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Epstein-Barr Virus-Associated Post-Transplantation Lymphoproliferative Disease in Patients Who Received Anti-CD20 after Hematopoietic Stem Cell Transplantation

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A B S T R A C T

Post-transplantation lymphoproliferative disease (PTLD) is a serious complication associated with Epstein-Barr virus (EBV) infection after hematopoietic stem cell transplantation (HSCT). Although anti-CD-20 therapy is now used as a preemptive strategy for EBV reactivation, PTLD still occurs in some patients. Here we analyzed outcomes and risk factors associated with PTLD transformation in 208 HSCT recipients who were diagnosed with EBV-DNAemia and received at least 1 course of rituximab. The median patient age was 42.52 years (range, 8.35 to 74.77 years), and the median duration of follow-up was 47.33 months (range, 3.18 to 126.20 months). The 2-year overall survival of the entire cohort was 62.8 (95% confidence interval [CI], 56.4 to 69.9), and the 2-year cumulative incidence function of PTLD was 6.3% (95% CI, 3.5% to 10.1%), for a median follow-up of patients diagnosed with PTLD of 37.85 months. Multivariable analysis identified 4 risk factors associated with PTLD: HSCT from an unrelated donor, recipient HLA-DRB1*11:01, fever at diagnosis of EBV infection, and donor-recipient sex-mismatched HSCT. The presence of more than 2 of these risk factors was associated with an increased risk of developing PTLD. This retrospective study identifies risk factors associated with PTLD in EBV-infected patients after HSCT and defines patient subgroups that may benefit from intensified preemptive strategies.

Key point 1

Despite preemptive therapy with anti-CD20 for EBV reactivation, PTLD still occurs. However, data on the incidence risk and outcomes of PTLD in the preemptive era are scarce.

Key point 2

Despite anti-CD20 therapy, the 2-year cumulative incidence of PTLD was 6.3%. Multivariable analysis identified 4 risk factors associated with PTLD: HSCT from an unrelated donor, recipient HLA-DRB1*11:01, fever at diagnosis of EBV infection, and donor-recipient sex-mismatched HSCT.

INTRODUCTION

Despite improvements in supportive care strategies for infectious complications in allogeneic hematopoietic stem cell transplantation (HSCT), Epstein-Barr virus (EBV)-related disease remains a persistent issue associated with significant morbidity and mortality [1-3]. Post-transplantation lymphoproliferative disease (PTLD) is the most feared complication associated with EBV

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infection in patients undergoing HSCT. The overall incidence of EBV-related PTLD varies among transplantation centers, ranging from 1% to 14%, depending on donor type, conditioning regimen intensity, use of T cell depletion, and stem cell source [4]. Before the era of preemptive strategies based on anti-CD20 targeting, the incidence of early and late EBV-related PTLD was very high [5].

The European Conference on Infections in Leukemia recently published guidelines for the prevention and treatment of EBV-related diseases occurring after HSCT, including PTLD [3]. Assessment of pre-transplantation EBV serology status and prospective monitoring of EBV-DNAemia by quantitative polymerase chain reaction (PCR) are highly recommended after high-risk allogeneic HSCT [3]. Significant (or high-load) DNAemia is defined as EBV >10,000 copies/mL in plasma or whole blood [3]. Persistent DNAemia may be associated with EBV-driven manifestations, such as EBV-driven organ disease or EBV-PTLD. Accepted histopathological criteria for EBV-PTLD are the presence of lymphoid infiltrates, with or without disruption of underlying cellular architecture and evidence of EBV infection in many cells by in situ hybridization [6]. The World Health Organization (WHO) classification recognizes 4 types of morphological lesions: polyclonal early lesions (nondestructive lymphoid hyperplasia), polymorphic, monomorphic (B cell or T/natural killer [NK] cell), and classical Hodgkin lymphoma-type PTLD [7].

Three main therapeutic approaches have been proposed for the prevention and treatment of EBV-PTLD: withdrawal of immunosuppression [8], administration of rituximab [4,9-12], and administration of EBV-specific cytotoxic T lymphocytes (CTLs) [13]. Rituximab therapy seems to be the most effective strategy in the preemptive (ie, decreasing the risk of PTLD transformation) and treatment settings. CTLs are not accessible in most transplantation centers, and the reduction of immunosuppression alone has shown limited efficacy and is not always possible in the context of graft-versus-host disease (GVHD). Therefore, prompt tapering of immunosuppression when possible in combination with preemptive rituximab administration has been the mainstay of treatment in patients with EBV infection [4].

New approaches to monitoring and preemptive treatment of EBV-DNAemia have reduced the incidence of EBV-PTLD and improved the outcomes of affected patients [8,9,14]. However, the mortality rate associated with this complication remains largely unsatisfactory [4], and risk factors for EBV-PTLD transformation in patients already diagnosed with EBV-DNAemia and receiving preemptive rituximab remain poorly defined.

In this single-center retrospective study, we analyzed the incidence and outcomes of EBV infection and risk factors associated with PTLD transformation despite the use of preemptive rituximab in recipients of allogeneic HSCT.

METHODS

Patient Population

We retrospectively analyzed all consecutive patients who received at least 1 course of preemptive rituximab treatment for significant EBV-DNAemia occurring after HSCT in our center between 2010 and 2017. The review of medical records was approved by the institutional Ethics Committee in agreement with the Helsinki Declaration of 1975, revised in 2008 [15]. Data collection was based on the European Society of Blood and Marrow Transplantation registry through the Project Manager Internet Server (ProMISe) central data processing system. All patients had been regularly followed until April 2018 (or death). Pertinent clinical data, including age, sex, disease diagnosis, comorbidities, type of transplantation, donor-recipient HLA match, conditioning regimen, GVHD prophylaxis, acute and chronic GVHD, and other clinical complications, were collected. Additional biological and clinical data, such as EBV DNA viral load in whole blood, inflammatory markers, symptoms at onset of EBV infection and PTLD transformation, histopathological characteristics, phenotypic characterization of lymphocyte subsets at the onset of EBV infection, number of courses of rituximab, and further treatment of PTLD, were also collected retrospectively. Objective clinical data, including ancillary testing, laboratory results, medical complications, and medication profiles, were abstracted through a standardized chart review after each visit.

Transplant Procedures

HLA-typing using high-resolution methods were used to select donors for allogeneic HSCT. Donor types included matched related, matched unrelated, mismatched unrelated, and haploidentical. Stem cell sources were peripheral blood, bone marrow, and cord blood. Myeloablative conditioning regimens included busulfan (3.2 mg/kg/day for 4 days) combined with cyclophosphamide (60 mg/kg/day for 2 days) [16] or with fludarabine (30 mg/m2/day for 5 days) or total body irradiation of 1200 cGy combined with cyclophosphamide (60 mg/kg/day for 2 days) [17] or busulfan (3.2 mg/kg/day for 2-3 days) in combination with thiotepa (5 mg/kg/day for 2 days) and fludarabine (40 mg/m^2/day for 4 days) [18]. Reduced-intensity conditioning regimens included fludarabine-based protocols, according to the disease and age of the recipient. Standard protocols for immunosuppression, including cyclosporine and short-term methotrexate or cyclosporine with mycophenolate mofetil were used for GVHD prophylaxis. In addition, recipients of unrelated donor transplants received rabbit antithymocyte globulin (ATG) or antilymphocyte globulin [19].

EBV Monitoring and EBV-PTLD Diagnosis

Each patient's EBV viral load was routinely monitored twice weekly by real-time quantitative PCR, as described previously [20] until infection or EBV disease resolution and according to the patient's risk profile [3]. Of note, a change in the method of EBV load determination was instituted during the study period to standardize molecular quantification of EBV to requirements of the WHO Expert Committee on Biological Standardization (Abbott RealTime EBV assay; Abbott Molecular, Des Plaines, IL). A conversion factor was applied to convert results expressed in copies/mL to international units (IU)/mL [20]. EBV infection (or reactivation) was defined as any positive detection of EBV DNA in blood. Significant EBV-DNAemia was characterized by an increase in EBV load above the threshold of 4.5 $\log_{10}$ copies/mL or 3.8 $\log_{10}$ IU/mL. For standardization, after conversion, all results were expressed in $\log_{10}$ IU/mL.

To rule out the possibility that patients with EBV reactivation had not already developed PTLD, a careful clinical examination, laboratory assessment (including a complete hematologic and biochemical profile, serum immunoglobulin quantification, and lymphocyte immunophenotyping), and computed tomography (CT) scan were performed.

Together with significant EBV-DNAemia, proven EBV-PTLD diagnosis was based on radiologic and histological findings according to the WHO's 2018 revised classification of lymphoid neoplasms [7]. In all cases, EBV-encoded RNA in situ hybridization was performed to confirm the presence of EBV infection. Diagnosis of probable EBV-PTLD was established when the EBV-DNAemia was associated with lymphadenopathy, hepatosplenomegaly, or any other organ involvement on CT findings, without tissue biopsy and in the absence of other documented causes.

Preemptive Therapy and Treatment of PTLD

Rituximab was administered preemptively to all patients with significant EBV-DNAemia at a dose of 375 mg/m^2 weekly until the EBV load fell below 2.5 $\log_{10}$ IU/mL.

Patients with rituximab-refractory EBV-DNAemia who developed PTLD received either intensified rituximab treatment (1000 mg total dose weekly until resolution of EBV-DNAemia and clinical signs) associated with a reduction of immunosuppression whenever feasible, or further systemic treatment with such agents as cyclophosphamide, etoposide, doxorubicin, brentuximab, or donor cellular products (eg, donor lymphocyte infusion [DLI], CD34$^+$-selected stem cell boost infusion).

Statistical Analysis

Data are presented as count and percentage or as median and range or interquartile range (IQR). Kaplan-Meier estimates [21] were used to determine the unadjusted probability of overall survival (OS) through 2 years post-transplantation, with differences between the curves determined using the log-rank test for univariate comparisons. OS was defined as the interval from transplantation to death from any cause. In the case of a nonevent, observations were censored at the time of last follow-up. The CIFs of acute and chronic GVHD, relapse, and PTLD were calculated in a competing-risk setting, with death considered the competing event [22,23].

Predictive analyses for PTLD were based on the proportional hazards model for subdistributions of competing risk. A set of independent predictors was constructed with a stepwise backward procedure. All predictors with a P value <.10 were considered and sequentially removed if the P value in the multivariable model was >.05.

Lymphocyte subgroups were analyzed as continuous and categorized variables, with cutoffs at the 50th percentile of the sample distribution (ie, 2 equal-sized groups). Fisher's exact test was used for categorical variables, and the Kruskal-Wallis test was used for continuous variables. Groups were compared using the nonparametric Mann-Whitney *U*test or Student's t test. All tests were 2–sided, and P < .05 was considered top indicate statistical

Table 1
Patient Characteristics

Characteristic	All Patients (N = 208)	PTLD$^+$ (N = 13)	PTLD$^-$ (N = 195)
Age, yr, median (range)	42.52 (8.35-74.77)	40.51 (17.49-74.77)	42.52 (8.35-69.13)
Follow-up, mo, median (range)	47.33(3.18-126.20)	37.85 (24.85-52.59)	47.80 (3.18-126.20)
Sex, n (%)			
Male	123 (58)	5 (38)	118 (60)
Female	84 (42)	8 (62)	76 (40)
Diagnosis, n (%)			
AML	61 (30)	1 (8)	60 (31)
ALL	43 (21)	4 (31)	39 (20)
MPN; CML	32 (15)	2 (14)	30 (15)
MDS	20 (10)	1 (8)	19 (10)
Lymphoma and CLL	19 (9)	1 (8)	18 (9)
BMF	24 (11)	4 (31)	20 (10)
Hemoglobinopathy	5 (2)		5 (3)
MM and plasma cell disorders, n (%)	4 (2)		4 (2)
Conditioning regimen, n (%)			
MAC with TBI	46 (22)	5 (38)	41 (21)
MAC without TBI	44 (21)	1 (8)	43 (22)
RIC	118 (57)	7 (54)	111 (57)
Busulfan-containing regimen	102 (49)	3 (23)	99 (50)
Fludarabine-containing regimen	118 (57)	7 (54)	111 (57)
Cyclophosphamide-containing regimen	113 (54)	10 (77)	103 (53)
Splenectomy before conditioning regimen	7 (13)	0	7 (14)
T cell-depleting therapy, n (%)			
Thymoglobulin	124 (60)	10 (77)	114 (59)
Lymphoglobulin	43 (20)	3 (23)	40 (20)
Alemtuzumab	3 (1)	0	3 (2)
GVHD prophylaxis, n (%)			
CSA-MTX	90 (43)	6 (46)	84 (43)
CSA-MMF	112 (54)	7 (54)	105 (54)
PTCy and CSA-MMF	6 (3)		6 (3)
Type of donor, n (%)			
MRD	48 (23)	1 (8)	47 (24)
MUD	59 (28)	5 (38)	54 (27)
MMUD	95 (46)	7 (54)	88 (45)
Haploidentical	6 (3)		6 (3)
Stem cell source, n (%)			
PB	169 (81)	9 (70)	160 (82)
BM	35 (17)	2 (15)	33 (17)
CB	4 (2)	2 (15)	2 (1)
CMV serologic status, n (%)			
D+/R+	67 (33)	4 (31)	63 (33)
D-/R -	58 (28)	5 (39)	53 (27)
D+/R -	32 (15)	2 (15)	30 (15)
D-/R+	51 (24)	2 (15)	49 (25)
EBV serologic status, n (%)			
D+/R+	175 (84)	10 (77)	165 (84)
D-/R -	4 (2)		4 (2)
D+/R -	6 (3)	1 (8)	5 (3)
D-/R+	23 (11)	2 (15)	21 (11)
Donor/recipient sex mismatch, n (%)			
Male/male	88 (42)	2 (15)	86 (45)
Female/female	28 (13)	2 (15)	26 (13)
Male/female	57 (28)	6 (46)	51 (26)
Female/male	35 (17)	3 (24)	32 (16)
ABO matching, n (%)			
ABO matched	123 (58)	5 (33)	118 (59)
ABO major mismatch	36 (17)	3 (25)	33 (17)

(*continued*)

Table 1 (*Continued*)

Characteristic	All Patients (N = 208)	PTLD$^+$ (N = 13)	PTLD$^-$ (N = 195)
ABO minor mismatch	36 (18)	5 (42)	31 (17)
ABO mixed incompatibility	13 (7)		13 (7)

ALL indicates acute lymphoblastic leukemia; AML, acute myelogenous leukemia; MPN, myeloproliferative neoplasm; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; CLL, chronic lymphocytic leukemia; BMF, bone marrow failure; MAC, myeloablative conditioning; TBI, total body irradiation; RIC, reduced-intensity conditioning; CSA, cyclosporine; MTX, methotrexate; MMF, mycophenolate mofetil; PTCy, post-transplantation cyclophosphamide; PB, peripheral blood; BM, bone marrow; CB, cord blood; MUD, matched unrelated donor; MMUD, mismatched unrelated donor; MM, multiple myeloma; CMV, cytomegalovirus; D, donor; R, recipient.

significance. Statistical analyses were performed using R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline Characteristics

Among 1024 recipients of allogeneic HSCT performed at Saint Louis Hospital, Paris between 2010 and 2017, 208 patients presented with significant EBV-DNAemia >3.8 $\log_{10}$ IU/mL in plasma. Patient characteristics and features of EBV infection are described in Tables 1 and 2, respectively.

The median age of the 208 patients with EBV reactivation was 42.52 years (range, 8.35 to 74.77), and the median follow-up for surviving patients was 47.33 months (range, 3.18 to 126.20 months). Diagnosis of EBV infection was made at a median of 35 days (range, 6 to 713 days) after HSCT. The leading indication for HSCT was acute leukemia (51%), whereas only 9% of the patients underwent HSCT for lymphoma. Conditioning regimens were more frequently of reduced intensity (57%) and fludarabine-based (57%). Most patients received anti-T cell serotherapy (80%) for GVHD prophylaxis. The majority of patients were transplanted with a donor matched for sex, ABO group, and cytomegalovirus and EBV serology. The main stem cell source was peripheral blood (81%), whereas bone marrow and cord blood were used in 17% and 2% of cases, respectively.

EBV-PTLD Cumulative Incidence and Subgroup Description

Thirteen patients developed an EBV-related PTLD despite receiving preemptive anti-B cell therapy with rituximab. The calculated cumulative incidence was 6.3% (95% confidence interval [CI], 3.5% to 10.1%) (Figure 1). Table 4 summarizes characteristics of the patients diagnosed with PTLD. The median age of the patients with PTLD was 40.51 years (range, 17.4 to 74.7 years), and the median duration of follow-up was 37.85 months (range, 24.85 to 52.59 months).

Patients received transplants mainly from an unrelated donor (7 from a mismatched unrelated donor and 5 from a matched

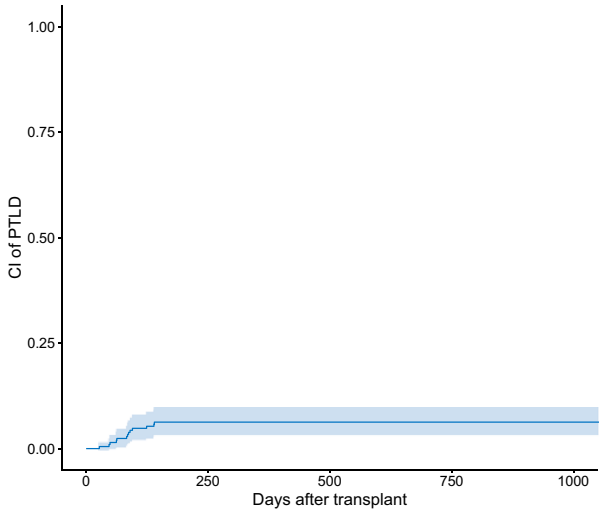

Figure 1. Cumulative incidence of PTLD in transplant recipients with significant EBV-DNAemia receiving rituximab preemptive therapy.

Table 2
EBV Infection Characteristics

Characteristic	All Patients	PTLD$^+$	PTLD$^-$
Time between transplantation and EBV infection, d, median (range)	35 (6-713)	35 (18-118)	36 (6-713)
EBV maximal viral load, log UI/mL, median (IQR)	4.68 (4.31-5.144)	5.55 (4.98-6.05)	4.64 (4.30-5.08)
EBV viral load before rituximab treatment, log UI/mL, median (IQR)	4.55 (4.19-5.05)	5.37 (4.95-6.05)	4.53 (4.17-5.02)
Time between EBV infection and PTLD transformation, d, median (range)		34 (9-105)	
Time between transplantation and PTLD transformation, d, median (range)		85 (27-140)	
Time between EBV infection and rituximab initiation, d, median (range)	14 (0-387)	14 (3-32)	14 (0-133)
Time between rituximab initiating and PTLD transformation, d, median (range)		19 (2-73)	
EBV viral load after first course of rituximab, median log (IQR)	2.8 (0-4.17)	4.66 (3.95-5.10)	2.68 (0-2.42)
Fever, n (%)*	38 (18)	4 (30)	34 (17)
Adenopathy, n (%)*	10 (5)	5 (45)	5 (2)
C-reactive protein, mg/L, median (IQR); normal level <2 mg/L*	3.0 (2.0-12.0)	7 (2-22.5)	3 (2-10)
Lactate dehydrogenase, U/L, median (IQR); normal range 240-480 U/L*	604 (399-702)	662 (452-819)	525 (400-688)
Ferritin, μg/L, median (IQR); normal range 13-150 μg/L*	2406 (1723-4953)	3092 (2390-5706)	2379 (1684-4930)
Hemoglobin, g/dL, median (IQR); normal range 12-16 g/dL*	10 (9.2-11.1)	9.2 (8.7-10.3)	10 (9.3-11.1)
Gammaglobulin, g/dL, median (IQR)*	5.40 (3.75-7.9)	5.40 (4.40-5.70)	5.40 (3.75-7.55)
Presence of monoclonal gamma protein, n (%)*	32 (15)	3 (23)	29 (14)
Lymphocytopenia (<.5 × 10^9/L), n (%)*	88 (50)	7 (63)	81 (44)
Neutropenia (<1.0 × 10^9/L), n (%)*	17 (9)	1 (9)	16 (9)
Thrombocytopenia (<100 × 10^9/L), n (%)*	115 (61)	8 (72)	107 (61)
Number of rituximab injections, median (range)	2 (1-11)	5 (2-11)	2 (1-6)
Negative EBV viral load after 1 mo of treatment, n (%)	184 (88)	5 (38)	179 (91)

* At the onset of EBV infection.

unrelated donor), and only 1 patient received a graft from an HLA-identical sibling donor. Chimerism analysis showed donor reconstitution in 10 patients, mixed chimerism in 2 patients, and autologous reconstitution in 1 patient. Seven patients had acute GVHD, and 9 had received corticosteroid treatment at PTLD onset. Extranodal involvement was seen in 10 patients, with central nervous system involvement in 2 patients. At PTLD presentation, 4 patients (30%) had fever >38.5 °C, and 5 patients (38%) had lymphadenopathy. Elevated serum lactate dehydrogenase level ≥1.5 times the upper limit of normal (480 U/L), elevated serum ferritin level (>3000 mg/L), thrombocytopenia ($<100 \times 10^9$ platelets/L), anemia (hemoglobin <10 g/dL), and lymphopenia ($<0.5 \times 10^9$/L), but not neutropenia, were frequent at disease onset. The median number of rituximab injections administered was 5 (range, 2 to 11). EBV-DNAemia became negative within 1 month in 5 out of 13 patients. The diagnosis of PTLD was histologically proven in 10 patients. Biopsy analysis showed monomorphic PTLD in 6 patients and a polymorphic subtype in 4 patients. Immunohistochemistry analysis revealed CD20 positivity in all patients with available histological samples. In 3 patients, the diagnosis was highly probable based on CT scan findings and tumor cytology. Patients developed PTLD at a median of 85 days (range, 27 to 140 days) after transplantation and 34 days (range, 9 to 105 days) after EBV reactivation.

Rituximab was initiated at a median of 14 days (range, 3 to 32 days) after the onset of EBV infection. The median number of rituximab courses administered before PTLD transformation was 2 (range, 1 to 4). In all patients who developed PTLD, immunosuppressive drugs were drastically withdrawn. In 3 patients, intensified rituximab treatment, together with reduced immunosuppression, allowed achievement of a complete response (CR) of PTLD. Five patients received other treatments, including etoposide, cyclophosphamide, doxorubicin, intrathecal methotrexate, and brentuximab. In the other 5 patients, no further treatment was possible because of poor performance status. DLI was performed in 1 patient, and 1 patient received a CD34-selected stem cell boost in the context of poor graft function.

The overall response rate of all treatments for PTLD was 46%; 6 patients experienced a complete remission, but 2 of them died due to other complications (1 with interstitial pneumonia and 1 with invasive aspergillosis). Overall, 9 patients with a diagnosis of PTLD died, for a mortality rate of 69%.

OS and Cumulative Incidence of Relapse, Acute GVHD, and Chronic GVHD

The 2-year OS of the entire cohort was 62.8% (95% CI, 56.4% to 69.9%) (Figure 2A**).**The CIF of relapse from the original disease was 12.7% (95% CI, 8.6% to 17.7%) at 1 year and 16.5 (95% CI,

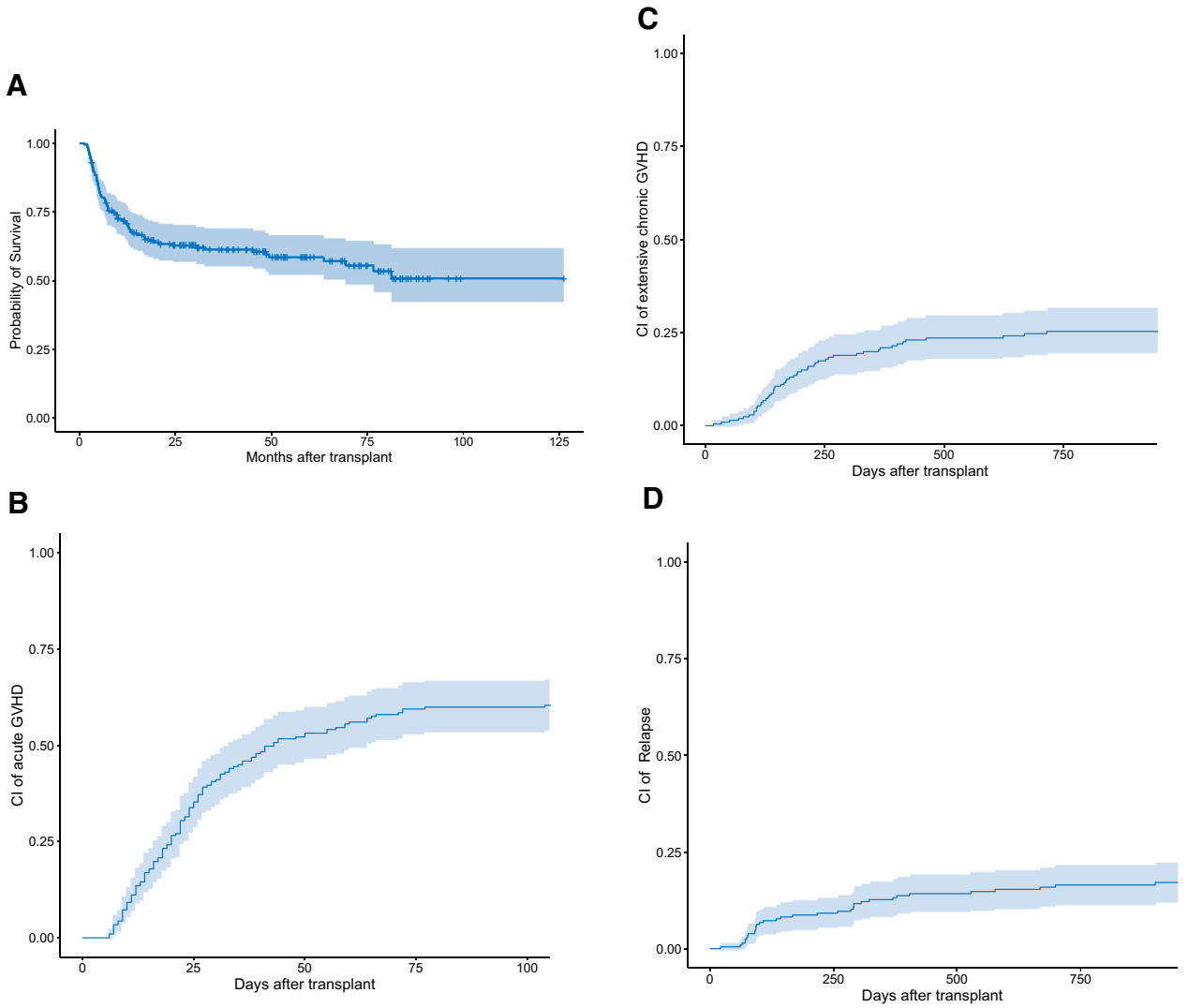

Figure 2. Outcomes of patients receiving at least 1 course of rituximab for an significant EBV-DNAemia. (A) OS. (B) Cumulative incidence of relapse. (C) Cumulative incidence of grade III-IV acute GVHD. (D) Cumulative incidence of extensive chronic GVHD.

Table 3
Univariable Analysis of Factors Influencing 2-Year OS

Factor	HR (95% CI)	*P* Value
Overall		
Age ≤42 yr		
Age >42 yr	2.69 (1.69-4.28)	<**.001**
Diagnosis		
Acute leukemia		
MDS and MPN	2.37 (1.47-3.81)	<**.001**
Lymphoma and CLL	1.06 (.47-2.39)	.878
Nonmalignant disease	.68 (.30-1.52)	.351
MM and plasma cell disorders	.70 (.09-5.15)	.731
Conditioning regimen		
MAC with TBI		
MAC without TBI	.49 (.27-.90)	**.022**
RIC	.51 (.28-.92)	**.026**
T cell-depleting therapy		
Thymoglobulin		
Lymphoglobulin	.88 (.53-1.46)	.629
Non T-depleted	.40 (.18-.86)	**.019**
Type of donor		
MRD		
MUD	.98 (.54-1.78)	.956
MMUD	1.04 (.60-1.80)	.861
Haploidentical	.93 (.21-4.01)	.934
Stem cell source		
PB		
BM	.55 (.25-1.03)	.062
CB	.29 (.04-2.15)	.994
CMV serologic status		
D+/R+		
D-/R -	.70 (.39-1.25)	.234
D+/R -	.78 (.40-1.54)	.489
D-/R+	1.13 (.65-1.94)	.651
EBV serologic status		
D+/R+		
Other EBV serologic status	.33 (.14-.76)	**.009**
Donor/recipient sex mismatch		
M/M		
F/F	.65 (.32-1.29)	.225
M/F	.70 (.41-1.17)	.179
F/M	.44 (.21-.90)	**.025**
ABO matching		
ABO matched		
ABO major mismatch	1.46 (.83-2.56)	.188
ABO minor mismatch	1.59 (.90-2.80)	.104
ABO mixed incompatibility	1.48 (.62-3.49)	.368
Fever at EBV infection onset		
No		
Yes	1.90 (1.12-3.23)	**.017**
EBV-DNAemia clearance within 1 month		
Yes		
No	2.78 (1.58-4.87)	<**.001**
Reduction of EBV-DNAemia		
>1 log UI/mL		
<1 log UI/mL	1.923 (1.20-3.07)	**.006**
Rituximab treatment		
<2 courses		
>3 courses	1.28 (.76-2.16)	.344
Presence of acute grade III-IV GVHD		
No		
Yes	2.42 (1.56-3.77)	<**.001**
Presence of extensive chronic GVHD		
No		
Yes	.54 (.31-.94)	**.032**

Significant *P* values are in bold type.

11.7% to 22.0%) at 2 years. The CIF of acute grade II-IV GVHD at day +100 was 59.9 (95% CI, 52.9% to 66.2%), and that of grade III-IV acute GVHD was 25.1% (95% CI, 19.4% to 31.2%). The CIF of chronic GVHD was 34.7 (95% CI, 28.2% to 41.2%) at 1 year and 41.4 (95% CI, 34.5% to 48.2%) at 2 years (Figure 2B-D).

Factors Affecting OS in EBV-Infected HSCT Recipients

Univariable analysis identified the following risk factors affecting the OS of transplant recipients diagnosed with EBV infection: age >42 years (hazard ratio [HR], 2.69; 95% CI, 1.69 to 4.28; *P*< .001), diagnosis of myelodysplastic syndrome or myeloproliferative neoplasm (HR, 2.37; 95% CI, 1.47 to 3.81; *P*< .001), and the presence of grade III-IV acute GVHD (HR, 2.42; 95% CI, 1.56 to 3.77; *P*< .001). The absence of T cell-depleted therapy, receipt of a reduced-intensity or myeloablative conditioning regimen without total body irradiation, EBV viral load clearance within 1 month, and EBV serologic status other than donor-positive/recipient-positive represented protective factors for survival. In a multivariable model, only age, EBV serologic status, and presence of grade III-IV acute GVHD influenced OS. EBV clearance was withdrawn from the model because of a violation of assumption of proportional hazard (Table 3 and Figure 3).

Risk Factor Analysis of PTLD Transformation

The univariable analyses identified 3 risk factors associated with PTLD transformation despite preemptive rituximab administration: donor/recipient sex mismatch (HR, 1.559; *P*= .039), presence of the HLA-DRB1*1:01 allele (HR, 4.850; *P*= .004), and EBV viral load clearance after 1 course of rituximab (HR, 6.556; *P*= .001) (Table 4).

In the multivariable model, EBV clearance after 1 rituximab course was removed because of a violation of assumption of proportionality basing on the scaled Schoenfeld residuals [24]. Significant risk factors associated with PTLD incidence in multivariable analysis were HSCT from an unrelated donor (HR, 2.11; 95% CI, 1.00 to 4.45; *P*= .05), recipient HLA-DRB1*11:01 allele (HR, 4.85; 95% CI, 1.57 to 14.97; *P*= .006), fever at onset of EBV infection (HR, 6.12; 95% CI, 1.74 to 21.58; *P* = .005), and donor/recipient sex-mismatched HSCT (HR, 4.69; 95% CI, 1.35 to 16.22; *P*= .015) (Figure 4).

A risk factor index was created; Figure 5 illustrates the cumulative incidence of PTLD according to the number of risk factors in the EBV-infected transplant recipients. At day +180 post-transplantation, the incidence of PTLD was very low in patients with 0 to 1 or 2 risk factors (1.9% [95% CI, .4% to 6.1%] and 4.6% [95% CI, 1.5% to 10.5%], respectively) and very high in those with 3 to 4 risk factors (43.8%; 95% CI, 18.7% to 66.5%).

Analysis of Lymphocyte Subsets

Supplementary Figures 1 and 2 show the distribution of $CD4^+$, $CD8^+$, B, and NK cells and a heatmap of the absolute number of all

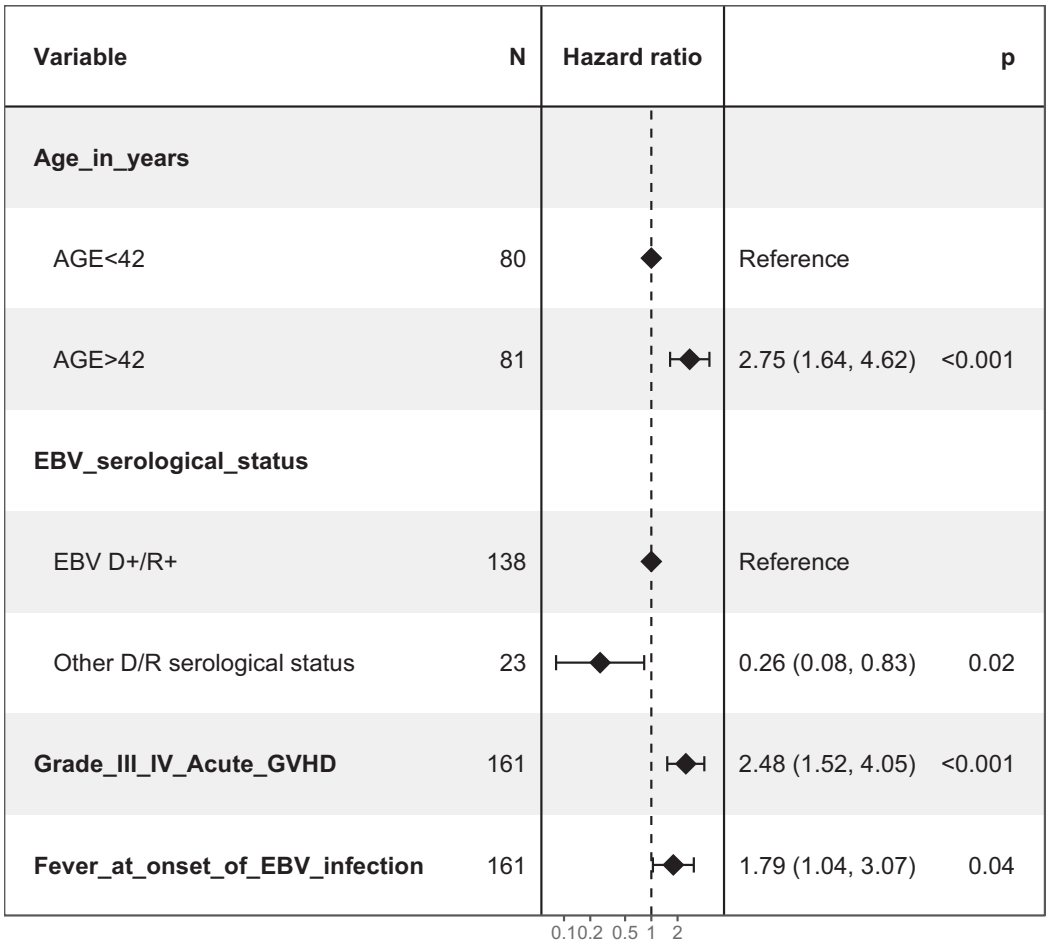

Figure 3. Forest plot of multivariable analysis of factors influencing OS.

Table 4
Univariable Analysis of Factors Influencing the Cumulative Incidence of PTLD*

Factor	HR	95% CI	*P* Value
Age	1.030	(.349-3.071)	.950
Diagnosis	1.290	(.855-1.960)	.220
Donor	1.295	(.760-2.200)	.140
Stem cell source	2.430	(.976-6.070)	.056
CMV serologic status	.873	(.565-1.350)	.450
EBV serologic status	1.640	(.461-5.830)	.689
Sex mismatch	1.559	(1.020-2.360)	**.039**
Conditioning regimen	1.330	(.670-2.631)	.420
T cell depletion	1.760	(.968-3.210)	.064
Cyclophosphamide-based conditioning	2.982	(.804-9.622)	.198
Fludarabine-based conditioning	.550	(.168-1.805)	.323
Grade III-IV acute GVHD	.534	(.119-2.382)	.413
Presence of HLA-DRB11:01 allele	4.850	(1.660-14.20)	**.004**
Fever at onset of EBV infection	2.810	(.924-8.531)	.069
Presence of polyclonal Ig	1.006	(.323-3.463)	.932
Presence of monoclonal Ig	1.820	(.491-6.760)	.370
Absence of EBV-DNAemia clearance within 1 month after rituximab initiation	2.660	(4.750-43.40)	<**.001**
Rituximab initiation <7 d from diagnosis of EBV infection	.395	(.122-1.281)	.120
T Lymphocyte number at onset of EBV infection	.996	(.991-1.124)	.063

Significant *P* values are in bold type.

the other lymphocyte subsets at the onset of EBV infection in patients with resolved EBV infection and patients developing PTLD. The absolute number of T lymphocytes was significantly higher in patients with resolved EBV, whereas the number of NK cells was higher in patients who developed PTLD (*P*= .016 and .049, respectively, not confirmed in multivariable analysis).

DISCUSSION

In the present study, we analyzed outcomes and risk factors associated with PTLD transformation in patients who developed EBV infection after HSCT despite preemptive administration of anti-CD20 therapy.

We were able to show that after preemptive rituximab administration, the cumulative incidence of PTLD after EBV infection was 6.3%, which is very close to previously reported incidence rates of PTLD [25,26]. In multivariate analyses, PTLD was associated with several risk factors, including HSCT with unrelated and sex-mismatched donors, recipient HLA allele DRB1*11:01, and fever at diagnosis of EBV infection. The presence of more than 2 risk factors was strongly associated with the risk of developing PTLD (>40% at 6 months post-HSCT).

Donor/recipient sex-mismatched HSCT, especially with male recipients and female donors, is well known to be associated with a higher incidence of GVHD and inferior survival [27,28]. Immune response against minor histocompatibility antigens encoded on the Y chromosome of a male recipient (H-Y antigens) may be the basis for this adverse effect [29]. H-Y antigens have been shown to elicit a coordinated B cell and T cell response [30].

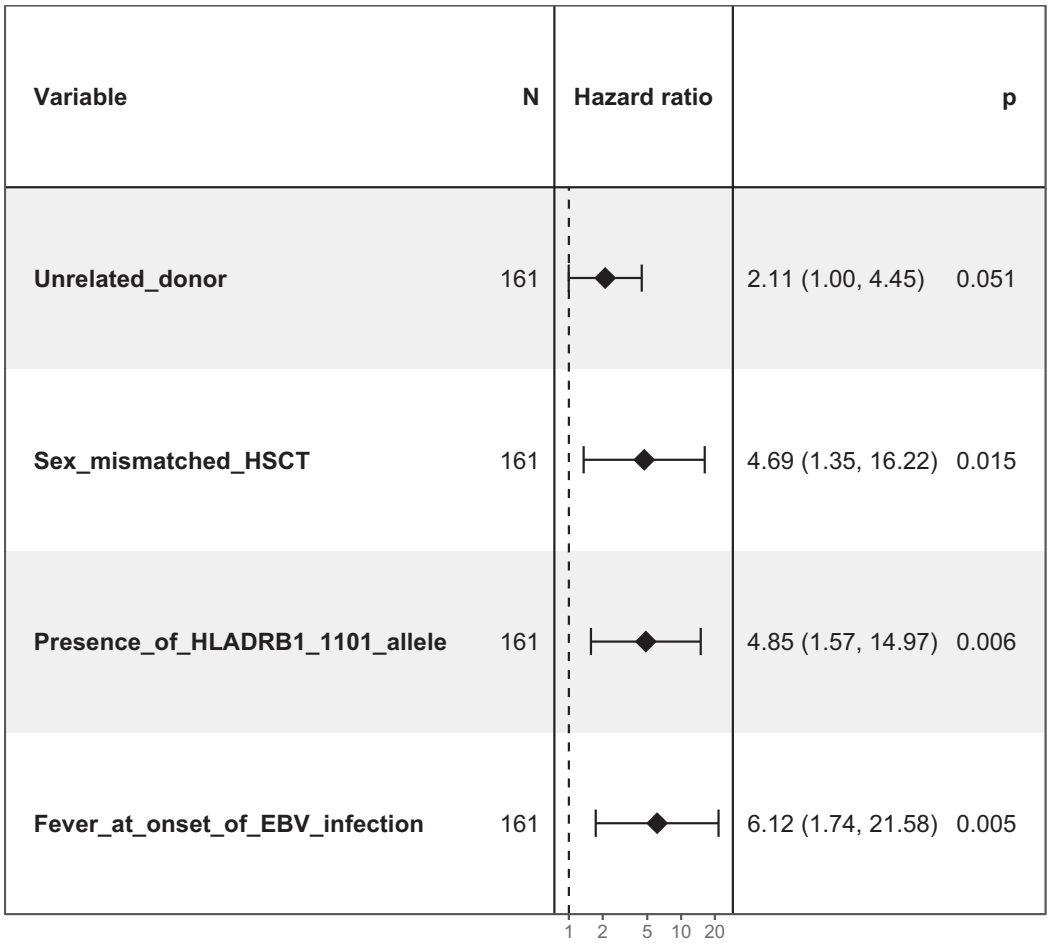

Figure 4. Forest plot of multivariable analysis of risk factors for the CIF of PTLD.

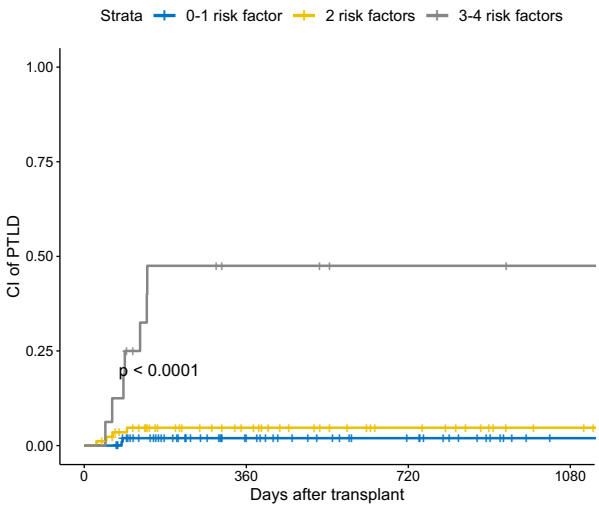

Figure 5. Cumulative incidence of PTLD in transplant recipients with significant EBV-DNAemia receiving rituximab preemptive therapy according to number of risk factors: (1) HSCT from an unrelated donor; (2) recipient HLA-DRB1*11:01; (3) fever at diagnosis of EBV infection; (4) sex-mismatched HSCT.

In our cohort, sex-mismatched transplantation was associated with increased risk of developing PTLD.

In multivariable analysis, presence of the HLA DRB1*11:01 allele was an independent predictive factor of EBV-PTLD. **Supplementary Figure 4** presents a dot chart showing the allele frequency of more representative class I and class II HLA alleles. DRB1*1101 was the most frequent HLA allele in the patients with PTLD (found in heterozygosis in 6 of 13 patients; 46%). Possible associations between certain HLA types and the risk of developing PTLD have been reported in other studies, especially in solid organ transplant recipients [31-34]. Associations also have been found between some HLA class II molecules and other EBV-related malignancies [35]. The most likely hypothesis for this effect is that HLA variants may affect the success of T cell surveillance for EBV and thereby influence a transplant recipient's predisposition to PTLD. It has been shown that HLA-DR mediates the interaction between EBV envelope glycoprotein gp42 and B cells participating in the viral envelope fusion necessary for virion entry in B cells [36]. The possibility that EBV might actively evade $CD4^+$ T lymphocytes through some HLA-DR polymorphisms has been suggested in some in vitro systems in which lytically infected B cells shed significant amounts of a soluble form of gp42 that can bind to mature HLA class II at the cell surface, protecting those cells from $CD4^+$ recognition [37]. Other proteins that play key roles in reactivation and viral genome persistence (eg, Zta, EBV nuclear antigens) also interact with HLA-DR to limit viral antigen presentation [38,39]. Epitopes selected according to HLA-DR polymorphisms may trigger different patterns of immune responses [38]. The best-characterized components of these mechanisms are specific CTLs directed against viral gene products of the latent state, which include EBV nuclear antigens

Table 5
Characteristics of Patients Developing PTLD

Patient	Sex	Initial Diagnosis	Age at HSCT	Stem Cell Source	Type of Donor/ Donor Sex	Acute GVHD Present (Maximum Grade)	Time between Rituximab Initiation and PTLD Transformation	Corticosteroid Use before Significant EBV-DNAemia	Time between HSCT and PTLD, d	PTLD Classification on Biopsy
196	F	BMF	55	BM	MMUD/M	No	73	Yes	124	Monomorphic PTLD B
197	F	PTCL NOS	34	PB	MUD/M	Yes; grade II	27	Yes	90	Monomorphic PTLD B
198	M	ALL-B	17	PB	MUD/F	No	9	No	47	No biopsy
199	F	AML	57	PB	MMUD/ M	No	7	No	62	Monomorphic PTLD B
200	F	MDS	62	PB	MUD/F	Yes; grade II	52	Yes	140	Monomorphic PTLD B
201	M	BMF	40	CB	MM/F	No	2	No	139	Only cytoaspiration
202	M	ALL-B	33	PB	MUD/M	Yes; grade II	28	Yes	83	Monomorphic PTLD B
203	M	ALL-B	19	PB	MMUD/ F	Yes; grade III	50	Yes	87	Polymorphic PTLD
204	M	MPN	74	PB	MMUD/ M	Yes; grade I	9	Yes	49	Polymorphic PTLD
205	F	MPN	56	PB	MMUD/ M	No	60	Yes	95	Monomorphic PTLD B
206	F	ALL-B	36	PB	MMUD/ M	Yes; grade III	19	Yes	63	Polymorphic PTLD
207	F	BMF	34	CB	MM/F	No	7	No	85	Polymorphic PTLD
208	F	BMF	52	BM	Identical sibling/ M	Yes; grade II	6	Yes	27	No biopsy

Patient	Sex	Initial Diagnosis	Positive Immunohistochemistry	Extranodal Involvement	PCR EBV max (log UI/mL)	No. of Rituximab Treatments	Other Treatments	Outcome PTLD	Status
196	F	BMF	CD20, CD79a	Liver, spleen	6.05	11	No	NR	Dead
197	F	PTCL NOS	CD20, CD79a, CD30,	Liver, spleen	5.65	4	Cyclophosphamide, etoposide, boost	CR	Dead
198	M	ALL-B	N/A	CNS	6.54	4	Etoposide, methotrexate IT	CR	Alive
199	F	AML	CD20, CD79a, MUM1	Liver, CNS	6.92	5	Etoposide, methotrexate IT	NR	Dead
200	F	MDS	CD20, CD79a	Colon	4.78	2	No	NR	Dead
201	M	BMF	CD20	No	4.30	2	No	CR	Alive
202	M	ALL-B	CD20, CD79a, CD30	Liver, spleen, kidney	5.55	4	Cyclophosphamide, doxorubicin, etoposide, brentuximab, DLI	CR	Dead
203	M	ALL-B	CD20, CD79a	Tonsils, cavum, kidney	5.37	7	No	CR	Alive
204	M	MPN	CD20, CD79a	No	4.98	2	No	NR	Dead
205	F	MPN	CD20, CD79a	Liver, bone	5.26	5	No	NR	Dead
206	F	ALL-B	CD20, CD79a, CD30, MUM1	Liver, cavum	6.94	8	Cyclophosphamide, etoposide, aracytine, brentuximab	NR	Dead
207	F	BMF	CD20, CD30, CD79a, CD138	Cavum	4.15	5	No	CR	Alive
208	F	BMF	N/A	No	5.93	5	No	NR	Dead

F indicates female; M, male; ALL-B, B cell ALL; PTCL NOS, peripheral T cell lymphoma not otherwise specified; N/A, not available; CNS, central nervous system; MM, mismatched; IT, intrathecal; CR, complete remission; NR, nonresponder.

1 to 6 and latent membrane proteins 1 and 2 [40]. It also has been reported that the lytic cycle of EBV correlates with the diminution of specific major histocompatibility complex (MHC) class I and class II molecules, and conversely, the down-regulation of surface MHC class I and II expression is maintained throughout the lytic cycle of EBV, with a significant effect on antigen presentation [39,41]. We can speculate that such mechanisms are the basis for the HLA-DRB1*11:01-associated risk of PTLD transformation after HSCT, but this obviously requires confirmation from other studies.

Hyperthermia at the onset of EBV infection was identified as the most clinically relevant risk factor associated with PTLD transformation in our cohort (HR, 6.12; $P = .005$). This finding is not surprising if we consider fever as a clinical feature consistent with probable or proven EBV disease [42]. However, despite the presence of fever in 4 out of 13 patients who further develop PTLD, none of those patients presented with clinical signs of lymphoproliferative disease at the beginning of EBV-DNAemia. In our cohort, fever at the onset of EBV-DNAemia also had an impact on survival outcome in univariable and multivariable analyses.

Unrelated donor transplant, T cell depletion, donor-positive and recipient-negative EBV serostatus, RIC regimens, grade II-IV acute GVHD, and splenectomy have been identified as risk factors for EBV-related PTLD in the general transplantation population [3,25,43]. In our cohort of patients already infected with EBV and receiving anti-CD20 preemptive treatment, T cell depletion, a reduced-intensity conditioning regimen, and EBV serologic status did not predispose toward an evolution to PTLD. Almost 80% of our patients received a T cell-depleted treatment before transplantation, and all patients with PTLD had received ATG or antilymphocyte globulin (and thus it was impossible to include this factor in multivariable analysis). Therefore, ATG may be considered a risk factor for EBV-DNAemia, but not a risk factor for PTLD. Clearance of EBV-DNAemia after 1 month is an independent factor impacting OS and PTLD incidence in univariable analysis, but its consideration in the multivariable model violated the assumption of proportionality [44]. Indeed, PTLD transformation per se is strongly associated with the persistence of EBV-DNAemia, but statistically, the relative effect of this covariate on the hazard function seems to change over time, likely due to the change in detection methods during the study period (see Methods).

The presence of a monoclonal immunoglobulin was not a risk factor for PTLD transformation in our cohort because it was also frequently present in patients with only EBV-DNAemia.

Interestingly, our immunohistochemistry analysis of the available samples revealed CD20 positivity in all patients diagnosed with PTLD. This is an important finding that leads us to speculate that the resistance to rituximab treatment in our cohort is merely clinical and should be considered a “breakthrough” mechanism, occurring with the tumor mass progression and EBV proliferation under immunosuppression, rather than a change in the molecular pathways of infected and transformed B cells. Undoubtedly, these results need to be confirmed with further in vitro or in vivo studies aimed at elucidating the mechanisms of resistance to anti-CD20 therapy in this particular setting.

The EBV-PTLD incidence in our cohort is quite low when we consider that our analysis was conducted in a potentially high-risk population. We can argue that this result depends on preemptive rituximab therapy, which is considered the best way to optimize outcomes in HSCT recipients with significant EBV-DNAemia [3]. The 3-year OS in patients not developing PTLD was nearly 70%, similar to the expected OS in the general HSCT recipient population [45]. Therefore, we can assume that EBV infection after HSCT, when occurring in the absence of risk factors for PTLD transformation, is not associated with poor outcomes. However, in our analysis, PTLD transformation was still associated with a high mortality rate (close to 70%), even with an overall response rate of patients developing EBV-PTLD of nearly 50%.

To date, no standard therapy has been accepted for rituximab-resistant EBV-PTLD. In the setting of rituximab failure, second-line therapy options include cellular therapy (DLI or CTLs) or rituximab associated with chemotherapy. Unselected DLI from an EBV-positive donor is used to restore broad T cell reactivity, including EBV-specific responses; however, this procedure can be associated with severe GVHD. CHOP-like [46] or dose-adjusted-EPOCH-R [47] regimens have been proposed, especially in the context of solid-organ PTLD. However, chemotherapy regimens are difficult to manage in the setting of HSCT. Brentuximab has been reported as a possible agent for PTLD with evidence of $CD30^+$ expression [48-50]. In our series, only 4 patients with PTLD were alive at the last follow-up time, including 1 patient after a chemotherapy regimen including etoposide and high-dose intrathecal methotrexate and 3 patients without receipt of second-line chemotherapy but with intensified rituximab treatment and drastically reduced immunosuppressive therapy. Patients receiving other chemotherapy regimens (CHOP-like) died with persistent disease. Brentuximab was associated with unsuccessful chemotherapy in 2 patients. One patient received DLI, and another received a $CD34^+$-selected boost in a context of poor graft function. These patients experienced CR but died several months later from other infectious complications (Table 5). Our group previously reported that preemptive rituximab is an important strategy to decrease the risk of EBV-related PTLD; however, the associated prolonged and profound B cell deficiency is associated with an increased risk of bacterial infection and mortality [51].

The retrospective nature and the limited number of PTLD cases are definitive drawbacks of this study, and thus our findings need further validation in larger cohorts. Despite these limitations, however, we believe that our results may contribute to better management of this post-transplantation complication and implementation of preemptive strategies, such as escalating rituximab doses or virus-specific T cell products, in selected patients presenting with multiple risk factors.

DECLARATION OF COMPETING INTEREST

There are no conflicts of interest to report.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.bbmt.2019.08.006.

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