3. Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic hematopoietic stem cell transplantation and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted stem cell transplantation.

Joost W.J. van Esser [1], Bronno van der Holt [2], Ellen Meijer [3], Hubert G.M. Niesters [4], Rudolf Trenschel [5], Steven F.T. Thijsen [6] , Anton M. van Loon [6], Francesco Frassoni [7], Andrea Bacigalupo [7] , Ulrich W. Schaefer [5] , Albert D.M.E. Osterhaus [4] , Jan Willem Gratama [8] , Bob Löwenberg [1], Leo F. Verdonck [3] , Jan J. Cornelissen [1]

Departments of Hematology [1], Statistics [2], Virology [4] and Medical and Tumor Immunology [8], University Hospital Rotterdam / Daniel den Hoed Cancer Center, The Netherlands. Departments of Hematology [3] and Virology [6], University Medical Center Utrecht, The Netherlands. Clinic of Bone Marrow Transplantation, University Hospital Essen, Germany [5]. Department of Hematology, Ospedale San Martino, Genoa, Italy [7].

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Abstract

Reactivation of the Epstein-Barr virus (EBV) after allogeneic hematopoietic stem cell transplantation (allo-SCT) may evoke a protective cellular immune response or may be complicated by the development of EBV-lymphoproliferative disease (EBV-LPD). So far, very little is known about the incidence, recurrence, and sequelae of EBV reactivation following allogeneic hematopoietic stem cell transplantation. EBV reactivation was retrospectively monitored in 85 EBV-seropositive recipients of a T-cell- depleted (TCD) allogeneic hematopoietic stem cell transplantation and 65 EBV-seropositive recipients of an unmanipulated allogeneic hematopoietic stem cell transplantation. Viral reactivation (more than 50 EBV genome equivalents geq/ml) was monitored frequently by quantitative real-time plasma polymerase chain reaction (PCR) until day 180 after stem cell transplantation. Probabilities of developing viral reactivation were high after both unmanipulated and TCD-allogeneic stem cell transplantation (31% ± 6% versus 65% ± 7%, respectively). A high $CD34^+$ cell number of the graft appeared as a novel significant predictor ($P = 0.001$) for EBV reactivation. Recurrent reactivation was observed more frequently in recipients of a TCD-graft, and EBV-LPD occurred only after TCD- stem cell transplantation. High-risk status, TCD, and use of ATG were predictive for developing EBV-LPD. Plasma EBV-DNA quantitatively predicted EBV-LPD. The positive and negative predictive values of a viral load of 1,000 geq/ml were, respectively, 39% and 100% following TCD. Treatment-related mortality did not differ significantly between TCD and non-TCD transplants, but the incidence of chronic graft-versus-host disease was significantly less in TCD-patients. It is concluded that EBV reactivation occurs frequently after TCD and unmanipulated allogeneic hematopoietic stem cell transplantation, especially in recipients of grafts with high $CD34^+$ cell counts. EBV-LPD, however, occurred only after TCD and EBV viral load quantitatively predicted EBV-LPD in recipients of a TCD graft.

1. Introduction

Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD) is a serious complication of allogeneic hematopoietic stem cell transplantation (allo-SCT) and solid organ transplantation.[1-3] Although the incidence of EBV-LPD is generally less than 2% after allogeneic hematopoietic stem cell transplantation, it may increase to 20% in patients with established risk factors, such as unrelated donor stem cell transplantation, the use of T-cell-depleted (TCD) allografts, use of antithymocyte globulin (ATG) and immunosuppression for prevention and treatment of graft-versus-host disease (GVHD).[4-8] EBV-LPD is associated with a poor prognosis despite the use of anti-B-lymphocyte monoclonal antibody therapy, donor lymphocyte infusion (DLI) and infusion of EBV-specific cytotoxic T cells (CTL).[9-15] Therefore, early diagnosis and preventive measures such as B-cell depletion of the donor graft, and pre-emptive therapy may be clinically useful.[4,7,16-24] We developed a real-time polymerase chain reaction (PCR) assay for the quantitative detection of EBV-DNA in plasma.[25] The assay accurately monitors viral load in plasma from patients with infectious mononucleosis and immunocompromised patients at risk of EBV-LPD or with established EBV-LPD.[25,26] In contrast to cytomegalovirus (CMV) antigenemia after allogeneic hematopoietic stem cell transplantation and the risk of developing CMV-disease, little is known about reactivation of EBV during the first 3 to 6 months after allogeneic hematopoietic stem cell transplantation and the predictive value of EBV reactivation for subsequent EBV-LPD. Although several studies have shown an association of viral load and a diagnosis of EBV-LPD, no study has longitudinally followed a larger cohort of allogeneic hematopoietic stem cell transplantation recipients with multiple risk factors.[27-43] We set out to monitor EBV reactivation by real-time PCR at regular time intervals after allogeneic hematopoietic stem cell transplantation. Incidences, risk factors, and sequelae of EBV reactivation were compared between patients receiving a TCD- stem cell transplantation and patients having transplantation with an unmanipulated stem cell graft. We show that subclinical EBV reactivation is a very frequent event after allogeneic hematopoietic stem cell transplantation and that quantification of EBV DNA appears useful to identify patients at risk of progression to overt EBV-LPD.

2. Patients and methods

Patients

The study population consisted of 152 consecutive patients treated at 4 transplant centers, who received stem cell transplants between March 1996 and June 1999. Patients underwent allografting at the department of hematology of the university hospitals of Utrecht (TCD stem cell transplantation) or Rotterdam (TCD stem cell transplantation), the Netherlands; Essen (non-TCD-stem cell transplantation), Germany; or Genoa (non-TCD-stem cell transplantation), Italy. Transplant protocols were approved by local institutional review

boards and all patients provided informed consent. Patient characteristics are presented in Table 1. Eighty-five patients received a TCD stem cell transplantation and 67 patients received a non-TCD stem cell transplantation. Median age was 41 years (range, 17-55 years) in the TCD group and 31 years (range, 17-56 years) in the non-TCD group ($P < 0.01$). Standard-risk patients had a diagnosis of acute lymphoblastic leukemia (ALL) in first complete remission (CR1), acute myeloid leukemia (AML) in CR1, chronic myeloid leukemia (CML) in first chronic phase and untreated (very) severe aplastic anemia (SAA), all other diagnoses were considered high risk. The non-TCD group included more patients with CML, and fewer patients with lymphoma, multiple myeloma or high risk disease ($P = 0.001$). Unrelated donor grafts were used more frequently in the non-TCD group ($P = 0.001$). The use of ATG added to the conditioning regimen for prevention of rejection was confined to patients having transplantation with TCD grafts from unrelated donors.

Transplantation

The conditioning regimen preceding a TCD-SCT consisted of cyclophosphamide (120 mg/kg) and total body irradiation (TBI) (12 Gy in 2 fractions). Rabbit ATG (Imtix Sangstat, Amstelveen, The Netherlands) was given for prevention of rejection prior to SCT in recipients of a TCD unrelated donor graft. 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). The conditioning regimen in case of an unmanipulated SCT consisted of cyclophosphamide (120 mg/kg) and TBI (10 Gy in 4 fractions or 10 Gy in 3 fractions).

Partial T-cell depletion was performed using sheep erythrocyte rosetting (n=53) or CD34 selection (CellPro, Wezembeek, Belgium) (n=32). Median T-cell numbers differed more than 2 logs between TCD and unmanipulated grafts (2.0×10^5/kg versus 510×10^5/kg), but numbers of granulocyte-macrophage colony-forming units (CFU-GM) and $CD34^+$ mononuclear cells (MNCs) did not differ significantly between the groups of patients. Peripheral blood-derived stem cells were used relatively more often than bone marrow-derived stem cells in patients receiving a TCD-graft as compared with patients receiving an unmanipulated graft ($P < 0.01$). Graft-versus-host (GVH) prophylaxis was cyclosporin A (3 mg/kg) from day –3 until day +100 after TCD stem cell transplantation, and the combination methotrexate (15 mg/m^2 on day 1; 10 mg/m^2 on day 3, 6 and 11) and cyclosporin A was used in recipients of an unmanipulated 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 to 360 after stem cell transplantation. Patients having transplantation in Utrecht (TCD stem cell transplantation) and Genoa (non-TCD stem cell transplantation) received long-term aciclovir prophylaxis from day 0 until day 360. Erythrocyte and platelet products for transfusion were filtered to remove leucocytes and subsequently irradiated (25 Gy).

Table 1. Patient characteristics

Characteristic	Allogeneic T-cell-depleted SCT (n=85)	Allogeneic non-T-cell-depleted SCT (n=67)	P-value
Sex male/female (n)	48/37	50/17	0.02
Age, y (median, range)	41 (17-55)	31 (17-56)	< 0.01
Diagnosis (n):			
AML CR1	11	3	
AML >CR1	8	8	
ALL CR1	5	6	
ALL >CR1	7	2	
ALL CR1 Ph^+	5	-	
MDS	3	1	
CML CP1	8	28	
CML >CP1	5	16	
SAA	5	-	
MM	15	1	
M. Hodgkin	2	-	
NHL	10	2	
CLL	1	-	
Risk status: SR/HR (n)	25/60	37/30	0.001
Donor type (n)			
Sib	61	30	0.001
MUD	24	37	
Conditioning regimen (n)			
Cy/TBI	59	67	
Cy/TBI/ATG	23	-	
Bu/Cy	2	-	
Bu/Cy/ATG	1	-	

Table 1. Patient characteristics (continued)

Characteristic	Allogeneic T-cell-depleted SCT (n=85)	Allogeneic non-T-cell-depleted SCT (n=67)	P-value
Graft characteristics: (median, range)			
MNC x 10^8/kg	0.13 (0.01-9.32)	3.43 (0.13-14.0)	
CD3 x 10^5/kg	2.0 (1.0-7.5)	510 (7.4-2195)	< 0.001
CFU-GM x 10^4/kg	16.7 (1.9-85.9)	14.1 (4.0-132)	0.6
CD34 x 10^6/kg	1.25 (0.06-6.43)	2.2 (0.04-14.1)	0.7
EBV-serology (n)			
D-R-	-	2	0.2
D+R-/D+R+/D-R+	85	65	
Stem cell source (n)			
BM	66	63	< 0.01
PB	19	4	

AML1 CR1 or >CR1 indicates acute myeloid leukemia in first or subsequent complete remission; ALL CR1 or >CR1, acute lymphoblastic leukemia in first or subsequent CR; ALL CR1 Ph^+ , ALL CR1 philadelphia chromosome-positive; MDS, myelodysplastic syndrome; CML CP1 or >CP1, chronic myeloid leukemia in first or subsequent chronic phase; SAA, severe aplastic anemia; MM, multiple myeloma; NHL, Non-Hodgkin's lymphoma; CLL , chronic lymphocytic leukemia; SR, standard risk; HR, high risk; Sib, HLA identical family donor; MUD, matched unrelated donor; Cy, cyclophosphamide; TBI, total body irradiation; Bu, busulphan; ATG, anti-thymocyte globulin; MNC, mononuclear cells; CFU-GM, granulocytes-monocyte colony-forming units; D+/-, EBV-seropositive / seronegative donor; R+/-, EBV-seropositive/seronegative recipient; BM, bone marrow; PB, peripheral blood.

Patients were hospitalized in reverse isolation and rooms with high-efficiency particulate-filtered air. All patients received food with a low microbial count until discharge, and parenteral alimentation was given in case of severe mucositis.

Real-time Taqman Assay

Taqman PCR primers were selected from the EBV-DNA genome encoding for the nonglycosylated membrane protein BNRF1-p143 and generated a DNA product of 74 basepairs, as described before. [25] A known EBV-DNA copy number based on a reference standard quantified by electron microscopy (ABI Advanced Biotechnologies, Columbia, MA, USA) was used for standardization. Serial dilutions ranging from 10 to 10^7 EBV-DNA genome equivalents per ml (geq/ml) were made to characterize linearity, precision,

specificity and sensitivity. The Taqman assay appeared to detect viral DNA in plasma over a linear span between 50 and 10^7 geq/ml with an average coefficient of variation of 1.56% (range, 0.7- 7.0%). Test results below 50 geq/ml were considered negative. No viral DNA was detected in plasma of healthy EBV-seropositive individuals. [25] EBV reactivation was defined as a plasma EBV-DNA level exceeding 50 geq/ml. Recurrent reactivation was defined by a positive PCR (more than 50 geq/ml) after (at least) two consecutive negative PCR results following a preceding episode of reactivation. Viral load was monitored in blood samples drawn at 2-week intervals starting at stem cell transplantation until day 180 after stem cell transplantation.

EBV-LPD diagnosis

A diagnosis of EBV-LPD was preferably based on lymph node histology or cytology and was classified according to the criteria of Knowles et al.[44] Immunohistology included antibody staining with CD19-specific (Becton Dickinson, San José, CA, USA), CD20-specific (DAKO, Glostrup, Denmark) and EBV latent membrane protein-1-specific (DAKO) monoclonal antibodies. Furthermore, clonality was assessed using immunohistochemical staining with monoclonal antibodies to kappa and lambda light chains (DAKO). In situ hybridization was performed to detect EBV-encoded small RNA molecules (EBV-EBER) using an EBV-EBER probe (DAKO) and PCR for detection of EBV-DNA encoding for the *Bam*HI fragment. EBV-LPD staging included physical examination, whole-body computed tomography scanning (CT) scanning, and flow cytometric detection of monoclonal B lymphocytes in blood, bone marrow, and, if indicated, cerebrospinal fluid.

Endpoints and statistical analysis

The data were analyzed as of January 2000. Patient characteristics of non-TCD patients and TCD-patients were compared using Fisher exact test or Pearson chi-square test, whichever was appropriate, in case of discrete variables, or the Wilcoxon rank-sum test in case of continuous variables. End points of the study included time to EBV reactivation, EBV-LPD, acute GVHD grades II to IV, chronic GVHD and treatment-related mortality (TRM). Time to first EBV reactivation was determined from the date of transplantation until day 180, and patients were censored at the date of last serum sample if this sample had been taken before day 180. Time to EBV-LPD was measured from SCT until EBV-LPD. Patients who died without EBV-LPD were censored at the date of death. Patients still alive at the date of analysis were censored at the last follow-up date. Two EBV-seronegative donor-recipient pairs were excluded from the analysis of EBV reactivation and EBV-LPD. GVHD was diagnosed and graded according to consensus criteria.[45] Chronic GVHD was evaluated among patients who survived at least 100 days after transplantation. TRM was defined according to standard criteria.[46] Time to EBV reactivation, EBV-LPD, acute and chronic GVHD, and TRM were estimated by the Kaplan-Meier method, and Kaplan-Meier curves were generated to illustrate differences between subgroups of patients.[47] The following

variables were included in the analysis of prognostic factors: sex, male patient and female donor, age, risk status, donor (sibling versus matched unrelated donor), source of stem cells (bone marrow versus peripheral blood), type of transplant (non-TCD versus TCD without ATG versus TCD with ATG) and graft characteristics (number of MNCs, number of $CD34^+$ cells, number of $CD3^+$ and CFU-GMs infused). Univariate survival analysis was performed using the log-rank test and Cox regression to see whether there was a difference between subgroups.[48,49] The variables that appeared significant in the univariate analysis were also included in a multivariate Cox regression. Moreover, Cox regression was performed using EBV reactivation within day 180 as a time-dependent covariate to assess whether EBV reactivation predicted EBV-LPD and TRM. All reported P-values are 2-sided and a significance level of α=0.05 was used.

3. Results

EBV reactivation

The probability of developing EBV reactivation was greater after TCD-allogeneic stem cell transplantation than after non-TCD stem cell transplantation (Figure 1, Table 2).

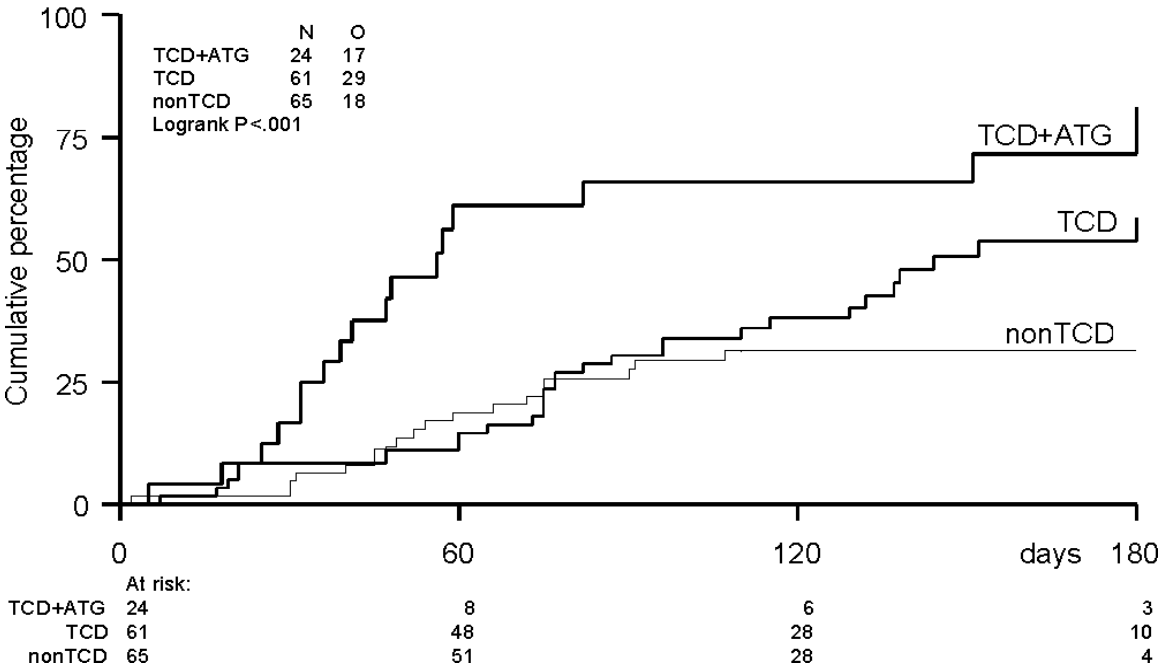

Figure 1. Incidence of EBV-reactivation. Incidence of EBV-reactivation after TCD-allogeneic hematopoietic stem cell transplantation with ATG (n=24), TCD stem cell transplantation without ATG (n=61), and non-TCD stem cell transplantation (n=65). Only TCD combined with ATG significantly increased the risk of EBV reactivation (P < 0.001).

Table 2. EBV reactivation and EBV-LPD

Parameter	T-cell-depleted allo-SCT (n=85)		Unmanipulated allo-SCT (n=65)	
No. of patients with EBV reactivation (%)	46	(54)	18	(28)
Time (d) to first EBV reactivation (median, range)	58	(5-180)	63	(2-107)
Maximum viral load (geq/ml) of first EBV reactivation (median, range)	535	(50-3,200,000)	808	(55-540,000)
No of patients (%) with recurrent EBV reactivation	14	(16)	2	(3)
No of patients (%) with EBV-LPD	10	(12)	-	
Time (d) from SCT to EBV-LPD (median, range)	87	(50-168)	-	
Time (d) from first EBV reactivation to EBV-LPD (median, range)	22	(13-120)	-	
EBV-LPD viral load (geq/ml, median, range)	110,0000	(1,800-790,000)	-	

geq/ml indicates genome equivalents EBV-DNA/ml. Other abbreviations are explained in Table 1.

That difference, however, could be largely attributed to the use of ATG in conjunction with TCD (Figure 1, Table 3). Probabilities of viral reactivation were not different between recipients of a non-TCD stem cell transplantation and recipients of TCD stem cell transplantation without concomitant ATG. Median time to first reactivation was 58 days (range, 5-180 days) in the TCD group and 63 days (range, 2-107 days) in the non-TCD

group (not significant). Plasma EBV-DNA levels measured at the peak of the first reactivation did not differ between the groups.
Recurrent reactivation was significantly more frequent after TCD (Table 2): 14 of 85 patients (16%) experienced multiple episodes of EBV reactivation after TCD stem cell transplantation, including 8 patients with 2 episodes, 5 patients with 3 episodes, and 1 patient showing 4 distinct periods of reactivation. This is exemplified for a recipient of a TCD donor graft who experienced 3 episodes of EBV reactivation without developing EBV-LPD (Figure 2).

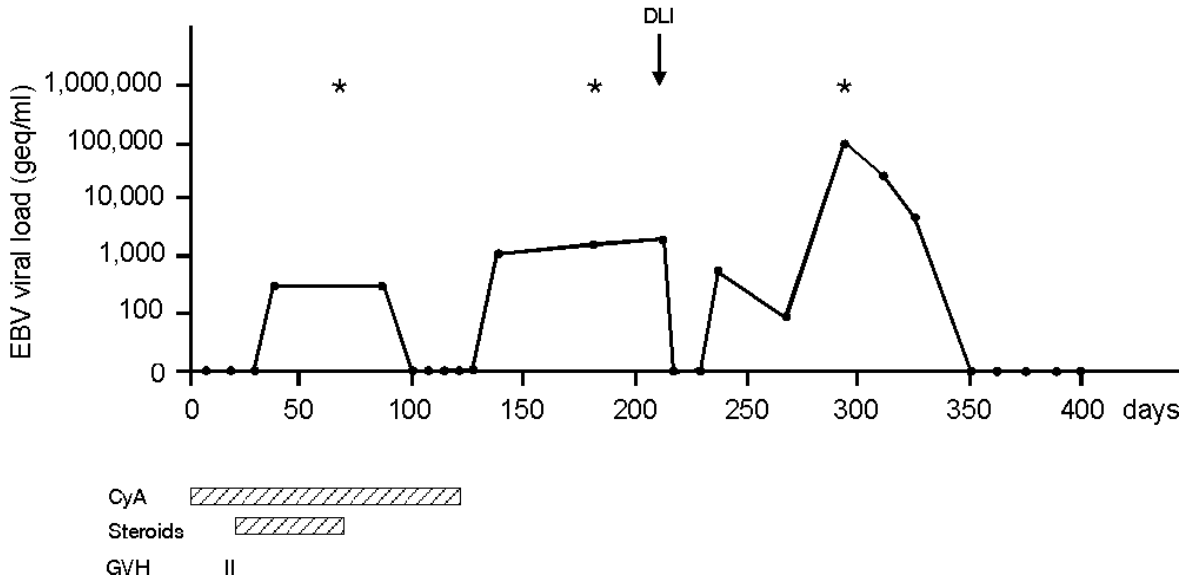

Figure 2. Monitoring EBV viral load after matched unrelated stem cell transplantation. A 16-year-old EBV-seropositive male with a philadelphia chromosome-positive (Ph^+) ALL in first complete remission received a TCD matched unrelated donor graft from an EBV-seropositive donor. Multiple EBV reactivations were observed; however, no EBV-LPD ensued. Frequent examination of bone marrow for the presence of monoclonal B cells and whole-body CT to detect lymphadenopathy were negative at various time points (*). At day 211, DLI (1.0 x 10^5 $CD3^+$ T-cells/kg) was administered because of molecular relapse of his Ph^+ ALL. Currently, the patient is free of disease and well at day 800 after SCT. CyA indicates cyclosporin A.

In contrast, only 2 of 65 patients (3%) receiving non-TCD grafts had a second period of reactivation. ATG appeared not to be associated with recurrent reactivation, as only 2 out of 14 patients with recurrent reactivation after TCD also received ATG as part of the conditioning regimen. Several risk factors predicted for first reactivation in univariate analysis (Table 3), including TCD ($P = 0.02$), use of ATG in the conditioning regimen ($P < 0.001$), transplantation of unrelated donor graft ($P = 0.02$), and a high $CD34^+$ cell number of the graft ($P = 0.001$) (Figure 3). Following multivariate analysis, only use of ATG and high $CD34^+$ cell count (> 1.35 x 10^6/kg) remained independently associated with EBV

reactivation (Table 3). Numbers of $CD34^+$ and $CD3^+$ cells were not associated with each other.

Table 3. Univariate and multivariate Cox regression analysis of risk factors for Epstein-Barr virus reactivation

Risk factor	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
T-cell depletion, no ATG	1.5	0.8-2.7	0.02	1.5	0.8-2.9	0.3
T-cell depletion, ATG	3.5	1.8-6.9	< 0.001	3.4	1.6-7.1	0.001
High-risk status	1.6	1.0-2.8	0.07	1.4	0.8-2.6	0.2
Unrelated donor	1.8	1.1-2.9	0.02	0.9	0.3-2.9	0.8
$CD34^+$ cell count of the graft (> 1.35 x 10^6/kg)	2.4	1.4-4.1	0.001	2.6	1.5-4.6	0.001

HR indicates hazard ratio; CI, confidence interval; ATG, antithymocyte globulin.

EBV-LPD

EBV-LPD was only observed following TCD stem cell transplantation (Table 2, Figure 4). Five patients developed EBV-LPD after HLA identical sibling SCT and 5 after unrelated donor stem cell transplantation (Table 4). Five of these patients had received ATG before unrelated donor stem cell transplantation, and 9 of them had been treated for high-risk disease. All EBV-LPD donor-recipient pairs were EBV seropositive. One donor had negative EBV serology before transplantation. Median time from first reactivation to EBV-LPD was 22 days (range, 13-120 days) (Table 2). Median EBV-DNA level at EBV-LPD diagnosis was 110,000 geq/ml (range 1,800-790,000). Histological proof of a diagnosis of EBV-LPD and classification according to the criteria of Knowles et al [44] were obtained in 8 patients. Patient 8 (Table 4), who received an HLA-identical sibling stem cell transplantation for multiple myeloma, was diagnosed with EBV-LPD by the presence of monoclonal B cells in his cerebrospinal fluid and an elevated plasma EBV-DNA level. Patient 9, who received an unrelated donor stem cell transplantation because of severe

aplastic anemia, was diagnosed with EBV-LPD because of massive lymphadenopathy on CT scanning and a highly elevated plasma EBV-DNA level.

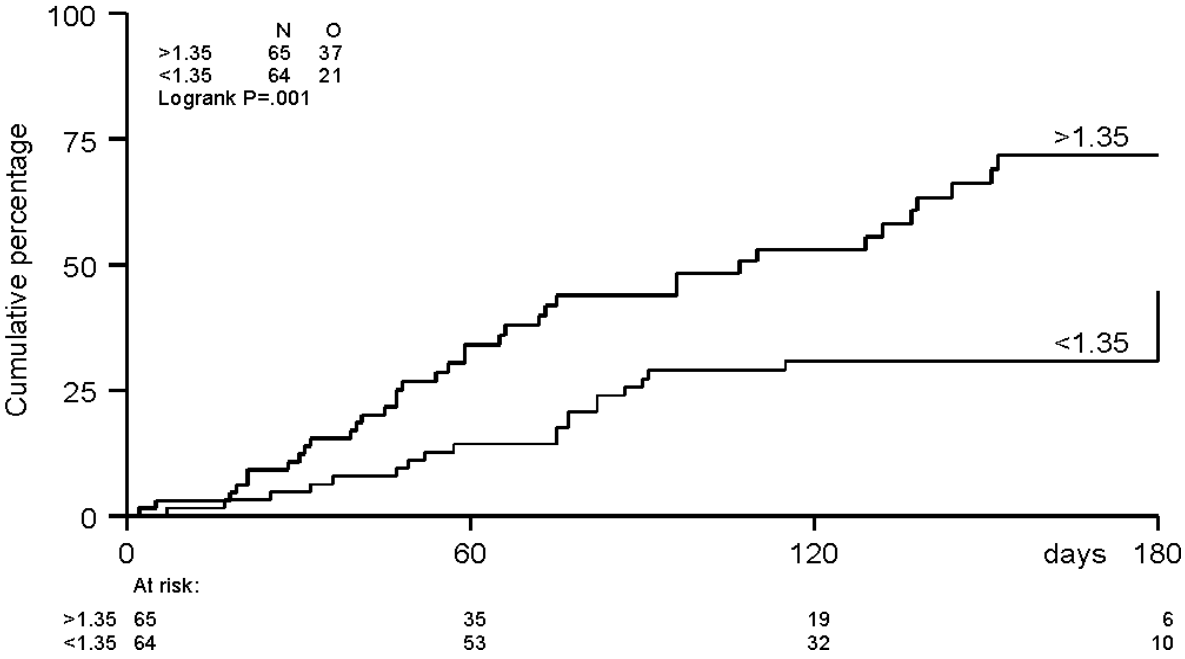

Figure 3. Incidence of EBV reactivation by number of CD34$^+$ cells in the graft. The median number of CD34$^+$ in the graft was 1.35 x 10^6/kg. Patients with grafts containing more than 1.35 x 10^6/kg were at higher risk (P = 0.001) of EBV reactivation.

Six patients received anti-B-cell monoclonal antibody therapy (rituximab), 5 patients received DLI, and immune suppression was discontinued in 8 patients (Table 4). Five patients obtained a complete remission and 5 other patients died of progressive EBV-LPD. Two responding patients are currently alive with a follow-up of 620 and 351 days. Three responding patients developed severe GVHD, 2 following DLI, and died due to GVHD-related complications.

Use of ATG, application of TCD, and high-risk status of underlying disease significantly predicted EBV-LPD in univariate analysis. Multivariate analysis was not performed because the latter 3 variables appeared strongly associated and the small number of events did not allow a reliable multivariate analysis. Several risk factors occurring after stem cell transplantation were evaluated for a possible association with EBV-LPD by time-dependent analysis. A lower lymphocyte count at first EBV reactivation appeared not predictive for developing EBV-LPD. In contrast, EBV load significantly predicted EBV-LPD in a quantitative manner. A stepwise increase of EBV DNA by 1 log (Table 5)

yielded a hazard ratio (HR) of 2.9 (95% confidence interval [CI], 1.7-4.8) for those patients receiving a TCD graft (P < 0.001).

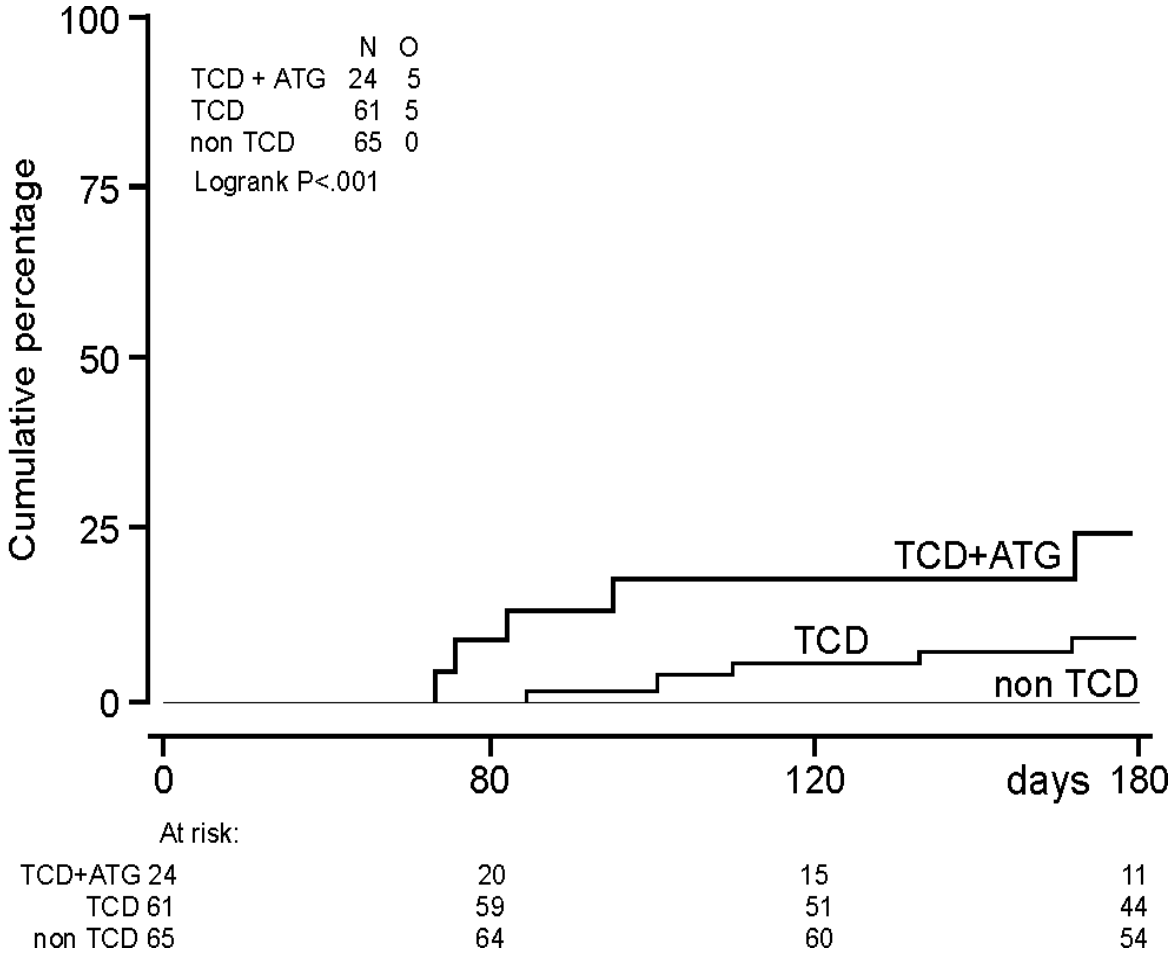

Figure 4. Incidence of EBV-LPD. Incidence of EBV-LPD (n=10) after TCD-allogeneic hematopoietic stem cell transplantation combined with ATG (n=24), TCD hematopoietic stem cell transplantation without ATG (n=61), and non-TCD hematopoietic stem cell transplantation (n=65).

Numbers of patients with a TCD stem cell transplantation with plasma levels of EBV-DNA exceeding a certain threshold value and the corresponding positive and negative predictive values for EBV-LPD for that subset of patients are shown in Table 5. Although the positive predictive value was 24% for patients with a copy number of 100 geq/ml or higher, it rose to 100% at the level of 500,000 geq/ml. However, only one patient with EBV-LPD reached that high number, and consequently the negative predictive value measured 89%.

Table 4. Epstein-Barr virus-lymphoproliferative disease following T-cell depleted allogeneic hematopoietic stem cell transplantation

Patient no	Donor Type	EBV-LPD diagnosis			Therapy			Outcome		
		Morphology	Clonality	Plasma EBV DNA (geq/ml)	SI	Anti-CD20	DLI	Response	Survival (d)	COD
1	Sib	III	Mono	1,800	+	-	+	PD	Dead	EBV-LPD
2	MUD	II	Poly	92,000	+	+	+	CR	Dead	GVHD
3	MUD	II	Poly	6,500	+	+	-	CR	Alive, 620^{+}	-
4	Sib	III	Mono	790,000	+	+	+	PD	Dead	EBV-LPD
5	Sib	III	Mono	128,000	+	+	-	CR	Alive, 351^{+}	-
6	Sib	II	Mono	74,000	+	+	-	CR	Dead	GVHD
7	MUD	III	Mono	133,000	+	+	+	PD	Dead	EBV-LPD
8	Sib	nd	Mono	7,900	+	-	-	PD	Dead	EBV-LPD
9	MUD	nd	nd	310,000	-	-	+	CR	Dead	GVHD
10	MUD	III	Mono	206,000	-	-	-	PD	Dead	EBV-LPD

SCT indicates stem cell transplantation; Sib, HLA identical family donor; MUD, matched unrelated donor; EBV-LPD, Epstein-Barr virus associated lymphoproliferative disease; I, plasmacell hyperplasia; II, polymorphic hyperplasia; III, Non-Hodgkin's lymphoma (criteria according to Knowles et al [44]); nd, not determined; Mono, monoclonal disease; Poly, polyclonal disease; SI, stop immunosuppression; anti-CD20, monoclonal anti B-cell therapy; DLI, donor lymhocyte infusion; PD, progressive disease; CR, complete remission; COD, cause of death; GVHD, graft-versus-host disease.

Table 5. Incidence of Epstein-Barr virus-lymphoproliferative disease by viral load following T-cell depleted allogeneic hematopoietic stem cell transplantation

EBV load (geq/ml)	No. of patients with specified reactivation	No. of patients with EBV-LPD	Predictive values	
			Positive (%)	Negative (%)
100	41	10	24%	100%
1,000	26	10	39%	100%
10,000	14	7	50%	96%
100,000	7	5	71%	94%
500,000	1	1	100%	89%

geq/ml indicates genome equivalents per ml. Other abbreviations are explained in Table 1.

Graft-versus-host disease

The actuarial probability of acute GVHD II-IV at day 100 was 57% ± 4% for the whole group and was not significantly different for patients receiving a TCD graft as compared with patients following unmanipulated allogeneic hematopoietic stem cell transplantation. An unrelated donor graft and a high $CD34^+$ cell count of the graft (independent from the number of $CD3^+$ T cells in the graft) were the only significant risk factors for developing acute GVHD following multivariate analysis. EBV reactivation was not associated with acute GVHD. Actuarial probabilities of chronic limited and extensive GVHD at 12 months post stem cell transplantation were significantly higher for non-TCD patients (83% ± 5%) than for TCD patients (38% ± 6%) ($P < 0.001$).

Treatment-related mortality

The actuarial probability of TRM was 29% ± 4% at 1 year for all patients and did not differ between TCD and unmanipulated allogeneic hematopoietic stem cell transplantation. Higher age and a higher $CD34^+$ cell count (> 1.35 x 10^6/kg) of the graft predicted higher TRM in multivariate analysis. Following time-dependent analysis, EBV reactivation (HR: 1.9, 95% CI: 1.0-3.3, P = 0.04) and acute GVHD grade I-IV (HR: 1.8, 95% CI: 1.0-3.3, P = 0.05) were associated with higher TRM. In addition, a higher lymphocyte count (> 0.6 x 10^9/l) at the time of first EBV reactivation significantly predicted less TRM (HR 0.3; 95% CI, 0.1-0.8; P = 0.02).

4. Discussion

This study demonstrates that EBV reactivation is a very frequent event after both TCD and unmanipulated allogeneic hematopoietic stem cell transplantation. In particular, recipients of stem cell grafts with high numbers of $CD34^+$ MNCs appeared to be at risk for EBV reactivation. However, patients receiving a TCD stem cell transplantation were at significantly higher risk for recurrent reactivation and only these patients developed EBV-LPD. The development of impending EBV-LPD in these patients could be predicted quantitatively by monitoring viral load in plasma at regular intervals during the first 6 months after SCT.

EBV reactivation was observed frequently after TCD stem cell transplantation and after unmanipulated allogeneic hematopoietic stem cell transplantation as well. The high incidence of first EBV reactivation after TCD stem cell transplantation could be largely attributed to the use of ATG and, as a result, TCD per se did not appear to be an independent risk factor for early EBV reactivation. However, patients receiving a TCD stem cell transplantation showed more recurrence of reactivation and EBV-LPD was observed only after TCD. Because the conditioning regimen has eradicated autologous EBV-specific immunity after both TCD and unmanipulated stem cell transplantation, early EBV reactivation may occur after both modes of allogeneic hematopoietic stem cell transplantation. [50,51] However, the significantly higher risks for recurrent EBV reactivation and EBV-LPD in TCD stem cell transplantation as compared with unmanipulated stem cell transplantation may be explained by the impaired capacity of patients receiving TCD grafts to mount an effective immune response to the reactivating virus. The strongly reduced numbers of EBV-specific memory T cells in TCD as compared with unmanipulated grafts may play a major role in this respect. [52,53]

Apart from the use of ATG as part of the conditioning regimen, we identified the number of $CD34^+$ cells in the graft as a novel independent risk factor for developing EBV reactivation (Table 3, Figure 3), and also for acute GVHD and TRM. Przepiorka et al [54] recently reported that recipients of peripheral blood stem cell grafts with high $CD34^+$ cell counts were at higher risk for acute GVHD, an effect that appeared independent of the number of $CD3^+$ T cells.[54] They suggested that GVHD at high $CD34^+$ cell doses may be exacerbated by cytokines released by the markedly expanding myeloid population at the time of engraftment. This explanation is supported by high levels of proinflammatory cytokines in patients with severe GVHD. [55-57] In the present study, acute GVHD significantly predicted TRM in a time-dependent analysis. Therefore, the association of $CD34^+$ cell dose and TRM might be explained by an increased incidence of GVHD. The association of $CD34^+$ cell dose and EBV reactivation is, however, less likely to be explained by more GVHD, as EBV reactivation preceded the onset of acute GVHD in a significant number of patients. Alternative explanations may include infusion of a higher number of EBV-infected B cells together with larger stem cell grafts, or stimulation of B-cell proliferation by cytokines produced by the higher number of rapidly maturing myeloid progenitors. The latter

explanation is supported by a number of preclinical as well as clinical studies showing that proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor α and -β, and IL-6, may very effectively stimulate the growth of EBV-infected B cells. [58] In particular, IL-6 may play an important role as a growth factor, promoting the progression toward overt EBV-LPD. [59-62] Apart from monocyte-macrophages and endothelial cells as an established source of proinflammatory cytokines, the rapid proliferating myeloid population of grafts containing high $CD34^+$ cell doses may add to cytokine release and thus contribute to viral reactivation.

A number of studies have demonstrated a correlation between high levels of viral load and a diagnosis of EBV-LPD after both stem cell transplantation and solid-organ transplantation. [26-43] No study, however, has longitudinally followed allogeneic hematopoietic stem cell transplantation recipients with multiple risk factors from day 0 until day 180 and reported positive and negative predictive values. Lucas et al [41] evaluated the predictive value of a quantitative PCR using DNA extracted from peripheral blood MNCs in a cohort of 195 patients receiving a solid-organ transplantation. [41] Although the negative predictive value appeared very high (100%), the positive predictive value was 38%. Our results observed in recipients of an allogeneic hematopoietic stem cell transplantation are in line with these findings. Considering both TCD and non-TCD transplants, the negative and positive predictive values of a copy number of 1,000 geq/ml were, respectively, 100% and 28%. Higher predictive values were obtained when the analysis was restricted to patients receiving a TCD stem cell transplantation. The positive predictive value of a high EBV-DNA level of more than 1,000 geq/ml and more than 10,000 geq/ml for patients receiving a T-cell depleted stem cell transplantation were 39% and 50%, respectively (Table 5).

Although highly significant, these predictive values also indicate that most patients (even recipients of TCD grafts) were able to mount an effective immune response and clear their viral reactivation. Monitoring of the reconstitution of HLA-specific T lymphocytes may add to the predictive value of viral load quantification. For this purpose, rapid assays are now available, such as the enumeration of EBV-specific T lymphocytes by tetramer binding or the induction of intracellular interferon-γ in T cells after specific stimulation. [63] The accurate prediction of impending EBV-LPD in patients at risk is important because pre-emptive therapy might be more effective than therapy of established EBV-LPD. Despite the application of new treatment modalities such as DLI and anti-B-cell immunotherapy, the mortality of patients with established EBV-LPD is still high. Ten patients developed EBV-LPD in the present study: 5 died due to progressive EBV-LPD and 3 patients secondary to GVHD following DLI, resulting in a 80% (8 of 10) mortality. Pre-emptive infusion of EBV-specific cytotoxic T cells has been shown to reduce viral load and may prevent the evolution toward EBV-LPD. [20] However, the preparation and use of such EBV-specific T cells is expensive and difficult to implement on a wide scale. B-cell depletion of the donor graft has been shown to effectively reduce the incidence of EBV-LPD. [7,16] Therefore, anti-B-cell immunotherapy aimed at in vivo B-cell depletion after stem cell transplantation in patients at

high risk of EBV-LPD might be a promising new means of pre-emptive therapy. A prospective phase II study with that specific aim is currently being performed. [64] Because the depletion of B cells may add to the impaired immune status of these patients, one may argue to restrict pre-emptive therapy to those patients at highest risk. A threshold of 1,000 geq/ml, as observed in our patient population, may thereby serve as a critical level of viral load to start pre-emptive therapy. Thus, pre-emptive therapy may be administered selectively to high-risk patients to prevent EBV-LPD and to avoid treatment of patients who have recovered their EBV-specific immunity to protective levels. The frequent monitoring of EBV load after allogeneic hematopoietic stem cell transplantation may therefore be considered for patients with a high risk profile for EBV-LPD and may preferably be combined with close monitoring of the reconstitution of EBV-specific T lymphocytes.

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