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Features of Epstein-Barr Virus (EBV) reactivation after reduced intensity conditioning allogeneic hematopoietic stem cell transplantation

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This single centre study assessed the incidence, kinetics and predictive factors of Epstein-Barr Virus (EBV) reactivation and EBV-related lymphoproliferative diseases (LPDs) in 175 consecutive patients who received a reduced-intensity conditioning (RIC) before allogeneic hematopoietic stem cell transplantation (allo-HSCT). The cumulative incidence of EBV reactivation at 6 months after allo-HSCT defined as an EBV PCR load above 1000 copies of EBV DNA/10^5 cells was 15%, and none of these patients experienced any sign or symptom of LPD. A total of 17 patients, who had EBV DNA levels exceeding 1000 copies/10^5 cells on two or more occasions, were pre-emptively treated with rituximab. With a median follow-up of 655 (range, 92–1542) days post allo-HSCT, there was no statistically significant difference in term of outcome between those patients who experienced an EBV reactivation and those who did not. In multivariate analysis, the use of antithymocyte globulin as part of the RIC regimen was the only independent risk factor associated with EBV reactivation (relative risk = 4.9; 95% confidence interval, 1.1–21.0; $P = 0.03$). We conclude that patients undergoing RIC allo-HSCT using anti-thymocyte globulin as part of the preparative regimen are at higher risk for EBV reactivation. However, this did not impact on outcome, as quantitative monitoring of EBV viral load by PCR and preemptive rituximab therapy allowed for significantly reducing the risk of EBV-related LPD.
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Introduction

Reduced-intensity conditioning (RIC) regimens are increasingly used before allogeneic hematopoietic stem cell transplantation (allo-HSCT) with the aim to decrease transplantation-related mortality (TRM) in elderly patients, heavily pretreated patients or in patients with medical comorbidities precluding the use of standard myeloablative preparative regimens.[1–5] The majority of these RIC protocols are designed to produce a state of profound immunosuppression rather than myeloablation.[6]

Epstein-Barr virus (EBV) is a latent γ-herpes virus with B lymphocyte-specific tropism that infects more than 90% of healthy individuals. Following allo-HSCT, EBV reactivation and EBV-related proliferations are well recognized complications.[7–12] EBV reactivation may be associated with a spectrum of clinical presentations, going from fever to lymphoproliferative diseases (LPDs), which arise as a consequence of an outgrowth of B cells latently infected with EBV in the setting of loss or suppression of normal cytotoxic T-cell surveillance. Established LPD post-allo-HSCT is associated with a significant mortality and morbidity.[13] LPD after allo-HSCT typically occurs within the first 6 months after transplant, usually before recovery of the EBV-specific cytotoxic T-lymphocyte response. Risk factors for EBV reactivation include the degree of mismatch between donor and recipient, manipulation of the graft to deplete T cells, degree and duration of immunosuppression used to prevent and treat graft-versus-host disease (GVHD).[14] On the other hand, early detection strategies with serial measurement of EBV-DNA load in peripheral blood samples have helped to identify high-risk patients and to diagnose early lymphoproliferation.[15–17] Moreover, pre-emptive anti-B-cell therapies are increasingly used and have been shown to be successful in preventing LPD.[18–21] To date, few data has been reported regarding the incidence and features of EBV-related disease following RIC allo-HSCT in adult patients with hematological diseases. Two recent reports suggested a higher incidence of LPD in pediatric patients who underwent RIC allo-HSCT including anti-thymocyte globulins (ATGs) or Campath.[22,23] The aim of this analysis was to define the incidence and potential risk factors predicting the development of EBV reactivation in the first 6 months following RIC allo-HSCT in 175 consecutive adult patients, and to assess its impact on clinical outcome.

Patients and methods

Study design

A total of 175 consecutive patients who received a RIC allo-HSCT for hematological malignancies in a single institution (University Hospital of Nantes, Nantes, France) between January 2005 and June 2009 were included in this retrospective study. Patient receiving RIC allo-HSCT using unrelated cord blood cells were excluded from this analysis.[24] In our transplant program, eligibility criteria for RIC allo-HSCT that preclude use of standard myeloablative allo-HSCT include: (1) patients older

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than 50 years of age; (2) heavily pretreated patients who received autologous HSCT and/or more than two lines of chemotherapy before allo-HSCT; and (3) patients with poor performance status because of significant medical comorbidities.[25] Written informed consent was obtained from each patient and donor. The study was performed according to institutional guidelines. Patients participated in investigational protocols approved by the Institutional Review Board, and the local ethical committee.

Conditioning regimen

The conditioning regimen associated fludarabine, busulfan and ATG in 107 cases (61%). The ATG-based RIC regimen included fludarabine with a daily dose of 30 mg/m^2 for 4–6 consecutive days, oral busulfan (4 mg/kg per day for 2 consecutive days) or intravenous busulfan (3.2 mg/kg per day for 2 or 3 consecutive days) and ATG (Thymoglobulin; Genzyme, Lyon, France; for a total dose of 5 mg/kg infused over 2 days).[25] In total, 32 patients (18%) received fludarabine 30 mg/m^2 for 3 consecutive days and low-dose total body irradiation 2 Gy.[3] Another 5 patients (3%) received fludarabine 30 mg/m^2 for 4 consecutive days and low-dose total body irradiation 2 Gy combined with cyclophosphamide (1200 mg/m^2) and ATG (7.5 mg/kg). The remaining 31 patient (18%) received different chemotherapy-based RIC regimens: (cytarabine 8000 mg/m^2—fludarabine (120 mg/m^2)—amsacrine (400 mg/m^2)—busulfan (12.8 mg/kg)—ATG (5 mg/kg) ($n=16$); fludarabine (90 mg/m^2)—melphalan (100 mg/m^2)—bortezomib (4 mg/m^2) ($n=10$; including one case with ATG 5 mg/kg)—fludarabine (150 mg/m^2)—treosulfan (36 g/m^2)—ATG (5 mg/kg) ($n=5$)). All patients received the preparative regimen in private rooms with laminar air flow devices, and remained hospitalized until hematopoietic and clinical recovery.

Grafts

All allogeneic grafts were obtained from human leukocyte antigen (HLA)-A, HLA-B, HLA-C, HLA-DR and HLA-DQ-matched donors. HLA-DP typing was not routinely performed at time of this study. A single HLA mismatched of 10 (at HLA-C) was allowed at the allele level. All donor/recipient pairs were typed at the allelic level. They were first typed at the two-digit level for HLA class I (HLA-A, HLA-B and HLA-Cw) and class II (HLA-DRB1 and HLA-DQB1) using published HLA class I PCR sequence-specific oligonucleotide and/or PCR sequence-specific primers typing protocols. HLA typing was performed according to the recommendations of the European Federation for Immunogenetics Histocompatibility Laboratory standards during the study period. In total, 165 patients (94%) received peripheral blood stem cells whereas 10 patients (6%) received a bone marrow graft collected under general anesthesia. All peripheral blood stem cell grafts were collected after donor mobilization with granulocyte colony-stimulating factor. A total of 84 grafts (48%) were obtained from HLA identical sibling donors, 80 (46%) from HLA-matched unrelated donors and 11 (6%) from one antigen HLA-mismatched unrelated donors. The day of bone marrow or peripheral blood stem cell infusion was designated as day 0. The median number of infused CD34+ cells was 6.1×10^6 CD34 cells/kg recipient body weight (range, 0.7–36.7)

GVHD prophylaxis and treatment

All patients received post-transplantation immunosuppression with either cyclosporine A alone (CsA, $n=77$; 44%; mainly patients transplanted from an HLA-identical sibling donor), CsA and mycophenolate mofetil (MMF, $n=93$; 53%) or CsA and short-course methotrexate ($n=5$; 3%). CsA was administered at a dose of 3 mg/kg by continuous intravenous infusion starting from day −3 or −2 and changed to twice daily oral dosing as soon as tolerated, adjusted to achieve blood levels between 150 to 250 ng/ml and to prevent renal dysfunction. MMF was administered at a fixed oral dose of 2 g per day starting from day 0.[26] Methotrexate was administered at 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3 and 6. In the absence of GVHD, MMF and CsA were tapered over 4 weeks starting from day 60 and day 90, respectively. Acute and chronic GVHD were graded according to the Seattle standard criteria. Grades II to IV acute GVHD were usually treated with corticosteroids 2 mg/kg per day, followed by a progressive taper in the absence of GVHD exacerbation. Extensive chronic GVHD was treated with the combination of CsA and corticosteroids (1 mg/kg per day) followed by a slow taper in the absence of GVHD exacerbation.

Infection prophylaxis, monitoring and supportive care

All patients received antiviral prophylaxis with intravenous acyclovir 250 mg thrice daily or oral valaciclovir 500 mg twice daily from the start of conditioning. No systematic antifungal and antibacterial prophylaxis was delivered before engraftment. Pneumocystis prophylaxis with cotrimoxazole was initiated following engraftment, as well as amoxicillin or penicillin for prevention of encapsulated bacterial infections.[27] Of note, supportive care was the same during the whole study period. Cytomegalovirus (CMV) infection management was also homogeneous. All blood products were filtered, irradiated and CMV screened. CMV, EBV, adenovirus (ADV) and human herpes virus 6 were routinely screened by quantitative PCR.[28]

EBV monitoring and therapy

During the first 6 months after allo-HSCT and in patients treated for GVHD, all patients were weekly DNA-PCR screened in the peripheral blood for EBV reactivation and were clinically monitored for clinical features attributable to EBV. Total nucleic acids were extracted from 200 µl EDTA of whole blood with a MagNAPure LC instrument and the MagNAPure LC DNA isolation kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's recommendations and stored in a final volume of 100 µl at −20 °C until further analysis. DNA quantifications were performed using real-time PCR procedures, as previously described.[29] Briefly, EBV (*BNRF1* gene) was quantified on 5 µl DNA extracts and viral loads were expressed as the number of viral DNA copies. EBV reactivation was defined as any EBV PCR load above 1000 copies of EBV DNA/10^5 cells. EBV LPD was defined as biopsy- or autopsy proven post-transplantation lymphoma, or reactivation along with computerized tomography nodal or soft-tissue abnormalities consistent with LPD. Patients with EBV viral load >1000 copies/10^5 cells on at least two consecutive occasions were treated with the anti-CD20 monoclonal antibody, rituximab at a dose of 375 mg/m^2 weekly until clearance of EBV reactivation (usually for a maximum of four infusions).[21] Computed tomography imaging was not performed before rituximab administration. For the purpose of this analysis, detailed data related to EBV were captured on designated report forms from medical charts by one of the coauthors (ZP).

Statistics

All time-related data were measured from the day of allo-HSCT. Complete remission and overall survival (OS) were defined

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according to standard criteria. OS was estimated with the Kaplan–Meier method and subgroups were compared with the log-rank test. TRM and EBV incidences were evaluated using the cumulative incidence method treating death as a competitive risk[30] and subgroups were compared using the Gray test.[31] Potential risk factors for EBV reactivation were compared between cases (patients with EBV reactivation) and controls (patients without EBV reactivation) using the Mann–Whitney test for continuous variables and the Fisher's exact test for categorical variables. Evaluated variables included patient age, patient and donor gender, CMV recipient and donor serologic status, EBV recipient and donor serologic status, diagnosis (myeloid or lymphoid malignancies), disease status (standard or high risk), stem cell source, donor type (matched related, matched unrelated or mismatch donor), conditioning regimen, GVHD prophylaxis, CD34+ cell count, time to neutrophil recovery, acute GVHD (0–I/II/III–IV, not time-dependent variable). Variables for which *P*-value was <0.30 in the univariate analysis were included in a multivariate regression analysis, using the semiparametric proportional hazards model of Fine and Gray.[32] All data were computed using the R package (R Development Core Team, 2006. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org.).

Results

Patients' characteristics and engraftment

Patients' characteristics are summarized in Table 1. Briefly, the median age of recipients was 56 (range 18–71) years. In all, 85 patients (49%) had a myeloid malignancy, whereas 86 (49%) patients were diagnosed with lymphoid malignancies. The remaining four patients (2%) were treated for severe aplastic anemia. According to their disease features, 145 (83%) patients were considered as high-risk. The median time to neutrophil recovery (absolute neutrophil count $>0.5\times10^9$/l) was 17 (range, 6–48) days. Clinically significant grade II–IV acute GVHD occurred in 61 cases (35%) and severe grade III–IV acute GVHD occurred in 37 cases (21%). Only one patient died during the first month of TRM and 154 (88%) patients were alive at day 100 after allo-HSCT. The cumulative incidence of TRM is shown in Figure 1a. Chronic GVHD was diagnosed in 59 of cases (34%), with extensive chronic GVHD occurring in 42 of these patients.

EBV-related events

The cumulative incidence of EBV reactivation at 6 months after allo-HSCT defined as an EBV PCR load above 1000 copies of EBV DNA/10^5 cells was 15% (95% confidence interval, 10–21%; Figure 1b). In 141 patients (81%), the EBV load remained <1000 EBV copies/10^5 cells at all time, and none of these patients experienced any sign or symptom of LPD. The remaining 34 patients (19%) experienced at least one EBV reactivation episode. EBV reactivation was observed at a median of 58 (range 0–930) days after allo-HSCT, with 27 (79%) of reactivations occurring during the first 6 months. The highest viral loads were measured between the second and twelveth weeks after transplantation. Among the 34 patients who experienced EBV reactivation, 17 patients experienced an EBV load superior to 1000/10^5 cells only at a single time point after allo-HSCT. In these 17 cases, there were no concomitant clinical symptoms and the EBV load normalized spontaneously.

Table 1 Study population characteristics and transplant-related events

Characteristic	N *(%)*
Patient age, median (range)	56 (18–71)
Patient gender	
Male	106 (61)
Female	69 (39)
Donor gender	
Male	96 (55)
Female	79 (45)
CMV serologic status	
Seronegative recipient and seronegative donor	61 (35)
Seropositive recipient or seropositive donor	114 (65)
EBV serologic status	
Seropositive recipient and seropositive donor	161 (92)
Seropositive recipient or seronegative donor	8 (5)
Seronegative recipient and seropositive donor	6 (3)
Seronegative recipient and seronegative donor	0
Diagnosis[a]	
Myeloid malignancies	85 (49)
Lymphoid malignancies	86 (49)
Severe aplastic anemia	4 (2)
Disease status[b]	
Standard risk	30 (17)
High risk	145 (83)
Stem cell source	
BM	10 (6)
PBSC	165 (94)
Donor type	
MRD	84 (48)
MUD	80 (46)
MIS	11 (6)
Conditioning regimen[c]	
With ATG	134 (77)
Without ATG	41 (23)
GVHD prophylaxis	
CsA alone	77 (44)
CsA and MMF	93 (53)
CsA and MTX	5 (3)
CD 34+ cell count ($\times10^6$/kg recipient BW) median (range)	6.1 (0.7–36.7)
Neutrophil recovery ANC$>0.5\times10^9$/l median (range)	17 (6–48)
Acute GVHD	
Grade 0–I	114 (65)
Grade II	24 (14)
Grade III–IV	37 (21)
Acute GVHD onset (days) after transplantation median (range)	34 (6–181)
Chronic GVHD	59 (34)
Limited	17 (10)
Extensive	42 (24)
Chronic GVHD onset (days) after transplantation median (range)	189 (100–768)

Abbreviations: ANC, absolute neutrophil count; ATG, antithymoglobulins; BM, bone marrow; BW, body weight; CMV, cytomegalovirus; CsA, cyclosporine A; EBV, Epstein-Barr Virus; GVHD, graft-vs-host disease; MIS, mismatched unrelated donor; MMF, mycophenolate mofetil; MRD, matched related donor; MUD, matched unrelated donor; MTX, methotrexate; PBSC, peripheral blood stem cell.

[a]Myeloid malignancies included 55 acute myeloid leukemias, 15 myelodysplastic syndromas (MDS), 12 myeloproliferative syndromes (MPS), 3 MDS/MPS lymphoid malignancies: 39 non-Hodgkin's lymphomas (NHL), 18 Hodgkin's disease, 13 chronic lymphocytic leukemias (CLL), 13 multiple myeloma, two acute lymphoblastic leukemias (ALL) and one prolymphocytic leukemia.

[b]Patients in complete remission, chronic phase or untreated were considered as standard risk, all others were considered as high risk.

[c]In this series, a RIC regimen was chosen as per institutional guidelines because one or several of the following reasons: age >50 years ($n=130$), and/or patients receiving previous transplantation ($n=72$), and/or presence of comorbidities precluding the use of a standard myeloablative regimen ($n=15$). According to our transplant program guidelines, all patients conditioned with Fludarabine and Busulfan received ATG. In other patients, ATG was added to enhance engraftment in those patients who had received less than two cycles of multiagent chemotherapy within the 2 months before transplantation.

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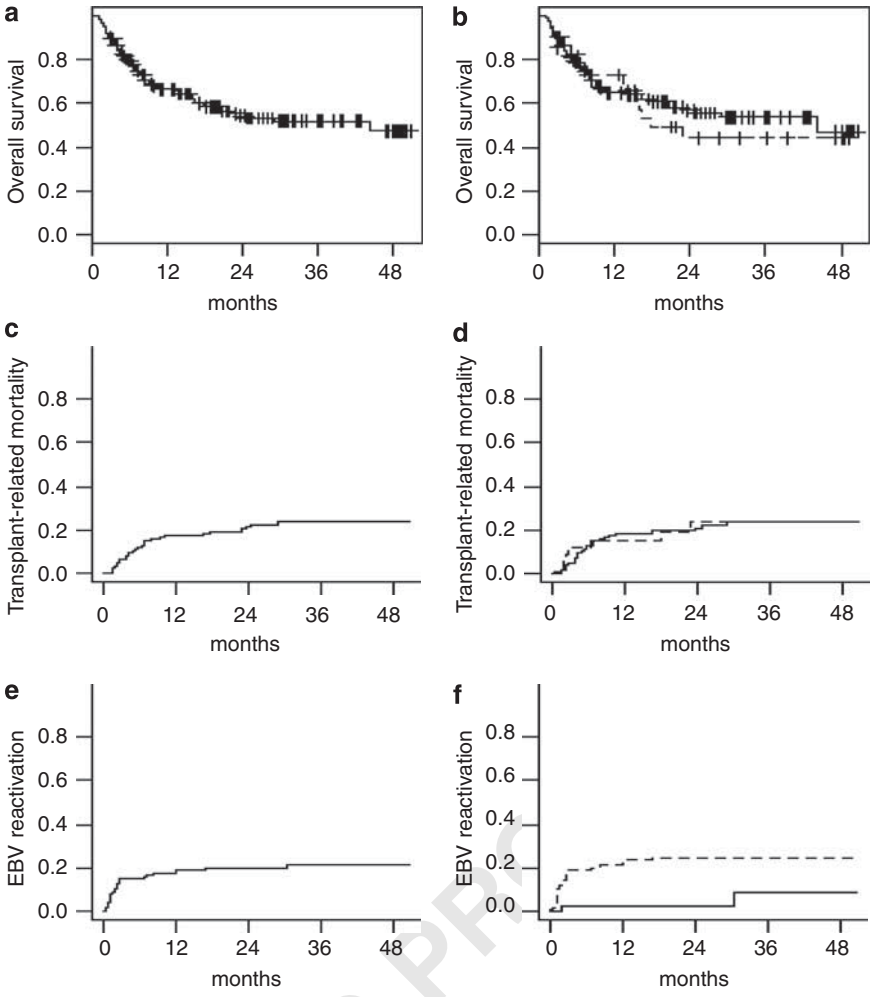

Figure 1 Outcome after reduced-intensity conditioning (RIC) allogeneic hematopoietic stem cell transplantation (allo-HSCT). Cumulative incidence of transplant-related mortality in the study population (**a**), Epstein-Barr Virus (EBV) reactivation in the study population (**b**), overall survival in the study population (**c**), overall survival according to EBV reactivation (dashed line: with EBV reactivation) (**d**), cumulative incidence of transplant-related mortality according to EBV reactivation (dashed line: with EBV reactivation) (**e**), and cumulative incidence of EBV reactivation according to the use of anti-thymocyte globulin (ATG) as part of the RIC regimen (dashed line: with ATG) (**f**). *x* axis: months post allo-HSCT.

The remaining 17 patients had EBV DNA levels exceeding 1000 copies/10^5 cells on two or more occasions, and were preemptively treated with a median number of 3 (range, 1–4) rituximab infusions, which resulted in complete clearance of EBV viremia in all, but one patient (97%). This patient was severely immunosuppressed, receiving three different immunosuppressive agents (CsA, MMF and weekly low-dose methotrexate), and experienced both EBV and ADV infection at day +34 after allo-HSCT from a mismatched unrelated donor. This patient had symptoms mainly related to the ADV infection and died of multiorgan failure. Eight of the 34 patients (24%) who experienced EBV reactivation (and before any therapeutic intervention) had other viruses detected in the same blood sample by PCR (EBV and CMV: $n=5$; EBV and ADV: $n=1$; EBV, CMV and ADV: $n=1$; EBV, CMV, ADV and human herpes virus 6: $n=1$). Only two patients experienced a second EBV reactivation episode.

Outcome and risk factors for EBV reactivation

With a median follow-up of 655 (range, 92–1542) days after allo-HSCT among surviving patients, 104 patients (59%) were still alive and the OS was 47% at 4 years (Figure 1c). In total, 69 patients died of disease progression ($n=37$) and transplant-related complications ($n=32$, of whom 22 deaths attributed to acute or chronic GVHD and 10 related to infectious-related causes) and two patients died of other reasons. There was no statistical difference in terms of chronic GVHD incidence between patients who received pre-emptive rituximab versus those who did not receive rituximab. Also, there was no statistically significant difference in terms of OS or TRM between those patients who experienced an EBV reactivation after allo-HSCT and those who did not (OS: log rank test, $P=0.62$, Figure 1d; TRM: Gray test, $P=0.99$, Figure 1e). Univariate analysis for risk factors associated with EBV reactivation is shown in Table 2. Only the use of ATG as part of the RIC regimen before allo-HSCT was significantly different between subgroups with and without EBV reactivation (Fisher's exact test, $P=0.006$). Variables with a P-value <0.30 were, respectively, diagnosis (lymphoid, myeloid malignancies and severe aplastic anemia; $P=0.14$), the conditioning regimen (ATG vs no ATG; $P=0.006$) and donor type (matched related donor, matched unrelated donor and mismatched unrelated donor; $P=0.19$) and were included into the regression analysis

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Table 2 Univariate analysis of risk factors for EBV reactivation comparing patients with and without EBV reactivation

Characteristic (%)	*Patients without EBV reactivation n = 141 (81)*	*Patients with EBV reactivation n = 34 (19)*	P
Patient age, median (range)	56 (18–71)	57 (23–70)	0.97
Patient gender			
Male	86 (61)	20 (59)	0.85
Female	55 (39)	14 (41)	
Donor gender			
Male	78 (55)	18 (53)	0.85
Female	63 (45)	16 (47)	
CMV serologic status			
Seronegative recipient and seronegative donor	50 (35)	11 (32)	0.84
Seropositive recipient or seropositive donor	91 (65)	23 (68)	
EBV serologic status			
Seropositive recipient and seropositive donor	129 (91)	32 (94)	1.00
Seropositive recipient and seronegative donor	7 (5)	1 (3)	
Seronegative recipient and seropositive donor	5 (4)	1 (3)	
Diagnosis			
Myeloid malignancies	64 (45)	21 (62)	0.14
Lymphoid malignancies	74 (53)	12 (35)	
Aplastic anemia	3 (2)	1 (3)	
Disease status			
Standard risk	24 (17)	6 (18)	0.91
High risk	117 (83)	28 (82)	
Stem cell source			
BM	7 (5)	3 (9)	0.41
PBSC	134 (95)	31 (91)	
Donor type			
MRD	71 (50)	13 (38)	0.19
MUD	63 (45)	17 (50)	
MIS	7 (5)	4 (12)	
Conditioning regimen			
With ATG	102 (72)	32 (94)	
Without ATG	39 (28)	2 (6)	**0.006**
GVHD prophylaxis			
CsA alone	62 (44)	15 (44)	0.85
CsA+MMF	74 (53)	19 (56)	
CsA+MTX	5 (3)		
CD 34+ cell count ($\times 10^6$/kg recipient BW) median (range)	6.0 (0.7–36.7)	6.9 (1.1–16.3)	0.52
Neutrophil recovery ANC $>0.5 \times 10^9$/l median (range)	17 (6–48)	17 (9–29)	0.32
Acute GVHD			0.36
Grade 0–I	91 (64)	23 (68)	
Grade II	21 (15)	3 (9)	
Grade III–IV	29 (21)	8 (23)	

Abbreviations: ANC, absolute neutrophil count; ATG, antithymoglobulins; BM, bone marrow; BW, body weight; CMV, cytomegalovirus; CsA, cyclosporine A; EBV, Epstein-Barr Virus; GVHD, graft-vs-host disease; MIS, mismatched unrelated donor; MMF, mycophenolate mofetil; MRD, matched related donor; MUD, matched unrelated donor; MTX, methotrexate; PBSC, peripheral blood stem cell.

Table 3 Multivariate regression analysis of risk factors for EBV reactivation using the Fine and Gray model

Factor	*Relative risk (RR)*	*Confidence interval (CI)*	P
ATG	**4.9**	**1.1–21.0**	**0.03**
Diagnosis			0.72
Lymphoid vs myeloid	1.3	0.4–1.5	
Donor type			0.16
MUD vs MRD	1.59	0.8–3.3	
MIS vs MRD	2.72	0.8–8.7	

Abbreviations: ATG, antithymoglobulins; EBV, Epstein-Barr Virus; MIS, mismatched unrelated donor; MRD, matched related donor; MUD, matched unrelated donor.

of competing risks. The cumulative incidence of EBV reactivation in patients receiving or not receiving ATG as part of their conditioning regimen is depicted in Figure 1f. In the Fine and Gray analysis (Table 3), the use of ATG remained the only independent risk factor associated with EBV reactivation (relative risk = 4.9; 95% confidence interval, 1.1–21.0; $P = 0.03$).

Discussion

This study assessed the incidence and features of EBV reactivation and EBV-related LPD in a series of 175 patients receiving RIC allo-HSCT. Previous studies reported an EBV reactivation incidence ranging between 0.6 and 26%, with this being higher in the context of T-cell depletion.[8,33,34] Furthermore, mortality rates following development of an EBV-related LPD can range between 50 and 80%.[35] The current study reports a cumulative incidence of EBV reactivation of 15 and 21% at 6 months and 3 years after allo-HSCT, respectively, but none of the patient experienced EBV-related LPD signs or mortality. In this series, the absence of EBV-related severe complications is likely due to both the strict policy of EBV monitoring during the first months after allo-HSCT and the systematic use of pre-emptive rituximab in those patients experiencing a viral load >1000 copies/10^5 cells as recently described by Blaes *et al.*[21] Indeed, numerous studies[17,18,20,22,36–38] already showed that EBV viral load monitoring in the peripheral blood may be of value in high-risk populations after allo-HSCT. Recent evidence-based guidelines recommended weekly screening of EBV-DNA for at least 3 months in high-risk allo-HSCT recipients.[13,39] In addition, an ever growing number of studies suggested that pre-emptive therapy with rituximab may be highly effective in controlling viral proliferation and avoiding progression into EBV-related LPD.[18,20,21,36] Of note, the efficacy of rituximab was mainly observed in the pre-emptive setting, but to a lesser extent once EBV-related LPD was fully established.[17] In the current series, the response rate to pre-emptive rituximab appeared to be similar to that previously reported in the literature,[40] with efficient and sustained control of EBV viral load in the great majority of cases (97%). As a matter of fact, our study did not include a control group. Thus, theoretically, it is not possible to know how many, if any, of these patients would have developed EBV-related LPD if not preemptively treated with rituximab.

The efficacy of B-cell specific antibodies relies on their capacity to target antigens present on the surface of EBV-transformed malignant cells. The most widely used antibody as prophylaxis and treatment for LPD is rituximab, a monoclonal anti-CD20

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antibody, with initial response rates ranging between 55 and 100%.[41] However, one should bear in mind that CD20 expression is not confined to the malignant cells, and normal B cells can be also destroyed. Also, neutropenia can occur after the repetitive use of rituximab. Therefore, patients receiving rituximab and who are already immunosuppressed are at increased risk of severe opportunistic infections.[42] However, in the current study, there was no apparent increased risk of infections in those patients receiving pre-emptive rituximab. In our study, we observed no significant difference in OS between patients who received and patients who did not receive pre-emptive treatment with rituximab (data not shown). In those rituximab-resistant patients, chemotherapy with regimens used in lymphoma, such as CHOP, remains a treatment option. Standard CHOP in adult patients with LPD can achieve an overall response rate of 65% and median overall and progression-free survivals of 13.9 and 42 months, respectively.[43] In patients who progress after initial maneuvers, options include T-cell therapies using EBV-specific cytotoxic T lymphocyte lines generated using EBV-transformed lymphoblastoid B-cell lines (38).[13,44] However, despite their efficacy and good safety profile, the use of cytotoxic T lymphocytes is still restricted to few transplant centers worldwide.

In terms of risk factors for EBV reactivation, this study showed that the use of ATG as part of the preparative regimen after RIC allo-HSCT was the most significant factor. As only a few patients received an ATG dosage different than our standard dosage of 5 mg/kg, it was not possible to have a meaningful assessment whether there is a correlation between ATG dose and EBV reactivation. Other studies found a correlation between EBV-reactivation and several factors, such as the degree of HLA mismatch between donor and recipient, manipulation of the graft to deplete T cells, degree and duration of immunosuppression used to prevent and treat GVHD, and the use of ATG and Campath.[22,23] A recent analysis by Savani *et al*.[45] suggested that EBV reactivation and the possible development of PTLD is reduced in patients with lymphoid malignancies treated with rituximab during the course of their disease before allo-SCT. Though this did not reach statistical significance, in our study there was a fewer number of patients with lymphoid malignancies in the group of patients who experienced an episode of EBV reactivation (Table 2).

In our series, the cumulative incidence of EBV reactivation in the subgroup of patients receiving ATG was 25% as compared with 9% in the remaining patients. This data supports the well established mechanism of action of ATG in terms of *in-vivo* partial T-cell depletion.[46] Interestingly, most of EBV reactivation episodes occurred mainly within the first 6 months after allo-HSCT, suggesting that reconstitution of the anti-EBV cytotoxic T lymphocyte-specific response is relatively quick after RIC allo-HSCT, as previously shown for CMV in the same setting.[47] EBV reactivations that occurred beyond 6 months after allo-HSCT were all found in severely immunosuppressed patients treated for extensive chronic GVHD or severe late-onset acute GVHD after donor lymphocyte infusion.

In all, we conclude that patients undergoing RIC allo-HSCT using ATG as part of the preparative regimen are at higher risk for EBV reactivation. However, this did not translate into a significant impact on outcome, as monitoring of EBV viral load using quantitative PCR and early systematic pre-emptive rituximab therapy allowed for significantly reducing the risk of EBV-related LPD.

Conflict of interest

The authors declare no conflict of interest.

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Author contributions

Z Peric: collected and assembled and analyzed data, wrote and revised the manuscript; X Cahu: collected data, performed statistical analysis, and helped writing and revising the manuscript; E Brissot, F Malard: collected and assembled data; T Guillaume, J Delaunay, P Chevallier, S Ayari, V Dubruille, S Le Gouill, B Mahe, T Gastinne, N Blin, JL Harousseau, P Moreau, N Milpied: recruited patients, and commented on the manuscript; B Saulquin: collected and assembled data; M Coste-Burel, BM Imbert-Marcille: performed viral monitoring and commented on the manuscript; M Mohty: supervised research, analyzed data, performed statistical analysis, wrote and revised the manuscript; All authors approved submission of the manuscript for publication purposes.

References

1 Giralt S, Estey E, Albitar M, van Besien K, Rondon G, Anderlini P *et al*. Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood* 1997; **89**: 4531–4536.
2 Slavin S, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G *et al*. Nonmyeloablative stem cell transplantation and cell therapy as an alternative to conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 1998; **91**: 756–763.
3 McSweeney PA, Niederwieser D, Shizuru JA, Sandmaier BM, Molina AJ, Maloney DG *et al*. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. *Blood* 2001; **97**: 3390–3400.
4 Storb R. Reduced-intensity conditioning transplantation in myeloid malignancies. *Curr Opin Oncol* 2009; **21**(Suppl 1): S3–S5.
5 Mohty M, Rocha V, Chevallier P, Harousseau JL, Nagler A. Reduced-intensity conditioning for allogeneic stem cell transplantation: 10 years later. *Curr Opin Oncol* 2009; **21**(Suppl 1): S1.
6 Storb R, Yu C, Wagner JL, Deeg HJ, Nash RA, Kiem HP *et al*. Stable mixed hematopoietic chimerism in DLA-identical littermate dogs given sublethal total body irradiation before and pharmacological immunosuppression after marrow transplantation. *Blood* 1997; **89**: 3048–3054.
7 Shapiro RS, McClain K, Frizzera G, Gajl-Peczalska KJ, Kersey JH, Blazar BR *et al*. Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. *Blood* 1988; **71**: 1234–1243.
8 Micallef IN, Chhanabhai M, Gascoyne RD, Shepherd JD, Fung HC, Nantel SH *et al*. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. *Bone Marrow Transplant* 1998; **22**: 981–987.
9 Hale G, Waldmann H. Risks of developing Epstein-Barr virus-related lymphoproliferative disorders after T-cell-depleted marrow transplants. CAMPATH Users. *Blood* 1998; **91**: 3079–3083.
10 Curtis RE, Travis LB, Rowlings PA, Socie G, Kingma DW, Banks PM *et al*. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. *Blood* 1999; **94**: 2208–2216.
11 Gross TG, Steinbuch M, DeFor T, Shapiro RS, McGlave P, Ramsay NK *et al*. B cell lymphoproliferative disorders following hematopoietic

stem cell transplantation: risk factors, treatment and outcome. *Bone Marrow Transplant* 1999; **23**: 251–258.
12 Juvonen E, Aalto SM, Tarkkanen J, Volin L, Mattila PS, Knuutila S *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**: 97–102.
13 Heslop HE. How I treat EBV lymphoproliferation. *Blood* 2009; **114**: 4002–4008.
14 Gottschalk S, Rooney CM, Heslop HE. Post-transplant lymphoproliferative disorders. *Annu Rev Med* 2005; **56**: 29–44.
15 Lucas KG, Burton RL, Zimmerman SE, Wang J, Cornetta KG, Robertson KA *et al.* 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.
16 Hoshino Y, Kimura H, Tanaka N, Tsuge I, Kudo K, Horibe K *et al.* Prospective monitoring of the Epstein-Barr virus DNA by a real-time quantitative polymerase chain reaction after allogenic stem cell transplantation. *Br J Haematol* 2001; **115**: 105–111.
17 Gartner BC, Schafer H, Marggraff K, Eisele G, Schafer M, Dilloo D *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**: 351–358.
18 van Esser JW, van der Holt B, Meijer E, Niesters HG, Trenschel R, Thijsen SF *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**: 972–978.
19 Lankester AC, van Tol MJ, Vossen JM, Kroes AC, Claas E. Epstein-Barr virus (EBV)-DNA quantification in pediatric allogenic stem cell recipients: prediction of EBV-associated lymphoproliferative disease. *Blood* 2002; **99**: 2630–2631.
20 Wagner HJ, Cheng YC, Huls MH, Gee AP, Kuehnle I, Krance RA *et al.* Prompt versus preemptive intervention for EBV lymphoproliferative disease. *Blood* 2004; **103**: 3979–3981.
21 Blaes AH, Cao Q, Wagner JE, Young JA, Weisdorf DJ, Brunstein CG. Monitoring and preemptive rituximab therapy for epstein-Barr virus reactivation after antithymocyte globulin containing non-myeloablative conditioning for umbilical cord blood transplantation. *Biol Blood Marrow Transplant* 2010; **16**: 287–291.
22 Cohen J, Gandhi M, Naik P, Cubitt D, Rao K, Thaker U *et al.* Increased incidence of EBV-related disease following paediatric stem cell transplantation with reduced-intensity conditioning. *Br J Haematol* 2005; **129**: 229–239.
23 Brunstein CG, Weisdorf DJ, DeFor T, Barker JN, Tolar J, van Burik JA *et al.* Marked increased risk of Epstein-Barr virus-related complications with the addition of antithymocyte globulin to a nonmyeloablative conditioning prior to unrelated umbilical cord blood transplantation. *Blood* 2006; **108**: 2874–2880.
24 Cahu X, Rialland F, Touzeau C, Chevallier P, Guillaume T, Delaunay J *et al.* Infectious complications after unrelated umbilical cord blood transplantation in adult patients with hematologic malignancies. *Biol Blood Marrow Transplant* 2009; **15**: 1531–1537.
25 Mohty M, Bay JO, Faucher C, Choufi B, Bilger K, Tournilhac O *et al.* Graft-versus-host disease following allogeneic transplantation from HLA-identical sibling with antithymocyte globulin-based reduced-intensity preparative regimen. *Blood* 2003; **102**: 470–476.
26 Malard F, Szydlo RM, Brissot E, Chevallier P, Guillaume T, Delaunay J *et al.* Impact of cyclosporine-A concentration on the incidence of severe acute graft-versus-host disease after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2010; **16**: 28–34.
27 Mohty M, Jacot W, Faucher C, Bay JO, Zandotti C, Collet L *et al.* Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin-based reduced intensity preparative regimen. *Leukemia* 2003; **17**: 2168–2177.
28 Chevallier P, Hebia-Fellah I, Planche L, Guillaume T, Bressolette-Bodin C, Coste-Burel M *et al.* Human herpes virus 6 infection is a hallmark of cord blood transplant in adults and may participate to delayed engraftment: a comparison with matched unrelated donors as stem cell source. *Bone Marrow Transplant* 2010; **45**: 1204–1211.
29 Bressollette-Bodin C, Coste-Burel M, Besse B, Andre-Garnier E, Ferre V, Imbert-Marcille BM. Cellular normalization of viral DNA loads on whole blood improves the clinical management of cytomegalovirus or Epstein Barr virus infections in the setting of pre-emptive therapy. *J Med Virol* 2009; **81**: 90–98.
30 Klein JP, Rizzo JD, Zhang MJ, Keiding N. Statistical methods for the analysis and presentation of the results of bone marrow transplants. Part I: unadjusted analysis. *Bone Marrow Transplant* 2001; **28**: 909–915.
31 Fine JP, Gray RJ. A proportional hazards model for subdistribution of a competing risk. *JASA* 1999; **94**: 496–509.
32 Scrucca L, Santucci A, Aversa F. Regression modeling of competing risk using R: an in depth guide for clinicians. *Bone Marrow Transplant* 2010; **45**: 1388–1395.
33 Antin JH, Bierer BE, Smith BR, Ferrara J, Guinan EC, Sieff C *et al.* Selective depletion of bone marrow T lymphocytes with anti-CD5 monoclonal antibodies: effective prophylaxis for graft-versus-host disease in patients with hematologic malignancies. *Blood* 1991; **78**: 2139–2149.
34 Wagner JE, Thompson JS, Carter SL, Kernan NA. Effect of graft-versus-host disease prophylaxis on 3-year disease-free survival in recipients of unrelated donor bone marrow (T-cell depletion trial): a multicentre, randomised phase II-III trial. *Lancet* 2005; **366**: 733–741.
35 Paya CV, Fung JJ, Nalesnik MA, Kieff E, Green M, Gores G *et al.* Epstein-Barr virus-induced posttransplant lymphoproliferative disorders. ASTS/ASTP EBV-PTLD task force and the mayo clinic organized international consensus development meeting. *Transplantation* 1999; **68**: 1517–1525.
36 van Esser JW, Niesters HG, Thijsen SF, Meijer E, Osterhaus AD, Wolthers KC *et al.* 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.
37 Clave E, Agbalika F, Bajzik V, Peffault de Latour R, Trillard M, Rabian C *et al.* Epstein-Barr virus (EBV) reactivation in allogeneic stem-cell transplantation: relationship between viral load, EBV-specific T-cell reconstitution and rituximab therapy. *Transplantation* 2004; **77**: 76–84.
38 Stevens SJ, Verschuuren EA, Verkuujlen SA, Van Den Brule AJ, Meijer CJ, Middeldorp JM. Role of Epstein-Barr virus DNA load monitoring in prevention and early detection of post-transplant lymphoproliferative disease. *Leuk Lymphoma* 2002; **43**: 831–840.
39 Styczynski J, Reusser P, Einsele H, de la Camara R, Cordonnier C, Ward KN *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**: 757–770.
40 Faye A, Quartier P, Reguerre Y, Lutz P, Carret AS, Dehee A *et al.* Chimaeric anti-CD20 monoclonal antibody (rituximab) in post-transplant B-lymphoproliferative disorder following stem cell transplantation in children. *Br J Haematol* 2001; **115**: 112–118.
41 Kuehnle I, Huls MH, Liu Z, Semmelmann M, Krance RA, Brenner MK *et al.* CD20 monoclonal antibody (rituximab) for therapy of Epstein-Barr virus lymphoma after hemopoietic stem-cell transplantation. *Blood* 2000; **95**: 1502–1505.
42 Suzan F, Ammor M, Ribrag V. Fatal reactivation of cytomegalovirus infection after use of rituximab for a post-transplantation lymphoproliferative disorder. *N Engl J Med* 2001; **345**: 1000.
43 Choquet S, Trappe R, Leblond V, Jager U, Davi F, Oertel S. CHOP-21 for the treatment of post-transplant lymphoproliferative disorders (PTLD) following solid organ transplantation. *Haematologica* 2007; **92**: 273–274.
44 Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA *et al.* Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 2010; **115**: 925–935.
45 Savani BN, Pohlmann PR, Jagasia M, Chinratanalab W, Kassim A, Engelhardt B *et al.* Does peritransplantation use of rituximab reduce the risk of EBV reactivation and PTLPD? *Blood* 2009; **113**: 6263–6264.
46 Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia* 2007; **21**: 1387–1394.
47 Mohty M, Mohty AM, Blaise D, Faucher C, Bilger K, Isnardon D *et al.* Cytomegalovirus-specific immune recovery following allogeneic HLA-identical sibling transplantation with reduced-intensity preparative regimen. *Bone Marrow Transplant* 2004; **33**: 839–846.