Medical Microbiology and Immunology (2019) 208:825–834
https://doi.org/10.1007/s00430-019-00629-2

ORIGINAL INVESTIGATION

Clinical utility of measuring Epstein–Barr virus-specific cell-mediated immunity after HSCT in addition to virological monitoring: results from a prospective study

Angela Chiereghin[1] · Giulia Piccirilli[1] · Tamara Belotti[2] · Arcangelo Prete[2] · Clara Bertuzzi[3] · Dino Gibertoni[4] · Liliana Gabrielli[5] · Gabriele Turello[1] · Eva Caterina Borgatti[1] · Francesco Barbato[6] · Mariarosaria Sessa[6] · Mario Arpinati[6] · Francesca Bonifazi[6] · Tiziana Lazzarotto[1]

Received: 27 March 2019 / Accepted: 29 June 2019 / Published online: 9 July 2019

Abstract

Lack of virus-specific cell-mediated immunity (CMI) is associated with worse viral infection outcome in hematopoietic stem cell transplantation (HSCT). We aimed to evaluate the role of immunological monitoring of Epstein–Barr virus (EBV) infection in addition to virological one in 33 adult and 18 pediatric allogeneic HSCT recipients. Virological monitoring of infection was performed on whole blood samples by a quantitative real-time PCR assay. Immunological monitoring was performed by Enzyme-linked ImmunoSPOT assay, evaluating EBV-specific CMI, at fixed time-points and when EBV DNAemia was ≥ 10,000 copies/mL. Fifty-one percent of patients developed a post-transplant EBV infection and reduced-intensity conditioning regimen was the only factor associated to infection ($P = 0.023$). Lack of EBV-specific CMI during active EBV infection was associated with a greater severity of infection. Patients without EBV-specific CMI showed higher median peak level of EBV DNAemia than patients with EBV-specific CMI ($P = 0.014$), and consequently received more frequently, at EBV DNAemia peak, anti-CD20 therapy (0 versus 54.5%, $P = 0.002$). No patients with EBV-specific CMI versus 27.2% without EBV-specific CMI developed EBV-related complications ($P = 0.063$), including two lethal EBV-related post-transplant lymphoproliferative disorders. Combined immunological and virological measurements could improve EBV infection management in HSCT, anticipating the beginning of preemptive treatment from the EBV DNAemia peak to the finding of the lack of EBV-specific CMI.

Keywords Epstein–Barr virus · Allogeneic hematopoietic stem cell transplantation · EBV DNAemia · Virus-specific cell-mediated immunity · EBV-related post-transplant lymphoproliferative disorders

Edited by Matthias J. Reddehase.

Francesca Bonifazi and Tiziana Lazzarotto contributed equally to this work.

✉ Tiziana Lazzarotto
tiziana.lazzarotto@unibo.it

[1] Department of Specialized, Experimental, and Diagnostic Medicine, Operative Unit of Clinical Microbiology, St. Orsola-Malpighi Polyclinic, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

[2] Unit of Pediatric Oncology and Hematology, Department of Pediatrics, St. Orsola-Malpighi Polyclinic, Via Massarenti 9, 40138 Bologna, Italy

[3] Department of Specialized, Experimental, and Diagnostic Medicine, Hematopathology Section, St. Orsola-Malpighi Polyclinic, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

[4] Department of Biomedical and Neuromotor Sciences, Unit of Hygiene and Biostatistics, University of Bologna, Via San Giacomo 12, 40126 Bologna, Italy

[5] Operative Unit of Clinical Microbiology, St. Orsola-Malpighi Polyclinic, Via Massarenti 9, 40138 Bologna, Italy

[6] Institute of Hematology "L. and A. Seràgnoli", St. Orsola-Malpighi Polyclinic, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

Introduction

In hematopoietic stem cell transplant recipients (HSCTRs), Epstein–Barr virus (EBV) infection may progress to the onset of post-transplant lymphoproliferative disorders (PTLD), a heterogeneous group of malignant diseases that are one of the most severe transplant-associated complications [1]. To date, prospective virological surveillance of EBV infection by quantitative PCR assay is recommended in HSCTRs at high risk for EBV-PTLD [1], since the development of EBV-PTLD is mostly accompanied by a significant increase of EBV-DNA levels in the blood of patients [2–4]. It was shown that whole blood (WB) and peripheral blood mononuclear cells (PBMCs) are equally informative in the assessment for the risk to develop EBV-PTLD [5, 6], while plasma has a low sensitivity, limiting its clinical use in the management of EBV infection [7]. Nevertheless, no specific EBV-DNA threshold levels for initiating preemptive therapy have yet been identified [1] and WB and PBMCs PCR assays do not provide information about the host immunological response against the EBV replication [8]. This point is crucial because the efficiency of specific T-cell responses has been already correlated with the outcome of viral infections [9, 10].

Here we report the results of a prospective study evaluating the role of immunological monitoring of EBV infection in the setting of allogeneic HSCT in addition to virological monitoring.

Materials and methods

Study design

This prospective observational study focused on the monitoring of EBV infection after allogeneic HSCT. The primary objective was to assess the usefulness of combined immunological–virological monitoring for improving the management of EBV infection in allogeneic HSCTRs. The potential risk factors for the development of EBV infection were also evaluated.

Inclusion criteria were: patients undergoing allogeneic HSCT from any donors and HSC sources, with a minimum follow-up of 2 months after transplant and written informed consent.

For all patients, the surveillance of EBV infection was performed, for at least 1 year post-transplant, with the following schedule. Virological surveillance was performed on WB samples by a quantitative real-time PCR every week during the first 100 days post-transplant and every 2 weeks until 180 days post-transplant. Blood samples were then investigated if clinically indicated. In the case of positive EBV DNAemia, monitoring was performed weekly for at least 1 month. Immunological surveillance using Enzyme-linked ImmunoSPOT (EliSpot) assay was performed on patient blood samples at four fixed timepoints: at days 60, 100, 180, and 360 after allogeneic HSCT. Afterwards, blood samples were investigated if clinically indicated. Finally, for any patients showing a value of EBV DNAemia $\geq$ 10,000 copies/mL WB, EliSpot assay was performed during the subsequent visit in addition to the fixed schedule.

The study was approved by the Independent Hospital Ethics Committee of the St. Orsola Polyclinic of Bologna and conducted according to the Helsinki declaration.

Definition of active EBV infection and disease

Since EBV-DNA is harbored within latently infected B cells, very low EBV-DNA levels may be present in WB samples in the setting of latent infection. Therefore, EBV reactivation/reinfection was defined as the detection of EBV-DNA > 500 copies/mL WB in at least two consecutive samples. In EBV-seronegative patients at the time of transplant, primary EBV infection was defined as the detection of any values of EBV DNAemia. Based on previous studies and local protocol [6, 11, 12] as well as on the GITMO (Italian Group of Bone Marrow Transplantation) guidelines for the diagnosis and treatment of EBV-PTLD in HSCTRs, any values of EBV-DNA $\geq$ 10,000 copies/mL WB were defined as high viral load. Probable and proven EBV disease was defined according to the European Conference on Infections in Leukemia (ECIL) guidelines [1]. Furthermore, cases of proven EBV-PTLD were classified according to the WHO criteria [13].

Virological and immunological EBV procedures

DNA extraction from WB samples collected in EDTA-anticoagulated tubes was performed using the QIAsymphony SP instrument (Qiagen, Hilden, Germany). Quantification of EBV-DNA was performed by real-time PCR assay (EBV ELITe MGB® kit, ELITech Group, Turin, Italy) on the ABI Prism 7500 real-time PCR System (PE Applied Biosystem, Foster City, CA, USA). The extraction and amplification protocols were previously described [6]. The assay's analytical sensitivity was ten copies of target DNA per amplification reaction. The assay's lower limit of quantification was 225 copies/mL WB.

EBV-specific CMI was assessed by EliSpot assay (EliSpot Interferon-γ Basis Kit; GenID GmbH, Strasburg, France) that enumerates IFN-γ secreting EBV-specific T-cells (both CD4+ and CD8+ cells), at a single cell level, upon in vitro stimulation with latent and lytic viral antigens. A mitogen stimulation and a negative control were included

to determine general T-cell responsiveness and background, respectively. The two commercially available latent and lytic EBV-specific peptide mix used (GenID GmbH, Strasburg, France) as well as the test's procedure and the results' interpretation were described elsewhere [14, 15]. Briefly, a positive EliSpot result identified a patient with detectable EBV-specific CMI; a negative result identified a patient without EBV-specific CMI but with global T-cell responsiveness; results were reported as indeterminate in non-responders of both EBV and mitogen stimulation.

Statistical analysis

Descriptive analysis of patient and transplant characteristics was given and comparisons between patients who developed an active post-transplant EBV infection and not-infected patients were performed by Chi square and Fisher's exact test, as appropriate. Survival estimates were calculated according to Kaplan–Meier method and survival comparisons were performed according to Log-rank test. To deal with the small study population size, the Log-rank test was replicated 10,000 times with random permutations of patients across the two conditions. An estimate of the Log-rank *P* value and of its 95% confidence interval was then provided by the number of times that the estimated Log-rank statistic was greater than the observed Log-rank, divided by the number of valid permutations [16].

For all tests, P values < 0.05 were considered statistically significant. Stata version 15.1 (StataCorp LP, College Station, TX, USA) was used for all analyses.

Results

Patients

Overall, 56 consecutive patients were enrolled between February 2014 and February 2015. Two adult (2/35; 5.7%) and three pediatric (3/21; 14.3%) allogeneic HSCTRs were excluded from the analysis because they died within 2 months post-transplant without developing post-transplant EBV infection. Patient and transplant characteristics of the 33 adult (mean age 40 years; range 18–59) and 18 pediatric (mean age 9 years; range 9 months–17 years) evaluable allogeneic HSCTRs are reported in Table 1. Adult patients received rabbit anti-lymphocyte globulin (ATLG) (Grafalon®, Neovii Biotech GmbH, Graefelfing, Germany), in a total dose ranging from 15 to 30 mg/kg, from day − 6 to − 2. A total of 12 pediatric patients (66.7%) received rabbit anti-thymocyte globulin (ATG) (Thymoglobuline®, Genzyme, Cambridge, MA, USA) in a total dose ranging from 6 to 9 mg/kg over 3 days, from day − 4 to − 2. For adult and pediatric patients, therapy for EBV infection foresaw tapering of immunosuppression when EBV-DNA load was over 10,000 copies/mL WB and then, if ineffective (i.e., increase of EBV-DNA levels and/or clinical progression), foresaw also the administration of anti-CD20 therapy with a maximum of four administrations at a dosage of 375 mg/m^2/week.

Active EBV infection and potential risk factors

Twenty-six (15 adult and 11 pediatric) allogeneic HSCTRs (26/51; 51%) developed an active post-transplant EBV infection. Median time of the first EBV DNAemia detection was 52 (range 20–474) and 48 (range 25–143) days post-transplant in adult and pediatric patients, respectively. Particularly, among the 15 EBV actively infected adult patients (15/33; 45.5%): 11 underwent allogeneic HSCT from unrelated donor (URD; 42.3%, 11/26 patients) and 4 were transplanted from HLA-identical-related donor (57.1%, 4/7 patients). One out of 15 patients (6.7%) was EBV-seronegative at the time of transplant and developed a primary EBV infection; the remaining 14 (14/15, 93.3%) developed EBV reactivation/reinfection. Among the 11 pediatric patients (11/18; 61.1%) who developed a post-transplant EBV reactivation/reinfection: 6 underwent allogeneic HSCT from URD (60%, 6/10 patients) and 5 were transplanted from HLA-identical related donor (62.5%, 5/8 patients); no pediatric patients were EBV-seronegative at time of transplant. Among the 26 actively EBV infected patients, 12 patients (9 adult and 3 pediatric; 46.2%) showed low values of EBV DNAemia (< 10,000 copies/mL WB), whereas 14 patients (6 adult and 8 pediatric; 53.8%) showed values of EBV DNAemia ≥ 10,000 copies/mL WB. Reduced-intensity conditioning regimen was the only factor associated to EBV infection (Table 2).

Combined virological–immunological monitoring of EBV infection as a possible predictive marker of EBV-related complications

The pattern of both T-cell immunity reconstitution and incidence of EBV infection during the first-year post-transplant is reported in Fig. 1a, b. The higher number of indeterminate EliSpot results was observed during the early post-transplant period. Specifically, after 2 months post-transplant (T1), 24.2% of adult and 11.1% of pediatric patients did not demonstrate a global T-cell responsiveness (8/33 and 2/18 patients, respectively). The number of indeterminate EliSpot results decreased in the subsequent time-points and the immune response became more sustained over time; concurrently the percentage of patients with active EBV infection clearly decreased. Notably, compared to adult recipients, pediatric allogeneic HSCTRs had faster CMI reconstitution. Finally, 5 adult patients (5/17; 29.4%) with positive

Table 1 Patient and transplant characteristics of the study population

Characteristics of patients and transplants	ADULT ($n=33$)	PEDIATRIC ($n=18$)	Total
Gender: male/female	21/12	13/5	51
Primary disease (%)			
AML	15 (45.4)	6 (33.3)	21
ALL	7 (21.2)	9 (50)	16
CML	3 (9.1)	–	3
MM	2 (6.1)	–	2
MDS	2 (6.1)	–	2
β-TM	–	2 (11.1)	2
Other	4[a] (12.1)	1[b] (5.0)	5
Donor type (%)			
Matched unrelated donor	26 (78.8)	10 (55.6)	36
Related	7 (21.2)	8 (44.4)	15
Hematopoietic stem cell (%)			
Peripheral blood stem cell	22 (66.7)	2 (11.1)	24
Bone marrow	8 (24.2)	16 (88.9)	24
Cord blood	3 (9.1)	–	3
EBV serology (%)			
D+/R+	23 (69.7)	10 (55.6)	33
D−/R+	4 (12.1)	3 (16.6)	7
D+/R−	1 (3.0)	–	1
Missing[c]	5 (15.2)	5 (27.8)	10
D−/R−	–	–	
Conditioning regimen (%)			
Busulfan-based myeloablative regimen	29 (87.9)	14 (77.8)	43
RIC regimen	3 (9.1)	3 (16.6)	6
TBI-based myeloablative regimen	1 (3.0)	–	1
Treosulfan-based myeloablative regimen	–	1 (5.6)	1
In vivo T-cell depletion (%)			
Yes	33 (100)	12 (66.7)	45
No	–	6 (33.3)	6
GVHD prophylaxis (%)			
CyA + MTX	31 (93.9)	10 (55.6)	41
CyA + MMF	2 (6.1)	1 (5.6)	3
Only CyA	–	7 (38.8)	7

AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; MM, multiple myeloma; MDS, myelodysplastic syndrome; β-TM, beta-thalassemia major; D, donor; R, recipient; +, positive; −, negative; RIC, reduced-intensity conditioning; TBI, total body irradiation; GVHD, graft-versus-host disease; CyA, cyclosporine; MTX, methotrexate; MMF, mycophenolate mofetil

[a]Hodgkin lymphoma, anaplastic large cell lymphoma, diffuse large B-cell lymphoma, myeloid sarcoma

[b]Severe aplastic anemia

[c]Missing: there were five pairs with recipient EBV-seropositive and unknown donor serology

EliSpot result at + 100 day post-transplant (T2) had negative result at the subsequent time-point (T3), showing not constant EBV-specific CMI. The analysis of EliSpot results in relation to both viral loads and patients' clinical outcome is reported in Fig. 2. The EliSpot results obtained during active EBV infection or at the time-point after the onset of infection were negative in 11 [6 adult and 5 pediatric, (11/26; 42.3%)] and positive in 15 [9 adult and 6 pediatric, (15/26; 57.7%)] patients. By comparing the 15 patients with and the 11 patients without detectable EBV-specific CMI, a statistically significant difference was observed regarding the detected median peak viral load [3412 copies/mL versus 55,769 copies/mL WB, respectively ($P=0.014$); data not shown]. In fact, the majority of patients (8/11; 72.7%) with negative EliSpot result showed values of EBV DNAemia ≥ 10,000 copies/mL WB. A statistically significant difference was also

Table 2 Patient characteristics and risk factors for EBV infection—adult and pediatric patients

	Total	EBV DNAemia-negative group	EBV DNAemia-positive group	χ^2 test, *P* value
Number of patients (%)	51 (100)	25 (49)	26 (51)	
Primary disease				
Acute lymphoblastic leukemia	16 (31.4)	7 (43.7)	9 (56.3)	0.924
Acute myeloid leukemia	21 (41.1)	11 (52.4)	10 (47.6)	
Chronic myeloid leukemia	3 (5.9)	1 (33.3)	2 (66.7)	
Other[a]	11 (21.6)	6 (55.5)	5 (45.5)	
Donor type				
Unrelated (matched unrelated donor)	36 (70.6)	19 (52.8)	17 (47.2)	0.406
Related (sibling)	15 (29.4)	6 (40)	9 (60)	
Hematopoietic stem cell				
Bone marrow	24 (88.9)	13 (54.2)	11 (45.8)	0.597[c]
Cord blood	3 (9.1)	2 (66.7)	1 (33.3)	
Peripheral blood	24 (11.1)	10 (41.7)	14 (58.3)	
Conditioning regimen				
Myeloablative[b]	45 (88.2)	25 (55.6)	20 (44.4)	**0.023**[c]
Reduced-intensity	6 (11.8)	0	6 (100)	
In vivo T-cell depletion with anti-lymphocyte/thymocyte immunoglobulin				
Yes	45 (88.2)	22 (48.9)	23 (51.1)	1.000[c]
No	6 (11.8)	3 (50)	3 (50)	
Cytomegalovirus (CMV) infection[d]				
Yes	29 (56.9)	13 (44.8)	16 (55.2)	0.492
No	22 (43.1)	12 (54.5)	10 (45.5)	
Acute graft-versus-host disease (< 100 days post-transplant) grading				
Absent	20 (39.2)	10 (50)	10 (50)	0.986
I	10 (19.6)	5 (50)	5 (50)	
≥ II	21 (41.2)	10 (47.6)	11 (52.4)	
Chronic graft-versus-host disease (> 100 days post-transplant) grading; data available for 50 patients				
Absent	41 (82)	21 (51.2)	20 (48.8)	0.467[c]
Mild-to-severe	9 (18)	3 (33.3)	6 (66.7)	

Statistically significant p-values are in bold typeface

[a]Severe aplastic anemia ($n=1$); beta-thalassemia major ($n=2$); Hodgkin lymphoma ($n=1$); anaplastic large cell lymphoma ($n=1$); multiple myeloma ($n=2$); diffuse large B-cell lymphoma ($n=1$); myeloid sarcoma ($n=1$); myelodysplastic syndrome ($n=2$)

[b]Myeloablative conditioning regimen includes both busulfan-based myeloablative regimen and total body irradiation-based myeloablative regimen

[c]Fisher's exact test

[d]All patients were monitored for CMV infection during the post-transplantation period. CMV real-time PCR was performed weekly during the first 3 months and then monthly until the 6 month. Afterwards, blood samples were processed if clinically indicated

observed regarding the number of patients that received, on the basis of the viral loads detected, anti-CD20 therapy: none with EBV-specific CMI versus 6/11 patients (54.5%) without EBV-specific CMI ($P=0.002$). Finally, none of the 15 patients with EBV-specific CMI versus 3/11 patients (27.2%) without EBV-specific CMI developed EBV-PTLD ($P=0.063$). Patient and transplant characteristics as well as virological, immunological, and clinical data of the six patients who received anti-CD20 therapy are reported in Table 3. In particular, four patients received anti-CD20 as preemptive therapy that successfully prevented EBV-PTLD in 3 of them, whereas one developed an early onset EBV-PTLD with nodal involvement. In these patients, preemptive therapy was administered at the peak of DNA levels. Of note, on the basis of the immunological results obtained, anti-CD20 therapy could have been initiated at least 7 days before (data not shown). Beside patient #1 (Table 3), in which the early use of the anti-CD20 monoclonal antibody successfully treated EBV-PTLD [14], anti-CD20 was administered for treatment in additional two patients failing

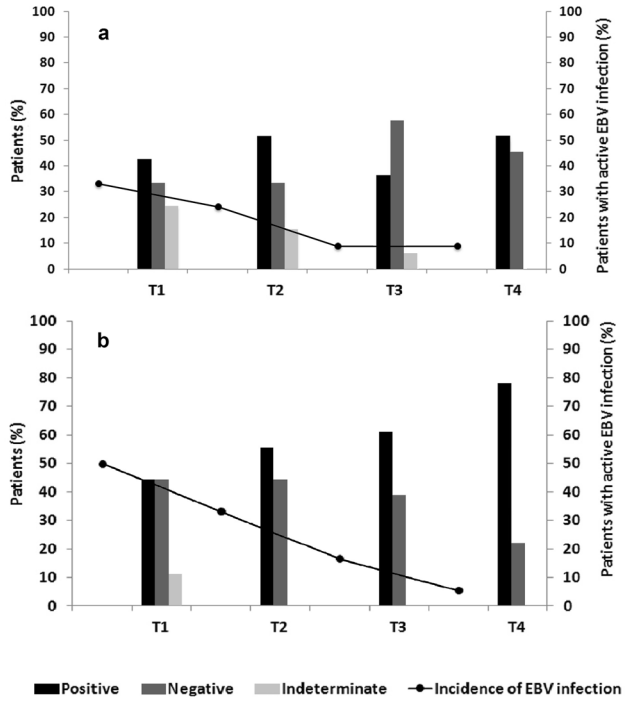

Fig. 1 EliSpot results and incidence of EBV infection—adult (**a**) and pediatric patients (**b**) T1 (+ 60 day), T2 (+ 100 day), T3 (+ 180 day) and T4 (+ 360 day). The EliSpot assay cutoff for positive response to EBV and mitogen stimulation was ≥ 5 spot-forming cells (SFCs)/2 × 10^5 PBMCs (EBV lytic and/or latent antigens minus negative control) and ≥ 50 SFCs/2 × 10^5 PBMCs (mitogen minus negative control), respectively

to resolve fatal EBV-PTLD. In patient #4 (Table 3), an early onset EBV-PTLD with a very aggressive clinical course and clinical signs of overt PTLD before preemptive therapy could be started was observed; the patient died after 13 days from the clinical onset. Patient #2 (Table 3) developed a mononucleosis-like syndrome after 474 days from cord blood transplant with complete response to anti-CD20 lasting 90 days, when a late-onset chemorefractory primary central nervous system EBV lymphoma was diagnosed and caused death 72 days after the onset.

Clinical outcome

During the study period, 10 patients (19.6%; 10/51)—7 adult and 3 pediatric—died at a median time of 227 days (range 58–636) after transplant. Specifically, the causes of death, besides EBV-PTLD ($n=2$), were relapse of the original disease ($n=4$), bacterial pneumonia (imaging diagnosis; $n=2$), sepsis ($n=1$) and renal and respiratory failure ($n=1$). The 2-year survival estimates were 54.2% (95% CI 18.1–80.3%) in actively EBV-infected patients and 76.4% (95% CI 33.5–93.6%) in non-infected patients (Fig. 3), and were not significantly different at the Log-rank test: $\chi^2=1.58$, $P=0.208$, which was confirmed by the permutation test ($P=0.209$, 95% CI 0.202–0.218).

Discussion

Despite the introduction of prospective virological surveillance of EBV infection, preemptive therapy and timely treatment with anti-CD20, EBV-PTLD-related mortality still remains high; approximately 1/3 of diagnosed patients [1, 17]. In this prospective study, we confirmed that in patients at higher risk of developing EBV-related complications immunological measurements, in addition to the virological one, could improve the management of EBV infection after HSCT providing useful information for clinical decision-making. In particular, it could be used to anticipate the initiation of the preemptive treatment.

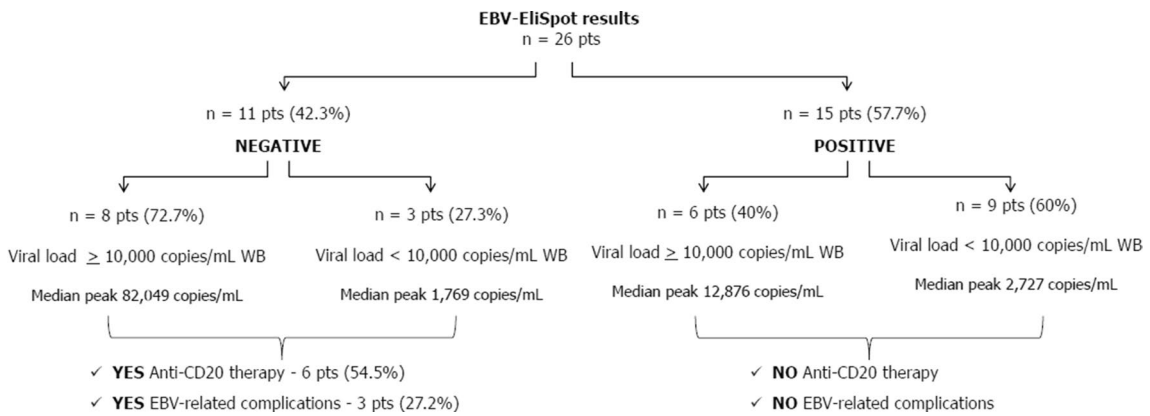

Fig. 2 Results of combined virological–immunological monitoring of EBV infection, management of infection and patient clinical outcome in the 26 EBV actively infected adult and pediatric patients (pts). The EliSpot results obtained during active EBV infection or at the time-point after the onset of infection were taken into account. *WB* whole blood

Table 3 Virological, immunological, and clinical data of the six patients who received anti-CD20 therapy

Pt	Age (years)	Sex	Donor type	Graft origin	EBV serostatus D/R	Onset of EBV infection—days post-TX	EBV DNAemia whole blood min–max copies/mL	EliSpot result[a]	Management of EBV infection Anti-CD20 therapy	EBV-related complications—days post-TX	Outcome
1	40	M	URD	BM	D?/R+	40	522–119,039	Negative	Preemptive (1 dose) Treatment (3 doses)	Nodal polymorphic and monomorphic DLBCL-like—65	Complete disease remission Alive
2	41	F	URD	CB	D?/R+	474	1837–74,374	Negative	Treatment (3 doses)	Mononucleosis-like syndrome (fever and pharyngitis)—474	Dead 636 days post-TX
								Negative	Anti-CD20 therapy + high dose methotrexate and cytarabine	Extra-nodal monomorphic DLBCL-like involving central nervous system—564	
3	34	F	URD	PBSC	D+/R+	62	4650–55,769	Negative	Preemptive (2 doses)	No	Alive
4	15	M	Related sibling	PBSC	D+/R+	33	872–2,011,688	Negative	Treatment (2 doses)	Nodal monomorphic DLBCL-like Extra-nodal PTLD involving stomach and large bowel—45	Dead 58 days post-TX
5	4	M	URD	BM	D+/R+	44	631–683,294	Negative	Preemptive (2 doses)	No	Alive
6	10	M	URD	PBSC	D+/R+	28	1120–89,724	Negative	Preemptive (2 doses)	No	Alive

Pt, patient; M, male; F, female; URD, unrelated donor; BM, bone marrow; CB, cord blood; PBSC, peripheral blood stem cell; D, donor; D?, donor not available; R, recipient; TX, transplant; DLBCL, diffuse large B-cell lymphoma

[a]Obtained during active EBV infection

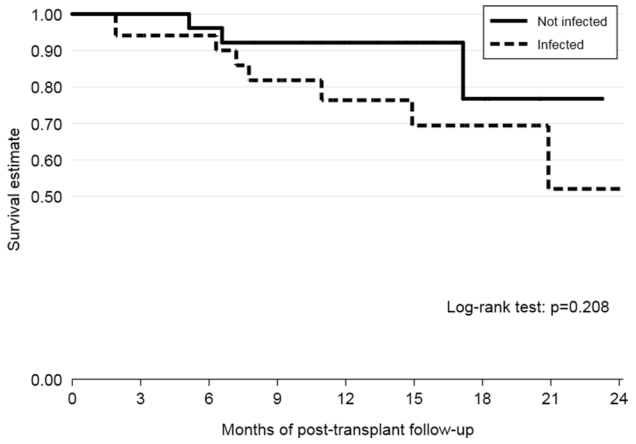

Fig. 3 Kaplan–Meier survival curves of adult and pediatric patients by EBV reactivation

As reported in other studies [18, 19], post-transplant active EBV infection was found to be a frequent event in the study population. Furthermore, EBV infection with high levels of EBV DNAemia was observed in 53.8% of cases. As expected, EBV infection occurred during the early post-engraftment phase. However, we found that even after a longer time after transplant, EBV DNAemia can be detected (Patient #2) and lead to a fatal EBV-PTLD. The finding of late-onset EBV-PTLD highlights the importance of continuing virological monitoring of EBV infection for patients who may be at high risk of developing EBV-related complications [11].

The role of in vivo T-cell depletion with ATLG/ATG is still controversial [20–22]. The impact of in vivo T-cell depletion depends on several factors such as the dose and the timing of ATLG/ATG [23, 24] and the age of transplant [22, 24], being more evident in the pediatric population. In our analysis, the impact of in vivo T-cell depletion is not associated with an increase of episodes of active post-transplant EBV infection. One possible explanation is that two-thirds of the study population is represented by adults treated with low doses of ATLG in accordance with previous reports [21, 24]. In our analysis, the only factor found to be significantly associated with higher frequencies of EBV infection was the intensity of conditioning. As other authors [12], we hypothesized that in reduced-intensity conditioning patients, the lower chemotherapy intensity left surviving recipient B cells, which are latently infected with EBV.

Consistent with the literature data [25], using anti-CD20 as preemptive therapy was effective in controlling viral proliferation and avoiding progression into EBV-PTLD in 75% of cases. An overall biopsy-proven EBV-PTLD frequency equal to 5.9% was observed, which is within the range of incidence reported in other studies [1, 26]. The overall treatment response to anti-CD20 was 33.3%. Styczynski et al. recently reported that the administration of anti-CD20 results in a positive outcome for approximately 65% of patients with EBV-PTLD [1]. Notably, two out of the three cases presented in our study, i.e., EBV-PTLD with fulminant feature and central nervous system EBV-PTLD, are very rare events [27–29]. Furthermore, PTLD with isolated central nervous system involvement is thought to be the most unfavorable localization with respect to outcome and no standard therapy has been actually accepted [1, 28, 29].

Regarding post-transplant CMI, as expected, the higher number of cases in which a complete absence of IFN-γ responses was obtained was at the first immunological measurement (+ 60 day post-transplant). The lack of global CMI could be attributed to the use of ATLG/ATG, which is known to may have a detrimental effect on T-cells immune recovery [30]. Of note, a faster CMI reconstitution was observed in pediatric patients compared with the adults. Particularly, age related involution of the thymus is a well-known physiologic phenomenon and many studies have demonstrated the impact of age on thymic output showing that the age was positively correlated with the onset of thymic activity after HSCT [31]. Such different timing of immune reconstitution was also observed by other investigators [32]. Regarding the virus-specific T-cell responses, at + 60 day post-transplant, almost the half of the patients showed EBV-specific CMI. Since it has been reported that viral infection may trigger the host immune response [33], this finding may reflect the rate of EBV infection occurred during the first 2 months after transplant. Notably, as reported by other authors in healthy individuals [33], not constant EBV-specific CMI was observed during the immunological post-transplant surveillance.

Despite the immunodominance hierarchies of EBV proteins are well established in healthy virus carriers [34], by analyzing the EliSpot responses to latent and lytic EBV-specific antigens in the study population, a distinct pattern of distribution of EBV lytic and latent-specific T-cell responses was not observed (data not shown). Similarly, other authors observed that the distribution of EBV-specific T-cell responses in solid organ transplant recipients was altered when compared to that observed in healthy individuals [35].

A greater severity of EBV infection was associated with lack of EBV-specific CMI during active EBV infection or at the time-point after the onset of infection. In fact, significantly higher median peak level of EBV DNAemia was detected in patients without detectable EBV-specific CMI, compared to the one detected in patients with detectable EBV-specific CMI. Moreover, anti-CD20 therapy administration was necessary for the majority of the patients without detectable EBV-specific CMI since they were not able to control EBV replication. Finally, 27.2% of these patients developed virus-related complications, including two lethal PTLD. Contrastingly, all patients with detectable

EBV-specific CMI controlled EBV replication without anti-CD20 therapy administration and none of the patients had EBV-related signs/symptoms. These results confirm that antiviral immune reconstitution after transplant is crucial for the control of EBV infection and, consequently, for the infection progression to overt disease [32, 36].

As in other studies [19, 37], adverse effects of EBV active infection on patient survival were not observed; the mortality due to EBV-PTLD was 3.9%. The relapse of the original disease was the major cause of death (44.4% of cases).

In conclusion, in light of the results obtained, we retain that high viral load (EBV DNAemia ≥ 10,000 copies/mL WB) without EBV-related signs/symptoms in combination with lack of EBV-specific T-cell response could represent the indication for timely preemptive interventions such as reduction of immunosuppression and/or administration of anti-CD20 therapy. This approach, combining virological and immunological monitoring, allows custom-tailored therapies against EBV infection after HSCT, with a fine-tuning of the clinical intervention strategies for each single transplant patient.

Acknowledgements This work is based on the PhD thesis of Dr. Angela Chiereghin, available at http://amsdottorato.unibo.it/7611/1/Chiereghin_Angela_Tesi.pdf. The authors thank the Linguistic Consultant, Lucy Scioscia, for editing the English language text.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The study was approved by the Independent Hospital Ethics Committee of St. Orsola-Malpighi Polyclinic, University of Bologna and was conducted in accordance with the 1964 Helsinki Declaration and its later amendments. Informed consent was obtained from all individual adult participants included in the study, as well as from the parents of the pediatric participants.

References

1. Styczynski J, van der Velden W, Fox CP, Engelhard D, de la Camara R, Cordonnier C et al (2016) Management of Epstein–Barr Virus infections and post-transplant lymphoproliferative disorders in patients after allogeneic hematopoietic stem cell transplantation: sixth European Conference on Infections in Leukemia (ECIL-6) guidelines. Haematologica 101:803–811
2. Gärtner BC, Schäfer H, Marggraff K, Eisele G, Schäfer M, Dilloo D et al (2002) 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 40:351–358
3. van Esser JW, van der Holt B, Meijer E, Niesters HG, Trenschel R, Thijsen SF et al (2001) 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 98:972–978
4. Kinch A, Oberg G, Arvidson J, Falk KI, Linde A, Pauksens K (2007) Post-transplant lymphoproliferative disease and other Epstein–Barr virus diseases in allogeneic haematopoietic stem cell transplantation after introduction of monitoring of viral load by polymerase chain reaction. Scand J Infect Dis 39:235–244
5. Baldanti F, Gatti M, Furione M, Paolucci S, Tinelli C, Comoli P et al (2008) Kinetics of Epstein–Barr virus DNA load in different blood compartments of pediatric recipients of T-cell-depleted HLA-haploidentical stem cell transplantation. J Clin Microbiol 46:3672–3677
6. Chiereghin A, Prete A, Belotti T, Gibertoni D, Piccirilli G, Gabrielli L et al (2016) Prospective Epstein–Barr virus-related post-transplant lymphoproliferative disorder prevention program in pediatric allogeneic hematopoietic stem cell transplant: virological monitoring and first-line treatment. Transpl Infect Dis 18:44–54
7. Lazzarotto T, Chiereghin A, Piralla A, Piccirilli G, Girello A, Campanini G et al (2018) Cytomegalovirus and Epstein–Barr virus DNA kinetics in whole blood and plasma of allogeneic hematopoietic stem cell transplantation recipients. Biol Blood Marrow Transplant 24:1699–1706
8. Calarota SA, Aberle JH, Puchhammer-Stöckl E, Baldanti F (2015) Approaches for monitoring of non virus-specific and virus-specific T-cell response in solid organ transplantation and their clinical applications. J Clin Virol 70:109–119
9. Remakus S, Sigal LJ (2013) Memory CD8(+) T cell protection. Adv Exp Med Biol 785:77–86
10. Sant AJ, McMichael A (2012) Revealing the role of CD4(+) T cells in viral immunity. J Exp Med 209:1391–1395
11. Patriarca F, Medeot M, Isola M, Battista ML, Sperotto A, Pipan C et al (2013) Prognostic factors and outcome of Epstein–Barr virus DNAemia in high-risk recipients of allogeneic stem cell transplantation treated with preemptive rituximab. Transpl Infect Dis 15:259–267
12. Uhlin M, Wikell H, Sundin M, Blennow O, Maeurer M, Ringden O et al (2014) Risk factors for Epstein–Barr virus-related post-transplant lymphoproliferative disease after allogeneic hematopoietic stem cell transplantation. Haematologica 99:346–352
13. Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H et al (2008) WHO classification of tumours of haematopietic and lymphoid tissues. Revised 4th edn. Volume 2
14. Chiereghin A, Bertuzzi C, Piccirilli G, Gabrielli L, Squarzoni D, Turello G et al (2016) Successful management of EBV-PTLD in allogeneic bone marrow transplant recipient by virological-immunological monitoring of EBV infection, prompt diagnosis and early treatment. Transpl Immunol 34:60–64
15. Chiereghin A, Gabrielli L, Zanfi C, Petrisli E, Lauro A, Piccirilli G et al (2010) Monitoring cytomegalovirus T-cell immunity in small bowel/multivisceral transplant recipients. Transplant Proc 42:69–73
16. Simon RM, Subramanian J, Li MC, Menezes S (2011) Using cross-validation to evaluate predictive accuracy of survival risk classifiers based on high-dimensional data. Brief Bioinform 12:203–214
17. Styczynski J, Gil L, Tridello G, Ljungman P, Donnelly JP, van der Velden W et al (2013) Response to rituximab-based therapy and risk factor analysis in Epstein Barr virus-related lymphoproliferative disorder after hematopoietic stem cell transplant in children and adults: a study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Clin Infect Dis 57:794–802
18. Carpenter B, Haque T, Dimopoulou M, Atkinson C, Roughton M, Grace S et al (2010) Incidence and dynamics of Epstein–Barr virus reactivation after alemtuzumab based conditioning for allogeneic hematopoietic stem cell transplantation. Transplantation 90:564–570

19. Cesaro S, Pegoraro A, Tridello G, Calore E, Pillon M, Varotto S et al (2010) A prospective study on modulation of immunosuppression for Epstein–Barr virus reactivation in pediatric patients who underwent unrelated hematopoietic stem cell transplantation. Transplantation 89:1533–1540
20. Finke J, Schmoor C, Bethge WA, Ottinger H, StelljesM Volin L et al (2017) Long-term outcomes after standard graft-versus-host disease prophylaxis with or without anti-human-T-lymphocyte immunoglobulin in haemopoietic cell transplantation from matched unrelated donors: final results of a randomised controlled trial. Lancet Haematol 4:e293–e301
21. Kröger N, Solano C, Wolschke C, Bandini G, Patriarca F, Pini M et al (2016) Antilymphocyte globulin for prevention of chronic graft-versus-host disease. N Engl J Med 374:43–53
22. Locatelli F, Bernardo ME, Bertaina A, Rognoni C, Comoli P, Rovelli A et al (2017) Efficacy of two different doses of rabbit anti-T-lymphocyte globulin to prevent graft-versus-host disease in children with haematological malignancies transplanted from an unrelated donor: a multicentre, randomised, open-label, phase 3 trial. Lancet Oncol 18:1126–1136
23. Admiraal R, Nierkens S, de Witte MA, Petersen EJ, Fleurke GJ, Verrest L et al (2017) Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a retrospective, pharmacodynamic cohort analysis. Lancet Haematol 4:e183–e191
24. Bonifazi F, Olivieri J, Sessa M, Dan E, Sinigaglia B, Rizzi S et al (2018) Low-dose anti-T lymphoglobulin as prophylaxis for graft-versus-host disease in unrelated donor transplantations for acute leukemias and myelodysplastic syndromes. Biol Blood Marrow Transplant 24:2450–2458
25. Styczynski J, Einsele H, Gil L, Ljungman P (2009) Outcome of treatment of Epstein–Barr virus-related post-transplant lymphoproliferative disorder in hematopoietic stem cell recipients: a comprehensive review of reported cases. Transpl Infect Dis 11:383–392
26. Rouce RH, Louis CU, Heslop HE (2014) EBV lymphoproliferative disease after hematopoietic stem cell transplant. Curr Opin Hematol 21:476–481
27. Gasiorowski R, Gibson J, Watson G, Trotman J, Larsen S (2013) Rapidly fatal post-allogeneic stem cell transplant lymphoproliferative disorder presenting with skin and bone marrow involvement. Pathology 45:81–83
28. Wróblewska M, Gil LA, Komarnicki MA (2015) Successful treatment of Epstein–Barr virus-related post-transplant lymphoproliferative disease with central nervous system involvement following allogeneic haematopoietic stem cell transplantation—a case study. Cent Eur J Immunol 40:122–125
29. Czyzewski K, Styczynski J, Krenska A, Debski R, Zajac-Spychala O, Wachowiak J et al (2013) Intrathecal therapy with rituximab in central nervous system involvement of post-transplant lymphoproliferative disorder. Leuk Lymphoma 54:503–506
30. Bosch M, Dhadda M, Hoegh-Petersen M, Liu Y, Hagel LM, Podgorny P et al (2012) Immune reconstitution after antithymocyte globulin-conditioned hematopoietic cell transplantation. Cytotherapy 14:1258–1275
31. Gaballa A, Sundin M, Stikvoort A, Abumaree M, Uzunel M, Sairafi D et al (2016) T cell receptor excision circle (TREC) monitoring after allogeneic stem cell transplantation; a predictive marker for complications and clinical outcome. Int J Mol Sci 17:1705
32. D'Aveni M, Aïssi-Rothé L, Venard V, Salmon A, Falenga A, Decot V et al (2011) The clinical value of concomitant Epstein Barr virus (EBV)-DNA load and specific immune reconstitution monitoring after allogeneic hematopoietic stem cell transplantation. Transpl Immunol 24:224–232
33. Vogl BA, Fagin U, Nerbas L, Schlenke P, Lamprecht P, Jabs WJ (2012) Longitudinal analysis of frequency and reactivity of Epstein–Barr virus-specific T lymphocytes and their association with intermittent viral reactivation. J Med Virol 84:119–131
34. Hislop AD, Taylor GS, Sauce D, Rickinson AB (2007) Cellular responses to viral infection in humans: lessons from Epstein–Barr virus. Annu Rev Immunol 25:587–617
35. Calarota SA, Chiesa A, Zelini P, Comolli G, Minoli L, Baldanti F (2013) Detection of Epstein–Barr virus-specific memory CD4+ T cells using a peptide-based cultured enzyme-linked immunospot assay. Immunology 139:533–544
36. Annels NE, Kalpoe JS, Bredius RG, Claas EC, Kroes AC, Hislop AD et al (2006) 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 42:1743–1748
37. Peric Z, Cahu X, Chevallier P, Brissot E, Malard F, Guillaume T et al (2011) Features of Epstein–Barr virus reactivation after reduced intensity conditioning allogeneic hematopoietic stem cell transplantation. Leukemia 25:932–938