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Brief communication

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

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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 IFN γ 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.

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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.

Objective: to analyze whether an effective EBV-specific immune response can be initiated after primary EBV-infection in a EBV-seronegative cardiac transplantation.

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. He was routinely monitored for the presence of EBV viral DNA, based on a real-time TaqMan™ EBV DNA PCR assay in ethylenediaminetetra acetic acid (EDTA)-plasma as described previously [16–18]. EBV seroconversion was determined based on the presence of IgM and IgG antibodies against the viral capsid antigen (VCA) or the EBV nuclear antigen (EBNA). During episodes of positive EBV-DNA PCR, antiviral therapy (Valaciclovir), was given at a dose of 3 × 1000 mg daily as well as reduction of the immunosuppression.

The post transplantation period remained uneventful. EBV load was measured weekly during 2 months post transplantation and

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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 for which written informed consent was obtained from the patient in accordance with the declaration of Helsinki. These blood samples were used to perform EBV load measurements and isolation of peripheral blood mononuclear cells (PBMC) for EBV-specific T-cell analysis. 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 1×10^6 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 permeabilized 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).

In addition, IFN γ producing EBV-specific T cells were enumerated using an IFN γ ELISpot assay as previously described [20]. In brief, 96 well multiscreen filter plates (Millipore, Volketswil, Switzerland) were coated overnight with 50 μ l of 15 μ g/ml anti-IFN γ monoclonal antibody (MABTECH, Stockholm, Sweden) in PBS. PBMC were incubated in triplicate at 1×10^5 cells/well in the presence of 10 μ g/ml of 15 mer 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 phytohemagglutinin (PHA) (Murex Diagnostics, Dartford UK). IFN γ production was detected using biotinylated anti-IFN γ monoclonal antibody (MABTECH). IFN γ producing cells were analyzed using an automated spot reader (AELVIS GmbH, Hannover, Germany). The number of EBV-specific IFN γ producing T cells was calculated per 1×10^6 PBMC after subtracting negative control values.

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 (Fig. 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 (Fig. 1A).

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 Fig. 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)(Fig. 1A). This fits with a study performed in pediatric recipients of a renal transplant that showed appearance

Fig. 1. A) EBV DNA load (copies/ml), depicted on the left y-axis (black line) and percentages of B57-VSF specific CD8⁺ T cells (gray 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 tetramer⁺ T cells in the blood within one month after transplantation (irrespective of EBV serostatus) [21]. 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 (Fig. 1A). All B57-VSF specific CD8⁺ T cells were of central memory phenotype both during primary infection and during reactivation (CD27⁺/CD45RO⁺) and were highly activated (CD38⁺/HLA-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-DR⁺) fluctuated throughout follow up, however, percentages of CD38⁺ and HLA-DR⁺ T cells increased during primary infection as well as during reactivation (Fig. 2A).

To analyze the potential cytotoxic capacity of the total CD8⁺ T cell population present we measured intracellular perforin expression directly ex vivo on whole blood. In healthy individuals the

Fig. 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-DR⁺ (gray triangles, gray dotted line), CD8⁺ (gray squares, gray line) and CD27⁺ (gray triangles, gray line) of CD8⁺ T cells. B) Percentage of perforin expressing CD8⁺ T cells throughout first year post transplantation (gray line, right y-axis) and EBV DNA load (copies/ml, black line, left y-axis). C) IFN γ producing T cells/ 1×10^6 PBMC (right y-axis) during first year post transplantation after stimulation in an ELISpot assay with BZLF-1 (gray triangles, gray dotted line), EBNA-1 (gray squares, gray striped line) or B57-VSF (gray circles, gray line) and EBV DNA load on the left y-axis (black line).

expression of perforin in CD8⁺ T cells ranged between 0.3 and 5.1% of CD8⁺ T cells [19]. Prior to onset of 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 total CD8⁺ T cells respectively (Fig. 2B). These data indicate that primary infection results in the development of the EBV-specific T cells with an effector phenotype (CD27⁺) while the total CD8⁺ T cell pool demonstrates phenotypic characteristics equivalent to antiviral responses as seen in healthy individuals.

To investigate the functional capacity of the EBV-specific T cell response (the cells being able to respond to EBV stimuli), and to broaden our analysis by investigating other targets, we measured IFN γ 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. IFN γ producing cells were readily detected after stimulation with the IE (immediate early) lytic protein BZLF1. At the onset of primary infection (week

15) 183 IFN γ producing cells/ 1×10^6 PBMC were detected. Directly after primary infection (week 19) the number of IFN γ producing T cells increased to 1009 cells/ 1×10^6 PBMC. Between primary infection and viral reactivation the number of IFN γ producing cells decreased to 237 cells/ 1×10^6 PBMC. However, upon viral reactivation (week 37) the number of BZLF-1 specific T cells increased to 2830 cells/ 1×10^6 PBMC. Responses against the latent protein EBNA-1 remained undetected throughout followup. (Fig. 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 [22] 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.

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 [23,24] 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 [25]. 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 [26]. 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 a study performed in pediatric recipients of kidney transplants, the majority was shown to develop EBV-specific T cells within the first month, suggesting that transplant recipients receiving immunosuppression can generate EBV-specific CD8⁺ T cell responses. However, most individuals in this study were EBV seropositive before transplantation [21].

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 [27]. 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 lifelong equilibrium with the EBV infected B cells.

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