Viro-immunological studies on the role of Epstein-Barr virus in the development of AIDS-related non-Hodgekin's lymphoma
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Chapter 7

Differential restoration of functional Epstein-Barr virus and Human immunodeficiency virus specific CD8+ T cells during antiretroviral therapy

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Differential restoration of functional Epstein-Barr Virus and Human immunodeficiency virus-1 specific CD8\(^+\) T cells during antiretroviral therapy

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In this study we investigated the effect of highly active anti-retroviral therapy (HAART) on HIV- and EBV-specific CD8\(^+\) T cells in 17 HIV-1 infected individuals, using a combination of both direct visualisation of virus-specific T cells with tetrameric HLA-peptide complexes, and functional analysis, using IFN\(\gamma\) elispot assays after peptide-stimulation. HAART induced a decrease of HIV-specific CD8\(^+\) T cells in most individuals, both in number and function, whereas numbers of EBV-specific T cells did not change. Numbers of IFN\(\gamma\) producing EBV-specific T cells increased in the majority of the individuals after HAART, resulting in higher numbers of functional EBV-specific CD8\(^+\) T cells as compared to HIV-specific T cells and leading to a higher percentage IFN\(\gamma\)/tetramer\(^+\) T cells. An increase in the number of EBV-specific IFN\(\gamma\)\(^+\) T cells was accompanied by a decrease in EBV load after HAART. In addition, a positive trend was found between CD4\(^+\) T cell numbers and the number of IFN\(\gamma\) producing EBV-specific CD8\(^+\) T cells in HIV-infected individuals with CD4\(^+\) T cell counts below 200/\mu l blood at initiation of HAART, suggesting that recovery of CD4\(^+\) T cells is important to improve the functional capacity of CD8\(^+\) T cells. These data indicate that loss of HIV antigenic pressure leads to a decline in HIV-specific T cell numbers in patients on HAART. In case of EBV, where load is not directly influenced by HAART, restoration of CD4\(^+\) T cell number leads to functional restoration of EBV-specific CD8\(^+\) T cells, resulting in a decrease in EBV load.

Introduction

In human viral infections, MHC class I-restricted CD8\(^+\) cytotoxic T cells (CTL) are the main mediators of viral clearance or control of viraemia.\(^1\)\(^2\) Primary infection with Epstein-Barr virus (EBV), a widespread human gamma herpesvirus, and reactivation of latently EBV-infected B lymphocytes is controlled by specific CTL responses.\(^3\) In contrast, in HIV-infection, despite the efficacy of HIV-specific CTL to suppress viral replication early in infection,\(^4\) CTL do not contain and eventually seem to lose control of HIV viral replication completely.\(^5\) Furthermore, during HIV-induced immunodeficiency, a higher rate of reactivation of EBV-infection occurs which may lead to uncontrolled lymphoproliferation,\(^6\) indicating that apart from HIV also other viruses like EBV can no longer be contained. Different techniques are now being employed which detect either presence or function of virus specific T cells.
Staining with tetrameric MHC-peptide complexes can enumerate CD8+ T cells with peptide specific T cell receptors, and their use revealed a much higher frequency of antigen-specific circulating T cells than estimated before by Limiting Dilution Analysis. 8-10 γCr release assays and cytokine detection assays, like IFNγ ELispot assays, assess the number of functionally reactive T cells at the peptide level. By detecting virus-specific CD8+ T cells by both tetrameric MHC-peptide complexes and IFNγ production, Zajac et al. showed that LCMV-infected CD4 knock out mice mounted substantial numbers of LCMV specific tetramer+ T cells, but had decreased numbers of IFNγ producing T cells compared to wild type mice. In addition, we have observed decreased numbers of IFNγ producing HIV-specific (Kostense et al. submitted) and EBV-specific CD8+ T cells in HIV-infected individuals (Van Baarle et al. submitted). In addition, HIV-infected individuals have higher EBV load and in HIV-infected individuals progressing to AIDS-related non-Hodgkin’s lymphoma (AIDS-NHL), loss of EBV-specific T cell function in the course of HIV-infection was paralleled by increasing EBV load (Van Baarle et al. submitted). Appay et al. showed that HIV-specific T cells indeed lacked perforin, an important effector molecule in killing virus infected cells. Because of the functional deficiency of CD8+ T cells, at this time for HIV-infected individuals antiretroviral drugs are the only means to suppress HIV RNA load. Although monitoring of treatment with combinations of antiretroviral drugs have mainly focused on suppression of viral load and reconstitution of CD4+ T cell numbers, improvement of CTL function is considered an important goal, especially in the context of the recently reported structured-treatment-interruption trials. Until now, evaluation of anti-HIV-specific CD8+ T cells has been dominated by observations of decreased HIV-specific CD8+ T cell numbers possibly due to decreased antigen burden without further detailed functional analysis. Furthermore, the effect of HAART on EBV immunity and EBV load, of which the latter is often high in HIV-infected individuals, has not been evaluated. Because of the reported positive correlation between CD4+ T cell numbers and effective antiviral CD8+ T cells, therapy-induced restoration of CD4+ T cell numbers is expected to lead to functional restoration of virus-specific CD8+ T cells.

In this study, we have used a combination of both direct visualisation, using tetrameric HLA-peptide complexes, and functional analysis, using IFNγ elispot assay, of HIV-specific CD8+ T cells and compared kinetics of HIV-specific T cells with EBV-specific CD8+ T cells after highly active antiretroviral therapy (HAART). Using these techniques simultaneously, also the fraction of functional cells (IFNγ producing T cells) of the total amount of antigen-specific T cells (tetramer+ T cells) was assessed. In addition, using real time quantitative PCR assay, the number of EBV virus particles was quantitated to study the possible impact of HAART on EBV load.

Materials and methods

Study population

This study was performed on participants of the Amsterdam Cohort
studies on AIDS and HIV-1 infection. We selected HIV-seropositive male individuals according to HLA-type A2, A11, B8, or B57. Peripheral blood mononuclear cells (PBMC) were cryopreserved according to a standard protocol in a computerized freezing device. We analyzed longitudinal PBMC samples from 17 HIV-infected individuals on HAART consisting of a triple drug regimen. Two of these individuals could only be investigated either for EBV or HIV responses alone, because of their HLA types. Several individuals were treated with 2 reverse transcriptase-inhibitors prior to addition of the protease inhibitors. Characteristics of the HIV-1 infected individuals are summarized in Table 1.

Table 1: Characteristic of HIV-1 infected homosexual men on HAART

<table>
<thead>
<tr>
<th>Patient</th>
<th>HLA</th>
<th>Baseline CD4+ T cell count / μl blood</th>
<th>Baseline viral load / μl serum</th>
<th>CD4 response</th>
<th>Load response</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2</td>
<td>120</td>
<td>180</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>A2</td>
<td>170</td>
<td>12</td>
<td>+</td>
<td>+</td>
</tr>
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<td>B8</td>
<td>150</td>
<td>93</td>
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</tr>
<tr>
<td>43</td>
<td>A2,B8</td>
<td>440</td>
<td>51</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>156</td>
<td>A11,B8</td>
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<td>A2,B8</td>
<td>270</td>
<td>54</td>
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<td>+</td>
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<td>270</td>
<td>A2,B8</td>
<td>100</td>
<td>81</td>
<td>+</td>
<td>+</td>
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<td>371</td>
<td>B57</td>
<td>310</td>
<td>166</td>
<td>+</td>
<td>+</td>
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<tr>
<td>545</td>
<td>A2,B8</td>
<td>350</td>
<td>90</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>557</td>
<td>A2,B8</td>
<td>130</td>
<td>17</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>585</td>
<td>A2</td>
<td>100</td>
<td>1</td>
<td>+</td>
<td>+</td>
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<tr>
<td>692</td>
<td>B8</td>
<td>170</td>
<td>340</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1113</td>
<td>B8,B57</td>
<td>280</td>
<td>66</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1194</td>
<td>A11,B8</td>
<td>350</td>
<td>110</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*HIV viral RNA copies/μl serum

# Increased CD4+ T cell numbers (to above 200/μl) after start of therapy = +; no change or decreased CD4+ T cell numbers = -.

$ Decreased HIV viral load after start of therapy = +; no change or increased viral load = -. 
Chapter 7

Tetrameric HLA-peptide complex formation

Refolding of HLA class I heavy chains and tetramer formation was performed as described previously. HLA class I heavy chains and β2 microglobulin were constructed in pET plasmids (Novagen) and expressed in BL21 E.coli strains. Heavy chain, β2m and peptides were refolded by dilution. Subsequently, MHC class I tetramers complexed with EBV and HIV-peptides were produced as previously described. The peptides used (Isogen, Maarssen, The Netherlands) are listed in table 2. Refolded HLA peptide complexes were biotinylated, FPLC purified using an H16/60 Superdex 200 column (Pharmacia). Purified complexes were bound to streptavidin-phycocyanin or streptavidin-APC (Sigma). Tetrameric product was FPLC purified and concentrated using amicon stir cells.

Flow cytometry and tetramer staining

Three- or four-color fluorescence analysis was performed as previously described. Briefly, PBMC were thawed and 1.5 x 10⁶ cells were stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (PBA) with MHC class I tetramers (PE and APC), PerCP conjugated Mab CD8 (Becton Dickinson, San José, California, USA) and anti-CD27-FITC (CLB, Amsterdam, The Netherlands). After staining, cells were washed with PBA and fixed in PBS/1% paraformaldehyde, and at least 250,000 events were acquired using a FACScalibur flow cytometer (Becton Dickinson).

To determine the percentage of dead cells in each sample, propidium iodide staining was performed. Lymphocytes were gated by forward and sideward scatter. Data were analyzed using the software program CELL Quest (Becton Dickinson).

ELISpot assay for single cell IFNγ-release

IFNγ producing antigen-specific T cells were enumerated using IFNγ specific ELISpot assays as previously described. Nylon-backed 96-well plates (Nunc, Roskilde, Denmark) were coated overnight with 50 µl of 15 µg/ml of anti-IFNγ mAb, 1-DIK (MABTECH, Stockholm, Sweden) in 0.1 M carbonate/bicarbonate buffer pH 9.6. After 6 wash steps with culture medium (RPMI1640, Gibco BRL, Life Technologies, Breda, The Netherlands) to remove unbound antibody, plates were blocked for 1 h with RPMI 1640 supplemented with 10% FCS. Subsequently, PBMC were added in triplicate wells at 1 x 10⁵ cells/well in the absence or presence of 2uM peptide (table 2). As a positive control to test the capacity of PBMC to produce IFNγ upon antigen independent stimulation, Phytohaemagglutinin (PHA) (Murex Diagnostics, Dartford, UK) was added. Cultures were incubated overnight at 37°C in 5% CO₂. The next day, cells were removed by washing with PBS/0.05% Tween 20 and the second biotinylated anti-IFNγ mAb, 7-B6-1 biotin (MABTECH), was added at 1 µg/ml in PBS and left for 3 h at room temperature, followed by streptavidin-conjugated alkaline phosphatase (MABTECH) for an additional 2 h. Individual cytokine-producing cells were detected as dark
Table 2. Peptides used in tetramer formation and ELISpot assay stimulation

<table>
<thead>
<tr>
<th>HLA type</th>
<th>EBV protein</th>
<th>peptide</th>
<th>HIV protein</th>
<th>peptide</th>
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<tbody>
<tr>
<td>A2</td>
<td>BMLF-1:</td>
<td>GLCTLVAML</td>
<td>Gag</td>
<td>SLYNTVATL</td>
</tr>
<tr>
<td>A11</td>
<td>EBNA-3B:</td>
<td>IVTDFSVIK</td>
<td>Pol</td>
<td>ILKEPVHG</td>
</tr>
<tr>
<td></td>
<td>EBNA-3B:</td>
<td>AVFDRKSDAK</td>
<td>nt</td>
<td>nt</td>
</tr>
<tr>
<td>B8</td>
<td>BZLF-1:</td>
<td>RAKFKQLL</td>
<td>Gag</td>
<td>EYKRWII</td>
</tr>
<tr>
<td></td>
<td>EBNA-3A:</td>
<td>FLRGRAYGL</td>
<td>Nef</td>
<td>FLKEKKG</td>
</tr>
<tr>
<td>B57</td>
<td>nt</td>
<td>Gag</td>
<td>KAFSPEVIPMF</td>
<td></td>
</tr>
</tbody>
</table>

*nt= none tested*

purple spots after a 10-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, Sigma, St. Louis, Missouri, USA). Reactions were stopped by extensive washing in water. Nylon membranes were dried and spots were counted after computerized visualization by a scanner (Hewlett Packard Company, Baise, Idaho, USA). The number of specific T cell responders per 10^6 PBMC was calculated after subtracting negative control values. Because the percentage of dead cells and the percentage of CD8^+ T cells was assessed in the same samples, the number of specific T cell responders/10^6 living CD8^+ T cells could be calculated. This assay was very reproducible when performed on multiple samples from EBV-positive donors, detecting as low as 1 positive cell per 1x10^6 PBMC (0.001%).

Viral load determination

HIV RNA load was quantitated in serum using NASBA (Organon Teknika, Boxtel, The Netherlands). For EBV load measurements in PBMC a real time quantitative Taqman assay was used.

PBMC (1x10^6) were lysed by addition of L6-lysis buffer. Genomic DNA was extracted by precipitation with isopropanol and DNA from 2x10^5 cells was amplified using PCR primers selective for the EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 p143. PCR amplification was performed as previously described using EBV/p143 forward and reverse primers resulting in a 74 basepairs DNA product. In the PCR reaction a fluorogenic EBV/p143-specific probe was added with a FAM reported molecule attached to the 5' end and a TAMRA quencher linked at the 3' end, to detect amplified DNA. Amplification and detection was performed with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, California, USA). 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 0.04. Each run contained several negative controls (no template or EBV-negative DNA), a positive control (a known amount of EBV copies) and a standard dilution of plasmid DNA.
containing the PCR product as insert, which was calibrated with an EBV quantified standard (Advanced Biotechnologies incorporated, Maryland, USA). The analyzed sensitivity of the assay was between 50 and 5x10^6 copies/ml. All reactions were performed in duplicate and only considered positive when both replications were above the threshold limit.

Statistical analysis

To compare HIV- or EBV-specific tetramer^+ and IFN^+ producing CD8^+ T cells before and after HAART Wilcoxon tests were performed. To compare the differences between HIV and EBV specific tetramer^+ T cells, IFN^+ T cells, delta IFN^+ T cells and fractions of IFN^+/tetramer^+ T cells, Mann-Whitney tests were performed. Correlations were tested using the Spearman’s non-parametric correlation test. All statistical analyses were performed using the software program SPSS 7.5 (SPSS Inc., Chicago, Illinois, USA).

Results

Viral load, CD4^+ T cells and EBV and HIV-specific CD8^+ T cell responses during treatment

To determine the effectiveness of the treatment strategies, we analysed HIV RNA load and CD4^+ T cell numbers before and after HAART. As also shown in several previous studies, HIV RNA load significantly decreased after start of HAART (Median 150 to 1 RNA copies/μl serum, p=0.007, Wilcoxon test) and CD4^+ T cell counts increased (median 245 to 288 T cells/μl blood) (p=0.012, Wilcoxon test) (Baseline values are listed in table 1). To investigate the changes in number and function of HIV- and EBV-specific CD8^+ T cells following start of therapy, we stained virus-specific CD8^+ T cells with specific HLA-peptide tetrameric complexes and performed IFN^+ elispot assays. In table 2 the HLA molecules and corresponding immunodominant viral peptides used to construct tetrameric complexes and to stimulate PBMC are shown. In figure 1 two HIV-infected individuals are shown; patient 1113 responded to therapy with increased CD4^+ T cell numbers, whereas patient 557 did not respond to therapy and showed decreasing CD4^+ T cell numbers. Each show quite distinct changes in numbers of tetramer^+ T cells and IFN^+ T cells or in the fraction of tetramer^+ T cells producing IFN^+ for HIV and EBV peptide-specific T cells after therapy. For individual 1113, the percentage tetramer^+ T cells specific for all HIV-peptides decreased, in contrast to stable or increased HIV-tetramer^+ T cells in patient 557 (figure 1, upper panels). The percentage EBV-specific tetramer^+ T cells, increasing in patient 1113 and decreasing in patient 557, showed kinetics opposite to HIV specific tetramer^+ T cells (figure 1 middle panels). Numbers of EBV and HIV specific IFN^+ T cells increased or remained stable in patient 1113, but decreased in patient 557. This resulted in an increase in the IFN^+ fraction of both EBV- and HIV-tetramer^+ T cells for patient 1113, whereas these fractions decreased for patient 557 (lower panels).
Therapy induced restoration of EBV- but not HIV-specific T cells

**Figure 1. Longitudinal kinetics of HIV and EBV specific T cells in 4 different HIV-infected individuals**

Individual HIV and EBV peptide-specific T cells of two HIV-infected individuals 1113 and 557 are shown. Patient 1113 illustrates kinetics of virus-specific T cells after therapy that was frequently observed in patients responding to therapy. For patient 557, therapy did not increase CD4+ T cell numbers and this patient showed different kinetics of virus specific T cells than responder 1113. The upper three panels indicate kinetics of virus peptide-specific T cells (% of CD8+ T cells) as measured by tetramer staining (solid symbols) and IFNγ secretion assays (open symbols). Individual peptide specific CD8+ T cells are plotted over time during therapy. Per graph is indicated whether HIV- or EBV-specific T cell percentages are depicted. Fractions of IFNγ+ T cells (functional T cells) of total tetramer+ T cells are indicated in the lowest panels and combine individual peptides per virus. Start of dual RT inhibitor therapy is indicated by arrows, and start of HAART is defined as t=0 and dashed vertical lines.

**Numbers of HIV-specific T cells but not EBV-specific T cells are decreased after therapy**

To investigate changes in the number of HIV-specific T cells and EBV-specific T cells in the total study group the sum of circulating (tetramer+) T cells was calculated from the individual peptide-specific CD8+ T cells and expressed as percentage of CD8+ T cells for 16 individuals. (figure 2) A decline in HIV-specific CD8+ T cells was observed in 15 out of 16 individuals (median from 1.05% before therapy to 0.58% after therapy, p=0.047, Wilcoxon test). (figure 2) In contrast, numbers of EBV-specific T cells did not change significantly (median from 0.85% before therapy to 0.77% after therapy, p=0.125, Wilcoxon) (figure 2).

**Figure 2. Tetramer+ HIV and EBV-specific T cells during therapy**

Baseline values (before) of HIV- (left panel) and EBV-specific (right panel) tetramer+ T cells as percentages of CD8+ T cells compared with averaged values after start of therapy (after) are depicted for 16 HIV-infected individuals. Tetramer-staining was performed as described in the Methods section.
Number of IFNγ producing EBV-specific T cells but not HIV-specific T cells increase after therapy

In a previous study we found decreased numbers of IFNγ producing HIV-specific (Kostense et al. submitted) and EBV-specific (van Baarle et al. submitted) CD8+ T cells in HIV-infected individuals, especially when these individuals progressed to AIDS or AIDS-NHL, respectively. To study whether the functionality of virus-specific CD8+ T cells was improved by HAART, we enumerated IFNγ producing CD8+ T cells before and after therapy, using the IFNγ elispot assay. To compare the kinetics of HIV-specific IFNγ producing T cells with EBV-specific IFNγ producing T cells before and after therapy for the total study group, the individual peptide-specific percentages of CD8+ T cells were summed for 16 individuals. As shown in figure 3a, in most HIV-infected individuals the percentage of IFNγ producing HIV-specific T cells decreased significantly after therapy (median from 0.11 before to 0.06% of CD8+ T cells after therapy, p=0.017, Wilcoxon), in parallel with the number of HIV-tetramer+ T cells (figure 2). In only 3 out of 16 individuals an increase in the number of functional HIV-specific T cells was observed. In contrast, the majority of the individuals in the study (10/16) showed an increase in the number of EBV-specific IFNγ producing T cells (median from 0.17 before to 0.24% after therapy), although not statistical significant (p=0.244, Wilcoxon test) (figure 3a) (figure 1, 1113). This resulted in higher percentages of functional EBV-specific CD8+ T cells (median=0.24%, p=0.025, Mann-Whitney test). These differential kinetics of EBV and HIV-specific IFNγ responses are illustrated by the differences in the delta IFNγ+ T cells in figure 3b, showing that in most patients, EBV specific responses improved to a higher extent than HIV-specific IFNγ responses (p=0.054, Mann-Whitney).

Figure 3. Number of IFNγ producing HIV and EBV-specific T cells during therapy
a. Baseline values (before) of HIV- (left panel) and EBV-specific (right panel) IFNγ producing T cells as percentages of CD8+ T cells compared with averaged values after start of therapy (after) are depicted for 16 HIV-infected individuals. The % of IFNγ producing T cells was measured using Elispot assays after peptide-stimulation and performed as described in the Methods section.

b. Difference in IFNγ producing T cells between baseline and average after HAART are indicated as delta IFNγ producing T cells for HIV- and EBV-specific CD8+ T cells.
Since the observed increase in the number of functional EBV-specific CD8\(^+\) T cells could be a reflection of increased CD4\(^+\) T cell help, we correlated the number of CD4\(^+\) T cells with the number of functional EBV-specific T cells. In individuals with low (below 200/\(\mu\)l) CD4\(^+\) T cell counts before treatment, a positive trend was observed between the percentage of functional EBV-specific CD8\(^+\) T cells and the number of CD4\(^+\) T cells before and during therapy (\(R=0.32, p=0.067\), figure 4). In addition, in individual 557 (figure 1), who does not show an improvement in the number of CD4\(^+\) T cells, decreasing numbers of IFN\(\gamma\) producing EBV-specific T cells were observed during HAART.

![Figure 4. Correlation between CD4\(^+\) and IFN\(\gamma\)+ CD8\(^+\) T cells.](image)

**Correlation between CD4\(^+\) T cell numbers (x-axis) and numbers of EBV-specific IFN\(\gamma\) producing T cells (y-axis) for HIV-infected individuals with less than 200 CD4\(^+\) T cells/\(\mu\)l at baseline (n=8) (Spearman, \(p=0.032\)).**

**Fraction of IFN\(\gamma\) / tetramer\(^+\) T cells during treatment**

In previous studies we have shown that not only the total number of IFN\(\gamma\) producing T cells, but also the percentage of IFN\(\gamma\)\(^+\) T cells of tetramer\(^+\) T cells (fraction of functional T cells) may give an indication of the functional status of virus-specific CD8\(^+\) T cells. (van Baarle, submitted and Kostense, submitted) Therefore, we calculated the percentage IFN\(\gamma\) producing T cells of the tetramer\(^+\) T cells for 11 individuals from whom we had the complete data set, i.e. we had both tetramer-staining data and IFN\(\gamma\) elispot data for each peptide epitope studied. We summed the individual peptide-specific percentages of functional tetramer\(^+\) CD8\(^+\) T cells. To investigate whether therapy, which induces suppression of viral load and an increase in CD4\(^+\) T cell numbers, could increase the fraction functional cells for EBV and HIV-specific CD8\(^+\) T cells, we compared the percentage functional T cells before with the average percentage after therapy. Although the fraction of functional HIV-specific T cells could increase after therapy in 3 individuals, it usually decreased (figure 5). In most of the individuals the increase in fraction functional HIV-specific T cells was not due to an increase in the number of HIV-specific IFN\(\gamma\) producing cells, but to a slower decline in IFN\(\gamma\) producing T cells as compared to the decline in tetramer\(^+\) T cells. In contrast, in 7 out of 11 patients an increase in the fraction functional EBV-specific T cells was observed (median from 31.4 before therapy to 39.2% after therapy) (figure 5). Before therapy, the fraction functional EBV-specific T cells was higher than the fraction functional HIV-specific T cells (\(p=0.054\), Mann-Whitney test), and this difference was even higher after therapy (median EBV =39.2 vs. median HIV=11.6%, \(p=0.019\)). Indeed the difference in fraction functional T cells (\(\Delta\)fraction) was higher for EBV-specific T cells (median= 7.45) in comparison with HIV-specific T cells (median= -0.90),
which was almost significant (p=0.074, Mann-Whitney) (data not shown).

![Figure 5](image)

**Figure 5.** Percentage IFNγ producing T cells of tetramer+ T cells for HIV and EBV-specific T cells during therapy

Baseline values (before) of HIV- (left panel) and EBV-specific (right panel) IFNγ producing T cells of tetramer+ T cells (fraction functional T cells) and averaged values after start of therapy (after) are depicted for 11 HIV-infected individuals. A fraction of 100% means that Elispot and tetramer staining identified equal numbers of peptide specific T cells.

Interestingly, individuals selected for having CD4+ T cell counts above 200/µl blood at initiation of HAART, showed a substantial increase in the fraction functional HIV-specific T cells shortly (within the first month) after therapy (median=6.9 before and 16.5% after therapy) (p=0.059, Wilcoxon test) (data not shown). This improvement was not observed after long-term therapy up to 1 year.

**Functional improvement of EBV-specific T cells and decreased EBV load after therapy**

To investigate whether the increase in EBV-specific IFNγ producing T cells leads to a reduction in EBV load, we measured EBV load using a real time quantitative-PCR assay before and after therapy in 15 HIV-infected individuals from whom we could obtain sufficient PBMC to isolate DNA.

![Figure 6](image)

**Figure 6.** Negative correlation between delta EBV-specific IFNγ+ T cells (y-axis) and delta EBV viral load (x-axis). (Spearman, p=0.096).

Overall, individuals with improved EBV responses tended to suppress their EBV load after therapy. (figure 6) In 9 out of 15 individuals EBV load decreased after start of therapy, sometimes after an initial transient increase. As shown in individual examples in figure 6, EBV load decreased following or simultaneous with the increase in EBV-specific IFNγ production. This decrease in EBV load occurred most outspoken in individuals with high CD4+ T cells after start of therapy (fig 7). One of the individuals that did not reach CD4+ T cell numbers well above 200/µl after therapy (and later progressed to AIDS), showed a further decrease in the number of EBV-specific IFNγ producing T cells and consequently an increase in EBV load. (fig 7, patient 585) Because EBV load differed enormously between individuals and ranged from 0 to 620.000 copies/10^6 PBMC, no statistical difference could be found before and after therapy (p=0.414, Wilcoxon test).
Therapy induced restoration of EBV- but not HIV-specific T cells

Hig hh CD4+ T cell numbers

Low CD4+ T cell numbers
(Progressor) 585

Figure 7. Kinetics of EBV load in relation to EBV-specific IFNγ+ T cells during therapy

EBV load (open circles, dashed line) in relation to EBV-specific IFNγ-producing T cells (filled circles and solid line) (lower panels) and CD4+ T cells (upper panels) are depicted during therapy (x-axis). Therapy is initiated at time point 0 and indicated by the dashed vertical lines. Individuals with high or increasing CD4+ T cell numbers (211, 692 and 43, left panels) are compared to one individual with low CD4+ T cell numbers who progressed to AIDS (585, right panel).

Discussion

In this study we compared HIV and EBV-specific CD8+ T cells after start of HAART, using both HLA-peptide tetrameric complexes and IFNγ elispot to determine the presence and function of these cells, respectively. As reported previously 14; 15; 23, we observed that in most individuals, therapy induced a marked decline of HIV-specific CD8+ T cells, both in number and function, whereas numbers of EBV-specific T cells did not change. The fraction of EBV-specific T cells producing IFNγ increased in the majority of the individuals after HAART, resulting in higher numbers of functional EBV-specific CD8+ T cells compared to HIV-specific T cells. Interestingly this increase was paralleled by a decrease in EBV load. Furthermore, a positive trend was found between CD4+ T cell numbers and the number of IFNγ producing EBV-specific CD8+ T cells in patients selected for CD4+ T cell counts below 200/μl blood before HAART.

It has been suggested that protease inhibitors (PI) reduce the number of HIV antigen-specific T cells as measured by tetramer staining, due to downmodulation of MHC-peptide surface expression 24. In this study several patients received dual RT inhibitors prior to PI treatment. In general, start of dual antiretroviral therapy already induced selective decreases in HIV-specific T cells. In addition, the number of EBV specific T cells did not substantially change after
initiation of HAART. Thus, HAART induced a selective decrease of HIV-specific CD8+ T cells instead of a general MHC class I modulated decrease of CD8+ T cells.

In this study, we utilized the fraction of IFNγ producing T cells within the tetramer+ T cells as an indicator of CD8+ T cell functionality. It appears that EBV specific T cell function is capable of improvement after therapy, whereas HIV specific T cell function is not improved. Earlier experiments indeed showed differential kinetics of HIV- and EBV-specific IFNγ secreting T cells during HAART. These findings have been explained by the differential reduction of viral antigen concentration. Combining the IFNγ assay with tetramer staining provides further information on the immune status, and indicates that CD8+ T cell functionality can improve without significant increase in tetramer+ T cell numbers.

Functional recovery of EBV-specific CD8+ T cells may be a reflection of improved CD4+ T cell help. Previous studies have shown that CD4+ T cells are important for maintaining the functional capacity of CD8+ T cells. When we correlated the number of CD4+ T cells with the number of functional EBV-specific T cells in individuals with low (<200/μl) CD4+ T cell counts before treatment, we found a positive trend consistent with this hypothesis (R=0.32, p< 0.067).

In co-infected patients, HIV- and EBV-specific CD4+ T helper cells are differently affected. HIV infection results in selective depletion of HIV-specific CD4+ T cells in the majority of the patients already during acute infection, although long term asymptomatics (and in our study, patients with CD4+ T cell counts over 200/μl) may preserve these CD4+ T cells. EBV-specific CD4+ T cells are already generated before HIV-infection, and are probably preserved for a longer period during chronic HIV-infection. The observed difference in functional recovery between EBV- and HIV-specific CD8+ T cells may thus be explained by the fact that HIV-specific CD4+ T cells may be irreversibly depleted already early in HIV-infection. Only individuals with relatively high numbers of CD4+ T cells may show some recovery of HIV-specific CD4+ T cells and thus concomitantly an increase in CD8+ virus specific IFNγ producers, and hence an increase in the fraction of IFNγ+ tetramer+ T cells. Moreover, since loss of antigen exposure results in decrease in HIV-specific T cells in the long term, this effect is observed only shortly (within the first month) after therapy. Indeed, patients selected for having CD4+ T cell counts above 200/μl blood at initiation of HAART, showed a significant increase in the fraction functional HIV-specific T cells shortly therapy. These results are in agreement with transient rises in CTLp frequencies observed after therapy.

In conclusion, we found a trend for EBV-but not HIV-specific recovery of IFNγ+CD8+ T cells in the majority of the HAART treated individuals that were responding to therapy. Although the total number of EBV-specific tetramer+ CD8+ T cells did not increase, the fraction of IFNγ producing T cells did improve, indicating that the function had improved on a per cell basis. This implies that immune reconstitution can be established and may prevent the occurrence of opportunistic infections or
malignancies against which antigen-specific CD4+ T cells are still present. This underscores the need to treat patients early in infection when HIV-specific CD4+ T cell responses may still be available. Early therapy, before HIV-specific CD4+ T cell helper is depleted, should improve HIV-specific CD8+ T cell responses. Assessment of the fraction IFNy producing T cells of tetramer+ T cells can be a helpful tool to monitor functional immune reconstitution.

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