Viro-immunological studies on the role of Epstein-Barr virus in the development of AIDS-related non-Hodgkin's lymphoma
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Chapter 5

Dysfunctional Epstein-Barr virus (EBV)-specific CD8+ T lymphocytes and increased EBV load in AIDS-related non-Hodgkin’s lymphoma patients

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Dysfunctional Epstein-Barr virus (EBV)-specific CD8+ T lymphocytes and increased EBV load in AIDS-related non-Hodgkin’s lymphoma patients

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AIDS-related Non-Hodgkin’s Lymphoma’s (AIDS-NHL) are thought to arise because of loss of Epstein-Barr Virus (EBV)-specific cytotoxic T lymphocytes. Here, we investigated whether cellular immunity to Epstein-Barr Virus (EBV) is lost due to physical loss or dysfunction and correlated these data with EBV load. For comparison, individuals who progressed to AIDS with Opportunistic Infections and long-term asymptomatics were studied. The number of virus-specific T cells was detected using tetrameric HLA-EBV-peptide complexes; function of these EBV-specific T cells was determined using the INFγ-ELIspot assay. We observed that EBV-specific CD8+ T cells were not physically lost but rather lost their capability to produce IFNγ. This loss of function correlated with lower CD4+ T cell numbers and was accompanied by increasing EBV load. In long-term asymptomatic HIV-1 infected individuals, in whom CD4+ T cell numbers were maintained, IFNγ producing EBV-specific T cells were stable and occasional bursts in EBV load were paralleled by increased numbers of functional T cells, suggestive of immune control. Our data suggest that functional loss of EBV-specific CD8+ T cells with a concomitant increase in EBV load may play a role in the pathogenesis of AIDS-NHL.

Introduction

Infection with Epstein-Barr virus (EBV), a widespread human gamma herpesvirus, leads to viral persistence in a latent form in B-lymphocytes. 1 The initial expansion and reactivation of these latently infected B lymphocytes is controlled by specific CD8+ MHC class I-restricted cytotoxic T-lymphocyte (CTL) responses. 2 Because reactivation may result in lytic antigen expression, 3 CTL control during this stage of reactivation may involve lytic antigen-specific effector T cells. 4 During immunodeficiency, a higher rate of reactivation of EBV-infection may lead to uncontrolled lymphoproliferation. 5 In human immunodeficiency virus (HIV)-infected individuals, the incidence of Non-Hodgkin’s lymphomas (NHL) is considerably increased and 75% of these lymphomas is EBV-positive. 6 These acquired immunodeficiency syndrome (AIDS)-related diffuse large cell NHL are therefore thought to arise because of loss of EBV-specific T cell immunity. 7 Until now, only few cross-sectional studies have been performed to study EBV-specific immunity in AIDS-NHL patients. Some studies show decreased
EBV-specific CTL activity in patients with AIDS and AIDS-related complex. In contrast, other studies by Carmichael et al. and Geretti et al. showed sustained EBV-specific CTL responses with declining HIV-specific CTL in advanced HIV-infection, suggesting selective loss of HIV-specific CTL. We previously reported a longitudinal study into the kinetics of HIV-1 and EBV-specific CTL responses in HIV-infected individuals using limiting dilution analysis (LDA) to determine the number of CTL precursors (CTLp). It revealed that in patients with AIDS-NHL, diagnosis of NHL was found to be preceded by a decrease in EBV-CTLp and an increase in the number of infected B cells as measured in an in vitro EBV transformation assay.

The underlying cause for this loss in EBV-specific CTLp is unknown. Recently, MHC class I-peptide tetrameric complexes, to directly visualize, and IFNγ ELISpot assays, to enumerate functional antigen-specific CD8+ T cells have been developed. These methods demonstrated much higher frequencies of antigen-specific T cells than estimated by LDA before. Moreover, Combination of these new methods allowed us to investigate at the peptide level whether EBV-specific CD8+ T cells become either deleted or dysfunctional in patients that have developed AIDS-NHL. In addition, using a real time quantitative PCR assay, the number of EBV virus particles was quantitated. Presence and function of CD8+ T lymphocytes specific for peptides from both latent and lytic EBV antigens were studied and EBV load was measured in the course of HIV-infection in AIDS-NHL patients. For comparison, HIV-infected individuals who progressed to AIDS with Opportunistic Infections (AIDS-OI) or were long-term asymptomatic (LTA) were studied.

Methods

Study population

This study was performed on participants of the Amsterdam Cohort studies on AIDS and HIV-1 infection. From these individuals, we selected HIV-positive male individuals according to duration of follow-up, availability of samples and HLA-type (A2 and/or B8). Blood samples from these (homosexual) individuals were collected every 3 months for HIV-1 serology and immunological studies. In addition, at all time points peripheral blood mononuclear cells (PBMC) were cryopreserved. We analyzed longitudinal PBMC samples from 5 HIV-infected individuals progressing to AIDS-related diffuse large cell Non-Hodgkin's Lymphoma (NHL), starting at or soon after HIV-1 seroconversion. For comparison, we studied PBMC samples from 3 HIV-infected individuals progressing to AIDS (PROG, classification of the Centers for Disease Control 1993) with Opportunistic Infections (OI) and 3 HIV-infected Long-term asymptomatic (LTA) individuals with CD4+ T cells counts >500/mm3 during more than 8 years of asymptomatic follow-up. Characteristics of the HIV-1 infected individuals are summarized in Table 1.

Flow cytometry and Tetramer staining

MHC class I tetramers complexed with EBV-peptides were produced as previously described. The peptides used
### Table 1. Characteristics HIV-1 infected individuals

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<th>participant</th>
<th>age*</th>
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<th>CD4-count§</th>
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*age in years at HIV-1 seroconversion or first seropositive visit; † CD4+ T cell numbers at AIDS-diagnosis or last time point studied for LTA; § seropositive follow-up, from HIV-1 seroconversion or first seropositive visit until AIDS-diagnosis or last time point studied; †† NHL = B cell non-Hodgkin’s lymphoma, PCP = Pneumocystis carinii pneumonia, Mycobact. = Mycobact. tuberculosis extrapulm.

were two immunodominant epitopes from EBV lytic cycle proteins, the HLA A2-restricted epitope GLCTLVAML (A2-GLC) from BMLF-1 and the HLA B8-restricted epitope RAKFKQLL (B8-RAK) from BZLF-1, and one immunodominant epitope from the latent antigen EBNA-3A, the HLA B8-restricted epitope FLRGRAYGL (B8-FLR). Biotinylated class I peptide complexes were tetramerized by addition of allophycocyanin or phycoerythrin-conjugated streptavidin. Two-color fluorescence analysis was performed as previously described. 13 Briefly, PBMC were thawed and 1.5 x 10^6 cells were stained in PBS supplemented with 0.5% (v/v) bovine serum albumin (PBA) with MHC class I tetramers and PerCP conjugated Mab CD8 (Becton Dickinson). Tetramer-staining was very reproducible because multiple stainings on PBMC, both frozen and fresh, from the same donor gave similar results.

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

T lymphocyte immunophenotyping for CD4 and CD8 membrane markers was performed in real time by flow cytometry.

**ELIspot assay for single cell IFNγ-release**

IFNγ producing antigen-specific T cells were enumerated using IFNγ specific ELIspot assays as previously described. 12 96-well nylon-backed 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 10^5 cells/well in case of HLA B8-restricted responses or 2 x 10^5 cells/well in case of HLA A2-restricted responses in the absence or presence of 2 μM peptide. As a positive control to test the capacity of PBMC to produce IFNγ in general, Phytohemagglutinin (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 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, both frozen and fresh, detecting as low as 1 positive cell per 1x10⁶ PBMC (0.001%), and nicely correlated with intracellular IFNγ staining (data not shown).

DNA extraction and Real-time quantitative TaqMan assay

PBMC (1x10⁶) were lysed by addition of L6-lysis buffer. Genomic DNA was extracted by precipitation with isopropanol and DNA from 2x10⁶ 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 14 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 $5 \times 10^6$ copies/ml. All reactions were performed in duplicate and only considered positive when both replications were above the threshold limit.

Statistical analysis

To test the relation between CD4$^+$ T cell numbers and EBV-specific functional CD8$^+$ T cells, EBV-tetramer$^+$ T cells or total CD8$^+$ T cells, regression analyses (mixed linear model) were performed after cube root transformation of all variables. To correct for a possible correlation between multiple observations from one person, compound symmetry (CS) was used as correlation structure using the PROC MIXED procedure of the software program SAS.

Results

Direct visualization and functional analysis of EBV-specific CD8$^+$ T lymphocytes

To investigate the cause of the defective EBV-immune surveillance in AIDS-NHL patients, we enumerated EBV-specific CD8$^+$ T cells using tetrameric HLA-EBV-peptide complexes and IFN$\gamma$-ELIspot assays in the course of HIV-infection. CD8$^+$ T cells specific for two epitopes from EBV lytic antigens, A2-GLC and B8-RAK, and for one epitope from a latent antigen, B8-FLR were studied. In preliminary studies using IFN$\gamma$ ELIspot assay, the selected EBV-epitopes were shown to be immunodominant, in that they were recognized by a high frequency of CD8$^+$ T cells (100 to 400 peptide-specific
T cells/10^6 PBMC) in all HIV-infected individuals studied. HLA-A2-restricted latent antigen specific T cells were not present or present in a very low range (data not shown). Therefore, HLA-A2-restricted T cells against latent epitopes were not studied. Once immunodominance was established the number and function of EBV-peptide specific CD8^+ T lymphocytes was determined in progressors to AIDS-NHL or AIDS-OI and LTA individuals. Detailed results from two study participants (1 LTA, 1 AIDS-NHL) are shown in figure 1. Overall, in HLA-A2^+ and HLA-B8^+ individuals most EBV-specific CD8^+ T cells (0.2% to 3.8% of total CD8^+ T cells) were directed against the lytic epitope B8-RAK (0.7% to 3.6% in fig. 1c), whereas less (0.1%-1.6%) T cells were directed against the latent epitope B8-FLR (0.1% to 1.1% in fig.1b). In HLA-A2^+ individuals 0.2% to 1.2% of the T cells were directed against the lytic epitope A2-GLC (0.6% to 0.8% in fig. 1d). Interestingly, the number of IFNγ producing EBV-specific T cells was relatively stable and high in LTA1160, whereas these numbers were lower and decreasing in the course of HIV-infection in AIDS-NHL patient NHL0308 for both lytic and latent antigens.

Loss of function of EBV-specific CD8^+ T lymphocytes in AIDS-NHL patients

The percentage of total circulating (tetramer^+) and total number of functional (IFNγ producing) EBV-specific CD8^+ T cells was calculated from the individual peptide-specific CD8^+ T cells (figure 2b, 3b, 4b and 5b). In this study population 0.2 to 6% of the CD8^+ T cells were EBV-

specific and approximately 10% of these T cells (range 4.1 to 25.8%) produced IFNγ in elispot assay in response to EBV peptides early in infection. (table 2) Similar results were obtained using intracellular IFNγ staining after peptide stimulation. (data not shown) In 4/5 AIDS-NHL patients (NHL6006, NHL0118, NHL6118 and NHL0308) only a moderate decline in the number of tetramer^+ T cells was observed in the course of HIV-infection after an initial rise in the first year(s) of HIV-infection (fig. 2b and 3b). One AIDS-NHL patient (NHL0139) even showed sustained increasing numbers of tetramer^+ T cells (fig. 3b). Interestingly, the function of these EBV-specific T cells decreased more rapidly in the course of HIV-infection than their numbers were falling. Sometimes almost no IFNγ-producing CD8^+ T cells were left after a few years of HIV-infection (patient NHL6006, fig. 2b,
Dysfunction of EBV-specific T cells in AIDS-NHL

Figure 2-3. Presence and function of EBV-specific CD8+ T cells and EBV load in 5 HIV-1 infected individuals with AIDS-NHL

On the x-axis follow-up is indicated in months after HIV-1 seropositive entry in the study. The arrows indicate the time of AIDS-NHL diagnosis, start of antiretroviral therapy (DDI) and HIV-1 seroconversion (HIV+). The vertical dotted line indicates the time point at which CD4+ T cell counts drop below 200/mm^3. The horizontal dotted line indicates the threshold for the number of IFNγ producing T cells.

Figure 2/3a. Longitudinal analysis of CD4+ and CD8+ T lymphocyte numbers.
Figure 2/3b. Longitudinal analysis of EBV-specific CD8+ T lymphocytes as assessed by tetramer staining as a composite of all tested tetramers (solid line) and IFNγ ELispot assay (dashed line), as described in Methods.
Figure 2/3c. Longitudinal analysis of EBV-load, expressed as the number of virus copies per 10^6 PBMC (solid line), in comparison with the number of functional EBV-specific CD8+ T lymphocytes per 10^6 CD8+ T cells (dashed line).

and B8-FLR-specific T cells in NHL0308, fig. 1b) or near lymphoma diagnosis (patient NHL0118, fig. 2b). In the course of HIV-infection, numbers of functional CD8+ T cells could be as high as 4000 /10^6 CD8+ T cells but eventually dropped below 250 for A2-restricted responses (NHL6006, NHL118), below 500 for B8-restricted responses (NHL0308) and below 800 for A2+B8-restricted responses (NHL6118, NHL0139) (fig. 2b and 3b, respectively). This loss of function was observed for both lytic and latent antigen-specific T cells (patient NHL0308, fig.1b and c).

Although the proportion of tetramer+ T
cells that produced IFNγ could be between 15 and 30% early in HIV-infection (NHL6006 and NHL0118, table 2), this percentage decreased in the course of HIV-infection, leaving less than 3% of the tetramer+ T cells to produce IFNγ already a few years before AIDS-NHL diagnosis (NHL6006, NHL0118, NHL0139 and NHL0308, table 2).

In LTA, the number of EBV-specific T cells decreased (LTA0036), increased (LTA1160) or remained stable (LTA0057) in the course of HIV-infection. (fig. 4b) However, absolute numbers of IFNγ producing T cells were stable in LTA compared to AIDS-NHL patients and ranged from +/- 250 to 400/10^6 CD8+ T cells for A2-restricted responses (LTA0057) and +/- 1100 to 7500 T cells/10^6 T cells for (A2+) B8-restricted responses (LTA0036 and LTA1160) (fig. 4b). Moreover, the percentage tetramer+ T cells that produced IFNγ was stable in the course of HIV-infection compared to AIDS-NHL (table 2).

Progressors to AIDS-OI showed low percentage of tetramer+ T cells (range 0.3-1.8%) and IFNγ producing T cells (range +/- 300 to 800/10^6 CD8+ T cells) specific for EBV (fig. 5b). But both number of tetramer+ T cells and IFNγ producing T cells were relatively stable in the course of HIV-infection. Functional capacity of EBV-specific CD8+ T cells only selectively decreased in slow progressor PROG0341 prior (3 months) to AIDS-diagnosis to almost zero (fig. 5b), at which the proportion of tetramer+ T cells that produced IFNγ was 0.5% (table 2). For fast progressor PROG0642 both number and function decreased only after AIDS-diagnosis (fig. 5b), and the percentage tetramer+ T cells that produced IFNγ did not decrease below
Dysfunction of EBV-specific T cells in AIDS-NHL

Figure 4-5. Presence and function of EBV-specific CD8+ T cells and EBV load in 3 HIV-1 infected LTA individuals (fig.4) and 3 HIV-1 infected individuals progressing to AIDS-OI (fig.5).

On the x-axis follow-up is indicated in months after HIV-1 seropositive entry in the study. The arrows indicate the time of AIDS-diagnosis (NHL or OI), start of antiretroviral therapy (DDI) and HIV-1 seroconversion (HIV+). The vertical dotted line indicates the time point at which CD4+ T cells count drop below 200/mm3. The horizontal dotted line indicates the threshold for the number of IFNγ producing T cells.

**Figure 3/4a.** Longitudinal analysis of CD4+ and CD8+ T lymphocyte numbers.

**Figure 3/4b.** Longitudinal analysis of EBV-specific CD8+ T lymphocytes as assessed by tetramer staining as a composite of all tested tetramers (solid line) and IFNγ ELISPOT assay (dashed line), as described in Methods.

**Figure 3/4c.** Longitudinal analysis of EBV-load, expressed as the number of virus copies per 10^6 PBMC (solid line), in comparison with the number of functional EBV-specific CD8+ T lymphocytes per 10^6 CD8+ T cells (dashed line).

3%. (table 2) PROG0232, a slow progressor to AIDS, even showed an increase in functionality just after start of highly active antiretroviral therapy (HAART) and just before AIDS-diagnosis (fig.5b).

This leads to an increase in the total number of IFNγ producing T cells from +/- 800 to 2400/10^6 CD8+ T cells and the percentage tetramer+ T cells that produced IFNγ from +/- 15 to 56% (table 2).
Table 2. IFNy producing CD8+ T cells as % of tetramer* T cells at all time points

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For each timepoint the percentage tetramer* CD8+ T cells that produced IFNγ is presented. The underlined numbers indicate the last timepoint before or timepoint at AIDS-diagnosis. Bold numbers indicate first timepoint after start of anti-retroviral therapy.

**EBV-specific CD8+ T lymphocyte function is dependent on CD4+ T cells**

We investigated whether there was a possible relation between CD4+ T cell numbers and the number of EBV-specific CD8+ T cells (fig.2a, 3a, 4a, 5a). In 4/5 AIDS-NHL patients, loss of functional EBV-specific CD8+ T cells (fig. 2b/c and 3b/c) was related in time to a drop in CD4+ T cell counts below 200/mm³ (fig. 2a and 3a). Moreover, when CD4+ T cell counts increased after anti-retroviral treatment (DDI) in patient NHL308, a parallel increase in the number of functional B8-FLR-specific CD8+ T cells was observed. (fig. 1b)

In LTA and PROG0232, who did not show a functional loss of EBV-specific CD8+ T cells (fig. 4b, 5b), CD4+ T cell numbers were stable during most time points of follow-up, slowly decreased at the end of follow-up, but never dropped below 200 CD4+ T cells/mm³ (fig. 4a, 5a).

HAART could increase the number of functional EBV-specific T cells. (fig. 5b) CD4+ T cell counts in progressors PROG0341 and PROG0642 dropped below 200/mm³ after 125 and 34 months, respectively (fig. 5a, 5b). Here, loss of function was initially paralleled by decrease in number, suggesting that loss of EBV-specific T cells is caused by physical deletion. Only at the last time point in patient P0341 function was selectively lost. (fig. 5b)

Indeed, when CD4+ T cell numbers measured at all timepoints were plotted against the number of EBV-specific CD8+ T cells that produced IFNγ a correlation was found (β = 0.076), which was highly significant (p<0.037), whereas no correlation was found between CD4+ T cell numbers and the number of tetramer* T cells (β = -0.043) (figure 7). In addition, no correlation was found between CD4+ T cell numbers and CD8+
T cell numbers, indicating that the correlation between CD4+ T cells and the number of functional EBV-specific T cells was not due to an increase in total CD8+ T cells (data not shown).

To investigate whether loss of functional T cells is associated with an increase in EBV load and whether this precedes the development of AIDS-NHL, EBV load/10^6 PBMC was determined using a real time quantitative PCR (Q-PCR). Indeed, as shown in figure 2c and 3c, loss of EBV-specific CD8+ T cell function was associated with an increase in EBV load in AIDS-NHL patients.

In patient NHL0139 and NHL0308 functional EBV-specific CD8+ T cells decreased after initial suppression of EBV load, resulting in an increase in EBV load near lymphoma diagnosis. (fig. 3c) After start of DDI treatment in patient NHL308, the increase in functional EBV-specific CD8+ T cells led to a small reduction in EBV load (fig.3c). In patient NHL0118 (fig. 2c) the gradual loss of function of EBV-specific CD8+ T cells is paralleled by a gradual increase in EBV load.

In patient NHL6006 and NHL6118, loss of function of EBV-specific CD8+ T cells was associated with high EBV peak loads. (fig. 2c, 3c) The subsequent increase in functional EBV-specific CD8+ T cells was followed by a decrease in EBV load, but EBV load increased again when T cell function was subsequently lost at AIDS-NHL diagnosis. (fig.2c and 3c). Especially for patient NHL6118 (fig. 3c), who had an EBV load of 9x10^5 copies at AIDS-NHL diagnosis, this leads to a very high EBV load per IFNγ producing T cell.

In contrast, in LTA occasional EBV peak loads were paralleled by an increase in the number of functional EBV-specific
CD8+ T cells after which EBV load decreased (fig. 4c). All three progressors to AIDS-OI also showed an initial increase in functional EBV-specific T cells together with an increase in total number of tetramer+ T cells, after which EBV load decreased. (fig. 5c) At AIDS-diagnosis, although functional EBV-specific T cells were lost in some patients (PROG0341), EBV load was low. HAART, which led to an increase in the number of IFNγ producing CD8+ T cells, resulted in a decrease of EBV load in PROG0232 (fig.5c).

Discussion

To investigate the cause of the defective EBV-immune control in AIDS-NHL patients, we studied number (using MHC-class I-tetramers) and function (using IFNγ elispot assay) of EBV-specific CD8+ T lymphocytes in the course of HIV-infection in relation to EBV load. This is, to our knowledge, the first longitudinal study demonstrating a dichotomy between direct visualization and functional assays enumerating IFNγ-producing antigen-specific T cells. The major conclusions from our study are (1) In AIDS-NHL patients EBV-specific CD8+ T cells are lost preferentially at the functional level and are not physically lost. (2) Loss of EBV-specific CD8+ T cell function is correlated with lower CD4+ T cell numbers. (3) Increasing EBV load correlates with loss of EBV-specific immunity. (4) The number of T cells directed against lytic antigens is higher than against latent antigens.

The observed correlation between loss of function of EBV-specific CD8+ T cells and lower CD4+ T cell numbers, indicates an important role for CD4+ T cells in maintaining the functional capacity of CD8+ T cells. Our data are in good agreement with studies on T helper dependence of LCMV-specific CTL in mouse models. 15-17 A critical role for CD4+ T cells has also been shown during immunization 18 and progressive loss of CTL in the absence of adequate helper cell function has been demonstrated for several murine viral infections. 19-21 Furthermore, CD4+ T cells also appear to be essential for long-term persistence of adoptively transferred virus-specific CTL in humans. 17, 22, 23

In the natural course of HIV-infection, it has been shown that progressors to AIDS lose CD8+ CTL when functional HIV-specific CD4+ T cells disappear. In contrast, in non-progressors, who have stable CD4+ T cell numbers, HIV-specific CTL responses can be sustained for long times 24-27 indicating that sustained HIV-specific helper activity is required for maintenance of functional CD8+ T cell responses. 24, 28, 29

The fact that the majority of EBV-specific CD8+ T cells were directed against lytic epitopes, suggests that these lytic antigen-specific T cells play a role not only during acute infection 30 but also in controlling EBV reactivation by eliminating virus-producing cells at an early stage. Loss of functional lytic-antigen specific CD8+ T cells could therefore lead to an increase in the number of virus particles as we indeed observed. As the pool of EBV-infected B cells grows, there is an increased risk for subsequent genetic hits resulting in malignant outgrowth of EBV-infected B cells. Because functional CD8+ T cells specific for latent antigens appear
to be lost as well newly developed tumor cells will not be destroyed. Our data suggest that loss of both lytic and latent antigen specific CD8⁺ T cells may be risk factors for development of AIDS-NHL.

In HIV-infection, 0.2 to 6% of the CD8⁺ T cells were found to be EBV-specific. In LTA approximately 6% (range 4.1-8.4%) of the HLA-A2-restricted EBV-specific T cells and 15% (range 5.5-28.5%) of the HLA-B8-restricted EBV-specific T cells produced IFNγ in response to EBV peptides. This is comparable to results recently reported for healthy HIV-seronegative EBV carriers, where the percentage IFNγ producing T cells ranged from 11 to 53% (avg of 25%). However, in AIDS-NHL patients and 2/3 other progressors to AIDS the number of IFNγ producing T cells was found to be lower already early in HIV-infection and to decrease rapidly in the course of HIV-infection in AIDS-NHL patients, resulting in a much lower percentage (<3%) of IFNγ producing T cells per tetramer⁺ T cell population. Therefore, our data indicate that next to tetramer staining, functional analysis of antigen-specific T cells is required to fully appreciate the role of CD8⁺ T cells in various clinical conditions. The observed discrepancy between number and function of EBV-specific CD8⁺ T cells is underscored by the finding that CD4⁺ T cell numbers correlated with the number of IFNγ producing EBV-specific CD8⁺ T cells but not with the number of tetramer⁺ CD8⁺ T cells.

The observed low percentage of IFNγ producing T cells is not likely to be a consequence of susceptibility to rapid activation-induced cell death in vitro, caused by pre-activation in vivo. Since tetramer-staining results before and after stimulation are virtually the same (data not shown), this indicates that antigen-specific cells are not lost after stimulation. In addition, when dead cells are included in the analysis no increase in the percentage of tetramer⁺ T cells was observed, suggesting that even if cells are lost, this is not occurring selectively in the EBV-specific CD8⁺ T cells. Furthermore, we studied expression of CD69, which has been shown to be highly expressed on non-responsive T cells in LCMV-infected CD4 knock-out mice. However, expression of CD69 was low, reaching no higher levels than 5% of the tetramer⁺ T cells. (data not shown) Indeed it has been shown that compared to healthy individuals a higher percentage of PBMC from HIV-infected individuals undergo apoptosis after overnight stimulation. However, death of T cells was not correlated with CD4⁺ T cell numbers or T cell function, not specifically observed in certain T cell subsets and was not confined to expression of T cell activation markers. Moreover, it has been shown that virus-specific CD8⁺ T cells are able to rapidly reinitiate cytokine production after recent stimulation. Thus, even if the EBV-specific T cells are recently activated or preactivated in vivo, this would result in a rapid production of IFNγ. Overall, these observations make a considerable death of EBV-specific T cells in vitro unlikely. The low percentage of IFNγ producing T cells was also not due to a suboptimal assay condition, since the elispot assay was shown to detect virtually all antigen-specific T cells when a T cell clone was analysed (data not shown). Furthermore, the percentage IFNγ producing T cells as assessed by
intracellular staining was similar to the percentage of IFNγ producers found by elispot assay in both healthy controls and patients (data not shown).

The phenomenon of dysfunction has also recently been shown for Hepatitis C virus\(^\text{35}\) during a period of acute infection, for tumor-specific T cells in melanoma patients\(^\text{36}\) and for HIV-specific T cells.\(^\text{37}\) This state of dysfunction has been shown to occur both at the level of IFNγ production and cytolytic activity.\(^\text{35, 36}\) In addition, in healthy individuals there is a correlation between IFNγ producing T cells and CTL precursor (CTLp) frequencies.\(^\text{31}\) Furthermore, our own observations indicate that the number of CTLp\(^\text{7}\) and IFNγ producing T cells correlate and both decreased in the course of HIV-infection in AIDS-NHL patients.

Thus, in AIDS-NHL patients we observed a loss of function of EBV-specific CD8\(^+\) T cells, sometimes occurring after an initial increase. During this increase a positive correlation with EBV load was observed, whereas loss of function was accompanied by increased EBV load leading to an inverse correlation. Overall, EBV load increased when the number of functional T cells dropped below 500/10\(^6\) T cells in case of B8-restricted responses and 250/10\(^6\) T cells in case of A2-restricted responses. This suggests that finally in AIDS-NHL patients indeed immune control over EBV seemed to be lost. Surprisingly, in LTA enormous transient bursts of EBV load were observed. These peaks in viral load were paralleled by expansions of functional CD8\(^+\) T cells specific for EBV. Since EBV load subsequently decreased these cells apparently were able to control EBV viremia. Although loss of function of CD8\(^+\) T cells specific for EBV was observed in one progressor to AIDS-OI, this was not paralleled by an increase in EBV load. It could however be that this individual eventually would have developed AIDS-NHL had he not developed AIDS-OI.

Because not all AIDS-NHL patients showed complete loss of function of EBV-specific T cells, there might be additional factors critical for development of lymphomas in HIV-1 infected individuals.

The development of AIDS-NHL is a multifactorial process involving at least virological and immunological parameters. Thus, both determinants are required to obtain a complete picture of the virus-host balance. We show that not just the total number of circulating EBV-specific CD8\(^+\) T cells, but mainly the number of functional EBV-specific CD8\(^+\) T cells are important in keeping EBV infection under control. When functional EBV-specific CD8\(^+\) T cells start to decrease, in most cases as a consequence of a decrease in CD4\(^+\) T cells, this decrease is paralleled by an increase in EBV load. To predict the occurrence of an AIDS-NHL, all these factors should be taken into consideration.

In conclusion, our data suggest that functional loss of EBV-specific CD8\(^+\) T cells with a concomitant increase in EBV load seems important in the pathogenesis of AIDS-NHL and that the defective function of EBV-specific CD8\(^+\) T cells in AIDS-NHL patients may be secondary to a lack of CD4\(^+\) T cell help.
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