This suggests loss of EBV-specific CD4⁺ T cells due to HIV infection, while HAART might restore this response. In addition, we found an increase in the EBNA1-specific CD8⁺ T cell response in HAART-treated subjects. Interestingly, numbers of EBV-specific CD4⁺ and CD8⁺ T cells were inversely correlated with EBV viral load, suggesting an important role also for EBV-specific CD4⁺ T cells in the control of EBV infection.

Received 27/10/04

Revised 17/1/05

Accepted 27/1/05

[DOI 10.1002/eji.200425792]

Key words:

EBV · CD4⁺ T cells
· HIV · Memory

Introduction

The Epstein-Barr virus (EBV) is a widespread human γ -herpesvirus. Primary infection with EBV is usually

asymptomatic, but can cause infectious mononucleosis when occurring at adolescence [1]. After primary infection, the virus persists for life in a latent form in resting memory B cells [2, 3]. Both primary infection and latent infection are thought to be controlled by CD8⁺ T cells [4, 5]. It has been shown both in animals and in humans that antigen-specific CD4⁺ T cell help is necessary both for generation and maintenance of effective specific CD8⁺ T cells [6, 7] directed against lymphocytic choriomeningitis virus [8, 9], murine

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Abbreviations: B-LCL: B-lymphoblastoid cell line · BZLF1: EBV lytic-switch protein ZEBRA · EBNA: EBV nuclear antigen ·

HAART: Highly active antiretroviral therapy ·

PHA: Phytohemagglutinin

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herpesviruses [10, 11], hepatitis C virus [12, 13], HIV [14, 15] and CMV [16, 17]. While during acute EBV infection EBV-specific CD4⁺ T cells can be detected *ex vivo* after short-term stimulation with EBV antigens, their frequencies during latency are below the detection limit in most donors [18, 19]. EBV nuclear antigen (EBNA)1 and EBNA3C appear to be immunodominant targets amongst the latent proteins [19, 20]. Furthermore, the lytic antigens EBV lytic-switch protein ZEBRA (BZLF1) and BMLF1 are recognized [18], and BMRF1 might also be an important CD4⁺ T cell target. Due to the difficulty to detect EBV-specific CD4⁺ T cells directly *ex vivo*, despite the use of very sensitive modern techniques, it remains unclear whether they play a role *in vivo* in the maintenance of control over EBV-infected cells.

In HIV-infected individuals, numbers of EBV-specific CD8⁺ T cells are generally well preserved [21, 22], although their ability to secrete IFN- γ in short-term antigen-specific stimulation assays is decreased [23]. Numbers of EBV-specific IFN- γ -producing CD8⁺ T cells correlated with absolute CD4⁺ T cell numbers, suggesting a role for CD4⁺ T cells in the maintenance of functional EBV-specific CD8⁺ T cells. To determine whether decreased EBV-specific CD8⁺ T cell function could be related to inadequate antigen-specific CD4⁺ T cell help, we developed an assay that allows the detection of central memory EBV-specific CD4⁺ T cells, able to expand and subsequently produce IFN- γ in response to an antigen, to both a latent and a lytic EBV protein, in a specific, reproducible way. This enabled us to study EBV-specific CD4⁺ T cell responses in both healthy and treated or untreated HIV-infected subjects.

Results

Low numbers of IFN- γ -producing EBV-specific CD4⁺ T cells after short-term stimulation with EBV peptide pools

To investigate whether we could detect IFN- γ -producing EBV-specific CD4⁺ T cells *ex vivo*, PBMC were stimulated overnight using overlapping EBV peptide pools as a stimulus. In accordance with earlier data [18, 19], we found low numbers of EBNA1-specific CD4⁺ T cells after overnight stimulation and measurement of IFN- γ -producing CD4⁺ T cells by either intracellular cytokine staining or ELISPOT (Fig. 1A, B, left panels, and data not shown). IFN- γ -producing CD4⁺ T cells after stimulation with EBNA1 peptide pools were detected only in 4/11 healthy donors (range 0.03–0.13% of CD3⁺CD4⁺ T cells), and 6/12 of highly active antiretroviral therapy (HAART)-treated HIV-infected EBV carriers (0.03–0.36%), using a cut-off value of two times above

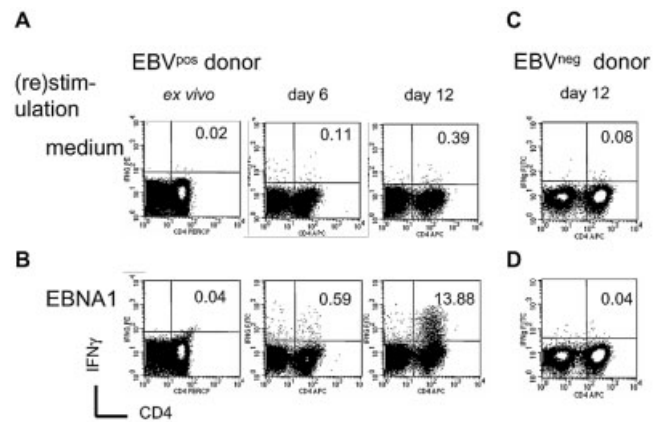


Fig. 1. Responses to EBV peptide pools directly *ex vivo* or after 6 or 12 days of expansion. Representative response of an EBV-seropositive donor *ex vivo* after 6 and 12 days. Cells were stimulated for 6 h with either medium (A) or EBNA1 peptide pool (B) directly *ex vivo* or after 6 or 12 days of culture with EBNA1 peptide pool and IL-2. The cells in the FACS plot were gated on CD3⁺ cells. Values in the upper right corner of the FACS plots indicate percentages of CD4⁺ T cells producing IFN- γ upon 6 h of restimulation with medium or peptide pool. (C, D) Representative response of an EBV-seronegative donor after 12 days of culture with EBNA1.

medium control. CD4⁺ T cell responses to BZLF1 were detected in 5/11 healthy (0.06–0.23%) and 2/12 HIV-infected individuals (0.15–0.29%). There was no significant difference in either the fraction of responders or the height of the response between healthy donors and HAART-treated HIV carriers (data not shown).

Antigen-specific expansion *in vitro* to study antigen-specific CD4⁺ T cell responses

As CD4⁺ T cell responses to EBNA1 and BZLF1 were hardly detectable directly *ex vivo*, an assay was developed in which specific T cells were expanded for 6–12 days by stimulation with EBV peptide pools. IL-2 was added only from day 3 onwards to give EBV-specific T cells a growth advantage. After expansion, cells were rested overnight in fresh medium and restimulated for 6 h with EBNA1 or BZLF1 peptide pools, and positive and negative controls were included. Fig. 1A (culture with EBNA1 pool, restimulation with medium) and Fig. 1B (culture and restimulation with EBNA1 pool) show the results for a donor in whom EBNA1-specific IFN- γ -producing CD4⁺ T cells were detected already on day 6. However, in most healthy donors, no response was detectable yet on day 6. On day 12, EBNA1-specific CD4⁺ T cells were detected in all healthy EBV seropositives (e.g. Fig. 1B). As a control, restimulation with overlapping peptide pools of HIV Env and Gag did not induce any responses in PBMC cultured with EBNA1 or BZLF1 peptide pools (data not shown). Furthermore,

even on day 12, no EBNA1- or BZLF1-specific CD4⁺ T cells were recovered from three EBV-seronegative donors and three cord blood samples (Fig. 1C, D).

Recognition of autologous EBV-transformed B-lymphoblastoid cell lines by EBNA1-specific CD4⁺ T cells

In order to confirm the EBV specificity of the polyclonal CD4⁺ T cell populations, recognition of autologous EBV-transformed B-lymphoblastoid cell lines (B-LCL) was tested. Cells from 12-day cultures were expanded for another week with phytohemagglutinin (PHA) and IL-2. After overnight rest in fresh medium, cells were stimulated in a 1:1 ratio with autologous B-LCL for 6 h. As control, uncultured PBMC were used as 'effectors'. Cells cultured with the EBNA1 peptide pool were able to recognize autologous B-LCL (Fig. 2A), as evidenced by IFN- γ production within the CD4⁺ T cells, while fresh PBMC were not (Fig. 2B). The B95–8 cell line, which does not express human MHC molecules, was not recognized. Moreover, the response could be inhibited by monoclonal antibodies against MHC class II (from 1.55 to 0.40% of IFN- γ -producing CD4⁺ T cells), but not by blocking of MHC class I (1.58% of IFN- γ -producing CD4⁺ T cells). These experiments further confirmed the EBV specificity and dependence on the MHC class II presentation pathway of CD4⁺ T cells expanded using peptide pools.

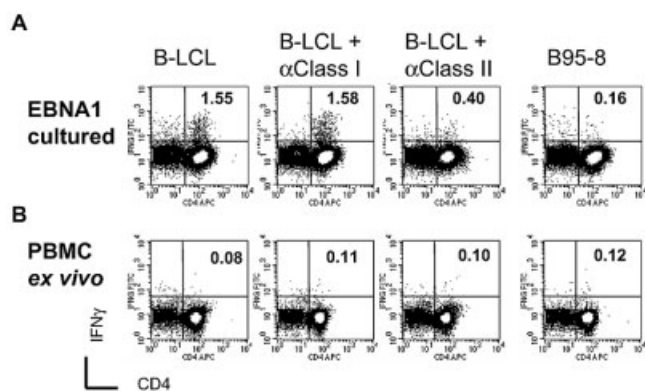


Fig. 2. Recognition of autologous B-LCL by EBNA1-specific CD4⁺ T cells via MHC class II. Cells cultured for 12 days in the presence of EBNA1 peptide pool and IL-2, and subsequently expanded for 1 week with PHA and IL-2 (A), and uncultured PBMC (B) were stimulated with autologous EBV-transformed B-LCL (left three panels) or B95–8 standard EBV B-LCL (right panel) for 6 h, in the presence or absence of antibodies to block MHC class I (W6/32) or class II (L243). FACS plots show the percentages of IFN- γ -producing CD4⁺ T cells, indicated in the upper right quadrant.

Interpretation of assay result

As the ratios of cellular subsets can vary a lot between individuals, especially when HIV seropositive, it was verified whether the depletion of CD4⁺, CD8⁺ T or B cells influenced the outcome of the assay. As shown in Fig. 3A and B for EBNA1 (donor 1) and Fig. 3B for BZLF1 (donor 2), the absence of CD8⁺ and CD19⁺ cells did not influence the fraction of EBV-specific cells measured within the CD4⁺ T cell subset, which remained around 20% in donor 1 and around 2% in donor 2. In donor 2, the depletion of CD4⁺ T cells was complete, so that no EBV-specific CD4⁺ T cells grew out, as expected. Interestingly, in donor 1, where after depletion 0.5% of CD4⁺ T cells remained and were put into culture, the outgrowth of specific cells within the CD4⁺ T cells after 12 days was similar to the percentage within the CD4⁺ T cell fraction of the total PBMC and CD8⁺ or CD19⁺ cell-depleted cultures (Fig. 3A, B). This indicates that the fraction of EBV-specific CD4⁺ T cells that grows out is independent of the total amount of CD4⁺ T cells in the culture.

The percentage of IFN- γ -producing cells within the CD3⁺CD4⁺ fraction is a commonly used value for describing the CD4⁺ T cell response to specific antigens. We wanted to determine a more absolute number of EBV-specific CD4⁺ T cells, enabling comparison of donors and patients with very different CD4⁺ T cell numbers. For this purpose, we calculated numbers of EBNA1- or BZLF1-specific IFN- γ -producing CD4⁺ T cells recovered out of 10⁶ PBMC put into culture on day 0. To illustrate the significance of these two values, donor PBMC were diluted in CD4-depleted PBMC, thereby diluting both specific and total CD4⁺ T cells, and then cultured for 12 days. As shown in Fig. 3C and D for two donors, the percentage of specific cells within the CD4⁺ T cells after 12 days remained constant in serial dilutions (left panels), whereas the absolute number of specific CD4⁺ T cells recovered did depend on the dilution of CD4⁺ T cells (right panels). Thus, expressing the results as a number of specific cells recovered after 12 days of culture gives a much better indication of the importance of the immune response than using the fraction of specific cells within the CD4⁺ T cell subset. We therefore choose to use this value for comparison of individuals or groups of individuals with each other. In addition, the assay was very reproducible, as shown in Fig. 3E for six donors with whom we were able to perform between two and five separate experiments.

CD4⁺ T cells specific for EBNA1 and BZLF1 in healthy and HIV-infected EBV carriers

To determine whether EBV-specific CD4⁺ T cells could be found in HIV-infected subjects, PBMC from

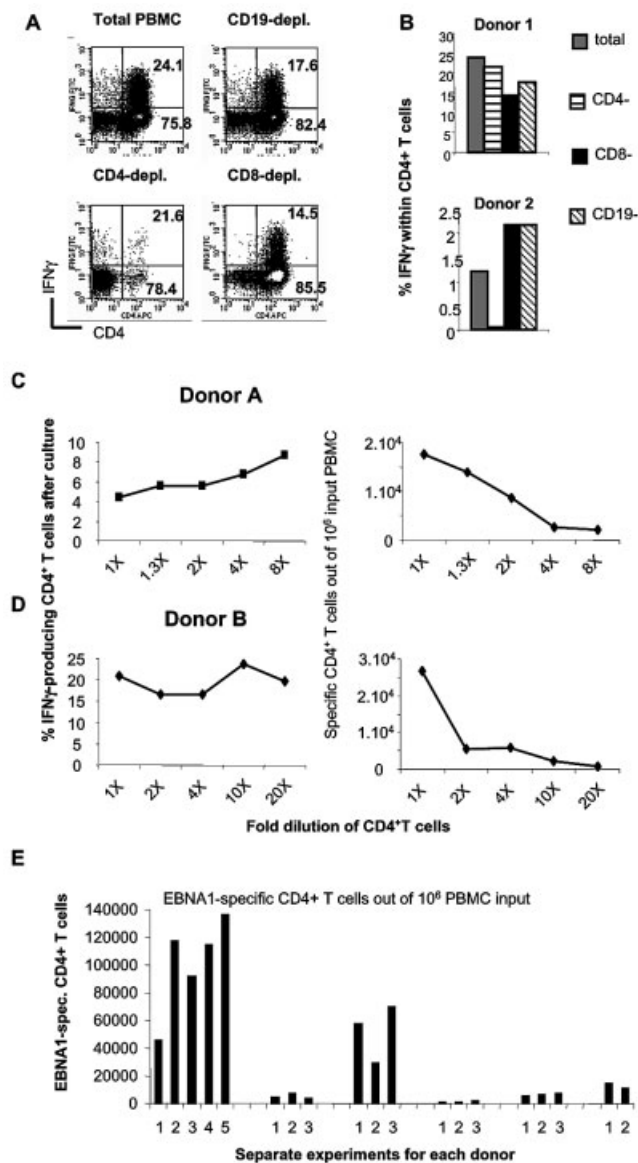


Fig. 3. Expression of results as percentage of the CD3 $^{+}$ CD4 $^{+}$ T cell subset or as the number of EBV-specific IFN- γ -producing CD4 $^{+}$ T cells per 10^6 PBMC put into culture. (A) FACS plots showing the effects of depletion of CD4 $^{+}$, CD8 $^{+}$ or CD19 $^{+}$ cells upon the response after 12 days of culture with EBNA1. Values indicate the percentages of IFN- γ -producing CD4 $^{+}$ T cells. (B) Overview of the effects of CD4/CD8/CD19 depletion on the expansion of EBNA1- (donor 1) and BZLF1- (donor 2) specific CD4 $^{+}$ T cells. (C, D) PBMC from two donors were mixed with CD4-depleted PBMC before a 12-day culture, to dilute EBNA1-specific CD4 $^{+}$ T cells. Left panels show the percentages of IFN- γ -producing cells within the CD3 $^{+}$ CD4 $^{+}$ T cell subset, which remains constant. Right panels show the numbers of IFN- γ -producing CD3 $^{+}$ CD4 $^{+}$ T cells recovered out of 10^6 PBMC put into culture at day 0, which is dependent on the number of CD4 $^{+}$ T cells put in. (E) The reproducibility of the assay was tested on six donors for EBNA1, with two to five separate experiments (indicated by the numbers on the x axis), whereas the y axis indicates the number of EBNA1-specific CD4 $^{+}$ T cells recovered out of 10^6 PBMC put into culture at day 0).

10 HAART-treated and 13 untreated HIV carriers were cultured. For comparison, PBMC from 14 healthy EBV carriers were used. EBNA1-specific CD4 $^{+}$ T cells were found in all (14) healthy donors (median 3,400 out of 10^6 input PBMC), all (10) HAART-treated HIV $^{+}$ (3,700 out of 10^6 input PBMC), and 9/13 untreated HIV-infected donors (500 out of 10^6 input PBMC) (Fig. 4A, C). Numbers of EBNA1-specific CD4 $^{+}$ T cells recovered out of 10^6 input PBMC were significantly lower in untreated HIV $^{+}$ compared to healthy donors ($p=0.043$, Mann-Whitney test). BZLF1-specific CD4 $^{+}$ T cells were

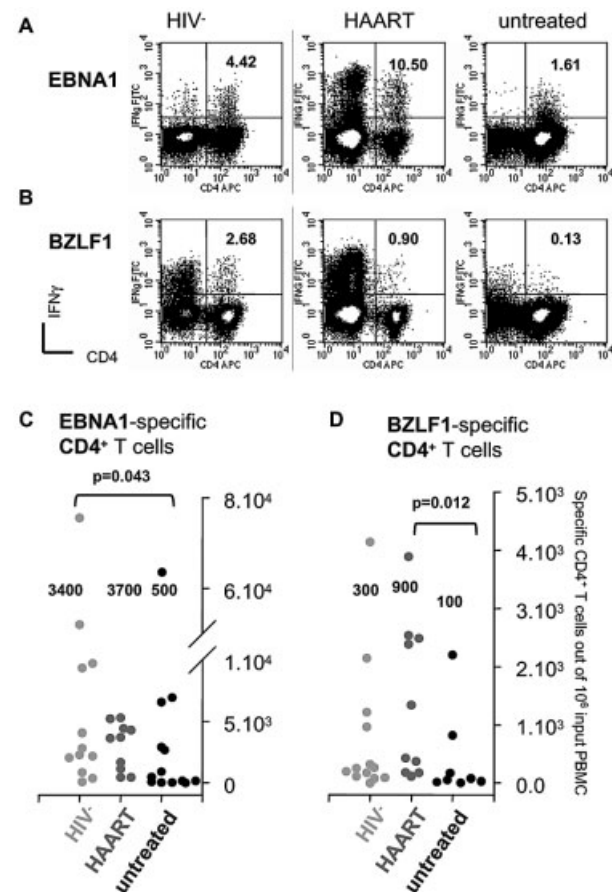


Fig. 4. Lower EBV-specific CD4 $^{+}$ T cell responses in untreated HIV $^{+}$ individuals compared to healthy donors and HAART-treated HIV $^{+}$ individuals after 12 days of expansion. (A, B) Representative FACS plots after 12 days of expansion of cells from a healthy EBV carrier (HIV $^{+}$), a HAART-treated HIV $^{+}$ individual (HAART) and an untreated HIV $^{+}$ individual (untreated), for culture and restimulation with EBNA1 (A) and BZLF1 (B). Indicated is the percentage of CD3 $^{+}$ CD4 $^{+}$ T cells producing IFN- γ upon a 6-h restimulation with peptide pool after 12 days of expansion. (C, D) Responses to EBNA1 (C) and BZLF1 (D) in healthy donors (HIV $^{+}$: $n=14$ for EBNA1 and $n=13$ for BZLF1), HAART-treated (HAART: $n=10$ for EBNA1 and BZLF1) and untreated HIV $^{+}$ individuals (untreated; $n=13$ for EBNA1 and $n=8$ for BZLF1). The values on the y axis indicate the number of IFN- γ -producing CD3 $^{+}$ CD4 $^{+}$ T cells recovered out of 10^6 PBMC put into the assay at day 0. Median values in the different groups are indicated above the dots.

measured in 9/13 healthy donors (300 out of 10^6 input PBMC), 9/10 HAART-treated (900 out of 10^6 input PBMC) and 4/8 untreated HIV⁺ donors (100 out of 10^6 input PBMC) (Fig. 4B, D). The recovery of BZLF1-specific CD4⁺ T cells was lower in untreated HIV⁺ compared to HAART-treated individuals ($p=0.012$), but not significantly lower than in healthy donors ($p=0.14$).

To verify that the results were not biased by differential survival of the PBMC under culture conditions, we calculated the ratio between the PBMC output and input. This did not differ between healthy donors (1.30 for EBNA1, 1.00 for BZLF1), HAART-treated (1.57 and 1.12) and untreated HIV carriers (0.95 and 0.90) (p values between 0.122 and 0.981). In addition, PHA-induced proliferation (for 13 untreated HIV carriers) and anti-CD2/CD28 or anti-CD3 antibody-induced proliferation (for two HAART-treated and nine untreated HIV carriers) did not correlate with the outcome of the 12-day assay for either EBNA1 or BZLF1 (data not shown), suggesting that a general defect in T cell proliferative capacity is not an explanation for these results.

Increased numbers of EBNA1-specific CD8⁺ T cells in HAART-treated HIV carriers

Together with specific CD4⁺ T cells, both EBNA1- and BZLF1-specific CD8⁺ T cells were expanded by culture with the corresponding peptide pools (Fig. 5A, B). Interestingly, higher numbers of EBNA1-specific CD8⁺ T cells were recovered from HAART-treated HIV⁺ individuals (8/10 responders) compared to healthy donors (8/14 responders; 7,800 vs. 800 out of 10^6 PBMC input, $p=0.031$; Fig. 5A, C) and untreated HIV carriers (8/13 responders; 7,800 vs. 800 out of 10^6 PBMC input, $p=0.036$; Fig. 5C). In contrast, the median recovery of BZLF1-specific CD8⁺ T cells was identical in healthy subjects (13/13 responders) and HAART-treated (10/10 responders) HIV carriers (7,800 vs. 9,400 out of 10^6 PBMC input, $p=0.563$), and not significantly lower in untreated (8/8 responders; 3,200 out of 10^6 PBMC input) compared to HAART-treated ($p=0.088$) and healthy donors ($p=0.311$) (Fig. 5B, D). Since an elevated EBNA1-specific CD8⁺ T cell response was observed earlier in EBV carriers with certain HLA class I alleles [24], we compared responses in HLA B7-, B35- or B53-positive subjects with those carrying other HLA alleles, but found no difference (data not shown). It thus appears unlikely that a bias in the distribution of HLA types could explain differences in EBNA1-specific CD8⁺ T cell responses.

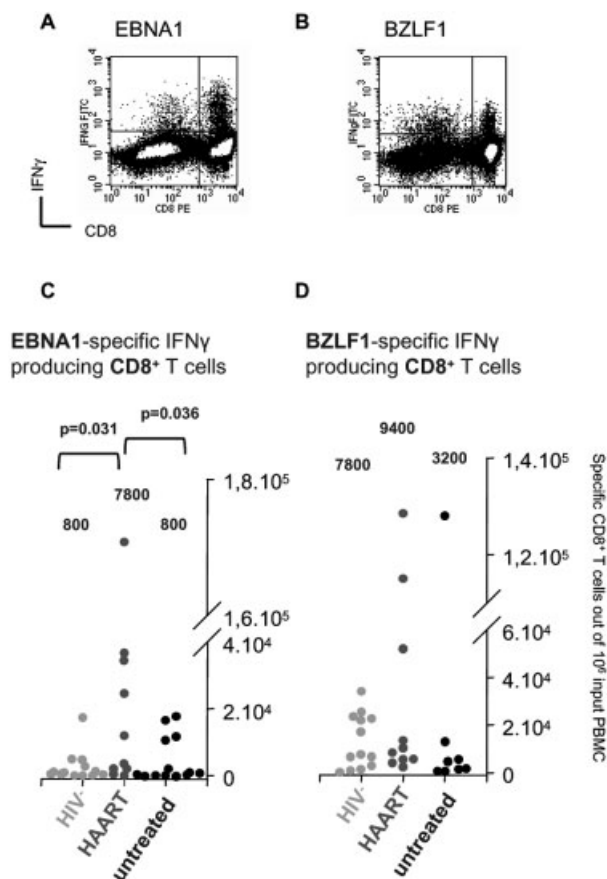


Fig. 5. Increased expansion of EBNA-specific CD8⁺ T cells from HAART-treated individuals. Examples of CD8⁺ T cell responses to EBNA1 (A) and BZLF1 (B) after a 12-day expansion of cells from a HAART-treated HIV⁺ individual. Responses to EBNA1 (C) and BZLF1 (D) in healthy donors (HIV: $n=14$ for EBNA1 and $n=13$ for BZLF1), HAART-treated (HAART: $n=10$ for EBNA1 and BZLF1) and untreated HIV⁺ individuals (untreated; $n=13$ for EBNA1 and $n=8$ for BZLF1). The values on the y axis indicate the number of IFN- γ -producing CD3⁺CD8⁺ T cells recovered out of 10^6 PBMC put into the assay at day 0.

Both CD4⁺ and CD8⁺ EBV-specific T cells correlate inversely with EBV viral load

Next, we investigated whether EBV-specific CD4⁺ and CD8⁺ T cell responses were related to EBV viral load. Only HIV-seropositive subjects were included in this analysis, as the healthy controls had no measurable EBV viral load. Interestingly, EBV load tended to be inversely correlated with EBNA1-specific CD4⁺ T cell responses (0.381, $p=0.089$; Spearman's correlation coefficient), and was inversely correlated with EBNA1-specific CD8⁺ (-0.496 , $p=0.022$) and both BZLF1-specific CD4⁺ (-0.664 , $p=0.005$) and CD8⁺ T cells (-0.500 , $p=0.048$) (Table 1). Furthermore, a role for CD4⁺ T cell help in the CD8⁺ T cell response was suggested by a weak correlation between CD4⁺ and CD8⁺ T cell responses to EBNA1 (0.442, $p=0.035$), while a lack of

Table 1. Correlations^{a)}

| Factor 1 | Factor 2 | Correlation coefficient ^{b)} | p value |
|--------------------------------|-----------|---------------------------------------|---------|
| EBV DNA ^{c)} | EBNA1-CD4 | −0.381 | 0.089 |
| | EBNA1-CD8 | −0.496 | 0.022 |
| | BZLF1-CD4 | −0.664 | 0.005 |
| | BZLF1-CD8 | −0.5 | 0.048 |
| HIV RNA ^{d)} | EBNA1-CD4 | −0.259 | 0.232 |
| | EBNA1-CD8 | −0.161 | 0.463 |
| | BZLF1-CD4 | −0.624 | 0.006 |
| | BZLF1-CD8 | −0.293 | 0.239 |
| EBV DNA | HIV RNA | 0.423 | 0.056 |
| Total CD4 ⁺ T cells | EBNA1-CD4 | 0.139 | 0.547 |
| | BZLF1-CD4 | 0.09 | 0.74 |
| EBNA1-CD4 | EBNA1-CD8 | 0.442 | 0.035 |
| BZLF1-CD4 | BZLF1-CD8 | 0.273 | 0.272 |

^{a)} Values in bold indicate significant correlations between factor 1 and factor 2. EBNA1-CD4 : EBNA1-specific CD4⁺ T cells, etc.

^{b)} Spearman's correlation coefficient.

^{c)} EBV DNA per 10⁶ PBMC.

^{d)} HIV RNA copies per ml plasma.

correlation between BZLF1-specific CD4⁺ and CD8⁺ T cell responses did not support this (0.273, $p=0.272$) (Table 1).

In addition, we investigated whether the EBV-specific T cell response was influenced by factors associated with HIV infection. Only BZLF1-specific CD4⁺ T cells were inversely correlated with HIV viral RNA (−0.624, $p=0.006$), while EBNA1-specific CD4⁺ and CD8⁺ and BZLF1-specific CD8⁺ T cells were not (Table 1). HIV and EBV viral load also tended to correlate (0.423, $p=0.056$). Interestingly, numbers of EBNA1- or BZLF1-specific cells were not correlated with absolute CD4⁺ T cell numbers (0.139, $p=0.547$ for EBNA1; 0.090, $p=0.740$ for BZLF1).

Discussion

In this study, we investigated whether a lack of specific CD4⁺ T cell help may explain a lower function of EBV-specific CD8⁺ T cells in HIV-infected subjects. To this end, we set up a specific, highly reproducible assay, combining *ex vivo* expansion of EBV-specific cells with flow cytometric analysis of IFN- γ production. This enabled us to measure EBV-specific CD4⁺ T cells in all healthy EBV carriers. We found: (1) comparable numbers of EBNA1- and BZLF1-specific CD4⁺ T cells in healthy EBV carriers and HAART-treated HIV⁺ individuals, while these responses were lower in untreated HIV⁺ subjects; (2) a higher frequency of EBNA1-specific

CD8⁺ T cells in HAART-treated subjects compared to both untreated HIV⁺ and healthy subjects; (3) that numbers of both CD4⁺ and CD8⁺ EBV-specific T cells were inversely correlated with EBV load. Therefore, measuring specific CD4⁺ T cells that are able to proliferate and subsequently respond by IFN- γ production most likely gives a good reflection of the EBV-specific immunological status of the patient.

As of this writing, few studies have reported *ex vivo* detection of EBV-specific CD4⁺ T cells [18, 19]. In a recent article, Amyes et al. report *ex vivo* detection of EBV-specific CD4⁺ T cells by stimulation with a lysate of PMA-reactivated EBV B-LCL in 23/28 healthy donors [25]. Here, we used overlapping peptide pools of EBNA1 and BZLF1 to enable measurement of responses independent of the donors' HLA type, (not yet well-defined) HLA restriction and immunodominance patterns of EBV epitopes, and independent of processing by APC *in vitro*. We observed detectable *ex vivo* CD4⁺ T cell responses to EBNA1 and BZLF1 only in a minority of healthy donors and HAART-treated HIV⁺ subjects. Therefore, an assay was developed combining an antigen-specific expansion step using overlapping peptide pools, with specific restimulation after 12 days. This assay presents several advantages as it enables detection of EBV-specific CD4⁺ T cells in all healthy EBV carriers, allowing determination of specificity at the protein level. Furthermore, this assay detects so-called “central memory T cells”, *i.e.* antigen-primed T cells that can proliferate and exert their function by cytokine produc-

tion upon re-encounter with this antigen. The outcome of such an assay depends on both precursor frequency and functional capacity of these T cells. It was shown in both hepatitis C virus- [26] and *Plasmodium falciparum*-infected individuals [27] that protection against infection and/or clearance of the pathogen correlated with IFN- γ -producing CD4⁺ T cells measured after *ex vivo* expansion, and not with the response measured directly *ex vivo*. Accordingly, *ex vivo* IFN- γ -producing HIV-specific CD4⁺ T cells were detected in subjects with both high and low HIV load, whereas CD4⁺ (and CD8⁺) T cells capable of proliferation were detected only in low-load carriers [15, 28]. Therefore, we believe the results presented here are a good reflection of the EBV-specific immunological status of the patient.

Using this assay to measure EBV-specific CD4⁺ T cells, we observed no difference between healthy EBV-positive donors and HAART-treated HIV⁺ persons, but a lower CD4⁺ T cell response to both EBNA1 and BZLF1 in untreated HIV-infected individuals. Interestingly, these responses (and the CD8⁺ T cell response measured in the same assays) tended to correlate inversely with EBV load, while HIV load correlated inversely only with BZLF1-specific CD4⁺ T cells, and no correlation was found with total CD4⁺ T cell numbers. This could reflect an impaired proliferation or exhausted EBV-specific memory T cell compartment due to chronic antigen exposure [15, 28]. As the HAART-treated subjects have both a lower EBV load and a higher EBV-specific T cell response in our assay, having antiretroviral treatment in itself seems to be the most important factor influencing EBV-specific memory responses.

Data from γ -herpesvirus infection in mice [10, 11] and many viral infection models in both mouse and man [6, 7, 12–17] suggest that CD4⁺ T cells most probably also play a role in the development and maintenance of an effective EBV-specific CD8⁺ T cell response. In addition, several studies investigated the recognition of EBV-infected cells by CD4⁺ T cells via recognition of latent [29–32] or lytic antigens [33] and showed that EBV-specific CD4⁺ T cells could directly control outgrowth of EBV-infected B cells *in vitro*. Similarly, EBNA1-specific CD4⁺ T cells were capable of recognizing autologous EBV-infected B cells in this study.

Interestingly, a higher EBNA1-specific CD8⁺ T cell response was measured in HAART-treated individuals compared to both untreated HIV carriers and healthy donors. In contrast to earlier reports that EBNA1 could not be directly presented via the MHC class I pathway [34], several recent studies show that EBNA1-specific CD8⁺ T cells are capable of directly recognizing EBV-infected B cells [35–39]. Increased numbers of EBNA1-specific CD8⁺ T cells after HAART, whether related to improved CD4⁺ T cell help or the antiretroviral therapy-

induced changes in proteasomal degradation pathways, might thus improve the immune control of EBV infection.

In conclusion, using a 12-day expansion protocol, we were able to investigate EBV-specific CD4⁺ T cells in healthy and HIV-infected subjects. Lower numbers of EBV-specific CD4⁺ T cells were found in untreated HIV-infected individuals, suggesting loss of EBV-specific CD4⁺ T cells due to HIV infection, while HAART might preserve EBV-specific CD4⁺ T cell immunity. Furthermore, we found indications for an inverse correlation between the recovery of EBV-specific CD4⁺ T cells after 12 days and EBV viral load, which is in accordance with recent data on HIV-specific CD4⁺ T cells [15, 28]. Therefore, we believe that our assay gives a good indication of an individual's ability to exert an EBV-specific CD4⁺ memory T cell response and that it will be a useful tool for studying the role of EBV-specific CD4⁺ T cells in various clinical settings.

Materials and methods

Study population

This study was performed on cryopreserved PBMC from 17 healthy blood bank donors. EBV seropositivity was tested by measurement of anti-viral charge antigen IgG. Included were 14 EBV-seropositive (median age 47) and 3 EBV-seronegative (median age 40) individuals. Furthermore, three cord blood samples were studied. EBV viral load was below detection limits in all healthy EBV-seropositive donors.

Samples from HIV-infected individuals were collected in the framework of the Amsterdam Cohort studies on AIDS and HIV-1 infection. Homosexual men at risk for HIV-1 infection were sampled every 3 months for HIV-1 serology and immunological studies. In addition, at all time points PBMC were cryopreserved. We selected ten HAART-treated individuals and 13 untreated cohort participants based on comparable CD4⁺ T cell numbers and availability of material. HAART was defined as a triple-drug regimen consisting of two nucleoside analogues and one protease inhibitor.

All individuals under study gave their informed consent according to the declaration of Helsinki. The characteristics of the HIV-infected individuals are shown in Table 2.

T cell stimulation

EBV-specific CD4⁺ T cells were stimulated using overlapping peptide pools. Peptides (15-mers) with 11-amino acid overlap spanning the immunogenic C-terminal region of EBNA1 (57 peptides) and the entire BZLF1 protein (59 peptides) were synthesized by Jerini AG (Berlin, Germany). Purity and sequences were verified by HPLC and mass spectrometry. Peptides were dissolved in DMSO and pooled at a final concentration of 1 mg/ml of each peptide.

Table 2. Characteristics of the individuals studied

| | HIV ⁺ HAART (n=10) | HIV ⁺ untreated (n=13) | p value ^{a)} |
|---------------------------------|---------------------------------|--|-----------------------|
| Time on HAART ^{b)} | 43 (3–52) | | na |
| Time from HIV SC ^{b)} | | 16.5 (2–53) (n=12) | na |
| Time from entry ^{b)} | 46 (3–88) | 18 (n=1) | na |
| Age | 40 (33–62) | 37 (24–61) | ns |
| CD4 per μ l | 540 (240–1,340) | 680 (80–950) | ns |
| HIV RNA ^{c)} | 400 (50–1.1 $\times 10^6$) | 28,000 (10 ³ –5.3 $\times 10^5$) | 0.001 |
| HIV RNA pre-HAART ^{d)} | 35,000 (400–1.7 $\times 10^5$) | na | ns |
| EBV DNA ^{e)} | 68 (0–451) | 500 (10–3,784) | 0.020 |

a) Mann-Whitney test; na: not applicable, ns: not significant.

b) Time in months; SC: seroconversion; from entry indicates time under study, when SC date is unknown.

c) HIV RNA copies per ml plasma.

d) HIV RNA load at start of HAART or 1 year before HAART, p value in comparison to untreated group.

e) EBV DNA per 10⁶ PBMC.

Detection of IFN- γ -producing EBV-specific T cells

IFN- γ -producing cells after stimulation with overlapping peptide pools were enumerated by intracellular cytokine staining (ICCS) [23, 40]. Briefly, 10⁶ PBMC were stimulated in 500 μ l medium containing 10% FCS for 18 h *ex vivo* (or 6 h after expansion in culture) with EBNA1 or BZLF1 peptide pools (at 2 μ g/ml of each peptide) and both anti-CD28 (2 μ g/ml) and anti-CD49d antibodies (1 μ g/ml) as costimuli, in the presence of 1:1,000 brefeldin A (Golgiplug; BD Biosciences, San José, CA) after 1 h. As a negative control, PBMC from HIV-negative individuals were either stimulated with medium and costimulation alone or with irrelevant peptide pools (HIV Env and Gag; NIH, Rockville, MD). As a positive control, PBMC were stimulated with 10 ng/ml PMA and 2 μ g/ml ionomycin. After stimulation, cells were washed in PBS, permeabilized (FACS Permeabilizing Solution; BD Biosciences), washed again and stained with antibodies specific for CD3, CD4, CD8 and IFN- γ (BD Biosciences). Cells were washed again and fixed (Cellfix; BD Biosciences), and 200,000 events were acquired on a FACSCalibur flow cytometer (Becton Dickinson). Lymphocytes were gated by forward and sideward scatter, and data were analyzed using the software program Cell Quest (Becton Dickinson). Responses were scored as positive when two times above the medium control value.

In pilot experiments, IFN- γ -producing T cells were also measured by ELISPOT, as described [23], either in whole PBMC or after depletion of CD8⁺ T cells using Dynabeads (DynaL GmbH, Hamburg, Germany), according to the protocol provided by the manufacturer.

Expansion of EBV-specific T cells

To expand EBV-specific T cells, PBMC were cultured for 12 days in the presence of the EBNA1 or BZLF1 peptide pool. Culture medium consisted of RPMI 1640 (Gibco Life Technologies, Breda, The Netherlands) supplemented with penicillin/streptomycin and 10% human pool serum. Cells were cultured at 2 $\times 10^5$ PBMC/well in 100 μ l medium in 96-well round-

bottom plates, at 37°C and 5%CO₂. Peptide pool (at 2 μ g/ml of each peptide) was added on days 0 and 6. IL-2 was added at 10 U/ml on days 3, 6, and 9. On day 12, cells were pooled, washed in RPMI and rested overnight in medium supplemented with 10% human pool serum, without peptides or IL-2. On day 13, cells were restimulated for 6 h using the protocol indicated in the preceding section, using human pool serum instead of FCS.

When indicated, CD4⁺, CD8⁺ or CD19⁺ cells were depleted from the PBMC using Dynabeads (DynaL GmbH, Hamburg, Germany), according to the protocol provided by the manufacturer. The efficacy of depletion usually reached less than 0.5% of target cells remaining, as verified by FACS analysis.

Recognition of autologous EBV-transformed B-LCL

To test recognition of autologous EBV-transformed B-LCL by EBNA1-specific CD4⁺ T cells, cells cultured for 12 days with EBV antigens were further expanded for 1 week with PHA (1 μ g/ml on the first day) and IL-2 (10 U/ml on day 3). Of these cultured PBMC, 5 $\times 10^5$ were then stimulated for 6 h with 5 $\times 10^5$ autologous B-LCL, and the assay was further performed as described above for peptide stimulations. MHC class I and II pathways were blocked using 10 μ g/ml of monoclonal antibodies W6/32 (anti-pan HLA class I; ATCC) and L243 (anti-HLA-DR; ATCC), respectively. The B95–8 cell line was used as a control for dependency on human MHC class II presentation, and uncultured donor PBMC were used to verify that recognition of B-LCL depended on expansion of EBNA1-specific CD4⁺ T cells.

Measurement of HIV load

HIV RNA load was measured in plasma by several assays. The NASBA HIV-1 QT assay (Organon Teknika, Bostel, The Netherlands), Amplicor HIV monitor (Roche Diagnostic Systems Inc., Branchburg, NJ) and Quantiplex bDNA 3.0 assay (Bayer Corporation, Tarrytown, NY) had detection limits

of 1,000, 400 and 50 copies/ml, respectively. Values of 1,000, 400 and 50 in the patients' characteristics (Table 2) indicate that HIV RNA was undetectable by the method used, the cut-off values corresponding to the assay that was used.

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

EBV load was measured in duplicate in DNA from 2×10^5 cells. Real-time PCR amplification was performed as described [41, 42]. The detection limit of this assay was initially reported as 50 copies/ 10^6 PBMC; however, values above 10 copies/ 10^6 PBMC were included when less than 10% variation was observed between duplicates, and otherwise scored as 0. As a control for input DNA, the amount of β -albumin DNA, a household gene present at 2 copies/cell, was also determined, using primers and probes as described [43].

Statistical analysis

For comparison of EBV-specific T cell numbers between different groups, Mann-Whitney tests were used. Correlations were calculated using Spearman's correlation tests. Software program SPSS 11.5 for Windows was used (SPSS Inc., Chicago, IL).

Acknowledgements: This study was part of the Amsterdam Cohort Studies on AIDS and HIV-1 infection, a collaboration of the Municipal Health Service, the Academic Medical Center and Sanquin Research at CLB. We thank Suzanne Jurriaans from the department of Human Retrovirology at the AMC for measurement of HIV RNA load and Maarten Koot from Sanquin Diagnostics for performing EBV serology. This work was supported by Grant CLBD2000–2164 from the Dutch Cancer Society (to D.v.B.).

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