A Prospective Study on Modulation of Immunosuppression for Epstein-Barr Virus Reactivation in Pediatric Patients Who Underwent Unrelated Hematopoietic Stem-Cell Transplantation

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Background. Posttransplant lymphoproliferative disease caused by Epstein-Barr virus (EBV-PTLD) is a severe complication after allogeneic hematopoietic stem-cell transplantation (HSCT). We evaluated whether the modulation of immunosuppression (IS) guided by quantitative polymerase chain reaction for EBV (EBV-PCR) was effective as a first-line therapeutic approach for EBV reactivation.

Methods. Eighty-nine pediatric patients who received an HSCT from an unrelated donor were prospectively assessed by quantitative EBV-PCR. The EBV-PCR threshold to modulate IS was set to more than 300 genomic copies (gc)/10^5 peripheral blood mononuclear cells.

Results. EBV-PCR positivity was observed in 56 (63%) of 89 patients at a median time of 44 days after HSCT. The variables associated with EBV-PCR positivity were bone marrow stem cells ($P=0.047$) and a lower total dose of nuclear cells reinfused ($P=0.03$). Thirty-one patients (35%) had more than or equal to 300 gc. IS was withdrawn or reduced in 18 (58%) and 13 (42%) of the 31 patients, respectively. EBV viral load (EBV-VL) less than 300 gc was achieved in 30 of these 31 patients at a median of 25 days. Only 1 (1%) of the 89 patients progressed to EBV-PTLD. The patients with EBV-VL more than 300 gc had a lower incidence of acute graft versus host disease III–IV than patients with EBV-VL less than 300 gc: 13% vs. 36%, $P=0.02$. No differences in terms of chronic graft versus host disease, overall survival, event-free survival and transplant-related mortality were observed between the two groups.

Conclusions. We conclude that PCR-guided modulation of IS may play a role in early intervention for EBV-PTLD and a prospective, randomized study is needed.

Keywords: EBV reactivation, Posttransplant EBV lymphoma, Hematopoietic stem-cell transplantation, Pediatric.

(*Transplantation* 2010;89: 1533–1540)

Epstein-Barr virus (EBV)-related lymphoproliferative disease is a severe complication in profoundly immunosuppressed patients who have undergone hematopoietic stem-cell transplantation (HSCT) (*1*). In these patients, several factors have been reported to increase the risk of developing posttransplant lymphoproliferative disease caused by EBV (EBV-PTLD), including degree of donor and recipient human leukocyte antigen (HLA) matching, the use of antithymocyte or antilymphocyte serum in the conditioning regimen, T-cell depletion of the graft, the severity of graft versus host disease (GVHD), the EBV serologic mismatch between donor and recipient, and the amount of immunosuppression (IS) used for the prevention or treatment of GVHD (*2–4*) In a study of the International Bone Marrow Registry on 18,014 patients from 235 HSCT centers, Curtis et al. (*5*) found an overall incidence of EBV-PTLD of 1% but the incidence was 0.5%, 1.7%, 8%, and 22.3%, according to the presence of no risk factors, or one, two, or more than three risk factors, respectively. Progression to EBV-PTLD has been reported to be more frequent in pediatric patients than in the adult population; 2.8% vs. 1% (*6*).

The development of EBV-PTLD still represents a life-threatening event, with mortality being as high as 80% to 90% (*2*, *6*). In the patients at high risk, such as those who undergo in vitro T-depletion of the graft, the use of ex vivo generated

The authors declare no conflict of interests.

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S.C. and G.T. designed the study, analyzed the data, and wrote the article; A.P. and S.V. collected the data; S.C., E.C., M.P., M.C., and C.M. were responsible of the patient clinical management; D.A. and C.M. were responsible of EBV-PCR data; All authors read and approved the article.

Received 14 January 2010. Revision requested 16 February 2010.

Accepted 11 March 2010.

ISSN 0041-1337/10/8912-1533

DOI: 10.1097/TP.0b013e3181dd6c0a

donor-EBV-specific cytotoxic T lymphocytes has been shown to be effective in preventing and treating EBV-PTLD (*7*, *8*). This technology is costly and time consuming, and it is not indicated for patients at lower risk of EBV-PTLD. Moreover, its efficacy needs to be assessed in patients who receive pharmacologic IS (cyclosporin, tacrolimus, or steroids) as GVHD prevention or treatment because these drugs can compromise the efficacy of immunotherapy.

In the last decade, it has been demonstrated that, irrespective of baseline risk factors, the posttransplant monitoring of EBV viral load (VL) in serum, plasma, or blood by polymerase chain reaction (PCR) is effective in predicting the patients at risk of EBV-PTLD (*9*–*12*). In particular, the use of weekly testing by quantitative EBV-PCR has shown that an increase of EBV-VL (EBV reactivation) occurs in up to 50% of allogeneic HSCT patients but progression to EBV-PTLD is limited in the patients with higher EBV-VL (*13*). The introduction of rituximab, a monoclonal anti-CD20 antibody, effective in reducing EBV-VL and as therapy for EBV-PTLD, combined with strict patient monitoring by quantitative EBV-PCR, has allowed transplanters to adopt a strategy of preemptive or earlier treatment of EBV-PTLD (*14*, *15*). Although the reduction or withdrawal of IS is a well-known first-line approach to control EBV reactivation in solid organ transplantation (*16*), its use in HSCT patients has been limited by the fear of inducing or worsening acute GVHD and by the speed of EBV replication (*17*, *18*). We analyzed the incidence and risk factors of EBV reactivation in pediatric patients who received an unmanipulated unrelated graft and report the results of a PCR-guided strategy of modulation of IS for the prevention of EBV-PTLD.

PATIENTS AND METHODS

Patients

From January 1, 1998, to December 31, 2007, 89 first unmanipulated allogeneic SCTs from an unrelated bone marrow (BM) or cord blood (CB) donor were performed in the Pediatric Hematology and Oncology, Department of Pediatrics of Padova, and represented the study group. Patients who received peripheral blood stem cells from an unrelated donor or graft manipulation (i.e., $CD34^+$ positive selection) were excluded from the analysis and any second allogeneic SCT. Fifty-seven of 89 patients were included in a previous report regarding EBV reactivation after allogeneic pediatric HSCT from both a related and an unrelated donor (*13*).

Table 1 shows the main demographic and clinical characteristics of the patients. There were 59 (66%) men and 30 (34%) women, with a median age at SCT of 9 years (range 0.7–18 years).

Eighty-five (96%) patients had a diagnosis of malignancy, whereas four (4%) were suffering from either aplastic anemia (*3*) or hemophagocytic lymphohystiocytosis (*1*). According to the type and the remission status of the underlying malignant disease, 55 (65%) patients were considered to be in the standard risk group. This group composed of 35 patients with acute lymphoblastic leukemia (ALL) in first and second complete remission (CR), 16 patients with acute myeloid leukemia in first CR, and four patients with chronic myeloid leukemia in first chronic phase. The remaining 30 (35%) patients were classified as the high-risk group and included three ALL patients in third CR, nine acute myeloid leukemia patients in second CR, two chronic myeloid leukemia patients in advanced phase, seven patients with myelodysplastic syndrome, six patients with non-Hodgkin lymphoma, and three patients affected by juvenile or chronic myelomonocytic leukemia. Transplant protocols were approved by the local institutional review board, and all parents or patients (where applicable) gave their informed consent. Standard criteria were used to define acute and chronic GVHD and transplant-related toxicity (*19*–*22*).

TABLE 1. Main demographic and clinical characteristics of patients

Gender	
M	59 (66%)
F	30 (34%)
Median age at SCT (yr)	9 (range, 0.7–18)
Period	
1998–2003	53 (60%)
2004–2007	36 (40%)
Stem cell source	
Cord blood	8 (9%)
Bone marrow	81 (91%)
Underlying disease	
Malignant	85 (96%)
Nonmalignant	4 (4%)
Risk group (according to the status of disease)	
Standard risk	55 (65%)
High risk	30 (35%)
HLA matching (according to antigen or allele matching for loci A, B, and DR)	
Full matched	46 (52%)
>1 Antigen/allele mismatched	43 (48%)
EBV D/R serology status	
+/+	53 (63%)
+/−	17 (20%)
−/−	5 (6%)
−/+	9 (11%)
CMV D/R[a] serology status	
+/+	32 (36%)
+/−	11 (12%)
−/−	12 (14%)
−/+	34 (38%)
Conditioning regimen	
TBI	60 (67%)
No TBI	29 (33%)
Median TNC infused $\times 10^8$/kg (bone marrow stem cells)	4 (range, 1.6–9.7)
Median TNC infused $\times 10^7$/kg (cord blood stem cells)	12.3 (range, 4.6–15.5)
Neutrophil engraftment[b] (d)	17.5 (range, 9–59)
Platelet engraftment[c] (d)	28 (range, 12–405)

[a] Data not available for five D/R pairs.
[b] Datum calculated on 86 patients (three patients were not eligible for engraftment assessment).
[c] Datum calculated on 75 patients (14 patients not eligible for platelet engraftment).
SCT, stem-cell transplantation; M, male; F, female; HLA, human leukocyte antigen; EBV, Epstein-Barr virus; CMV, cytomegalovirus; TBI, total body irradiation; TNC, total nuclear cell; D/R, donor/recipient.

Supportive Care and Preventive Measures

All patients were nursed in high-efficiency particulate-filtered air rooms during the neutropenic phase, and standard measures were adopted to prevent infectious complications, that is, nonabsorbable antibiotics (paramomycin and nystatin) for gut decontamination; fluconazole for antifungal prophylaxis; and acyclovir for prophylaxis of herpes simplex virus and vari-

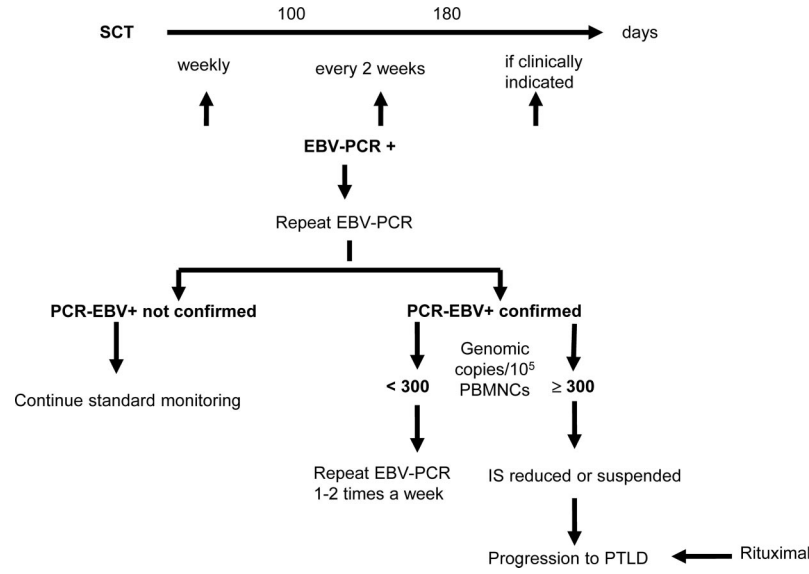

FIGURE 1. Protocol of EBV reactivation monitoring and treatment. PCR indicates polymerase chain reaction; EBV, Epstein-Barr virus. SCT, stem-cell transplantation; EBV, Epstein-Barr virus; PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cell; PTLD, posttransplant lymphoproliferative disease.

cella zoster virus and cotrimoxazole for *Pneumocystis* infections. Erythrocyte and platelet products were filtered to remove leukocytes and irradiated (25 Gy). Routine surveillance of cytomegalovirus (CMV) reactivation was based on the CMV pp65 antigen test or, from 2004, real-time PCR. EBV reactivation was monitored with real-time PCR performed before SCT, weekly from day +15 to day +100, every 2 weeks from day +101 to day +180, and thereafter, only if clinically indicated.

Transplant Data

All patients received a conditioning regimen containing rabbit antithymocyte serum (Thymoglobulin; Genzyme, Milan, Italy) at a median cumulative dose of 9 mg/kg (range 5–23 mg/kg). The donor stem-cell source was BM in 81 (91%) patients and CB in 8 (9%) patients. Based on the high-resolution typing for class I (A and B) and II (DR), 46 (52%) HSCT were performed with a complete donor/recipient HLA match, whereas in 43 (48%) HSCT, the pairs were HLA mismatched for at least one allele or antigen. Total body irradiation or a busulfan-based conditioning regimen were used, respectively, in 60 (67%) and 29 (33%) SCTs.

GVHD prophylaxis with cyclosporine and short methotrexate was used in 77 (86%) patients, whereas cyclosporine alone or cyclosporine plus other drugs were used in 6 (7%) and 6 (7%) patients, respectively. The median number of total nuclear cells (TNCs) infused was 4×10^8/kg (range 1.6–9.7) for BM and 12.3×10^7/kg (range 4.6–15.5) for CB. Neutrophil and platelet engraftment were established at the first of three consecutive days on which neutrophil and platelet counts exceeded 0.5×10^9/L and 50×10^9/L, respectively. Neutrophil engraftment occurred in 86 of 89 SCTs after a median of 17.5 days (range 9–59 dyas); platelet engraftment was recorded in 75 of 89 SCTs after a median of 28 days (range 12–405 days).

Diagnosis of Epstein-Barr Virus Reactivation and Its Management

The protocol of EBV-VL monitoring has been reported elsewhere (*13*) and is shown in Figure 1. The cut-off level of EBV-VL adopted to decide on the reduction or withdrawal of IS within 7 days was more than 300 gc/10^5 peripheral blood mononuclear cells (PBMCs). This low VL threshold was chosen to modulate the IS preemptively, before PTLD was established. Suspension of IS was permitted in patients with an acute GVHD score of 0 to I or no chronic GVHD at the time of EBV reactivation, whereas a reduction of at least 25% of the previous immunosuppressive therapy was allowed in the other cases. The rate of IS reduction was expressed as the decrease in the dose per kilogram per day for patients receiving one drug (cyclosporine or tacrolimus or steroid), or the withdrawal of one or more drugs for patients on combination therapy; for instance, the suspension of steroid in a patient previously receiving cyclosporine and steroid or cyclosporine, mycophenolate, and steroid, was rated as 50% and 30%, respectively. Low-dose steroid therapy (0.2 mg/kg in patients <40 kg of body weight or 10 mg/d in patients with >40 kg of body weight) was not modulated irrespective of the EBV-VL. Patients with increasing EBV-VLs or worsening clinical condition or overt PTLD were treated with the monoclonal anti-CD20 antibody rituximab at a weekly dose of 375 mg/m^2 (*23*).

The diagnosis of EBV-PTLD was based on clinical and radiologic assessments together with lymph node or other tissue histology. PTLD was classified according to Knowles criteria (*24*).

Real-Time Taqman Assay for Epstein-Barr Virus Quantitation

The real-time quantitative PCR assay used has been described in detail elsewhere (*13–18*). Briefly, 3 mL of peripheral blood samples were collected in standard EDTA tubes, and PBMCs were isolated by Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation. Cells were counted in a Coulter counter. DNA, extracted from 2×10^6 PBMCs by the proteinase K and phenol-chloroform method, was resuspended in 200 μL of water and stored at −20°C until use. Five microliters of the extracted DNA was amplified and quantified with the ABI PRISM 7700 Detection System (Applied Biosystems, Foster City, CA) in 50 μL of PCR mixture containing 25 μL of TaqMan Universal Master Mix (Applied Biosystem), 30 pmol of each primer, and 10 pmol of probe. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A six-point standard curve was obtained using serial dilutions (from 50 to 5×10^6 copies) of pTA-EBV in a genomic DNA solution (0.05 μg/μL) derived from an EBV-seronegative donor. This standard curve was linear throughout the above-mentioned limits. The detection threshold was 10 genomic copies (gc)/10^5 PBMCs. The sensitivity is higher than that of an "in-house" quantitative competitive PCR. The specificity was 100%, with no cross-reaction being observed with other human *Herpesviridae*. Intratest variability never exceeded 10%. The EBV genomic copy number of the clinical samples was automatically calculated by 7700 ABI PRISM SDS software and expressed as copy number/10^5 PBMCs. Each sample was processed and assayed in duplicate.

Statistical Analysis

Follow-up data were analyzed up to April 30, 2008. Patient characteristics were compared using chi-square or Fisher's exact test (as appropriate) in the case of discrete variables, and the *t* test or Mann-Whitney test in the case of continuous variables. The primary endpoint of the study was the occurrence of EBV reactivation; the secondary endpoints were the incidence of PTLD;

the grade of acute GVHD; the rate of chronic GVHD; the transplant-related mortality (TRM); the overall survival (OS), and the event-free survival (EFS). The time to EBV reactivation was calculated from the date of SCT to the date of the first of at least two consecutive positive PCR results. The time to EBV-PTLD was measured from the date of SCT to the date of histologically proven PTLD. Chronic GVHD was assessed only in patients who survived at least 100 days after SCT. TRM and OS were calculated from the date of SCT to the date of any nonrelapse death, or any death due to any cause, respectively; or to the date of last follow-up. EFS was calculated from the date of SCT to the date of the first event (death due to any cause, relapse, or second malignancy) or to the date of last follow-up. TRM, OS, and EFS were estimated by the Kaplan-Meier method, with differences between patients with or without EBV reactivation compared by log-rank test. The following variables were included in the analysis of risk factors for EBV-VL more than 300 gc/10^5 PBMCs: recipient gender (M vs. F), median age at SCT, period of SCT (1998–2003 vs. 2004–2007), underlying disease (neoplastic vs. non-neoplastic), risk group for neoplastic disease (standard risk vs. high risk), stem-cell source (BM vs. CB), recipient and donor HLA A-B-DR match (matched vs. mismatched), recipient and donor EBV serostatus, recipient and donor CMV serostatus, use of total body irradiation, median number of TNCs infused, median time to neutrophil engraftment, and median time to platelet engraftment. The variables proving significant at univariate analysis were included in a multivariate Cox regression analysis. All reported *P*-values were two-sided, and a significance level of $\alpha=0.05$ was used. Data analysis was performed using SAS software, version 9.1 (SAS Institute Inc., Cary, NC) and NCSS software (NCSS LLC, Kaysville, Utah).

RESULTS

EBV reactivation was diagnosed in 56 of 89 SCTs (63%) and EBV-VL reached the threshold limit value for IS modulation, that is, more than 300 gc/10^5 PBMCs in 31 of these 56 (55%) patients. Seventeen (55%) of the 31 patients presented with signs or symptoms consistent with EBV infection and that were not explained by other causes: fever (10), fever and laterocervical lymphadenopathy (4), fever and hepatomegaly (1), fever and hepatosplenomegaly (1), and fever, laterocervical lymphoadenopathy, and hepatomegaly (1) (Table 2). Only one patient progressed to EBV-PTLD.

The median interval time to an EBV-VL more than 300 gc/10^5 PBMCs was 49 days from SCT (range 27–87), the median EBV-VL at that time being 948 gc/10^5 PBMCs (range 300–5000). In these patients, the peak value of EBV-VL was achieved after a median of 16 days (range 0–52 days) and the median peak value was 2896 gc/10^5 PBMCs (range 300–142,725 gc/10^5).

TABLE 2. Incidence of EBV-PTLD according to the class of risk identified by different thresholds for EBV-VL

	PTLD (%)
All SCT	1/89 (1.1)
EBV-VL ≥300 g.c./10^5 PBMCs	1/31 (3.2)
EBV-VL ≥1000 g.c./10^5 PBMCs	1/24 (4.2)
EBV-VL ≥1000 g.c./10^5 PBMCs+symptoms[a]	1/16 (6.3)
EBV-VL ≥10.000 g.c./10^5 PBMCs[a]	1/8 (12.5)
EBV-VL ≥20.000 g.c./10^5 PBMCs[a]	1/5 (20.0)

[a] All these patients had symptoms consistent with EBV infection, such as unexplained fever, lymphadenopathy, and hepatosplenomegaly.

SCT, stem-cell transplant; EBV-VL, Epstein-Barr virus viral load; EBV-PTLD, Epstein-Barr virus posttransplant lymphoproliferative disease; g.c., genomic copies; PBMC, peripheral blood mononuclear cell.

Risk Factors for Epstein-Barr Virus Reactivation

Among several factors analyzed, only the source of stem cells and the number of TNCs infused were significantly associated with an EBV-VL more than or equal to 300 gc/10^5 PBMCs. None of the eight patients who received CB stem cells had a significant increase in EBV-VL more than or equal to 300 gc/10^5 PBMCs vs. 31 (38%) of 81 patients who received BM stem cells ($P=0.047$). Of note, six of the eight CB recipients were EBV seropositive before HSCT. Moreover, only 11 (24%) of 46 patients who received TNCs more than the median dose had an EBV-VL more than or equal to 300 gc/10^5 PBMCs vs. 20 (47%) of 43 patients who received TNCs less than the median dose ($P=0.03$). The results of the risk factor analysis are shown in Table 3.

Modulation of Immunosuppression

All but four of 31 patients with an EBV-VL more than or equal to 300 gc/10^5 PBMCs were receiving double or triple immunosuppressive treatment based on cyclosporine or tacrolimus together with prednisone (19 patients), and/or extracorporeal photopheresis (5 patients), and/or mycophenolate mofetil (3 patients). The other four patients were receiving only cyclosporine.

Patients had their immunosuppressive treatment either withdrawn (18 patients) or reduced (13 patients) within 10 days. In the latter group, the degree of IS reduction was 75% in two patients, 66% in one patient, 50% in nine patients, and 25% in one patient. The median time to achieve an EBV-VL less than 300 gc/10^5 PBMCs was 25 days (range 4–179 days) among 30 of the 31 patients who did not develop EBV-PTLD.

Table 4 depicts the need for immunosuppressive therapy at 3 and 6 months in comparison with the baseline at the time of starting modulation. Overall, the reduction or withdrawal of IS was not associated with a later rebound of need for IS and a progressive decrease in the number of patients receiving two or more IS drugs was observed at 3 and 6 months, as expected.

Clinical Outcome

Posttransplant Lymphoproliferative Disease Caused by Epstein-Barr Virus

EBV-PTLD occurred in a 9-year-old boy affected by ALL in second CR who was grafted with an HLA mismatched unrelated donor. From day +24 postSCT, prednisone was added to cyclosporin for acute grade II GVHD. EBV-VL exceeded 300 gc/10^5 PBMCs from day +48. At this stage, prompt reduction of IS was not possible because of unstable control of acute GVHD. On day +57, the EBV-VL peaked to 25,762 gc/10^5 PBMCs and was associated with a progressive clinical deterioration (high fever, laterocervical lymphoadenopathy, and hepatomegaly). IS was first reduced by 50% with a partial decrease of EBV-VL (15,792 gc/10^5 PBMCs) and then withdrawn (day +62), and the first dose of rituximab was given (day +63). Unfortunately, the patient died on day +67 from interstitial pneumonia and multiorgan failure.

Acute Graft Versus Host Disease

Eighty-six of 89 patients were eligible for acute GVHD assessment whereas three patients did not achieve engraft-

TABLE 3. Result of analysis of risk factors for EBV reactivation

Patient characteristics	EBV-VL <300 copies	EBV-VL ≥300 copies	*p*
Gender			
M	38 (64%)	21 (36%)	0.8
F	20 (67%)	10 (33%)	
Median age at SCT			
>8.97 yr	28 (62%)	17 (38%)	0.6
<8.97 yr	30 (68%)	14 (32%)	
Period of SCT			
1998–2003	35 (66%)	18 (34%)	0.8
2004–2007	23 (64%)	13 (36%)	
Underlying disease			
Malignant	55 (65%)	30 (35%)	1
Non malignant	3 (75%)	1 (25%)	
Risk group			
Standard risk	35 (64%)	20 (36%)	0.8
High risk	20 (67%)	10 (33%)	
Stem cell source			
Cord blood	8 (100%)	0	0.047
Bone marrow	50 (62%)	31 (38%)	
HLA matching			
Full A, B, DR matched	28 (61%)	18 (39%)	0.4
At least 1 allele or antigen mismatched	30 (70%)	13 (30%)	
Donor EBV status			
−	12 (86%)	2 (14%)	0.1
+	45 (63%)	26 (37%)	
Recipient EBV status			
−	17 (71%)	7 (29%)	0.5
+	40 (63%)	23 (37%)	
Donor CMV status[a]			
−	31 (67%)	15 (33%)	0.6
+	27 (63%)	16 (37%)	
Recipient CMV status[a]			
−	15 (65%)	8 (35%)	1
+	43 (65%)	23 (35%)	
Conditioning regimen			
TBI	36 (60%)	24 (40%)	0.1
No TBI	22 (76%)	7 (24%)	
Median TNC infused (bone marrow)			
≥4×10^8/kg	31 (74%)	11 (26%)	0.02
<4×10^8/kg	19 (49%)	20 (51%)	
Median TNC infused (cord blood)			
≥12.3×10^7/kg	4 (100%)	0 (0%)	−
<12.3×10^7/kg	4 (100%)	0 (0%)	
Dose of TNC infused[b]			
≥Median[b]	35 (76%)	11 (24%)	0.03
<Median[b]	23 (53%)	20 (47%)	
Median time to PMN engraftment[c]			
≥17.5 d	25 (58%)	18 (42%)	0.3
<17.5 d	30 (70%)	13 (30%)	
Median time to PLT engraftment[d]			
≥28 d	23 (61%)	15 (39%)	0.9
<28 d	22 (59%)	15 (41%)	

[a] Data not available for five D/R pairs.
[b] Median TNC dose was 4×10^8/kg TNC in bone marrow SCT and 12.3×10^7/kg TNC in cord blood transplant.
[c] Datum calculated for 86 patients (three were not eligible for PMN engraftment).
[d] Datum calculated for 75 patients (14 were not eligible for PLT engraftment).
SCT, stem-cell transplant; EBV-VL, Epstein-Barr virus viral load; D/R, donor/recipient; M, male; F, female; HLA, human leukocyte antigen; CMV, cytomegalovirus; TBI, total body irradiation; TNC, total nuclear cell; PMN, polymorphonuclears; PLT, platelet.

TABLE 4. Summary of the need for IS therapy at 3 and 6 mo after the start of modulation

	No. Patients (%)				
	No IS	1 IS	2 IS	3 IS	Total
Baseline (EBV-VL ≥300 g.c.$\times10^5$ PBMCs)	0	4 (13)	21 (68)	6 (19)	31 (100)
At 3 mo[a]	4 (14)	12 (41)	11 (38)	2 (7)	29 (100)
At 6 mo[b]	5 (18)	15 (56)	4 (15)	3 (11)	27 (100)

[a] Two patients deceased within 3 mo.
[b] Four patients deceased within 6 mo.
EBV-VL, Epstein-Barr virus viral load; IS, immunosuppressive treatment; g.c., genomic copies; PBMCs, peripheral mononuclear cells.

TABLE 5. Acute and chronic GVHD according to EBV reactivation

	EBV ≥300		
	No	Yes	*p*
Acute GVHD			
Grade 0–II	35 (64%)	27 (87%)	0.02
Grade III–IV	20 (36%)	4 (13%)	
Chronic GVHD			
Absent or limited	25 (56%)	16 (53%)	0.8
Extensive	20 (44%)	14 (47%)	

EBV, Epstein-Barr virus; GVHD, graft versus host disease.

ment. Table 5 shows that moderate to severe acute grade III–IV GVHD was significantly lower in patients with EBV-VL more than 300 gc/10^5 PBMCs versus patients with EBV-VL less than 300 gc/10^5 PBMCs; 13% vs. 36%, $P=0.02$.

Chronic Graft Versus Host Disease

Seventy-five of 89 patients were assessable for chronic GVHD whereas the remaining 14 patients were not evaluated

because they did not achieve a stable engraftment or died before day +100 post-SCT. Overall, 45 (60%) of 75 patients developed chronic GVHD of whom 11 had a limited and 34 had an extensive form. No significant difference was found in terms of limited or extensive form of chronic GVHD between patients with or without EBV-VL more than 300 gc/10^5 PBMCs (Table 5).

Overall Survival, Event-Free Survival, and Transplant-Related Mortality

After a median follow-up of 4.5 years (range 0.33–9.5 years), the projected 5-year and 10-year OS rates were 60% (95% confidence interval [CI] 49–70) and 53% (95% CI 35–68), respectively. Overall, 34 patients died during the observation period, 18 from relapse or progression of underlying disease or both and 16 from transplant complications, as follows: infection (7), hemorrhage (3), multiorgan failure (2), GVHD (1), respiratory insufficiency or fibrosis (2), and veno-occlusive disease (1). The 5- and 10-year projected EFS was 60% (95% CI 49–70) and 56% (95% CI 43–67), respectively. Five-year TRM was 20% (95% CI 13–31).

The occurrence of an EBV-VL more than versus less than 300 gc/10^5 PBMCs was associated with a trend, though not significant, of a better OS (71% vs. 57%, $P=0.2$) and of a lower EFS (71% vs. 55%, $P=0.1$) but had no impact on TRM (13% vs. 21%, $P=0.4$). The modulation of IS was not associated with a difference in OS, EFS, and TRM (data not shown).

DISCUSSION

The major aim of this study was to assess the incidence of EBV reactivation and its progression to EBV-PTLD using a PCR-guided strategy for the reduction or withdrawal of IS as an early preventative intervention of EBV-PTLD. The reduction of IS is recommended by recent guidelines of European Group for Blood and Marrow Transplantation as preemptive treatment of increasing EBV-VL, but its level of evidence is limited to a single-center experience and lower than that published for the use of rituximab; moreover, the choice or the priority of the three possible preemptive interventions for an increasing EBV-VL, that is, use of rituximab, reduction of IS, and administration of EBV-specific cytotoxic T lymphocytes, is left to the discretion of the clinician (*25*).

According to Curtis et al. (*5*), the patients included in this study had a medium risk of developing EBV-PTLD. In fact, all patients were grafted with an unrelated donor and underwent in vivo T-cell depletion by the use of antithymocyte globulin in the conditioning regimen; in addition, almost half of them were HLA-mismatched at least one of the A, B, or DR loci, or received a regimen with two to three immunosuppressive drugs for the control of acute GVHD more than or equal to II.

The incidence of EBV reactivation was 35% (31 of 89), which is comparable to that reported previously (*13*, *26*) and occurred early, at a median time of 49 days after SCT. In our analysis, the factors significantly associated with EBV reactivation were the type of stem-cell source and the number of TNCs infused.

The adoption of CB versus BM as a stem-cell source was demonstrated to be protective with regard to EBV reactivation despite that six of eight CB recipients were EBV seropositive before HSCT. This datum needs to be confirmed because in our study the patients having CB SCT represented only 9% of the total HSCT, but it is consistent with the low incidence of EBV-PTLD in both pediatric and adult series of CB SCT (*27*, *28*) and with the donor's origin of EBV infection in HSCT patients (*4*). This information, therefore, could play a role in the choice of the donor source, when multiple donor options are available for a patient.

The role of higher TNCs dose in preventing EBV reactivation is not easily explained. We speculate that patients with a higher TNCs inoculum may have a more rapid EBV-specific immunoreconstitution. Historically, the expected incidence of EBV-PTLD in patients who have undergone an unmanipulated unrelated allogenic HSCT, including antilymphocyte or thymocyte serum as GVHD prophylaxis regimen was around 8% without any intervention (*5*, *13*).

Conversely, our strategy to modulate IS as the first measure to control EBV reactivation was associated with a lower incidence (1%) of EBV-PTLD (1 of 89). The modulation of IS is a well-known intervention in solid organ transplantation (*17*, *27*) but is much less frequently adopted in SCT patients for the fear of worsening acute GVHD and increasing TRM (*17*). This has led to a broad use of anti-CD20 monoclonal therapy in patients with increasing EBV-VL (*14*, *15*, *17*). Despite its efficacy, the prophylactic or preemptive use of rituximab may expose the patients to overtreatment (*29*), and to other potential late effects such as a delay in B lymphocyte reconstitution (*30*, *31*), neutropenia (*32*, *33*), severe viral infections (*34*), and acute liver toxicity (*35*). Lastly, the broader use of rituximab may contribute to an increase in health care costs.

Interestingly, the incidence of EBV-PTLD in our study (1%) was similar to that found recently by Ahmad et al. (*29*) (0.8%) who used rituximab treatment preemptively guided by EBV quantitative PCR determination in 19 (16.5%) of the 115 patients studied. On the other hand, in our study, reduction or withdrawal of IS was adopted in 31 (84%) of 37 patients as a first-line measure to control the progressive increase of EBV-VL. This strategy proved to be safe because the patients did not experience either a major incidence of grade III–IV acute GVHD or a major incidence of chronic GVHD. Moreover, a trend to an overall decrease in need for IS was observed at 3 and 6 months after EBV reactivation as expected in patients without refractory or resistant GVHD. Finally, the classical transplant outcomes were not affected, the figures for OS, EFS, and TRM being comparable both between patients with or without EBV reactivation and between patients with or without IS modulation.

Considering the baseline risk factors and the fact that the incidence of EBV-PTLD was lower (1%) than that previously reported (8%–9%) (*5*, *13*), we would suggest that our approach is reliable and has a favorable risk-benefit ratio. Moreover, we found that the combination of EBV-VL and symptoms of EBV infection enabled us to define groups at increasing risk of developing PTLD. In particular, the incidence rate of EBV-PTLD approached that expected historically without any intervention when the EBV-VL was more than 1000 gc/10^5 PBMCs, especially if the patient was symptomatic, whereas it was higher than historically expected when the EBV-VL was more than 10,000 gc/10^5 PBMCs.

In these two groups, the modulation of IS was successful in all patients but one. Considering that rapid and frequent development of PTLD is described in patients with high EBV-VL, and that a high fatality rate is reported once PTLD is established, we have decided that, in future, a preemptive approach with rituximab will be justified in our patients with an EBV-VL more than 10.000 gc/10^5 PBMCs.

Despite its efficacy, the success rate of rituximab once PTLD is established is in the range of 60% to 70% (*36*, *37*). Our patient who died of EBV-PTLD initially had a temporary reduction in EBV-VL with IS modulation. Despite this, the subsequent deterioration in his clinical condition and a further increase in EBV-VL necessitated the introduction of rituximab. Unfortunately, this patient had a rapid progression to overt PTLD and died before receiving a second dose of rituximab.

To prevent this event, earlier treatment with rituximab is advocated although the complete prevention of EBV-PTLD is not assured. Ahmad et al. (*29*) found that the preemptive use of rituximab guided by quantitative EBV-PCR was effective in reducing EBV-VL in 17 (89%) of the 19 patients treated for EBV reactivation but the remaining two patients died of acute GVHD without achieving a negative EBV-VL. In the future, the study of EBV-specific T-cell recovery may represent a further useful criterion to decide the timing and duration of preemptive treatment with rituximab, as shown by Annels et al. (*38*).

In addition, adoptive immunotherapy showed a high efficacy as prophylaxis or treatment of EBV-PTLD but the time needed for the preparation of EBV-specific cytotoxic T lymphocytes and the costs have limited its use mainly to T-cell-depleted HSCT (*7*). Recently, it has been showed that it is possible to prepare within days EBV-specific T cells, using a parent as donor. This method could allow a broader use of adoptive immunotherapy in patients with EBV-PTLD not responsive to rituximab (*39*).

In conclusion, this study has suggested that the modulation of IS may represent an effective and safe first-line preemptive approach in SCT patients with increasing EBV-VL. This strategy may help reduce the overuse of rituximab in patients who have undergone in vivo T-cell depletion with antithymocyte globulin, but weekly monitoring by quantitative PCR is needed. The broader extension of these results needs confirmation by a prospective controlled trial.

ACKNOWLEDGMENTS

The authors thank Stefania Gallo for her skilful assistance in editing the manuscript; Judith Kingston, M.D., for the useful comments and the review of the English style; Maria Vittoria Gazzola, Ph.D., and Roberta Destro, Ph.D., for graft processing; and all the medical and nursing staff of Pediatric Hematology of Padova for the collaboration and dedication to our patients.

REFERENCES

1. Juvonen E, Aalto SM, Tarkkanen J, et al. High incidence of PTLD after non-T-cell-depleted allogeneic haematopoietic stem cell transplantation as a consequence of intensive immunosuppressive treatment. *Bone Marrow Transplant* 2003; 32: 97.
2. Gross TG, Steinbuch M, DeFor T, et al. B cell lymphoproliferative disorders following hematopoietic stem cell transplantation: Risk factors, treatment and outcome. *Bone Marrow Transplant* 1999; 23: 251.
3. Buyck HC, Ball S, Junagade P, et al. Prior immunosuppressive therapy with antithymocyte globulin increases the risk of EBV-related lymphoproliferative disorder following allo-SCT for acquired aplastic anaemia. *Bone Marrow Transplant* 2009; 43: 813.
4. Sundin M, Le Blanc K, Ringden O, et al. The role of HLA mismatched, splenectomy, and recipient Epstein-Barr virus seronegativity as risk factors in posttransplant lymphoproliferative disorder following allogeneic hematopoietic stem cell transplantation. *Haematologica* 2006; 91: 1059.
5. Curtis RE, Travis LB, Rowlings PA, et al. Risk of lymphoproliferative disorders after bone marrow transplantation: A multi-institutional study. *Blood* 1999; 94: 2208.
6. Ocheni S, Kroeger N, Zabelina T, et al. EBV reactivation and post transplant lymphoproliferative disorders following allogeneic SCT. *Bone Marrow Transplant* 2008; 42: 181.
7. Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus induced lymphoma in allogeneic transplant recipients. *Blood* 1998; 92: 1549.
8. Gustafsson A, Levitsky V, Zou JZ, et al. Epstein-Barr virus (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.
9. Aalto SM, Juvonen E, Tarkkanen J, et al. Epstein-Barr viral load and disease prediction in a large cohort of allogeneic stem cell transplant recipients. *Clin Infect Dis* 2007; 45: 1305.
10. Gärtner BC, Schäfer H, Marggraff K, et al. Evaluation of use of Epstein Barr viral load in patients after allogeneic stem cell transplantation to diagnose and monitor posttransplant lymphoproliferative disease. *J Clin Microbiol* 2002; 40: 351.
11. Hoshino Y, Kimura H, Tanaka N, et al. Prospective monitoring of the Epstein-Barr virus DNA by a real-time quantitative polymerase chain reaction after allogeneic stem cell transplantation. *Br J Haematol* 2001; 115: 105.
12. van Esser JW, van der Holt B, Meijer E, et al. 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. *Blood* 2001; 98: 972.
13. Cesaro S, Murrone A, Mengoli C, et al. The real-time polymerase chain reaction-guided modulation of immunosuppression enables the preemptive management of Epstein-Barr virus reactivation after allogeneic haematopoietic stem cell transplantation. *Br J Haematol* 2005; 128: 224.
14. van Esser JW, Niesters HG, van der Holt B, et al. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. *Blood* 2002; 99: 4364.
15. Gruhn B, Meerbach A, Hafer R, et al. Pre-emptive therapy with rituximab for prevention of Epstein-Barr virus-associated lymphoproliferative disease after hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2003; 31: 1023.
16. Taylor AL, Marcus R, Bradley JA. Post-transplant lymphoproliferative disorders (PTLD) after solid organ transplantation. *Crit Rev Oncol Hematol* 2005; 56: 155.
17. Heslop HE. How I treat EBV lymphoproliferation. *Blood* 2009; 114: 4002.
18. Biasolo MA, Calistri A, Cesaro S, et al. Case report: Kinetics of Epstein-Barr virus load in a bone marrow transplant patient with no sign of lymphoproliferative disease. *J Med Virol* 2003; 69: 220.
19. Bearman SI, Appelbaum FR, Back A, et al. Regimen-related toxicity and early posttransplant survival in patients undergoing marrow transplantation for lymphoma. *J Clin Oncol* 1989; 7: 1288.
20. Sullivan KM. Acute and chronic graft-versus-host disease in man. *Int J Cell Cloning* 1986; 4: 42.
21. McDonald GB, Hinds MS, Fisher LD, et al. Veno-occlusive disease of the liver and multiorgan failure after bone marrow transplantation: A cohort study of 355 patients. *Ann Intern Med* 1993; 118: 255.
22. Farag SS. Chronic graft versus host disease: Where do we go from here? *Bone Marrow Transplant* 2004; 33: 569.
23. Kuehnle I, Huls MH, Liu Z, et al. CD20 monoclonal antibody (rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoietic stem-cell transplantation. *Blood* 2000; 95: 1502.
24. Knowles DM, Cesarman E, Chadburn A, et al. Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of

posttransplantation lymphoproliferative disorders. *Blood* 1995; 85: 552.
25. Styczynski J, Reusser P, Einsele H, et al. Management of HSV, VZV and EBV infections in patients with hematological malignancies and after SCT: Guidelines from the Second European Conference on Infections in Leukemia. *Bone Marrow Transplant* 2009; 43: 757.
26. Clave E, Ablika F, Bajzik V, et al. Epstein-Barr virus reactivation in allogeneic stem cell transplantation: Relationship between viral load, EBV-specific T-cell reconstitution and rituximab therapy. *Transplantation* 2004; 77: 76.
27. Wagner JE, Barker JN, DeFor TE, et al. Transplantation of unrelated donor umbilical cord blood in 102 patients with malignant and non-malignant diseases: Influence of CD34 cell dose and HLA disparity on treatment-related mortality and survival. *Blood* 2002; 100: 1611.
28. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. *N Engl J Med* 2004; 351: 2276.
29. Ahmad I, Cau NV, Kwan J, et al. Preemptive management of Epstein-Barr virus reactivation after hematopoietic stem-cell transplantation. *Transplantation* 2009; 87: 1240.
30. Corti P, Peters C, Balduzzi A, et al. Reconstitution of lymphocyte subpopulations in children with inherited metabolic storage diseases after haematopoietic cell transplantation. *Br J Haematol* 2005; 130: 249.
31. Buser A, Stern M, Arber C, et al. Impaired B-cell reconstitution in lymphoma patients undergoing allogeneic HSCT: An effect of pretreatment with rituximab? *Bone Marrow Transplant* 2008; 42: 483.
32. Ram R, Ben-Bassat I, Shpilberg O, et al. The late adverse events of rituximab therapy—Rare but there! *Leuk Lymphoma* 2009; 50: 1083.
33. Aksoy S, Dizdar O, Hayran M, et al. Infectious complications of rituximab in patients with lymphoma during maintenance therapy: A systematic review and meta-analysis. *Leuk Lymphoma* 2009; 50: 357.
34. Freim Wahl SG, Folvik MR, Torp SH. Progressive multifocal leukoencephalopathy in a lymphoma patient with complete remission after treatment with cytostatics and rituximab: Case report and review of the literature. *Clin Neuropathol* 2007; 26: 68.
35. Qazilbash MH, Qu Z, Hosing C, et al. Rituximab-induced acute liver failure after an allogeneic transplantation for chronic myeloid leukemia. *Am J Hematol* 2005; 80: 43.
36. Faye A, Quartier P, Reguerre Y, et al. Chimeric anti-CD20 monoclonal antibody (rituximab) in post-transplant B-lymphoproliferative disorder following stem cell transplantation. *Br J Haematol* 2001; 115: 112.
37. Svoboda J, Kotloff R, Tsai DE. Management of patients with post-transplant lymphoproliferative disorder: The role of rituximab. *Transpl Int* 2006; 19: 259.
38. Annels NE, Kalpoe JS, Bredius RG, et al. Management of Epstein-Barr virus (EBV) reactivation after allogeneic stem cell transplantation by simultaneous analysis of EBV DNA load and EBV-specific T cell reconstitution. *Clin Infect Dis* 2006; 42: 1743.
39. Uhlin M, Okas M, Gertow J, et al. A novel haplo-identical adoptive CTL therapy as a treatment for EBV-associated lymphoma after stem cell transplantation. *Cancer Immunol Immunother* 2010; 59: 473.

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