Viro-immunological studies on the role of Epstein-Barr virus in the development of AIDS-related non-Hodgekin's lymphoma
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In HIV-infected individuals the incidence of high grade malignant B cell NHL is considerably increased (5-10%). Although these NHL are thought to arise because of uncontrolled EBV-driven proliferation of B cells, the cellular and molecular basis for the defective EBV-immunity in HIV-infection is largely unknown. In the studies presented in this thesis we aimed to obtain more insight into the role of the Epstein-Barr virus and EBV-specific immunity in the pathogenesis of AIDS-related NHL. We aimed to define parameters that may predict the occurrence of AIDS-NHL and elucidate the role of (defective) EBV immunity in the pathogenesis of AIDS-NHL. To this end both virological studies (part I) and immunological studies (part II) were performed.

I: Virological studies

To define parameters that may predict the occurrence of AIDS-NHL, we studied (super)infection with EBV type(s) and EBV load in the blood of HIV-infected individuals. The decrease in EBV-specific immunity in the course of HIV infection was suggested to result in superinfection with additional EBV-strains, which are less frequent among healthy individuals. In addition, the reported high frequency of EBV type 2 positive tumors has led to the suggestion that superinfection with EBV type 2 might contribute to the development of lymphomas. Therefore, we developed a type-specific nested PCR for direct EBV-typing in PBMC to analyze the prevalence of EBV-types 1 and 2 in several well-defined groups of HIV-1 infected individuals. A high prevalence of EBV type 2 infection and superinfection with both type 1 and 2 was observed (chapter 2), confirming earlier published observations from Yao et al. However, our observations that neither type 2 infection nor superinfection correlated with a failing immune system and that superinfection was also present in healthy persons, indicate that superinfection is not related to immunodeficiency. Moreover, acquisition of additional EBV strains (superinfection) seems a common observation and can occur in time, as also has been suggested by Brooks et al. Furthermore, neither type 2 EBV infection nor superinfection proved to be related to the development of AIDS-NHL. Therefore, the high frequency of EBV type 2 positive tumors may be merely a reflection of the high prevalence of EBV type 2 in HIV-infected homosexual individuals. This has also been suggested for the high frequency of EBV type 2 Burkitt's lymphomas (BL) in Central Africa where EBV type 2 is common. Moreover, EBV gene polymorphisms detected in EBV isolates from healthy virus carriers occurred with similar frequency in virus-associated tumors from the same geographical region. Further studies including different HIV-negative populations showed that EBV
type 2 prevalence was high among homosexual men in general and caused by sexual transmission. (chapter 3)  

A second possible predictor of AIDS-NHL we studied was EBV load. (chapter 4) Using a quantitative real-time PCR assay (to determine the number of EBV copies) we showed that in HIV+ homosexual men the EBV load was 100-fold higher than in HIV+ homosexual men, who in turn had a higher EBV load than HIV+ heterosexual individuals. Furthermore, we compared EBV load in PBMC and serum in the course of HIV-1 infection in subgroups of HIV-infected individuals. Overall, EBV load in PBMC was high, displayed considerable fluctuations over time and proved to be non-predictive of the development of AIDS-NHL. EBV was detectable in serum at some time points, but at a lower level and it did not predict the occurrence of AIDS-NHL. A possible explanation for the observations that HIV+ homosexual men had higher EBV load than HIV-homosexual men, who in turn had a higher EBV load than HIV-heterosexuals, may be the occurrence of increased frequencies of superinfection with additional EBV types. Since superinfection with EBV type 1 and 2 is a frequent observation in HIV-infected and HIV-uninfected homosexual individuals, EBV load may be increased by these additional EBV strains. In addition, the higher EBV load may be explained by the degree of immunodeficiency. However, our data show that the degree of immunodeficiency, as reflected in the number of CD4+ T cells is not related to the height of EBV load. A third possibility which may explain the high EBV load in HIV+ versus HIV-individuals and homosexuals versus heterosexuals may be chronic antigen stimulation. This may lead to polyclonal B cell expansions and increased frequencies of EBV-reactivation. This immune activation may be antigen-specific and be induced by HIV, superinfection with additional EBV strains and infections with other common viruses and less common viruses and virus strains. Especially cytomegalovirus (CMV) has been implicated in EBV reactivation. Furthermore, immune activation can also be induced via cytokines or by viral gene products like the HIV-protein Nef. 

Since HIV-homosexual men had higher EBV load than HIV-heterosexual individuals suggests that chronic antigen stimulation might be related to sexual behavior. Increased numbers of sexual intercourse partners could lead to acquisition of other EBV strains and viruses like human herpesvirus 8 (HHV), hepatitis B and C virus (HBV and HCV). This may lead to increased stimulation of the immune system and thereby evoke higher rates of EBV-reactivation, which may lead to higher EBV load. 

At the start of our studies, we hoped to find a correlation between high EBV load and the development of AIDS-NHL, as has been shown for EBV load in PTLD. However, we observed that total numbers of EBV copies were not predictive for lymphoma development.
(Chapter 4) This could be due to the fact that the total number of EBV copies, which the Q-PCR detects, is determined both by the number of infected B cells and the number of copies per cell. The number of infected cells can be measured by a spontaneous B cell transformation assay (SBT), which is based on the transforming capacity of the EBV strains present. Using this assay it was shown that the number of EBV-infected B cells did predict AIDS-NHL development, suggesting that the total number of EBV copies may not reflect the number of infected B cells. Recently, EBV load in post-transplant patients was investigated using a Q-PCR in combination with in situ-hybridisation to determine the total number of EBV copies and study the number of EBV copies per cell. One study showed that the increase in EBV copies seemed to be due to higher numbers of episomes per B cell and not to an increase in the number of infected B cells. Another study showed that the increase in viral copies was not due to virus replication, suggesting that also the number of infected B cells was rising. These somewhat different results indicate that the exact relation between the total number of EBV copies and the number of EBV-infected B cells still needs to be established.

EBV load in serum did not correlate with EBV load in PBMC, especially because enormous peaks of EBV DNA can be found in serum, despite low EBV load in PBMC. This and the fact that EBV load in serum does not reflect the actual viral burden of the patient, indicate that EBV load should be measured in PBMC. Although we anticipated that virological factors would be important in the pathogenesis of AIDS-NHL, our data show that virological characteristics like EBV type and load are neither related to nor predictive of AIDS-NHL development.

II: Immunological studies

It was previously shown by our group that the occurrence of AIDS-NHL is preceded by decreasing EBV-CTLprecursors and increasing numbers of EBV-infected B cells. To investigate by what mechanism EBV-specific CTLs are lost, we studied whether cellular immunity to EBV is lost due to physical loss or due to dysfunction. Moreover, we correlated these data on cellular immunity with the number of EBV-particles. (Chapter 5) We determined both number (using HLA-EBV-peptide tetrameric complexes), phenotype (using monoclonal antibodies against CD45RO and CD27) and function (using IFNγ Elispot assay) of virus-specific T cells. We observed that EBV-specific CD8+ T cells did not disappear but rather lost their capability to produce IFNγ. (Chapter 5) This loss of function of EBV-specific CD8+ T cells correlated with lower CD4+ T cell numbers, was accompanied by increasing numbers of EBV copies and was characterized by lack of differentiation into CD27− effector T cells. In LTA individuals, in whom CD4+ T cell numbers were maintained and EBV-specific T cells differentiated into the CD27− effector phenotype (Chapter 6), IFNγ producing EBV-specific T cells were stable. (Chapter 5) In LTA, occasional bursts in EBV load were paralleled by increased numbers of...
functional T cells, suggestive of immune control. 
Our data indicate that loss of function was related to lack of CD4+ T cell help, indicating that CD4+ T cells are important in maintaining the function of CD8+ T cells. Functional CD4+ T cells may be necessary to enable CD27+ memory CD8+ T cells to differentiate into CD27− effector T cells, either directly or indirectly by cytokine production and CD70-CD27 interaction. These CD27− effector T cells have a higher functional capacity and therefore are important to delay disease progression. Highly active antiretroviral therapy (HAART) was found to increase CD4+ T cell numbers and thereby leading to higher numbers of EBV-specific IFNγ producing CD8+ T cells and a subsequent reduction in EBV load. (chapter 7)

**EBV-specific CTL and EBV load: a delicate balance**

Absolute EBV load fluctuates over time and does not seem to predict AIDS-NHL development, although EBV load tends to increase in the course of HIV-1 infection in AIDS-NHL patients. (chapter 4) In addition, we observe lower EBV-specific (functional) immunity in progressed to AIDS and decreasing EBV-specific immunity in AIDS-NHL patients in the course of HIV-infection. (chapter 5) This suggests that there is a delicate balance between virus and the immune system. Functional loss of EBV-specific CD8+ T cells with a concomitant increase in EBV indicates a disturbed balance, which seems critical in the pathogenesis of AIDS-NHL. (figure 1)

During acute viral infection, an increase in viral load induces a virus-specific CD8+ T cell response. 33 Depending on the height of the viral load, the speed of CD8+ T cells to increase and the subsequent decrease in viral load, a "viral set-point" is reached in which the viral load is in balance with the CTL response. The level of this viral setpoint can differ between individuals. 36-39 Upon infection with HIV, continuous antigen-stimulation leads to a renewed viral set point, at which the viral load is at a higher level than in healthy controls. (chapter 4) With the loss of functional EBV-specific CD8+ T cells occurring in the course of HIV-1 infection 40 (chapter 5) and the increased frequencies of EBV reactivation, it will be increasingly difficult to suppress EBV load. This leads to accumulating amounts of EBV particles in the B cell pool of HIV-infected individuals. (chapter 4) As long as there is still sufficient EBV-specific (functional) immune surveillance, the virus and CTL are in balance. (chapter 5, figure 1 first panel) But when there is loss of functional EBV-specific T cells (as in NHL patients), EBV load gradually increases. (chapter 4 and 5) (figure 1, second panel)

Our data indicate that only studying EBV load may not give a complete picture. Risk assessment requires the evaluation of both EBV load and the number and function of EBV-specific CD8+ T cells. High or low EBV load with sufficient CTL response infers a low risk of developing NHL, whereas high or low but increasing EBV load with low CTL responses infers a high risk of developing AIDS-NHL suggesting that
the system is out of balance. (Fig. 1, second panel) With a further increase in EBV load and loss of function of EBV-specific T cells, this could lead to AIDS-NHL development. (Fig. 1, third panel) The immune system may try to compensate for this loss of EBV-specific immunity by increasing the numbers of EBV-specific CD8+ T cells. (Fig. 1, fourth panel)

Lack of differentiation into CD27-effectector T cells as a progression marker

We show that differentiation into CD27-effectector CD8+ T cells is essential in controlling chronic virus infections. Individuals that show an accumulation of antigen-specific CD27- T cells have delayed disease progression, due to the high effector functions of CD27- T cells. Thus, lack of these CD27- effector T cells results in a relatively poorly functional HIV- or EBV-specific T cell population leading to faster progression to AIDS or progression to AIDS-NHL, respectively. Since poor response to virus seems to co-exists with poor differentiation into CD27- effector T cells, CD27 expression on virus-specific T cells may be used as a disease progression marker in HIV-infection but possibly also in other clinical settings. In addition, it may be used as read-out for improvement after therapy or vaccination.

![Disturbed balance diagram](image)

**figure 1. Disturbance of the balance between virus and immunity**

Three mechanisms by which the balance between virus and immunity can be disturbed are depicted. The filled triangles indicate EBV-specific CD8+ T cells, of which the black triangles are the functional ones. White triangles indicate EBV load. The balance between virus and immunity is depicted in the first panel. This balance can get disturbed when (a) EBV-specific CD8+ T cells lose their functional capacity or (b) EBV-specific functional T cells are lost or c) when EBV load increases. (Phase I: second panel) This disturbed balance leads to an increase in EBV load and concomitantly an increased risk of developing a lymphoma. (Phase II, third panel) The immune system may attempt to compensate for the loss of function of EBV-specific T cells by increasing numbers (gray triangles) of EBV-specific CD8+ T cells. (fourth panel)
**Model of EBV-induced AIDS-NHL**

The development of AIDS-NHL is a multifactorial process involving at least virological and immunological parameters. Chronic antigen stimulation induced by HIV-infection (direct or indirect) and possibly (super)infection with other EBV types or other viruses leads to increased frequencies of EBV-reactivation, causing an initial EBV-driven proliferation of B cells. (fig.2, 1) In addition, higher levels of TGFβ, which have been reported to occur in HIV-infected individuals, 45 can induce the EBV lytic cycle by increasing ZEBRA expression leading to higher levels of EBV-reactivation.

Reactivation of EBV probably leads to entry into the lytic cycle, with concomittant lytic antigen expression, 46 and subsequently latent antigen-expression. Therefore, lytic-antigen-specific T cells may be important in controlling the initial reactivation of EBV-infected B cells, and may thus be the key determinator for the expansion of EBV-infected B cells. (fig.2, 7)

Although lytic antigens 47; 48 are sometimes expressed on tumors, the overall consensus is that most tumors express only latent antigens. 49; 50 An outgrowth of latently infected B cells is therefore controlled by latent-antigen specific T cells. (fig.2, 9)

An increase in EBV load (fig.2, 2) should drive EBV-specific T cells into a more effector phenotype. Lack of CD4* T cell help (fig.2, 3), possibly necessary to enable CD27* memory T cells to differentiate into CD27− effector T cells (fig.2, 4), results in maintenance of a memory phenotype and loss of function. Functional loss of both lytic and latent EBV antigen-specific CD8* T cells together with an increase in EBV load is critical in the pathogenesis of AIDS-NHL. Therefore, a combination of CTL and EBV load may be favored to predict the occurrence of AIDS-NHL.

HIV-infection is characterized by disturbances in cytokine expression regulation, 51; 52 leading to higher levels of immunosuppressive and tumor-promoting cytokines, which could contribute to the pathogenesis of AIDS-NHL. It has been shown that B cells from HIV-infected patients constitutively express IL-6, which is known to promote the growth of EBV-positive B cells. 53; 54 Furthermore, EBV-positive AIDS-related BL have been shown to express IL-10, 55 another cytokine which may not only promote the growth of EBV-positive B cells, 56 but also interferes with CTL-generation and thus could contribute to CTL dysfunction. Furthermore, TGFβ has been shown to be transcribed in NHL cells from SIV-infected rhesus monkeys, 57 and may act as an autocrine growth stimulus.

Overall, the balance between virus and the immune system is a critical factor in the pathogenesis of AIDS-NHL. Loss of this balance may favor the development of a malignant lymphoma, by increasing the chance of acquisition of additional specific genetic changes, such as activation of oncogenes, inactivation of suppressor genes. Chromosomal translocations in AIDS-NHL frequently involve bcl-6, a proto-oncogene that affects B cell maturation 58; 59 and c-myc, which can cause tumorigenic conversion in lymphoblasts. In addition, point-mutations in N-ras and K-ras have been found. 60 Furthermore, the tumor suppressor gene P53 can be inactivated by mutations. 61; 62 (figure 2)
Model of EBV-induced lymphomagenesis in HIV infection

figure 2. Model of EBV-induced lymphomagenesis in HIV infection
A model is depicted to explain the development of EBV-related non-Hodgkin's lymphomas in HIV-infected individuals. Chronic antigen-stimulation in the lymphoid tissue can lead to clonal expansion of EBV-infected B cells (1) and increased EBV load in the peripheral blood. (2) Because of lack of CD4+ T cell help (3), as a consequence of HIV-infection, memory CD8+ T cells do not differentiate into CD27- effector T cells. (4) EBV-specific CD8+ T cell function decreases, whereby EBV load in the blood can increase, either by increased numbers of EBV-infected cells (5), or increased numbers of EBV copies per B cell (6). Dysfunctional EBV-specific T cells can not control the expansion of EBV-infected B cells. (7) Additional genetic hits may subsequently result in a malignant outgrowth of these EBV-infected B cells (8), which can also not be controlled by specific T cells (9), resulting in an AIDS-related non-Hodgkin’s lymphoma (10).

Implications for therapeutic approaches
The studies presented in this thesis shed some light on the mechanism by which EBV-positive AIDS-NHL develop. These new insights into AIDS-NHL pathogenesis can be translated into
possible therapies to prevent or cure AIDS-NHL.
Adoptive transfer of EBV-specific CTL has been shown to result in decreased EBV load and regression of PTLDs in transplant recipients. 63-65 However, reinfusions of EBV-CTL were necessary to maintain a low level of EBV load. 64 To restore the balance between CTL and EBV load in AIDS-NHL patients and to lyse the tumor, adoptive transfer of EBV-specific CTL is a possible candidate. However, CD4+ T cells are important to sustain the functional capacity of antigen-specific CD8+ T cells. 66-71 Because of the underlying CD4+ T cell defect in HIV-infected individuals, adoptive transfer of EBV-specific CD8+ T lymphocytes would only be temporarily successful and long-term EBV-specific immunity would not be restored.

Infusion of CTL may eradicate the tumor as shown for PTLD patients, in whom at the same time also immunosuppressive therapy is reduced. In HIV-infected individuals, CD4+-specific immunity seems irreversibly affected. Addition of (EBV-specific) CD4+ T cells or addition of a cytokine (IL-2, IL-12, IL-15) restoring or replacing the function of CD4+ T cells may be an option to consider.

We observed that EBV-specific immunity improves after start of HAART. In particular the number of IFNγ producing EBV-specific T cells increased leading to a higher % of IFNγ producing tetramer+ T cells (IFNγ/tetramer ratio). This improved cellular immunity was probably caused by an increase in the number of CD4+ T cells, thereby probably restoring EBV-specific CD4+ T cell help, and led to a decrease in EBV load. Therefore, HAART seems a good candidate therapy to improve EBV-specific immunity and thereby reduce the risk on AIDS-NHL. Furthermore, a combination of HAART therapy and infusion of EBV-specific CTL may be an option. Initial infusion of EBV-specific CTL to lyse part of the tumor and subsequent boost of the immune response with HAART may lead to control of EBV load and thereby decrease the risk of NHL.

Since NHL is often a late complication of AIDS, in the early days the incidence was expected to increase due to the prolonged survival of AIDS patients as a result of antiretroviral therapy (AZT) and effective treatment and prophylaxis of opportunistic infections. 72, 73 Since HAART leads to higher CD4+ T cell numbers, general immune reconstitution 74 and general immune reconstitution 75 and improvement of EBV-specific T cell immunity, there are indications that the incidence of AIDS-NHL is beginning to decrease. 76, 77

Future studies

Future studies should be aimed at unraveling the exact role of CD4+ T cells in supporting CD8+ T cell function in EBV infection. It is relevant to study the quantity of EBV-specific CD4+ T cell responses and investigate whether physical loss of these T cells by HIV-induced killing or initial loss of function of these virus-specific CD4+ T helper cells causes a lack in CD8+ T cell function. Furthermore, studies should focus on the need of costimulation in CD8+ and CD4+ T cell function. When the exact role of CD4+ T cell is established, therapeutic strategies to prevent or cure AIDS-NHL can be developed.
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