Controlling herpesviruses after allogeneic stem cell transplantation: Predictive features of T-cell immunity

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Controle van herpesvirussen na allogene stam cel transplantatie:
Voorspellende aspecten van T cel immuniteit

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

Introduction and scope of thesis

INTRODUCTION

Infections with herpesviruses such as cytomegalovirus (CMV) and Epstein-Barr virus (EBV) occur mostly during early childhood and usually have an asymptomatic course. CMV, a β-herpesvirus infects 35-70% of the population in developed countries[1] while the prevalence in poor socioeconomic groups exceeds 90%[2]. CMV is a large virus consisting of a 230 kb genome and expresses three gene sets during replication; immediate early, early and late genes[3]. The most studied CMV proteins are the immediate early protein IE-1 and the tegument protein pp65. During latency CMV resides in many different tissues and cell types such as macrophages, granulocytes and dendritic cell precursors[4].
EBV is a ubiquitous γ-herpesvirus which infects over 90% of the human population during life[5]. In order to establish and maintain infection, EBV uses several transcription programmes which all express a certain subset of genes (reviewed in chapter 2). During its true latent phase, which is required for lifelong persistence, the virus resides in peripheral-blood memory B cells where none of the EBV proteins are expressed. Only the protein EBNA-1 is expressed when the infected memory B cell divides[6]. When primary EBV infection is delayed until adolescence, it can induce infectious mononucleosis. This symptomatic disease is characterized by severe fatigue, sore throat and lymphadenopathy[7]. After primary infection, both viruses persist lifelong in the host where there is a tightly regulated balance between virus infected cells and control through cytotoxic T-cell responses[5;8;9]. However, this balance can be disrupted in immunocompromised individuals such as HIV infected individuals or allogeneic stem cell transplantation recipients resulting in severe complications.

Allogeneic haematopoietic stem cell transplantation (SCT) is a treatment for a wide variety of both malignant and non-malignant haematological diseases. After conditioning the patient with chemotherapy and/or total body irradiation, stem cells of the donor are infused[10]. Early complications after SCT are acute graft versus host disease (caused by donor T cells recognising minor histocompatibility antigens on host cells)[11] on one hand and infectious complication such as viral reactivations due to the immunosuppressed state of the host on the other hand.

During the immunosuppressed state following allogeneic stem cell transplantation, primary infection or reactivation of the already persisting virus can result in EBV associated post-transplant lymphoproliferative disorders (PTLD)[12] or CMV disease. CMV disease can manifest itself in for example pneumonia, colitis or retinitis. Early reconstitution of an adequate CMV or EBV-specific T-cell response after SCT can prevent viral reactivation and subsequent complications. Therefore it is of utmost importance to identify early parameters of proper EBV- and CMV-specific T-cell reconstitution.

SCOPE OF THESIS

This thesis focuses on identifying features of T-cell immunity which can predict adequate or on the other hand lack of control, of viral reactivations. First we provide an overview of the development of EBV associated PTLD in immunocompromised individuals, as non-Hodgkin lymphomas, in chapter 2.
To elucidate the role of EBV-specific T-cell reconstitution in development of EBV reactivations after SCT we investigated the EBV-specific $CD4^+$ and $CD8^+$ T-cell reconstitution during the first year after SCT between patient with and without EBV reactivation. To detect the EBV-specific T cells early after SCT when absolute T-cell numbers are still reconstituting we applied a novel expansion technique to measure these EBV-specific T-cell responses. The results of this study are described in chapter 3.

Recipients of solid organ transplantation are at risk of PTLD due to their immunosuppressed state following transplantation. This risk is even higher for EBV seronegative individuals. Therefore we were therefore interested in the EBV-specific T-cell response in EBV seronegative patients receiving a seropositive transplantation. In chapter 4 we followed an EBV seronegative recipient of an EBV seropositive cardiac transplantation and we describe the kinetics of an EBV-specific T-cell response capable of adequate control of a primary EBV infection in the presence of severe immunosuppression.

Identifying allogeneic SCT recipients at risk of viral reactivation and subsequent complications early after transplantation can reduce risk of EBV and CMV related complications by early induction of pre-emptive therapy. On the other hand, identifying patients that develop adequate antiviral responses capable of viral control can prevent overtreatment. In chapter 5 we investigated the prognostic capacity of perforin expression on $CD8^+$ T cells in predicting onset and severity of EBV and CMV reactivations early after SCT. To this end we also analyzed absolute numbers of T cells early after SCT and tried to elucidate whether early viral reactivation triggers T-cell reconstitution or whether early adequate T-cell reconstitution prevents viral reactivation (chapter 6).

Chapter 7-9 focuses on CMV reactivation after allogeneic SCT. The kinetics of CMV-specific T cells during the first year after SCT are described in chapter 7. Because CMV seropositivity of the recipient and/or donor plays an important role in transplantation outcome, we retrospectively analyzed differences in the onset and severity of CMV reactivation between recipients of CMV seropositive and seronegative grafts (chapter 8). The contribution of CMV specific T cells in the graft to the subsequent development and severity of viral reactivation is studied in chapter 9. Finally, the results of the studies described in this thesis are summarized and discussed in chapter 10.

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Chapter 2

Immune Surveillance of EBV Infected B cells and the development of NHL in immunocompromised patients

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ABSTRACT

After infection with the Epstein-Barr virus, a common gammaherpes virus which infects and persists in the B cells, an equilibrium is established in which newly infected and differentiating B cells are controlled by cytotoxic T-cell (CTL) responses. Disturbance of this equilibrium, which can occur in immunocompromised situations, can lead to uncontrolled lymphoproliferation and subsequent development of non-Hodgkin Lymphomas (NHL). Here we review the role of immunesurveillance of EBV infected B cells and two situations where immunesurveillance is altered due to immunodeficiencies, transplantation recipients and HIV infection, which can lead to EBV mediated NHL. In transplant recipients, immunosuppression prior and during transplantation can lead to lack of immunesurveillance and results in proliferation of infected B cells, which would normally be controlled by CTL responses. Interestingly, in HIV infection both deregulation of the normal B cell biology and a reduction in immunity play a role in developing NHL. Therefore, the nature of EBV infection in HIV-positive subjects is very different from that in transplanted individuals, in whom (re-)appearance of EBV-specific $CD8^+$ T cells – either by a decrease in immune suppression or infusion of donor lymphocytes - immediately leads to a decrease in EBV load.

INTRODUCTION

Epstein-Barr virus (EBV) is a widespread gamma-herpes virus which infects over 90% of the human population during life. Infection usually occurs during childhood where it occurs asymptomatically. However, when primary infection is delayed until adolescence, EBV can induce Infectious Mononucleosis (IM), a symptomatic course of the infection characterized by severe fatigue, fever, sore throat and lymphadenopathy[1,2]. The immense T-cell response in IM patients, where over 50% of the lymphocytes may be EBV specific, and the subsequent cytokine release are thought to be the cause of the symptoms [3,4]. After primary infection EBV persists as a lifelong latent infection in the memory B cell compartment[5].

EBV was first isolated from Burkitt's lymphoma in 1964 by Epstein *et al.* and it was soon to be described to have oncogenic capacities[6]. EBV is associated with several malignancies like Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma and Non Hodgkin Lymphoma's (NHL) in immunocompromised individuals[7]. Non Hodgkin lymphoma is a collective name for several lymphoid malignancies with different pathological and biological backgrounds[8]. A classification of NHL was established in 1993 by the International Lymphoma Study Group called the Revised European-American Lymphoma (REAL) classifications[9].A major risk factor for development of EBV $^{+}$ NHL is altered immunity[10]. This can occur in primary immunodeficiencies like the X-linked lymphoproliferative syndrome. Furthermore these NHL have been shown to occur in solid organ transplantation (SOT) due to risk of primary infection in combination with immunosuppressive therapy, stem cell transplantation (SCT), due to immunosuppression and the necessity of developing a new immune system and other acquired immunocompromised settings like HIV infection[10,8]. NHL can also occur in immunocompetent hosts, however, in these individuals it is not clear whether EBV contributes to the development of the NHL, or is a passenger virus that is merely detected in the lymphoma [11]. Table I gives an overview of the most frequently occurring EBV^{+} NHL in both immunocompromised and immunocompetent hosts [11].

In this review, EBV persistence and the subsequent immunesurveillance of the infected B cells in healthy individuals will be discussed, as well as the role of immunesurveillance in development of NHL. Since EBV has been directly implicated in the development of NHL in immunocompromised hosts, we will focus on two immunocompromised situations. First we will discuss Post Transplant Lymphoproliferative Disorders (PTLD), a NHL type which occurs after haematopoietic stem cell transplantation or solid organ transplantation. Secondly, immune deficiencies through HIV resulting in NHL will be discussed.

Tabel I. EBV-associated NHL

Type of NHL	Target cell	EBV Frequency	Latency
Immunosuppressed Patients			
NHL associated with primary immunodefiency	B cell	>95%	III
Post transplant lymphoma			
Hematopoietic SCT	B cell	>95%	III
Solid organ transplant	B cell	>95% in first year post transplant	Usually III but late lymphomas may be I or II
HIV associated lymphomas			
Primary CNS lymphoma	B cell	>95%	III
Primary effusion lymphoma	B cell	>90%	I
Diffuse large cell lymphoma	B cell	30%-60%	II or I
Burkitt's lymphoma	B cell	30%-60%	I
Methotrexate induced lymphomas	B cell	>95%	III
Immunocompetent Patients			
Extranodal NK-T cell lymphoma	NK cell with some T cell-associated antigens	>95%	II
Aggressive NK leukemia lymphoma	NK cell with some T cell-associated antigens	30%-60%	II or I
Endemic Burkitt lymphoma	B cell	>95%	II
Diffuse large B cell lymphoma	B cell	10-35%	II
Angioimmunoblastic lymphoma	B cells and T cells	>80%	II

Adapted from Ref.11. This research was originally published in Blood, Heslop, Biology and treatment of Epstein-Barr virus associated non-hodgkin lymphomas. Blood (Hematology) 2005;260-266 © American Society of Hematology. CNS, central nervous system; HIV, human immunodeficiency virus.

EBV life cycle

After primary infection, EBV remains as a lifelong persistent infection of the memory B cells[5]. Understanding how EBV is able to maintain infection in vivo is crucial in investigating the immunesurveillance of these infected B cells and how lack of immune-surveillance can result in uncontrolled lymphoproliferation. The biology of EBV infection in the B cell system and the route whereby EBV accesses the B cell memory cells are still poorly understood. The best described model to date is the Thorley-Lawson model of EBV persistence shown in figure 1.

EBV infects the lymphoid tissue of Waldeyer's ring where the lytic phase of the infection results in continuous viral replication, production of all viral proteins and viral shedding. Subsequently the latent phase is induced, where after EBV infected cells exit the tonsils

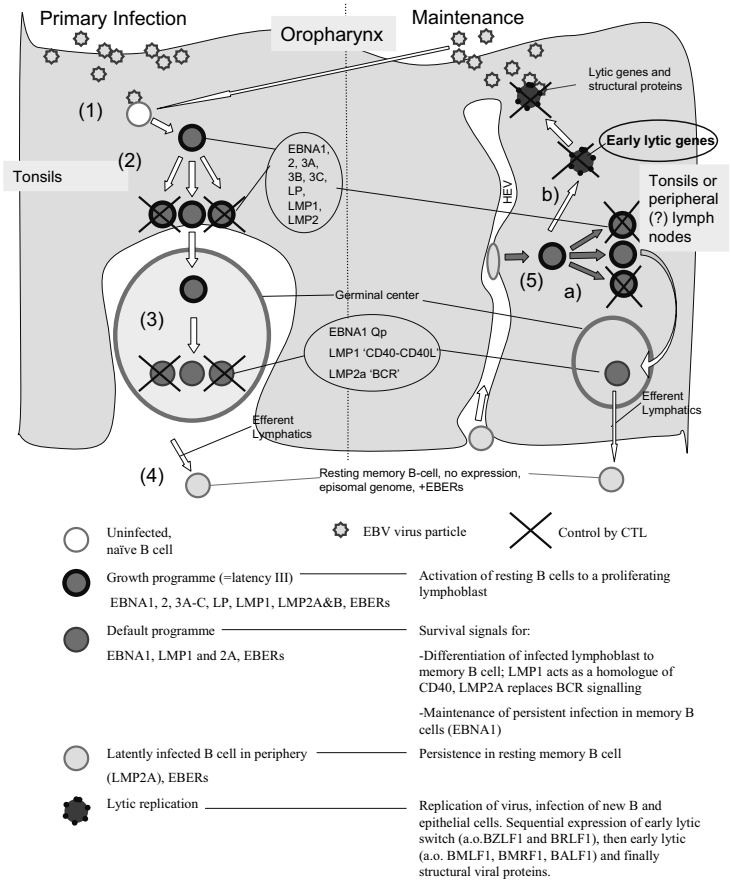

Figure 1. Schematic respresentation of the EBV life cycle

1) EBV generally enters the host via the epithelium of the oropharynx, where it infects naïve B cells either directly or possibly via infection of epithelial cells first 2) During primary EBV-infection B-cells are driven into proliferative lymphoblasts with full expression of EBV latent genes. 3) After down-regulation of most latent genes, EBV-infected B cells enter the germinal centre (GC) reaction, where they differentiate into memory B cells. 4) As the infected memory B cells enter the peripheral circulation through the efferent lymphatics, EBV gene expression is virtually shut down. The cells are then undetectable by the immune system and can persist in a quiescent state in the periphery. EBNA1 is transiently expressed when homeostatic division of memory B cells occurs, in order to duplicate the episomal genome. 5) Cell can subsequently migrate out of the peripheral blood trough the high endothelial venules (HEV), to (tonsillar or other) lymph nodes, where they can either a) switch to the growth programme and go through a GC reaction again, or b) differentiate into plasma cells, which induces lytic replication of EBV. In the latter case new viruses are produced which can either infect new B cells or epithelial cells, or be shed into the saliva. During clearance of acute infection and later during latency, $CD8^+$ T cells eliminate EBV-infected B cells with excessive gene expression, indicated by strike-troughs in the picture.

into the peripheral blood. However, these latently infected B cells can travel back to the tonsils and re- enter the lytic phase[12]. It is debated which cells are first infected by EBV. Whilst some studies indicate viral replication in epithelial cells in the oropharynx and subsequent B cell infection[13], other studies suggest direct infection of B cells in the oropharynx[14]. Recent findings by Shannon-Lowe *et al* show that EBV efficiently binds to the B cell surface where it can both initiate latent infection in the B cells and access the epithelium by transfer infection[15]. Once the B cell is infected the cells differentiate while undergoing several transcription programmes and finally enter the periphery as latently infected memory B cells. During this whole cycle the virus uses the biological differentiation cycle of normal B cells[12]. The gene expression pattern during the different stages of the infection with respect to the B cell differentiation cycle/programme is described below.

Thorley-Lawson describes a model for EBV persistence in vivo which provides an explanation for the ability of EBV to maintain persistent infection and its specificity for the memory B cell pool[16,12]. This model contains four different transcription programmes with different viral gene expression patterns. During the lytic stage of the infection virus is shed for transmission. There is a continuous number of cells in the lytic phase in the tonsils shedding virus[17]. The lytic cycle involves expression of immediate early (IE) proteins, like BZLF1, which are transactivators of viral gene expression. This is followed by the expression of early (E) proteins, for example BMRF1, which are essential for viral DNA replication. Finally late (L) proteins, as VCA (viral capsid antigen), are expressed which encompass structural virion proteins [18].

After the lytic infection the cells enter the latent phase. The other three transcription programmes all comprise the latent phase of the infection. The growth programme, also known as latency type III, activates the infected B cell to differentiate into a resting memory B cell. During the growth programme all Epstein-Barr Nuclear Antigens (EBNAs) are expressed and the latent membrane proteins LMP1 and LMP2A-B. In the default programme, latency type II, only three viral proteins are expressed, EBNA1 for replication of the viral genome during cell division [19] and LMP1 and LMP2a. These latter two viral genes provide important survival signals. They can mimic signals required to rescue the activated B cell into the memory pool. Together these genes allow the infected lymphoblast to differentiate into memory B cells and then persistently maintain infection[17]. Once the infected cell has reached the true latency phase, latency type 0, it will exit the tonsils and circulate in the peripheral blood. During this phase no viral proteins are expressed , allowing the infected memory B cell to remain undetected by the immune system[5,20]. Table 2 gives an overview of proteins expressed during the latent differentiation stages of the EBV infected B cell. When the latently infected memory

B cell divides, latency type I, which occurs as part of the normal B cell homeostasis and is not virus driven, EBNA1 is expressed [20].Thorley-Lawson speculates that circulating latently infected B cells can travel back to the tonsils where they will receive signals that allow re-expression of LMP1 and LMP2a ensuring prolonged survival[12]. Kuppers *et al* describe a role for $EBNA2^{+}/LMP1^{-}$ cells which do not fit into the classical latency programmes described above. This phenotype is seen in the interfollicular region in IM infected tonsils as well as in AIDS and transplant associated tumours. This may indicate the use of another virus programme [21].

Table II. Transcription programmes used by EBV to establish and maintain infection

Type of infected B cell*	Program	Genes expressed	Function of the program
Naive cell	Growth (III)	EBNA-1 through EBNA-6 LMP-1, LMP-2A and LMP-2B	Activates B cells
Germinal-center cell	Default (II)	EBNA-1, LMP-1 and LMP-2A	Differentiates activated B cell into memory cell
Peripheral-blood memory cell	Latency (0)	None	Allows lifetime persistence
Dividing peripheral-blood memory cell	EBNA-1 only (I)	EBNA-1	Allows viral DNA in latency-program cell to divide
Plasma cell	Lytic	All lytic genes	Replicates virus in plasma cell

Adapted from Ref. 12. Copyright © 2004 Massachusetts Medical Society. All rights reserved. EBNA, EBV nuclear antigen; LMP, latent membrane protein. * Except where indicated, the types of cells are primarily restricted to the lymphoid tissue of Waldeyer's ring.

In summary, EBV will infect B cells in the lymphoid tissue of Waldeyer's ring where they will differentiate into resting memory B cell using the normal B cell differentiation programme together with EBV's transcriptional programme. Once differentiated into memory cells, they will enter the circulation. No viral replication occurs in the periphery, but continuous viral shedding and replication occurs in the oesophageal lymphoid tissue. Infected memory B cells can travel from the periphery back to the tonsils where prolonged survival signals are given [5,17].

Immunesurveillance of EBV infected B cells

Whilst antibody mediated immune responses do play a role in EBV control, especially gp350 specific antibodies which may contain viral spread because of their important capacity of being virus-neutralising, cellular mediated responses are much more important. The common understanding is that during primary infection, EBV is mainly controlled by $CD8^{+}$ T cells[1,22]. Most of what we know about these T-cell responses comes from studies performed in IM patients as these individuals present themselves

with clinical signs of a primary infection. This is shown to be associated with a large T-cell response [18].

The $CD8^+$ T-cell response to EBV has been well characterized. In the early days it was shown that the CTL response towards latent antigens is dominant and mainly directed against EBNA3A, EBNA3B and EBNA3C epitopes[22]. CTL responses against EBNA-1 were shown to be rare. This was described to be caused by an immune evasive strategy of EBV in which an internal glycine-alanine (gly-ala) repeat domain protects EBNA-1 from HLA-class I presentation [23]. Recently this was explained as it was postulated that the gly-ala repeat works as a brake on EBNA-1 mRNA translation [25]. Despite this, $CD8^+$ T-cell responses have been described against EBNA-1[24]. How these responses are elicited and whether they contribute to protection and control remains to be elucidated.

A major part of the $CD8^+$ T-cell response was shown to be directed against IE (like BZLF1 and BRLF1) and E (like BMLF1 and BMRF1) proteins [22]. EBV specific $CD8^+$ T cells against these lytic proteins are detected early during primary infection and remain present throughout latency[26]. Lytic protein specific $CD8^+$ CTL responses were first described after the introduction of tetramers. In IM the massive expansion of T cells was shown to constitute mostly specific T cells. EBV-specific $CD8^+$ T cells against a peptide derived from the lytic protein BZLF1 restricted through HLA-B8, RAKFKQLL, formed up to 44% of the peripheral blood lymphocyte compartment in IM patients [3]. After resolution of acute infection, a high number of EBV-specific memory $CD8^+$ T cells are still detected against lytic antigens, but in time these were shown to decrease more than those towards latent proteins. As the latent-antigen responses are less expanded during the primary phase, the specific $CD8^+$ T cells against lytic antigens remain more abundant in the memory phase [18].

$CD4^+$ T cells are both indispensable for priming $CD8^+$ T cells and for keeping $CD8^+$ T-cell memory. However, the role of $CD4^+$ T cells is much less well studied than $CD8^+$ T cells. Lack of perturbation of Vbeta usage suggested that $CD4^+$ T-cell responses are lower than $CD8^+$ T-cell responses[27]. During acute infection EBV-specific T cells can be detected by intracellular cytokine staining directly ex vivo. $CD4^+$ T-cell responses against both BZLF1 and EBNA-1 were detected during primary infection [26], although during latency $CD4^+$ T cells remain below the detection limit. In an acute IM patient, followed after kidney transplantation, the $CD4^+$ T-cell response spiked just prior to the specific $CD8^+$ T-cell response. This was seen after the viral load increased, but prior to the onset of IM symptoms [28]. In another study, $CD4^+$ T cells could be measured after resolution of acute infection. The response was shown to be dominant against EBNA-1 and, although less extensive, to EBNA3C[29]. Still, the level of detected $CD4^+$ T cells remained quite low[30].

Thus, whilst $CD8^+$ T cells show a preference for lytic proteins early during infection, $CD4^+$ T cells recognize both latent and lytic proteins. Precopio *et al* suggest that the difference in $CD4^+$- and $CD8^+$ T-cell recognition in primary infection is due to differences in availability to processing pathways or antigen presenting cells and not due to differences in the time span in which the proteins are expressed. A large contrast was found between the responses against BZLF1. This response declined rapidly for $CD4^+$ T cells after infection and increased for $CD8^+$ T cells, remaining detectable during latency[26].

$CD4^+$ T-cell responses during the latent EBV infection phase are yet to be clarified, both in the role of $CD4^+$ effector function as helper function. Long *et al* have investigated the capacity of $CD4^+$ T cells to recognize infected B cells and function as effectors similar to $CD8^+$ cytotoxic T cells in vitro. Several CD4 epitopes were identified for EBNA-1,-2-3A and -3C. $CD4^+$ T-cell effector function is only demonstrated against a minority of peptides which are most abundantly presented[31]. Recently, $CD4^+$ T-cell responses against late lytic (structural) antigens of EBV were shown to be easily detected, differently from $CD8^+$ T-cell responses against these antigens which are very weak, suggesting that these structural antigen-specific responses are the ones to look for during primary infection [32]. The role of specific $CD4^+$ T-cell in the function of $CD8^+$ T cells could help to understand the immunesurveillance of EBV. New techniques to stimulate and expand these specific $CD4^+$ T cells have been developed by Piriou *et al* [33]. Using EBNA-1 and BZLF1 overlapping peptide pools specific $CD4^+$ T cells were readily detected in latently infected healthy individuals. EBNA-1 specific $CD4^+$ T cells were detected in all healthy donors, BZLF1 specific $CD4^+$ T cells were detected in 9 out 13 healthy donors. Also EBNA-1 specific T cells were more abundant in the total lymphocyte pool than BLZF1 specific T cells [33].

In summary, the EBV gene repertoire which is expressed is based on the location and phase of the infected B cell and leads to an equilibrium between virus-infected cells and the immune system, through cytotoxic T-cell (CTL) responses,. In this review we will focus on the consequences when the dynamic equilibrium between infection of B cells and immunesurveillance by T cells is disturbed.

POST TRANSPLANT LYMPHOPROLIFERATIVE DISORDERS

Due to the immunosuppressive state, transplant recipients are susceptible to viral infections post SCT. This includes both primary infections as well as reactivations of viruses which are already present. Infection or reactivation with EBV can lead to uncontrolled lymphoproliferation and subsequently to NHL. NH lymphomas in transplant recipients,

which usually are EBV driven, are referred to as post transplant lymphoproliferative disorders (PTLD). PTLD is in its turn also a collective name for all lymphoproliferative conditions occurring post solid organ or stem cell transplantation ranging from EBV driven polyclonal proliferation to monomorphic proliferation resembling diffuse B cell lymphomas [34]. The range of lymphomas which PTLD encompasses are classified in the Society of Hematopathology classification system [35,36]. Here we will solely focus on EBV associated PTLD, however it has to be noted that PTLD occurring late after transplantation can also have an EBV-unrelated origin[37]. Even though the early onset EBV-associated lymphoproliferative disease seen in post-transplant patients can present with a range of histologies, it is probably one disease presenting at different stages of evolution. However it should be treated separately from those other lymphomas (rare cases of Burkitt's Lymphoma, EBV-negative diffuse large B cell lymphomas, T-cell lymphomas) that arise occasionally in post-transplant patients, usually many years after transplantation.

Most PTLD contain EBV infected cells which display the growth programme and express all latent genes [38]. As described previously, infected cells undergoing the growth programme are therefore susceptible to CTL responses. In order for these infected B cells to become lymphomas, two events must take place. First, there must be a lack of T-cell control. Secondly, the EBV infected cell which expresses the growth programme must not be able to exit the cell cycle to become a resting B cell. Thorley-Lawson *et al.* proposes that PTLD is initiated when another B cell (not a naïve cell) is infected in the Waldeyer's ring and subsequently expresses the growth programme. This infected cell can then not exit the growth programme and due to a lack of T-cell control it will continuously proliferate, resulting in PTLD. Since this is a rare occurrence, it also explains why not all immunosuppressed patients who undergo viral infection or reactivation develop PTLD [12]. Most transplant recipients are EBV seropositive at time of transplantation and have a risk of EBV-reactivation. However, EBV seronegative recipients are at a higher risk of developing PTLD due to primary EBV infection[39]. This also explains the higher incidence of PTLD in children, where the percentage of seronegative transplant recipients is larger[40].

Stem cell transplantation

Stem cell transplantation (SCT) is indicated for several malignant and non-malignant conditions. Because SCT is associated with a high level of morbidity and mortality due to graft versus host disease (GVHD), a condition in which T cells of the graft attack cells of the donor, this often implies the use of T-cell depletion during and after transplantation. However, this T-cell depletion is a risk factor for viral reactivation which can lead to

uncontrolled lymphoproliferation. T-cell depletion is therefore the major risk factor for development of PTLD/NHL. The T-cell suppressive state can be achieved through chemotherapy and/or radiation prior to transplantation (conditioning regime) as well as the use of T-cell suppressive, as cyclosporine, or even depleting drugs like antithymocyte globulin (ATG) or anti-CD3 therapy. Several other transplant related risk factors have been identified for viral reactivation and subsequent development of PTLD, however, most of these risk factors influence the choice of immunosuppressive treatment, for example, HLA mismatched donors which indicate use of ATG[38,41].

Solid organ transplantation

In solid organ transplantation (SOT) recipients receive immunosuppressive treatment to prevent graft rejection. In these SOTs the incidence of PTLD varies between the types of organ transplanted. This can be explained by the degree of immunosuppression used which varies for different organs [42]. Interestingly, Everly *et al* suggest that the amount of lymphoid tissue transplanted, as therefore the amount of virus in the lymphoid tissue differs, increases the risk of PTLD due to the risk of primary infection[43]. The incidence of PTLD is high after small bowel transplantation and lung transplantation recipients, whereas the prevalence of PTLD in heart and liver recipients is much lower. The high incidence of PTLD after small bowel transplantation may be partly due to the fact that most patients are (EBV-seronegative) infants. The lowest incidence of PTLD was shown in renal transplantation[43]. However, since kidneys are the most abundantly transplanted organs, PTLD post renal transplantation is seen and studied most frequently[35,43].

EBV load as a determinant for PTLD

Epstein-Barr viral load is frequently used as a diagnostic tool to predict the onset of PTLD[44-49]. However, the role of EB viral load alone as a diagnostic tool to predict onset of PTLD and need for pre-emptive therapy is controversial, as well as the DNA source which can be whole blood, plasma or PBMCs. Several studies describe the correlation between occurrence of PTLD and EBV load level both in stem cell transplantation [45,48,49] and solid organ transplantation[50,46,47]. On the other hand, Oertel *et al*. showed that EBV load level does not correlate with PTLD treatment in patients that received a solid organ transplant (SOT) and therefore cannot be used to predict PTLD relapse [51]. Furthermore, the source of material for EBV-DNA PCR is disputable. EBV-DNA can be measured in PBMCs (peripheral blood mononuclear cells), whole blood samples or in plasma. Sensitive assays can detect EBV DNA in PBMCs of some healthy carriers. Loads above the normal range in at-risk patients will, in most circumstances,

reflect latent virus infection of the B cells and occasionally a lytically infected cell in an immunocompromised individual. EBV genomes are never amplifiable above the detection limit from plasma in healthy individuals. In patients who have already developed PTLD, genome signals in plasma could come from viral DNA shed from dying PTLD cells, as in nasopharyngeal carcinoma patients. In tumour-free individuals DNA in plasma could have been released from sites of virus replication or even from the site of growth-transformation in the oesopharyngeal lymphoid tissues. Such plasma signals are seen transiently in very acute mononucleosis patients but the source is still not clear. Total blood viral load is a composite of viral load in PBMCs and plasma and therefore one has to be careful interpreting these data. The preferred source is discussed in several studies and may play a role in predictive impact[52,53,54].

The viral load level which implies onset of PTLD and subsequent induction of pre-emptive therapy is debated. Stevens *et al.* depict a viral cut-off of 2000 EBV genome equivalents (geq)/ml. in whole blood. In their study, 78% of PTLD patients showed higher Epstein-Barr viral load levels than 2000 geq/ml, whilst in non-PTLD patients, only 3,4% exceeded this viral load cut-off level[47]. Pre-emptive therapy to prevent EBV associated lymphoproliferative disease (LPD) is discussed by Gruhn *et al*. They suggest that an increase of EB viral load greater than 10^4 EBV genome copies/ 10^5 PBMC predicts EBV-LPD risk and indicates pre-emptive therapy[55]. Van Esser *et al* denominate EB viral reactivations measured in plasma exceeding 1000 geq/ml as high risk for development of PTLD and indication for pre-emptive therapy[48,49]. They show a positive predicative value of 38% when using 1000 geq/ml as a determinant for PTLD. Thus, EBV load can be a useful tool to determine whether patients are at risk for development of PTLD and may indicate the need of pre-emptive therapy. Since the threshold of viral copies as well as the EBV-DNA source is debatable, determination of other factors that could influence PTLD development, like T-cell reconstitution data could provide necessary additional tools.

General and EBV-specific immune reconstitution post transplantation

A number of patients are unnecessarily treated when diagnosis is solely based on EB viral load[56,49]. As described above, the threshold of EBV viral load level as an indicator for therapy is also heavily debated. Therefore several studies have investigated the beneficial value of T-cell reconstitution data in combination with viral load to predict incidence and outcome of PTLD as well as indication of therapy for both solid organ transplantation and stem cell transplantation. Various studies have described the general T-cell reconstitution post solid organ and stem cell transplantation. Here we describe some recent findings.

The EBV-specific- and absolute- $CD4^+$ /$CD8^+$ T-cell numbers in PTLD patients following SOT show a significantly lower absolute $CD4^+$ T-cell count compared to patients not developing PTLD, while absolute $CD8^+$ T-cell counts varied. This suggested that absolute $CD4^+$ T-cell levels post transplantation could indicate PTLD risk[57]. However, more studies into absolute $CD4^+$ T-cell counts post transplantation are needed to substantiate these data further. Absolute total T-cell reconstitution data were recently shown to contribute to identifying patients at risk for PTLD. A $CD3^+$ T-cell count during initial EBV reactivation of at least 300 cells/µl was shown to be a good threshold to determine whether a patient, together with viral load data, requires pre-emptive therapy[56]. Furthermore, the ratio between viral load and EBV-specific T lymphocytes, as determined by enzyme-linked immunospot assay (ELIspot), was proposed to be a measure to calculate the risk for PTLD post SOT. This ratio has been shown to be of prognostic value especially in EBV seronegative recipients[58]. However, only functional aspects of the T cells can be determined by ELIspot. Other studies have investigated immune reconstitution post SCT using EBV-specific MHC class I tetramers. In SCT recipients, viral load data was combined with absolute T-cell numbers and EBV-specific $CD8^+$ T-cell numbers to determine the need for therapy for PTLD. No therapy was required for patients who had detectable EBV-specific $CD8^+$ T cells concomitant with detectable EBV-DNA, since they could develop an efficient immune response[59]. In another study, undetectable EBV-specific $CD8^+$ T-cell levels post SCT have shown to be a risk for EBV reactivation and PTLD. If EBV-specific $CD8^+$ T cells reach insufficient levels during six months post SCT, patients were at high risk of EBV reactivation [60]. Using EBV specific $CD8^+$ T cells numbers in combination with viral load increases the positive predictive value from 38%[48], using viral load threshold of 1000 geq/ml, to 100% when combining undetectable EBV-specific $CD8^+$ T cells (<0,5 cells /µL) and viral load (>1000 geq/ml) [60].

Thus, a number of studies have quantitatively investigated T-cell reconstitution after both solid organ and stem cell transplantation of which we have discussed several above. Similar to the discussion on which EBV viral load threshold treatment initiation should be based, also with respect to the T-cell compartment still additional data are required to define such a threshold. However, instead of quantitative aspects, as the number of EBV-specific T cells, qualitative aspects, as the functionality of these EBV-specific $CD8^+$ T cells could play a role in development of PTLD. Studies investigating the functional aspects of these EBV-specific $CD8^+$ T cells are yet to be performed.

CONTROL OF EBV-RELATED MALIGNANCIES IN HIV-INFECTION

The incidence of EBV-related malignancies is considerably increased in untreated HIV-infected individuals, due to a combination of factors that can lead to uncontrolled outgrowth of EBV-transformed B cells. Most of these NHL (70%) are Diffuse large cell lymphoma (DLCL) [61,62]. These DLCL are mostly systemic or primary central nervous system lymphomas (PCNSL), of monoclonal B-cell origin[61,62,63] and are EBV positive in 70 to 80% of cases [64,65,66]. Although Burkitt Lymphomas are also increased in HIV^+ individuals, these often occur much earlier and may result from chronic B cell stimulation during primary HIV infection. Moreover, these BL are mostly EBV-negative.

An elevated EBV load is not predictive of malignancies in HIV+ subjects

As indicated in the previous section, it has been demonstrated that both in solid organ [44-47], as well as in bone-marrow transplanted subjects [48,49], the number of EBV-infected cells and/or EBV copies in a particular volume of blood, is useful for discriminating patients at risk of developing EBV-related malignancies. In contrast, in HIV-infected individuals, EBV viral load is often elevated, independent of the degree of overall immune deficiency and absolute height of EBV load does not discriminate patients at risk from those that will not develop an EBV-related malignancy [67,68]. Furthermore, EBV-specific $CD8^+$ T-cell responses are relatively preserved in asymptomatic HIV carriers [69,70,67].

At the individual level we recently showed that EBV load increased within the first year following HIV infection, whereas $CD8^+$ T-cell responses were maintained at pre-HIV seroconversion levels, suggesting that EBV load could dramatically increase despite the presence of an EBV-specific $CD8^+$ T-cell response [71]. This implies that the pathogenesis of EBV-related lymphoma is quite different in HIV-infected subjects compared to transplant recipients, in whom iatrogenic suppression of EBV-specific immunity can directly allow uncontrolled outgrowth of EBV-infected B-cells, and thus an increase in EBV load directly reflects uncontrolled outgrowth of EBV-infected B cells. We postulated that in HIV-infected subjects the development of malignancies results from a gradual degradation of EBV-specific immunity, together with chronic stimulation of B cells [72,73,70,67]. Thus, in HIV infection an elevated EBV load reflects chronic stimulation of B cells, leading to EBV-reactivation, and can occur in the presence of a strong EBV-specific $CD8^+$ T-cell response.

There are several indications that HIV may induce more frequent reactivation of EBV. HIV infected individuals show greater EBV replication in the oropharynx which is clinically presented as oral hairly leukoplakia lesions [74]. Two studies by Doisne *et al.* and Pap-

agno *et al.* report increased EBV-specific $CD8^+$ T cells during acute HIV infection [75,76]. In addition, we observed an increase in $CD8^+$ T-cell numbers specific for the early lytic switch protein BZLF1 following HIV-seroconversion [71]. Increased levels of antibodies to lytic EBV antigens in HIV-infected individuals also suggest a more active EBV infection [77,78]. Chronic hyper immune activation is one of the hallmarks of HIV infection and the level of several immune activation markers are highly predictive of HIV progression [79,80,81]. In addition to physical loss of $CD4^+$ T cells, their responsiveness to polyclonal and specific stimuli *in vitro* is altered [82,83]. Similarly normal B-cell biology is altered, leading to a decline in the fraction of memory B cells [84] and poor responsiveness to T-cell help and stimulation *in vitro* [82,85-89]. As EBV is strictly dependent on the B-cell biology of its host [17], we hypothesized that increased stimulation of B cells might be an important factor leading to higher levels of EBV DNA in PBMC. Indeed, we found that EBV load was more elevated in individuals with a higher degree of immune activation, and increased over time in parallel with immune activation[90]. Most interestingly, inter-individual differences in EBV setpoint before HIV infection [91] were conserved after HIV seroconversion and during chronic HIV, even after antiretroviral treatment. This suggests that immune activation leads to an increase in the number of EBV-infected B cells, resulting in a pool of EBV-infected B cells which is proportional to the number of EBV-infected B cells before HIV seroconversion (as schematized in figure 2A). An important alteration of the regulation of B-cell memory generation and more frequent stimulation of these cells is very likely to affect the normal life cycle of EBV, which may lead to an increase in the number of EBV-infected B cells. Thus, a new EBV setpoint is attained after HIV

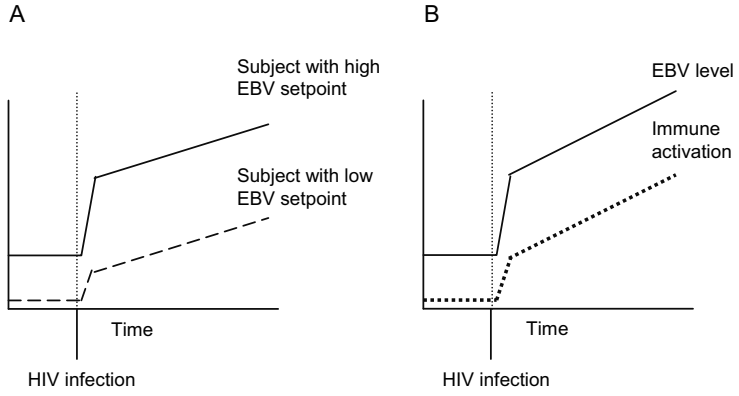

Figure 2. Changes in EBV DNA level after HIV infection

A) An individual with a low EBV setpoint before HIV infection (indicated by a dashed line) will also have a lower setpoint after HIV infection, compared to an individual with a higher EBV setpoint (solid line).

B) After HIV seroconversion, EBV load (solid line) increases due to increased immune activation (dotted line), and continues to rise during HIV infection, in parallel with the level of immune activation.

infection, consisting of a higher EBV load together with a maintained EBV-specific $CD8^+$ T-cell response, whereby the relative differences in EBV level between subjects are conserved. Importantly, these inter-individual differences – whether intrinsic differences in EBV-specific immune control, chronic immune activation, virulence of the particular EBV strain(s) carried or any other individual factor - could mask the predictive value of EBV DNA load[90].

EBV-specific immunity in HIV-infected individuals

EBV-specific $CD8^+$ T-cell numbers are easily detected in asymptomatic HIV carriers[69,70], although their function as measured by the ability to produce IFNγ is lower than in healthy EBV carriers [67]. In HIV-infected subjects progressing to EBV positive NHL, a loss of EBV-specific $CD8^+$ T-cell precursors was found [70]. More recently, a loss of EBV-specific $CD8^+$ T-cell function (using *ex vivo* IFNγ production upon stimulation with EBV peptides as a read-out) was observed, whereas EBV-specific $CD8^+$ T-cell numbers (measured using HLA peptide tetramers) were preserved [67]. Loss of functional EBV-specific $CD8^+$ T-cell function correlated with total $CD4^+$ T-cell numbers, suggesting that a loss of (specific) $CD4^+$ T-cell help might play a role in progression towards EBV-related NHL. Evidence for the importance of specific $CD4^+$ T-cell help to control human viral infections is accumulating [92,93,94,95,96] and it is very likely that during and after the acute HIV infection, $CD4^+$ T-cell help is needed for expansion of EBV-specific $CD8^+$ T cells. A possible mechanism of help is the secretion of cytokines such as IL-2 [97]. Another possibility is that during chronic infection, new naïve $CD8^+$ T-cell precursors have to be primed regularly in order to maintain a sufficient pool of effective $CD8^+$ T cells. Especially during chronic stimulation of EBV-specific $CD8^+$ T cells in HIV-infected subjects this may require regular recruitment of new T-cell responses from the naïve pool.

Using a recently described expansion assay which depends on the precursor frequency of specific cells and on their ability to proliferate in response to antigen, and exert effector functions after antigen-driven expansion [33], we found a decline in EBV-specific $CD4^+$ T cells – especially CD4+ T cells directed against the latent antigen EBNA1 - in HIV-infected subjects [98]. Furthermore, progressors to NHL suffered the most severe loss of both EBNA1-specific $CD4^+$ and $CD8^+$ T cells, whereas these cells were relatively preserved in a number of progressors to other AIDS-defining events and in slow progressors. In contrast T cells recognizing the lytic antigen BZLF-1 were more stable during HIV-infection, and even increased in a longitudinal follow-up of HIV-infected individuals [99]. This suggests that EBNA1-specific T cells might be important for control of EBV-infected B cells. This may be related to recognition of EBNA1-expressing B cells, but could also reflect the function of latent antigen-specific T cells in general. In contrast, lytic antigen-specific T

cells might be driven by B cells and epithelial cells expressing lytic EBV antigens, and be a reflection of more frequent EBV-reactivation and more important in controlling viral spread, but not the development of EBV-transformed malignant B-cell clones. In line with this highly active anti-retroviral therapy (HAART) was shown to lead to recovery of EBNA1-specific $CD4^+$ T cells, whereas BZLF1-specific T cells decreased after HAART [99]. This suggests that latent antigen-specific T-cell responses are restored in parallel with polyclonal proliferative capacity and lytic antigen-specific T-cell numbers decline due to a decreased rate of stimulation (figure 3). Further studies should shed light on the generality of these findings for one latent and one lytic antigen to the whole scala of latent and lytic antigens. With the use of this new assay and the insights from these studies in HIV-infected individuals, a more complete picture could be generated in the future for EBV control in transplant recipients. In this situation EBV-specific T-cell immunity, especially $CD4^+$ T cells, has not been studied in this much detail yet.

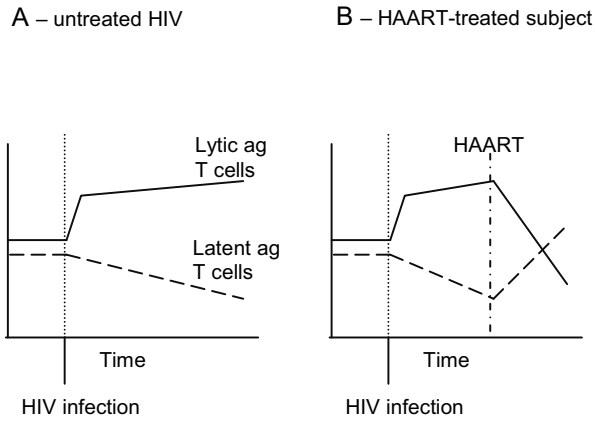

Figure 3. Differential effect of HIV-infection and HAART on EBV latent and lytic antigen-specific T-cell responses.

A) After HIV seroconversion, an increased level of EBV reactivation induces an increased immune response to lytic EBV antigens. This is not observed for responses to latent antigens, which slowly decline during HIV infection. B) When HAART is applied, latent antigen-specific responses are restored, together with a restoration of general proliferative capacity. Lytic antigen-specific responses decline, presumably due to a decreased rate of EBV reactivation.

CONCLUSION

EBV infection in healthy individuals consists of a very tightly regulated equilibrium between EBV and the host immune system. When either of these components is disturbed,

as in transplant patients where the host immune system is altered or in HIV-infection where both the normal B cell biology as well as the host immune system is disturbed, NHL can occur. Immunesurveillance of EBV in healthy individuals is mostly controlled by T cells, with a distinct role for $CD8^+$ T cells in both acute infection and latent infection. The role of $CD4^+$ T cells in acute infection has been established, however, the role of $CD4^+$ T cells in latency is yet to be thoroughly investigated. Sebelin-Wulf *et al.* suggest that frequency and function of circulating EBV-specific $CD8^+$ T cells are dependent on absolute $CD4^+$ T-cell counts, implicating an important role for the $CD4^+$ T cells in priming $CD8^+$ T-cell responses[57]. Future studies should reveal the importance of CD4+ T-cell responses in the reconstitution of functional EBV immunity after solid organ and stem cell transplantation.

Besides the CTL response, there might be a role for NK cells in controlling EBV. Williams *et al* describe a role for NK cells in the control of EBV during IM. A high NK cell response is associated with a lower viral load and high levels of $CD56^{bright}$ cells increase symptoms of IM by stimulating EBV specific CTL responses [100]. However, in control of persistent EBV infection the importance of NK cells is questionable. In immunocompromised patients post stem cell transplantation PTLD occurs several months after transplantation, when the NK cell compartment has reconstituted whilst the T-cell compartment is still deficient[101], suggesting that the EBV control by NK cells does not play a role in PTLD development. However, studies investigating the role of NK cells in PTLD development have not been performed yet.

Both components of the EBV equilibrium are disturbed in HIV infection. First, more frequent stimulation of B cells and alterations in cytokine levels will lead to more frequent reactivation of EBV from B cells. In response to this altered EBV-B-cell biology, EBV-specific $CD8^+$ T cells are initially able to adapt and control this new situation early in HIV infection. This results in an altered EBV equilibrium, which is maintained as long as functional and physical exhaustion of $CD8^+$ T cells is avoided by the presence of specific $CD4^+$ T-cell help. Over time however, both the overall loss of $CD4^+$ T-cell numbers and function, and antigen-specific $CD4^+$ T-cell exhaustion, will lead to a loss of $CD4^+$ T-cell help, and ultimately of EBV-specific $CD8^+$ T-cell function. This will allow malignant outgrowth of EBV-infected B cells.

The nature of EBV infection in HIV-positive subjects is very different from that in transplanted individuals, in whom (re-)appearance of EBV-specific $CD8^+$ T cells immediately leads to a decrease in EBV load, which is most probably due to the clearance of all EBV-infected B cells with aberrant expression of EBV antigens.

Therapy indicated for prevention of PTLD or treatment of PTLD is different between SOT recipients and SCT recipients. SOT patients are usually treated for PTLD by lowering the immunosuppressive drugs which mostly restores the immune response against EBV. However, SCT patients are conditioned with radiation or chemotherapy, which destroys most of their immune system prior to transplantation, requiring a whole new immune system to be developed. Prevention and treatment of PTLD in SCT patients is therefore based on restoring the T-cell function, reducing the B-cell mass and targeting EBV[38]. As infusion of donor lymphocytes may additionally increase risk for GvHD, future therapeutic strategies may involve infusion of EBV-specific (CD4+ and CD8+) T cells to avoid GvHD. A phase II multicenter clinical trial using allogeneic CTLs has recently been performed, overcoming the need for CTL-cell lines to be grown for each individual patient and may be promising in the future[31].

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Chapter 3

Decreased functional CD4$^+$ T-cell and CD8$^+$ T-cell reconstitution in allogeneic stem cell transplantation recipients with high-level EBV reactivation

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ABSTRACT

In this study we investigated the contribution of Epstein-Barr virus (EBV)-specific $CD4^+$ T cells in controlling EBV reactivation after allogeneic stem cell transplantation (SCT). Because of low numbers of circulating EBV-specific $CD4^+$ T cells early after SCT we used a 12-day expansion assay to retrospectively analyse $CD4^+$ and $CD8^+$ T-cell responses against a lytic (BZLF-1) and a latent (EBNA-1) EBV protein in 12 patients during the first year after SCT. We found that functional T-cell responses after non-specific in-vitro restimulation were impaired in patients with high-level EBV reactivation. EBV-specific $CD8^+$ T-cell responses were readily detected from 2 months onward, while EBV-specific $CD4^+$ T cells remained low throughout follow-up. EBNA-1 specific T cells were only present in patients with low-level reactivations, while BZLF-1 responses were mainly detected in patients with more severe reactivations. In conclusion, functional capacity is impaired in patients with high-level EBV reactivation. Since EBV-specific $CD8^+$ T-cell responses specific for BZLF-1 are abundant in patients with EBV reactivation suggests that this response is not capable of adequate viral control.

INTRODUCTION

EBV is a widespread γ-herpes virus which is normally controlled by cytotoxic $CD8^+$ T-cell responses[1;2]. Reactivations of EBV, which may progress to EBV-associated post transplant lymphoproliferative disorders (EBV-PTLD), are a rare but serious complication in recipients of allogeneic stem cell transplants. EBV-PTLD can present with varying clinical symptoms and signs, ranging from fever to a mononucleosis-like illness with fever, pancytopenia and lymphadenopathy to a rapidly progressive lymphoma. Early diagnosis and treatment is considered crucial in order to prevent EBV-PTLD related mortality. To that end, early diagnosis based on EBV DNA monitoring has been shown important, since the presentation of EBV-PTLD can be rather non-specific [3]. Van Esser et al, however, showed that the positive predictive value of an EBV DNA level of ³ 1000 copies/mL was limited to only 39%[4]. The combined use of EBV load and EBV-specific $CD8^+$ T-cell monitoring increased the positive predictive value for development of EBV-PTLD [5].
The importance of contribution of EBV-specific $CD4^+$ T-cell reconstitution in controlling and predicting EBV infection has not been investigated in detail yet. Several studies have investigated the kinetics of EBV-specific $CD4^+$ T cells during Infectious Mononucleosis, showing an increase of EBV-specific $CD4^+$ T cells during acute infection, which was followed by a rapid decline of EBV-specific $CD4^+$ T-cell numbers after recovery. [6;7]. In solid organ transplant (SOT) patients, low $CD4^+$ T-cell counts were associated with onset of LPD. The study by Sebelin-Wulf *et al* suggests that the frequency and function of EBV-specific $CD8^+$ T cells is associated with absolute $CD4^+$ T-cell counts[8] which may implicate an important role for EBV-specific $CD4^+$ T cells in EBV-specific $CD8^+$ T-cell control. Several studies have investigated the role of specific $CD4^+$ T cells in controlling cytomegalovirus infections. Gamadia *et al* have shown that CMV-specific effector-memory $CD4^+$ T cells seem necessary for a functional $CD8^+$ T-cell response in primary infections [9]. Deficient CMV-specific $CD4^+$ T-cell immunity is associated with a high risk of viral reactivation and CMV end-organ disease in recipients of allogeneic stem cell transplantation and HIV-infected patients, respectively[10;11].

In this study we set out to investigate the role of EBV-specific $CD4^+$ T cells in controlling EBV reactivations after allogeneic SCT. Studying EBV-specific $CD4^+$ T cells directly *ex vivo*, by means of tetramer analysis among others, is hampered by the very low number of specific cells. To be able to detect virus specific $CD4^+$ T cells, a 12-day in-vitro expansion assay has been developed [12]. In addition, since $CD8^+$ T-cell numbers are diminished as well shortly after SCT, this expansion assay was used to analyse EBV-specific $CD8^+$ T-cell numbers. This expansion assay enabled us to evaluate multiple functional features of the EBV-specific T-cells (by in parallel measuring proliferative capacity as well as effector function by using IFNγ production as readout) while tetramer analysis only detects the

presence of the specific T cells. We retrospectively analysed the EBV-specific $CD4^+$ and $CD8^+$ T-cell responses against a lytic (BZLF-1) and a latent (EBNA-1) EBV protein as well as the general T-cell reconstitution in 12 allogeneic SCT patients throughout the first year after transplantation. These analyses aim to shed light on the role of EBV-specific CD4+ T cells in CD8+ T-cell recovery following allogeneic stem cell transplantation.

MATERIAL AND METHODS

Study population

Twelve allogeneic stem cell transplant recipients were retrospectively included in this study. Patients were transplanted between January 2006 and February 2008 at the department of Hematology of the University Medical Center Utrecht, Utrecht, the Netherlands. Patient selection for this study was based on several criteria. First, the patient and donor both had to be EBV seropositive prior to transplantation. Second, we excluded patients with CMV reactivation during follow up. Finally, PBMC samples had to be available at all time points during follow up. Patients were subsequently subdivided into EBV reactivation categories; no viral reactivation, low-level viral reactivation (detectable EBV DNA load not exceeding 1000 copies/ml in plasma) and high-level reactivation (EBV DNA load exceeding 1000 copies/ml) since the positive predictive value for EBV-PTLD with 1000 copies/ml has been shown to be 39% [4;5].The second selection criteria could not be met completely for the high-level reactivation group since concurrent CMV reactivation was so abundant in this group. Therefore we selected three high-level reactivating individuals with concurrent CMV reactivation. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki. Patient and transplantation related characteristics are described in table 1. All patients received a peripheral blood stem cell graft which was HLA mismatched in two cases. Three patients received a myeloablative conditioning regimen (consisting of Cyclophosphamide and 2 x 6 Gy TBI), 9 patients were treated with a non-myeloablative regimen (fludarabine and 1 x 2 Gy TBI) and 8 patients received in vivo T-cell depletion with antithymocyte globulin (ATG). Acute graft versus host disease (aGVHD) developed in 4 and chronic graft versus host disease (cGVHD) in 8 patients, which was limited in 3 and extensive in 5 cases. A relapse occurred in 4 patients (table 1).

CMV and EBV monitoring

CMV and EBV monitoring was based on a real-time TaqMan™ CMV or EBV DNA PCR assay in ethylenediaminetetra acetic acid (EDTA)-plasma[13-16], which was performed routinely

in all patients until 6 months post transplantation. Patients were treated pre-emptively with valganciclovir (900 mg twice daily) when CMV-DNA load exceeded 500 copies/ml and with Rituximab 375 mg/m^2 when EBV DNA exceeded 1000 copies/ml. Valaciclovir prophylaxis was given to all patients (500 mg twice daily). Viral infection was defined as EBV viral load exceeding the detection limit of 50 copies/ml in plasma.

Antigen-specific T-cell expansion

To expand the number of EBV-specific T-cells we cultured PBMC in the presence of EBV overlapping peptide pools and IL-2 as described before[12]. Overlapping peptide pools consisted of 15-mer peptides with an 11 amino acid overlap. We used both the entire BLZF1 proteome (59 peptides) and the immunogenic C-terminal region of EBNA-1 (57 peptides) (JPT Peptide Technologies GmbH, Berlin, Germany). Peptides were pooled at a final concentration of 1mg/ml of each peptide in DMSO. After 12 days of culture with 1 µg/ml of peptide pool, cells were rested and subsequently restimulated with either peptide pool (1µg/ml) and αCD28 (1µg/ml); or αCD28 (1µg/ml) alone as a negative control. After 1 hour, 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, Calfiornia, USA) was added and cells were incubated for an additional 5 hours. Samples were washed with phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 0.02% sodiumazide and stained for CD3-Pacific Blue (eBioscience Inc., San Diego, California, USA), CD8-PerCP (BD), and CD4-PE-Cy7 (eBioscience). After permeablisation (FACS Permeabilization Solution and FACS Lysis Solution, BD) cells were stained with IFNγ-FITC (BD). Cells were acquired on a LSR-II flow cytometer and analysed using FACSdiva software (BD). EBV-specific CD4$^+$ and CD8$^+$ T cells were determined by the percentage of IFNγ producing CD4$^+$ and CD8$^+$ T cells after stimulation with either BZLF-1 or EBNA-1. PBMC from 6 healthy individuals were analysed for normal range of BZLF-1 and EBNA specific CD4$^+$ and CD8$^+$ T cells.

Total T-cell functionality

PMA/ionomycin stimulation reflects the total capacity of a T-cell to become activated independent of TCR engagement and costimulation[18]. Therefore we used the capacity of CD4$^+$ and CD8$^+$ T cells to produce IFNγ upon PMA/ionomycin restimulation as a measure of total function T-cell reconstitution following SCT. PBMC were stimulated during 12 days in the presence of EBNA-1 or BZLF-1 peptide pools as described above. Since the total T-cell population is expected to respond to PMA/ionomycin stimulation, regardless of their specificity, we used cells of either EBNA-1 or BZLF-1 stimulated cultures to determine total T-cell functionality. After 12 days of culture cells were rested and subsequently restimulated with PMA (10ng/ml), ionomycin (2 µg/ml) and αCD28 (1µg/ml), or

αCD28 (1µg/ml) alone as a negative control. After 1 hour, 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, Calfiornia, USA) was added and cells were incubated for an additional 5 hours. Samples were stained and analyzed as described above.

Absolute T-cell numbers

To determine the absolute number of $CD3^+$, $CD4^+$ and $CD8^+$ T cells per ml whole blood, TRUcount™ tubes (BD) were used according to manufacturers protocol as described before[17]. In brief, 50µl of whole blood was incubated with CD45-PerCP and CD3-Pacific Blue CD4-PE-Cy7 and CD8-APC-Cy7 (eBioscience). Thereafter erythrocytes were lysed (BD lysisbuffer) and samples were measured on LSR-II FACS machine. At least 2000 lymphocytes were measured (identified as CD45 positive and SSC low) and analysed with FACSdiva software (BD). Besides patient material, blood samples anti-coagulated with EDTA were drawn from 15 healthy volunteers as a control for normal range of T cells.

Statistical analysis

Correlation between T-cell functionality and absolute T-cell counts was determined by Spearman correlation. Comparison of general T cell reconstitution at 1 year post SCT was determined using Mann-Whitney U-test. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA) software.

RESULTS

Patient population

Patients were selected based on the presence or absence of EBV reactivation (and sample availability) and subsequently divided into a reactivation category based on the peak EBV viral load during the first 6 months post transplantation (table 1). Four patients did not develop an EBV reactivation during the first 6 months post SCT (#1,2,3,4). Three patients, (#5,6,8) had multiple episodes of detectable EBV DNA, however, the viral load never exceeded the detection limit of 50 copies/ml. Patient #7 had a maximum viral load of 283 copies/ml. We categorized these four patients as low-level reactivations. In patients # 9,10,11 and 12 a high-risk EBV infection was seen with viral loads above 1000 copies/ml and subsequently anti-CD20 treatment at a dose of 375 mg/m^2(Rituximab, Roche) was given. One of these patients developed a PTLD (#11) (this patient is further discussed in detail below).Concurrent CMV reactivation occurred in 3 patients (#9,10 and 12).

Table I. Patient characteristics

		sex	age	disease	stem cell source	donor	HLA mismatch	Conditioning	ATG	relapse/ progression	aGVHD	cGVHD
	1	f	47,3	AML	PB	unrelated	-	NMA	-	-	II - IV	-
no	2	m	43,8	AML	PB	related	-	NMA	-	6 months	II - IV	extensive
reactivation	3	m	22,6	ALL	PB	unrelated	-	MA	yes	-	none - I	limited
	4	m	52,3	NHL	PB	related	yes	NMA	yes	-	none - I	limited
	5	f	32,1	AML	PB	unrelated	-	MA	yes	-	none - I	-
low	6	m	28,4	ALL	PB	unrelated	-	NMA	yes	-	none - I	extensive
reactivation	7	m	47,9	MM	PB	unrelated	-	NMA	yes	14 months	none - I	-
	8	m	59,3	AML	PB	unrelated	yes	NMA	yes	< 1 month	II - IV	extensive
	9	m	44,3	AML	PB	related	-	MA	-	-	none - I	extensive
high	10	m	18,8	NHL	PB	unrelated	-	NMA	yes	-	none - I	-
reactivation	11	m	57,1	NHL	PB	unrelated	-	NMA	yes	-	none - I	limited
	12	m	51,8	NHL	PB	related	-	NMA	-	< 1 month	II - IV	extensive

Abbreviations: AML, acute myeloid leukaemia; ALL, acute lymphoid leukaemia; NHL, non-Hodgkin lymphoma; MM, multiple myeloma; PB, peripheral blood; NMA, nonmyeloablative; MA, myeoloablative; ATG, anti-thymocyte globulin; aGVHD, acute graft versus host disease; cGVHD, chronic graft versus host disease

Lower T-cell functionality in patients with high-level reactivations

We investigated whether the total T-cell functionality after non-specific restimulation with PMA/ionomycin was different between patients with or without EBV reactivation. To this end, PBMC from 12 patients at 5 time points (prior to SCT and at 2,4,6 and 12 months post SCT) were stimulated with EBNA-1 or BZLF-1 peptide pools for 12 days. Representative FACS plots displaying IFNγ producing $CD4^+$ and $CD8^+$ T cells after PMA/ionomycin restimulation for 3 patients in each of the reactivation categories (without, low-level and high-level reactivation) after 12 day stimulation with BZLF-1 peptide pool are shown in figure 1. Total T-cell functionality for four patients without or with low EBV reactivation and four patients with high-level EBV reactivation are shown in figure 2a. Patients without or with low-level EBV reactivation have higher percentages of IFNγ producing $CD4^+$ (median 6.9%) and $CD8^+$ T cells (median 30.4%) throughout follow-up compared to patients with a high-level EBV reactivation ($CD4^+$ T cells; median 2.8%, range 0-21.7%, $CD8^+$ T cells; median 8.9%, range 0-23.4%). In all patients without or with a low reactivation, the percentage of IFNγ producing $CD8^+$ T cells reconstituted rapidly (median 30.4% throughout follow up; range 0-75.2%) and was higher compared to the percentage of IFNγ producing $CD4^+$ T cells (median 6.9% throughout follow up; range 0-46.2%). However, for patients with high-level reactivations the percentages of IFNγ producing $CD4^+$ and $CD8^+$ T cells remained low throughout follow up although percentages of IFNγ producing $CD8^+$ T cells were slightly higher compared to $CD4^+$ T cells. At one year post transplantation, the T-cell functionality was significantly higher in patients without or with low EBV reactivation compared to patients with high-level reactivations. A median of 16.6% of all T cells produced IFNγ upon PMA/ionomycin restimulation against only 6.0% of T cells in the high-level reactivation group (p=0.005) (figure 2b). We subsequently analysed the percentages of IFNγ production within either the $CD4^+$ or $CD8^+$ T-cell compartment and found that patients with high-level reactivations had lower percentages of IFNγ producing T-cells for both $CD4^+$ and $CD8^+$ T cells. This difference was especially evident in the BZLF-1 stimulated $CD8^+$ T cells (36.7% versus 6.9%, p 0.02) (figure 2c).

EBV reactivation despite high BZLF-1 specific T-cell responses

The EBV-specific functional T-cell reconstitution following SCT was determined by IFNγ production in $CD4^+$ and $CD8^+$ T cells after restimulation with either EBNA-1 or BZLF-1 peptide pools following expansion. Representative FACS plots of three patients in each of the reactivation categories after stimulation with BZLF-1 is shown in figure 3A. The BZLF-1 specific $CD8^+$ T-cell reconstitution after SCT occurs faster in all patients compared to the BZLF-1 reconstitution in $CD4^+$ T cells. For the three representative patients shown

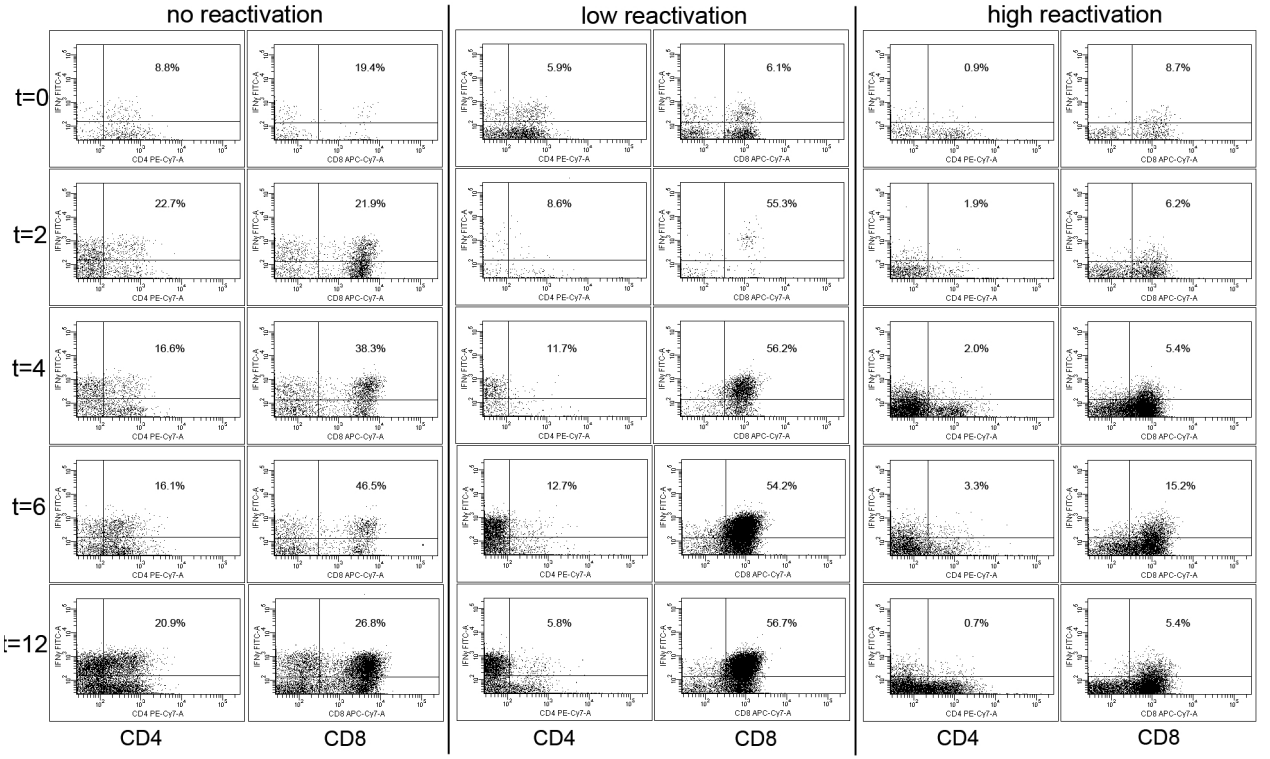

Figure 1. Representative FACS plots of total T-cell functionality

Representative FACS plots of $CD4^+$ and $CD8^+$ T cells in patients without EBV reactivation (two left columns), low EBV reactivation (two center columns) and high EBV reactivation (two right columns) throughout follow up (t=0,2,4,6 and 12). IFNγ production was measured after restimulation with PMA/ionomycin following 12 day stimulation with BZLF-1 peptide pool. FACS plots show $CD4^+$ (left panel for each patient) or $CD8^+$ (right panel for each patient) expression on x-axis and IFNγ production on y-axis. Percentages in upper right quadrant indicate the percentage of IFNγ producing $CD4^+$ or $CD8^+$ T cells.

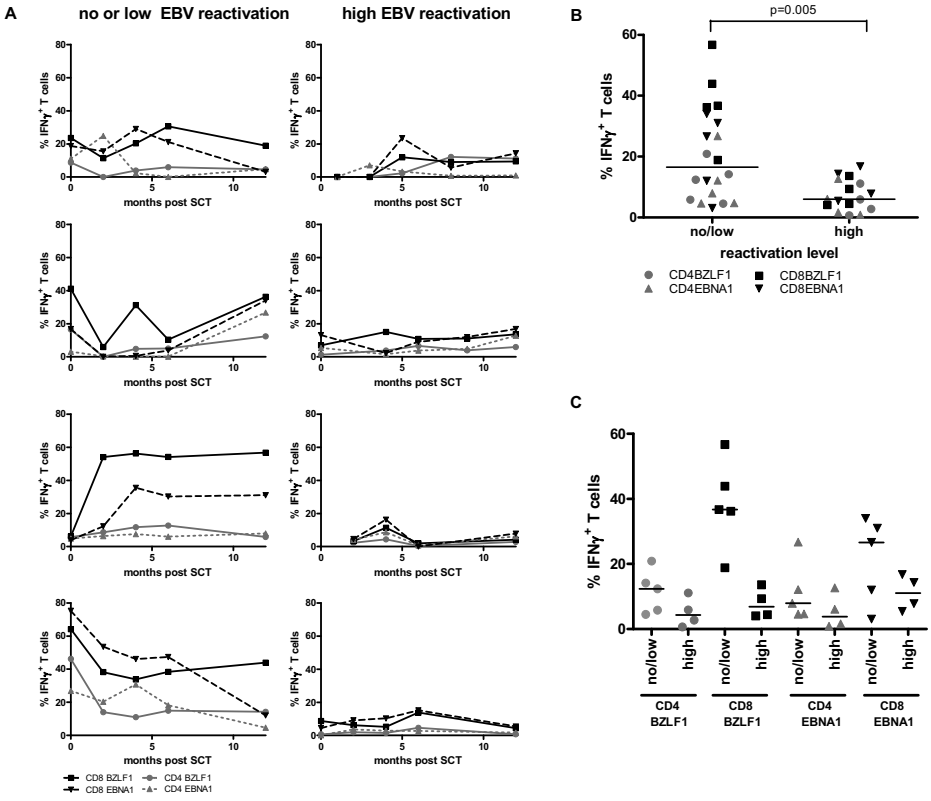

Figure 2. T-cell functionality is impaired in patients with high-level EBV reactivations

A) Representative graphs of 4 patients without or with low EBV reactivation (left panel) and 4 patients with high-level reactivation (right panel). %IFNγ CD4$^+$ or CD8$^+$ T cells after PMA/ionomycin restimulation following BZLF-1 or EBNA-1 stimulation are depicted on y-axis. Months post SCT is depicted on x-axis.

B) Percentages of IFNγ$^+$ T cells at 1 year post SCT. Median percentages of IFNγ$^+$ T cells are significantly higher 1 year post SCT in patient without or with a low viral reactivation (black squares: BZLF-1 stimulated CD8$^+$ T cells; black triangles: EBNA-1 stimulated CD8$^+$ T cells; grey circles: BZLF-1 stimulated CD4$^+$ T cells; grey triangles: EBNA-1 stimulated CD4$^+$ T cells) (p=0.005).

C) Percentages of IFNγ$^+$ T cells at 1 year post SCT subdivided over the different peptide pools stimuli and CD4$^+$/CD8$^+$ T cells.

in figure 3A, levels of BZLF-1 specific CD8$^+$ T cells were detectable as early as 2 months post SCT and remained detectable throughout follow up, while BZLF-1 specific CD4$^+$ T cells remained undetectable until 12 months post SCT. Also, the EBNA-1 specific CD8$^+$ T cells reconstituted before the CD4$^+$ T cells. Comparison of BZLF-1 and EBNA-1 reconstitution for the 3 representative patients is shown in figure 3b. Interestingly, the representative patient without EBV reactivation developed EBNA-1 specific CD8$^+$ T cells

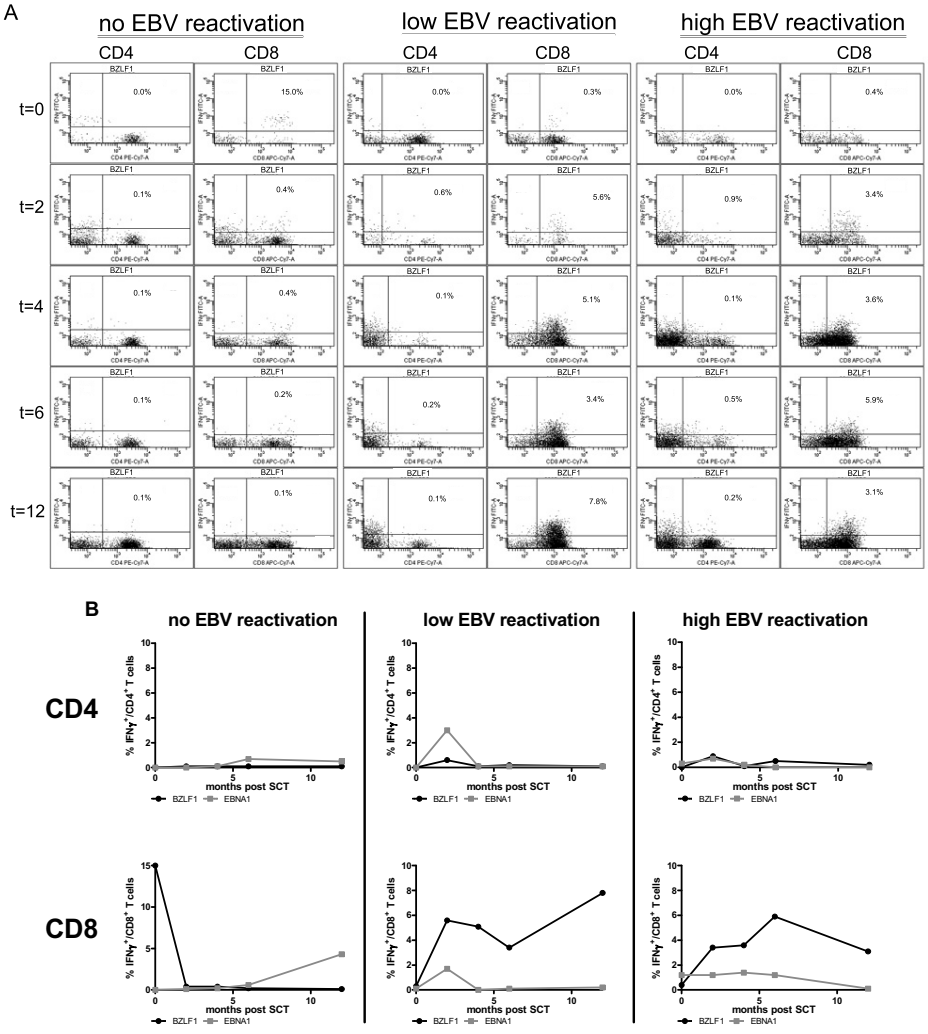

Figure 3. A) Impaired EBNA-1 specific although high BZLF-1 specific T-cell responses in EBV reactivations Representative FACS plots of $CD4^+$ and $CD8^+$ T cells for 3 patients (without EBV reactivation (two left columns), low EBV reactivation (two center columns) and high EBV reactivation (two right columns) throughout follow up (t=0,2,4,6 and 12). IFNγ production was measured after BZLF-1 peptide pool stimulation. FACS plots show $CD4^+$ (left panel for each patient) or $CD8^+$ (right panel for each patient) expression on x-axis and IFNγ production on y-axis.

B) Representative graphs of IFNγ production following either BZLF-1 or EBNA-1 stimulation for 3 patients (without, low and high EBV reactivation). Top 3 graphs show IFNγ production by $CD4^+$ T cells after EBNA-1 (grey lines) or BZLF-1 (black lines) stimulation (y-axis) and months post SCT on x-axis. Bottom 3 graphs show IFNγ production by $CD8^+$ T cells after EBNA-1 (grey lines) or BZLF-1 (black lines) stimulation (y-axis) and months post SCT on x-axis.

1 year post SCT, while BZLF-1 levels remain undetectable. This is in contrast to the low and high-reactivating patients, which developed a high proportion of BZLF-1 specific $CD8^+$ T-cell responses early after SCT that remained high throughout follow-up.

EBV-specific T cells before and one year after SCT are lower compared to healthy individuals

We compared the magnitude of the EBV-specific T-cell responses prior to transplantion to a cohort of healthy controls (n=6), as the condition of the patients prior to SCT (either due to their haematological disease , prior treatments and/or prior conditioning regime) could influence their (EBV-specific) immune responses. All healthy individuals showed responses against BZFL-1 and EBNA-1 (figure 4a). IFNγ producing $CD4^+$ and $CD8^+$ T cells were slightly lower in our patient group compared to the healthy controls. However, this was only significant for $CD4^+$ T-cell responses against EBNA-1 (median HC 3.0% (range 0.56-5.00%), median patient group 0.35% (range 0.0-2.1%) p=0.03).

To determine next whether our patients reconstitute their (EBV-specific) immune responses to 'normal healthy' levels one year post SCT, we compared IFNγ producing $CD4^+$ and $CD8^+$ T-cell levels 1 year post SCT to our healthy controls (figure 4a). At 1 year post SCT, there was considerable variation in the range of IFNγ producing $CD4^+$ and $CD8^+$ T cells following EBNA-1 or BZLF-1 stimulation. The median percentages in SCT recipients were overall lower compared to healthy controls (0.50% versus 2.97% for EBNA-1 specific $CD4^+$, 0.30% versus 0.43% for EBNA-1 specific $CD8^+$ and 0.10% versus 0.51% and 1.95 versus 4.03% for BZLF-1 specific $CD4^+$ and $CD8^+$ T cells, respectively). This implies that EBV-specific T cells have not reconstituted to 'normal' levels 1 year post SCT. This difference was especially visible for EBNA-1 specific $CD4^+$ T cells which were significantly lower (0.50% versus 2.97%) 1 year post SCT compared to healthy controls (p 0.03).
Subsequently we analysed EBV-specific T-cell function at 1 year post SCT for each of the viral reactivation categories. While the overall percentages of functional EBV-specific T cells were lower compared to healthy individuals, we found this was mainly caused by the patients without or with low reactivations. Patients with high level reactivations had higher levels of BZLF-1 specific $CD4^+$ (median 1.9%, range 0.0-6.4%) and $CD8^+$ T cells (median 5.6%, range 0.0-158%) compared to patients without (median $CD4^+$= 0.1%, range 0.0-0.8%; median $CD8^+$=0.5%, range 0.1-20.5%) or with low (median $CD4^+$= 0.0%, range 0.0-0.1%; median $CD8^+$=0.8%, range 0.0=7.8%) reactivations (figure 4b). Unfortunately, these findings could not be statistically verified due to the small group size for each reactivation category.

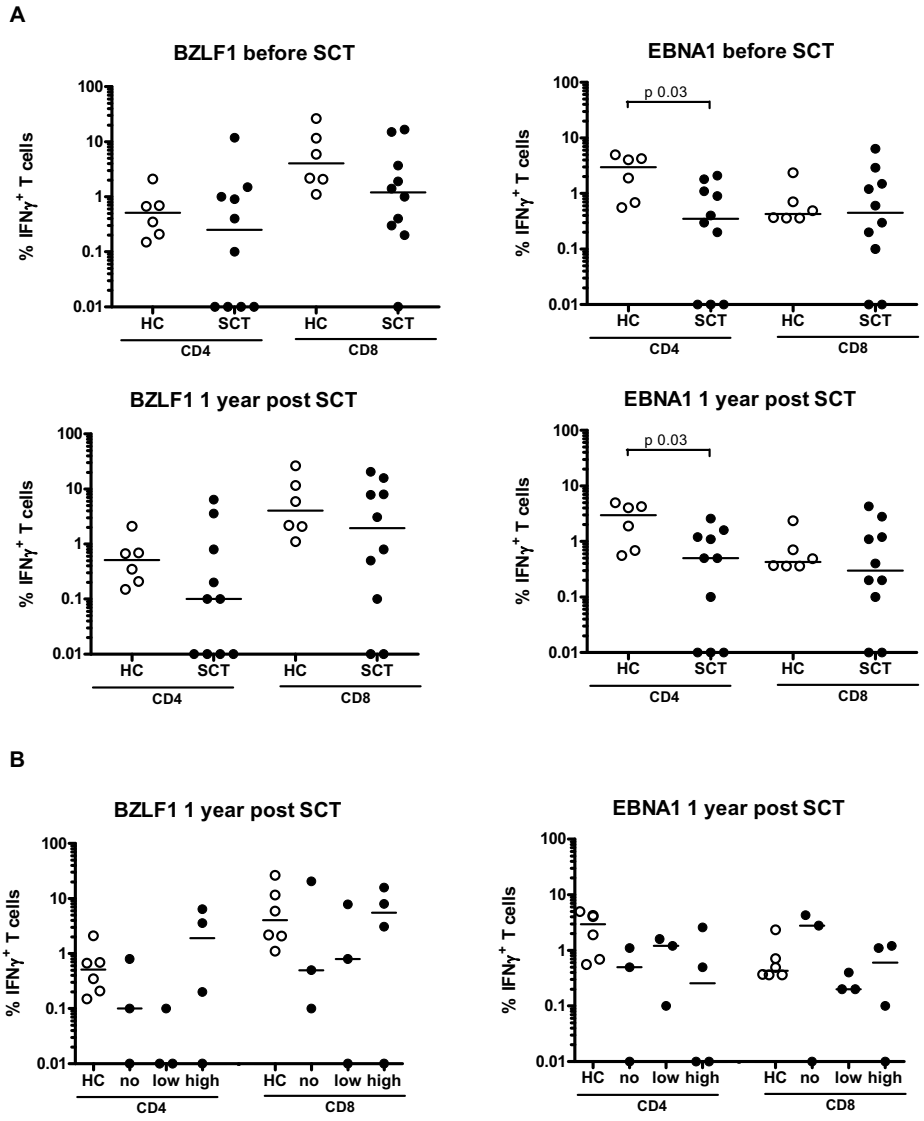

Figure 4. IFNγ producing T cells pre and post SCT

A) Dot plot comparing IFNγ production after BZLF-1 stimulation (upper left graph) or EBNA-1 stimulation (upper right graph) between healthy controls and pre SCT samples and comparing IFNγ production after BZLF-1 stimulation (lower left graph) or EBNA-1 stimulation (lower right graph) between healthy controls and 1 year post SCT samples. EBNA-1 specific $CD4^+$ T cells were impaired in patients both before and 1 year post SCT compared to healthy controls (p=0.03).

B) Dot plot comparing BZLF-1 (left graph) or EBNA-1 (right graph) 1 year post SCT for the different reactivation categories.

Post transplant lymphoproliferative disease

One patient (#11) developed a post transplant lymphoproliferative lymphoma. This 57 year old male patient was diagnosed with mantle cell lymphoma. He received a nonmyeloablative SCT from a matched unrelated donor after a conditioning regimen of antithymocyte globulin (2mg/kg/day for 4 days), fludarabin (30mg/m^2/day for 3 days) and total body irradiation (2Gy). At day 111 post SCT the first detectable EBV load of 462 copies/ml was measured, which increased to 1939 copies/ml at day 118. 127 days post SCT he presented with cervical lymphadenopathy. Histological examination of the lymphnode confirmed an EBV-PTLD Treatment consisted of rituximab (4x 375 mg/m^2) after which immunosuppressive therapy was reduced.

IFNγ producing BZLF-1 and EBNA-1 specific CD4$^+$ and CD8$^+$ T cells were undetectable prior to viral reactivation. BZLF-1 specific CD8$^+$ T cells became detectable shortly after the onset of EBV reactivation (5 months post SCT) (figure 5a) and remained detectable until 1 year post SCT. Also the percentage of BZLF-1 specific CD4$^+$ T cells increased after the onset of viral reactivation. Percentages of both EBNA-1 specific CD4$^+$ T cells and CD8$^+$ T cells remained low after viral reactivation, not exceeding 1.6% of CD4$^+$ T cells (at 8 months post SCT) and 1.1% of CD8$^+$ T cells (at 12 months post SCT). In addition we analysed the absolute number of T cells following SCT, depicted in figure 5C. All T-cell counts remain very low throughout follow-up. The CD3$^+$ T-cell count 1 year post SCT was 233 cells/µl, while the median value measured in 15 healthy individuals was 1292 cells/µl. Although the percentages of IFNγ producing CD4$^+$ and CD8$^+$ T cells increased after viral reactivation, the absolute number of T cells remained very low, resulting in a very low and therefore possibly inadequate absolute number of virus specific T cells. The poor T-cell reconstitution in this patient is confirmed by the lack of IFNγ producing CD4$^+$ and CD8$^+$ T cells after restimulation with PMA/ionomycin (figure 5d).

DISCUSSION

Generation of virus-specific CD8$^+$ T cells is dependent on CD4+ T-cell help, as has been shown for several viruses such as HIV[19;20] and CMV[9-11;21]. We studied the role of EBV-specific CD4$^+$ and CD8$^+$ T cells in controlling EBV reactivation after allogeneic stem cell transplantation. Sebelin-Wulf *et al* showed that in the setting of SOT patients the frequency and function of circulating EBV-specific CD8+ T cells were dependent on absolute CD4+ T-cell counts [8]. *Ex vivo* detection of EBV-specific CD4$^+$ T cells has been shown to be very difficult due to low numbers of IFNγ-producing EBV-specific CD4$^+$ T cells after short-term stimulation with EBV peptide pools[12]. Since T-cell counts are very low shortly after

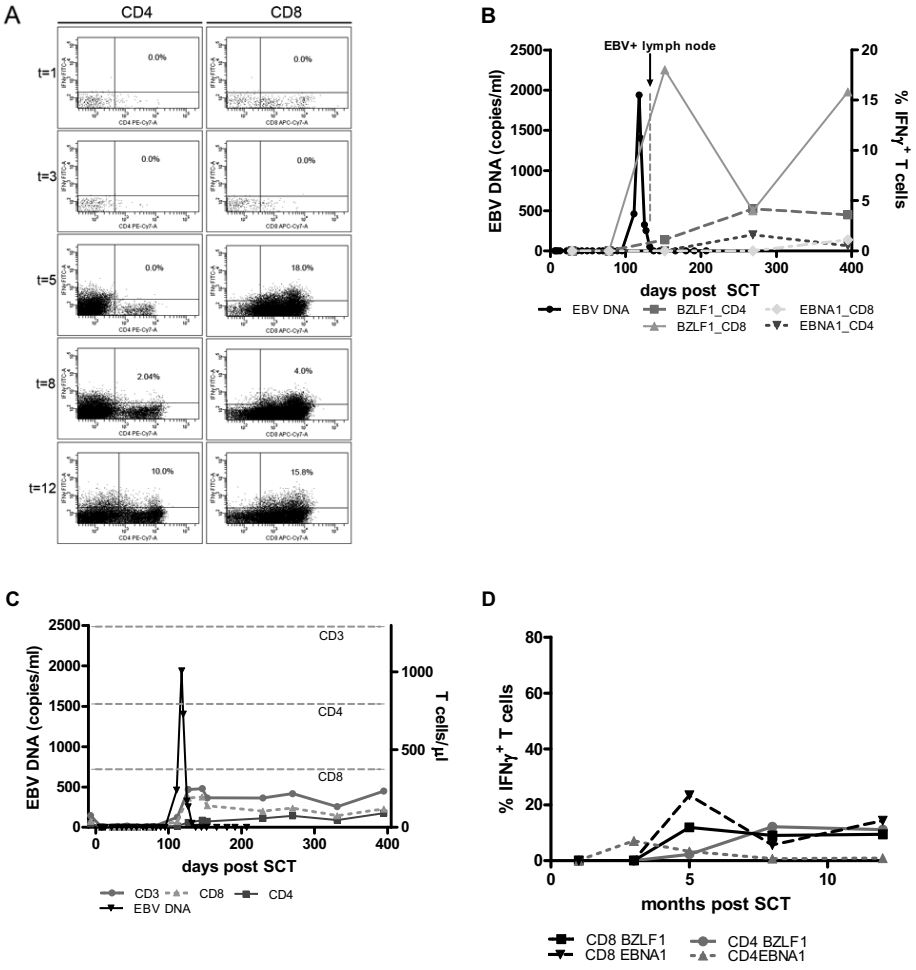

Figure 5. Patient with Post Transplantation Lymphoproliferative Disease

A) Representative FACS plot of IFNγ production after BZLF-1 stimulation throughout follow up for $CD4^+$ T cells (left panel) and $CD8^+$ T cells (right panel).

B) Graph showing the viral load (EBV DNA in copies/ml; black line, left y-axis) and the % IFNγ producing T cells (right y-axis, dark grey squares: BZLF-1 stimulated $CD4^+$ T cells; grey triangles: BZLF-1 stimulated $CD8^+$ T cells; light grey dashed line: EBNA-1 stimulated $CD8^+$ T cells; dark grey dashed line: BZLF-1 stimulated $CD4^+$ T cells) during follow up (months post SCT, x-axis).

C) Graph showing the viral load (EBV DNA in copies/ml; black line, left y-axis) and the absolute number of T cells/µl blood (right y-axis, dark grey circles, $CD3^+$ T cells; dark grey squares, $CD4^+$ T cells; light grey triangles, $CD8^+$ T cells) during follow up (months post SCT, x-axis).

D) Graph showing percentages of IFNγ producing T cells (y-axis) after restimulation with PMA/ionomycin following stimulation with BZLF-1 ($CD4^+$ T cells, grey circles; $CD8^+$ T cells, black squares) or EBNA-1 ($CD4^+$ T cells, grey triangles; $CD8^+$ T cells, black triangles) during follow up (months post SCT (x-axis).

SCT, this provides an extra hurdle in detecting EBV-specific CD4+ T-cell responses after SCT. For HIV-infected individuals an EBV-specific CD4+ T-cell detection method has been designed stimulating PBMCs during 12 days in the presence of EBV-specific overlapping peptide pools[12]. Here, we applied this technique for the first time in SCT patients to investigate both the general as well as EBV-specific CD4+ and CD8+ T-cell reconstitution. This technique has a second advantage: as opposed to tetramer staining, the detection of specific T cells is independent of HLA type and rather qualitative than quantitative.
Here we used two approaches for measuring the functional CD4+ and CD8+ T-cell responses after SCT. First, we determined total T-cell functionality by measuring IFNγ production after two week stimulation with EBV-specific peptide pools followed by restimulation with PMA/ionomycin. Second, we determined EBV-specific T-cell functionality by restimulation with the corresponding EBV-specific peptide pool. The first approach resulted in high levels of IFNγ producing T cells, especially in the CD8+ T-cell compartment. These levels are much higher compared to the IFNγ production after peptide specific restimulation suggesting that although EBV-specific CD4+ and CD8+ T cells have expanded and compose the majority of the T-cell pool, variation in avidity of the T cells results in lower percentages of IFNγ after more subtle peptide restimulation. In addition, some T cells might not have fully differentiated to the effector stage resulting in lower IFNγ production after peptide pool restimulation.

Using the expansion assay, we showed that non-specific functional T-cell reconstitution is hampered in patients with high-level viral reactivations. In all 4 high-level reactivation patients the non-specific functional T-cell reconstitution was impaired and no reconstitution was observed until 12 months post SCT. This is in line with findings by Annels *et al.* who suggested that pre-emptive intervention is necessary only in patients that lack an expansion of memory T cells during the initial phase of the reactivation[22]. The benefit of rapid general T-cell reconstitution following SCT with respect to prevention and control of viral reactivation has been stressed before[23].
In our study, BZLF-1 specific responses were predominantly detected in patients with EBV reactivation. This is in line with data from acute EBV infections where up to 44% of CD8+ T cells were shown to be specific for a BZLF-1 HLA-B8 restricted peptide RAKFKQLL[24]. EBNA-1 evades recognition by cytotoxic CD8+ T cells by preventing HLA-class I presentation due to an internal glycine-alanine (gly-ala) repeat domain[25], although, more recent studies have shown that EBNA-1 is an important target for both CD4+ and CD8+ T cells[26-28]. Our data suggest that although a BZLF-1 specific T-cell response is evoked during viral reactivation, EBNA-1 specific T cells remain low throughout follow up in all patients. However, we detected EBNA-1 specific CD4+ and CD8+ T cells more readily in patients without or with a low-level viral reactivation suggesting that EBNA-1 specific T cells could play an important role in controlling the viral reactivation. This

is supported by the finding that EBNA-1 specific T cells do not reconstitute to 'normal' values 12 months post SCT. Lack of adequate EBNA-1 specific T cell responses have been described to be associated with progression to EBV related NHL[29]. These findings are reflected in our PTLD patient, where very few EBNA-1 specific $CD8^+$ T cells were detectable throughout follow up against 18% of $CD8^+$ T cells producing IFNγ after BZLF-1 stimulation at the first time point following onset of PTLD. BZLF-1 specific T cells appear more readily in high-level reactivations and thus seem not capable of viral control. This is in line with a recent study using the 12 day expansion assay in PTLD. High $CD8^+$ T-cell responses against BZLF-1 were measured that were shown to be dominant compared to EBNA-1 specific responses[30].

The combined measurement of general T-cell functionality together with EBV-specific functionality provides an extra tool in analyzing the T-cell reconstitution following SCT. Our data suggests that sufficient general T-cell reconstitution is necessary for a robust EBV-specific T-cell response and that although EBV-specific $CD8^+$ could be detected in patients with high-level reactivations, their general T-cell reconstitution was severely hampered resulting in lack of viral control. Since all of the high-level reactivating patients were given αCD20 treatment upon viral loads exceeding 1000 copies/ml, we have no data on the percentage of high-level reactivating patients that would have been capable of clearing the reactivation themselves. However, our data do show that patients with high-level reactivations already have an altered total and EBV-specific T-cell reconstitution prior to onset of viral complications and therapeutic intervention.

The importance of a robust T-cell reconstitution has been described before. A rapid reconstitution to at least 300 $CD3^+$ T cells/μl blood was shown to distinguish between patients with viral control and patients at risk of reactivation [22]. Also, for CMV it has been recently shown that a threshold number of absolute CMV specific T cells can be used to identify patients at risk of CMV disease [31]. However, here we show that not only the number of T cells is of importance but the functional capacity, i.e. the capability of IFNγ production following general stimulation, is impaired in individuals that develop a high-level EBV reactivation.

Both total and EBV-specific $CD8^+$ T cells show a more rapid recovery compared to $CD4^+$ T cells. In patients without or with low-level reactivations the T-cell functionality was higher for $CD8^+$ T cells compared to $CD4^+$ T cells. However, in the high-level reactivating patients the functionality was similar for $CD4^+$ and $CD8^+$ T cells. Also for the EBV-specific T cells there was a difference between $CD4^+$ and $CD8^+$ T cells. In most patients EBV-specific $CD8^+$ T cells were detectable from 2 months onwards, while EBV-specific $CD4^+$ T cells remained low throughout follow up. Interestingly, absolute $CD4^+$ T-cell numbers do not

reconstitute slower in our patients compared to $CD8^+$ T cells(data not shown). This suggests that the EBV-specific functional $CD8^+$ T-cell response is more important in controlling an EBV reactivation than the $CD4^+$ T-cell response. However, in our cohort there was a high incidence of non-myeloablative conditioning and in-vivo T-cell depletion through ATG, which have both been described to impair EBV-specific $CD8^+$ T-cell reconstitution post SCT[5;32]. This in combination with the low number of patients included in this study requires our findings to be verified in a larger patient cohort which enables correction of the data for transplantation associated factors as conditioning regimen.

In conclusion, T-cell functionality is impaired in patients with high-level reactivations. EBV-specific $CD8^+$ T cells responses can be detected as early as 2 months post SCT while $CD4^+$ T cells responses remain low throughout follow up. EBV-specific $CD8^+$ T-cell responses are mostly aimed at BZLF-1 in patients with high-level reactivations suggesting that despite a dominant EBV-specific $CD8^+$ T-cell response viral reactivation occurred.

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Chapter 4

Adequate control of primary EBV infection and subsequent reactivations after cardiac transplantation in an EBV seronegative patient

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Submitted

ABSTRACT

EBV seronegative recipients of cardiac transplantation are at risk for development of post transplant lymphoproliferative disease following primary EBV infection due to the ongoing treatment with immunosuppressive drugs. Here we present detailed kinetics of the EBV-specific T-cell response following cardiac transplantation in an EBV seronegative recipient who developed a primary EBV infection 15 weeks post transplantation and subsequent viral reactivations throughout follow up. The patient developed an EBV-specific $CD8^+$ T-cell response within 24 days after first detection of the primary infection. Subsequently, an increased EBV-specific $CD8^+$ T-cell response developed upon viral reactivation, indicated by a threefold increase of EBV-specific $CD8^+$ T cells and increased IFNy production after stimulation with EBV-specific peptide pools. These data indicate that an EBV-specific T-cell response capable of adequate control of a primary EBV-infection and subsequent viral reactivations can develop in an EBV seronegative cardiac transplant recipient in the presence of severe immunosuppression.

INTRODUCTION

Epstein-Barr virus is a widespread gamma herpes virus which infects over 90% of the human population during life. Infection usually occurs during childhood where it has an asymptomatic course, however when infection is delayed until adolescence it can induce infectious mononucleosis[1,2]. After primary infection EBV persists as a lifelong latent infection in the memory B cell compartment[3]. The common understanding is that EBV infection is mainly controlled by cytotoxic $CD8^+$ T cells[1,4] establishing a lifelong equilibrium between virus-infected cells and the immune response. Disturbance of this equilibrium in immunocompromised hosts (i.e. cardiac transplant recipients) can lead to uncontrolled lymphoproliferation and subsequent post transplant lymphoproliferative disease (PTLD). The primary risk factor for development of PTLD after cardiac transplantation (HTx) is EBV seronegativity of the patient prior to transplantation [5,6]. Monitoring of EBV-DNA in transplant recipients is a widespread tool for detecting patients at risk of PTLD[7-11]. However, EBV DNA levels have shown to be insufficient as a sole prognostic factor for PTLD. Since PTLD arises as a result of an imbalance between T-cell control and infection, the number of EBV-specific T cells, which indicate control of infection, could contribute to prediction of PTLD [11-15].

Here we present the EBV-specific T-cell kinetics of a primary EBV infection and subsequent reactivations in an EBV seronegative cardiac transplant recipient in whom a T-cell response against EBV developed during primary infection resulting in adequate control of EBV infection throughout follow up.

MATERIAL AND METHODS

Patient characteristics

A 60 year old male (HLA A1, A30, B51, B57) underwent a cardiac transplantation in June 2007 for ischemic cardiac failure. Immunosuppressive therapy following transplantation consisted of Tacrolimus, Mycophenolate mofetil and Prednisolone. Prednisolone was tapered and withdrawn at 9 months post HTx, according to clinical guidelines. During episodes of positive EBV-DNA PCR, antiviral therapy (Valaciclovir), was given at a dose of 3x 1000mg daily as well as reduction of the immunesuppression. The post transplantation period remained uneventful. EBV load was measured weekly during 2 months post transplantation and approximately monthly until 12 months post transplantation, thereafter incidentally on out-patient visits until 3 years post transplantation. During the first year post transplantation, blood samples were drawn at each visit to perform

EBV load measurements and isolation of peripheral blood mononuclear cells (PBMC) for EBV-specific T-cell analysis. Written informed consent was obtained from the patient in accordance with the declaration of Helsinki

EBV DNA monitoring and serology

EBV monitoring was based on a real-time TaqMan™ EBV DNA PCR assay in ethylenediaminetetra acetic acid (EDTA)-plasma as described previously[16-18]. Determination of EBV seroconversion was based on the presence of IgM and IgG antibodies against the viral capsid antigen (VCA) or the EBV nuclear antigen (EBNA) as part of the diagnostic routine.

Tetramer and phenotype stainings

HLA-B57 tetramer complexed with an EBV peptide derived from the latent antigen EBNA-3B (VSFIEFVGW) was used to identify EBV-specific $CD8^+$ T cells (Sanquin, Amsterdam, the Netherlands). At least $1x10^6$ PBMC were stained with the HLA-B57 EBV tetramer, conjugated to PE, co-stained with CD3-Pacific Blue (eBioscience Inc., San Diego, California, USA), CD8- PerCP, CD45RO- FITC (Invitrogen, Carlsbad, California, USA) and CD27-APC Alexa Fluor 750 (Ebioscience) for phenotypical analysis or co-stained with HLA-DR-APC-Cy7 (BD Biosciences (BD), San José, California, USA), CD38 PE-Cy7, CD80-FITC and CD25 APC (eBioscience) for activation marker analysis. Perforin expressing $CD8^+$ T cells were measured directly on fresh whole blood samples as described previously[19]. In brief, erythrocytes were lysed and samples were washed and incubated with CD3-PerCP, CD8-APC-Cy7, CD56-APC (BD) and CD16 Pacific Blue (eBioscience). Cells were permeabilised and lysed and subsequently incubated with FITC conjugated perforin (BD). For all stainings at least 200000 events were acquired on a LSRII flowcytometer and analyzed using FACSdiva software (BD).

IFNγ ELIspot assay

IFNγ producing EBV-specific T cells were enumerated using an IFNγ ELIspot assay as previously described[20]. In brief, 96 well multiscreen filter plates (Millipore, Volketswil, Switserland) were coated overnight with 50 µl of 15µg/ml anti-IFNγ monoclonal antibody (MABTECH, Stockholm, Sweden) in PBS. PBMC were incubated in triplicate at $1x10^5$ cells/well in the presence of 10 µg/ml of 15mer peptide pools with 11 amino acid overlap. We used both the immunogenic C-terminal region of the latent protein EBNA-1 and the entire lytic protein BZLF-1 (JPT Peptide Technologies GmbH, Berlin, Germany). As a control the cells were cultured in the absence of stimuli or in the presence of phyto-

hemoagglutinin (PHA) (Murex Diagnostics, Dartford UK). IFNγ production was detected using biotinylated anti-IFNγ monoclonal antibody (MABTECH). IFNγ producing cells were analysed using an automated spot reader (AELVIS GmbH, Hannover, Germany). The number of EBV-specific IFNγ producing T cells was calculated per $1x10^6$ PBMC after subtracting negative control values.

RESULTS

Virological characteristics

EBV serology and DNA load were monitored following cardiac transplantation in an EBV seronegative recipient. The patient had no detectable IgM or IgG antibodies against viral capsid antigen (VCA) or EBNA at 1 year prior to HTx and on the day of transplantation. The transplant donor was EBV seropositive (IgM VCA negative, IgG VCA, early antigen (EA) and EBNA positive). EBV DNA in plasma was detectable at day 102 post transplantation (50 copies/ml). This increased to 553 copies/ml on day 117 and decreased below detection limit on day 133 (figure 1a). An EBV reactivation subsequently occurred on day 249 post transplant with a maximum viral load of 107 copies/ml on day 256. EBV seroconversion was observed 270 days post transplantation, as evidenced by the appearance of IgM and IgG antibodies against VCA (figure 1a).

B57-VSF specific $CD8^+$ T cells develop rapidly following primary infection and expand upon reactivation

Kinetics of EBV-specific $CD8^+$ T cells were visualized using a HLA-B57 tetramer complexed with the EBV epitope VSFIEFVGW (derived from the latent antigen EBNA3B) throughout the first year post transplantation. In healthy individuals (HLA-B57 and EBV seropositive) the range of VSF specific $CD8^+$ T cells is 0.08-1.40% (data not shown). Representative tetramer stainings in our transplant patient are shown in figure 1b. EBV B57-VSF specific $CD8^+$ T cells were rapidly detectable after the onset of primary infection. Within 24 days after first detection of EBV-DNA in plasma, we could detect EBV-specific $CD8^+$ T cells (0.38% of $CD8^+$ T cells)(figure1a). After the primary infection resolved (undetectable EBV DNA load at day 133), the B57-VSF specific $CD8^+$ T cells ranged from 0.38% to 0,47% of $CD8^+$ T cells until day 229. During the subsequent viral reactivation, the amount of B57-VSF specific $CD8^+$ T cells rapidly increased. On day 249, EBV DNA was detectable at 62 copies/ml and B57-VSF specific $CD8^+$ T cells accounted for 0,93% of total $CD8^+$ T cells. This increased to 1,5% (week 37) during viral reactivation and decreased again to 0,3% when the reactivation resolved (figure 1a). All B57-VSF specific $CD8^+$ T cells were

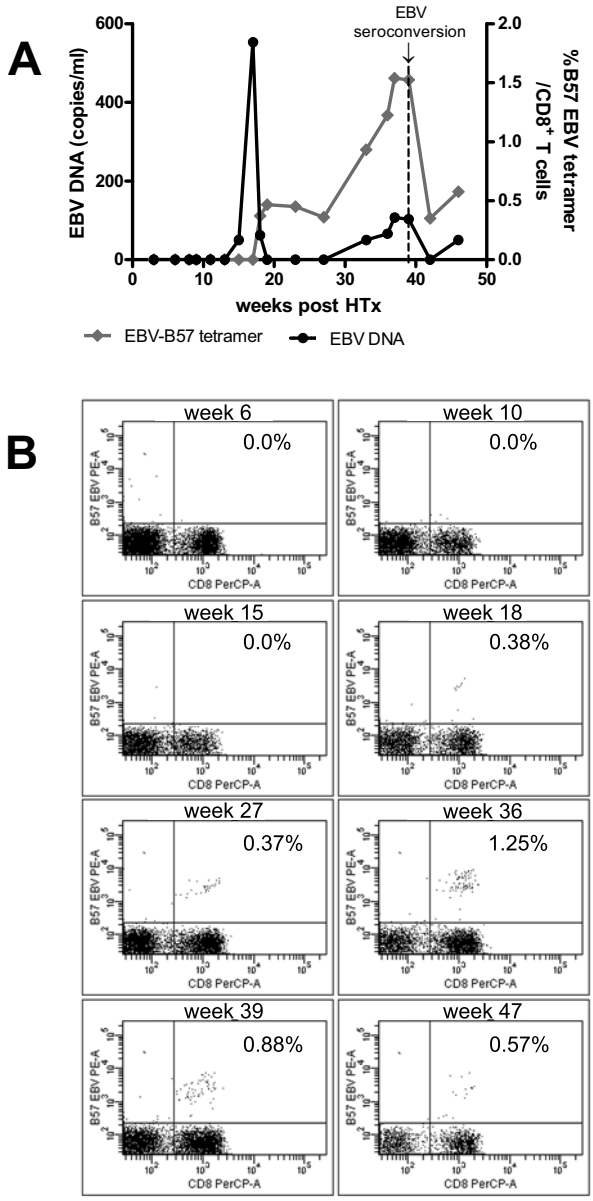

Figure 1.

A) EBV DNA load (copies/ml), depicted on the left y-axis (black line) and percentages of B57-VSF specific CD8$^+$ T cells (grey line) (right y-axis) throughout follow up. Thin dotted line indicates time point of seroconversion (detectable IgM and IgG antibodies against VCA) at 39 weeks post transplantation.
B) Representative FACS plots of HLA B57-VSF specific CD8$^+$ T cells at different time points following cardiac transplantation. Percentages in upper right corner of each FACS plot indicate the percentage of B57-VSF specific cells/CD8$^+$ T cells.

of central memory phenotype both during primary infection and during reactivation ($CD27^+/CD45RO^+$) and were highly activated ($CD38^+/HLA\text{-}DR^+$). Percentages of $CD8^+$ T cells expressing effector phenotype ($CD27^-/CD8^+$) rapidly increased upon viral reactivation and peaked during onset of reactivation (53.7% of $CD8^+$ T cells). Percentages of activation markers on $CD8^+$ T cells ($CD38^+$ and/or $HLA\text{-}DR^+$) fluctuated throughout follow up, however, percentages of $CD38^+$ and $HLA\text{-}DR^+$ T cells increased during primary infection as well as during reactivation (figure 2a).

To analyze the cytotoxic capacity of the $CD8^+$ T cell response we measured intracellular perforin expression directly ex vivo on whole blood. In healthy individuals the expression of perforin in $CD8^+$ T cells ranged between 0.3-5.1% of $CD8^+$ T cells[19]. Prior to onset of

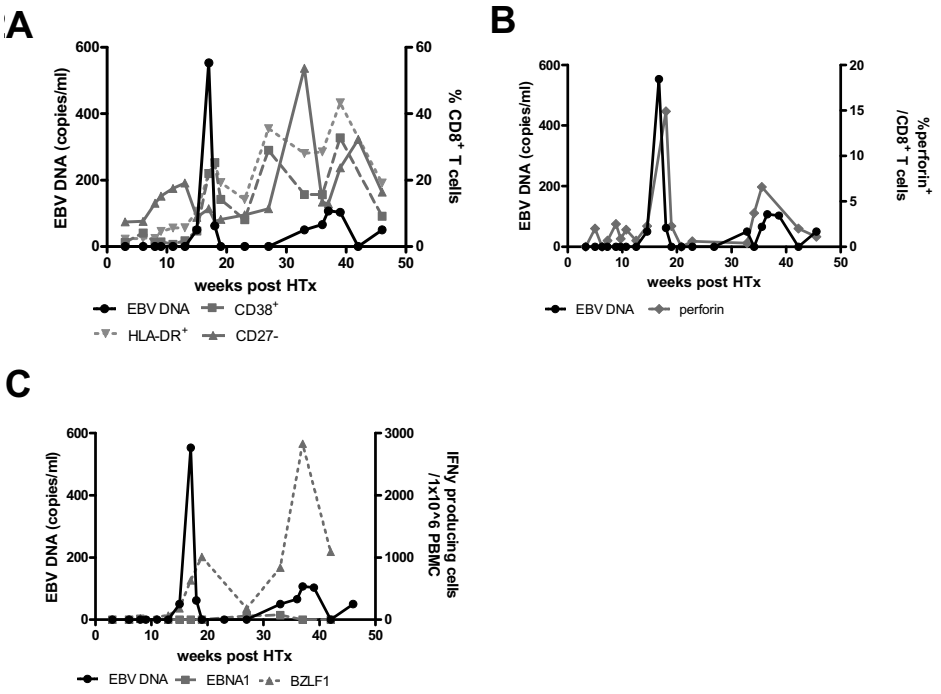

Figure 2.

A) EBV DNA load (copies/ml) throughout first year post transplantation (black line, left y-axis). Depicted on the right y-axis is the percentage of $HLA\text{-}DR^+$ (grey triangles, grey dotted line), $CD38^+$ (grey squares, grey line) and $CD27^-$ (grey triangles, grey line) of $CD8^+$ T cells.

B) Percentage of perforin expressing $CD8^+$ T cells throughout first year post transplantation (grey line, right y-axis) and EBV DNA load (copies/ml, black line, left y-axis).

C) IFNy producing T cells/ $1x10^6$ PBMC (right y-axis) during first year post transplantation after stimulation in an ELIspot assay with BZLF-1 (grey triangles, grey dotted line), EBNA-1 (grey squares, grey striped line) or B57-VSF (grey circles, grey line) and EBV DNA load on the left y-axis (black line).

primary EBV infection, perforin expression fluctuated between 0,0% and 2,5% of $CD8^+$ T cells in the patient. Following primary infection and during reactivation, perforin levels increased to 14,9 and 6,6% of $CD8^+$ T cells respectively (figure 2b). These data indicate that primary infection results in the development of the EBV-specific T cells while the total $CD8^+$ T cell pool demonstrates phenotypic characteristics equivalent to antiviral responses as seen in healthy individuals.

IFNγ production by EBV-specific T cells

To investigate the functional capacity of the EBV-specific T-cell response, and to broaden our analysis by investigating other targets, we measured IFNγ production by EBV-specific T cells using an ELIspot. We stimulated cells overnight with EBV-specific overlapping peptide pools against the latent protein EBNA-1 or the lytic protein BZLF-1. IFNγ producing cells were readily detected after stimulation with the IE (immediate early) lytic protein BZLF1. At the onset of primary infection (week 15) 183 IFNγ producing cells/$1x10^6$ PBMC were detected. Directly after primary infection (week 19) the number of IFNγ producing T cells increased to 1009 cells/$1x10^6$ PBMC. Between primary infection and viral reactivation the number of IFNγ producing cells decreased to 237 cells/$1x10^6$ PBMC. However, upon viral reactivation (week 37) the number of BZLF-1 specific T cells increased to 2830 cells/$1x10^6$ PBMC. Responses against the latent protein EBNA-1 remained undetected throughout follow up. (figure 2C). Since EBV-specific T-cell responses were abundantly present after stimulation with BZLF-1, we screened the BZLF-1 protein for HLA-B57 immunodominant epitopes. We used peptide prediction programs as described previously [21] to identify potential HLA-B57 specific epitopes in the BZLF1 protein. This analysis indeed showed the presence of a potential B57-restricted epitope, [VSTAPTGSW], at position 66-74 of the BZLF1 protein. Future studies should reveal whether this newly identified epitope is recognized by CD8+ T cells in other patients.

DISCUSSION

Here we describe in detail the kinetics of the EBV-specific T-cell response during primary EBV infection and subsequent viral reactivations following cardiac transplantation. We followed an initially seronegative recipient of a seropositive cardiac transplant throughout the first year post transplantation. During this period the patient developed a primary EBV infection at 3 months post transplantation, with a peak EBV DNA load of 553 copies/ml in plasma, and subsequently several viral reactivations throughout follow up. EBV-specific T cells against the VSF epitope of the latent EBNA3B protein were detectable three weeks after onset of primary infection. Onset of viral reactivation resulted in

a threefold expansion of B57-VSF specific T cells. In our study we detected EBV-specific T cells against the immunodominant HLA-B57 VSF epitope of the latent protein EBNA3B in up to 1,5% of all $CD8^+$ T cells during reactivation. However, in the ELIspot experiments the response against the lytic protein BZLF-1 was much higher compared to the response against the VSF epitope.

The development of an EBV-specific T-cell response following asymptomatic primary EBV infection has not been studied widely. Most studies are conducted in infectious mononucleosis patients[22,23] or in PTLD patients after transplantation. A vigorous cytotoxic T-cell response, in which up to 44% of $CD8^+$ T cells are specific for an EBV epitope derived from the lytic protein BZLF1, has been described in primary EBV infection[24]. In solid organ transplant recipients, Sebelin-Wulf *et al* detected EBV-specific $CD8^+$ T cells in PTLD patients at a similar rate to healthy individuals but found increased numbers of EBV-specific T cells in patients with EBV reactivation without PTLD[25]. In allogeneic hematopoietic stem cell transplantation, rapid recovery of EBV-specific $CD8^+$ T cells after transplantation has shown to reduce the risk of EBV reactivation[14]. These studies suggest that an adequate EBV-specific T-cell reconstitution following transplantation can prevent PTLD development.
In conclusion, the rapid increase of EBV-specific $CD8^+$ T cells upon viral reactivation suggests that despite the immunosuppressive regime the patient was capable of initiating an adequate anti-viral response and resolved the primary infection and subsequent EBV reactivations without additional antiviral or anti B cell therapy. The administration of valaciclovir during EBV-DNA positive episodes could have contributed to the prevention of PTLD. Effect of valaciclovir has been described in prevention of PTLD. In EBV seronegative lung transplantation recipients continuous antiviral prophylaxis has been shown to reduce the incidence of PTLD[26]. The rapid declines in EBV DNA load upon viral reactivations and the parallel appearance of EBV-specific T-cell responses suggest that the patient developed a robust EBV-specific T-cell response capable of establishing a life long equilibrium with the EBV infected B cells.

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Chapter 5

High perforin expression in T cells: An early prognostic marker for severity of herpesvirus reactivation after allogeneic stem cell transplantation in adults

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ABSTRACT

Background: Epstein-Barr virus and Cytomegalovirus reactivations are frequent complications following hematopoeitic allogeneic stem cell transplantation (SCT) due to lack of T-cell control following immunosuppression. Early diagnosis of reactivation and subsequent pre-emptive therapy relies on frequent viral load detection. Additional virus-specific T-cell reconstitution data could improve the predictive value of viral load detection for viral complications following transplantation. Here we studied perforin-expression in $CD8^+$ T cells as a measure of cytotoxic T-cell capacity in relation to occurrence of viral reactivation.

Methods: In a prospective study we followed 40 patients during the first 3 months post transplantation and measured viral load levels in combination with intracellular perforin-expression in $CD8^+$ T cells.

Results: Median perforin-expression in $CD8^+$ T cells throughout follow-up was higher in patients with viral reactivation (4.9%) compared to patients without viral reactivation (2.3%)(p=0,001). Median percentage of perforin-expressing $CD8^+$ T cells in patients with high viral reactivations exceeding 1000 copies/ml was significantly higher (10,7%) than in patients with minor reactivations of 50-1000 copies (4.0%), patients with detectable EBV loads that did not exceed the detection limit of 50 copies/ml (2.9%) and patients without reactivation (0.8%). Patients with high viral reactivations reached a high percentage of perforin-expressing $CD8^+$ T cells (>10,2%) more often and faster than patients with low viral loads (below 1000 copies/ml) or without viral reactivation. High perforin-expression preceded high viral loads.

Conclusion: Perforin-expressing CD8+ T cells may be useful as an easy to measure prognostic marker for identifying patients at risk of severe viral reactivations very early after SCT.

INTRODUCTION

Due to an impaired immune system, herpesvirus reactivations are frequently observed after allogeneic hematopoeitic stem cell transplantation (SCT) and can cause severe complications. Both the conditioning regime as well as T-cell depletion in vivo (anti-thymocyte globulin) or in vitro together with the post-transplantation application of immunosuppressive drugs, result in an impaired immune system[1]. Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) are the two most common causes of viral illnesses following SCT[1;2]. Both are widespread herpesviruses which persist as lifelong latent infections which are normally controlled by cytotoxic $CD8^+$ T-cell responses[3;4;5;6]. Frequent monitoring of EBV and CMV-DNA loads post transplantation is used to detect viral reactivations and infections providing a basis for pre-emptive therapy to prevent clinical complications (reviewed in [7;8]). Although viral load increase was shown to be a good indicator for clinical complications [9;10], the power of EBV and CMV load as the sole diagnostic tool is debated and overtreatment can occur [11]. Moreover, for CMV it has been shown that early treatment of CMV reactivation can result in severe CMV disease at a much later stage because CMV-specific T-cell reconstitution is interfered with due to antiviral therapy [12]. Measuring the cytotoxic T-cell response early after transplantation could help to predict the course of viral reactivation and indicate whether pre-emptive therapy can be postponed in case of an adequate T-cell response. Given the fact that measuring virus-specific T cells is very patient specific because HLA specific reagents are required, we sought for a marker that represents cytotoxic T-cell responses and can be measured with routine laboratory techniques. Perforin is one of the cytotoxic molecules released by $CD8^+$ T cells and natural killer (NK) cells to kill target cells[13;14]. Its expression upon T-cell maturation is tightly regulated after activation and effector T cells were shown to express the highest levels of intracellular perforin[15]. As these cells are the ones most important in restraining viral reactivation, we postulate that the level of perforin in $CD8^+$ T cells could indicate the functional capacity of an antiviral response. To this end we performed a prospective study in 40 patients undergoing SCT during the first 3 months following transplantation and analysed the kinetics of perforin-positive T cells in relation to viral reactivation.

PATIENTS, MATERIALS AND METHODS

Patient and transplantation characteristics

Forty patients receiving allogeneic SCT between January 2007 and October 2007 were prospectively followed during 3 months post SCT at the department of Hematology of the University Medical Center Utrecht. Patient and transplantation related characteris-

tics are described in table 1. Patients received an allogeneic stem cell transplant from either a related (n=13) or an unrelated (n=27) donor. The stem cell source was mostly peripheral blood (n=36) and in most patients a nonmyeloablative conditioning regime was used (n=35). In vivo T-cell depletion consisting of ATG was added to the conditioning regimen in patients receiving grafts from unrelated donors or human leukocyte antigen (HLA) mismatched donors. Whole blood samples were routinely drawn weekly from all patients to determine EBV and CMV load. After removal of plasma for EBV and CMV PCR analyses, the leftover whole blood was used in this study. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki.

CMV and EBV monitoring

CMV and EBV monitoring was based on a real-time TaqMan™ CMV or EBV DNA PCR assay in ethylenediaminetetra acetic acid (EDTA)-plasma[16-19], which was performed weekly in all patients until 4 months post transplantation. Patients were treated pre-emptively with valganciclovir (900 mg twice daily) when CMV-DNA load exceeded 500 copies/ml and with Rituximab 375 mg/m^2 when EBV DNA exceeded 1000 copies/ml. Valaciclovir was given to all patients prophylactic (500 mg twice daily). Viral reactivations and/or infections were defined as EBV and or CMV viral load exceeding the detection limit of 50 copies/ml in plasma.

Intracellular perforin staining and absolute CD8$^+$ T-cell determination

After whole blood samples were obtained, erythrocytes were lysed using a solution containing ammoniumchloride, potassium hydrogen carbonate and EDTA. Samples were washed with phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 0.02% sodiumazide. 0.5x10^6 Cells were incubated with CD3-PerCP, CD8-APC-Cy7, CD56-APC (BD Biosciences (BD), San José, California, USA) and CD16 Pacific Blue (eBioscience Inc., San Diego, California, USA). Cells were subsequently permeabilised and lysed for intracellular perforin staining (BD perm/lysis). After permeabilisation cells were incubated with FITC conjugated perforin (BD). Samples were measured on LSR-II and at least 100000 events per sample were acquired. Perforin expression was analysed when at least 50 CD8$^+$ T cells were acquired (using FACSdiva software (BD). The median number of CD8+ T cells acquired was 1038, and most samples contained > 100 events in the CD8+ T cell gate. Besides patient material, blood samples anti-coagulated with EDTA were drawn from 15 healthy volunteers as a control for normal range of perforin levels in T cells.

To determine the absolute number of CD8$^+$ T cells per ml whole blood, TRUcount™ tubes (BD) were used according to manufacturers protocol. In brief, 50µl of whole blood

was incubated with CD45-PerCP and CD8-APC-Cy7 (BD). Thereafter erythrocytes were lysed (BD lysisbuffer) and samples were measured on LSR-II FACS machine. At least 2000 lymphocytes were measured (identified as CD45 positive and SSC low) and analysed with FACSdiva software (BD).

Statistical analysis

All transplantation related risk factors for the development of CMV or EBV reactivation were assessed using a Fisher's Exact test. The prognostic value of perforin on the onset and severity of viral reactivation was determined using Mann-Whitney U-tests and Kaplan-Meier graphs and logistic regression analysis. Risk factors for high perforin were analysed using a Fisher's Exact test for univariate analysis and Cox regression for multivariate analysis. A probability level of 5% ($p<0.05$) was considered significant in all analyses. All statistical analysis were performed with SPSS version 12.1 (SPSS Inc, Chicago, USA) and GraphPad Prism 3.0 (GraphPad Software Inc., San Diego, USA) software.

RESULTS

Patient population

Forty patients who received allogeneic haematopoietic stem cell transplantation during January 2007 and October 2007 were prospectively followed during the first 12 weeks post transplantation. All transplantation-related factors that could influence the onset of EBV and/or CMV reactivation were assessed in univariate analysis (table I). Only CMV serostatus prior to transplantation was significantly associated with viral reactivation. Patients were categorized according to occurrence of reactivation and the peak viral load of either EBV or CMV that was reached during the first 12 weeks post transplantation. Patients in which both viruses reactivated were classified according to the highest viral load reached of either virus . An EBV and/or CMV reactivation occurred in 17 patients (42.5%). Subdivision in the different reactivation categories is shown in figure 1. Representative viral load graphs throughout follow-up of patients with low and high reactivations are displayedin figure 2b. No differences between related (MRD) and unrelated (MUD) donors were observed in reactivation severity (data not shown).

Intracellular perforin in $CD8^+$ T cells is increased in patients with viral reactivations

Intracellular perforin expression was measured in $CD8^+$ T cells and NK cells weekly until 12 weeks post SCT. Figure 2a shows representative FACS plots of perforin expression in

$CD8^+$ T cells for a patient without viral reactivation and a patient with a high reactivation. Perforin expressing $CD8^+$ T cells and CMV viral load levels at corresponding time points throughout follow-up are shown in figure 2b. Median perforin expression of $CD8^+$ T cells of healthy individuals was 2.7% (range 0.3-5.1%) (data not shown). In patients

Table I. Patient characteristics

		Total	Reactivation (%)	No reactivation (%)	univariate
	N=	40	17 (42,5%)	23 (57,5%)	
Sex	M	22	8 (36,4%)	14 (63,6%)	
	F	18	9 (50,0%)	9 (50,0%)	p=0,523
Median age (range)		51,5 (21,6-65,9)	53,7 (22,9-65,9)	51,4 (21,6-61,2)	p=0,594
Disease	AA	2	2 (100%)	0 (0%)	
	ALL	5	2 (40,0%)	3 (60,0%)	
	AML	12	4 (33,3%)	8 (66,7%)	
	CLL	1	0 (0%)	1 (100%)	
	CML	3	2 (66,7%)	1 (33,3%)	
	Hodgkin	1	0 (0%)	1 (100%)	
	MDS	3	1 (33,3%)	2 (66,7%)	
	MM	7	4 (57,1%)	3 (42,9%)	
	NHL	6	2 (33,3%)	4 (66,7%)	p=0,745
Stem cell source	Cordblood	3	2 (66,7%)	1 (33,3%)	
	Peripheral blood	36	14 (38,9%)	22 (61,1%)	
	Bonemarrow	1	1 (100%)	0 (0%)	p=0,371
Donor	Related	13	4 (30,8%)	9 (69,2%)	
	Unrelated	27	13 (48,1%)	14 (51,9%)	p=0,333
HLA mismatch	Yes	10	6 (60,0%)	4 (40,0%)	
	No	30	11 (36,7%)	19 (63,3%)	p=0,274
Conditioning	NMA	35	15 (42,9%)	20 (57,1%)	
	MA	5	2 (40,0%)	3 (60,0%)	p=>.99
ATG	Yes	29	15 (51,7%)	14 (48,3%)	
	No	11	2 (18,2%)	9 (81,1%)	p=0,079
EBV serology (R/D)	+/+	35	15 (42,9%)	20 (57,1%)	
	+/-	5	2 (40,0%)	3 (60,0%)	p=>.99
CMV serology (R/D)	+/+	11	9 (81,1%)	2 (18.2%)	
	+/-	15	6 (40,0%)	9 (60,0%)	
	-/+	4	1 (25,0%)	3 (75,0%)	
	-/-	10	1 (10,0%)	9 (90,0%)	p=0,006

Abbreviations: AA, aplastic anemia; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; HLA, human leukocyte antigen; NMA, non myeloablative; MA, myeloablative; ATG, anti-thymocyte globulin; EBV, Epstein-Barr virus; CMV, cytomegalovirus; R/D, recipient/donor.

Comparison between reactivation and no reactivation group: Mann-Whitney U t-test for age, other univariate analysis using Fisher's Exact test.

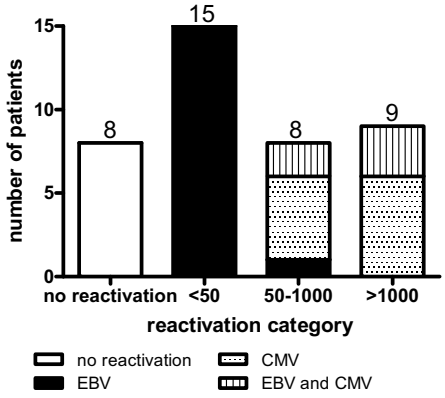

Figure 1. EBV and CMV reactivations

Patients were categorized in reactivation categories based on the peak viral load of either virus in plasma during 12 weeks post SCT. Patients with undetectable viral load (n=8) or with EBV load not exceeding detection limit (50 copies/ml plasma) (n=15) were classified as not reactivating. Patients with viral loads that did not exceed 1000 copies/ml were low reactivators (n=8). 5 Patients only had a CMV reactivation, 1 patient an EBV reactivation and 2 patients had both EBV and CMV reactivations. The high reactivation group encompassed 9 patients with loads exceeding 1000 copies/ml. Of these 9 patients, 6 patients only had CMV viral loads exceeding 1000 copies, 2 patients had besides CMV viral loads exceeding 1000 copies/ml also EBV viral load >50 copies/ml and <1000 copies/ml. 1 Patient had both EBV and CMV viral loads exceeding 1000 copies/ml.

without viral reactivation the percentage of perforin expressing $CD8^+$ T cells almost never exceeded the maximum expression of perforin in healthy individuals of 5.1%. All patients with viral reactivations had percentages of perforin expressing $CD8^+$ T cells over 5.1% during follow-up reaching as high as 30% of $CD8^+$ T cells (figure 2a). Furthermore, percentages of perforin expressing T cells fluctuated during follow-up, especially in the high reactivation group (figure 2a). In addition, also the onset of reactivation occurred at a different time point after SCT for each patient. In order to circumvent these fluctuations in perforin expression and the variable occurrence of reactivation in relation to time point of transplantation, we compared patients based on the median perforin expression throughout follow-up. Median perforin expression in NK cells did not differ between patients with and without viral reactivation. Both for $CD3^-/CD16^+$ cells (32.4% of $CD16^+$ cells expressed perforin in the reactivation group and 30.1% in the no reactivation group p=0,74) and for $CD3^-/CD56^+$ T cells there was no significant difference (40.7% in reactivation group and 41.4% in the no reactivation group, p=0.75). However, in $CD8^+$ T cells, the median perforin levels was 2.3% of $CD8^+$ T cells in the group without reactivation, and 4.9% of $CD8^+$ T cells for patients with viral reactivation (p= 0,001) (figure 3a).

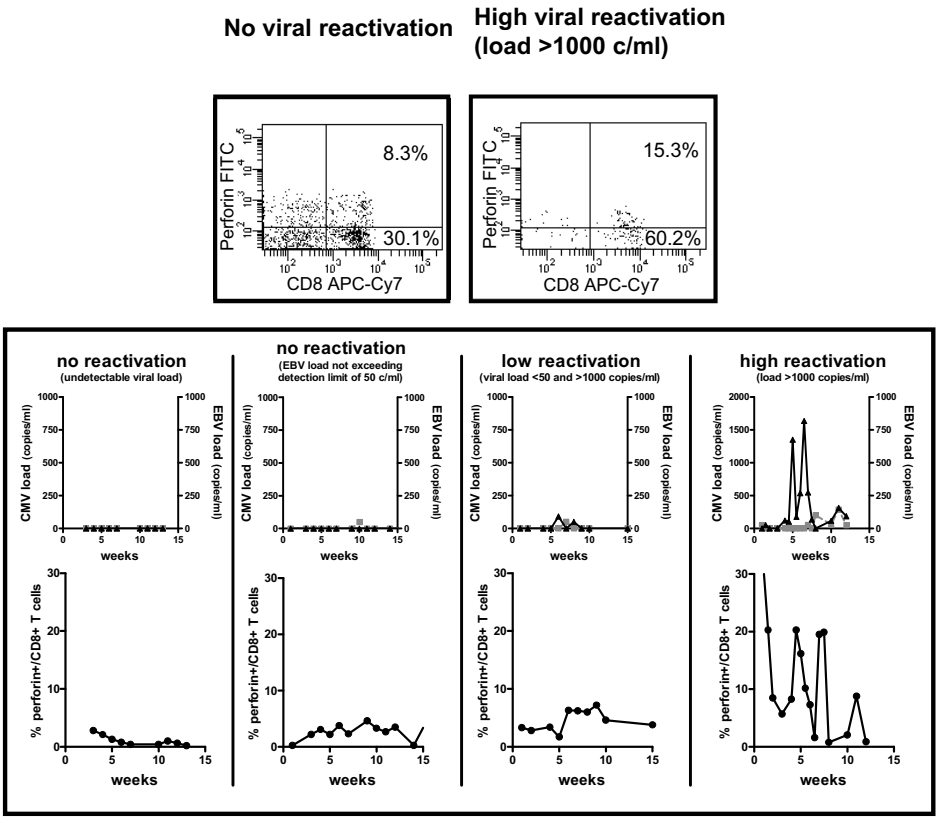

Figure 2. Representative graphs of viral load and perforin expression in CD8$^+$ T cells in patients from different reactivation groups

Representative FACS plots of perforin FITC (y-axis) and CD8- APC-Cy7 (x-axis) expression for 2 different patients. Left dot plot shows a patient without viral reactivation, at 58 days post transplantation. Right dot plot shows a patient with high viral reactivation at 29 days post SCT. CMV reactivation is starting at this time point (viral load 106 copies/ml). Percentages indicated in the upper right quadrant indicate the % perforin+ CD8+ T cells.

Representative graphs of viral reactivation (top panel) and perforin-expression in CD8$^+$ T cells (bottom panel) are shown for each reactivation group; patients without viral reactivation, patient with EBV reactivation not exceeding detection limit, patients with either CMV or EBV reactivation not exceeding 1000 copies/ml or patients with EBV or CMV reactivation exceeding 1000 copies/ml.

Top panel: CMV viral load (black triangles, on left y-axis) and EBV viral load in copies/ml (grey squares, on right y-axis) throughout follow up in weeks post SCT (x-axis). Bottom panel: percentage of perforin producing CD8$^+$ T cells (black circles, on y-axis) throughout follow up in weeks post SCT (x-axis).

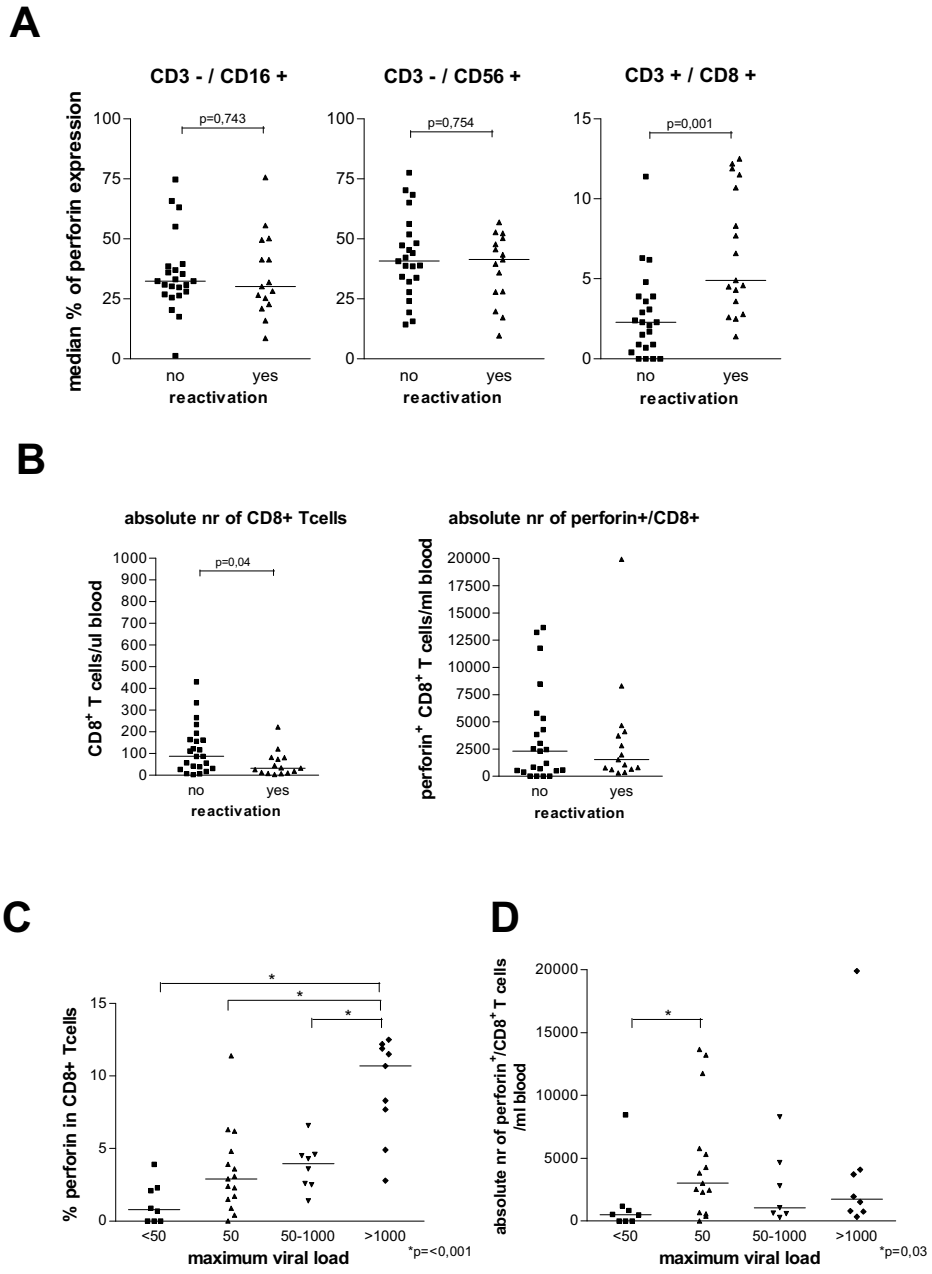

Figure 3. Perforin expression increases with viral reactivation in T cells, not in NK cells

A) Median percentage of perforin expressing cells throughout 12 weeks post SCT (y-axis) are plotted for patients with (viral load of EBV or CMV exceeding detection limit) or without reactivation (viral load of EBV or CMV not exceeding detection limit) for NK cells (CD3-/CD16+, CD3-/CD56+, left panels) and T cells (CD3+/CD8+, right panel). Perforin expression in NK or T cells was determined by intracellular FACS

staining of perforin combined with CD56, CD16 or CD8. Median value of perforin expression in each cell type was calculated for each patient.
B) Median absolute $CD8^+$ T cells/ul blood, as determined by trucount analysis, throughout 12 weeks post SCT (y-axis), are plotted for patients with or without viral reactivation (left panel). Median absolute $perforin^+/CD8^+$ T cells/ml blood (y-axis) are plotted for patients with or without viral reactivation (right panel). Perforin expression in T cells was determined by intracellular FACS staining of perforin combined CD8 and calculated to absolute numbers using trucount data.
C) Median percentage of perforin expressing $CD8^+$ T cells plotted for each patient in the different reactivation groups (no reactivation, patients with EBV reactivation not exceeding detection limit, EBV or CMV reactivation not exceeding 1000 copies/ml or patients with EBV or CMV reactivation exceeding 1000 copies/ml).
D) Median absolute number of perforin expressing $CD8^+$ T cells/ ml whole blood plotted for each patient in the different reactivation groups (no reactivation, patients with EBV reactivation not exceeding detection limit, EBV or CMV reactivation not exceeding 1000 copies/ml or patients with EBV or CMV reactivation exceeding 1000 copies/ml).

Thus, perforin expression in $CD8^+$ T cells and not in NK cells is significantly increased in patients with viral reactivations.
Patients undergoing a viral reactivation, however, did have significantly lower absolute numbers of CD8+ T cells throughout the first 3 months post-SCT compared to patients without viral reactivation (figure 3b). Therefore, the absolute number of perforin+ CD8+ T cells does not differ between reactivating and non-reactivating patients (figure 3b). To further explore the observed differences in % of perforin-expressing CD8+ T cells as a potential marker associated with viral reactivation, we analysed whether this percentage correlated with the level of viral reactivation. We subdivided the patients in the earlier defined categories with low (peak viral load between 50-1000 copies/ml) and high reactivations (peak viral load >1000 copies/ml) and compared the median perforin level throughout follow-up in each group. Median percentage of perforin expressing $CD8^+$ T cells in the group with high reactivations (10.7%,figure 3c) was significantly higher than patients with minor reactivations (4.0%, p=0,003), patients with detectable EBV loads that did not exceed the detection limit (2.9%, p=0,0016) and patients without reactivation (0.8%, p=0.0002) (figure 3c). There were no differences between the 4 reactivation categories for the absolute numbers of $perforin^+$ $CD8^+$ T cells. (figure 3d) Interestingly, patients without viral reactivation, that occasionally have detectable viral titers not exceeding detection limit (50 copies/ml) have significantly higher absolute numbers of perforin expressing $CD8^+$ T cells compared to patients without viral reactivation. This observation in this specific subgroup is being investigated further in a larger cohort (manuscript in preparation). Thus, not absolute numbers but percentages of $perforin^+$ $CD8^+$ T cells seem associated with the severity of viral reactivations.

High perforin levels are associated with high viral loads

To find a cut-off value of perforin expression in $CD8^+$ T cells that could distinguish patients with and without viral reactivation we used the normal range of perforin expression in $CD8^+$ T cells in healthy individuals (0.3-5.1%). Perforin levels that exceeded twice the maximum percentage found in healthy individuals (10.2%) were classified as high. Using Kaplan Meier survival analysis we compared the occurrence and time of onset of viral reactivation between patients with low intracellular perforin expression (never exceeding 10.2% of $CD8^+$ T cells throughout follow-up) and high intracellular perforin expression (exceeding 10.2% of $CD8^+$ T cells at some time point during 12 weeks post SCT). Patients with high intracellular perforin expression (n=25) reactivated more frequently than patients with low intracellular perforin levels (n=15) (p=0.006) (figure 4a). Next we investigated the time-point after SCT at which this high level of perforin expression is reached in the different reactivation groups. To this end we subdivided our patients into the different reactivation categories. The group without any DNAemia during 12 weeks post SCT almost never exceeded 10.2% $perforin^+$ / $CD8^+$ T cells (figure 4b).The incidence of high perforin expressing $CD8^+$ T cells in the low reactivators (50-1000 copies/ml) and the group with EBV loads not exceeding detection limit was similar. In these groups, 10.2% of perforin expression is observed in several cases, however mostly relatively late during follow up (median day 23, range = 3-64 days). In contrast, all patients in the high reactivation group had perforin expression exceeding 10.2% of their $CD8^+$ T-cell population shortly after transplantation (median day 7, range = 3-28 days) (figure 4b).
Subsequently, we investigated the prognostic capacity of perforin measured within the first 3 weeks post SCT on high viral reactivations. Although the median onset of viral reactivation in the high reactivation group (26 days after SCT, range 4-84 days),did not significantly differ from the occurrence of high (>10.2%) perforin expression (median of 7 days after SCT, range 3-28 days) (figure 5), high perforin expression always preceded viral loads exceeding 1000 copies/ml (median of 56 days, range 10-84 days) (figure 4b and 5).

DISCUSSION

In immunocompromised individuals, such as SCT recipients, viral complications are thought to occur due to a lack of T-cell control. We investigated whether the presence or lack of perforin in $CD8^+$ T cells, as a measure for potential $CD8^+$ T-cell cytotoxicity, could predict the efficacy of the antiviral response. Strikingly, we observed that increased perforin levels were not a measure for adequate antiviral capacity but that perforin levels in $CD8^+$ T cells were increased in patients with viral reactivations compared to

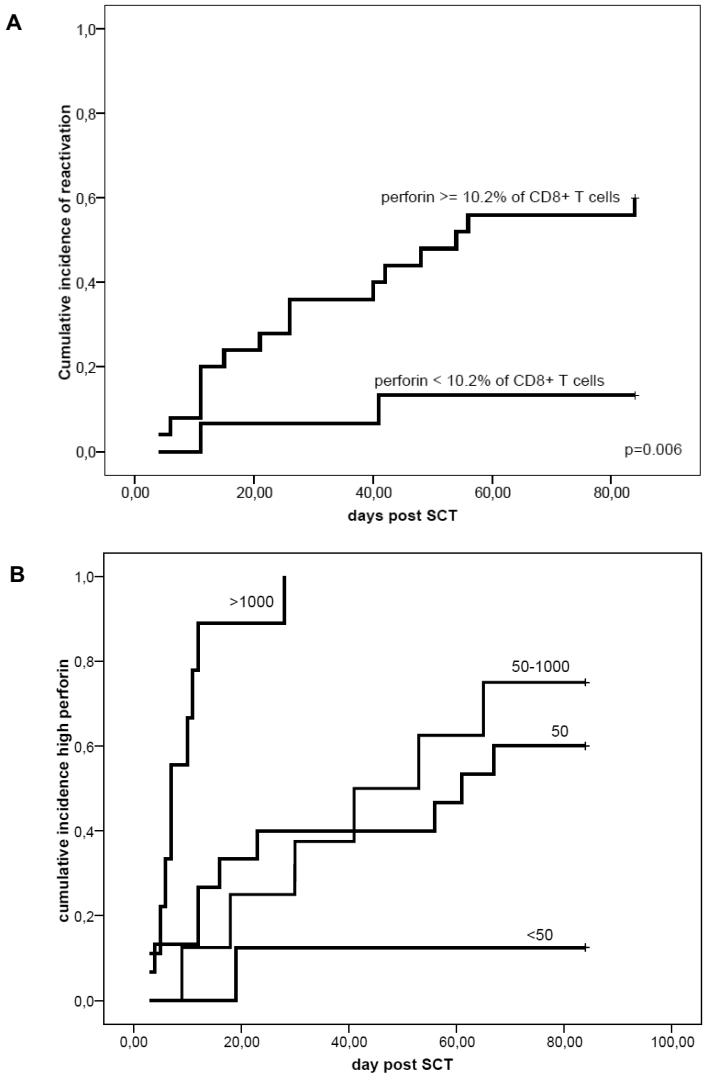

Figure 4. High perforin levels are associated with high viral loads

A) Patients were grouped based upon whether they had perforin expression exceeding 10.2% of $CD8^+$ T cells at any time point during their 12 week follow-up period. Kaplan meier plot show the incidence of viral reactivation (on y-axis) during followup (on x-axis) in SCT recipients having less than 10.2% of perforin expressing $CD8^+$ T cells or more than 10.2% perforin expressing T cells at any time point during their 12-week follow-up.

B) Patients were divided into groups based upon their maximum viral load throughout follow-up. The Kaplan-Meier plot shows the incidence of the percentage of perforin expressing $CD8^+$ T cells (on y-axis) during follow-up (on x-axis) in SCT recipients divided in the different reactivation categories (0 copies/ml, max 50 copies/ml, 50-1000 copies/ml or > 1000 copies/ml).

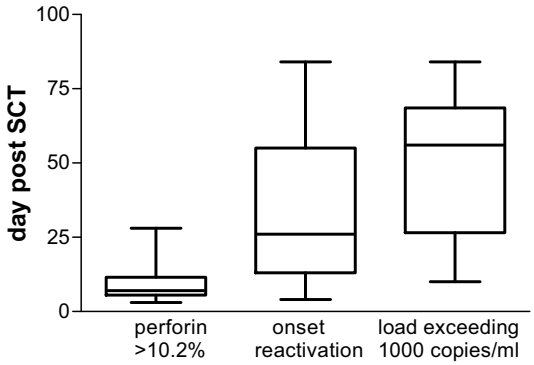

Figure 5. Prognostic value of perforin expression in CD8+ T cells
Boxplot of patients in the high reactivation group plotting the day post SCT (y-axis) at which perforin exceeds 10.2% of CD8+ T cells (perforin>10,2%), at which the onset of viral reactivation occurs (onset reactivation) and at which viral load exceeds 1000 copies/ml (load exceeding 1000 copies/ml).

patients without viral reactivations. In addition we found that perforin levels in CD8+ T cells increased with reactivation severity. Furthermore a defined threshold of high perforin expression (>10.2% of CD8+ T cells) was reached much quicker and more often in patients with viral reactivation. This enables the use of perforin as a prognostic marker to predict reactivation severity early after transplantation.

Other studies also have identified perforin expression as a marker for disease progression. In HIV infection a negative correlation between perforin expressing HIV specific CD8+ T cells and the peripheral blood CD4+ T cell count was shown [20]. Also a correlation between EBV-DNA load and perforin expression has been suggested earlier by Yoshimi *et al*. They showed that perforin expression increases with EBV-DNA load in two bone marrow transplant recipients[21]. This is in line with our findings in a much larger patient cohort.
The early occurrence of the peak in perforin expressing CD8+ T cells, suggests that another transplantation related factor may be the cause of the increase in perforin expression Shortly after transplantation the graft has to fill the new compartment created through the conditioning regime and has to win space over resident blood cells until full donor chimerism is established[22]. This could trigger immune responses which could be reactive against the host, i.e. alloreactivity. Concurrent with this hypothesis is the association of perforin with acute rejection in solid organ transplantation[23-25]. The use of perforin and granzyme expression as diagnostic markers for activated cytotoxic T cells in solid organ transplantation has already been suggested in 1991. Perforin expressing lymphocytes infiltrating transplanted tissues in cardiac transplant recipients

were identified in relation to rejection[23]. Also the ratio between perforin expression in peripheral blood in T and NK cell subpopulations has been suggested as a predictor for acute rejection in renal transplant recipients[24]. An elevated expression of perforin and granzyme B measured by RT-PCR in peripheral blood has been associated with both cell mediated and antibody mediated rejection[25].

The transplantation related factor which activates immune responses (T cells) may also be the cause of the viral reactivation. As this immune response may involve a multitude of immune cells (including for example B cells) one could envisage that also (B) cells containing latent herpesviruses (EBV) could be activated which may lead to reactivation of the latent viruses. This would fit with previous studies suggesting an active role for HIV-1 for triggering and/or dysregulating B cell responses during acute HIV-infection, when the immune system is still intact[26]. Also for CMV it could be that activation of endothelial cells (a common target cell in GvHD) could result in reactivation of CMV. In this light, we checked whether high percentages of perforin+ T cells were associated with aGvHD. However, aGvHD was not a risk factor for high % perforin+ T cells, neither did the early peaks of perforin correlate with onset of aGvHD (data not shown).

On the other hand, the viral reactivation obviously already starts prior to it being detectable in plasma. Thus, the $CD8^+$ T cells expressing perforin early after SCT could be a commencing anti-viral CTL response against a very preliminary viral reactivation. Given the lower absolute CD8+ T-cell counts, these reactivating patients could be compensating these low absolute numbers of $CD8^+$ T cells by a higher percentage of their $CD8^+$ T cells that express perforin compared to patients without reactivation. However, preliminary results of an ongoing study investigating EBV and CMV specific T-cell responses using Interferon-γ ELIspot assays show EBV and CMV specific T cells only appearing after the onset of viral reactivation (data not shown). Also, a study by Meij *et al* has shown that EBV specific T cells do not appear before one month post SCT[27].

We recently performed similar analyses in a pediatric SCT setting and surprisingly we observed higher perforin-expressing CD8+ T cells during viral load clearance. (de pagter et al, submitted for publication) There are important differences between the two patient cohorts, which may explain the seeming discrepancy between the two data sets. First of all, the reactivations occurring in the pediatric cohort exist mainly of reactivation of HHV6. Second of all, this reactivation occurs very early after SCT[28]. Thirdly, in children mostly myeloablative conditioning regimes are used, whereas the majority of the adults received non-myeloablative conditioning. We previously observed that only MA conditioned adult patients had similar HHV6 reactivation incidence as the children (de Pagter submitted for publication). Since in our adult patient group most patients were NMA conditioned, we believe that the MA-conditioning resulting in early HHV6-reactivation, may constitute the difference between the observed data.

In conclusion, here we have shown that high levels of perforin expressing $CD8^+$ T cells are associated with viral reactivation and that the height of the initial peak of perforin producing $CD8^+$ T cells can predict reactivation severity. We hypothesize that the level of perforin producing $CD8^+$ T cells is the result of an initial transplant related trigger which also is associated with poor T cell reconstitution that results in the severity of the viral reactivation. Although we may not be able to use perforin as a measure of 'induced immunity' it can be used to predict the severity of a viral reactivation very early after SCT which could make long term viral load monitoring unnecessary. In patients with low intracellular perforin levels following SCT, viral load monitoring can be diminished whilst in patients with high intracellular perforin levels early after SCT there is a high chance of severe viral reactivation and monitoring should proceed. Future clinical studies should be performed to prove this.

Acknowledgments

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Chapter 6

CD8+ T-cell reconstitution early after allogeneic SCT: Cytomegalovirus, but not Epstein-Barr virus reactivation drives CD8+ T-cell expansions

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Submitted

ABSTRACT

Epstein-Barr virus and Cytomegalovirus reactivations are frequently observed after allogeneic stem cell transplantation. We investigated the hypothesis that poor or slow T-cell reconstitution might be related to onset and subsequent severity of EBV and CMV reactivation. 116 patients were prospectively sampled for absolute $CD8^+$ T-cell numbers weekly post SCT during the first 3 months and thereafter monthly until 6 months post SCT together with viral load monitoring. Onset of viral reactivation was not associated with poor early $CD8^+$ T-cell reconstitution. On the contrary however, viral reactivation seemed to influence subsequent the T-cell reconstitution resulting in higher absolute T-cell counts 6 months post SCT in patients with high-level reactivation. Interestingly, these relatively high absolute T cell counts 6 months after SCT were mainly observed after high-level CMV reactivations but not EBV reactivations. These data indicate that especially high-level CMV reactivations drive T-cell reconstitution, which should be taken in to account in evaluation of T-cell numbers during immune reconstitution after SCT.

INTRODUCTION

Herpesvirus reactivations, both Epstein-Barr virus (EBV) and Cytomegalovirus (CMV), occur frequently after allogeneic hematopoietic stem cell transplantation (SCT). EBV is a ubiquitous γ-herpesvirus that infects over 90% of the population during life[1]. CMV is a β-herpesvirus infecting 35-70% of people in developed countries and up to 90% in poor socioeconomic groups[2;3]. Both viruses persist lifelong in the host with a tightly regulated balance between the virus infected cells and control by cytotoxic T-cell responses[1;4;5]. However, due to the immunosuppressive state following SCT, viral reactivation can cause severe complications. EBV reactivations can progress to EBV-associated post transplant lymphoproliferative disorders (EBV-PTLD). CMV reactivations can lead to CMV disease which manifests itself in for example pneumonia, colitis or retinitis [6]. Early diagnosis and treatment is considered crucial in order to prevent EBV-PTLD and CMV disease related mortality.

Frequent monitoring of EBV and CMV-DNA loads post transplantation is used to detect viral reactivations and infections providing a basis for pre-emptive therapy to prevent clinical complications (reviewed in [7;8]). For EBV it has been shown that including T-cell reconstitution data increased the predictive value of laboratory data for development of EBV-PTLD[9]. Measuring the T-cell response early after transplantation could help to predict the course of viral reactivation and could identify patients with adequate T-cell reconstitution in which pre-emptive therapy can be withheld. Annels *et al* have indicated an arbitrary value of sufficient T-cell reconstitution for which pre-emptive therapy can be withheld upon EBV reactivation[10]. Most studies link T-cell reconstitution data to development of viral complications (i.e. EBV-PTLD). Since EBV and CMV are mainly controlled by cytotoxic $CD8^+$ T cell responses it is generally believed that a poor T-cell reconstitution after SCT is the cause of viral reactivation. We investigated the impact of 1) $CD8^+$ T-cell numbers early after SCT on the onset of viral reactivation, 2) viral reactivation on T-cell reconstitution after resolving the viral reactivation . To this end we monitored 116 allogeneic SCT recipients during the first 6 months post SCT for viral reactivations as well as absolute T-cell numbers both early (weekly during first 3 months post SCT),the time during which most viral reactivations occur, and late (6 months post SCT) after SCT, after which most reactivations have resolved.

PATIENTS, MATERIALS AND METHODS

Patient and transplantation characteristics

116 Patients receiving allogeneic SCT between January 2007 and June 2009 were prospectively followed during 6 months post SCT at the department of Hematology of the University Medical Center Utrecht. Patient and transplantation related characteristics are described in table 1. Patients received an allogeneic stem cell transplant from either a related (n=35) or an unrelated (n=81) donor. The stem cell source was mostly peripheral blood (n=106) and in most patients a nonmyeloablative conditioning regime was used (n=106). In vivo T-cell depletion consisting of ATG was added to the conditioning regimen in patients receiving grafts from unrelated donors or human leukocyte antigen (HLA) mismatched donors (n=87). Whole blood samples were routinely drawn weekly from all patients to determine EBV and CMV load. After removal of plasma for EBV and CMV PCR analyses, the leftover whole blood was used in this study. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki.

CMV and EBV monitoring

CMV and EBV monitoring was based on a real-time TaqMan™ CMV or EBV DNA PCR assay in ethylenediaminetetra acetic acid (EDTA)-plasma[11-13]which was performed weekly in all patients until 4 months post transplantation. Patients were treated pre-emptively with valganciclovir (900 mg twice daily) when CMV-DNA load exceeded 500 copies/ml and with Rituximab 375 mg/m^2 when EBV-DNA exceeded 1000 copies/ml. Valaciclovir prophylaxis was given to all patients (500 mg twice daily). Viral reactivations and/or infections were defined as EBV and or CMV viral load exceeding the detection limit of 50 copies/ml in plasma.

IFNγ ELIspot assay

IFNγ producing EBV- and CMV-specific T cells were enumerated using an IFNγ ELIspot assay as previously described[14]. In brief, 96 well multiscreen filter plates (Millipore, Volketswil, Switserland) were coated overnight with 50 µl of 15µg/ml anti-IFNγ monoclonal antibody (MABTECH, Stockholm, Sweden) in PBS. PBMC were incubated in triplicate at 1x10^5 cells/well in the presence of 10 µg/ml of 15mer peptide pools with an 11 amino acid overlap. We used the immunogenic C-terminal region of the latent EBV protein EBNA-1, the entire lytic EBV protein BZLF-1 (JPT Peptide Technologies GmbH, Berlin, Germany) and the CMV protein pp65 (138 peptides) (kindly provided by the NIH reagent program). As a control the cells were cultured in the absence of stimuli or in

the presence of phytohemoagglutinin (PHA) (Murex Diagnostics, Dartford UK). IFNγ production was detected using biotinylated anti-IFNγ monoclonal antibody (MABTECH). IFNγ producing cells were analyzed using an automated spot reader (AELVIS GmbH, Hannover, Germany). The number of EBV and CMV-specific IFNγ producing T cells was calculated per $1x10^6$ PBMC after subtracting negative control values.

Absolute T-cell counts

To determine the absolute number of $CD3^+$ and $CD8^+$ T cells per µl whole blood, TRU-count™ tubes (BD Biosciences (BD), San José, California, USA) were used according to manufacturers' protocol. In brief, 50µl of whole blood was incubated with CD45-PerCP (BD), CD3-Pacific Blue (eBioscience Inc., San Diego, California, USA), and CD8-APC-Cy7 (BD). Thereafter erythrocytes were lysed (BD lysisbuffer) and samples were measured on LSR-II FACS machine. At least 2000 lymphocytes were measured (identified as CD45 positive and SSC low) and analysed with FACSdiva software (BD).

Statistical analysis

All transplantation related risk factors for the development of CMV or EBV reactivation were assessed using a Fisher's Exact test. Comparison of absolute T-cell counts between different reactivation categories was performed with Mann-Whitney U-tests. A probability level of 5% ($p<0.05$) was considered significant in all analyses. All statistical analysis were performed with SPSS version 15 (SPSS Inc, Chicago, USA) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA) software.

RESULTS

Patient population

116 Patients receiving allogeneic stem cell transplantation between January 2007 and June 2009 were prospectively followed during the first 6 months post SCT for EBV and/or CMV infections or reactivations. Transplantation associated risk factors that could influence the onset of viral reactivation were assessed in a univariate analysis (table I). Only CMV serostatus of patient and donor (p=0,000) and the administration of ATG (p=0,030) were significantly associated with onset of viral reactivation.

Table I. Patient characteristics

		total	reactivation		univariate
	N =	116	54	(46,6%)	
Sex	M	71	30	(42,3%)	p=0,258
	F	45	24	(53,3%)	
median age (range)		49,8 (17,6-70,6)	50,7 (17,6-68,5)		p=0,427
Disease					
	AA	5	3	(60,0%)	p=0,951
	ALL	11	4	(36,4%)	
	AML	38	15	(39,5%)	
	CLL	10	5	(50%)	
	CML	3	2	(66,7%)	
	Hodgkin	1	1	(100%)	
	MDS	5	2	(40,0%)	
	MM	21	11	(52,4%)	
	NHL	16	7	(43,8%)	
	other	1	1	(100%)	
Stemcell source					
	cordblood	1	0	(0,0%)	p=0,300
	peripheral blood	106	48	(45,3%)	
	bonemarrow	9	6	(66,7%)	
Donor	Related	35	13	(37,1%)	p=0,225
	Unrelated	81	4	(4,9%)	
HLA mismatch		20	11	(55,0%)	p=0,465
Conditioning					
	NMA	106	50	(47,2%)	p=0,749
	MA	10	4	(40,0%)	
ATG		87	46	(52,9%)	**p=0,030**
EBV serology (R/D)					
	+/+	94	42	(44,7%)	p=0,842
	+/-	5	3	(60,0%)	
	-/+	4	2	(50,0%)	
	-/-	11	6	(54,5%)	
CMV serology (R/D)					
	+/+	39	28	(71,8%)	**p=0,000**
	+/-	37	18	(48,6%)	
	-/+	10	2	(20,0%)	
	-/-	28	5	(17,9%)	
aGVHD		64	29	(45,3%)	p=0,831
	unknown	19			

Abbreviations: AA, aplastic anemia; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NMA, non myeloablative; MA, myeloablative; ATG, anti-thymocyte globulin; EBV, Epstein-Barr virus; CMV, cytomegalovirus; R/D, recipient/donor; aGVHD, acute graft versus host disease.

Comparison between reactivation and no reactivation group: unpaired t-test for age, univariate analysis using Fisher's Exact test.

Viral reactivation

Patients were monitored during 6 months post SCT for both EBV and CMV DNA in plasma. Viral infection or reactivation was defined as viral load exceeding the detection limit of 50 copies/ml plasma. Patients were subdivided into low-level or high-level viral reactivation based on whether the peak viral load exceeded 1000 copies/ml. Subdivision of all patients over the different reactivation categories is shown in figure 1. Viral reactivation was diagnosed in 54 patients (46.6%), of which 28 developed a low-level (load <1000 copies/ml) viral reactivation (CMV reactivation in 21 patients, EBV reactivation in 4 patients and both EBV and CMV reactivation in 3 patients). A high-level (maximum load >1000 copies/ml) occurred in 26 patients, of which 15 were CMV alone, 5 EBV and 6 both EBV and CMV reactivations (figure 1)

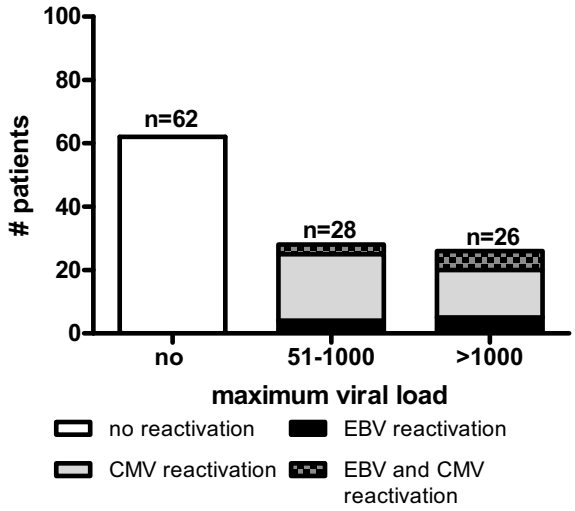

Figure 1. Viral reactivations in our patient cohort

Patients were categorized in reactivation categories based on their peak viral load of either EBV and/or CMV DNA in plasma during 6 months post SCT. White bar indicates patients without viral reactivation; grey bars patients with CMV reactivation alone; black bars EBV reactivation alone; and checkered bars indicate patients with both EBV and CMV reactivations.

Enhanced T- cell reconstitution after viral reactivation

EBV and CMV are normally controlled by a tightly regulated cytotoxic $CD8^+$ T cell response.

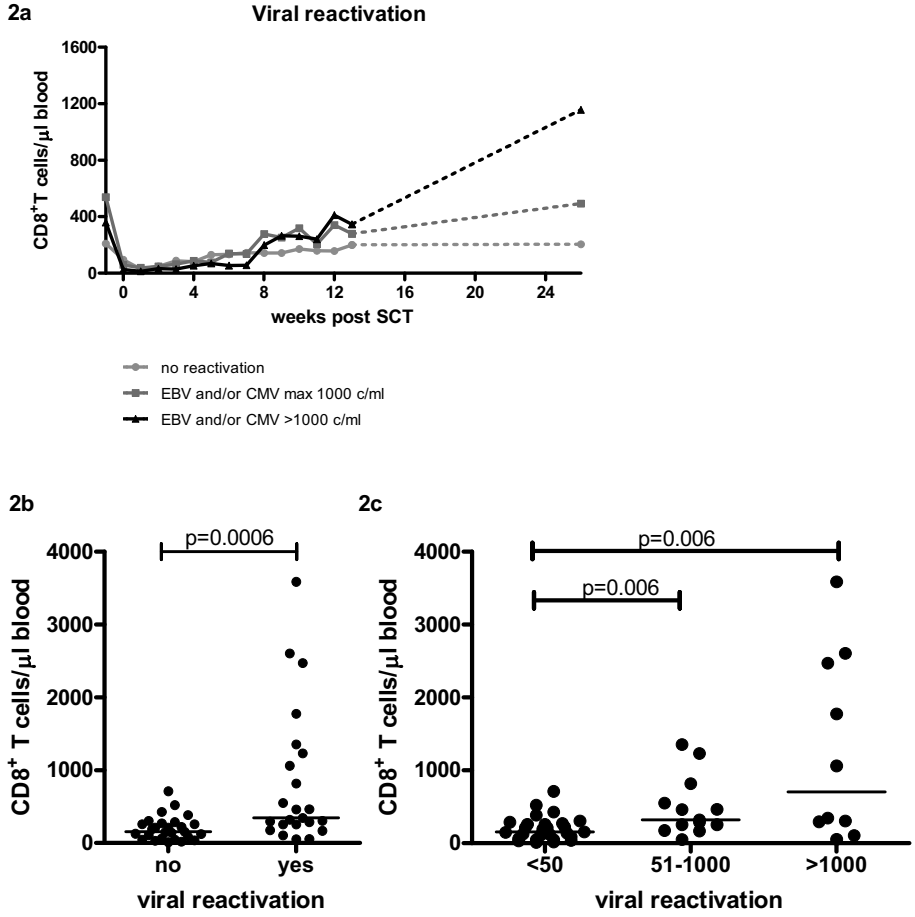

Figure 2. Longitudinal analysis CD8+ T-cell counts for a patient without, with low-level or high-level viral reactivation

A) Median CD8+ T-cell reconstitution for each reactivation category. Patients are subdivided based on their peak viral load of either EBV and or CMV. The median number of CD8+ T cells/µl blood is plotted weekly during first 3 months post SCT and at 6 months post SCT.

B) Dot plot showing the median number of CD8+ T cells/ µl at 6 months post SCT for patients without or with viral reactivation (EBV and/or CMV) reactivation.

C) Median CD8+ T-cell counts at 6 months post SCT for the different viral reactivation categories (<50: no detectable EBV and/or CMV viral load; 51-1000: detectable EBV and/or CMV load not exceeding 1000 copies/ml; >1000: detectable EBV and/or CMV load exceeding 1000 copies/ml)

To investigate whether poor T-cell reconstitution after SCT influences the onset of viral we analyzed the absolute number of CD8+ T cells early after SCT. The majority of patients started to reactivate within the first three months post SCT.[15] Therefore, we plotted the

median number of $CD8^+$ T cells at each weekly time point during the first three months post SCT in figure 2a. Patients were categorized based on the occurrence of either EBV and/or CMV reactivation and on the peak viral load exceeding 1000 copies/ml of either virus. Analysis of the median $CD8^+$ T-cell count of patients in each reactivation category did not show any significant difference between patients without, with low or with severe viral reactivation at all time points during the first 3 months post SCT (figure 2a).

Since there was no difference in early absolute $CD8^+$ T-cell numbers around the onset of viral reactivation we investigated the absolute $CD8^+$ T-cell count at 6 months post SCT, when most viral reactivations had resolved. Interestingly, at 6 months post SCT, patients that had developed a viral reactivation had significantly higher $CD8^+$ T-cell numbers (median 346 $CD8^+$ T cells/µl; range 50-3589 $CD8^+$ T cells/µl) compared to patients without viral reactivation (155 $CD8^+$ T cells/µl; range 12-713 $CD8^+$ T cells/µl) (p=0.0006) (figure 2b). When subdivided into low-level and high-level reactivations, the higher number of $CD8^+$ T cells was mainly observed in patients with high-level reactivations (704 $CD8^+$ T cells/µl; range= 54-3589 $CD8^+$ T cells/µl) (figure 2c). This implies that although T-cell numbers did not differ in patients with or without a viral reactivation in the first 3 months, viral reactivation promoted subsequent $CD8^+$ T cell reconstitution after SCT.

Increased $CD8^+$ T cell reconstitution after 3 months post SCT is CMV but not EBV driven

Patients who presented with viral reactivation (EBV and/or CMV) have higher absolute $CD8^+$ T-cell counts 6 months post SCT compared to patients that did not develop viral reactivation. To analyze whether this expansion of $CD8^+$ T cells is observed after both EBV and CMV reactivation, we investigated the impact of the viral reactivation on $CD8^+$ T-cell counts separately. Patients were categorized for each virus separately into no reactivation, low-level reactivation and high-level reactivation, not taking the other virus into consideration. Time dependent T-cell reconstitution was analyzed as indicated previously. For EBV the $CD8^+$ T-cell reconstitution developed similarly for all three reactivation categories (figure 3a). In contrast to the above data on combined viral infections, the patients without EBV reactivation had higher $CD8^+$ T-cell numbers (259 $CD8^+$ T cells/µl; range= 12-3589 $CD8^+$ T cells/µl)at 6 months post SCT compared to patients with EBV reactivation (109 $CD8^+$ T cells/µl; range 50-553 $CD8^+$ T cells/µl) (figure 3b). The group of patients with EBV reactivation (n=7) was too small to verify this statistically. We subsequently studied the distribution of CMV reactivation within the EBV reactivation subgroups (figure 3c). All of the patients with high $CD8^+$ T-cell numbers in the EBV no reactivation group were patients that had high-level CMV reactivation (open triangles in figure 3c) suggesting that CMV-specific reconstitution played an important role in the

growth of absolute CD8+ T-cell counts in the patients without EBV reactivation but with high CD8+ T-cell counts.

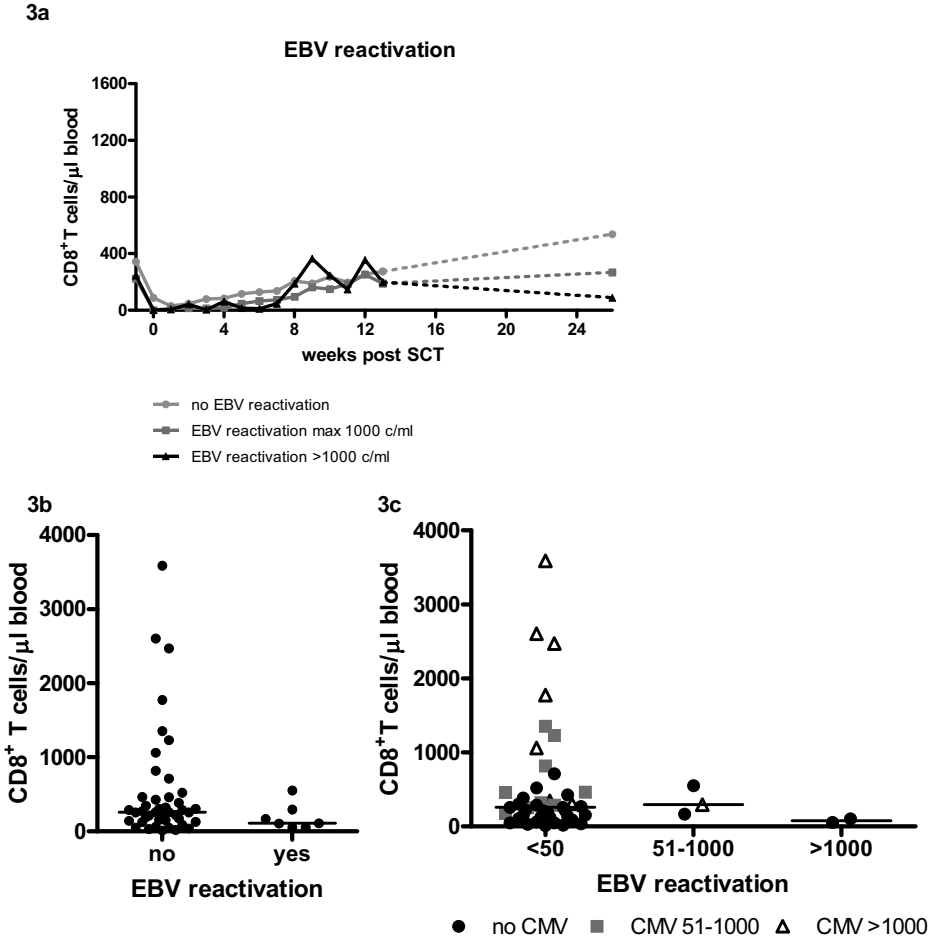

Figure 3. Longitudinal analysis of CD8+ T-cell count for a patient without, with low-level or high-level EBV reactivation

A) Patients are subdivided based on their peak EBV load. The median number of CD8+ T cells/µl blood is plotted weekly during first 3 months post SCT and at 6 months post SCT.

B) Dot plot showing the median number of CD8+ T cells/ µl at 6 months post SCT for patients without or with EBV reactivation.

C) Median CD8+ T-cell counts at 6 months post SCT for the different viral reactivation categories (<50: no detectable EBV viral load; 51-1000: detectable load not exceeding 1000 copies/ml; >1000: detectable EBV exceeding 1000 copies/ml)

Time dependent analysis of $CD8^+$ T-cell reconstitution within CMV reactivation categories showed that patients developing a high-level CMV reactivation showed an increase in $CD8^+$ T cell numbers around 9 weeks, which continued to expand until the end of follow up (figure 4a). At 6 months post SCT patients with CMV reactivation have significantly higher numbers of $CD8^+$ T cells (287 $CD8^+$ T cells/µl; range = 50-3589 $CD8^+$ T cells/µl) compared to patients without CMV reactivation (155 $CD8^+$ T cells/µl; range= 12-713 $CD8^+$ T cells/µl) ($p<0.0001$)(figure 4b). Upon subdivision between low and high-level CMV reactivation it is evident that the high numbers of $CD8^+$ T cells at 6 months post SCT occur in patients with high-level CMV reactivation (median 1419 $CD8^+$ T cells/µl; range= 295- 3589 $CD8^+$ T cells/µl) (figure 4c). In a previous study we determined that healthy individuals have an average $CD8^+$ T-cell number of 395 cells/µl blood. Our high-level CMV reactivating patients show a threefold increase in $CD8^+$ T-cell numbers compared to healthy controls. This shows that CMV reactivation triggers T- cell reconstitution after SCT resulting in high numbers of $CD8^+$ T cells after high-level viral reactivation, while EBV reactivation seems to play a minimal role in the $CD8^+$ T-cell reconstitution.

The majority of $CD8^+$ T cells 6 months post SCT in patients with high-level CMV reactivation are CMV-specific

Since high-level CMV reactivation results in high numbers of $CD8^+$ T cells at 6 months post SCT, we next investigated whether these cells were CMV-specific. To this end we analyzed PBMC from 15 patients at 2 and 6 months post SCT in an IFNγ ELIspot after stimulation with EBV (BZLF-1 and EBNA-1) and CMV (pp65) peptide pools. Responses were defined as positive when at least 100 IFNγ producing T cells/ $1x10^6$ PBMC were detected (after subtraction of negative control) (table II). Representative ELIspot results of 3 patients at 2 and 6 months post SCT after stimulation with pp65 or BLZF-1 are shown in figure 5. EBV-specific T cells (BZLF-1) were detected in only 1 patient at 2 months post SCT (157 $IFN\gamma^+$ cells/$1x10^6$ PBMC) (patient # 6). At 6 months post SCT 3 patients (#7,8,13) had EBNA-1 specific T cells (range 100-140 $IFN\gamma^+$ cells/$1x10^6$ PBMC) and 3 patients (#8,9,13) had BZLF-1 specific T cells (range157-1463 $IFN\gamma^+$ cells/$1x10^6$ PBMC) , while 8 patients developed an EBV reactivation (of which 5 were high-level). CMV-specific responses were more abundantly detected. At 2 months post SCT already 5 patients (#3,6,7,10,14) had pp65 specific responses (range 100- 907 $IFN\gamma^+$ cells/$1x10^6$ PBMC). This increased to 8 patients (range 157-2557 $IFN\gamma^+$ cells/$1x10^6$ PBMC) at 6 months post SCT, of which most (n=7) developed high-level CMV reactivation and 1 patient a low-level CMV reactivation. Another patient (#10) developed a severe CMV reactivation with a maximum viral load of 1520000 copies/ml. CMV-specific T cells were detected at 2 months post SCT, however at 6 months they became undetectable (figure 5a). This patient was diagnosed with CMV disease and died of respiratory insufficiency probably

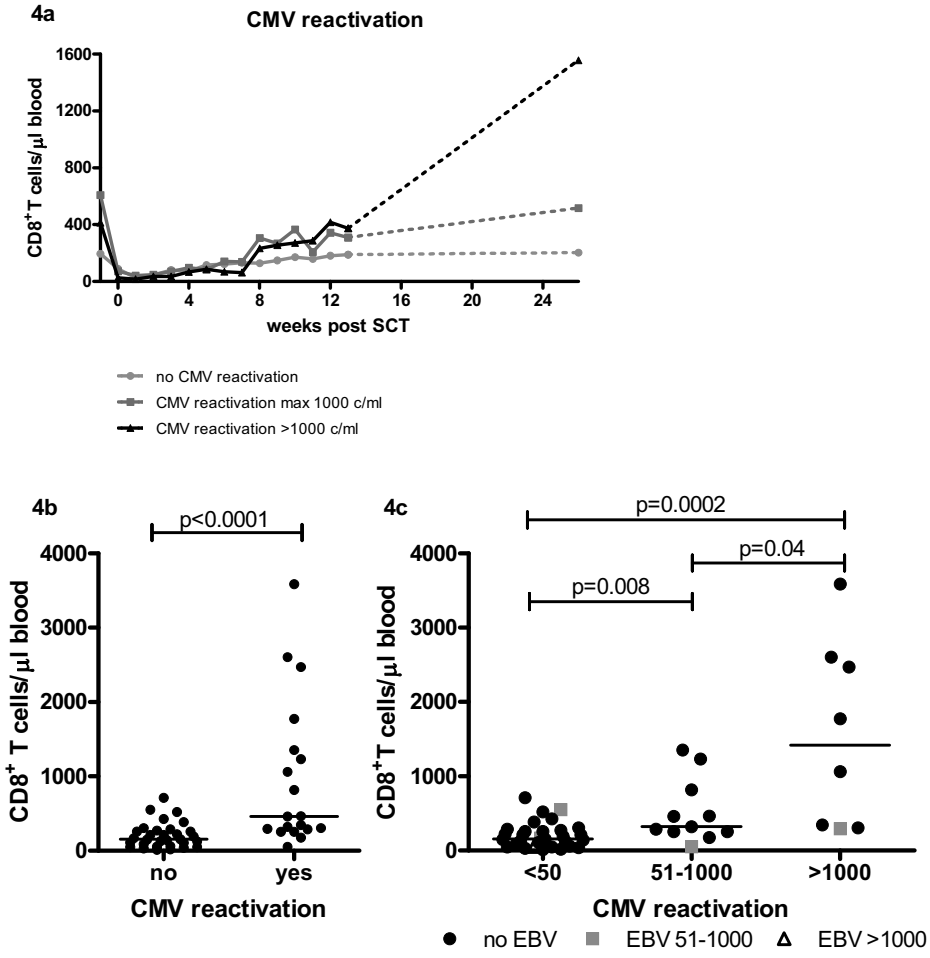

Figure 4. Longitudinal analysis of CD8+ T-cell count for a patient without, with low-level or high-level CMV reactivation

A) Patients are subdivided based on their peak CMV load. The median number of CD8+ T cells/µl blood is plotted weekly during first 3 months post SCT and at 6 months post SCT.

B) Dot plot showing the median number of CD8+ T cells/ µl at 6 months post SCT for patients without or with CMV reactivation.

C) Median CD8+ T-cell counts at 6 months post SCT for the different viral reactivation categories (<50: no detectable CMV viral load; 51-1000: detectable load not exceeding 1000 copies/ml; >1000: detectable CMV exceeding 1000 copies/ml)

caused by CMV pneumonia 8 months post SCT. Absence of CMV specific-T cells in this patient can be considered as evidence for a lack of viral control resulting in the severe complication. Since most patients with high-level CMV reactivation have CMV-specific

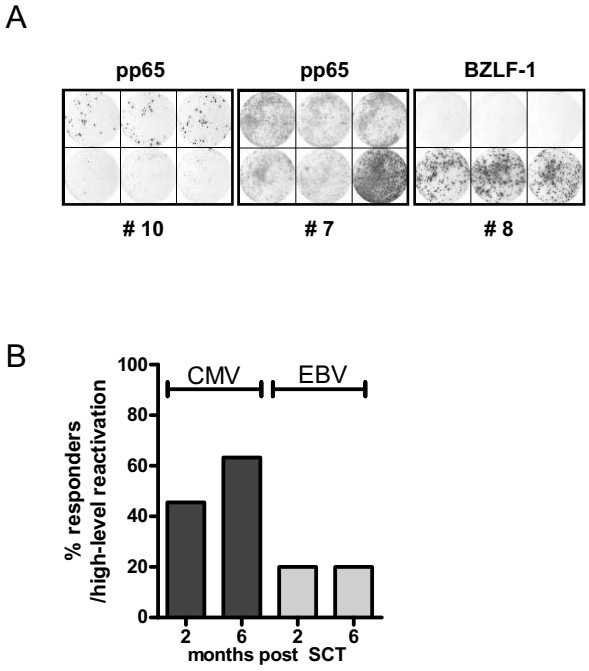

Figure 5. CMV and EBV specific T cells early and late after SCT

A) Representative ELIspot of IFNγ producing T cells (in triplicate) following stimulation with pp65 (two left panels) and BZLF-1 (right panel). Top row show IFNγ production 2 months post SCT, bottom row is of 6 months post SCT. Samples are from patients number 10, 7 and 8 respectively.

B) Percentages of high-level reactivating patients that have virus specific responses at 2 and 6 months post SCT. Responses against CMV (pp65) at 2 and 6 months are shown in the two left bars, responses against EBV (BZLF-1 or EBNA-1) in the two right bars (t=2 and t=6).

responses 6 months post SCT (63,6%) while only 1 patient with high-level EBV reactivation has EBV-specific T cells detectable at 6 months post SCT (20%) (figure 5b), this also suggest that CMV reactivation has a strong influence on T-cell reconstitution post SCT and that rapid expansion of CMV-specific T cells after high-level reactivation results in high $CD8^+$ T cell numbers post SCT.

DISCUSSION

Persistent viruses, like EBV and CMV, are normally controlled through cytotoxic T cell responses[1;4;5]. Adequate T-cell reconstitution after SCT is therefore crucial in preventing viral reactivation progressing to severe complications. This has been demonstrated by Meij *et al*, who showed that incorporating EBV-specific T-cell reconstitution data with

viral load monitoring increases the positive predictive value for EBV-associated PTLD from 39% to 100%[9]. Another study, by Annels *et al* used an arbitrary threshold of 300 $CD3^+$ T cells/µl blood during the initial phase of EBV reactivation to determine adequate T-cell reconstitution and the subsequent need for pre emptive therapy[10]. However, these studies investigated the role of T-cell reconstitution in prevention of viral complications. In our study, we were interested in the role of the T cells in the onset of the viral reactivation itself and whether lack of early T-cell reconstitution triggers development of viral reactivation and plays a role in subsequent reactivation severity (identified by maximum viral load).

In contrast to the general belief, we found that early T-cell reconstitution after SCT does not play a role in the onset of viral reactivation as numbers of $CD8^+$ T cells during the first 3 months post SCT are similar in patients with or without viral reactivation. However, other studies have shown that the conditioning regime prior to SCT as well as in-vivo T-cell depletion around time of transplantation play an important role in the T-cell reconstitution rates after SCT. In-vivo T-cell depletion through alemtuzumab has been shown to delay both $CD4^+$ and $CD8^+$ T-cell reconstitution and ATG administration results in a delayed $CD4^+$ T-cell reconstitution[16]. In the latter study the total $CD8^+$ T cell count was also investigated in relation to CMV infection and no significant association was seen between infection and $CD8^+$ T cell numbers at 1 month post SCT suggesting that even earlier differences (prior to 1 month post SCT) in T-cell reconstitution might contribute to an increase risk for CMV reactivation. In our study the earliest time point is 1 week post SCT. Our longitudinal analysis also showed no difference in T-cell reconstitution rates this early after SCT. However, also in our study, we did observe a significant difference in T-cell counts early after SCT between patients who received ATG and patients that did not. During the time of average onset of viral reactivation, there was no significant difference in T-cell counts between patients that did or did not receive ATG (data not shown). However, we cannot exclude that viral reactivation was already initiated earlier after SCT and not detected yet.

Based on these findings, we postulate that onset of viral reactivation is not influenced solely by a lack of $CD8^+$ T-cell control. In line with this, we showed in a previous study that CMV reactivations occur more frequently in recipients of CMV seropositive grafts (F.L. Pietersma *et al*, manuscript in press). Therefore, we hypothesize that re-infections with CMV strains from donor origin may be an important source of viral reactivation post SCT. Further studies could elucidate the origin of the viral reactivation. Even though we showed that early T-cell reconstitution does not influence the onset of viral reactivation, the outcome of viral reactivation, determined by severity and disease, has been shown to be determined through T-cell control. Several studies have described the association between virus-specific T cells and CMV or EBV related complications[9;10;17;18]. In our study we show a strong impact of high-level CMV reactivation on the number of total $CD8^+$ T

cells following reactivation (at 6 months post SCT). Even more, these $CD8^+$ T-cell counts are much higher compared to healthy individuals. In a previous study we determined absolute T-cell counts in a cohort of healthy individuals (n=15) (F.L. Pietersma *et al*, manuscript submitted). The median $CD8^+$ T-cell count was 374 $CD8^+$ T cells/µl blood. However, our high-level CMV reactivating patients had a median $CD8^+$ T-cell count of 1556 $CD8^+$ T cells/µl blood. Studies in healthy individuals have shown that CMV infection results in higher absolute $CD8^+$ T-cell numbers as well as a more differentiated phenotype of the T-cell pool[19-22]. This is in concordance with our findings that high-level CMV reactivation results in a rapid expansion of the $CD8^+$ T-cell pool. This could result in a false positive perception of adequate T-cell reconstitution. Although high numbers of T cells are present, this T-cell pool has a restricted repertoire which may be detrimental for the host. Therefore, monitoring T-cell reconstitution alone could lead to a skewed interpretation of adequate T-cell immunity since high-level CMV reactivation can result in a restricted T-cell repertoire. Investigating the reconstitution of the naïve T cell compartment could provide a better indication of T-cell reconstitution post SCT.

Surprisingly, the rapid expansion of T cells was not seen in patients with high-level EBV reactivations, suggesting that this rapid expansion is not a phenomenon caused by other viruses but is CMV-specific. Patients that developed high-level EBV reactivation have much lower $CD8^+$ T-cell numbers compared to patients that developed high-level CMV reactivation, even though high number of EBV-specific T cells can be reached for example during Infectious Mononucleosis[23]. One explanation why CMV drives T-cell expansion after viral reactivation to a much larger extend than EBV could be the broader infection targets. CMV is capable of infecting a wider range of cell types and moreover, capable of infecting dendritic cells[24] which can stimulate $CD8^+$ T cells directly, resulting in better priming of the $CD8^+$ T-cell pool.

Most studies investigating T-cell reconstitution after allogeneic SCT focus on one virus alone. In our study we analysed data with respect to EBV and CMV separately as well as combined which provides a much better view of the contribution of each virus to the general T-cell reconstitution. Given these data, the impact of other viruses on the T-cell reconstitution cannot be omitted/excluded. Further studies should involve other common viruses such as HHV-6 and infection with adenovirus to analyse their impact on general T-cell reconstitution post SCT. Even more, our data indicate that absolute T-cell numbers can provide a biased view on the T-cell reconstitution especially considering the quality of the T-cell response. Therefore, we suggest that functional and phenotypical markers should be included when analyzing general T-cell reconstitution post SCT.

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Chapter 7

CMV-reactivation influences T-cell differentiation and CMV-specific T-cell reconstitution after stem cell transplantation

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Submitted

ABSTRACT

Background: CMV-specific T cells were shown to be important for protection against CMV-disease in SCT recipients. Here we investigated specific T-cell features like effector cell differentiation and perforin-expression as well as CMV-specific T cells after SCT in relation to CMV-reactivation.

Methods: $CD4^+$ and $CD8^+$ T-cell characteristics (differentiation, activation and functional CMV-specific immunity) of SCT patients with (n=13) or without (n=8) CMV-reactivation were analysed longitudinally by flow cytometry. CMV-specific IFNγ-production as measured by intracellulair staining and proliferation as measured by CFSE dye dilution were analysed after stimulation with overlapping peptide pools of the tegument protein pp65 and immediate early antigen 1.

Results*:* A more differentiated phenotype, up-regulation of the activation markers CD38 and HLA-DR on $CD4^+$ T cells and increased expression of perforin on $CD8^+$ T cells was more frequently observed in patients with CMV-reactivation compared to patients without reactivation. Interestingly, these T-cell features were often already different early after SCT. In addition, CMV-specific $CD8^+$ T-cell responses, both based on IFNγ-production as well as proliferation, directed against both pp65 and IE1 tended to be present more frequently in patients with CMV-reactivation compared to patients without reactivation.

Conclusion: These data suggest that CMV-reactivation influences CMV-specific T-cell reconstitution after SCT and that early T-cell differentiation differences may be helpful in predicting viral reactivations.

INTRODUCTION

CMV is a widespread persistent human β–herpesvirus (1-3). Serious illness can develop during primary infection or reactivation of the virus in immunocompromised patients, such as after SCT. Timely reconstitution of CMV-specific T-cell responses has been reported to be important for protection against disease in SCT (4-9) and SOT(10-12). A similar pattern of reconstitution of CMV-specific $CD4^+$ and $CD8^+$ T cells after SCT was observed.(13), indicating that both $CD4^+$ and $CD8^+$ T cells are important for control of CMV-infection. It is known that CMV-infection drives T cells to an effector phenotype in healthy individuals(14). In renal transplant patients a dramatic change in phenotype has been observed after primary infection, resulting in increased numbers of CMV-specific $CD45RA^+CD27^-CCR7^-$ or $CD45RO^+CD27^-CCR7^-$ $CD8^+$ T cells(15). Differences in $CD8^+$ T-cell function were demonstrated to depend on differentiation status(16).
As it is unclear whether CMV-reactivation is required for CMV-specific T-cell reconstitution or whether absence of CMV-specific T cells is responsible for lack of viral control, we investigated specific T-cell features like effector cell differentiation and perforin expression as well as CMV-specific T cells after SCT in relation to CMV-reactivation.

PATIENTS AND METHODS

Study population and conditioning regimen

Between October 2005 and September 2006, 65 patients received a SCT at the University Medical Center Utrecht. Patients were included when either donor or recipient serostatus was CMV-positive (n=56) and when bloodsamples were available from at least two time points (n=53). Only patients without EBV-reactivation were included (n=22), to be able to observe effects of CMV-reactivation only. As a result 14 SCT patients with CMV-reactivation and 8 without reactivation were included. Patient characteristics are shown in Table 1*. Blood samples were drawn just before conditioning for SCT and 2, 4, 6, 9 and 12 months after SCT. PBMC were isolated by Ficoll-hypaque density centrifugation and cryopreserved. The study was approved by the local ethical committee and all participants gave written informed consent.

CMV and EBV monitoring

CMV and EBV-monitoring was based on realtime TaqMan™ CMV or EBV DNA PCR assay in EDTA anticoagulated plasma(30-33) and performed weekly in all patients until day 120 post-transplantation. Pre-emptive antiviral therapy with valganciclovir (twice daily 900

mg) was initiated when CMV-DNA load exceeded 500 copies/ml plasma. Valaciclovir was given to all patients prophylactic (2x 500 mg daily). Viral reactivation and/or infection were defined as CMV load exceeding 50 copies/ml plasma.

Analysis of lymphocyte markers by flow cytometry

Differentiation and activation status of lymphocytes was analysed by six-colour fluorescence flowcytometry. To this end, PBMC were stained with CD3-PerCP, CD4-PE Cy7, CD8-APC Cy7, in combination with either CD45RO-PE, CD27-APC and PD-1 FITC or HLA-DR FITC (Becton Dickinson, San José, California, United States) and CD38 PE (Sanquin, Amsterdam, The Netherlands). Before staining with Perforin-FITC (BD) cells were permeabilized (FACS Permeabilizating Solution and FACS Lysis Solution, BD), washed and stained with specific antibodies. 100,000 cells were acquired by the LSRII flow cytometer (BD). Based on the expression of CD45RO and CD27, T cells were divided into naïve ($CD27^+CD45RO^-$), central-memory ($CD27^+$, $CD45RO^+$) and effector-memory ($CD27^-CD45RO^+$)or effector ($CD27^-CD45RO^-$) T-cell populations (34).

T-cell stimulation

PBMC were stimulated with overlapping peptide pools consisting of 15-mer peptides with 11 amino acid overlap. Peptide pools spanned the entire pp65 or IE-1 protein (138 and 120 peptides, respectively) (Mimotopes, Melbourne, Australia) and consisted of a final concentration of 1 mg/ml of each peptide, dissolved in DMSO.

Intracellular cytokine staining after antigenic stimulation

1-2*10^6 PBMC, were stimulated for 6 hours with pp65 or IE1 peptide pool (2 µg/ml) and anti-CD28 (1 µg/ml) as costimulation at 37˚C. After one and half hour 1:1000 Monensin was added (Golgistop, BD Biosciences) to allow accumulation of cytokines in the cytosol.. As a negative control PBMC were stimulated with medium and co-stimulation alone. As a positive control PBMC were stimulated with PMA (10 ng/ml) and ionomycin (2 µg/ml). PBMC were washed and stained with CD3-PerCP and CD4-APC Cy7(BD), permeabilized (FACS Permeabilizating Solution and FACS Lysis Solution, BD), washed again and stained with specific antibodies for IFNγ-APC and IL-2-PE (BD). 200,000 cells were acquired by the LSRII flow cytometer (BD) and data were analysed by BD FACSDiva software. In case of IFNγ- and IL-2-measurements, the number of responding T cells was calculated after subtraction of the negative control values.

Antigen-specific T-cell proliferation

PBMC were pelleted at a final concentration of $6*10^6$ PBMC/ml and labelled with CFSE (Molecular Probes) according to the manufacturer's protocol. $1\text{-}2*10^6$ PBMC were stimulated with 2 µg/ml pp65 or IE-1 peptide pool or with anti-CD3 and anti-CD28 (positive control) or medium (RPMI with pen/strep and 10% human pooled serum) alone (negative control) for 5 days at 37˚C. PBMC were stained with CD3 PerCP, CD8 APC (BD) and CD4 APC Cy7 (BD Pharmingen), and 200,000 cells were acquired by the LSRII flow cytometer (BD) and analysed by BD FACSDiva software. Stimulation indices (SI) were calculated by dividing the percentage proliferating cells after stimulation with antigen by the percentage of proliferating cell after culture with medium.

Statistical analysis

Statistics were computed by SPSS 15.0 for Windows (SPSS, Inc., Chicago, Illinois, USA). Differences between groups were compared using the Fisher's Exact test in case of discrete variables. In case of continues variables the Mann-Whitney test was used. *P*-values <0.05 were considered significant.

RESULTS

Characteristics of SCT recipients with and without CMV reactivation

CMV-reactivation occurred in 14 out of 22 SCT patients (64%), mostly within 2 months after SCT (86%).(Table 1). Only donor and recipient CMV-serostatus were associated with CMV-reactivation after SCT (p=0.010).

CMV-reactivation skews to a more differentiated T-cell phenotype

As CMV-infection drives T cells to a differentiated phenotype in healthy subjects (14), we investigated the influence of CMV-reactivation on phenotype differentiation of $CD4^+$ and $CD8^+$ T cells after SCT. Figure 1A shows a representative T-cell phenotype development after SCT of a patient with and without CMV-reactivation. Interestingly, $CD8^+$ T cells from patients without CMV-reactivation consisted for the major part of naïve ($CD45RO^-/CD27^+$, range 54-65% post-SCT) T cells and showed a slow recovery of naïve and memory T cells (Fig. 1A, lower panel). Percentages of naïve T cells after SCT were significantly higher in patients without reactivation compared to those with CMV-reactivation (p=0.008, Fig. 1B). $CD8^+$ T cells from reactivating patients mainly

Table I. Patient characteristics

		n	%	n	%	n	%	univariate p-value
total included		22		14		8		
Sex	**male**	11	50	8	57	3	38	0.659
	female	11	50	6	43	5	63	
median age	**(range)**	56	(18-66)	58	(19-66)	52	(18-61)	0.165
diagnosis	**AML**	8	36	5	36	3	38	0.423
	MM	5	23	2	14	3	38	
	NHL	6	27	5	36	1	13	
	hodgkin	1	5	1	7	0	0	
	ALL	1	5	1	7	0	0	
	MDS	1	5	0	0	1	13	
HLA mismatch		2	9	2	14	0	0	0.515
Donor	**related**	17	77	11	79	6	75	>.99
	unrelated	5	23	3	21	2	25	
Conditioning	**NMA**	19	86	13	93	6	75	0.527
	MA	3	14	1	7	2	25	
ATG		7	32	4	29	3	38	>.99
CMV	**R+/D+**	8	36	8	57	0	0	0.010
serostatus	**R+/D-**	9	41	5	36	4	50	
	R-/D+	5	23	1	7	4	50	
	R-/D-	0	0	0	0	0	0	

AML, acute myeloid leukemia; MM, multiple myeloma; NHL, non-hodgkin lymphoma; ALL, acute lymphatic leukemia; MDS, myelodysplastic syndrome; NMA, non-myeloablative; MA, myeloablative; ATG, anti-thymocyte globulin; CMV, cytomegalovirus. Differences between groups were compared using Fisher's Exact test for discrete variables and Mann-Whitney U-test for continuous variables.

consisted of effector-memory ($CD45RO^+/CD27^-$) and effector ($CD45RO^-/CD27^-$) T cells which recovered shortly after SCT and increased further after SCT (Fig 1A, upper panel). Percentages of effector-memory T cells after SCT were significantly increased in the patient group with CMV-reactivation (p=0.006, Fig 1B). Already before SCT, percentages of effector-memory $CD8^+$ T cells, but not $CD4^+$ T cells, were increased in patients with CMV-reactivation (p=0.002, Fig 1B), although there is no knowledge of CMV-reactivation at that time.

Therefore, we investigated the impact of CMV-serostatus of donor and recipient on the differentiation of $CD8^+$ T cells, which may explain the observed effector-memory T-cell dominance pre-SCT. The group with detectable CMV load post SCT was represented by only 1 R^-/D^+ patient who developed CMV-disease and was left out of this analysis. We compared the R^+/D^+ and R^+/D^- patients with CMV-reactivation (n=8 and n=5, respectively) and the R^+/D^- and R^-/D^+ patients without CMV-reactivation (n=4 and n=4, respectively; Fig 1C). The most differentiated phenotype was observed in the R^+/D^+ patients with CMV-reactivation. After SCT, percentages of naïve ($CD45RO^-/CD27^+$) and

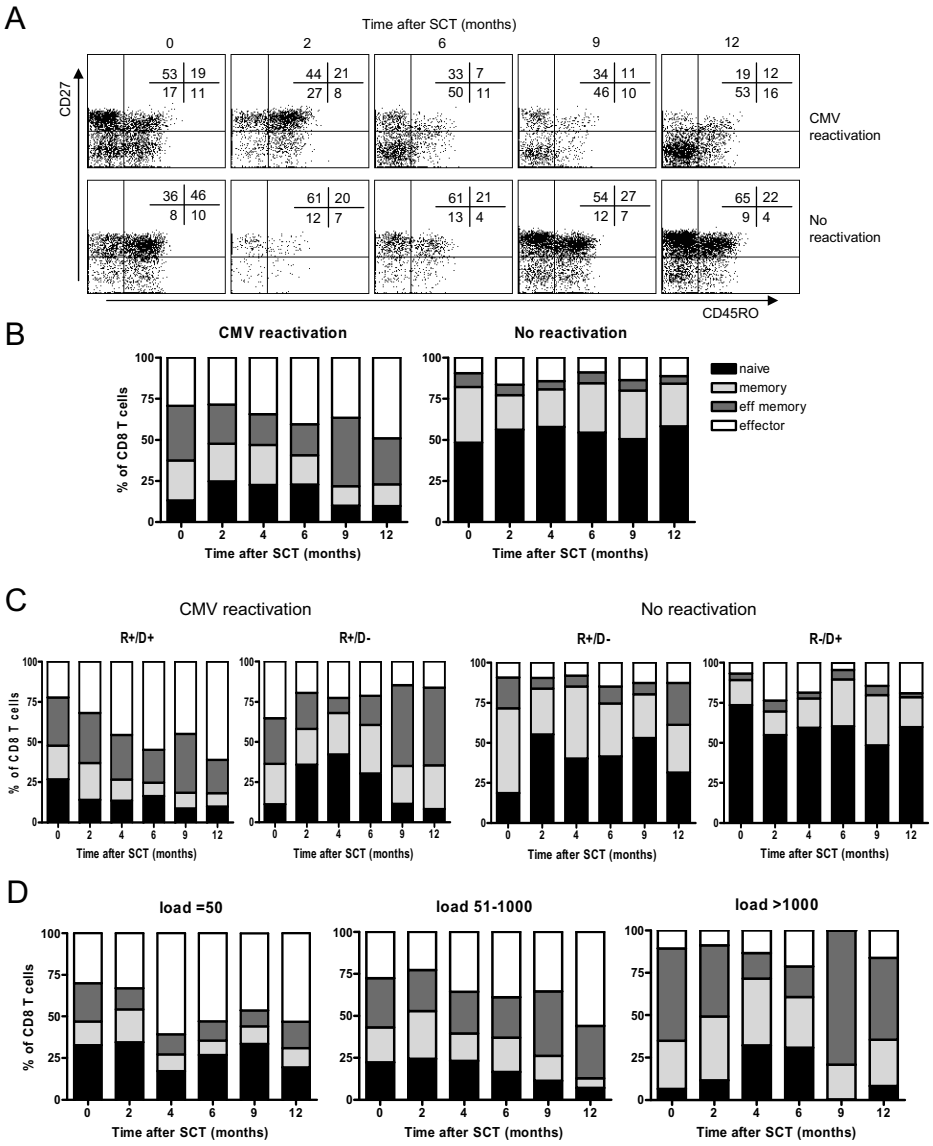

Figure 1. Longitudinal phenotype differentiation after SCT in patients with and without CMV reactivation.

A) Representative examples of phenotype differentiation of $CD8^+$ T cells from a patient with (upper panel) and without CMV-reactivation (lower panel). Percentages in each quadrant are shown in the upper right corner.

B) Mean percentage of naïve ($CD45RO^-/CD27^+$, black), central-memory ($CD45RO^+/CD27^+$, light grey), effector-memory ($CD45RO^+/CD27^-$, dark grey) and effector ($CD45RO^-/CD27^-$, white) $CD8^+$ T cells in patients with and without CMV-reactivation.

C) Phenotype differentiation of $CD8^+$ T cells depending on recipient and donor CMV-serostatus in patients with and without CMV-reactivation.
D) Phenotype differentiation of patients with CMV reactivation depending of the height of the peak viral load within one year post-SCT (copies/ml).

central-memory ($CD45RO^+/CD27^+$) $CD8^+$ T cells were significantly decreased in the R^+/D^+ patients compared to the R^+/D^- patients with CMV-reactivation (p=0.030 and p=0.045, respectively).

To determine the effect of the viral load level on differentiation of $CD8^+$ T cells, patients with CMV-reactivation where divided into three groups based on their peak viral load level within one year post-SCT (Fig. 1D). Interestingly, the patient group with a low (detectable, $\leq$50 copies/ml, n=3) or intermediate (50-1000 copies/ml, n=7) peak viral load tended to differentiate towards the so-called terminally-differentiated effector ($CD45RO^-/CD27^-$) $CD8^+$ T cells, whereas in the group with high viral peak load (>1000 copies/ml, n=3) more effector-memory ($CD45^+/CD27^-$) $CD8^+$ T cells were observed.

Effect of CMV-reactivation on immune activation and expression of perforin

Next, generalized activation of T lymphocytes by expression of both HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells was measured. Increased activation was apparent in $CD4^+$ T cells, but not in $CD8^+$ T cells, in patients with CMV-reactivation after SCT (p=0.037; fig. 2A). This difference in activation was already present before SCT (T0, p=0.011). Programmed death (PD)-1 receptor, a negative regulator of T-cell activity which is up-regulated after activation, has been suggested to be a prognostic indicator of CMV-disease in solid-organ transplant patients(17). Figure 2B shows that PD-1 expression was similar between both groups in $CD4^+$ as well as $CD8^+$ T cells. Interestingly, high PD-1 expression (MFI >500) was only observed in patients with a viral load >1000 copies/ml on $CD4^+$ as well as $CD8^+$ T cells (data not shown).
The expression of perforin was measured in $CD8^+$ T cells, as a marker for effector $CD8^+$ T cells with cytolytic activity. Figure 2C shows representative FACS stainings of perforin-expression by $CD8^+$ T cells from a patient with (left panel) and without CMV-reactivation (right panel), two months post SCT. In patients with CMV-reactivation, perforin-expression was significantly increased (p=0.045, median perforin-expression by CD8+ T cells from all time points: 23.9%) compared to patients without CMV-reactivation (median perforin-expression by $CD8^+$ T cells from all time points: 9.0%; Fig 2C and D).

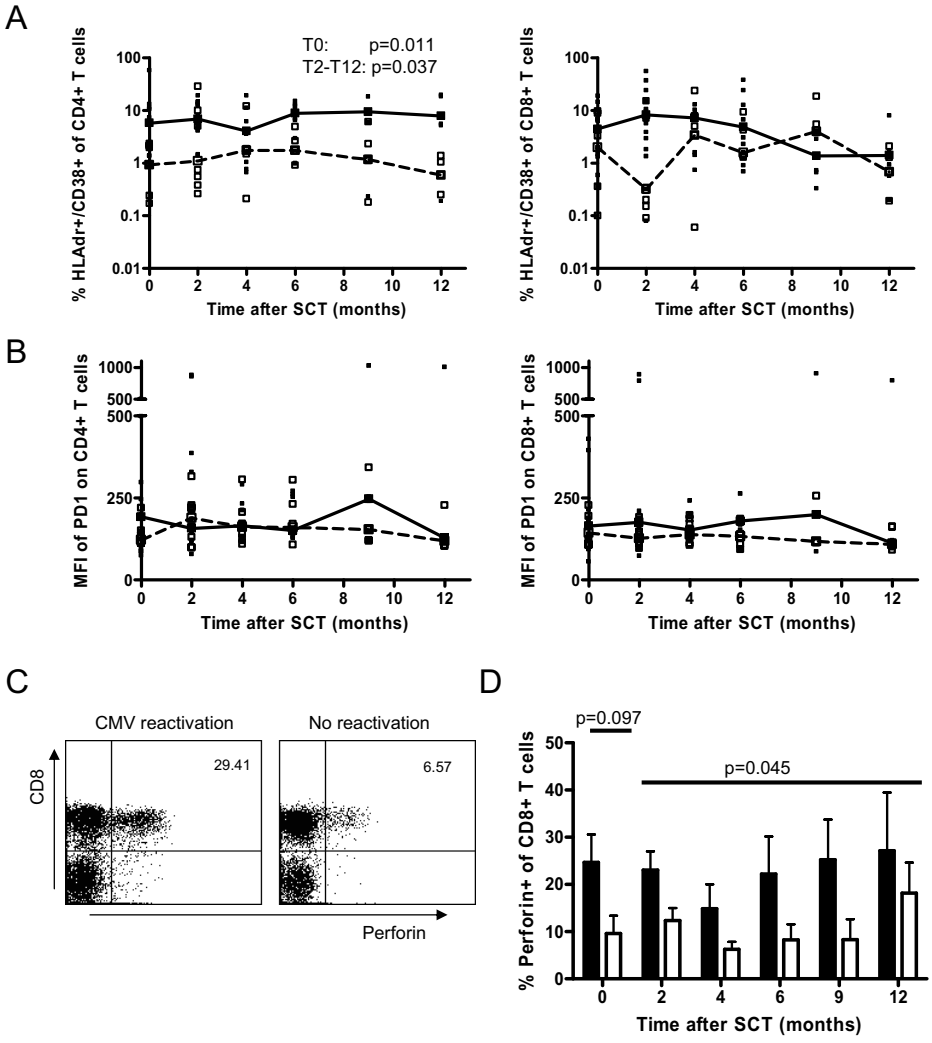

Figure 2. T-cell activation, PD-1 and perforin expression in patients with and without CMV reactivation Percentage of HLAdr$^+$/CD38$^+$ (A) and MFI of PD-1 (B) in CD4$^+$ and CD8$^+$ T cells of patients with (black square; median straight line) and without (open square; median dashed line) CMV-reactivation. C) Representative example of perforin-expression by CD8$^+$ T cells in a patient with and without CMV-reactivation, two months after SCT. Percentages of perforin$^+$ T cells within the CD8$^+$ T cells are indicated in the upper right corner. D) Mean (and S.E.M.) percentage of perforin-expression in patients with (black bars) and without (white bars) CMV-reactivation.

CMV-specific T-cell responses are dominated by pp65

We measured CMV-specific $CD4^+$ and $CD8^+$ T-cell responses towards pp65 and IE1 overlapping peptide pools in patients with and without CMV-reactivation. The maximum T-cell response against CMV-antigen within one year post-SCT is shown in figure 3A. Pp65-specific $CD8^+$ T-cell responses tended to be more frequently (8 out of 13 patients, 62%) above background (0,2% of $CD8^+$ T cells, measured in healthy CMV-seronegative controls) in patients with CMV-reactivation compared to patients without reactivation (2 out of 8 patients, 25%). Although less pronounced, also IE1-specific $CD8^+$ T-cells responses tended to be more regularly detected in patients with CMV-reactivation (5 out of 13 patients, 38%) compared to patients without CMV-reactivation (2 out of 8 patients, 25%). Differences in $CD4^+$ T-cell responses directed against either pp65 or IE1 were not observed. In addition, IL-2 production by $CD4^+$ and $CD8^+$ T cells was very low in both groups (data not shown).

IE1-specific T-cell proliferation is mainly present in patients with CMV-reactivation

Proliferative capacity in response to either pp65 or IE1 was studied by CFSE dye dilution. Proliferative capacity in response to pp65 was observed in $CD4^+$ and $CD8^+$ T cells in both patients with and without CMV-reactivation (Fig 3B)[#]. However, responses towards IE1 were absent or very low in patients without CMV-reactivation, whereas proliferative capacity was present in both $CD4^+$ and $CD8^+$ T cells in approximately half of the patients with CMV-reactivation.

Immunological features of a SCT patient with primary CMV-infection

We analysed features and function of T-cell responses during primary CMV-infection in a patient who was CMV-seronegative before transplantation and was transplanted with stem cells from a CMV-seropositive donor[&]. $CD8^+$ T cells differentiated to predominantly effector-memory and effector T cells (Fig. 4A) and were highly activated, as measured by the expression of both HLA-DR and CD38 (reaching up to 78% of $CD8^+$ T cells). During acute CMV-infection the percentage $perforin^+CD8^+$ T cell strongly increased (from undetectable to 58% of $CD8^+$ T cells), higher than patients with CMV-reactivation (median 10,7% of $CD8^+$ T cells), and remained high for at least one year post-SCT, though viral load was undetectable after treatment. Two and four months post SCT no CMV-specific T-cell responses were observed. More than four months after SCT CMV-specific immunity started to reconstitute. Although IL-2 producing $CD4^+$ and $CD8^+$ T cells were hardly present (Fig. 4D), both $CD4^+$ and $CD8^+$ T cells produced IFNγ (Fig 4B) and displayed proliferative capacity in response to pp65 and IE1 (Fig. 4C). Especially $CD8^+$ T-cell responses directed towards IE1 were high and remained at least until one year post-SCT (Fig 4D).

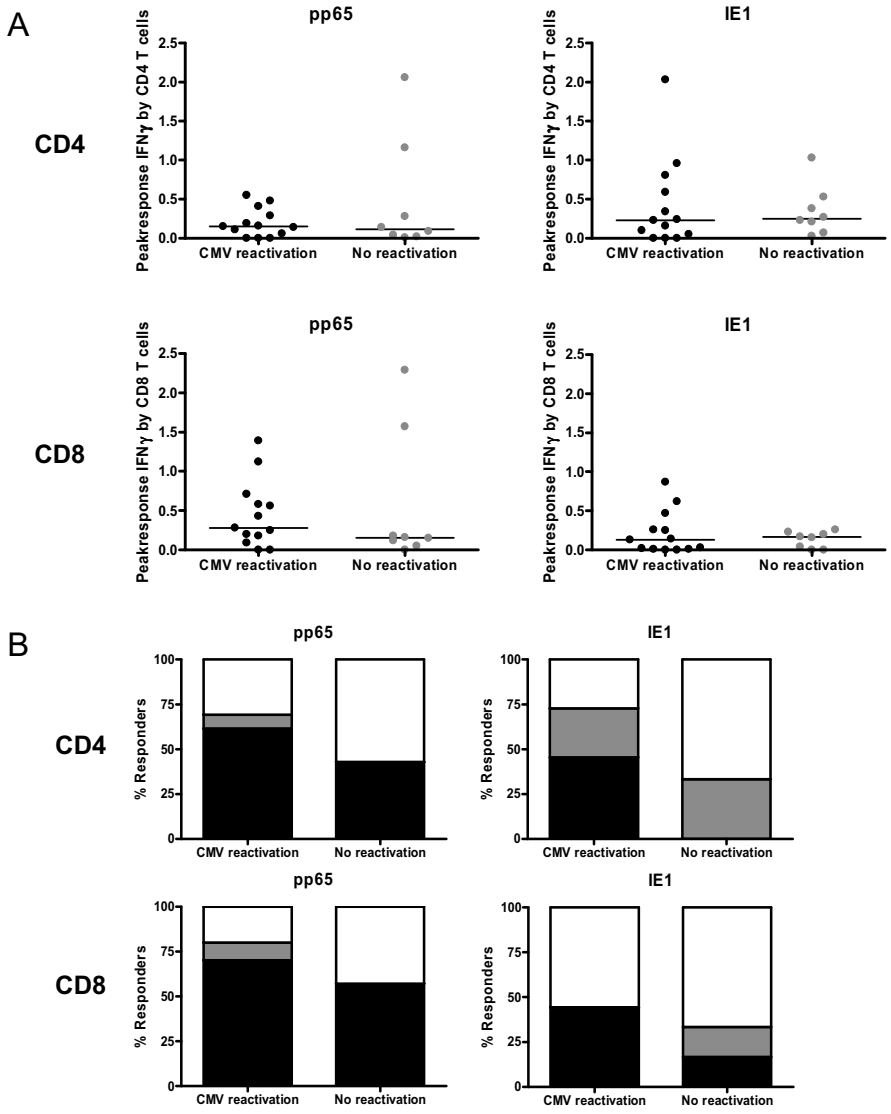

Figure 3. CMV-specific IFNγ-production and proliferative capacity

A) IFNγ-production by $CD4^+$ and $CD8^+$ T cells in response to pp65 or IE1 in patients with and without CMV-reactivation after SCT were measured by ICCS. Presented are the maximum T-cell responses within one year post-SCT. The median is indicated by a straight line.

B) Proliferative capacity of $CD4^+$ and $CD8^+$ T cells in response to pp65 or IE1 was measured by CFSE-labelling. At each time point SI was determined and categorized into good (S.I.>2.0), intermediate (1.5<S.I.<2.0)or no proliferative capacity (S.I.<1.5). Presented is the percentage of patients with most responses after SCT in the represented category. Good proliferative capacity S.I.>2.0, black; Intermediate proliferative capacity 1.5<S.I.<2.0, grey; No proliferative capacity S.I.<1.5, white.

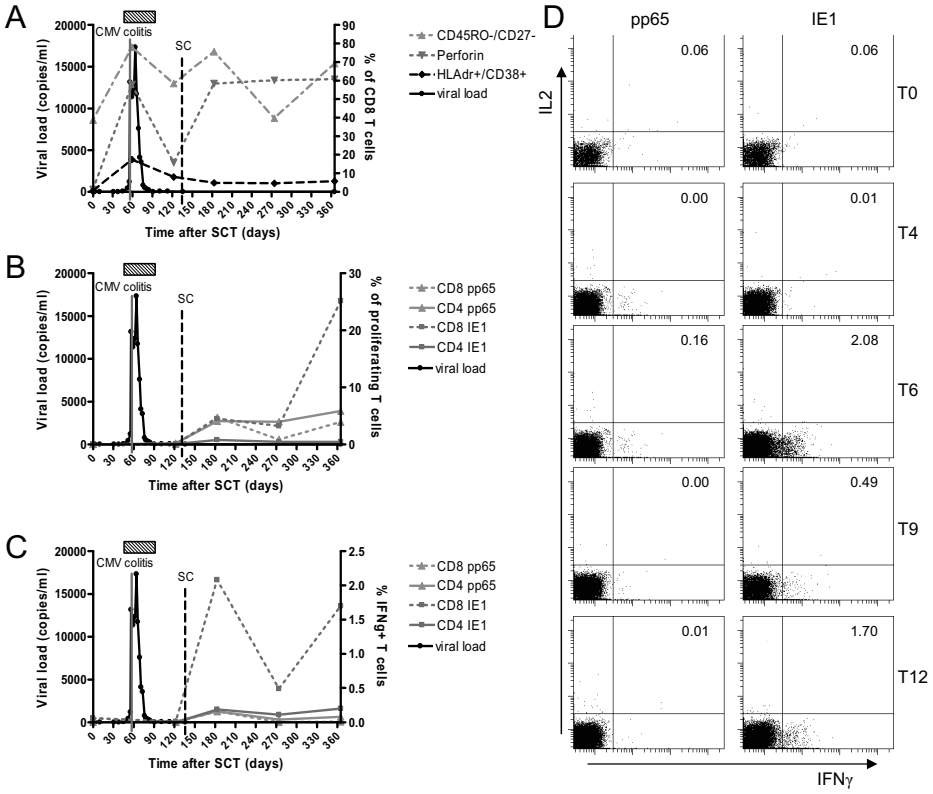

Figure 4. Immunological features of a patient with primary CMV-infection and subsequent development of CMV-colitis

A) Viral load (copies/ml), straight line, black; Percentage of activated $CD8^+$ T cells ($HLAdr^+/CD38^+$), dashed line, black; Percentage of perforin-expression by $CD8^+$ T cells, dashed line, dark grey; Percentage effector cells ($CD45RO^-/CD27^-$) of $CD8^+$ T cells. Percentages of proliferating (B) and IFNγ-producing (C) $CD4^+$ and $CD8^+$ T cells in response to pp65 or IE1. Viral load (copies/ml), straight line, black; Percentage responding $CD4^+$ (straight line) and $CD8^+$ (dashed line) T cells in response to pp65 (light grey) and IE1 (dark grey). S.C. CMV-seroconversion, striped box: treatment with ganciclovir. D. FACS staining on several time points post-SCT of the production of IFNγ and IL-2 by $CD8^+$ T cells after stimulation with pp65 or IE1. Percentages of $IFN\gamma^+CD8^+$ T cells are indicated.

This patient was transplanted with stem cells from a matched, related, CMV-seropositive donor after non-myeloablative conditioning. After SCT, CMV load became detectable on day 45 and the patient subsequently developed CMV-colitis after 58 days, as diagnosed by histology of sigmoid biopsies. The patient had not developed GVHD at time of diagnosis of CMV-colitis. CMV load reached a maximum viral load of $1.7*10^4$ copies/ml on day 64. The patient was treated with intravenous ganciclovir from day 56 till day 92. From day 90 onwards CMV load remained undetectable for at least one year. Serology was performed to confirm primary CMV-infection. IgM and IgG became detectable three months after the first detection of CMV load (day 136 post SCT)

DISCUSSION

To investigate the causal relationship between CMV-reactivation and reconstitution of CMV-specific T-cell responses, immunological features of SCT patients with and without CMV-reactivation were determined.

We observed a more differentiated phenotype of $CD8^+$ T cells, but not of $CD4^+$ T cells, in patients with CMV-reactivation compared to those without, which fits with the knowledge that CD8+ T cells are more pronounced in their differentiation status compared to CD4+ T cells. (14) Interestingly, this difference in T-cell phenotype is already observed before SCT, suggesting that parameters prior to SCT may influence T-cell phenotype. In line with the observation that CMV drives $CD8^+$ T-cell differentiation, in the patient group with CMV-reactivation recipients were already CMV-seropositive before transplantation, whereas only half of the patients without CMV-reactivation were CMV-seronegative pre-SCT and displayed therefore a more naïve phenotype. After SCT the antigenic drive for T-cell differentiation is absent in patients without CMV-reactivation and reconstitution mainly results from thymic output. In the R^+/D^- pairs from the patient group with CMV-reactivation the development of a more differentiated T-cell phenotype is therefore somewhat delayed. In the R^+/D^+ pairs CMV-specific T cells from the donor will expand rapidly, whereas in the R^+/D^- pairs reconstitution of a new CMV-specific T-cell pool is required.

The CMV load level did not appear to have a major impact on T-cell differentiation, as a relatively small amount of virus is already sufficient for a differentiated T-cell phenotype. Interestingly, the high viral load group tended to display a more effector-memory phenotype, whereas the patients with low or intermediate viral load level showed a more effector phenotype. As high viral load levels were accompanied by recurrent reactivation, this may have led to differences in $CD8^+$ T-cell differentiation. A recent study showed lack of $CD45RA^+CD27^-CD28^-$ T cell in patients with uncontrolled CMV-reactivations(18). Furthermore, Gamadia et al.(11) showed in renal transplant patients loss of CD45RO only when CMV load had dropped, suggesting that CMV drives T cells to an effector-memory phenotype during periods of antigen exposure, whereas T cells differentiate into terminally-differentiated effector type T cells only when antigen levels are reduced.

Prolonged CMV-reactivation may lead to exhaustion of T cells as has been suggested for other antigens(19,20). Interestingly, high expression of the exhaustion marker PD-1 (MFI>500) was only observed in patients with a viral load above 1000 copies/ml. Previously Barber *et al*.(21) showed only upregulation of PD-1 on $CD8^+$ during the late phase of chronic infection.

Activation of $CD4^+$ T cells was increased in patients with CMV-reactivation; however this difference was also observed before SCT. No significant differences in activation of $CD8^+$ T cells where observed, probably due to low numbers of patients in both groups. Especially early after SCT (2 months), CD8+ T-cell activation is different between the two groups, but this difference is lost during follow-up. Increased $CD8^+$ T-cell activation was observed clearly during acute infection in a patient subsequently developing CMV colitis, which may represent activation of specific immunity. As a measure for potent $CD8^+$ T-cell function, perforin levels were increased in patients with CMV-reactivation compared to those without reactivation. Previous studies described high levels of perforin-expression by CMV-specific $CD8^+$ T cells(15,21,22), indicating that high levels of $perforin^+CD8^+$ T cells measured in this study may be a consequence of high levels of CMV-specific T cells. Remarkably, perforin-expression tended to be increased in patients with CMV-reactivation already before reactivation. Since effector T cells, which are preferentially present in CMV-seropositive individuals, express more perforin compared to T cells with a more early phenotype, the difference in perforin-expression present before SCT might reflect the more differentiated phenotype of T cells in patients who are CMV-seropositive before SCT. Alternatively, perforin-expression may indicate subclinical reactivation or alloreactivity.

$CD8^+$ T-cell responses against pp65 and IE1 were observed more frequently in patients with CMV-reactivation, compared to patients without reactivation. This suggests that the recent encounter of virus leads to increased IFNγ-production by CMV-specific $CD8^+$ T cells, a characteristic of effector T cells, which were abundantly present(16). Furthermore, pp65-specific $CD8^+$ T-cell responses were increased compared to IE1-specific T cells, which is in accordance with previous findings (23-25). However, these pp65-specific responses were not correlated with protection against reactivation (24,25). Furthermore, IFNγ production after CMV-antigen stimulation by $CD3^+$ T cells was reported to correlate with lower peak viral loads four weeks after SCT (26). Sacre et al.(27) reported a correlation between IE1-specific T-cell reconstitution in the first three months post SCT and protection against CMV-replication. Low pp65-specific $CD4^+$ T-cell responses and low IE1-specific $CD8^+$ T-cell responses have been reported to correlate with recurrent CMV-reactivation after SCT, whereas in patients with single or no reactivations better responses were observed(24).

In the patient who developed a primary CMV-infection after SCT, delayed reconstitution of CMV-specific responses, as measured by IFNγ-production, may have led to the development of CMV-colitis. In line with our observation, previous studies reported suppressed (28) or lack of IFNγ-production by $CD4^+$ and $CD8^+$ T cells in patients with CMV-disease (28). In line with Morita-Hoshi et al.(28) we observed an increase in responses with recovery of disease.

In conclusion, in this study a more differentiated phenotype, up-regulation of the activation markers CD38 and HLA-DR on $CD4^+$ T cells and increased expression of perforin on $CD8^+$ T cells was more frequently observed in patients with CMV-reactivation compared to patients without reactivation. This suggests that active CMV-replication plays a role in immune activation, thereby influencing T-cell features as phenotype differentiation and perforin-expression. The early occurrence of these differences, some already detectable before SCT, may indicate these parameters as potential prognostic or diagnostic markers. Interestingly in this light is our recent observation that $perforin^+$ CD8+ T cells very early after SCT (first 3 weeks) predict the severity of viral-reactivation in a prospective cohort study (29).

In addition, CMV-specific $CD8^+$ T-cell responses directed against both pp65 and IE1 tended to be present more frequently in patients with CMV-reactivation compared to those without reactivation. These data fit with earlier studies showing that activation of CMV is a potent stimulator of T-cell function [23,24] and suggest that CMV-antigen may be required for reconstitution of CMV-specific T-cell responses. Future studies should aim to identify the main determinant of CMV-reactivation in the interaction between CMV load and CMV-specific immunity in the immunocompromised host.

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Chapter 8

Donor CMV status influences severity of viral reactivation after allogeneic SCT in CMV seropositive recipients

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ABSTRACT

We investigated the role of donor CMV-serostatus on CMV-reactivation in CMV^{pos} transplant recipients. CMV-reactivation occurred more frequently in patients receiving a CMV^{pos} graft, but was less severe compared to patients receiving a CMV^{neg} graft. These data suggest roles for both virus as well as CMV-specific immunity present in the graft.

INTRODUCTION

Cytomegalovirus infection is a major cause of morbidity and mortality after allogeneic stem cell transplantation[1]. Studies have shown that the use of a CMV^{NEG} graft in a CMV^{POS} patient is linked to the occurrence of late CMV reactivation[2] in patients with preceeding early CMV reactivation as well as provided some support for an association with the development of CMV-related disease[3,4]. In accordance with this it was recently described that CMV^{POS} SCT recipients receiving a CMV^{NEG} transplant are twice as likely to require antiviral therapy.[5,6] However, no data exists on the actual association of donor CMV serostatus and the onset and severity of CMV-reactivation. Therefore, we investigated whether CMV serostatus of the donor influences the onset of viral reactivation as well as the magnitude of the viral reactivation defined by maximum viral load reached early after SCT in CMV^{POS} recipients.

METHODS

Consecutive CMV^{POS} patients were prospectively followed for 6 months after allogeneic stem cell transplantation. Patients were transplanted between October 2005 and January 2009 at the department of Hematology of the University Medical Center Utrecht, Utrecht, the Netherlands. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki. Patient characteristics are summarized in table 1. Patients received an allogeneic stem cell transplant from either an unrelated (n= 61) or a related donor (n=47). A non-myeloablative conditioning regime was used in most patients (n=96). Anti-thymocyte globulin was added to the conditioning regime for in-vivo T-cell depletion in patient receiving
a graft from an unrelated donor or a graft from a HLA-mismatched donor (n=65).
CMV serostatus of patients and donors was determined as part of the standard diagnostic routine: 50 patients received a graft from a CMV^{POS} donor and 56 from a CMV^{NEG} donor (2 unknown). CMV monitoring was based on a real-time TaqMan CMV-DNA PCR assay in EDTA-treated plasma[7]. This assay was performed weekly in all patients as part of the diagnostic routine until 6 months post transplantation and enabled identification of early reactivations. Patients were treated pre-emptively with valganciclovir (900 mg/twice daily) when the CMV-DNA load exceeded 500 copies/ml. Valaciclovir was given prophylactically (500 mg/twice daily) to all patients. Based on this clinical treshold for antiviral therapy, we studied the magnitude of the viral reactivation (< or > 500copies/ml) as a read out for reactivation severity.
All transplantation risk factors for CMV-reactivation were assessed using a Chi-square test and cox regression test for multivariate analysis. Incidence of CMV reactivation was

determined using a Kaplan-Meier graph and log rank test. Differences between viral loads were assessed by Mann-Whitney U-tests. A probability level of 5% ($p < 0.05$) was considered significant. All statistical analysis were performed with SPSS version 12.1 (SPSS Inc. Chicago, USA) and GraphPad Prism 5.0 (Graphpad Software Inc. San Diego, USA).

RESULTS AND DISCUSSION

108 CMV ^{POS} allogeneic stem cell recipients were prospectively monitored for viral reactivation during the first 6 months post SCT. Viral reactivation was defined as CMV-DNA load exceeding the detection limit of 50 copies/ml. Reactivations were categorized into low and high reactivation based on the peak viral load exceeding a clinically relevant threshold of 500 copies/ml plasma, which is based on the threshold for pre-emptive therapy. Viral reactivation occurred in 62 patients (57,4%). A low reactivation developed in 29 cases (46.7%) and high reactivation in 32 (51.6%). Transplant-related factors that could influence the onset of viral reactivation were assessed (table I). Age (p=0.035), conditioning regime (p=0.016) and CMV
status of the donor (p=0,000) were all predictors of viral reactivation. All transplant related factors with $p < 0.2$ were analysed in a multivariate cox regression test. Transplantation factors that were significantly associated with the onset of viral reactivation were conditioning regime (p=0.019, HR=0.220, CI=0.060-0.798), ATG administration (p=0.006 HR=0.426 CI=0.231-0.786), aGVHD (p=0.017 HR=0.516 CI= 0.300-0.886) and the CMV serostatus of the donor (p=0.001, HR= 0.387 CI=0.219-0683). The association of conditioning regime with CMV reactivation can be explained by the limited number of patients receiving a myeloablative transplantation in our study (n=12), of which 3 developed a CMV-reactivation.

Next, we analyzed the relation between donor CMV serostatus and the occurrence of CMV reactivation early after SCT. We found that in patients receiving a CMV^{POS} graft, CMV reactivation occurred much more frequently than in patients receiving a CMV^{NEG} graft (figure 1a). Within 6 months post SCT, CMV reactivation occurred in 79.0 % of the patients receiving a graft from a CMV^{POS} donor, against 39.3% of patients transplanted with a CMV ^{NEG} graft ($p < 0.001$). There was no difference in onset of viral reactivation between the two groups. Patients receiving a CMV ^{NEG} graft developed CMV reactivation at a median of 17 days post SCT (range 0-136 days) versus 22 days for patients receiving a CMV^{POS} graft (range 3-104 days) (p 0.45). To investigate whether donor CMV status influences the severity of the viral reactivation, we subsequently analyzed the peak CMV-DNA load reached by each patient undergoing viral reactivation within 6 months

Table I. Patient characteristics

		total	reactivation(%)	no reactivation(%)	univariate	multivariate	Hazard ratio (CI)
	N =	108	62 (57,4 %)	46 (42,6 %)			
median age		52,1	56,2	47,6	p=0,035	p=0,756	1,004(0,979-1,030)
Sex	M	64	38 (59,4 %)	26 (40,6 %)	p=0,618		
	F	44	24 (54,5 %)	20 (45,5 %)			
Disease	AA	3	3 (100,0 %)	0 (0,0 %)	p=0,743		
	ALL	6	6 (100,0 %)	0 (0,0 %)			
	AML	31	17 (54,8 %)	14 (45,2 %)			
	CLL	5	4 (80,0 %)	1 (20,0 %)			
	CML	3	1 (33,3 %)	2 (66,7 %)			
	Hodgkin	2	1 (50,0 %)	1 (50,0 %)			
	HES	1	0 (0,0 %)	1 (%)			
	XLP	1	1 (100,0 %)	0 (0,0 %)			
	Myelofibrosis	1	1 (100,0 %)	0 (0,0 %)			
	MDS	7	3 (42,9 %)	4 (57,1 %)			
	MM	20	11 (55,0 %)	9 (45,0 %)			
	NHL	20	12 (60,0 %)	8 (40,0 %)			
	other	1	1 (100,0 %)	0 (0,0 %)			
Stemcell source	peripheral blood	101	58 (57,4 %)	43 (42,6 %)	p=0,907		
	bonemarrow	4	2 (50,0 %)	2 (50,0 %)			
	cord blood	3	2 (66,7 %)	1 (33,3 %)			
Donor	Related	47	24 (51,1 %)	23 (48,9 %)	p=0,242		
	Unrelated	61	38 (62,3 %)	23 (37,7 %)			
HLA	yes	17	13 (76,5 %)	4 (23,5 %)	p=0,083	p=0,230	1,509(0,771-2,953)
mismatch	no	91	49 (53,8 %)	42 (46,2 %)			
Conditioning	NMA	96	59 (61,5 %)	37 (38,5 %)	p=0,016	p=0,021	0,220(0,060-0,798)
	MA	12	3 (25,0 %)	9 (75,0 %)			
ATG	yes	65	42 (64,6 %)	23 (35,4 %)	p=0,063	p=0,006	0,426(0,231-0,786)
	no	43	20 (46,5 %)	23 (53,5 %)			
CMV serology	positive	50	38 (76,0 %)	12 (24,0 %)	p=0,000	p=0,001	0,387(0,219-0,683)
donor	negative	56	22 (39,3 %)	34 (60,7 %)			
aGVHD	yes	43	29 (67,4 %)	14 (32,6 %)	p=0,064	p=0,017	0,516 (0,300-0,886)
	no	61	30 (49,2 %)	31 (50,8 %)			
survival	yes	57	30 (52,6 %)	27 (47,4 %)	p=0,289		
> 1 year	no	51	32 (62,7 %)	19 (37,3 %)			

Abbreviations: AA, aplastic anemia; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; HES, Hypereosinofil syndrome; XLP, X-linked lymphoproliferative disease; MM, multiple myeloma; NHL, non-hodgkin lymphoma; NMA, non-myeloablative; MA, myeloablative; ATG, anti-thymocyte globulin; CMV, cytomegalovirus; aGVHD, acute Graft Versus Host disease. Comparion between reactivation and no reactivation group: Fisher's Exact test, Mann-Whitney U t-test for age, multivariate analysis using Cox regression.

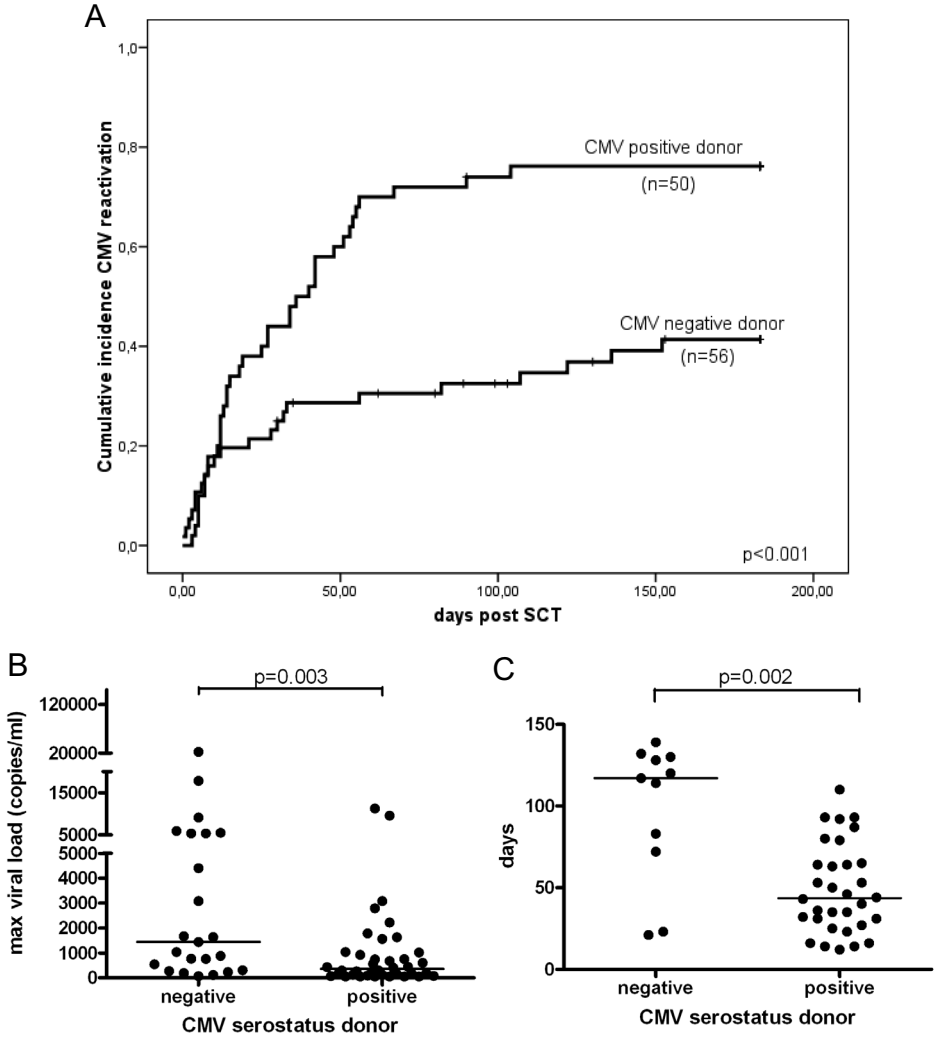

Figure 1.

A) Viral reactivation (detectable CMV load in plasma within 6 months post SCT) occurs more frequently in patients receiving a CMV positive graft. Kaplan Meier graph shows the cumulative incidence of viral reactivation after SCT. 79% recipients of CMV positive grafts reactivate within 6 months against 39.2% of negative graft recipients (p=0.001).

B) Higher viral loads after SCT with a CMV negative graft. The peak viral load measured during 6 months post SCT in plasma is plotted for each patient that reactivates. The median viral load for negative graft recipients is 1439 copies/ml versus 355 copies/ml in positive graft recipients (p=0.003).

C) Prolonged CMV DNA positivity in recipients of CMV^{neg} grafts. The median duration of CMV DNA positivity was 117 days for patients receiving a CMV^{neg} graft versus 43,5 days for patients receiving a CMV^{pos} graft (p=0.002)

post SCT (n=62). The percentage of patients with high reactivations (n=33, loads >500 copies/ml) was significantly
higher in the group receiving a CMV^{NEG} graft (72.7% of reactivations) than in the group receiving a CMV^{POS} graft (42.1% of reactivations) (p=0.032). We plotted the maximum viral load obtained within 6 months post SCT for patients receiving a CMV^{NEG} or CMV^{POS} transplant (figure 1b). SCT recipients receiving a CMV^{NEG} graft reached significantly higher peak viral loads (median 1439 copies/ml, range 66-22811 copies/ml) than patients receiving a CMV^{POS} graft (median 354,5 copies/ml, range 50-11257 copies/ml) (p=0.003) (figure 1b). We subsequently investigated the duration of the viral reactivations. For each patient we calculated the number of days of CMV-reactivation during the first 6 months post SCT by combining the duration of all episodes of positive CMV-DNA PCR. We excluded patients that deceased within this timeframe (n=17). Patients receiving a CMV^{NEG} graft had more and longer episodes of CMV reactivation, median 117 days of positive CMV DNA (range 21-139), against 43.5 days (range 12-110) for patients receiving a CMV^{POS} graft (p=0.002)(figure 1c). Thus, although CMV reactivation occurred more often in CMV^{POS} patients after transplantation with a CMV ^{POS} graft, the viral reactivation is less severe and short-lasting compared to SCT with a CMV^{NEG} graft. The higher viral loads and prolonged timeframe of CMV DNAemia is in concordance with a recent publication by Zhou *et al,* in which recurrent and prolonged use of antiviral therapy was shown for CMV^{POS} recipients of a CMV^{NEG} graft[5].

We postulate that the more frequent CMV detection in recipients of a CMV^{POS} graft may be caused by (re)activation of the CMV strain of the donor. This has previously been shown to occur for Epstein-Barr virus (EBV) infections. Active EBV infections after allogeneic SCT were shown to frequently result from re-infection with an exogenous EBV strain instead of being a true reactivation of the endogenous strain[8]. This hypothesis should be further explored in a future study, which is specifically designed to tackle this question by for example taking saliva samples of the donor before transplantation and comparing CMV strains in these specimens with the initially present CMV strain in saliva of the donor and the reactivating CMV strain (in either blood or saliva). However, retrospective identification of the CMV strain in the donor is very difficult if not impossible because of undetectable CMV levels in the blood. Furthermore, lower viral loads occurring in recipients of a CMV^{POS} graft could be due to the transfer of CMV-specific immunity from the graft. Presence of CMV-specific T cells in the graft could result in a faster CMV-specific T-cell reconstitution in the recipient resulting in better containment of viral reactivation. This transferred immunity has been previously shown for herpes simplex virus reactivation after SCT[9]. Ganepola *et al* suggest that a delay in CMV-specific T-cell immunity in seropositive recipients of a CMV^{NEG} donor causes frequent CMV infections and disease[10].

Gratama *et al* have also suggested that CMV-specific T cells in the CMV^{POS} graft could protect against progressive CMV infection as they have shown that the number of CMV- specific CD8+ T cells in the graft inversely correlated with the number of recurrent CMV infections requiring pre-emptive antiviral treatment[11]. More recently it was shown that the reconstitution of CMV-specific T cells after SCT was determined by the characteristics of the CMV-specific T cells in the donor. The same CMV antigens were targeted in both the donor and the recipient. In addition, less differentiated CMV-specific T-cell responses in the donor persisted in the recipient, whilst terminally differentiated cells in the donor decreased after SCT in the recipient[12]. Evenmore, Zhou *et al* showed rapid reconstitution of multifunctional CD8+ T cells in CMV^{POS} recipients after SCT with a CMV^{POS} donor, suggesting that this early expansion is derived from the CMV-specific T cells of the donor[5].

Unfortunately, the clinical group studied is heterogeneous, with respect to the use of related versus unrelated donor grafts and diagnosis, previously shown to be associated with a higher risk for CMV reactivation.[2] Therefore, a less heterogeneous patient population (e.g., of only matched siblings, or of patients with more similar diseases) would improve the analysis.

In summary, the higher incidence of viral reactivation in CMV^{POS} recipients of CMV^{POS} stem cell grafts may be caused by reactivation of the donor CMV strain, as this reactivation may be subsequently controlled by CMV-specific T cells from the graft, this would lead to only mild viral reactivations. On the other hand, we observe more severe viral reactivations occur with CMV^{NEG} grafts. Due to lack of CMV-specific T cells in the graft, control of CMV reactivation is likely dependent on reconstitution and activation of naïve T cells, which take more time to develop. These data suggest roles for both virus as well as CMV-specific immunity present in the graft.

Acknowledgements

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Chapter 9

Detailed analysis of CMV-specific CD8$^+$ T cells in the graft of allogeneic stem cell transplantation recipients

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ABSTRACT

CMV seropositive recipients of allogeneic stem cell transplantation have more frequent, but less severe viral reactivations after transplantation with a CMV seropositive donor compared to recipients of CMV seronegative grafts. Therefore, we investigated whether donor CMV seropositivity influenced the T-cell constitution of the graft and subsequent development and/or severity of viral reactivation. Also we investigated whether the role of CMV-specific $CD8^+$ T cells herein. We found that CMV seropositive stem cell grafts contain a more differentiated $CD8^+$ T-cell pool and that recipients of CMV seropositive grafts have higher absolute $CD8^+$ T-cell numbers during the first few months after transplantation. However, the presence or absence of CMV-specific $CD8^+$ T cells in the graft was not associated with subsequent viral reactivation. CMV-specific $CD8^+$ T cells 2 months post SCT were only detectable in patients that received CMV seropositive grafts, suggesting that transfer of immunity plays an important role in the early antiviral response.

INTRODUCTION

Cytomegalovirus infections in allogeneic stem cell transplantation recipients are a major cause of morbidity and mortality[1]. The importance of the recipient CMV serostatus on the outcome of SCT has been established[2-4]. Seropositive patients have lower survival rates post SCT and seropositivity is a predictor for treatment-related mortality (TRM)[5]. However, controversy exists on the impact of the donor CMV serostatus on mortality. Ljungman *et al* described improved 5-year survival, improved event-free survival and reduced transplant-related mortality after SCT with an unrelated CMV^{pos} donor[6]. On the other hand, Kollman *et al* did not show a difference in 5 year-survival after SCT (with an unrelated donor) between a seronegative or positive donor[7]. Besides having an impact on TRM and mortality, the serostatus of the donor also seems to play a role in the development and severity of CMV reactivation post SCT. Several studies have shown that CMV positive recipients of a CMV negative graft are more likely to develop late CMV reactivation, CMV related diseases and require prolonged antiviral therapy[6-9]. In a recent study we have shown that CMV seropositive recipients of allogeneic stem cell transplantation have more frequent, but less severe viral reactivations after transplantation with a CMV seropositive donor. We postulated that this could be due to transferred CMV-specific immunity in the graft (F.L. Pietersma, manuscript in press). Ganepola *et al* suggested that a delay in CMV-specific T-cell immunity in recipients of a negative donor results in more frequent CMV infections and disease[10]. Also, it has previously been shown that the number of CMV-specific $CD8^+$ T cells in the graft is inversely correlated to the number of recurrent CMV infections that require pre-emptive antiviral therapy[11]. Moreover, a rapid reconstitution of multifunctional $CD8^+$ T cells in CMV positive recipients of CMV positive grafts suggests that the early expansion of these cells is derived from the donor[9]. Here we investigated whether donor CMV seropositivity influenced the T-cell constitution of the graft and whether the presence or absence of CMV-specific $CD8^+$ T cells plays a role in development and/or severity of viral reactivation.

METHODS

Patient and transplantation characteristics

In a previous retrospective database study (F.L. Pietersma, manuscript in press) we analysed CMV serology and subsequent viral reactivations in 108 CMV seropositive stem cell transplantation recipients. Patients were transplanted between December 2005 and August 2009 at the department of Hematology of the University Medical Center Utrecht, Utrecht, the Netherlands. From 40 of these patients graft samples were avail-

able. These patients were included in this study. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki. Patient characteristics are summarized in table 1. Patients received an allogeneic stem cell transplant from either an unrelated (n= 33) or a related donor (n=7). A non-myeloablative conditioning regime was used in most patients (n=38). Anti-thymocyte globulin was added to the conditioning regime (n=34) for in-vivo T-cell depletion in patients receiving a graft from an unrelated donor or a graft from a HLA mismatched donor (n=8). CMV serology of patient and donor was determined as part of the standard diagnostic routine, 18 patients received a graft from a CMV positive patient and 22 from a CMV negative patient. 37 patients received GCSF-mobilized peripheral blood stem cells and 3 patients received bone marrow stem cells. Cryopreserved PBMC from 2 months post SCT were available for 12 patients.

CMV monitoring

CMV monitoring was based on a real-time TaqMan CMV DNA PCR assay in EDTA-treated plasma[12]. This assay was performed in all patients as part of the diagnostic routine until 6 months post transplantation. Patients were treated pre-emptively with valganciclovir (900 mg twice daily) when the CMV DNA load exceeded 500 copies/ml. Valaciclovir was given prophylactically (500 mg twice daily) to all patients. Viral reactivation or infection was defined as CMV DNA load exceeding the detection limit of 50 copies/ml. Viral reactivations were divided into low and high reactivation based on the peak viral load exceeding 1000 copies/ml plasma with 6 months post SCT.

CMV-specific T-cell staining and absolute $CD8^+$ T-cell counts

After transplantation, remaining graft material was cryopreserved until further use. Samples were thawed and washed in phosphate buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 0.02% sodiumazide. MHC class I tetramers complexed with specific peptides were produced as previously described[13]. One HLA-A2 restricted peptide NLVPMVATV and one HLA-B7 restricted peptide TPRVTGGAM derived from the CMV-pp65 protein were used. Biotinylated class I peptide complexes were tetramerized by addition of APC (for B7-TPR) and PE (for A2-NLV) conjugated streptavidin. Approximately 1.0x 10^6 cells were incubated with either tetramer together with CD3 eFluor (eBioscience Inc, San Diego, California, USA), CD8 PerCP (BD biosciences (BD) San José, California, USA) CD45RO-FITC (Caltag Laboratories),CD27 APC-Cy7 (eBioscience) and CD57-PE (eBioscience). Samples were measured on a LSR-II FACS machine (BD),in most samples at least 100000 events were acquired. To determine the absolute number of $CD8^+$ T cells per ul whole blood, TRUcount ™ tubes (BD) were used according to

manufacturers protocol. In brief, 50 µl of whole blood was incubated with CD45 PerCP (BD), CD3 Pacific Blue (eBioscience), CD4 PE-Cy7 (BD) and CD8 APC-Cy7 (BD). Thereafter erythrocytes were lysed and samples were measured on a LSR-II FACS machine. At least 2000 lymphocytes, identified as CD45 + and SSC low, were measured, All data was analysed with FACSdiva software (BD).

Statistical analysis

All transplantation risk factor for CMV reactivation were assessed using a Chi-square test. Cox regression was used for multivariate analysis. Incidence of CMV reactivation was determined using a Kaplan-Meier graph and log rank test. Differences between viral loads and T-cell features between different groups were assessed by Mann-Whitney U tests. A probability level of 5% ($p>0.05$) was consideren significant in all analyses. All statistical analysis were performed with SPSS version 12.1 (SPSS Inc. Chicago, USA) and GraphPad Prism 4.0 (Graphpad Software Inc. San Diego, USA).

RESULTS

Patient population

40 CMV seropositive allogeneic stem cell transplantation recipients were prospectively monitored for viral reactivation during 6 months post SCT. Viral reactivation was defined as CMV-DNA load exceeding the detection limit of 50 copies/ml plasma during the first 6 months after SCT. Viral reactivations were subsequently categorized into low or high reactivation based on whether the peak viral load exceeded 1000 copies/ml within 6 months post SCT. CMV reactivation occurred in 26 patients (65%). A low-level reactivation developed in 16 cases (61,5% of reactivations) and high-level reactivation in 10 (38,5%). Transplant-related factors that could influence the onset of viral reactivation were assessed (table I). Only the conditioning regime was significantly associated with development of CMV reactivation ($p=0,048$). All factors with $p<0.2$ were analysed in a multivariate cox regression test. None of the listed transplantation related risk factors were associated with CMV reactivation. However, the limited number of patients included in this study makes it difficult to elucidate significantly associated risk factors. In our larger patient cohort CMV seropositivity of the donor was significantly associated with a higher incidence of viral reactivation as well as conditioning regime, ATG administration and onset of acute graft versus host disease (F.L. Pietersma, manuscript submitted).

Table I. Patient characteristics

		total	reactivation(%)		univariate	multivariate	Hazard ratio (CI)
	N =	40	26	(65,0 %)			
median age		55,8	57,4		p=0,127	p=0,824	0,996(0,957-1,036)
Sex	M	26	19	(73,1 %)	p=0,144	p=0,191	1,876(0,730-4,818)
	F	14	7	(50,0 %)			
Disease	AA	1	1	(100,0 %)	p=0,655		
	ALL	2	2	(100,0 %)			
	AML	12	7	(58,3 %)			
	CLL	4	3	(75,0 %)			
	CML	1	0	(0,0 %)			
	Hodgkin	0	0	(0,0 %)			
	HES	1	0	(0,0 %)			
	XLP	1	0	(0,0 %)			
	Myelofibrosis	1	1	(100,0 %)			
	MDS	4	2	(50,0 %)			
	MM	8	5	(62,5 %)			
	NHL	5	4	(80,0 %)			
Stemcell source	peripheral blood	37	24	(64,9 %)	p=0,950		
	bonemarrow	3	2	(66,7 %)			
	cord blood	0	0	(0,0 %)			
Donor	Related	7	4	(57,1 %)	p=0,631		
	Unrelated	33	22	(66,7 %)			
HLA mismatch	yes	8	6	(75,0 %)	p=0,112	p=0,850	1,104(0,397-3,065)
	no	32	20	(62,5 %)			
Conditioning	NMA	38	26	(68,4 %)	p=0,048	p=0,985	0,000(0-.)
	MA	2	0	(0,0 %)			
ATG	yes	34	23	(67,6 %)	p=0,403		
	no	6	3	(50,0 %)			
CMV serology donor	positive	18	13	(72,2 %)	p=0,386		
	negative	22	13	(59,1 %)			
aGVHD	yes	15	12	(80,0 %)	p=0,082	p=0,051	0,409(0,167-1,003)
	no	23	12	(52,2 %)			
survival > 1 year	yes	18	11	(61,1 %)	p=0,641		
	no	22	15	(68,2 %)			

Abbreviations: AA, aplastic anemia; ALL, acute lymphatic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphatic leukemia; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; HES, hypereosinofil syndrome; XLP, X-linked lymphoproliferative disease; MM, multiple myeloma; NHL, non-Hodgkin lymphoma; NMA, non-myeloablative; MA, myeloablative; ATG, anti-thymocute globulin; CMV, cytomegalovirus; aGVHD, acute Graft Versus Host Disease. Comparison between reactivation and no reactivation group: Mann-Whitney U t-test for age, other univariate analysis using Fisher's Exact test. Multivariate analysis using Cox regression.

CMV seropositive grafts contain more differentiated T cells

In healthy individuals it has been demonstrated that CMV infection drives T cells towards an effector phenotype[14]. Several studies have shown that CMV seropositive individuals have a more differentiated $CD8^+$ T-cell pool compared to CMV seronegative individuals[15-17]. To this end we investigated the phenotype of the $CD8^+$ T-cell pool in stem cell grafts. CMV seropositive grafts contained a larger portion of effector ($CD27^-/CD45RO^-$) $CD8^+$ T cells (median= 13,25%) compared to CMV seronegative grafts (median=5.75%, p=0.003)(figure 1a).This difference in number of effector $CD8^+$ T cells did not influence the development of CMV reactivation in the corresponding graft recipients. The percentage of effector $CD8^+$ T cells in the graft was not different between the no reactivation group (8,25%); the low reactivation group (7,90%); and the high reactivation group (10,6%) (figure1b). Expression of CD57 on $CD8^+$ T cells is associated with replicative senescence as well as antigen-induced apoptotic death in chronic viral infections[18]. However, even though CMV seropostive grafts clearly contained more differentiated $CD8^+$ T cells (figure 1b) there was no difference in expression of CD57 in the $CD8^+$ T cells between CMV seronegative (median=4,1%) and CMV seropositive grafts (median= 8,7%)(figure 1c).
We subsequently investigated whether the serostatus of the graft has an impact on the absolute $CD8^+$ T-cell reconstitution early after SCT. We measured absolute T-cell numbers in the patients in fresh whole blood samples weekly during 3 months post SCT. The absolute number of $CD8^+$ T cells during the first 3 months after SCT was higher in patients receiving CMV seropositive grafts compared to CMV seronegative grafts. The median number of $CD8^+$ T cells during 3 months post SCT was 27 $CD8^+$ T cells/µl after transplantation with a CMV seronegative graft and 102 $CD8^+$ T cells/µl blood after SCT with a seropositive graft (p=0,01) (figure 2a). However, the number of $CD8^+$ T cells shortly after SCT is also influenced by the onset and the severity of CMV reactivation. Patients developing a high-level CMV reactivation have significantly less $CD8^+$ T cells compared to patients with a low-level CMV reactivation (p=0.03) (figure 2b).

Presence or absence of CMV-specific T cells in graft and subsequent viral reactivation

We investigated whether differences in percentages of CMV-specific $CD8^+$ T cells in the graft could predict the onset and or severity of the subsequent viral reactivation in CMV seropositive recipient/donor pairs. A2-NLV and B7-TPR tetramer stainings were performed on all HLA-A2 and B7 CMV seropositive grafts (n=14) (table II). Representative tetramer stainings are shown in figure 3. CMV-specific $CD8^+$ T cells were detectable in 7 grafts (50%). A2-NLV specific $CD8^+$ T cells were detected in 2 out of 10 grafts (20%) while B7-TPR specific $CD8^+$ T cells were detected in 5 out of 7 samples (71,4%). Recipients of grafts containing CMV-specific $CD8^+$ T cells (n=7) developed a CMV reactivation in 4

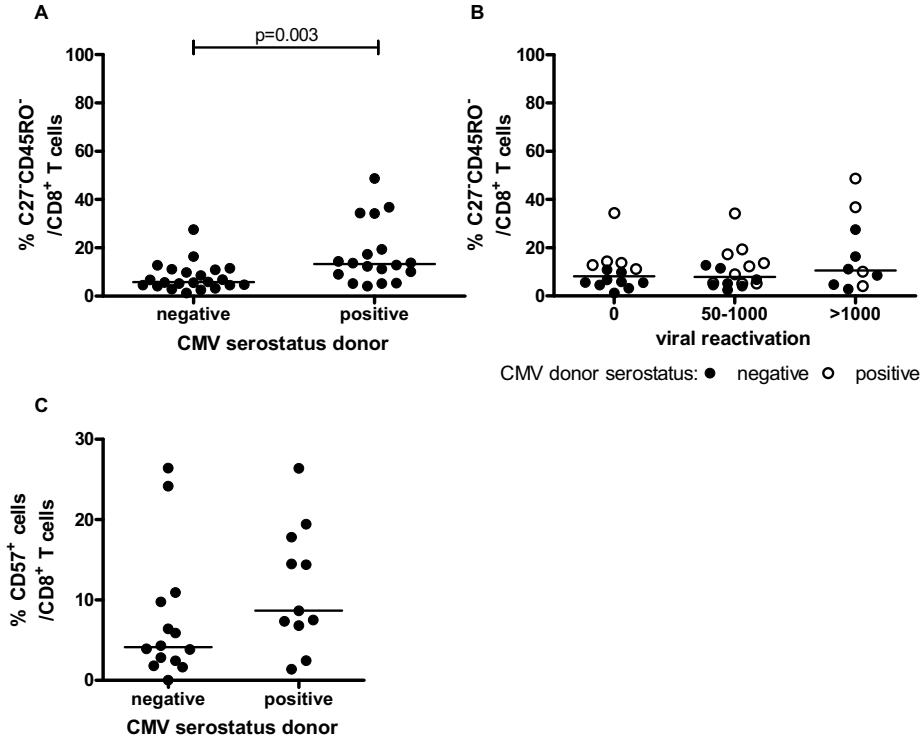

Figure 1. More differentiated CD8+ T cells in CMV seropositive grafts

A) Dot plot showing a higher percentage of effector ($CD27^-/CD45RO^-$) $CD8^+$ T cells in stem cell grafts for recipients of a CMV seropositive graft compared to recipients of a CMV seronegative graft (p=0.003).

B) Dot plot showing the percentages of effector($CD27^-/CD45RO^-$) $CD8^+$ T cells in the graft of patients without viral reactivation and patients who subsequently develop low or high-level CMV reactivation. Patients in the different viral reactivation categories are subdivided into whether they received a CMV seropositive graft (open circles) or a CMV seronegative graft (closed circles).

C) Dot plot showing the percentages of $CD57^+$ expression on $CD8^+$ between CMV seropositive grafts and CMV seronegative grafts.

cases, all with maximum viral loads below 1000 copies/ml, and in three individuals no CMV reactivation occurred. CMV-specific $CD8^+$ T cells could not be detected in the grafts of the patients that developed high-level viral reactivation (n=2).

Presence or absence of CMV-specific T cells in graft does not correlate with presence or absence of CMV-specific T cells 2 months post SCT

Next, we investigated whether the presence of A2-NLV or B7-TPR specific T cells in the graft is predictive of A2-NLV or B7-TPR specific $CD8^+$ T cells in the patient at 2 months

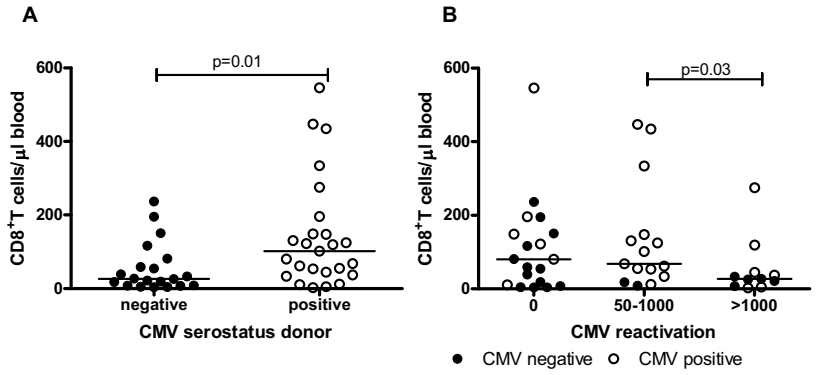

Figure 2. Absolute T-cell counts in the patients early after SCT

A) Median absolute $CD8^+$ T cell counts (cells/µl whole blood) during first 3 months post SCT in patients. Patients receiving a CMV seropositive graft have higher $CD8^+$ T cell counts (p=0.01).

B) Absolute $CD8^+$ T cell counts during first 3 months post SCT subdivided over the different reactivation categories. Patients developing a high-level viral reactivation have lower $CD8^+$ T cell numbers early after SCT compared to patients developing a low-level viral reactivation (p=0.03). Open circles indicate recipients of CMV seropositive grafts, closed circles indicate recipients of CMV seronegative grafts.

post SCT. PBMC from 12 patients 2 months post SCT were stained with A2-NLV or B7-TPR tetramers. Representative FACS stainings are shown in figure 4. In four patients we were able to detect CMV-specific $CD8^+$ T cells. All of these patients developed a low-level CMV reactivation (maximum viral loads ranged from 130-432 copies/ml). However, A2-NLV or B7-TPR specific $CD8^+$ T cells in the patient at 2 months post SCT did not correlate with the presence of A2-NLV or B7-TPR in the graft (figure 4). CMV-specific $CD8^+$ T cells were detectable either in the patient but not in the graft (upper 3 graphs), or in the graft but not in the patient (lower graph). Only in 2 cases CMV-specific $CD8^+$ T cells were detected both in the graft and in the recipient. Interestingly, the patients with A2-NLV or B7-TPR specific $CD8^+$ T cells at 2 months post SCT were all transplanted with a CMV seropositive graft, suggesting that CMV-specific $CD8^+$ T cells were likely to be present in the graft, however below the detection limit of our assay. None of the patients transplanted with a CMV negative graft had A2-NLV or B7-TPR specific $CD8^+$ T cells detectable at 2 months post SCT.

DISCUSSION

This study was based on a retrospective database study we conducted previously (F.L. Pietersma *et al,* manuscript in press) in which we investigated the role of the CMV

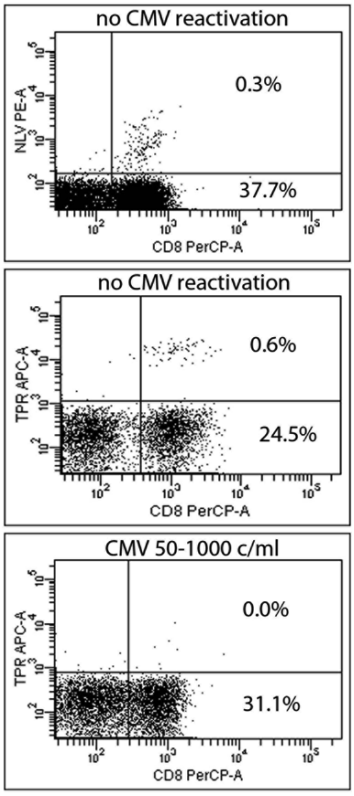

Figure 3. CMV-specific CD8$^+$ T cells in stem cell grafts

Representative FACS staining of A2-NLV or B7-TPR tetramer in 3 stem cell grafts. Upper plot shows a graft with 0,8% of A2-NLV specific CD8$^+$ T cells transplanted into a patient that subsequently did not develop a CMV reactivation. Middle panel shows a graft containing 2,4% of B7-TPR specific CD8$^+$ T cells transplanted into a patient without reactivation. Lower panel shows no B7-TPR specific CD8$^+$ T cells in a graft of a patient subsequently developing a low-level viral reactivation.

serostatus of the donor on development and severity of CMV reactivation in CMV seropositive allogeneic stem cell transplantation recipients. We found that although patients receiving a CMV seronegative graft reactivate less frequently compared to patients receiving a CMV seropositive graft, the reactivations are more severe (i.e. higher viral loads and prolonged episodes of CMV-DNAemia are measured). We postulated that while transferring CMV in the graft results in more frequent reactivations, the simultaneous transfer of CMV-specific CD8$^+$ T-cell immunity results in less severe viral reactivations. Therefore, here we retrospectively investigated whether presence of CMV-specific T cells in the graft is predictive of viral control and whether T-cell constitution of the graft varies between CMV seronegative and seropositive donors. We retrospectively included 40 patients from which graft material was stored after SCT. Previously described risk

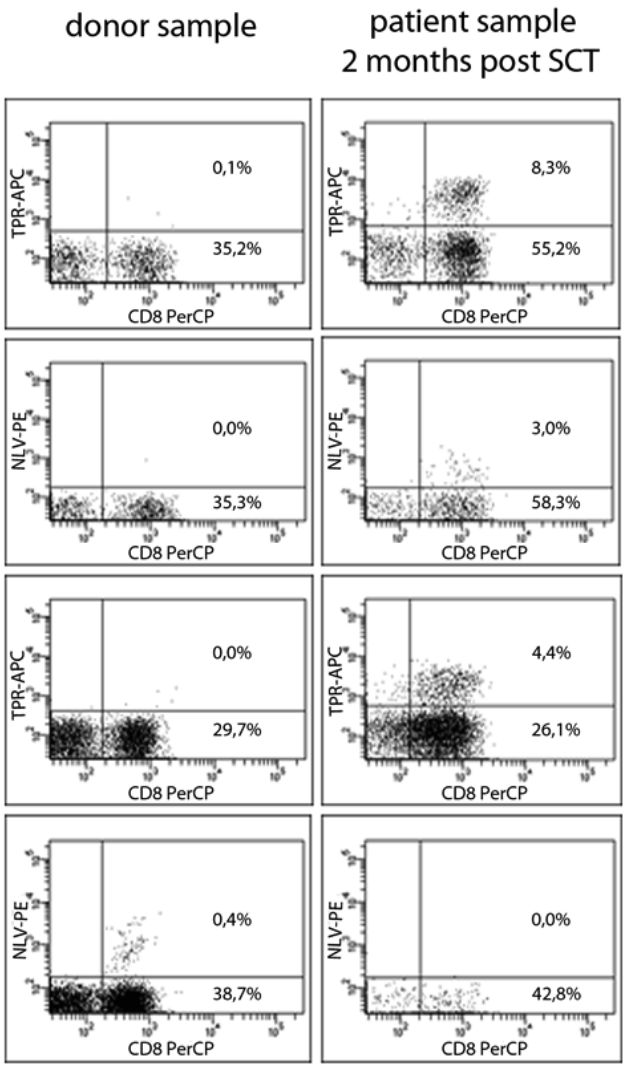

Figure 4. CMV-specific CD8$^+$ T cells in graft and recipient

Representative FACS stainings of CMV-specific A2-NLV or B7-TPR CD8$^+$ T cells in graft samples (left column) and in the corresponding patient 2 months post SCT (right column). Top three plots are of patients developing a low-level viral reactivation, lower plots are of a patient without viral reactivation.

factors for the development of CMV reactivation after allogeneic stem cell transplantation could however not be distilled from our patient group. The 40 patients included in our study seem to be a biased group resulting in a skewed perception of the risk of CMV reactivation. In the larger study (n=108) conducted by our group (F.L. Pietersma *et al*, manuscript in press), which also encompassed the 40 patients in this study, we did observe SCT related factors which were significantly associated with the develop-

ment of viral reactivation in concordance with literature. One explanation could be the overrepresentation of unrelated donors. In the larger patient cohort, 56.5% of patients received transplantation from an unrelated donor, against 82.5% of patients included in this study. The portion of CMV seronegative unrelated donors is over represented in our smaller cohort (45% of patients against 29% of patients in the large cohort), while the percentage of related CMV seropositive donors is much lower (7.5% against 20% in the large cohort). This effect has been previously described by Zhou *et al* where it is suggested that this discrepancy can be caused by similarity of the CMV status amongst siblings[9]. Since our study encompassed mainly unrelated donors this effect could have resulted in the skewed associated of CMV donor serostatus and viral reactivation.

The skewed distribution of patients in this study makes it very difficult to analyse the differences between recipients of CMV seropositive and seronegative graphs in respect of the subsequent development of CMV reactivation.

The more differentiated $CD8^+$ pool in healthy CMV seropositive individuals[14-17] is also reflected in the stem cell grafts derived from these individuals. We found that the $CD8^+$ T-cell pool in a seropositive graft is of a more differentiated phenotype (% of $CD27^-$/$CD45RO^-$/$CD8^+$ T cells). Even though CMV seropositive grafts clearly contained more differentiated $CD8^+$ T cells there was no difference in expression of CD57 in $CD8^+$ T cells. This could be explained by a recent finding that the regulation of CD57 is TCR independent, in contrast to the TCR-driven differentiation of CD45RA expression within antigen-specific responses[19].

However, we were not able to correlate the difference in phenotype to the development of CMV reactivation in the recipients of these grafts. This is most likely caused by absence of the association of CMV donor serostatus with CMV reactivation due to the biased patient group. The T-cell constitution of the graft seems to play a more important role in the T-cell reconstitution early after SCT. We found that patients receiving a CMV seropositive graft have significantly higher absolute numbers of $CD8^+$ T cells during the first 3 months post SCT. Subsequently, the majority of patients that developed a low level viral reactivation received a CMV seropositive graft (86,7%) and these individuals had higher $CD8^+$ T cells numbers compared to patients that developed a high-level viral reactivation (of which only 54,5% received a CMV seropositive graft). This is in line with our previous findings that transplantation with a CMV seropositive graft results in less severe (lower maximum viral loads) reactivations (F.L. Pietersma, submitted) Further studies should elucidate whether the phenotypical constitution as well as absolute $CD8^+$ T-cell numbers of the donor are reflected in the $CD8^+$ T reconstitution and phenotype of

the recipient irrespective of viral reactivation, or whether viral reactivation is also likely to trigger $CD8^+$ T-cell differentiation and increase in T- cell numbers.

Presence of CMV-specific $CD8^+$ T cells in the stem cell graft could result in a faster CMV-specific T-cell reconstitution in the recipient and this could lead to better containment of the CMV reactivation. Previous studies have shown that the number of CMV-specific $CD8^+$ T cells in the graft is inversely correlated with the number of recurrent CMV infections[11]. Also, a more rapid reconstitution of multifunctional CMV-specific $CD8^+$ T cells was described in patients receiving a CMV seropositive graft[9]. Scheinberg *et al* showed recently that the reconstitution of CMV-specific $CD8^+$ T cells after SCT was determined by the phenotypical characteristics of the CMV-specific T cells in the donor. They found that less differentiated CMV-specific T-cell responses found in the donor persisted in the patient, while the terminally differentiated CMV-specific $CD8^+$ T cells found in the graft decreased after SCT in the patient[20].
In our study, we were limited in patient numbers since CMV-specific $CD8^+$ T cells could only be analysed in recipients of CMV seropositive grafts that were HLA-A2 or HLA-B7 positive (n=14). Since only 2 patients in this group developed a high-level viral reactivation we cannot draw conclusions on the protective immunity transferred by CMV-specific $CD8^+$ T cells in the graft despite the absence of CMV-specific $CD8^+$ T cells in these 2 patients. However, the presence of CMV-specific $CD8^+$ T cells in our patients 2 months post SCT only in recipients of CMV seropositive grafts does suggest that these cells are of donor origin and therefore transfer of CMV-specific immunity has taken place.

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Chapter 10

Controlling herpesviruses after allogeneic stem cell transplantation: Predictive features of T-cell immunity General discussion

Allogeneic haematopoeitic stem cell transplantation (SCT) is a treatment for a wide range of malignant and non-malignant hematological diseases. Donor stem cells, either of mobilized peripheral blood origin, bonemarrow or cord blood are infused after conditioning the patient with chemotherapy and/or total body irradiation[1]. However, the immunosuppressed state of the patient following SCT can give rise to a wide arrange of complications. Common early complications are on the one hand acute graft versus host disease, in which donor T cells recognize and react to minor histocompatibility antigens on host cells[2] and on the other hand viral complications. These viral complications, which can be both primary infections as well as viral reactivation from latency of already encountered persistent viruses, are thought to arise due to a lack of immune control caused by the immunosuppressed condition of the patient.
Epstein-Barr virus and Cytomegalovirus are two common causes of viral complications after allogeneic SCT. Both viruses infect the majority of the population during early childhood[3;4] and persist lifelong in the host in whom there is a tightly regulated balance between the virus infected cells and cytotoxic T cell control [3;5;6]. Reactivation from latency or primary infection of these viruses after allogeneic SCT can result in EBV-associated post transplant lymphoproliferative disorders (PTLD) or CMV disease. Early identification and pre-emptive treatment of viral infection/reactivation is therefore crucial in patients after allogeneic SCT. In the studies conducted in this thesis approximately 49% of all patients had detectable EBV and or CMV DNA in plasma during follow up (chapter 5,6 and 8). However, only a small percentage of these patients developed clinical complications (PTLD or CMV disease). We were interested in two different aspects of the viral reactivation. First, we investigated the onset of the viral reactivation to address the question what the trigger for viral reactivation is and whether we can predict the subsequent course of the reactivation. Second, we investigated total T-cell and EBV- and CMV-specific immunity and its effect on the course of the viral reactivation to address the question whether quantity or quality of the T-cell reconstitution matter in prevention of severe viral reactivation and subsequent complications.

Viral load monitoring and clinical outcome post SCT

Viral load monitoring is crucial in studying T-cell reconstitution after SCT in the light of viral complications. Prior to transplantation, patients and donors are serologically screened for EBV and CMV. Following transplantation patients are monitored closely for EBV and CMV-DNA by real-time TaqMan™ PCR assay[7-10] in plasma, whole blood or peripheral blood mononuclear cells (PBMC)[11-13]. This results in different thresholds for determining viral reactivation, since viral DNA in whole blood is the total of both plasma and PBMC and even more, sensitive assays can detect EBV-DNA in PBMC of healthy carriers (reviewed in chapter 2). For monitoring patients at risk of CMV reactivation, not only

detection of CMV-DNA but also detection of CMV pp65 antigen in leukocytes is often used for viral monitoring[14]. This results in large variation of viral load levels between studies conducted in allogeneic SCT recipients and subsequently in the interpretation and threshold for instituting pre-emptive therapy (reviewed in [15]).
Differences in viral load monitoring, variation in outcome used in different studies together with the changing conditioning regime following SCT make it very difficult to compare studies investigating T-cell immunity after allogeneic SCT. In the studies described in this thesis, we defined viral reactivation when EBV or CMV-DNA exceeded the detection limit of 50 copies/ml in plasma during follow up. Reactivating patients were subsequently subdivided into low-level or high-level viral reactivation based on whether their peak viral load during a certain follow up period exceeded 1000 copies/ml. We chose 1000 copies/ml since this level has been described to be predictive for development of EBV associated PTLD [16] and this is the threshold for pre-emptive rituximab therapy. For CMV pre-emptive therapy is given above 500 copies/ml in our institution but this threshold is more flexible (reviewed in[16]). Therefore we classified CMV into low and high-level reactivations also based on the threshold of 1000 copies/ml.

Previous studies investigating viral reactivation after allogeneic stem cell transplantation often have viral complication (i.e. CMV disease or PTLD) as outcome. The role of adequate T-cell immunity in preventing and predicting these complications has been widely demonstrated[17;18]. However, in the era of T-cell undepleted grafts together with reduced intensity conditioning regime (non-myeoablative) used in most patients, the incidence of viral complications has decreased. In the studies conducted in this thesis approximately half of all patients developed viral reactivation (loads exceeding detection limit of 50 copies/ml) and 20% developed high-level viral reactivation (loads exceeding 1000 copies/ml). Clinical complications were not evaluated in our studies since T-cell undepleted grafts used in our hospital, in combination with the mostly non-myeoblative conditioning regime together with close viral load monitoring resulted in few cases of CMV disease or PTLD. However, in chapter 3 and 7 we describe virus-specific responses in a patient with CMV disease (chapter 7) and a patient with PTLD (chapter 3). In both patients there is a clear lack of both general and virus specific immunity following SCT resulting in CMV colitis and PTLD, which is in line with previous studies investigating CMV disease and PTLD development [17-20].

What triggers viral reactivation?

It is generally accepted that CMV and EBV related complications following SCT are caused by a lack of adequate virus specific T-cell immunity after SCT[3;5;6]. In line with this we show that patients that develop high-level EBV and/or CMV reactivation, and are therefore

at risk of viral complications, have a poorer functional virus specific T-cell reconstitution compared to patient without viral reactivation (chapter 3 and 7). However, here we specifically aimed to study the role of lack of T-cell immunity as the actual trigger for the onset of viral reactivation. Interestingly, we observed no difference in absolute numbers of $CD8^+$ T cells during the first 3 months post SCT between patients with and without viral reactivation (chapter 6), suggesting that it is not lack of T cell reconstitution that causes viruses to reactivate per se. This is supported by the fact that despite low T-cell numbers present in all patients shortly after SCT due to the condition regime, not all patients reactivate (approximately 50% in the studies conducted in this thesis). This implies that other factors contribute to viral reactivation.

In chapter 5 we investigated perforin expression as a marker for $CD8^+$ T-cell cytotoxicity and whether this could predict onset of viral reactivation and/or severity. Interestingly, high levels of perforin expressing $CD8^+$ T cells were associated with development of high-level viral reactivation. However, this perforin expression preceded high viral loads and often even preceded the onset of viral reactivation. Within 3 weeks post SCT, high levels of perforin expressing $CD8^+$ T cells were measured in patients that subsequently developed high-level viral reactivations. Although we were not able to identify the specificity of these cells due to the very low numbers of T cells this early after SCT, Meij *et al* have shown that EBV-specific T cells do not appear prior to 1 month post SCT[18]. Therefore we do not assume that these perforin expressing cells are virus specific.

We hypothesise that the perforin expression is caused by a transplantation associated trigger which also can provide the trigger for onset of viral reactivation. An explanation could be that viral reactivations can occur due to activation of the immune response which occurs because the stem cell graft has to win space over resident blood cells to establish full donor chimerism[21]. This activation of the immune system could also trigger a multitude of immune cells such as latent EBV infected B cells or activation of endothelial cells in the case of CMV resulting in viral reactivation. In HIV infected individuals, such an active role for triggering and/or dysregulating B-cell responses by HIV-1 despite an intact immune system has been shown[22]. Also, acute graft versus host disease (aGVHD) is a common risk factor for the development of CMV reactivation[23] and could be explained by the activation of (CMV infected) endothelial cells which are a common target in aGVHD.

Despite similar T-cell reconstitution rates between patients with or without detectable viral loads, the role of the T-cell in onset of viral reactivation cannot be completely omitted. In several studies conducted in this thesis, in-vivo T-cell depletion through ATG administration has been shown to be a risk factor for viral reactivation (chapter 6 and 8).

As described in literature, ATG administration, results in a slower $CD8^+$ T-cell reconstitution after SCT[24]. Furthermore, the association between ATG and the development of EBV reactivation and subsequent higher risk of PTLD has been described previously[16]. We observed this slower rate of $CD8^+$ T-cell reconstitution also in our studies. As expected, patients that received ATG have lower absolute $CD8^+$ T-cell counts directly after SCT (figure 1a). These numbers remained lower during the first few weeks post SCT compared to patient without ATG. However, just after the (average) onset of viral reactivation (median 26 days, range= 4-84; chapter 5) numbers of T cells expanded and patients that received ATG reached higher absolute $CD8^+$ T-cell counts from 7 weeks onward compared to patients that did not receive ATG (figure 1a). This increase in $CD8^+$ T-cell counts is observed at the same time point when patients that develop high-level CMV reactivations start to show rapid expansion of the absolute $CD8^+$ T cell pool (figure 1b)(and chapter6).
Therefore we hypothesise a two hit model for onset of viral reactivation, in which another trigger is necessary to initiate viral reactivation, next to lack of T-cell control. This is in line with data from Thorley-Lawson *et al* showing that two events must take place before a post transplant lymphoma develops. In brief, EBV infection of another type of B-cell (not the naïve B cell which is the case under normal conditions) in the Waldeyer's ring leads to expression of the growth programme from which it cannot exit. Subsequently, lack of T-cell control due to the immunosuppressive state results in continuous replication of this infected B cell resulting in PTLD[25].

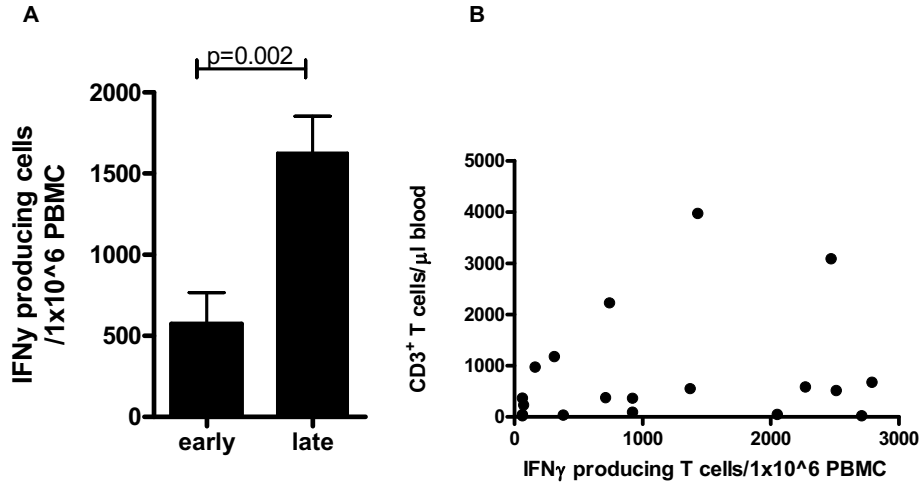

Figure 1. Functional capacity does not correlate with T-cell numbers

A) Median number of IFNy producing T cells/1x10^6 PBMC after stimulation with PHA as measured by Elispot assas early after SCT (2 months) and late after SCT (6 months)

B) No correlation between number of IFNy producing T-cells/1x10^6 PBMC and absolute number of $CD3^+$ T cells (r= 0.269, p= 0,265)

Viral infection, reactivation or re-infection?

EBV and/or CMV DNA detected in plasma can not only be caused by reactivation of the patients own virus, but also by re-infection with the donor strain or primary infection in the case of a seronegative patient. However, in most studies, when EBV or CMV DNA is detected after allogeneic stem cell transplantation it is often referred to as viral reactivation. The role of patient serostatus for both EBV and CMV has been widely studied. EBV seronegative patients are at higher risk of developing PTLD due to primary infection after SCT [26], while CMV seropositivity is a predictor for transplantation related mortality[27]. Since the majority of patients are EBV seropositive (90%) and a large portion are CMV seropositive (35-70%) prior to transplantation[3;4], the generally used term for viral load increase after SCT is reactivation. However, another possibility is re-infection with the viral strain originated from the donor which can be transferred within the graft. It is unclear whether potential re-infection would be different from reactivation of the already present virus with respect to onset and height of viral load levels and the extent of re-infections compared to reactivations. In chapter 8 we analyzed patient and recipient CMV serostatus and the onset together with subsequent severity of viral reactivation. Here we found that CMV seropositive recipients of a CMV seropositive graft reactivate more frequently compared to recipients of a CMV seronegative graft. However, the magnitude of the viral reactivation was much higher in recipients of CMV seronegative grafts. Even more, the total duration of the viral reactivation was longer in recipients of CMV seronegative grafts. Other studies have also shown that CMV seronegative grafts result in occurrence of late CMV reactivations[28], higher incidence of CMV-related disease [23;29] and that these patients are more likely to require anti-viral therapy[30] than recipients of CMV seropositive grafts. Therefore, we postulate that after receiving a CMV seropositive graft, re-infection with the donor strain can occur, which may lead to a higher frequency of viral load detection in this group of patients, but transferred immunity within the graft results in adequate viral control, which leads to less severe 'reactivations'. In recipients of CMV seronegative grafts, the main source for viral load detection is the patients own strain, but is more difficult to control due to the absence of CMV specific immunity which is destroyed by the condition regime. However in all patients, CMV re-infection with a strain not originated from donor or recipient but from for example household contact cannot be excluded. In this light, studies investigating strain variation between donor and recipient and the strain which subsequently reactivates could be very interesting. One study investigating the strain variation after transplantation was conducted in recipients of solid organ transplantation originated from similar donors by comparing glycoprotein B genotypes. Strains were compared based on variation between reactivating seronegative or seropositive patients that received organs from the same donor. Their data suggest that in seropositive recipients viral reactivation with

the patient's own strain occurs in half of the cases and re-infection with donor strain in the other half. Moreover, they show that multiple CMV strain can be transferred from donor to recipient and that these can reactivate simultaneously or sequentially[31]. Meijer *et al* conducted a small study with 6 recipient/donor pairs and found that in half of the patients reactivations were re-infections with donor strains. Moreover, the infections caused by exogenous strains were more severe compared to infections by endogenous strains[7]. However, future studies in a larger patient cohort should confirm these data.

These data suggest that less severe viral reactivations occurred in patients that reactivated their endogenous strain, which can be explained by transfer of CMV specific immunity within CMV seropositive graft (chapter 9). We found that in concordance with studies conducted in healthy individuals[32-35], CMV seropositive grafts contain a more differentiated T-cell pool and CMV-specific T cells were detected in 50% of the grafts. We also observed that this leads to a more differentiated T-cell pool after SCT in the patient compared to recipients of CMV seronegative grafts (chapter 7). However we could not find a correlation between the presence of CMV specific T cells in the graft and the subsequent onset or severity of viral reactivation (chapter 9). In addition, we could not find an association with viral reactivation or severity. Neither did the presence of CMV-specific immunity in the graft correlate with the presence of CMV-specific $CD8^+$ T cells in the patient 2 months post SCT. However, this study was limited by a very biased patient group. A larger patient study, conduction in a more heterogeneous group of transplantation patients should be conducted in order to draw further conclusion on the benefit of transferred immunity in recipients of CMV seropositive grafts.

T cells after SCT, does quantity or quality matter?

In chapter 6 we investigated whether absolute $CD8^+$ T-cell numbers early after SCT could predict onset and subsequent severity of viral reactivation. However, we found that there were no differences in $CD8^+$ T-cell numbers throughout the first 3 months post SCT between patient with or without viral reactivation, regardless of the severity. However, absolute numbers of T cells after SCT have been shown to be a good predictor for clinical complications. Annels *et al* determined a threshold of absolute $CD3^+$ T cells during onset of viral reactivation at which pre-emptive therapy can be withheld due to the capability of the patients own T-cell response.
Interestingly, despite not finding any differences in absolute $CD8^+$ T-cell numbers around the onset of viral reactivation, we did find large differences in $CD8^+$ T-cell numbers 6 months post SCT. Patients that developed high-level CMV reactivation had disproportional high numbers of $CD8^+$ T cells at 6 months post SCT. The median number of $CD8^+$ T cells 6 months post SCT in these patients was more than three times higher compared to

healthy individuals. This rapid expansion of $CD8^+$ T cells was only observed in high-level CMV reactivating patients, not in high-level EBV reactivating patients. The high numbers of absolute $CD8^+$ T cells after high-level CMV reactivation can give a false positive perception of adequate T-cell reconstitution after SCT because these $CD8^+$ T cells likely have a restricted repertoire. Therefore, investigating functional and phenotypical data alongside absolute T-cell numbers is important in determining adequate T-cell immunity.
We investigated whether absolute T cell numbers correlated with functional capacity of the T cells. To this end we performed an IFNγ ELIspot assay after stimulation with a non-specific stimulus (phytohemagglutinin, (PHA)) in the high-level reactivating patients described in chapter 6. We found that the amount of IFNγ producing T cells/$1x10^6$ PBMC was significantly lower at 2 months post SCT (early) compared to 6 months post SCT (late) (figure 2a). Absolute $CD3^+$ T-cell counts were also determined in these patients at 2 and 6 months post SCT and interestingly, there was no correlation between the absolute number of $CD3^+$ T cells and the functional capacity (figure 2b). This suggest that the increased functionality at 6 months post SCT is not caused by an increase in T-cell numbers but an increase in the functional capacity of the T cells providing a rationale for measuring general T-cell functionality as a measure of adequate T-cell reconstitution. In line with this, we observed that in patients without or with low-level EBV viral reactivations, total T-cell functionality, as identified after PMA/ionomycin stimulation, reconstituted rapidly after SCT and remained high, while patients with high-level viral reactivation had much lower percentages of IFNγ $CD4^+$ and $CD8^+$ T cells after SCT. (chapter 3)

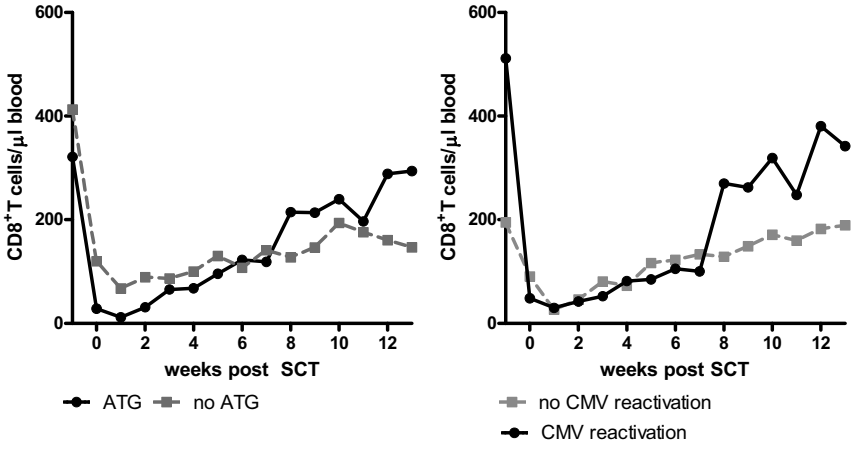

Figure 2. Role of ATG in early T-cell count recovery

A) Average number of $CD8^+$ T cells during 3 months post SCT for patients (n=.87) that received ATG (black dots) and patients that did not receive ATG (grey squares)

B) Average number of $CD8^+$ T cells/µl blood during 3 months post SCT for patients (n=45) that developed CMV reactivation (black dots) and patients that did not develop CMV reactivation (grey squares)

The studies described in chapter 3 and 7 are one of the first studies investigating the functional capacity of virus specific T cells after SCT. Several studies have investigated the EBV and CMV specific reconstitution after allo-SCT in numbers by use of tetramers and describe the subsequent link between poor virus specific T-cell reconstitution and onset of disease [17;18]. For CMV it has been shown that there is a suppressed production of IFNγ production by both $CD4^+$ and $CD8^+$ T cells in patients with CMV disease [19;20]. Since the presence of EBV and CMV specific T-cells after SCT has been linked to the prevention of disease, these cells are expected to be functional[17;18]. However, previous studies in HIV infected individuals showed a discrepancy between the functional capacity and absolute numbers of EBV-specific $CD8^+$ T cells[36]. This is in line with our data described above in which functional capacity of the T cells is not directly correlated with the absolute number of T cells. Moreover, our results indicate that functional capacity of the T cells may play a more important role than the absolute number of T-cell in viral reactivation severity. Therefore, studies investigating the functional T-cell reconstitution after allogeneic SCT, by means of novel techniques as the 12 day expansion assay used in chapter 3 could provide more insight in adequate T-cell immunity after allogeneic SCT.

Concluding remarks

In conclusion, the studies described in this thesis all focussed on controlling herpesvirus reactivation early after allogeneic stem cell transplantation. In figure 3 we incorporated the factors discussed in this thesis that influenced both onset and/or severity of viral reactivation. Two different aspects addressed in this model are: 1) factors that can influence the onset of the viral reactivation and 2) factors influencing the severity of the viral reactivation. Onset can be influenced by the patient/donor serostatus prior to transplantation (both primary infections or re-infections), conditioning regime and complications such as aGVHD. However, all these factors might also have an impact on the subsequent severity of the viral reactivation. Factors influencing the severity all contribute to proper T-cell reconstitution post SCT. The serostatus of the donor can give rise to viral reactivation due to re-infection with the donor strain on one hand, but on the other hand it could enhance T-cell reconstitution by transfer of virus specific immunity within the graft. Also the conditioning regime might influence viral reactivation when in-vivo T-cell depletion by ATG administration is given as well as causing a slow T-cell reconstitution on the long term through the conditioning regime (myeloablative versus non-myeloablative). However, close monitoring of patients both through viral load detection and measuring T-cell reconstitution (both in numbers and function), upon which preemptive therapy can be installed can prevent viral reactivation developing into viral complications.

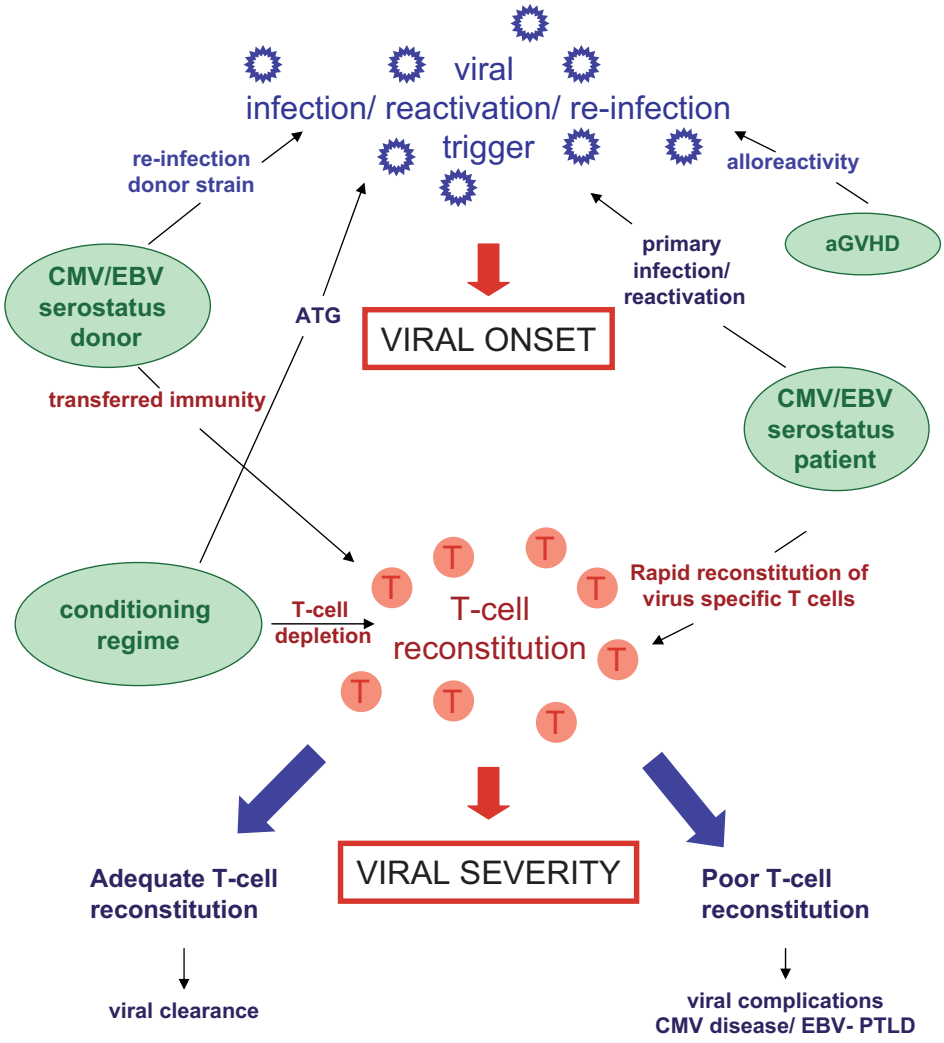

Figure 3. Transplantation factors involved in onset and severity of viral reactivation

Model incorporating several transplantation associated factors that influence the onset and/or severity of viral reactivation described in this thesis.

Future directions

The studies described in this thesis have provided insights in the role of T-cell immunity in onset of viral reactivation after allogeneic stem cell transplantation as well as the role of general and virus specific T-cell reconstitution in reactivation severity. However, these data also resulted in more questions regarding the T-cell control of herpesvirus reactitvation after SCT. The importance of CMV serostatus of patient and donor in severity of viral reactivation suggest that reactivation or re-infection results in a different course of the reactivation. Therefore, it would be interesting to study strain variation of CMV and EBV in both the recipient and the donor and investigate whether reactivation or re-infection is the major cause of severe viral reactivation and complications.

The prognostic capacity of early perforin expression in $CD8^+$ T-cell can provide a new tool for identifying patients at risk of viral complication early after SCT. A patient study, in which viral monitoring is reduced in patients with low perforin expression and increased in patients with high expression as well as withholding pre-emptive therapy in reactivating patients without early perforin expression could provide the basis for implementing perforin as a diagnostic marker in the clinic. Also, the very early expression of perforin suggests that the trigger for viral reactivation starts early after SCT. Therefore studies investigating the early innate immune response, by measuring for example absolute numbers of NK cells after SCT could provide more insight into early triggers of viral reactivation. In addition, the high intracellular levels of perforin do not say anything about the cytotoxic capacity of these cells. Further studies could investigate the potential of these perforin positive cells to excrete the perforin by ELISA. Even more, the specificity of these cells could be elucidated by using viral specific and non-specific stimuli to trigger perforin excretion.
We hypothesized that measuring T-cell functionality is more important in determining adequate T-cell immunity than measuring absolute T-cell counts. Moreover, we showed that a restricted repertoire of $CD8^+$ T cells after CMV reactivation can provide a false positive interpretation of adequate reconstitution. Therefore, studies investigating phenotypical characteristics of the T-cell reconstitution (i.e. naive T cells through thymic output) alongside absolute T-cell counts, would provide important insights in the induction of a multi-functional immune response capable to respond to a multitude of potential pathogens. Also, analysis of absolute $CD4^+$ T-cell numbers in addition to the $CD8^+$ T-cell reconstitution data described in this thesis could provide more insight in controlling herpesvirus reactivations after allogeneic SCT.

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Chapter 11

Nederlandse samenvatting: Controle van herpesvirussen na allogene stam cel transplantatie: Voorspellende aspecten van T-cel immuniteit

INTRODUCTIE

Een allogene stamceltransplantatie is een therapie, veelal gebruikt als laatste redmiddel bij een verscheidenheid aan maligne en niet-maligne aandoeningen zoals leukemie, non-hodgkin lymfomen, ziekte van Hodgkin, ziekte van Kahler of aplastische anemie. Hierbij ontvangt de patiënt stamcellen van een donor die verkregen zijn uit beenmerg, navelstrengbloed of gemobiliseerd perifeer bloed. Voordat de patiënt de stamcellen ontvangt wordt het eigen immuunsysteem geheel of gedeeltelijk uitgeschakeld door middel van chemotherapie en radiotherapie. Hierbij worden ook de cellen van het afweersysteem van de patiënt uitgeschakeld en moet het immuunsysteem opnieuw opgebouwd worden uit de stamcellen van de donor. Door deze immuungecomprimeerde situatie van de patiënt kunnen er verschillende complicaties optreden. Zowel primaire virale infecties als het reactiveren van virussen die de patiënt al met zich meedraagt kunnen zorgen voor ernstige complicaties.
Twee virussen die voor problemen in de vroege post transplantatie periode kunnen zorgen zijn het Epstein-Barr virus en het cytomegalovirus. Het Epstein-Barr virus is een gammaherpes virus dat meer dan 90% van de bevolking infecteert gedurende het leven. Normaliter vindt besmetting in de vroege jeugd plaats en verloopt dit asymptomatisch. Als besmetting pas tijdens de adolescentie plaatsvindt, kan er infectieuze mononucleosis, ook wel bekend als de ziekte van Pfeiffer, optreden. Deze aandoening kenmerkt zich door extreme vermoeidheid, keelpijn en gezwollen lymfeklieren. Cytomegalovirus is een betaherpesvirus dat bij 35-70% van de bevolking aanwezig is en bij meer dan 90% in onderontwikkelde landen. Beide virussen blijven levenslang in de gastheer aanwezig waar ze in bedwang worden gehouden door het immuunsysteem en in het bijzonder door de cytotoxische $CD8^+$ T cellen.

In de immuungecomprimeerde situatie die gecreëerd is na een stamceltransplantatie kan een reactivatie of primaire infectie van het Epstein-Barr virus resulteren in een posttransplantatie lymfoom. De manier waarop een posttransplantatie lymfoom kan ontstaan na SCT wordt beschreven in hoofdstuk 2 van dit proefschrift. Reactivatie of infectie met het cytomegalovirus kan leiden tot CMV ziekte. Afhankelijk van de betrokken organen kan CMV ziekte zich in verschillende ziektebeelden uiten zoals longontsteking, darmontsteking of een ontsteking van het netvlies.

DIT PROEFSCHRIFT

In dit proefschrift is er gekeken naar verschillende factoren vroeg na de stamceltransplantatie die het ontstaan en de eventuele ernst van een virale reactivatie kunnen voor-

spellen. Allereerst hebben we gekeken naar het ontstaan van de virale reactivatie om meer inzicht te kunnen krijgen in de daadwerkelijke aanzet van de reactivatie evenals het verloop van de reactivatie. Daarnaast hebben we de vroege EBV en CMV specifieke T-cel reconstitutie bestudeerd en de effecten hiervan op het verloop van de reactivatie alsmede de vraag of het de kwantiteit van de virus specifieke respons of de kwaliteit van de virus specifieke respons is die zorgt voor adequate T-cel immuniteit.

Het monitoren van patiënten na stamceltransplantatie voor het ontstaan van virale problemen gebeurt door middel van detectie van virus deeltjes in het plasma. Dit is onderdeel van de wekelijkse diagnostische routine tot een half jaar na SCT. Hoofdstuk 2 geeft een overzicht van de literatuur met betrekking tot het ontstaan en diagnosticeren van EBV reactivaties na stamceltransplantatie. In het patiënten cohort, dat voor de studies die beschreven staan in dit proefschrift gebruikt is, komen weinig ernstige virale complicaties voor. Dit wordt mede veroorzaakt door mildere conditionering voorafgaande aan de transplantatie (zogenoemde niet-myeloablatieve conditionering) evenals het gebruik van ongedepleteerde transplantaten (hierbij worden de T cellen niet verwijderd uit het transplantaat). In hoofdstuk 3 en hoofdstuk 7 beschrijven we 2 patiënten die respectievelijk een post transplantatie lymfoom (hoofdstuk 3) en een CMV colitis (hoofdstuk 7) ontwikkelen ten gevolge van een EBV reactivatie en een primaire CMV infectie. In beide gevallen is er een duidelijk gebrek aan virus specifieke immuniteit kort na SCT wat resulteerde in de ernstige complicaties. In hoofdstuk 4 laten we juist de andere kant zien, hier hebben we een patiënt gevolgd die een adequate T-cel response laat zien na een primaire EBV infectie ten gevolge van een harttransplantatie (EBV positief hart getransplanteerd in een EBV negatieve patiënt). Het snelle ontstaan van de EBV specifieke T-cel response na het ontstaan van de primaire infectie ondanks de immuunsuppressie zorgt in dit geval voor een goede controle van de infectie en voorkomt het ontstaan van virale complicaties.

Vroege detectie van het ontstaan en ernst van de virale reactivaties

In dit proefschrift hebben we onderzocht of we kort na SCT het ontstaan en/of de ernst van de virale reactivatie kunnen voorspellen op basis van verschillende aspecten van T-cel immuniteit. In hoofdstuk 5 hebben we de voorspellende waarde van perforine onderzocht. Perforine is een molecuul dat door geactiveerde $CD8^+$ T cellen wordt uitgescheiden met als doel gaatjes te maken in de celwand van de virus geïnfecteerde cel zodat andere moleculen die de cel kapot kunnen maken naar binnen kunnen gaan. We hebben de hoeveelheid perforine positieve $CD8^+$ T cellen gemeten kort na transplantatie (gedurende de eerste 12 weken) en dit gecorreleerd aan de ernst van de virale reactivatie (zowel EBV and CMV). Patiënten die kort na transplantatie een hoog percentage perfo-

rine+/CD8+ T cellen hebben, hebben een grote kans op het ontwikkelen van een ernstige virale reactivatie (virus titer > 1000 kopieën/ml plasma). Daarnaast hebben we onderzocht of het absolute aantal T cellen vroeg na transplantatie een rol speelt in het ontstaan en de ernst van de reactivatie. In hoofdstuk 6 hebben we de absolute CD8+ T cellen in het bloed van een groep patiënten gemeten gedurende het eerste half jaar na transplantatie. Er waren geen verschillen meetbaar in de T-cel reconstitutie in de patiënten die een virale reactivatie ontwikkelden en patiënten zonder. Wel zagen we, op een half jaar na SCT, dat patiënten die een ernstige CMV reactivatie hebben ondergaan (virus titer >1000 kopieën/ml plasma) significant meer CD8+ T cellen per ml bloed hebben. Deze CD8+ T cellen lijken allemaal CMV specifiek te zijn, waardoor een beperkt T-cel repertoire is ontstaan. Doordat het absolute aantal CD8+ T cellen hoog is, is er een schijnveiligheid van adequate T cel reconstitutie gecreëerd, terwijl deze reconstitutie juist beperkt is qua diversiteit.

Virus specifieke T cellen, is kwantiteit of kwaliteit belangrijk?

Behalve de T-cel reconstitutie in absolute aantallen hebben we in dit proefschrift ook gekeken naar de functionele aspecten van T cellen kort na transplantatie. Hiervoor hebben we de productie van interferon-gamma (IFNγ) gemeten. IFNγ wordt geproduceerd door de T cellen en heeft een antivirale werking. Het percentage T cellen dat na stimulatie (zowel algemene stimulatie als virus specifieke stimulatie) IFNγ produceert zegt iets over de functionele capaciteit van de T cellen. Om kort na transplantatie, als er maar weinig T cellen zijn, de totale functionaliteit en de virus specifieke T-cel functionaliteit te meten hebben we een nieuwe techniek gebruikt die staat beschreven in hoofdstuk 3 en 7. Door de cellen gedurende 12 dagen te kweken in de aanwezigheid van virale eiwitten of algemene T-cel stimuli, groeit het aantal (virus specifieke) T cellen tot een detecteerbaar niveau. Patiënten met een CMV (hoofdstuk 7) of EBV (hoofdstuk 3) reactivatie lieten allemaal een functionele virus specifieke response zien. Maar de totale functionaliteit, weergegeven door de totale IFNγ productie na algemene stimulatie, is veel lager in patiënten met een ernstige EBV reactivatie (hoofdstuk 3). Dit zou het gebrek aan virale controle in deze patiënten kunnen verklaren. Deze resultaten geven aan dat het meten van T-cel functionaliteit (zowel algemeen als virus specifiek) nieuwe inzichten kan geven in adequate T-cel reconstitutie en een belangrijkere rol kan spelen in het voorspellen van virale reactivaties en complicaties dan het meten van absolute aantallen.

Virale re-infectie of reactivatie?

In hoofdstuk 8 en 9 hebben we onderzocht of er een verschil is in ernst van de virale reactivatie na transplantatie van een CMV seropositieve of juist een CMV seronegatieve donor. In hoofdstuk 8 hebben we een database onderzoek gedaan in 108 CMV seropo-

sitieve SCT patiënten waarbij we gekeken hebben naar de CMV serostatus van de donor en het ontstaan en de ernst van een eventuele CMV infectie. Hier hebben we gevonden dat patiënten die een transplantatie krijgen van een CMV seropositieve donor vaker een CMV reactivatie ondergaan, maar deze is minder ernstig dan patiënten die een transplantatie van een CMV seronegatieve donor krijgen. Dit heeft geleid tot onze hypothese dat er vaker reactivaties optreden ten gevolge van CMV overdracht wat eigenlijk resulteert in een CMV re-infectie met het virus van de donor, maar dat ten gevolge van overdracht van CMV specifieke immuniteit met het transplantaat deze re-infecties minder ernstig verlopen. Om deze hypothese te testen hebben we in hoofdstuk 9 gekeken naar de aanwezigheid van CMV specifieke immuniteit in het transplantaat zelf. Hier vonden we dat CMV seropositieve transplantaten een meer doorgedifferentiëerd T-cel repertoire hebben (in hoofdstuk 7 laten we zien dat dit ook leidt tot een meer doorgedifferentiëeerd T-cel repertoire in de patiënt). Een correlatie tussen de aanwezigheid van CMV specifieke T cellen in het transplantaat en het ontstaan en/of ernst van de virale reactivatie konden we helaas niet aantonen.

CONCLUSIE

De studies beschreven in dit proefschrift hebben meer inzicht gegeven in de controle van herpesvirus reactivaties kort na allogene stamceltransplantatie. We hebben gekeken naar de rol van T-cel immuniteit in het ontstaan van de virale reactivatie, alsmede de rol van T-cel immuniteit in het verloop en de ernst van de reactivatie. In het ontstaan van de virale reactivatie speelt de serostatus van de patiënt een belangrijke rol. Een reactivatie van het virus van de patiënt, of een re-infectie met virus van de donor lijkt voor een ander verloop te zorgen mede door overdracht van virale immuniteit van de donor. In dit kader zouden studies die de virale stammen van de donor en de patiënt onderzoeken zeer interessant zijn. Voor het verloop van de virale reactivatie is de functionele capaciteit van de T cellen belangrijker dan het absolute aantal. De eventuele ernst van de virale reactivatie kan al vroeg voorspeld worden door middel van de productie van perforine. De totale T-cel functionaliteit is verstoord in patiënten die een ernstige virale reactivatie ondergaan terwijl het absolute aantal T cellen die verschilt van patiënten met een milde of helemaal geen virale reactivatie. Het volgen van patiënten, niet alleen door middel van virale titers in plasma maar juist ook door het monitoren van de T-cel reconstitutie (zowel in aantal en in functie), is belangrijk in het voorspellen welke virale reactivaties ernstig kunnen verlopen en daarmee kunnen virale complicaties voorkomen worden. De manier waarop T-cel reconstitutie in de patiënt gevolgd kan worden en de mate waarin dit naast de virale titer een rol kan spelen in de eventuele behandeling van de patiënt zou in de toekomst door middel van een prospectieve patiënten studie uitgezocht moeten worden.

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Liefs,

Floor

Curriculum Vitae

Floor Dessing- Pietersma werd geboren op 10 juli 1982 te Den Haag. In 2000 behaalde ze haar International Baccalaureate diploma aan het Kungsholmens Gymnasium te Stockholm, Zweden, waarna ze begon aan de studie Farmacie aan de Universiteit Utrecht. Na het behalen van het bachelor diploma in 2004 is ze begonnen aan de master Immunity & Infection van de opleiding Biomedische Wetenschappen aan de Universiteit Utrecht. Tijdens deze studie liep zij stage op de afdeling vaccin ontwikkeling van het Nederlands Vaccin Instituut onder supervisie van dr. C. van Els. De tweede stage voerde zij uit op de afdeling Medische Microbiologie van het LUMC onder begeleiding van dr. M. Ressing. In 2006 behaalde ze haar master titel waarna ze begon aan haar promotieonderzoek onder leiding van dr. D. van Baarle en dr. E. Meijer op de afdeling Immunologie van het UMC Utrecht. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Floor en Thomas Dessing hebben samen twee kinderen, Frederieke (Juli 2009) en Willem (December 2010).