

Epstein-Barr Virus Reactivation does not Impact Total T-Cell Reconstitution but is associated with Decreased Functional T Cells after Stem Cell Transplantation

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Abstract

Epstein-Barr virus reactivations are frequently observed after allogeneic stem cell transplantation (SCT). We investigated the role of total as well as EBV-specific T-cell reconstitution in relation to onset and severity of EBV reactivation. To this end, 116 patients were prospectively sampled for viral load and absolute T-cell numbers weekly post-SCT during the first 3 months and thereafter monthly until 6 months post-SCT. In addition, we retrospectively analysed EBV-specific T-cell responses in 12 patients during the first year after SCT.

In contrast to the general belief, we found that early T-cell reconstitution after SCT does not play a role in the onset of EBV reactivation as numbers of CD4⁺ and CD8⁺ T cells during the first 3 months post SCT are similar in patients with or without viral reactivation. However, functional T-cell responses after non-specific in-vitro restimulation were impaired in patients with high-level EBV-reactivation. Although EBV-specific CD8⁺ T-cell responses were readily detected from 2 months onward, EBV-specific CD4⁺ T cells remained low throughout follow-up, and especially EBNA-1-specific CD4⁺ T cells did not normalize to healthy control levels one year post-SCT.

In conclusion, EBV-reactivation does not influence total T-cell reconstitution, but functional capacity is impaired in patients with high-level EBV-reactivation.

Keywords: EBV; SCT; T cells; Reactivation

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. Early diagnosis based on EBV-DNA monitoring has been shown important to prevent EBV-PTLD related mortality [3]. However, the positive predictive value of an EBV-DNA level of ≥ 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].

In solid organ transplant (SOT) patients low CD4⁺ T-cell counts were associated with onset of lymphoproliferative disorders and both frequency and function of EBV-specific CD8⁺ T cells was shown to be associated with absolute CD4⁺ T-cell counts [6] which may indicate an important role for EBV-specific CD4⁺ T cells in EBV-specific CD8⁺ T-cell control. Similarly, several studies have investigated the role of specific CD4⁺ T cells in controlling cytomegalovirus infections. CMV-specific effector-memory CD4⁺ T cells seemed necessary for a functional CD8⁺ T-cell response in primary infections [7]. 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 (SCT) and HIV-infected patients, respectively [8,9].

Adequate T-cell reconstitution after SCT is thus crucial in preventing viral reactivation progressing to severe complications [9,10]. Annels et al used an arbitrary threshold of 300 CD3⁺ T cells/ μ l blood during the initial phase of EBV-reactivation to determine adequate T-cell reconstitution and the subsequent need for pre-emptive therapy [11]. However, this study investigated the role of T-cell reconstitution in prevention of viral complications. In our study, we were interested in the role of 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 addition, we set out to investigate the role of EBV-specific CD4⁺ T cells in controlling EBV reactivations after allogeneic SCT. Because studying EBV-specific CD4⁺ T cells directly ex vivo is challenging due to the very low number of specific cells, a 12-day in-vitro expansion assay has been developed [10]. This enabled us to evaluate multiple functional features (both proliferative capacity as well as effector function by using IFN γ production as readout of the EBV-specific T-cells), in parallel in CD4⁺ and CD8⁺ 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, which previously showed to induce both CD4⁺ as well as CD8⁺ T cell responses [11] despite the fact that EBNA-1 was thought to be immunosilent by interfering with antigen-processing. Recent studies indicate that the mechanisms of EBNA1 antigen

processing require reevaluation and that it is an important target for CD4⁺ and CD8⁺ EBV-specific T-cell responses [12-14].

Analysis of the role of total as well as EBV-specific T-cell reconstitution in relation to onset and severity of EBV reactivation revealed that total T cell numbers were not associated with the occurrence of EBV reactivation, whereas functional T cell responses were affected by the presence of (severe) EBV reactivation.

Material and Methods

Study population

Since January 2006, patients receiving an allogeneic stem cell transplantation were followed at the department of Hematology of the University Medical Center Utrecht, Utrecht, the Netherlands. From January 2007 - June 2009, 116 patients were prospectively followed

during the first 6 months post SCT for EBV DNA load and T-cell reconstitution kinetics (Table 1A). From the complete set of patients, we selected patients to analyse EBV-specific immunity for whom both patient and donor were EBV-seropositive prior to transplantation, had a follow-up of at least a year and had PBMC samples available at 5 time points during follow up (before transplantation and at 2, 4, 6 and 12 months after transplantation). Twelve allogeneic stem cell transplant recipients could be retrospectively included (Table 1B). Although the patients were heterogeneous with respect to donor type, GvHD incidence, ATG usage and conditioning, none of these factors that may influence T-cell reconstitution was overrepresented in either of the reactivation groups. As a control group we included 6 healthy virus-carriers who had no evidence of viral reactivation (data not shown) to analyse T-cell responses. Written informed consent was obtained from all patients in accordance with the declaration of Helsinki.

| | | total | EBV reactivation | | univariate |
|---|------------------|------------------|------------------|---------|------------|
| | N = | 116 | 18 | (16.4%) | |
| Sex | M | 71 | 13 | (18.3%) | p=0.180 |
| | F | 45 | 5 | (11.1%) | |
| median age (range) | | 49.8 (17.6-70.6) | 48.5 (17.6-68.5) | | p=0.445 |
| Stemcell source | cordblood | 1 | 0 | (0.0%) | p=1.000 |
| | peripheral blood | 106 | 17 | (16.0%) | |
| | bonemarrow | 9 | 1 | (11.1%) | |
| Donor | Related | 35 | 1 | (2.9%) | p=0.015 |
| | Unrelated | 81 | 17 | (21.0%) | |
| HLA mismatch | | 20 | 6 | (30.0%) | p=0.561 |
| Conditioning | NMA | 106 | 15 | (14.2%) | p=0.359 |
| | MA | 10 | 3 | (30.0%) | |
| ATG | | 87 | 18 | (20.7%) | p=0.004 |
| EBV serology (R/D) | +/+ | 94 | 18 | (19.1%) | p=0.429 |
| aGVHD | | 64 | 12 | (18.8%) | p=1.000 |
| Abbreviations: HLA: Human Leukocyte Antigen; NMA: Non Myeloablative; MA: Myeloablative; ATG: Anti-Thymocyte Globulin; EBV: Epstein-Barr Virus; R/D: Recipient/Donor. Comparison between reactivation and no reactivation group: Fisher's Exact test. | | | | | |

Table 1A: Patient characteristics prospective study

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[15-18], which was performed routinely in all patients until 6 months post transplantation. Patients were treated preemptively with valganciclovir (900 mg twice daily) when CMV-DNA load exceeded 500 copies/ml and with Rituximab 375 mg/m² 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 (15-mer peptides with an 11 amino acid overlap from the entire BZLF1 proteome and the immunogenic C-terminal region of EBNA-1, JPT Peptide Technologies GmbH, Berlin, Germany) and IL-2 as described before [10].

Peptides were pooled at a final concentration of 1 mg/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 (1 µg/ml) and αCD28 (1 µg/ml); or αCD28 (1 µg/ml) alone as a negative control. After 1 hour, 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, 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 permeabilisation (FACS Permeabilization Solution and FACS Lysis Solution, BD) cells were stained with IFN γ -FITC (BD). A minimum of 100.000 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 IFN γ 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-1 specific CD4⁺ and CD8⁺ T cells.

| | | Sex | Age | Disease | stem cell source | Donor | HLA mismatch | Conditioning | ATG | relapse/progression | aGVHD | cGVHD |
|-------------------|----|-----|------|---------|------------------|-----------|--------------|--------------|-----|---------------------|----------|-----------|
| no reactivation | 1 | F | 47.3 | AML | PB | Unrelated | - | NMA | - | - | II - IV | - |
| | 2 | M | 43.8 | AML | PB | Related | - | NMA | - | 6 months | II - IV | Extensive |
| | 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 |
| low reactivation | 5 | F | 32.1 | AML | PB | Unrelated | - | MA | Yes | - | none - I | - |
| | 6 | M | 28.4 | ALL | PB | Unrelated | - | NMA | Yes | - | none - I | Extensive |
| | 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 |
| high reactivation | 9 | M | 44.3 | AML | PB | Related | - | MA | - | - | none - I | Extensive |
| | 10 | M | 18.8 | NHL | PB | Unrelated | - | NMA | Yes | - | none - I | - |
| | 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 Leukemia; ALL: Acute Lymphoid Leukemia; NHL: Non Hodgkin Lymphoma; MM: Multiple Myeloma; PB: Peripheral Blood; NMA: Non-Myeloablative; MA: Myeloablative; ATG: Antithymocyte Globulin; aGVHD: Acute Graft Versus Host Disease; cGVHD: Chronic Graft Versus Host Disease.

Table 1B: Patient characteristics retrospective study

Total T-cell functionality

PMA/ionomycin stimulation reflects the total capacity of a T-cell to become activated independent of TCR engagement and co-stimulation [19]. Therefore we used the capacity of CD4⁺ and CD8⁺ T cells to produce IFN γ upon PMA/ionomycin restimulation as a measure of total functional 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 (10 ng/ml), ionomycin (2 μ g/ml) and α CD28 (1 μ g/ml) or α CD28 (1 μ g/ml) alone as a negative control. After 1 hour, 1:1000 Brefeldin A (Golgiplug, BD Biosciences (BD), San José, California, 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, TRUcountTM tubes (BD) were used according to manufacturer's protocol as described before [20]. In brief, 50 μ l 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.

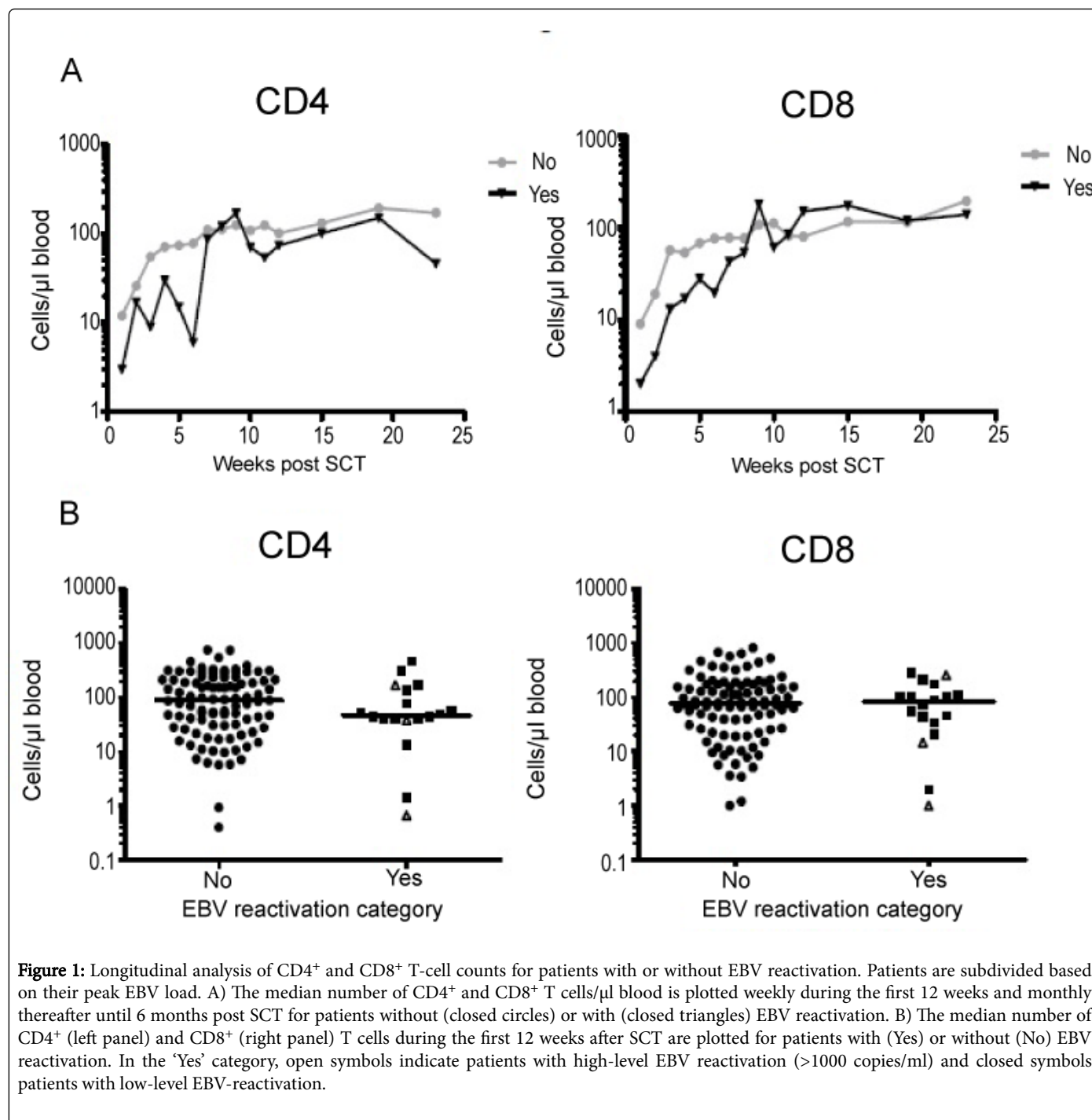
Statistical analysis

Comparison of total T-cell numbers between EBV-reactivation and non-reactivation groups as well as functional T-cell reconstitution 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

Similar T-cell reconstitution during the first 3 months post SCT irrespective of EBV reactivation

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-infections or reactivations by monitoring EBV DNA in plasma. EBV reactivation (viral load exceeding 50 copies/ml plasma) was diagnosed in 18 patients (16.4%). Seventeen of these received a SCT from an unrelated donor, and all patients received ATG (Table 1A).



Patients were subdivided into low- or high-level viral reactivation based on whether the peak viral load exceeded 1000 copies/ml. Fifteen patients developed a low-level (load < 1000 copies/ml) viral reactivation. A high-level EBV reactivation (maximum load > 1000 copies/ml) occurred in 3 patients. (data not shown) The median time to EBV reactivation was 6 weeks post SCT (range 3-56).

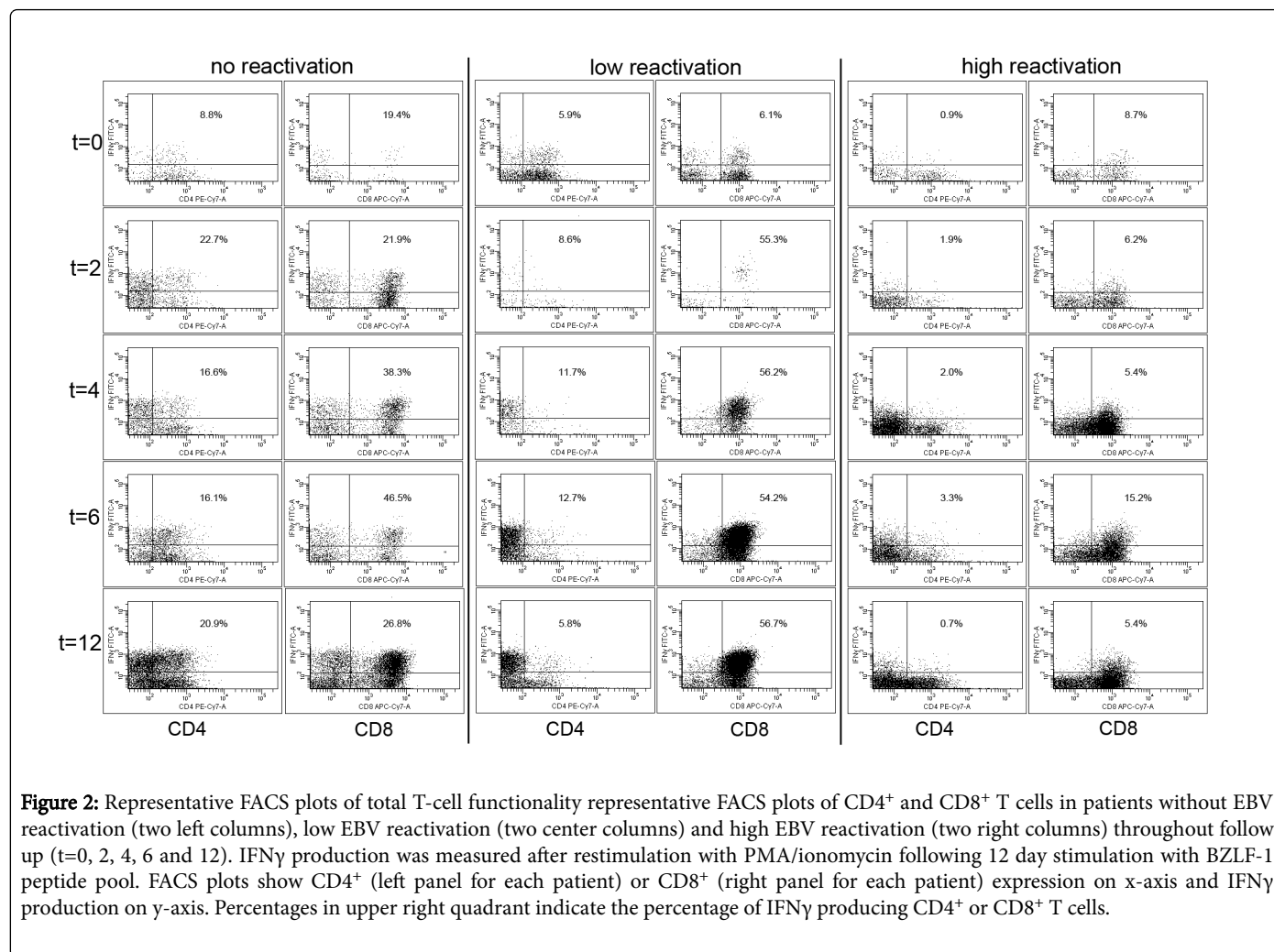
We investigated the impact of EBV reactivation on total CD4⁺ and CD8⁺ T-cell counts. CD4⁺ and CD8⁺ T-cell reconstitution dynamics (as assessed by the increase in T cell counts during the first 12 weeks) were similar for patients with or without EBV reactivation (Figure 1a).

To circumvent fluctuations in T-cell counts and the variable occurrence of reactivation during SCT, we compared patients based on the median CD4⁺ and CD8⁺ T-cell count throughout the first 12 weeks of follow-up. No difference was observed in median CD4⁺ T-cell ($p=0.24$) and median CD8⁺ T-cell numbers ($p=0.66$) between individuals that reactivated EBV (CD4⁺ 47 cells/ μ l, CD8⁺ 82 cells/ μ l) or not (CD4⁺ 89 cells/ μ l, CD8⁺ 78 cells/ μ l) (Figure 1B). Patients with high level EBV-reactivation showed a similar distribution as the individuals with low level EBV reactivation.

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

We next 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, selected from the complete set of patients followed from January 2006 onwards, were analysed at 5 time points (prior to SCT and at 2, 4, 6 and 12 months post SCT) for T-cell responses (Table 1B). 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/m² (Rituximab, Roche) was given. One of these patients developed a PTLD (11). Concurrent CMV-reactivation occurred in 3 patients (9,10 and 12).



Cells were stimulated with EBNA-1 or BZLF-1 peptide pools for 12 days. We previously have shown differential kinetics of T-cell responses towards these antigens, which may present readouts for control of infection (EBNA-1 specific T-cell responses) versus active viral replication (BZLF-1 specific responses) [21]. Representative FACS plots displaying IFN γ producing CD4⁺ and CD8⁺ T cells after PMA/ionomycin restimulation for 3 patients, one in each of the reactivation categories (without, low-level and high-level reactivation) are shown in Figure 2. 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 3a. As functional patterns were similar for patients without or with low reactivation, we analysed them together. In all patients without or with a low reactivation, the percentage of IFN γ producing CD8⁺ T cells reconstituted rapidly (Figures 2 and 3A) and

was higher (median 30.4%) compared to the percentage of IFN γ producing CD4⁺ T cells (median 6.9%) (Figures 3A and 3B). However, for patients with high-level reactivations the percentages of IFN γ producing CD4⁺ and CD8⁺ T cells remained low (Figure 3A), although percentages of IFN γ producing CD8⁺ T cells were slightly higher compared to CD4⁺ T cells (CD4⁺ T cells; median 2.8%, CD8⁺ T cells; median 8.9%). At one year post transplantation, T-cell functionality was significantly higher in patients without or with low EBV-reactivation (16.5%) compared to patients with high-level reactivations (6%) (p=0.005) (Figure 3B).

EBV reactivation impacts the number of functional EBV-specific T cells

EBV-specific functional T-cell reconstitution following SCT was determined by IFN γ production in CD4 $^+$ and CD8 $^+$ T cells after restimulation with either EBNA-1 or BZLF-1 peptide pools following expansion. (Figure 4A for representative FACS plots) BZLF-1 specific CD8 $^+$ T-cells after SCT occurred faster in all patients (detectable as early as 2 months) compared to the BZLF-1-specific CD4 $^+$ T cells, which remained undetectable until 12 months post SCT. Also, EBNA-1 specific CD8 $^+$ T cells reconstituted before the CD4 $^+$ T cells in most cases. To determine whether our patients reconstitute their (EBV-specific) immune responses to 'normal healthy' levels, we compared

the magnitude of EBV-specific T-cell responses at one year post-SCT (Figure 4B). All healthy individuals showed responses against BZLF-1 and EBNA-1 (figure 4B) after 12-day expansion with EBV-specific peptide libraries. At 1 year post SCT, there was considerable variation in the level of IFN γ producing CD4 $^+$ and CD8 $^+$ T cells following EBNA-1 or BZLF-1 stimulation. Overall, median percentages in SCT recipients were lower compared to healthy controls. This difference was especially visible for EBNA-1 specific CD4 $^+$ T cells which were significantly lower (0.50%) 1 year post SCT compared to healthy controls (2.97%, p 0.03). The low and high reactivation patients were equally distributed (data not shown).

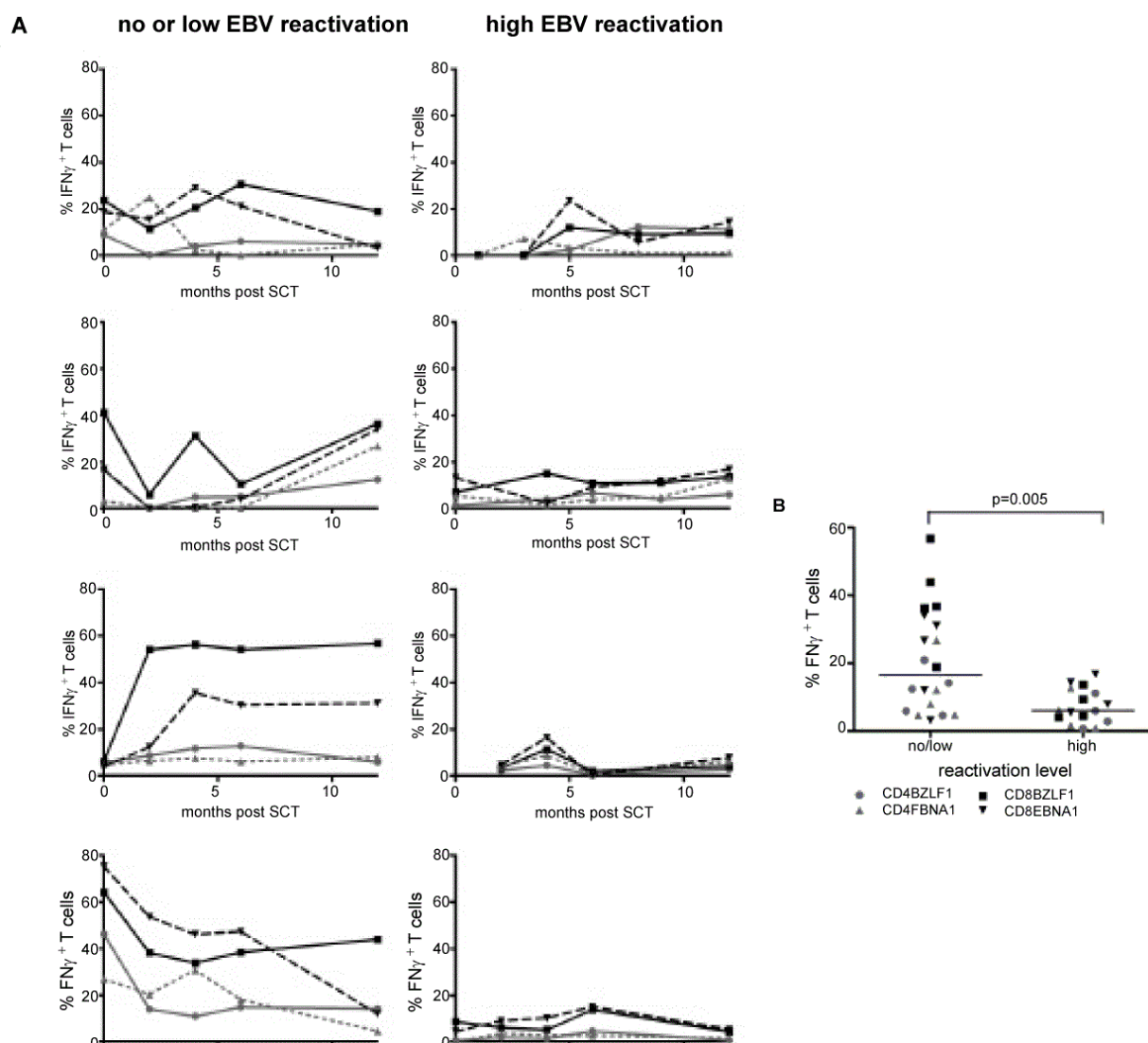


Figure 3: T-cell functionality is impaired in patients with high-level EBV reactivations. Representative graphs of 4 patients without or with low EBV reactivation (left panel) and 4 patients with high-level reactivation. %IFN γ CD4 $^+$ or CD8 $^+$ T cells after PMA/ionomycin restimulation following BZLF-1 or EBNA-1 stimulation are depicted on y-axis. Months post SCT is depicted on x-axis. Percentages of IFN γ $^+$ T cells at 1 year post SCT. Median percentages of IFN γ $^+$ 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) ($p=0.005$).

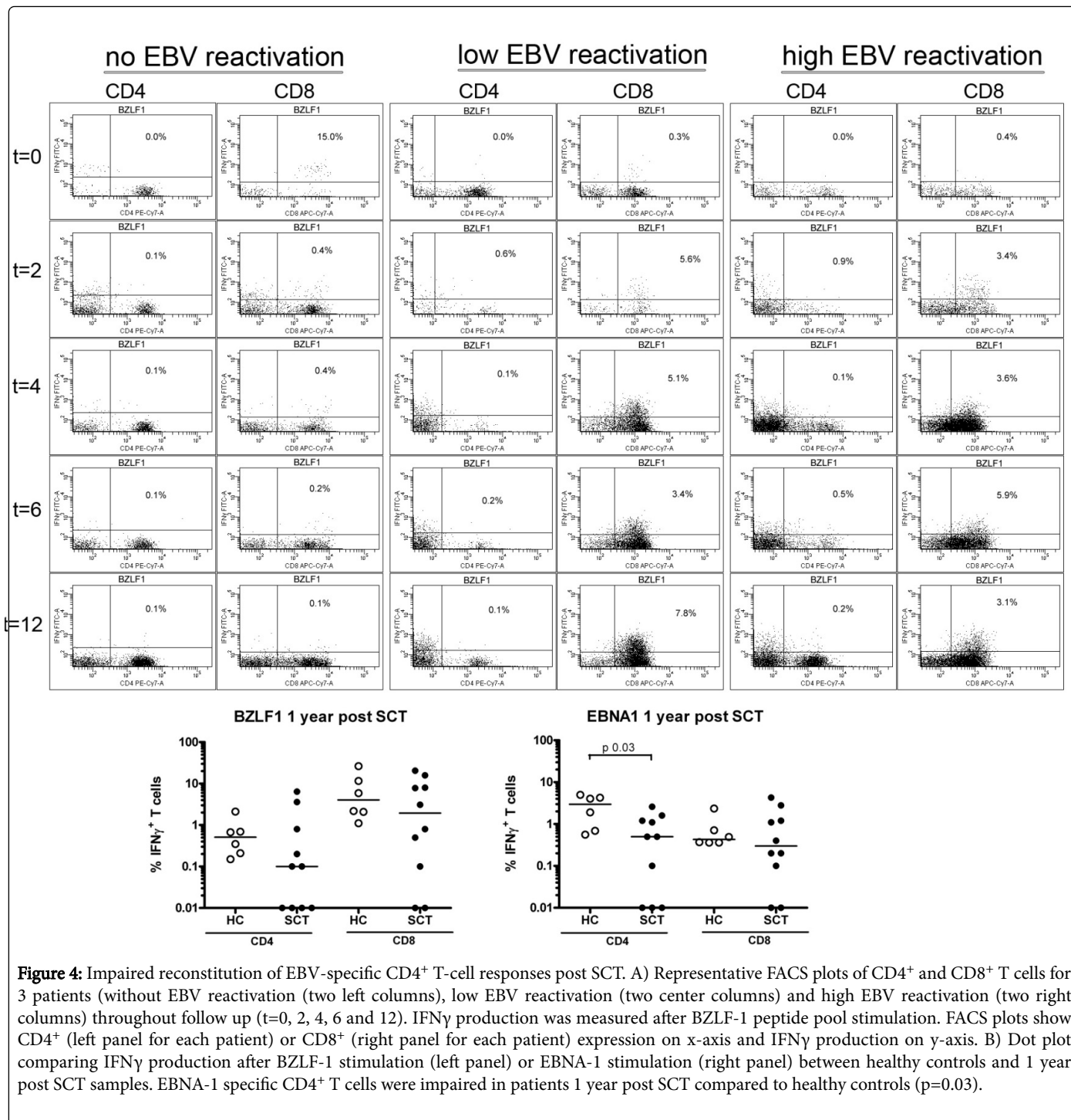


Figure 4: Impaired reconstitution of EBV-specific CD4⁺ T-cell responses post SCT. A) Representative FACS plots of CD4⁺ and CD8⁺ T cells for 3 patients (without EBV reactivation=two left columns), low EBV reactivation (two center columns) and high EBV reactivation (two right columns) throughout follow up (t=0, 2, 4, 6 and 12). IFN γ 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 IFN γ production on y-axis. B) Dot plot comparing IFN γ production after BZLF-1 stimulation (left panel) or EBNA-1 stimulation (right panel) between healthy controls and 1 year post SCT samples. EBNA-1 specific CD4⁺ T cells were impaired in patients 1 year post SCT compared to healthy controls (p=0.03).

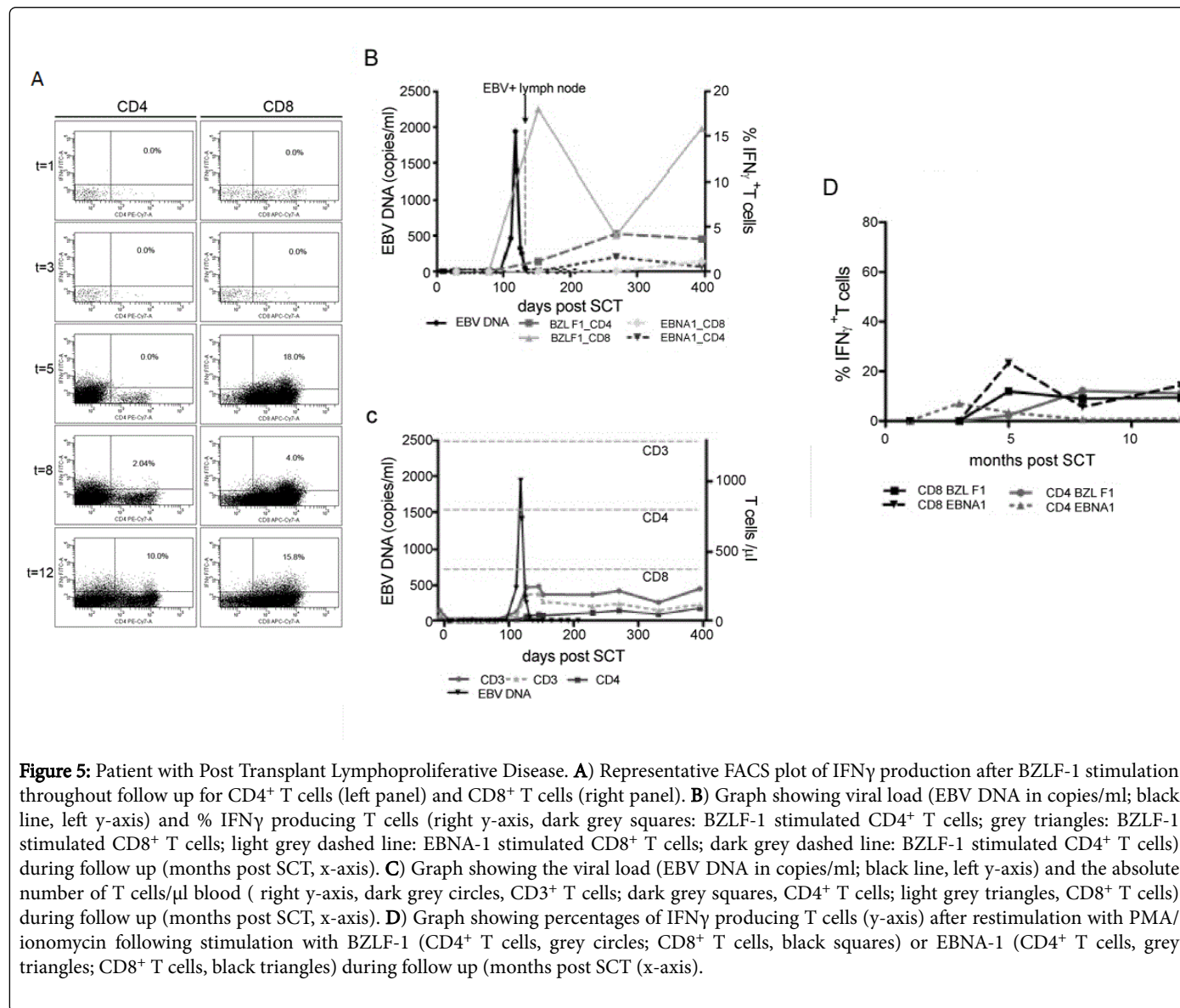
Post-transplant lymphoproliferative disease

One patient (11) developed a post-transplant lymphoproliferative lymphoma. 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 (4 × 375 mg/m²) after which immunosuppressive therapy was reduced. IFN γ 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 until at least day 200, despite high viral loads at day 100. EBNA-1 specific T cells were undetectable except for a peak at 8 months post-SCT of 1.6% of CD4⁺ T cells. EBNA-1-specific CD8⁺ T cells only increased after 8 months and peaked at 12 months post SCT to 1.1% of CD8⁺ T cells (Figure

5B). In addition, we analysed the absolute number of T cells following SCT (Figure 5C). All T-cell counts remain very low throughout follow-up. The CD3⁺ T-cell count 1 year post SCT was 233 cells/ μ l compared to 1292 cells/ μ l in 15 healthy controls. The poor T-cell reconstitution in

this patient was confirmed by the low level of IFN γ producing CD4⁺ and CD8⁺ T cells after restimulation with PMA/ionomycin (Figure 5D).



Discussion

Persistent viruses, like EBV and CMV, are normally controlled through cytotoxic T cell responses [1,2,22] and complications can be cured through adoptive T-cell transfer [23,24]. Adequate T-cell reconstitution after SCT is therefore crucial in preventing viral reactivation progressing to severe complications [9,25].

We found that early T-cell reconstitution after SCT does not play a role in the onset of viral reactivation as numbers of CD4⁺ and CD8⁺ T cells during the first 3 months post SCT are similar in patients with or without EBV reactivation. 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 [26]. 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 [27]. Indeed, we did observe a significant difference in T-cell counts early after SCT between patients who received ATG and patients that did not (data not shown). During the time of average onset of viral reactivation (8 weeks after SCT), 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 initiated already earlier after SCT and not detected yet, nor can we exclude differences in phenotypic markers that may explain divergent outcomes with respect to viral reactivation.

Generation of virus-specific CD8⁺ T cells is dependent on CD4⁺ T-cell help, as has been shown for several viruses such as HIV [28,29] and CMV [7-9,30]. We studied the role of EBV-specific CD4⁺ and CD8⁺ T cells in controlling EBV-reactivation after allogeneic stem cell

transplantation. We applied a 12day expansion protocol, which has been shown to be very useful to measure low numbers of specific T cells in several situations [21], as well as in SCT patients to investigate both the general as well as EBV-specific T-cell reconstitution. This technique also enabled us to compare reconstitution of CD4⁺ and CD8⁺ T cells using a similar qualitative assay. As read-out of functionality we choose IFN γ as this is the last cytokine to be lost upon exhaustion, and thus has the ability to reveal T cells still able to produce (this) cytokine. IFN γ is therefore the most robust cytokine and enabled us to measure the majority of cytokine-producing T cells without underestimation of specific T cells due to loss of IL-2 production as a result of exhaustion. As we grow the T cells in IL-2 supplemented medium however, we also skew the cells to a more Th1 phenotype, making the production of IL4 and IL10 less likely.

Although the study population is small, we showed- using the expansion assay- that non-specific functional T-cell reconstitution is hampered in patients with high-level EBV reactivations. In all 4 high-level reactivation patients 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 [31].

In our study, EBNA-1 specific T cells remain low throughout follow up in all patients and specifically EBNA-1 specific CD4⁺ T cells do not reconstitute to 'normal' values 12 months post SCT. In contrast, 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. Lack of adequate EBNA-1 specific T cell responses have been described to be associated with progression to EBV-related NHL[21]. These findings are reflected in our PTLD patient, in whom few EBNA-1 specific CD8⁺ T cells were detectable throughout follow up also when high viral loads were present. In contrast, we observed 18% of CD8⁺ T cells producing IFN γ 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 [32]. Also Hislop et al. previously reported that BZLF-1 responses are very immunodominant [33]. These data suggest that during reconstitution, outgrowth of EBNA-1 specific T cells is required to control EBV-reactivation.

Despite the small patient group, 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 functional 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 EBV 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 α CD20 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 functional and EBV-specific T-cell reconstitution prior to onset of viral complications and therapeutic intervention. A possible explanation for our findings could be that during the reconstitution process, where only a selective

number of T cells will slowly reconstitute, T cells with lower avidity are activated by the viral reactivation. These cells will be of lower functionality and may not contain the reactivation. Because of the still low number of T cells, this will be reflected in both the total functional T-cell pool as well as the EBV-specific one. In addition, exhaustion may play a role in the face of high antigenic loads, leading to less ability of T cells to proliferate in vitro.

The importance of a robust T-cell reconstitution has been described before. A rapid reconstitution to at least 300 CD3⁺ T cells/ μ l blood was shown to distinguish between patients with viral control and patients at risk of reactivation [31]. 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 [34]. However, here we show that not so much the number of T cells is of importance but the functional capacity, i.e. the capability of IFN γ production following general stimulation, is impaired in individuals that develop a high-level EBV reactivation. We cannot exclude the possibility that in patients with an EBV-reactivation T cells have become more clonally exhausted and therefore may not have been able to proliferate in our in vitro system. We unfortunately did not measure markers of exhaustion.

Thus, both total functional and EBV-specific CD8⁺ T cells show a more rapid recovery compared to functional CD4⁺ T cells. EBV-specific CD8⁺ T-cell responses can be detected as early as 2 months post SCT, while EBV-specific CD4⁺ T cells responses remain low throughout follow up, even though both CD8⁺ and CD4⁺ T-cell responses are broadly targeted across all eight latent proteins[33]. In addition, up to half of the currently defined CD4 epitopes are derived from EBNA1 and there is an equal distribution of epitope recognition among the early, immediate early and late lytic proteins [35]. Interestingly, absolute CD4⁺ T-cell numbers do not reconstitute slower in our patients compared to CD8⁺ T cells. Why the EBV-specific CD4⁺ T cell response is not recovering to the extent the EBV-specific CD8⁺ T-cell response is recovering is unclear, but may lie in differences in ability of clonal expansion, especially in the situation of SCT or the repertoire of T cells involved. The fact that EBNA-1-specific CD4⁺ T cells did not normalize to healthy control levels one year post-SCT and were undetectable up to at least 8 months in the PTLD patient, however, do not rule out a role for EBV-specific CD4⁺ T cells in control of EBV-reactivations.

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