6. Impaired recovery of Epstein-Barr virus (EBV)-specific CD8+ T-cells after allogeneic hematopoietic stem cell transplantation identifies patients at high risk for progressive EBV reactivation and lymphoproliferative disease

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Abstract

The cytotoxic T-cell immune response towards Epstein-Barr virus (EBV) is considered pivotal for prevention of lymphoproliferative disease (LPD) in recipients of an allogeneic hematopoietic stem cell transplantation (allo-SCT). The aim of this study was to evaluate the recovery of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation and to study the relation between EBV-specific $CD8^+$ T-cells, EBV reactivation and EBV-LPD. EBV-specific immunity was studied using a panel of 11 HLA class I tetramers presenting peptides derived from 7 EBV proteins. Blood samples were taken at regular intervals after allogeneic hematopoietic stem cell transplantation in 61 patients and EBV-DNA levels were assessed by real-time polymerase chain reaction (PCR). Forty-five patients showed EBV-reactivation, including 25 with high-level reactivation (≥ 1,000 geq/ml). Nine of these patients progressed to EBV-LPD. $CD8^+$ T-cells specific for latent and lytic EBV epitopes repopulated the peripheral blood at similar rates. Absolute numbers of EBV-specific $CD8^+$ T-cells increased after allogeneic hematopoietic stem cell transplantation to normal levels within 6 months in the majority of patients. Concurrently, the incidence of EBV reactivation strongly decreased. Patients with insufficient recovery were at higher risk for EBV reactivation in the first 6 months after stem cell transplantation. Absence of EBV-specific $CD8^+$ T-cells in patients with high-level reactivation was significantly associated with the subsequent development of EBV-LPD (P=0.048). Thus, the earlier defined positive predictive value of approximately 39%, based on high-level viral reactivation only, increased to 100% in patients without EBV-specific T-cells. These results show that the absence of EBV-specific $CD8^+$ T-cells in patients with high-level viral reactivation may identify a subgroup of patients at extremely high risk for EBV-LPD, and support that EBV-specific $CD8^+$ T-cells may protect allogeneic hematopoietic stem cell transplantation recipients from progressive EBV-reactivation and EBV-LPD.

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous γ-herpesvirus that infects more than 90% of the world population. Following primary infection in the oropharynx, EBV remains latently present in B-cells. [1] Latent EBV infection is normally controlled by a cell-mediated immune response and $CD8^+$ T lymphocytes directed against the immunodominant latent proteins EBNA3A, -3B, 3C and the lytic proteins BZLF1 and BMLF1 can be detected in the circulation in the majority of healthy EBV seropositive individuals. [2-4] EBV-infected B-cells may evolve to lymphoproliferative disease (EBV-LPD) in immuno-suppressed transplant recipients, due to inhibition of immunological control of latently infected B-cells. EBV-LPD is a serious complication following allogeneic hematopoietic T-cell depleted allogeneic hematopoietic stem cell transplantation (allo-SCT) and solid-organ transplantation and may be associated with considerable mortality.[5-8] Quantifying EBV-DNA in plasma, currently allows for the monitoring of EBV reactivation in transplant recipients.[9-11] We recently reported a longitudinal study that revealed EBV reactivation as a frequent event after allogeneic hematopoietic stem cell transplantation.[11] In addition, EBV-LPD could be quantitatively predicted by the frequent monitoring of viral load in plasma. A viral load of 1,000 genome equivalents per millilitre (geq/ml) proved to be a level of EBV reactivation associated with a positive predictive value of 39 % and a negative predictive value of 100%.[11]

The cytotoxic T-cell immune response towards EBV is considered important for controlling EBV in allogeneic hematopoietic stem cell transplantation recipients and the prevention of EBV-LPD.[1,3] Especially, the clinical success of adoptive cellular immunotherapy of EBV-LPD using EBV-specific cytotoxic T lymphocytes (CTL) has indicated the pivotal role of these CTL in controlling EBV in allogeneic hematopoietic stem cell transplant recipients.[12-14] While the overall recovery of $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation has been studied extensively, little is known as regards the recovery of EBV-specific $CD8^+$ T-cells in allogeneic hematopoietic stem cell transplantation patients.[15-18] Antigen-specific $CD8^+$ T-cells can be enumerated by staining with tetrameric MHC-class I-peptide complexes. It has revealed a much higher frequency of antigen-specific circulating T cells than estimated before by limiting dilution assays. [2,19-21] The aim of this study was to evaluate the recovery of EBV-specific $CD8^+$ T-cells using tetramer technology and to evaluate the relationship between regeneration of EBV-specific T-cells and viral reactivation and progression to EBV-LPD.

2. Patients and methods

Patients

The study population included 61 consecutive patients, who received a T-cell depleted allogeneic hematopoietic stem cell transplantation between January 1998 and December 2000. Patient, donor and graft characteristics are presented in Table 1.

Table 1. Patient characteristics

Parameter	Study population (n=61)
Sex male/female (n)	32/29
Median age (y, range)	38 (16-55)
Diagnosis (n):	
AML	17
ALL	10
MDS	1
CML	8
SAA	1
MM	8
Lymphoma	16
Donor type (n)	
Sib	39
MUD	22
Conditioning regimen (n)	
Cy/TBI	35
Cy/TBI/ATG	23
Bu/Cy	3
Graft characteristics: (median, range)	
CD3 x 10^5/kg	2.0 (1.0-7.5)
CD34 x 10^6/kg	1.6 (0.5-8.8)
EBV-serology (n):	
D+R	3
D+R+	55
D-R+	3
Stem cell source (n):	
BM	51
PB	10

Legend to table 1.
AML indicates acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; MDS, myelodysplastic syndrome; CML , chronic myeloid leukaemia; SAA, severe aplastic anaemia; MM, multiple myeloma; Sib, HLA identical family donor; MUD, matched unrelated donor; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; ATG, anti-thymocyte globulin; D+/- , EBV-seropositive/seronegative donor; R+/-, EBV seropositive/seronegative recipient; BM, bone marrow; PB, peripheral blood.

Patients were positive for at least one of the following HLA-alleles: A*0201, A*1101, B*0702, B*0801 and B*3501. The distribution of these HLA-alleles, corresponding to the EBV-tetramers, is shown in Table 2. The HLA-A*0201 allele was present in 35 patients, HLA-A*1101 in 7 patients, HLA-B*0702 in 17 patients, HLA-B*0801 in 17 patients and HLA-B*3501 in 12 patients. All patients were conditioned with cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (12 Gy in 2 fractions with partial lung shielding). Rabbit ATG (Imtix Sangstat, Amstelveen, The Netherlands) was given for prevention of rejection in recipients with an unrelated donor at days –7 to –4 preceding transplantation (total dose: 8 mg/kg). If patients had previously been treated with locoregional irradiation, the conditioning regimen consisted of oral busulfan (4 mg/kg on each of 4 successive days) and cyclophosphamide (120 mg/kg). Partial T-cell depletion was performed using sheep erythrocyte rosetting (n=31) or CD34 selection (Miltenyi, Bergisch Gladbach, Germany) (n=30). Ten patients received peripheral blood-derived stem cells and 51 patients received bone marrow-derived stem cells. Cyclosporin A (3 mg/kg) was given as graft-versus-host prophylaxis from day –3 until day + 100 after allogeneic hematopoietic stem cell transplantation. All patients received ciprofloxacin and fluconazole for prevention of infection during neutropenia, and cotrimoxazole was given after neutrophil recovery until day 180 after allogeneic hematopoietic stem cell transplantation. Erythrocyte and platelet products for transfusion were filtered to remove leukocytes and subsequently irradiated (25 Gy).

Quantitative EBV-specific PCR

EBV-DNA levels were measured as described previously. [22] Briefly, Taqman PCR primers were selected from the EBV-DNA genome encoding for the non-glycosylated membrane protein BNRF1-p143 and generated a DNA product of 74 basepairs. A standard with a fixed EBV copy number (ABI Advanced Biotechnologies, Columbia, MD, USA) was used for standardisation of the assay. Serial dilutions ranging from 10 to 10^7 EBV-DNA genome equivalents per millilitre (geq/ml) were made to characterise linearity, precision, specificity, and sensitivity. The Taqman assay detects viral DNA in plasma over a linear range between 50 and 10^7 geq/ml. Test results below 50 geq/ml were considered negative. Plasma samples for quantification were obtained at weekly intervals in patients without EBV-LPD and daily in patients with established EBV-LPD. EBV reactivation was defined as EBV DNA levels in plasma of > 50 geq/ml, EBV DNA levels of <1,000 geq/ml were

defined as low-level reactivation and EBV DNA levels of ≥ 1,000 geq/ml were defined as high-level reactivation in this particular group of patients.[11] As from January 1999, patients with high-level reactivation were treated pre-emptively with rituximab (375mg/m^2) (Roche, Basel, Switzerland) as recently described. [23]

Table 2. HLA-allele distribution

HLA-allele	n
A*0201	17
A*0201 A*1101	1
A*0201 B*0702	4
A*0201 B*0702 B*3501	2
A*0201 B*0702 B*0801	2
A*0201 B*0801	8
A*0201 B*3501	1
A*1101	5
A*1101 B*3501	1
B*0702	6
B*0702 B*0801	1
B*0702 B*3501	2
B*0801	5
B*0801 B*3501	1
B*3501	5

EBV-LPD diagnosis

EBV-LPD was diagnosed using histology and/or cytology and was classified according to the criteria of Knowles et al. [24] Immunohistology included staining with monoclonal antibodies specific for CD19 (BD Biosciences, San Jose, CA), EBV-encoded latent membrane protein 1 (LMP1) and kappa and lambda light chains (all from Dako, Glostrup, Denmark). In situ hybridisation was performed to detect the expression of EBV encoded RNA's (EBER) using an EBV-EBER probe (Dako). PCR was performed for detection of EBV-DNA for the BamHI fragment. Furthermore, EBV-LPD staging included physical examination, whole-body computed tomography (CT) scanning and detection of monoclonal B-lymphocytes in blood and bone marrow derived mononuclear cell suspensions using flow cytometry. Patients with a diagnosis of EBV-LPD were treated

with interruption of immunosuppressive drugs and rituximab guided by viral load as described. [25] Patients were treated with donor leukocyte infusions, if no response ensued following rituximab. [25]

Enumeration of $CD8^+$ T lymphocytes specific for class I HLA-restricted EBV-encoded epitopes

Heparinised blood samples were obtained from transplant recipients at 2, 3, 6, 9, 12, 18 and 24 months after allogeneic hematopoietic stem cell transplantation and from healthy controls, i.e. 37 laboratory workers and 39 allogeneic hematopoietic stem cell transplantation donors prior to mobilisation of bone marrow donation. Peripheral blood mononuclear cells (MNC) were isolated using Ficoll-Isopaque density grade centrifugation and cryo-preserved until required. In parallel, absolute numbers of $CD8^+$ T lymphocytes were assessed in whole blood samples within 6 hours after venipuncture. $CD8^+$ T-cells were enumerated using a 3-color, single platform whole blood immunostaining technique. [26,27] The following monoclonal antibodies were used: CD45 (clone 2D1, conjugated with fluorescein isothiocyanate (FITC); BD Biosciences (BD), San Jose, CA), CD8 (clone SK1 conjugated with phycoerythrin (PE); BD) and TCR PAN α/β (clone BMA031 conjugated with PE-Cy5; Immunotech, Marseille, France). Of each sample 50,000 leukocytes were acquired using a FACSCalibur flow cytometer (BD). During data analysis, $CD8^+$ T-lymphocytes were defined as events with low to medium forward light scatter, low sideward light scatter, $CD45^+$, $TCR\alpha/\beta^+$ and $CD8^+$. The proportion of EBV-specific $CD8^+$ T-cells was assessed using tetramer technology on cryo-preserved and thawed MNC. EBV tetramers used in this study have been characterized as described before and are summarised in Table 3. [28-31] Mononuclear cell suspensions were incubated with EBV tetramers and CD8 (clone SK1 conjugated with allophycocyanin) for 30 min on melting ice. After 1 wash, cells were resuspended in PBS containing 1µg/ml 7-aminoactinomycin D (7-AAD; Sigma, St. Louis, MO). Following acquisition of 20,000 living $CD8^+$ T-lymphocytes (defined as having low to intermediate forward light scatter, low sideward light scatter as well as being $7\text{-}AAD^-$ and $CD8^+$), the proportions of living $CD8^+$ T-cells binding the tetramer(s) was assessed. The absolute number of circulating tetramer-binding $CD8^+$ T-cells was calculated from the proportion of $CD8^+$ T-cells binding the tetramer and the simultaneously obtained absolute $CD8^+$ T-cell count. [27] The lower limit of detection was 0.1 EBV-specific $CD8^+$ T-cells/µl.

Statistical analysis

Regeneration kinetics as a function of time were described for the individual EBV-specific $CD8^+$ T-cell subsets. As the regeneration kinetics of $CD8^+$ T-cells specific for latent and lytic EBV epitopes did not differ significantly (see results), these data were pooled for the subsequent analyses addressing the interaction between recovery of EBV-specific $CD8^+$ T-

cells, EBV reactivation and development of EBV-LPD. In these analyses, each patient was entered at each time point with a single EBV-specific $CD8^+$ T-cell count. For patients studied with multiple tetramers, the result of the EBV-specific $CD8^+$ T-cell subset with the highest absolute count was used for these analyses. EBV-specific $CD8^+$ T-cell recovery was considered effective when any EBV-specific $CD8^+$ T-cell subset had reached the threshold level of ≥ 0.5 cells/μl. Fisher's exact test was used to analyse 2-by-2 tables. Statistical evaluation of the differences between two groups was performed using the non-parametric Mann-Whitney U ranking test. P values < 0.05 were considered significant.

3. Results

Clinical outcome of EBV reactivation

EBV serology was positive in both donor and recipient in 55 out of 61 patients. In 3 out of 61 patients, EBV serology was positive in the donor and negative in the recipient. EBV serology was negative in the donor and positive in the recipient in the 3 remaining patients. Forty-five patients showed EBV reactivation (> 50 geq/ml). The median time to first reactivation was 65 days (range, 4 - 447). High-level EBV reactivation (≥ 1,000 geq/ml) was apparent in 25 of the latter 45 patients. Ten of these 25 patients were pre-emptively treated with anti-B-cell monoclonal antibody therapy (rituximab). [23] None of these patients progressed to overt EBV-LPD. Among the 15 patients not treated pre-emptively, 9 progressed to EBV-LPD. Seven LPD-patients responded to therapy, the 2 non-responders died from progressive EBV-LPD (Table 4). The median follow-up for EBV-specific $CD8^+$ T-cell recovery and EBV reactivation was 10 months (range 2 to 38 months following stem cell transplantation).

Repopulation of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation

Recovery of EBV-specific $CD8^+$ T-cells was evaluated by measuring these cells at 2, 3, 6, 9, 12, 18 and 24 months after allogeneic hematopoietic stem cell transplantation using a panel of 11 EBV-specific tetramers (Table 2, Table 3). Figure 1 shows the recovery patterns of $CD8^+$ T-cell subsets with different EBV specificities. As only few datapoints for the epitopes AVF and IVT were available, data for these epitopes are not shown. Normal values were defined for each epitope as the 5 to 95 percentile values assessed in 20-25 healthy EBV-seropositive donors (Table 3). The recovery directed against both lytic and latent epitopes appeared to be relatively fast. Most EBV-specific $CD8^+$ T-cell subsets returned to their respective normal ranges within 3 – 6 months after allogeneic hematopoietic stem cell transplantation in the majority of patients (Figure 1). With respect to lytic antigen derived epitopes, high median numbers were observed for GLC and RAK specific T-cells at 3 months post- stem cell transplantation.

Table 3. EBV tetramers used in this study

EBV antigen (co-ordinates)	HLA-restriction	Sequence	Reference	Healthy donors* Median (5^{th}-95^{th} percentile) [n]
Latent				
EBNA1 (407-417)	B*3501	HPVGEADYFEY	30	2.0 (0.2 - 5.2) [20]
EBNA3A (379-387)	B*0702	RPPIFIRRL	29	1.8 (0.3 - 4.7) [20]
EBNA3A (502-510)	B*0702	VPAPAGPIV	29	0.6 (<0.5-3) [20]
EBNA3A (325-333)	B*0801	FLRGRAYGL	28,29	1.4 (0.4 - 3.9) [21]
EBNA3A (458-466)	B*3501	YPLHEQHGM	30	1.3 (0.1 – 50) [20]
EBNA3B (399-408)	A*1101	AVFDRKSDAK	ur	1.2 (0.9 – 4.0) [11]
EBNA3B (416-424)	A*1101	IVTDSVIK	ur	0.6 (0.4 – 1.5) [11]
EBNA3C (284-293)	A*0201	LLDFVRMGV	29	0.9 (0.5 - 5.1) [20]
LMP2 (426-434)	A*0201	CLGGLLTMV	29	0.5 (0.2 - 5.7) [22]
Lytic				
BZLF1 (190-197)	B*0801	RAKFKQLL	28	2.9 (0.2 – 32) [20]
BZLF1 (54-64)	B*3501	EPLPQGQLTAY	31	5.0 (0.2 – 43) [20]
BMLF1 (280-288)	A*0201	GLCTLVAML	28	1.3 (0.4 – 10) [25]

** Median and 5^{th} and 95^{th} percentile of absolute numbers of EBV-specific $CD8^+$ T-cells determined in healthy EBV-seropositive donors. Between brackets the number of healthy donors analysed; ur indicates unpublished results D. van Baarle, F. Miedema.*

Table 4. Patient outcome

Parameter	No. of patients (n=61)
No EBV reactivation	16
EBV reactivation	45
50 – 1000 geq/ml	20
≥ 1000 geq/ml	25
EBV-LPD	9
EBV-LPD-mortality	2

As regards the latent epitopes, the $CD8^+$ T-cells directed against most of these epitopes also repopulated to normal levels within 3-6 months after allogeneic hematopoietic stem cell transplantation, but T-cells with specificity for the LLD-epitope, derived from EBNA-3C, recovered by 9 months after stem cell transplantation. Of note, GLC-, FLR- and HPV-specific T-cells recovered to supranormal levels within 3-6 months in 5 patients and also remained at those levels. Only 1 out of these 5 patients developed EBV reactivation, but the EBV viral load in that particular patient was rapidly cleared and did not exceed the low level of 80 geq/ml. The recovery of EBV-specific $CD8^+$ T-cells was delayed in patients who received ATG as part of the conditioning regimen. The effect of ATG on the recovery of RAK-specific T-cells is illustrated in Figure 2. It shows that patients, who didn't receive ATG as part of the conditioning regimen, had recovered RAK-specific T-cells to normal levels by 3 months after stem cell transplantation, while these levels had not been reached in patients pretreated with ATG until 9 months after stem cell transplantation. A similarly delayed recovery of EBV-specific $CD8^+$ T-cells following ATG was also observed for the lytic epitope EPL and the latent epitopes CLG, RPP, FLR, HPV and YPL (results not shown).

EBV-specific $CD8^+$ T-cells and EBV reactivation

The relation between the recovery of EBV-specific $CD8^+$ T-cells and EBV reactivation was studied by evaluating patients at risk in time intervals, starting from a particular time-point of T-cell monitoring until the next time-point. Results are presented in Table 5. At each time-point, only those patients were included of whom EBV-specific $CD8^+$ T-cells had been enumerated at least once and for whom follow-up for EBV reactivation was effectively monitored until the next time-point of evaluation of T-cell recovery. It is shown that the number of patients who recovered EBV-specific $CD8^+$ T-cells rapidly increased in time.

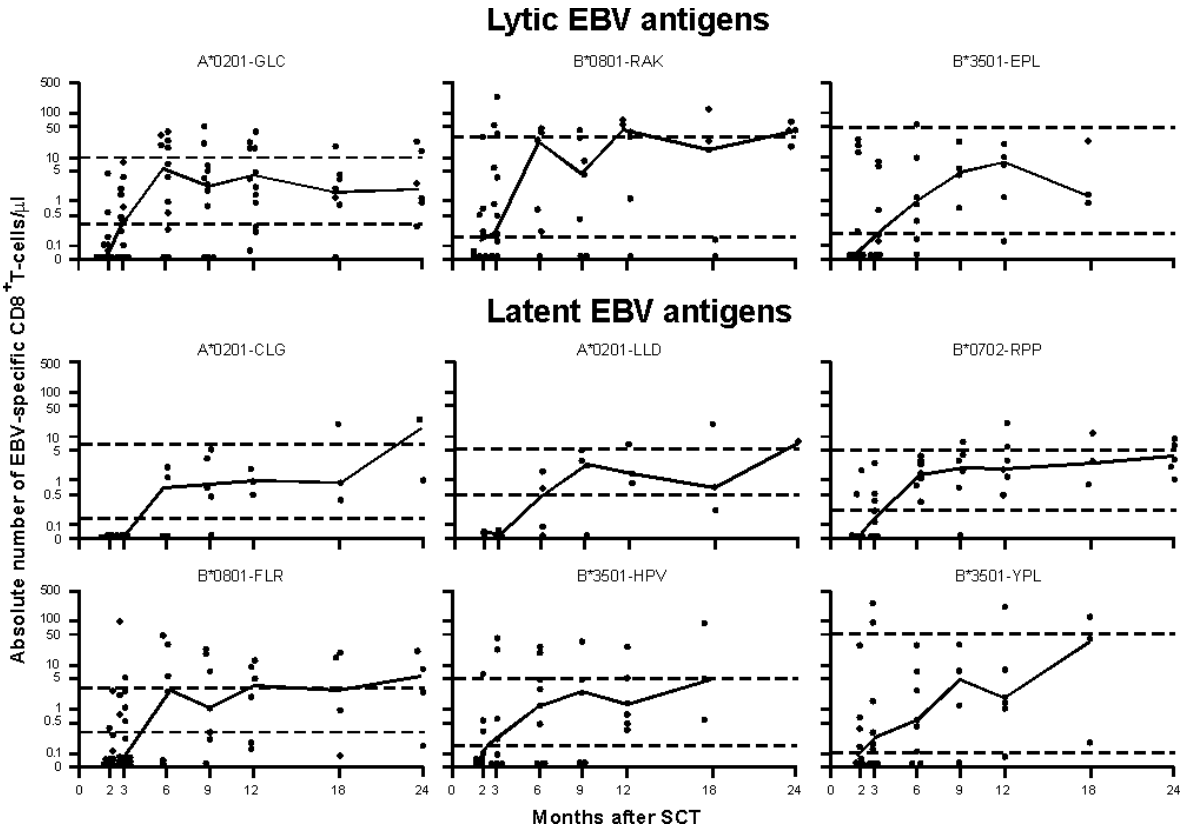

Figure 1. Repopulation of EBV-specific CD8$^+$ T-cells after allogeneic T-cell depleted hematopoietic stem cell transplantation. Each panel represents the recovery of CD8$^+$ T-cells directed against a single EBV-specific epitope as measured by tetramer technology. IVT and AVF are not included, since only few datapoints were available. For each EBV-encoded epitope, the median values per time point are connected with a line. Horizontal lines indicate the normal ranges as defined by the 5th and the 95th percentiles of EBV-specific CD8$^+$ T-cells as measured in healthy EBV-seropositive donors.

Seven out of 35 patients (20%) had recovered EBV-specific CD8$^+$ T-cells by 2 months and 73% (22/30) of patients had done so by 6 month following SCT. This increase was accompanied by an increase in the median level of EBV-specific T-cells during the first 6 months after allogeneic hematopoietic stem cell transplantation, which remained at a stable level thereafter (Table 5, Figure 1). Concurrently, the incidence of EBV reactivation decreased from 30-40% at 2-3 months to less than 20% of patients after 6 months post-stem cell transplantation. These data show that most EBV reactivations occurred during the period of insufficient EBV-specific CD8$^+$ T-cell recovery. A trend towards a correlation between insufficient recovery and EBV reactivation was observed for those patients, who had been monitored for T-cell recovery at 2 and/or 3 months post- stem cell transplantation and monitored for EBV reactivation up to the 6 month time-point.

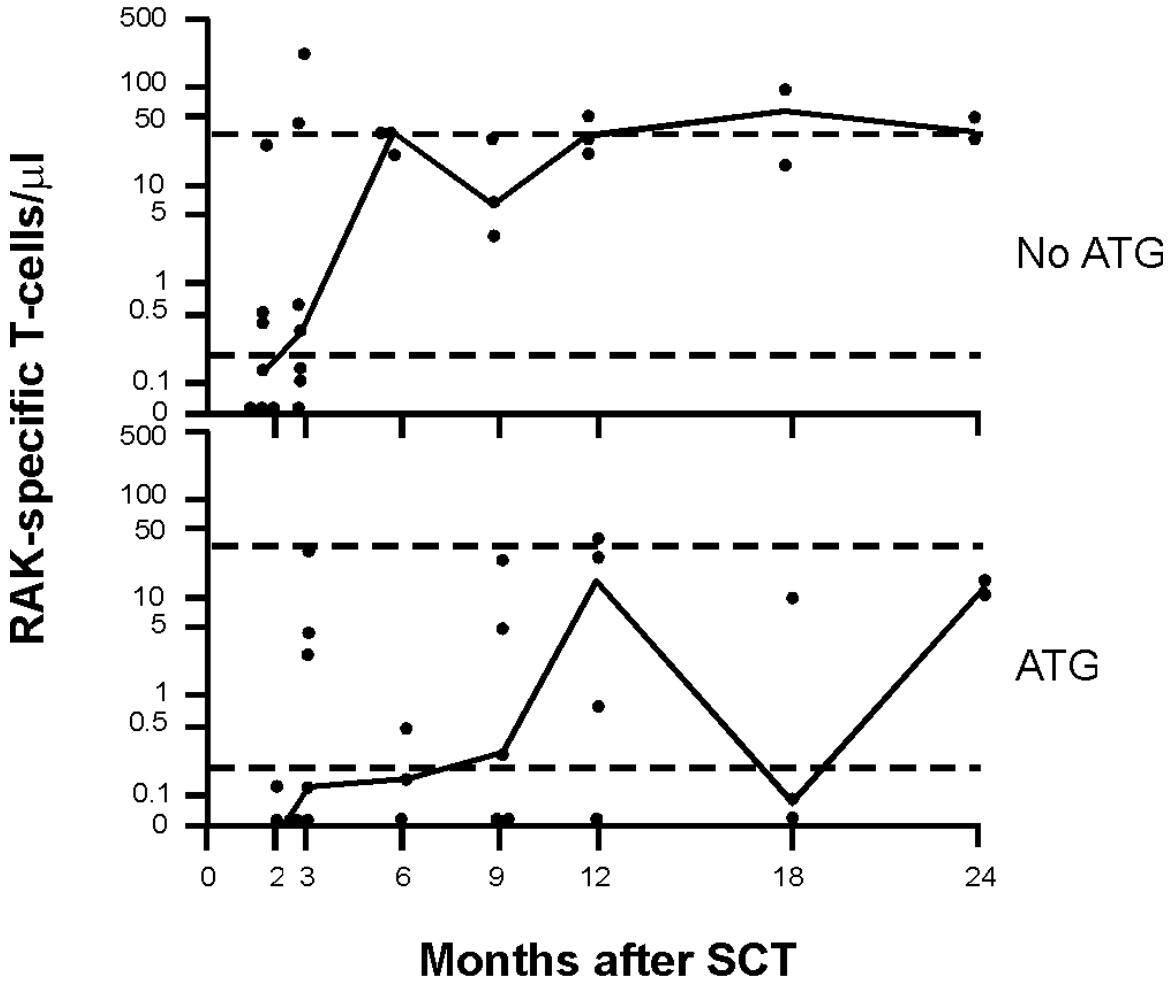

Figure 2. ATG delays the recovery of RAK-specific CD8$^+$ T-cells after allogeneic hematopoietic stem cell transplantation. RAK-specific T-cells were measured following allogeneic hematopoietic stem cell transplantation in patients who did not receive ATG (upper panel) and patients who received ATG as a part of the conditioning regimen (lower panel). See further legend to Figure 1.

Sixteen out of a total of 43 patients, studied at 2 and 3 months, effectively recovered an EBV-specific CD8$^+$ T-cell response before 6 months and only 3 of them (19%) developed EBV reactivation. No detectable EBV-specific CD8$^+$ T-cells were found in 27 of 43 patients (63%), and 13 of these 27 patients developed EBV reactivation (48% versus 63%, P = 0.053). Thus, patients with recovery of EBV-specific CD8$^+$ T-cells at 2 –3 months after allogeneic hematopoietic stem cell transplantation are at lower risk to of EBV reactivation, as compared to patients with an impaired EBV-specific CD8$^+$ T-cell response.

Table 5. EBV-specific $CD8^+$ T-cell recovery and EBV reactivation

Parameter	Time-point after transplantation (months)						
	2	**3**	**6**	**9**	**12**	**18**	**24**
EBV-specific T-cell recovery							
• No. of patients recovered / No. of patients at risk**	7/35	13/37	22/30	16/22	15/17	12/14	3/3
• Level of recovery (T-cell/µl) Median (range)	< 0.1 *(0.1-28.9)*	0.16 *(0.1-253)*	3.6 *(0.1-54.9)*	4.52 *(0.1-166)*	6.3 *(0.1-224)*	3.44 *(0.1-151)*	12 *(3.5-23.6)*
EBV reactivation*							
• No. of patients reactivating / No. of patients at risk**	11/35	15/37	6/30	4/22	2/17	1/14	0/3

**) patients were evaluated in time intervals, starting from the time-point indicated until the next time-point,*

***) numbers of patients at risk pertime-point were determined by (1) availability of tetramer tests and (2) follow-up for EBV reactivation until next time-point*

EBV-specific CD8$^+$ T-cells in patients with high-level EBV reactivation

We subsequently evaluated whether the absence of EBV-specific CD8$^+$ T-cells would correlate with a high quantitative level of EBV reactivation (≥ 1,000 geq/ml), since these patients have been shown to be at high risk to develop EBV-LPD. [11] Patients with high-level EBV reactivation (≥ 1,000 geq/ml) and patients with low-level EBV reactivation (< 1,000 geq/ml) were compared. Twenty-four patients, in whom EBV-specific T-cells had been assessed prior to the onset of EBV reactivation, were studied. As shown in Table 6, only 2 of 15 patients (13%) developing high-level EBV reactivation had recovered EBV-specific T-cells preceding EBV reactivation, while 6 of 9 patients (67%) with low-level EBV reactivation had done so (13% versus 67%, P=0.02). These differences in absolute numbers of EBV-specific T-cells did not relate to a difference in absolute numbers of CD8$^+$ T-cells. Absolute numbers of CD8$^+$ T-cells preceding EBV reactivation were similar for both groups (< 1000 geq/ml and ≥ 1000 geq/ml) (data not shown).

Table 6. EBV low- and high-level reactivation and EBV-specific CD8$^+$ T-cells

EBV reactivation (geq/ml)	No. of patients with EBV-specific CD8$^+$ T-cells		P-value
	< 0.5/µ versus	≥ 0.5/µ	
50 -1000	3	6	0.02
≥ 1000	13	2	

High-level reactivation was defined as an EBV copy number ≥ 1,000 geq/ml

EBV-specific CD8$^+$ T-cells and EBV-LPD

Next we addressed the question whether the presence or absence of EBV-specific CD8$^+$ T-cells could improve the positive predictive value as earlier defined by quantitative viral load. [11] The presence of EBV-specific CD8$^+$ T-cells was analysed in 9 patients with high viral load (≥ 1000 geq/ml), who had not been treated pre-emptively with rituximab. Table 7 shows that EBV-LPD developed in all 5 patients with high viral load reactivation with no effective recovery of EBV-specific CD8$^+$ T-cells. In contrast, only 1 of 4 patients, who had effectively repopulated EBV-specific T-cells in the face of high viral load, developed EBV-LPD (P=0.048). Thus, the positive predictive value increased from 39% to 100% in patients with high-level reactivation without detectable EBV-specific T-cells. Conversely, the positive predictive value was reduced to 25% in patients with high-level reactivation, who actually did recover EBV-specific T-cells up to a level of ≥ 0.5 T-cells/µl. Of note, the earlier defined negative predictive value of a viral load of < 1,000 geq/ml was 100% and

therefore remained unchanged. [11] Next we studied the recovery of EBV-specific $CD8^+$ T-cells prior and after a diagnosis of EBV-LPD in further detail. Only results of the subset of EBV-specific T-cells with the highest absolute counts per patient and per time interval preceding and following a diagnosis of EBV-LPD are depicted (Figure 3). Nine EBV-LPD-patients were studied after LPD-diagnosis and 6 patients of them could also be studied before diagnosis (Table 7).

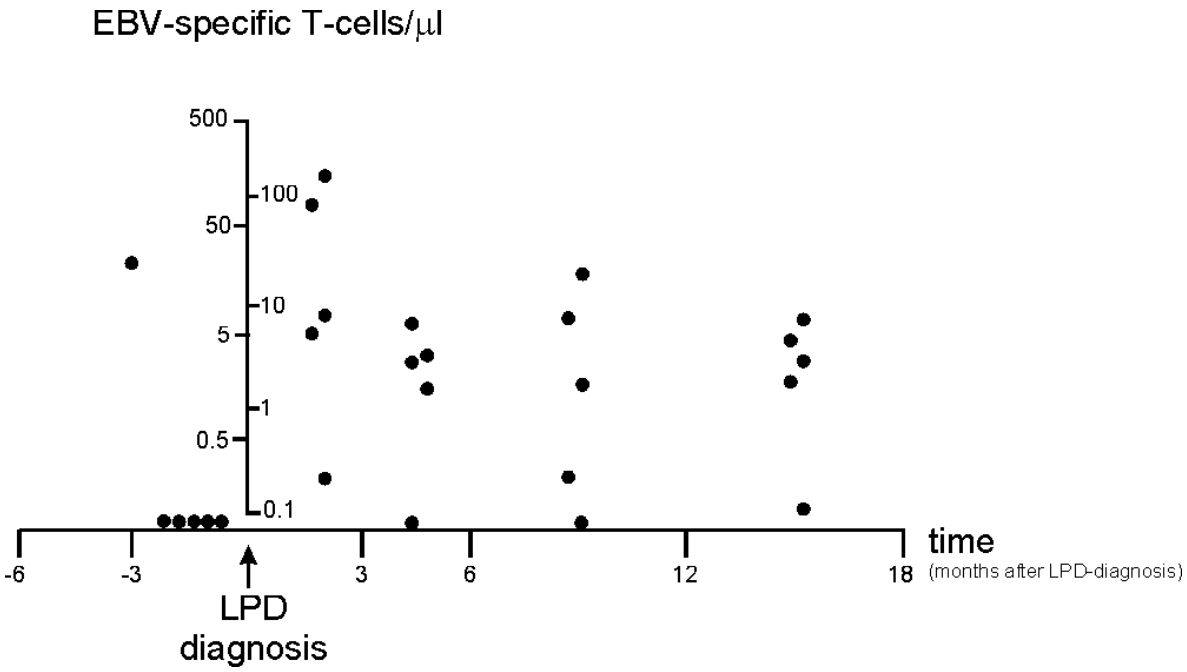

Figure 3. Recovery of EBV-specific $CD8^+$ T-cells prior and after a diagnosis of EBV-LPD. Absolute numbers of EBV-specific T-cells preceding and following EBV-LPD diagnosis in 9 patients. Six patients were evaluated prior to EBV-LPD diagnosis. Only the single highest value of the various tetramer-binding T-cells per patient are depicted.

Table 7. EBV-LPD and EBV-specific $CD8^+$ T-cells

Presence of EBV-specific T-cells (≥ 0.5/µl)	No. of patients with EBV-DNA ≥ 1000 geq/ml**		P-value
	Without EBV-LPD	Progressing to EBV-LPD	
Absent	0	5	0.048
Present	3	1	

*Absolute numbers of EBV-specific $CD8^+$ T-cells were monitored from allogeneic hematopoietic stem cell transplantation until day 180 or until diagnosis and treatment of EBV-LPD. **Patients with high-level reactivation receiving pre-emptive rituximab were excluded.*

EBV-specific T-cells were present in 1 of 6 patients preceding EBV-LPD. All patients were treated with a combination of rituximab and interruption of immunosuppression and 3 patients received donor lymphocytes as well. Seven out of 9 patients responded to therapy and EBV-load gradually decreased to become undetectable after a median of 17 days (range 5 – 59 days). All 7 responding patients had detectable EBV-specific $CD8^+$ T-cells post EBV-LPD, which recovery could already be detected within 4 weeks from treatment in 5 out of 7 patients. Two patients rapidly died due to progressive disease at respectively 10 and 41 days from EBV-LPD diagnosis. One of these 2 patients was investigated 5 days post EBV-LPD and had no detectable EBV-specific $CD8^+$ T-cells at that time.

4. Discussion

In the present study we evaluated the recovery of EBV-specific $CD8^+$ T-cells directed against lytic or latent antigens in recipients of T-cell depleted allogeneic hematopoietic stem cell grafts. We were particularly interested in the relation between the EBV-specific immune response, the incidence of EBV reactivation, and the development of EBV-LPD. It is shown that most EBV reactivations occur during the time lag before recovery of EBV-specific $CD8^+$ T-cells to normal levels. Furthermore, we also show a lack of EBV-specific $CD8^+$ T-cells in the majority of patients developing high-level reactivation and EBV-LPD. The absence of EBV-specific $CD8^+$ T-cells in patients with high-level EBV reactivation was significantly associated with EBV-LPD, indicating that the recovery of EBV-specific $CD8^+$ T-cells after allogeneic hematopoietic stem cell transplantation protects against uncontrolled reactivation and the development of EBV-LPD. Thus, the absence of EBV-specific $CD8^+$ T-cells strongly improved the positive predictive value of a viral load > 1,000 geq/ml. The earlier defined positive predictive value of a viral load > 1,000 geq/ml increased from 39% to 100% in patients without detectable EBV-specific $CD8^+$ T-cells. [11] Conversely, the positive predictive value was reduced to 25% in patients, who effectively recovered EBV-specific $CD8^+$ T-cells up to a level of at least 0.5 T-cells/µl. These data compare well to those of Smets et al, who monitored EBV-specific T-cells in recipients of liver allografts using interferon-γ ELISPOT stimulating peripheral blood lymphocytes with autologous EBV-derived lymphoblastoid cell lines. [32] Similarly, the combination of an elevated EBV viral load and the absence of EBV-specific $CD8^+$ T-cells resulted in a positive predictive value of 100% for the development of EBV-LPD in their patients. [32]

We did not evaluate the functional characteristics of the tetramer binding T-cells *in vitro*, nor did we evaluate the recovery of EBV-specific $CD4^+$ T-cells. However, the strong association between the level of EBV-specific $CD8^+$ T-cells and the observed protection against uncontrolled reactivation suggests that the EBV-specific T-cells detected are fully functional *in vivo* in the majority of our patients. Comparable results were obtained in

recipients of an allogeneic hematopoietic stem cell transplantation monitored for the recovery of CMV-specific $CD8^+$ T-cells by use of tetramers. [27] The mere presence of CMV-specific T-cells already proved to be strongly associated with in-vivo function, i.e. protection against CMV-disease. In contrast, van Baarle *et al.* evaluated numbers and function of EBV-specific $CD8^+$ T-cells in AIDS-related non-Hodgkin's lymphoma patients and showed that EBV-specific $CD8^+$ T-cells were not physically lost but rather lost their functionality. [28] This loss of function correlated with lower $CD4^+$ T-cell numbers and increasing viral load. Such a discrepancy between presence and function could possibly explain the high incidence of viral reactivation in recipients of unmanipulated allogeneic stem cell grafts, which patients were shown to experience as much reactivation as recipients of T-cell depleted grafts. [11] Despite higher peripheral T-cell numbers, recipients of unmanipulated stem cell grafts may experience viral reactivation during the time period in which they are treated with cyclosporin, which drug effectively inhibits the function of $CD4^+$ T-cells and thereby abrogates the indispensable help to $CD8^+$ T-cells. [33]

The EBV-specific $CD8^+$ T-cell response recovered within 3 – 6 months after allogeneic hematopoietic stem cell transplantation. Early recovery was already noted at 1 month after allogeneic hematopoietic stem cell transplantation in some of the patients. The rapid recovery of the EBV-specific T-cells corresponds well to the pattern of viral reactivation as monitored by our plasma real-time PCR. The median time to first reactivation was 2 months and early reactivation was already noted in the first month after allogeneic hematopoietic stem cell transplantation in 11 patients. These findings correspond well with those of Marshall et al, who described rapid repopulation of $CD8^+$ T-cells for latent and lytic antigens after allogeneic hematopoietic stem cell transplantation. [29] Early recovery of T-cells may be explained by oropharyngeal EBV-excretion, which has been demonstrated to occur already during the first month after allogeneic hematopoietic stem cell transplantation, thereby providing an antigenic stimulus immediately following transplantation. [34,35] From a pathophysiological point of view, early oropharyngeal EBV-excretion may represent a critical phase in which B-cells of the donor graft may become infected and transformed. [36] The T-cell response directed to lytic antigens may play a critical role in that early phase by limiting oropharyngeal lytic infection and thus subsequent B-cell infection.

Recovery of EBV-specific $CD8^+$ T-cells was delayed in patients, who had been treated with ATG. As shown in Figure 2, a delayed recovery lasting > 3 months was apparent for RAK-specific $CD8^+$ T-cells. A similar delay was observed for the other EBV-epitope-specific T-cells as well. ATG has been recognized as a particularly strong risk factor for the development of EBV-LPD. Several studies have reported increased hazard ratio's ranging from 5 to >10. [7,8,37] We have recently reported that ATG was associated with early reactivation of EBV and a higher probability of progression to EBV-LPD. [11] Based on the results of the present study, we postulate the probable elimination of recipient and graft derived EBV-specific T-cells by ATG which may lead to reduced levels of EBV-specific

$CD8^+$ T-cells in the early time-period post transplant. It further suggests that an early recovery of EBV-specific T-cell immunity is critical for prevention of EBV-LPD.
In conclusion, monitoring the recovery of EBV-specific $CD8^+$ T-cells by use of tetramers was shown to significantly enhance the predictive value of an increased viral load after allogeneic hematopoietic stem cell transplantation. Thereby, a more accurate identification of patients at high risk for EBV-LPD has become possible by combining the 2 assays. It may enable us to further narrow pre-emptive treatment and avoid over-treatment of recipients, who are able to mount an immune response that controls the proliferation of EBV-infected B-cells.

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