Mechanisms of decreasing HIV-1 specific CD8+ T cell activity during progression to AIDS
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Kinetics of tetramer$^+$ T cells and IFN$^+$ T cells specific for Human Immunodeficiency Virus and Epstein-Barr Virus during treatment of HIV-1 infection


We investigated the effect of highly active anti-retroviral therapy (HAART) on HIV- and EBV-specific CD8$^+$ T cells in 14 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$^+$ elispot assays after peptide-stimulation. HAART induced a decrease of HIV-specific CD8$^+$ T cells in most individuals as measured by tetramer staining, whereas numbers of EBV-specific tetramer$^+$ T cells did not change. Although no significant changes were found for IFN$^+$ producing HIV-specific or EBV-specific T cells, the relative percentages of IFN$^+$ producing T cells increased compared to the total number of both HIV- and EBV-specific T cells. This relative improvement of EBV specific T cells did not significantly suppress EBV load. These data suggest that antiretroviral therapy decreases the magnitude of HIV-specific T cells, but improves the antigen responsiveness of HIV- and EBV-specific T cells.

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 (3,190). 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 (224). In contrast, in HIV-infection, despite the efficacy of HIV-specific CTL to suppress viral replication early in infection (13), CTL do not contain and eventually seem to lose control of HIV viral replication completely (225). Furthermore, during HIV-induced immunodeficiency, a higher rate of reactivation of EBV-infection occurs which may lead to uncontrolled lymphoproliferation (226), 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 (47) 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 (127,128,227). $^{51}$Cr release assays and cytokine detection assays, like IFN$^+$ Elispot assays (214), 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 IFN$^+$ 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 IFN$^+$ producing T cells compared to wild type mice (146). In addition, we have observed decreased numbers of IFN$^+$ producing HIV-specific (189) and EBV-specific CD8$^+$ T cells in HIV-infected individuals (185). Furthermore, HIV-infected individuals have higher EBV load than HIV seronegative individuals, 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 (185).

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 focussed 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 (228,229). 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 (230) without further detailed functional analysis (89). Furthermore, the effect of HAART on EBV immunity and EBV load has not been evaluated. Because of the reported positive correlation between CD4$^+$ T cell numbers and effective antiviral CD8$^+$ T cells (146,163), 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 ratio of functional cells (IFNγ producing T cells) relative to 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.

**Table 1: Group characteristic of HIV-1 infected homosexual men on HAART**

<table>
<thead>
<tr>
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<th>Before HAART</th>
<th>Average during HAART</th>
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<tbody>
<tr>
<td>CD4 counts*</td>
<td>285 (128-440)</td>
<td>396 (143-570)</td>
</tr>
<tr>
<td>HIV RNA load*</td>
<td>106 (1-370)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>EBV load*</td>
<td>4.77 (0.5-79)</td>
<td>4.39 (0.91-4.66)</td>
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* CD4+ T cells / µl blood; median (range)  
* HIV viral RNA copies / µl serum; median (range)  
$ Log EBV copies / 10^6 PBMC; median (range)

**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 or B8. Peripheral blood mononuclear cells (PBMC) were cryopreserved according to a standard protocol in a computerised freezing device.

We analyzed longitudinal PBMC samples from 14 HIV-infected individuals on HAART. The samples included at least one sample before start of any treatment. During therapy, at least 2 samples were drawn; one during the first 3 months; one after 6 to 15 months after start of HAART. For comparisons of samples before and after start of HAART, samples before treatment were averaged and compared to the average of all samples after start of HAART.

For each individual, the drug regimen consisted of 1 protease inhibitor (saquinavir, indinavir or ritonavir) in combination with two RT inhibitors (DDI, 3TC, AZT or D4T). Ten of the 14 individuals started dual therapy 3 months before the addition of a protease inhibitor. Group characteristics of the HIV-1 infected individuals are given in table 1, in which baseline values of CD4+ T cells and viral load are compared with averages of two or three measurements after start of HAART.

Ten individuals were HLA-A2 positive, 9 were HLA-B8 positive, 5 of these expressed both HLA-A2 and -B8. One individual did not respond to therapy and had increasing viral load and decreasing CD4+ T cell counts. One individual had progressed to AIDS shortly before start of therapy.

**Tetrameric HLA-peptide complex formation**

Refolding of HLA class I heavy chains and tetramer formation was performed as described previously (47). 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 (167). Subsequently, MHC class I tetramers complexed with EBV and HIV-peptides were produced as previously described (47). The peptides used (Isogen, Maarssen, The Netherlands) are listed in table 2. Refolded HLA peptide complexes were biotinylated, PFLC purified using an H16/60 Superdex 200 column (Pharmacia). Purified complexes were bound to streptavidin-phycocerythrin or streptavidin-APC (Sigma). Tetrameric product was PFLC purified and concentrated using amicon stir cells.

**Flow cytometry and tetramer staining**

Three- or four-color fluorescence analysis was performed as previously described. (231) Briefly, PBMC were thawed and 1.5 x 10^6 cells were stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (BPA) with MHC class I tetramers (PE and APC), PerCP conjugated Mab CDB (Becton Dickinson, San José, California, USA) and anti-CD27-FITC (CLB, Amsterdam, The Netherlands). After staining, cells were washed with BPA 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).
tetrazolium (BCIP/NBT, Sigma, St. Louis, Missouri, 4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT, Sigma, St. Louis, Missouri, USA). Reaction 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 1x10^5 PBMC (0.001%).

Intracellular cytokine staining

PBMC were stimulated and stained for intracellular IFNγ as described previously (189). Briefly, 2x10^6 PBMC were incubated with 1μg/ml peptide in the presence of monensin. Incubation without peptide, and incubation with PMA and ionomycin were used as negative and positive controls respectively. After 4 hours, cells were washed, stained for CD8 and tetramer, fixed, permeabilised, and stained for IFNγ and CD69. CD69^+ IFNγ^+ T cells were compared with the number of tetramer^+ T cells in the negative control sample.

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 (169). 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 (232,233). PCR amplification was performed as previously described (234) 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. The variation between duplicates was as low as 7.5% (Van Baarle et al. submitted).

Results

Viral load and CD4^+ T cells during treatment

To determine the effectiveness of the treatment strategies, we analysed HIV RNA load and CD4^+ T cell numbers before and after HAART. We compared the average of measurements before with the averaged measurements after start of treatment. As expected, CD4^+ T cell counts increased (median 275 to 397 T cells/µl blood; p=0.004, Wilcoxon signed rank test) after start of HAART. HIV RNA load significantly decreased (Figure 1) (Median 106 to 1 RNA copies/µl serum, p=0.006, Wilcoxon). One individual had decreasing CD4^+ T cell numbers and increasing viral load after start of therapy. EBV load did not significantly change during HAART (Figure 1) (Median 4.8 to 4.4 log EBVcopies/10^6 PBMC, p=0.79, Wilcoxon).

EBV- and HIV-specific CD8^+ T cell responses during treatment

To investigate the dynamics 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 IFNγ ellispot 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 2, two treated HIV-infected individuals are shown; subject 1113 responded to therapy with decreasing viral load and increasing CD4^+ T cell numbers, whereas subject 557 did not respond to therapy and showed decreasing numbers of CD4^+ T cells and increasing viral load. Each show quite distinct changes in numbers of tetramer^+ T cells and IFNγ^+ T cells. For subject 1113, the percentage tetramer^+ T cells specific for all HIV- and EBV-specific IFNγ^+ T cells and IFNγ^+ T cells. For subject 557, the percentage EBV-specific tetramer^+ T cells, increasing in subject 1113 and decreasing in subject 557, showed kinetics opposite to HIV-specific tetramer^+ T cells. Numbers of EBV and HIV specific IFNγ^+ T cells increased or remained stable in subject 1113, but decreased in subject 557. This resulted in an increased ratio IFNγ^+ /
Figure 3. Tetramer* T cells, IFNy* T cells and IFNy/tetramer percentages of HIV and EBV-specific T cells during therapy.

For each individual on HAART, total HIV (left panels) or EBV (right panels) specific responses were calculated by the sum of all responses from each peptide tested. Before and after start of HAART are the averages of samples before (usually one), and the average of two or three measurements after start of HAART. Successfully treated subjects are depicted as closed circles; open circles indicate the subject with decreasing CD4+ T cells and increasing HIV load; open triangles indicate the subject progressing to AIDS before start of HAART. Statistics were performed with the Wilcoxon signed rank test. P values are based on all subjects, except in part C were one individual who did not show increased CD4+ T cell numbers was excluded.

A. Percentage tetramer* T cells of CD8+ T cells. B. IFNy* T cells from Elispot assays transformed to percentages of CD8+ T cells. C. Ratio IFNy / tetramer* T cells expressed as percentage.

Numbers of HIV- but not EBV specific T cells decrease during therapy

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 each of the 14 individuals. Since no significant changes were observed from samples drawn early and late after start of HAART, we calculated the average of all samples after start of HAART. A significant decline in HIV-specific CD8+ T cells was observed in most individuals (median from 1.08% before therapy to 0.61% after therapy, p=0.030, Wilcoxon), except for the non-responding subject 557 (open circle). In contrast, numbers of EBV-specific T cells did not change significantly after start of HAART (median from 0.91% before therapy to 0.75% after therapy, p=0.24, Wilcoxon) (figure 3A).

Numbers of IFNy producing T cells during therapy

To study whether the functionality of virus-specific CD8+ T cells was improved by HAART, we enumerated IFNy producing CD8+ T cells before and after therapy, using the IFNy elispot assay. To compare the kinetics of HIV-specific IFNy producing T cells with EBV-specific IFNy producing T cells before and after therapy for the total study group, the individual peptide-specific percentages of CD8+ T cells were summed for 14 individuals. As shown in figure 3B, no significant changes were observed for HIV-specific (median from 0.14 before to 0.17% of CD8+ T cells after therapy) (p=0.45, Wilcoxon), or EBV-specific IFNy producing T cells (median from 0.19 before to 0.24 after therapy, p=0.40, Wilcoxon).

To confirm that the Elispot assay showed relevant changes in IFNy producing CD8+T cells, we tested one individual simultaneously with the Elispot assay and intracellular cytokine staining (49). Although intracellular cytokine staining detected somewhat higher numbers of IFNy+ T cells, the dynamics were identical for both assays (Figure 4).

Ratio IFNy+ / tetramer+ T cells during treatment

Besides the absolute number of IFNy producing T cells, also the ratio IFNy+ T cells relative to the number of tetramer+ T cells may give an indication of the functional status of virus-specific
CD8+ T cells (189). Therefore, we calculated the ratio of IFNγ producing T cells of the tetramer+ T cells expressed as percentage. We summed the individual peptide-specific percentages of functional tetramer+ CD8+ T cells, and compared the percentage functional T cells before with the average percentage after therapy. The percentage of functional virus-specific T cells increased in most individuals for both HIV- and EBV-specific T cells (median from 16 to 31; p=0.03, and 23 to 39%; p=0.07, respectively; figure 3C).

One subject showed an increase in viral load and a decrease in CD4+ T cell numbers (open circle in figure 3). Another subject responded to therapy but progressed to AIDS shortly before start of therapy (open triangle). Indeed these individuals showed several distinct changes in T cells, compared to the majority of the individuals, who responded positively to treatment. When excluding the one individual with decreasing CD4+ T cells, a significant improvement was found for both HIV and EBV specific T cells (Wilcoxon: p=0.010, and p=0.028, respectively).

Influence of HAART on EBV load
Antiretroviral therapy in principle does not interfere with EBV replication. Therefore changes in EBV load during HAART are likely an effect of immune-reconstitution. The average EBV load did not significantly change after start of HAART (median from 4.8 to 4.4 log EBV-copies/10^6 PBMC), however, substantial longitudinal changes were observed. Several subjects showed a temporal increase in EBV load shortly after start of therapy, followed by a reduction. In some occasions, temporal peaks in EBV load were accompanied by parallel increases in IFNγ+ T cells (Figure 5, subjects 523, 211). In other subjects EBV-specific IFNγ+ T cells and EBV load showed inverse dynamics in time (Figure 5, 585, 43). However, no definite trend was observed.

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 (89,230,235), we observed that in most individuals, therapy induced a marked decline of HIV-specific CD8+ T cells, whereas numbers of EBV-specific T cells did not change. The number of virus specific T cells as detected by Elispot assays differed substantially from tetramer staining results confirming earlier reports which suggests that not all tetramer+ T cells are capable of IFNγ production (189,192,236). The ratio of HIV- and EBV-specific T cells that produced IFNγ, increased in the majority of the individuals after HAART, which suggests that the function of virus specific T cells is improved in individuals successfully treated with HAART.

![Figure 4. Comparison of Elispot and Intracellular cytokine staining in one HAART treated subject. One individual tested for virus specific IFNγ responses before, shortly after and long after start of HAART. Elispot and intracellular cytokine staining was performed after stimulation with HLA-B8 restricted epitopes as described in table 2. Results are the sum of two tested HIV peptides (left panels) and two EBV peptides (right panels). Elispot results are transformed from number of spots to percentage of CD8+ T cells. Percentage CD8+ T cells positive for IFNγ in Elispot assays or intracellular cytokine staining after stimulation with HIV or EBV derived peptides show similar dynamics.](image-url)
viremia may be controlled. A reduction in EBV-specific T cells may allow EBV load to increase; in contrast, a rise in EBV load is likely to trigger the expansion of EBV specific T cells. Both sorts of interactions were observed in the current study population. Overall, it appears that the interaction between the immune system and the viral load may be more complex during antiretroviral therapy, than anticipated. In addition, since the EBV load assay measures the total number of EBV copies/10^6 PBMC, increase in EBV load may indicate to an increase in copies per cell or an increase in infected B cells. Since the first would be the result of EBV replication with associated lytic antigen expression, lytic antigen-specific T cells would be more important in control of EBV load. Expansion of latently infected B cells may be solely controlled by latent antigen-specific T cells. However, as we observed similar kinetics for both lytic and latent antigen-specific T cells during HAART, the influence of both may be the same on EBV load.

Functional recovery of virus-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. Indeed in our study, without an increase in CD4^+ T cells no improvement of T cell function was observed. However, the initial rise in CD4^+ T cells is likely to be caused by redistribution (79), which implies that these cells were present before therapy. Likewise, IFNγ producing CD8^+ T cells may have been present before therapy in lymph-nodes, and redistributed to the blood after therapy. However, the number of EBV-specific T cells does not seem to be affected by redistribution, as numbers of EBV-tetramer^+ T cells remain relatively stable. Nonetheless the relative proportion of IFNγ producing T cells does improve. Alternatively, suppression of HIV load enhances polyclonal T cell responsiveness to anti-CD3 antibodies (79,238). This may directly or indirectly improve CD8^+ T cell responsiveness after antigen-specific stimulation.

In this study, we utilized the percentage of IFNγ producing T cells within the tetramer^+ T cells as an indicator of CD8^+ T cell functionality. 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. Although the total number of virus-specific tetramer^+ CD8^+ T cells decreased and the total number of IFNγ^+ T cells did not significantly change, the ratio IFNγ/tetramer^+ 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 help to prevent the occurrence of opportunistic infections or malignancies. Assessment of the percentage IFNγ producing T cells of tetramer^+ T cells can be a helpful tool to monitor functional immune reconstitution.

Acknowledgements
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