Chapter 4

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

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Abstract

Reactivation of the Epstein-Barr virus (EBV) after allogeneic stem cell transplantation (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 SCT. EBV reactivation was retrospectively monitored in 85 EBV-seropositive recipients of a T cell depleted (TCD) SCT and 65 EBV-seropositive recipients of an unmanipulated SCT. Viral reactivation (more than 50 EBV copies (c) /ml) was monitored frequently by quantitative real-time plasma polymerase chain reaction (PCR) until day 180 after SCT. Probabilities of developing viral reactivation were high after both unmanipulated and TCD allogeneic SCT ($31\% \pm 6\%$ versus $65\% \pm 7\%$, 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 SCT. High-risk disease status, TCD, and use of Antithymocyteglobulin (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 1000 c/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 SCT, 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.

Introduction

Epstein-Barr virus-associated lymphoproliferative disease (EBV-LPD) is a serious complication of allogeneic stem cell transplantation (SCT) and solid organ transplantation[1-3]. Although the incidence of EBV-LPD is generally less than 2% after SCT, it may increase to 20% in patients with established risk factors, such as unrelated donor SCT, the use of T cell depleted (TCD) grafts, use of Antithymocyteglobulin (ATG) and immuno-suppression 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 SCT and the risk of developing CMV-disease, little is known about reactivation of EBV during the first 3 to 6 months after SCT 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 SCT recipients with multiple risk factors[27-43]. We set out to monitor EBV reactivation by real-time PCR at regular time intervals after SCT. Incidences, risk factors and sequelae of EBV reactivation were compared between patients receiving a TCD SCT and patients transplanted with an unmanipulated stem cell graft. We show that subclinical EBV reactivation is a very frequent event after SCT and that quantification of EBV DNA appears useful to identify patients at risk of progression to overt EBV-LPD.

Methods

Patients The study population consisted of 152 consecutive patients treated at 4 transplant centres, who received stem cell transplants between March 1996 and June 1999. Patients underwent allografting at the department of haematology of the university hospitals of Utrecht (TCD SCT) or Rotterdam (TCD SCT), the Netherlands; Essen (non-TCD SCT), Germany; or Genoa (non-TCD SCT), 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 SCT and 67 patients received a non-TCD SCT. 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). Low-risk patients had a diagnosis of acute lymphoblastic leukaemia (ALL) in first complete remission (CR1), acute myeloid leukaemia (AML) in CR1, chronic myeloid leukaemia (CML) in first chronic phase and untreated (very) severe aplastic anaemia (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 transplanted 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 (Thymoglobulin™, 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 CD34+ cells 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

Table 1 Patient characteristics

Characteristics	TCD SCT (n=85)	nonTCD SCT (n=67)	P
Age, y (range)	41 (17-55)	31 (17-56)	<0.01
Diagnosis			
AML CR1	11	3	
AML >CR1	8	8	
ALL CR1	10	6	
ALL >CR1	7	2	
MDS	3	1	
CML CP1	8	28	
CML >CP1	5	16	
SAA	5	0	
MM	15	1	
NHL	10	2	
Other	3	0	
Risk status: low/high	25/60	37/30	0.001
Donor: MRD/MUD	61/24	30/37	0.001
Graft characteristics			
T cells (range; x 10^5/kg)	2.0 (0.01-9.32)	510 (7.40-2195)	<0.001
CD34+ cells (range; x 10^6/kg)	1.25 (0.06-6.43)	2.2 (0.04-14.10)	
Conditioning regimen			
Cy/TBI	59	67	
Cy/TBI/ATG	23	0	
Other	3	0	
EBV serostatus			
R-/D-	0	2	
R+/D+, R+/D-, R-/D+	85	65	
Stem cell source: BM/PB	66/19	63/4	<0.01

Data are no. of patients, unless otherwise indicated; AML CR1 or >CR1 = acute myeloid leukaemia in first or subsequent complete remission; ALL CR1 or >CR1 = acute lymphoblastic leukaemia in first or subsequent CR; MDS = myelodysplastic syndrome; CML CP1 or >CP1 = chronic myeloid leukaemia in first or subsequent chronic phase; SAA = severe aplastic anaemia; MM = multiple myeloma; NHL = Non-Hodgkin's lymphoma; MRD = matched related donor; MUD = matched unrelated donor; Cy = cyclophosphamide; TBI = total body irradiation; ATG = Antithymocyteglobulin; R/D = recipient/donor; BM = bone marrow; PB = peripheral blood.

unmanipulated graft (p<0.01). Graft-versus-host (GVH) prophylaxis was cyclosporin A (3 mg/kg) from day -3 until day +100 after TCD SCT, 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 SCT.

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 SCT. Patients transplanted in Utrecht (TCD SCT) and Genoa (non-TCD SCT) received long-term acyclovir prophylaxis from day 0 until day 360. Blood products were leukocyte depleted and subsequently irradiated (25 Gy). Patients were hospitalised 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 non-glycosylated membrane protein BNRF1-p143 and generated a DNA product of 74 base-pairs, 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 c/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 c/ml with an average coefficient of variation of 1.56% (range, 0.7- 7.0%). Test results below 50 c/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 c/ml. Recurrent reactivation was defined by a positive PCR (more than 50 c/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 SCT until day 180 after SCT.

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 hybridisation was performed to detect EBV-encoded small RNA mole-

cules (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 analysed 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 (MRD versus MUD), 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 mononuclear cells, number of CD34+ cells, number of CD3+ cells and granulocyte-macrophage colony forming units infused). Univariate survival analysis was performed using the log-rank test[48]. The variables that appeared significant in the univariate analysis were included in a multivariate Cox regression[49]. 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 p=0.05 was used.

Results

EBV-reactivation The probability of developing EBV reactivation was greater after TCD allogeneic SCT than after non-TCD SCT (Figure 1, Table 2). 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 SCT and recipients of TCD SCT 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 SCT, 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). 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.

Table 2 EBV reactivation and EBV-LPD

Parameter	TCD SCT (n=85)	non-TCD SCT (n=65)
No. of patients with EBV reactivation (%)	46 (54)	18 (28)
Days post-transplant to first EBV reactivation	58 (5-180)	63 (2-107)
Maximum VL (c/ml) of first reactivation	535 (50-3200000)	808 (55-540000)
No. of patients with recurrent reactivation (%)	14 (16)	2 (3)
No. of patients with EBV-LPD (%)	10 (12)	0
Days post-transplant to EBV-LPD	87 (50-168)	0
Days from first EBV reactivation to EBV-LPD	22 (13-120)	0
EBV-LPD VL (c/ml)	110000 (1800-790000)	

Data are expressed as median (range), unless otherwise indicated; VL = viral load; other abbreviations: see Table I.

Figure 1

Upper panel: Incidence of EBV-reactivation after TCD allogeneic SCT with ATG (n=24), TCD SCT without ATG (n=61), and non-TCD SCT (n=65). Only TCD combined with ATG significantly increased the risk of EBV reactivation (p<0.001).

Lower panel: Incidence of EBV reactivation by number of CD34+ cells in the graft. The median number of CD34+ cells was 1.35 x 10^6/kg. Patients with grafts containing more than 1.35 x 10^6/kg CD34+ cells were at higher risk (p=0.001) of EBV reactivation.
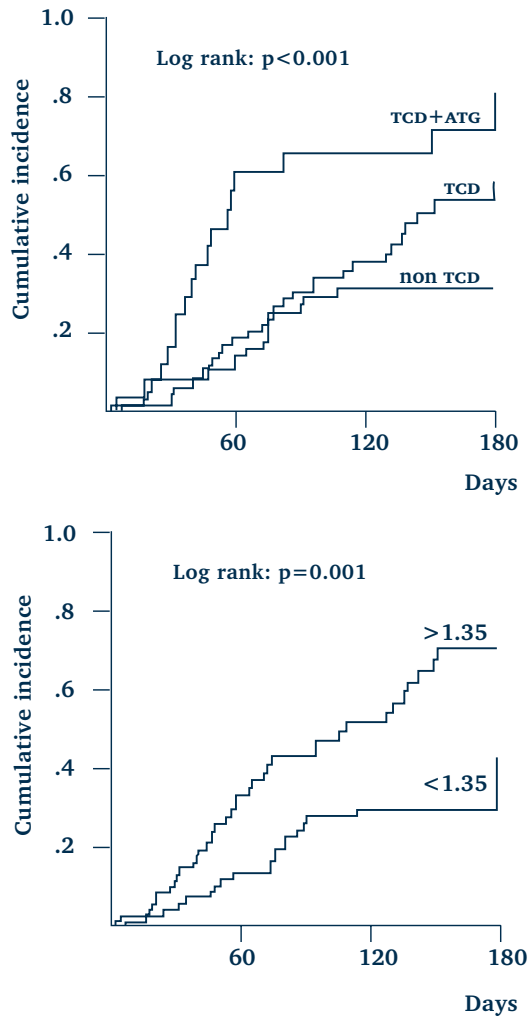
Figure 2

Monitoring of EBV viral load after a matched unrelated SCT. 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.
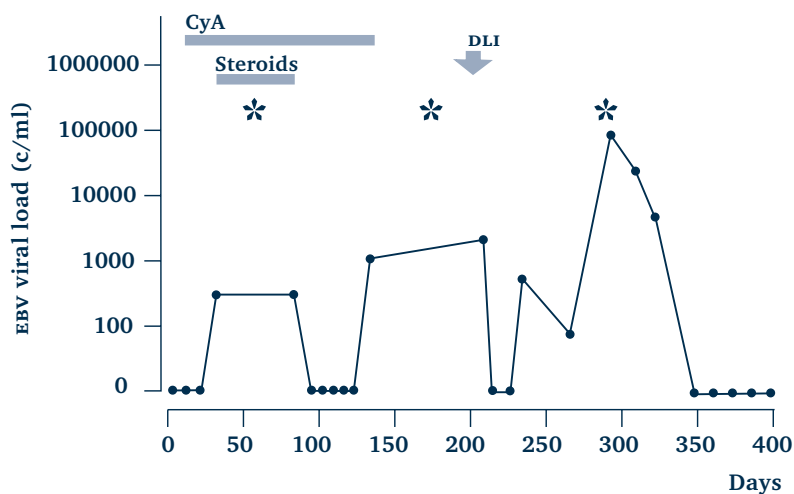
Several risk factors predicted for first reactivation in univariate analysis (Table 3), including TCD (p=0.02), use of ATG in the conditioning regimen (p<0.001), transplantation of unrelated donor graft (p=0.02), and a high CD34+ cell number of the graft (p=0.001) (Figure 1). Following multivariate analysis, only use of ATG, a high CD34+ cell count (>1.35x10^6/kg) and a high-risk disease status remained independently associated with EBV reactivation (Table 3). Numbers of CD34+ and CD3+ cells were not associated with each other.

EBV-LPD EBV-LPD was only observed following TCD SCT (Table 2, Figure 3). Five patients developed EBV-LPD after HLA identical sibling SCT and 5 after unrelated donor SCT (Table 4). Five of these patients had received ATG before unrelated donor SCT, and 9

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

Risk factor	Univariate Analysis			Multivariate analysis		
	RR	95% CI	P	RR	95% CI	P
TCD, no ATG	1.5	0.8-2.7	0.02	1.5	0.8-2.9	0.3
TCD, 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	0.02
MUD	1.8	1.1-2.9	0.02	0.9	0.3-2.9	0.8
CD34+ cell count*	2.4	1.4-4.1	0.001	2.6	1.5-4.6	0.001

* = >1.35 x 10^6/kg.

Figure 3

Incidence of EBV-LPD (n=10) after TCD allogeneic SCT combined with ATG (n=24), TCD SCT without ATG (n=61), and non-TCD SCT (n=65).
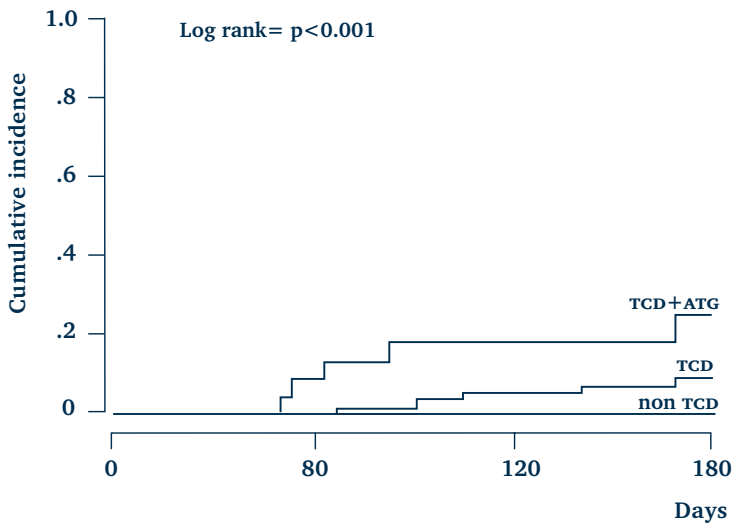
Table 4 Epstein-Barr virus-lymphoproliferative disease following TCD SCT

Patient no.	Donor	EBV-LPD diagnosis			Therapy			Outcome		
		Morpho-logy	Clonality	Viral load	SI	anti CD20	DLI	Response	Survival, d	COD
1	MRD	III	Mono	1800	+	-	+	PD	Dead	LPD
2	MUD	II	Poly	92000	+	+	+	CR	Dead	GVHD
3	MUD	II	Poly	6500	+	+	-	CR	Alive, 620	
4	MRD	III	Mono	790000	+	+	+	PD	Dead	LPD
5	MRD	III	Mono	128000	+	+	-	CR	Alive, 351	
6	MRD	II	Mono	74000	+	+	-	CR	Dead	GVHD
7	MUD	III	Mono	133000	+	+	+	PD	Dead	LPD
8	MRD	ND	Mono	7900	+	-	-	PD	Dead	LPD
9	MUD	ND	ND	310000	-	-	+	CR	Dead	GVHD
10	MUD	III	Mono	206000	-	-	-	PD	Dead	LPD

SCT indicates stem cell transplantation; MRD = matched related 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[45]); ND = not determined; Mono = monoclonal disease; Poly = polyclonal disease; SI = stop immunosuppression; anti-CD20 = monoclonal anti B cell therapy; DLI = donor lymphocyte infusion; PD = progressive disease; CR = complete remission; COD = cause of death; GVHD = graft-versus-host disease.

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 110000 c/ml (range 1800-790000). 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 SCT 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 SCT because of SAA, was diagnosed with EBV-LPD because of massive lymphadenopathy on CT scanning and a highly elevated plasma EBV-DNA level. 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 SCT 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 ($p<0.001$). Numbers of patients with a TCD SCT 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 c/ml or higher, it rose to 100% at the level of 500000 c/ml. However, only one patient with EBV-LPD reached that high number, and consequently the negative predictive value measured 89%.

Table 5   Incidence of EBV-LPD by viral load

EBV viral load (c/ml)	No. of patients with reactivations	No. of patients with EBV-LPD	Predictive value Positive (%)	Negative (%)
100	41	10	24	100
1000	26	10	39	100
10000	14	7	50	96
100000	7	5	71	94
500000	1	1	100	89

Abbreviations: see 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 SCT. 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 SCT 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 SCT. 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).

Discussion

This study demonstrates that EBV reactivation is a very frequent event after both TCD and unmanipulated SCT. In particular, recipients of stem cell grafts with high numbers of CD34+ cell counts appeared to be at risk for EBV reactivation. However, patients receiving a TCD SCT 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 SCT and after unmanipulated SCT as well. The high incidence of first EBV reactivation after TCD SCT 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 SCT 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 SCT, early EBV reactivation may occur after both modes of SCT[50,51]. However, the significantly higher risks for recurrent EBV reactivation and EBV-LPD in TCD SCT as compared with unmanipulated SCT 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 1), 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 pro-inflammatory 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 num-

ber 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 SCT and solid-organ transplantation[26-43]. No study, however, has longitudinally followed SCT 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 SCT are in line with these findings. Considering both TCD and non-TCD transplants, the negative and positive predictive values of a copy number of 1000 c/ml were, respectively, 100% and 28%. Higher predictive values were obtained when the analysis was restricted to patients receiving a TCD SCT. The positive predictive value of a high EBV-DNA level of more than 1000 c/ml and more than 10000 c/ml for patients receiving a TCD SCT 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 SCT 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 1000 c/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 SCT 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.

References

1   Shapiro RS, McClain K, Frizzera G, Gajl-Peczalska KJ, Kersey JH, Blazar BR, Arthur DC, Patton DF, Greenberg JS, Burke B, Ramsay NKC, Filipovich AH. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. Blood 1988; 71: 1234-1243.

2   Zutter MM, Martin PJ, Sale GE, Shulman HM, Fisher L, Thomas ED, Durnam DM. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988; 72: 520-529.

3   Hanto DW, Frizzera G, Gajl-Peczalska KJ, Simmons RL. Epstein-Barr virus, immunodeficiency, and B-cell lymphoprolifera-tion. Transplantation 1985; 39: 461-472.

4   Gross TG, Steinbusch M, DeFor T, Shapiro RS, McGlave P, Ramsay NKC, Wagner JE, Filipovich AH. B cell lymphoprolifera-tive disorders following hematopoetic stem cell transplantation: risk factors, treatment and outcome. Bone Marrow Transplant 1999; 23: 251-258.

5   Micallef INM, Chhanabhai M, Gascoyne RD, Shepherd JD, Fung HC, Nantel SH, Toze CL, Klingemann H-G, Sutherland HJ, Hogge DE, Nevill TJ, Le A, Barnett MJ. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. Bone Marrow Transplant 1998; 22: 981-987.

6   Curtis RE, Travis LB, Rowlings PA, Socié G, Kingma DW, Banks PM, Jaffe ES, Sale GE, Horowitz MM, Witherspoon RP, Shriner DA, Weisdorf DJ, Kolb H-J, Sullivan KM, Sobocinski KA, Gale RP, Hoover RN, Fraumeni JF, Deeg HJ. Risk of lym-phoproliferative disorders after bone marrow transplantation: a multi-institutional study. Blood 1999; 94: 2208-2216.

7   Hale G, Waldmann H for CAMPATH Users. Risks of developing Epstein-Barr virus-related lymphoproliferative disorders after T-cell-depleted marrow transplants. Blood 1998; 91: 3079-3080.

8   Walker RC, Marshall WF, Strickler JG, Wiesner RH, Velosa JA, Habermann TM, McGregor CGA, Paya CV. Pretransplantation assessment of the risk of lymphoproliferative disorder. Clin Infect Dis 1995; 20: 1346-1353.

9   Fischer A, Blanche S, Le Bidois J, Bordigoni P, Garnier JL, Niaudet P, Morinet F, Le Deist F, Fischer A. Anti-B-cell mono-clonal antibodies in the treatment of severe B-cell lymphoproliferative syndrome following bone marrow and organ trans-plantation. N Engl J Med 1991; 324: 1451-1456.

10   Faye A, Van den Abeele T, Peuchmaur M, Mathieu-Boue A, Vilmer E. Anti-CD20 monoclonal antibody for post-transplant lymphoproliferative disorders. Lancet 1998; 352: 1285.

11   Milpied N, Vasseur B, Parquet N, Garnier JL, Antoine C, Quartier P, Carret AS, Bouscary D, Faye A, Bourbigot B, Reguerre Y, Stoppa AM, Bourquard P, Hurault de Ligny B, Dubief F, Mathieu-Boue A, Leblond V. Humanized anti-CD20 monoclonal antibody (Rituximab) in post transplant B-lymphoproliferative disorder: a retrospective analysis on 32 patients. Ann Oncol 2000; 11: S113-S116.

12   Kuehnle J, Huls MH, Liu Z, Semmelmann M, Krance RA, Brenner MK, Rooney CM, Heslop HE. CD20 monoclonal antibody (Rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoetic stem-cell transplantation. Blood 2000; 95: 1502-1505.

13   Papadopoulos E, Ladanyi M, Emmanuel D, Mackinnon S, Boulad F, Carabasi MH, Castro-Malaspina H, Childs BH, Gillio AP, Small TN, Young JW, Kernan NA, O'Reilly RJ. Infusions of donor lymphocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. N Engl J Med 1994; 330: 1185-1191.

14   Heslop HE, Ng CYC, Li C, Smith CA, Loftin SK, Krance RA, Brenner MK, Rooney CM. Long term restauration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. Nat Med 1996; 2: 551-555.

15 Rooney CM, Smith CA, Ng CYC, Loftin S, Li C, Krance RA, Brenner MK, Heslop HE. Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. Lancet 1995; 345: 9-13.

16 Cavazzana-Calvo M, Bensoussan D, Jabado J, Haddad E, Yvon E, Moskwa M, Tachet des Combes A, Buisson M, Morand P, Virion JM, Le Deist F, Fischer A. Prevention of EBV-induced B-lymphoproliferative disorder by ex vivo marrow B-cell depletion in HLA-phenoidentical or non-identical T-depleted bone marrow transplantation. Br J Haematol 1998; 103: 543-551.

17 McDiarmid SV, Jordan S, Lee GS, Toyoda M, Goss JA, Vargas JH, Martin MG, Bahar R, Maxfield AL, Ament ME, Busuttil RW. Prevention and pre-emptive therapy of posttransplant lymphoproliferative disease in pediatric liver recipients. Transplantation 1998; 66: 1604-1611.

18 Green M, Kaufmann M, Wilson J, Reyes J. Comparison of intravenous ganciclovir followed by oral acyclovir with intravenous ganciclovir alone for prevention of cytomegalovirus and Epstein-Barr virus disease after liver transplantation in children. Clin Inf Dis 1997; 25: 1344-1349.

19 Kuo PC, Dafoe DC, Alfrey EJ, Sibley RK, Scandling JD. Posttransplant lymphoproliferative disorders and Epstein-Barr virus prophylaxis. Transplantation 1995; 59: 135-138.

20 Gustafsson A, Levitsky V, Zou JZ, Frisan T, Dalianis T, Ljungman P, Ringden O, Winiarski J, Ernberg I, Masucci MG. Epstein-Barr (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: prophylactic infusion of EBV-specific cytotoxic T cells. Blood 2000; 95: 807-814.

21 Davis CL, Harrison KL, McVicar JP, Forg PJ, Bronner MP, Marsh CL. Antiviral prophylaxis and the Epstein-Barr virus-related post-transplant lymphoproliferative disorder. Clin Transplant 1995; 9: 53-59.

22 Trigg ME, Finlay JL, Sondel PM. Prophylactic acyclovir in patients receiving bone marrow transplants [letter]. N Engl J Med 1985: 26: 1708-1709.

23 Darenkov IA, Marcarelli MA, Basadonna GP, Friedman AL, Lorber KM, Howe JG, Crouch J, Bia MJ, Kliger AS, Lorber MI. Reduced incidence of Epstein-Barr virus-associated posttransplant lymphoproliferative disorder using pre-emptive antiviral therapy. Transplantation 1997; 64: 848-852.

24 Green M, Reyes T, Jabbour N, Yunis E, Putnam P, Todo S, Rowe D. Use of quantitative PCR to predict onset of Epstein-Barr viral infection and post-transplant lymphoproliferative disease after intestinal transplantation in children. Transplant Pro 1996; 28: 2759-2760.

25 Niesters HGM, Van Esser J, Fries E, Wolthers KC, Cornelissen JJ, Osterhaus ADME. Development of a real-time quantitative assay for detection of Epstein-Barr virus. J Clin Microbiol 2000; 38: 712-715.

26 Van Esser JWJ, Niesters HGM, Thijsen SFT, Meijer E, Osterhaus ADME, Wolthers KC, Boucher ChAB, Gratama JW, Budel LM, Van Der Holt B, Van Loon AM, Löwenberg B, Verdonck LF, Cornelissen JJ. Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. Br J Haematol 2001; 113: 814-821.

27 Lucas KG, Burton RL, Zimmerman SE, Wang J, Cornetta KG, Robertson KA, Lee CH, Emanuel DJ. Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. Blood 1998; 91: 3654-3661.

28 Savoie A, Perpête C, Carpentier L, Joncas J, Alfieri C. Direct correlation between the load of Epstein-Barr virus-infected lymphocytes in the peripheral blood of pediatric transplant patients and risk of lymphoproliferative disease. Blood 1994; 83: 2715-2722.

29 Beck R, Westdörp I, Jahn G, Schäfer H, Kanz L, Einsele H. Detection of Epstein-Barr virus DNA in plasma from patients with lymphoproliferative disease after allogeneic bone marrow transplantation or peripheral blood stem cell transplantation [letter]. J Clin Microbiol 1999; 37: 3430-3431.

30   Limaye AP, Huang M-L, Atienza EE, Ferrenberg JM, Corey L. Detection of Epstein-Barr virus DNA in sera from transplant recipients with lymphoproliferative disorders. J Clin Microbiol 1999; 37: 1113-1116.

31   Rooney CM, Loftin SK, Holladay MS, Brenner MK, Krance RA, Heslop HE. Early identification of Epstein-Barr virus-associated post-transplantation lymphoproliferative disease. Br J Haematol 1995; 89: 98-103.

32   Hoshino Y, Kimura H, Kuzushima, Tsurumi T, Nemoto K, Kikuta A, Nishiyama Y, Kojima S, Matsuyama T, Morishima T. Early intervention in post-transplant lymphoproliferative disorders based on Epstein-Barr viral load. Bone Marrow Transplant 2000; 26: 199-201.

33   Rowe DT, Qu L, Reyes N, Jabbour N, Yunis E, Putnam P, Todo S Green M. Use of quantitative competitive PCR to measure Epstein-Barr virus genome load in the peripheral blood of pediatric transplant patients with lymphoproliferative disorders. J Clin Microbiol 1997; 35: 1612-1615.

34   Kenagy DN, Schlesinger Y, Weck K, Ritter JH, Gaudreault-Keener MM, Storch GA. Epstein-Barr virus DNA in peripheral blood leucocytes of patients with posttransplant lymphoproliferative disease. Transplantation 1995; 60: 547-554.

35   Telenti A, Marshall WF, Smith TF. Detection of Epstein-Barr virus by polymerase chain reaction. J Clin Microbiol 1990; 28: 2187-2190.

36   Yamamoto M, Kimura H, Hironaka T, Hirai K, Hasegawa S, Kuzushima K, Shibata M, Morishima T. Detection and quantification of virus DNA in plasma of patients with Epstein-Barr virus associated diseases. J Clin Microbiol 1995; 33: 1765-1768.

37   Kimura H, Morita M, Yabuta Y, Kuzushima K, Kato K, Kojima S, Matsuyama T, Morishima T. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. J Clin Microbiol 1999; 37: 132-136.

38   Riddler SA, Breinig MC, McKnight LC. Increased levels of circulating Epstein-Barr virus (EBV)-infected lymphocytes and decreased EBV nuclear antigen antibody responses are associated with the development of posttransplant lymphoproliferative disease in solid-organ transplant recipients. Blood 1994; 84: 972-984.

39   Green M, Cacciarelli TV, Mazariegos GV, Sigurdsson L, Qu L, Rowe DT, Reyes J. Serial measurement of Epstein-Barr viral load in peripheral blood in pediatric liver transplant recipients during treatment for posttransplant lymphoproliferative disease. Transplantation 1998; 66: 1641-1644.

40   Barkholt LM, Dahl H, Enbom N, Lindé. Epstein-Barr virus DNA in serum after liver transplantation - surveillance of viral activity during treatment with different immunosuppressive agents. Transpl Int 1996; 9: 439-445.

41   Lucas KG, Filo R, Heilman DK, Lee CH, Emmanuel DJ. Semiquantitative Epstein-Barr virus polymerase chain reaction analysis of peripheral blood from organ transplant patients at risk for the development of lymphoproliferative disease. Blood 1998; 92: 3977-3978. Letter.

42   Krieger NR, Martinez OM, Krams SM, Cox K, So S, Esquivel CO. Significance of detecting Epstein-Barr-specific sequences in the peripheral blood of asymptomatic pediatric liver transplant recipients. Liver Transplantation 2000; 6: 62-66.

43   Baldanti F, Grossi P, Furione M, Simoncini L, Sarasini A, Comoli P, Maccario R, Fiocchi R, Gerna G. High levels of Epstein-Barr virus DNA in blood of solid organ transplant recipients and their value in predicting posttransplant lymphoproliferative disorders. J Clin Microbiol 2000; 38: 613-619.

44   Knowles DM, Cesarman E, Chadburn A, Frizzera G, Chen J, Rose EA, Michler RE. Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders. Blood 1995; 85: 552-565.

45   Przepriorka D, Weisdorf D, Martin P, Klingemann HG, Beatty P, Hows J, Thomas ED. 1994 consensus conference on acute GVHD grading. Bone Marrow Transplant 1995; 15: 825-829.

46   Clift R, Goldman J, Gratwohl A, Horowitz M. Proposals for standardized reporting of results of bone marrow transplantation for leukaemia. Bone Marrow Transplant 1989; 4: 445-451.

47   Kaplan EL, Meier P. Nonparametric estimation for incomplete observations. J Am Stat Assoc 1958; 53: 457-481.

48   Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966; 50: 163-170.

49   Cox DR. Regression models and life-tables (with discussion). J R Stat Soc B 1972; 34: 187-220.

50   Gratama JW, Oosterveer MAP, Zwaan FE, Lepoutre J, Klein G, Ernberg I. Eradication of Epstein-Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. Proc Natl Acad Sci USA 1988; 85: 8693-8696.

51   Gratama JW, Oosterveer MAP, Lepoutre JMM, Van Rood JJ, Zwaan FE, Vossen JMJJ, Kapsenberg JG, Richel D, Klein G, Ernberg I. Serologic and molecular studies of Epstein-Barr virus infection in allogeneic marrow graft recipients. Transplantation 1990; 49: 725-730.

52   Lucas KG, Small TN, Heller G, Dupont B, O'Reilly RJ. The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. Blood 1996; 87: 2594-2603.

53   Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu Rev Immunol 1997; 15: 405-431.

54   Przepiorka D, Smith TL, Folloder J, Khouri I, Ueno NT, Mehra R, Körbling M, Huh YO, Giralt S, Gajewski J, Donato M, Cleary K, Claxton D, Braunschweig I, Van Besien K, Andersson BS, Anderlini P, Champlin R. Risk factors for acute graft-versus-host disease after allogeneic blood stem cell transplantation. Blood 1999; 94: 1465-1470.

55   Ferrara JL. Cytokine inhibitors and graft-versus-host disease. Ann N Y Acad Sci 1995; 770: 227-236.

56   Rus V, Svetic A, Nguyen P, Gause WC, Via CS. Kinetics of Th1 and Th2 cytokine production during the early course of acute and chronic murine graft-versus-host disease. Regulatory role of donor CD8+ T cells. J Immunol 1995; 155: 2396-2406.

57   Hill GR, Crawford JM, Cooke JR, Brinson YS, Pan L, Ferrara JLM. Total body irradiation and acute graft-versus-host disease: the role of gastrointestinal damage and inflammatory cytokines. Blood 1997; 90: 3204-3213.

58   Tosato G, Teruya-Feldstein J, Setsuda J, Pike SE, Jones KD, Jaffe ES. Post-transplant lymphoproliferative disease (PTLD): lymphokine production and PTLD. Springer Semin Immunopathol 1998; 20: 405-423.

59   Tosato G, Tanner J, Jones KD, Revel M, Pike SE. Identification of interleukin-6 as an autocrine growth factor for Epstein-Barr virus-immortalized B-cells. J Virol 1990; 64: 3033-3041.

60   Scala G, Quinto I, Ruocco MR, Arcucci A, Mallardo M, Caretto P, Forni G, Venuta S. Expression of an exogenous interleukin 6 gene in human Epstein Barr virus B cells confers growth advantage and in vivo tumorigenicity. J Exp Med 1990; 172: 61-68.

61   Tosato G, Jones KD, Breinig MK, McWilliams HP, McKnight JLC. Interleukin-6 production in posttransplant lymphoproliferative disease. J Clin Invest 1993; 91: 2806-2814.

62   Jones K, Rivera C, Sgadari C, Franklin J, Max EE, Bhatia K, Tosato G. Infection of human endothelial cells with Epstein-Barr virus. J Exp Med 1995; 182: 1213-1221.

63   Marshall NA, Howe JG, Formica R, Krause D, Wagner JE, Berliner N, Crouch J, Pilip I, Cooper D, Blazar BR, Seropian S, Pamer EG. Rapid reconstitution of Epstein-Barr virus-specific T lymphocytes following allogeneic stem cell transplantation. Blood 2000; 96: 2814-2821.

64   Cornelissen JJ, Van Esser JWJ, Van Der Holt B, Gratama JW, Löwenberg B, Trenschel R, Schaefer UW, Frassoni F, Bacigalupo A, Meijer E, Verdonck LF, Osterhaus ADME, Niesters HGM. Pre-emptive anti B-cell immunotherapy guided by quantitative PCR effectively reduces the incidence and mortality of Epstein-Barr virus (EBV) lymphoproliferative disease (LPD) after allogeneic T-cell depleted (TCD) stem cell transplantation (SCT). Blood 2000; 96: S507a. Abstract.