Management of Epstein–Barr virus-related post-transplant lymphoproliferative disorder after allogeneic hematopoietic stem cell transplantation

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Abstract: Epstein–Barr virus-related post-transplant lymphoproliferative disorder (EBV-PTLD) is a rare but life-threatening complication after allogeneic hematopoietic stem cell transplantation (allo-HSCT). T-cell immunodeficiency after transplantation and EBV primary infection/reactivation play major roles in the pathogenesis. Unspecific clinical manifestations make the diagnosis difficult and time consuming. Moreover, this fatal disease usually progresses rapidly, and leads to multiple organ dysfunction or death if not treated promptly. Early diagnosis of EBV-DNAemia or EBV-PTLD generally increases the chances of successful treatment by focusing on regular monitoring of EBV-DNA and detection of symptomatic patients as early as possible. Rituximab ± reduction of immunosuppression (RI) is currently the first-line choice in preemptive intervention and targeted treatment. Unless patients are suffering from severe graft *versus* host disease (GvHD), it is better to combine rituximab with RI. Once a probable diagnosis is made, the first-line treatment should be initiated rapidly, along with, or ahead of, biopsy, although histopathologic confirmation is requisite. In addition, EBV-specific cytotoxic T lymphocytes (EBV-CTLs) or donor lymphocyte infusion (DLI) has shown promise in cases of suboptimal response. Chemotherapy ± rituximab might lend more opportunities to refractory/relapsed patients, who might also benefit from ongoing clinical trials. Herein, we discuss our clinical experience in detail based on the current literature and our five cases.

Keywords: allogeneic hematopoietic stem cell transplantation, Epstein–Barr virus, management, post-transplant lymphoproliferative disorder

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Introduction

Post-transplant lymphoproliferative disorder (PTLD), ranging from nondestructive lymphoplasmacytic proliferation to malignant lymphoma, is strongly related to Epstein–Barr virus (called EBV-PTLD) under the status of immunodeficiency that occurs following hematopoietic stem cell transplantation (HSCT) or solid organ transplantation (SOT).[1,2] Recipients of allogeneic HSCT (allo-HSCT) are at a particular risk of PTLD due to their profound immunodeficiency. EBV-PTLD develops in fewer than 1% of recipients without risk factors, but in more than 10% of those with several risk factors.[3–5] PTLD after allo-HSCT is almost exclusively EBV positive, although rare cases of EBV-negative PTLD are reported.[2,6,7] With the growing number of allo-HSCT recipients, post-HSCT EBV-PTLD has attracted increasing attention.[2]

Both diagnostic and therapeutic strategies for EBV-PTLD have evolved over time. Although clinical manifestations are unspecific, monitoring the EBV-DNA load in peripheral blood offers an

Table 1. Clinical characteristics of cases.

Case number	Gender	Age (years)	Underlying disease	Pre-transplant risk factors	Post-transplant risk factors	Time of EBV-DNA ≥1000 copies/ml	Diagnostic basis	Diagnosis	Therapeutic strategies	Clinical outcome
Case 1	F	60	CMML-2 (NR)	(1) Haplo-HSCT; (2) ATG use	(1) grade III acute GvHD	day +99	(1) Abnormal EBV-DNA loads in the peripheral blood	EBV-DNAaemia	(1) Rituximab (2) Donor-derived EBV-CTLs	Alive
Case 2	F	23	SAA	(1) ATG use	–	day +40	(1) Fever and Lymphadenopathy; (2) Abnormal EBV-DNA loads in the peripheral blood; (3) Histopathologic examination with EBER+	Proven EBV-PTLD	(1) Rituximab + RI	Alive
Case 3	M	36	AML (CR1)	(1) Haplo-HSCT; (2) ATG use	(1) grade III acute GvHD	NA	(1) bloody stools; (2) Histopathologic examination with EBER+	Proven EBV-PTLD	(1) Rituximab	Alive
Case 4	M	21	AML (CR1)	(1) Haplo-HSCT; (2) ATG use	–	day +157	(1) Abnormal EBV-DNA loads in the peripheral blood and stool; (2) Extranodal lesions in the liver and the spleen showed by CT and 18F-FDG-PET/CT; (3) Histopathologic examination with EBER+	Proven EBV-PTLD	(1) Rituximab; (2) Donor-derived EBV-CTLs; (3) R-COP	Alive
Case 5	F	20	ALL (CR1)	(1) MUD; (2) ATG use	–	day +49	(1) Fever, headache, severe emesis and lymphadenopathy; (2) Abnormal EBV-DNA loads in the peripheral blood and CSF; (3) Histopathologic evidence of post-transplant DLBCL; (4) symmetrical, extensive, supra- and infra-tentorium lesions showed by MRI	Proven EBV-PTLD	Rituximab (intravenously and intrathecally administered)	Alive

ALL, acute lymphocytic leukaemia; AML, acute myelogenous leukaemia; ATG, anti-thymocyte globulin; CMML-2, type 2 chronic myelomonocytic leukemia; CR1, first complete remission; CSF, cerebrospinal fluid; CT, computed tomography; DLBCL, diffuse large B-cell lymphoma; EBER+, EBV-encoded RNA positive; EBV, Epstein–Barr Virus; F, female; 18F-FDG-PET/CT, radionuclide fluorine 18-fluorodeoxyglucose-positron emission tomography/computed tomography; EBV-CTLs, EBV-specific cytotoxic T lymphocytes; GvHD, graft *versus* host disease; HLA, human leukocyte antigen; M, male; MRI, magnetic resonance imaging; MUD, matched unrelated donor; NA, not available; NR, not remitted; R-COP, rituximab + chemotherapy regimen of cyclophosphamide, vincristine and prednisone; SAA, severe aplastic anaemia.

indication for preemptive intervention.[2] Preemptive rituximab ± reduction of immunosuppression (RI) is effective in preventing EBV-DNAemia from progressing to EBV-PTLD, but it is hard to define the optimal point of therapy initiation with minimal adverse effects. Histopathologic evidence is vital, but accurate histopathologic classification is not always available due to the overlap of characteristics and coexistence of multiple subtypes, even within a single biopsy sample.[1] Rituximab ± RI, adoptive cellular therapy utilizing EBV-specific cytotoxic T lymphocytes (EBV-CTLs) or donor lymphocyte infusion (DLI) and chemotherapy have achieved favorable effects.[2,8] However, this disease progresses very rapidly and often leaves limited time for diagnosis and treatment; long-term survival of EBV-PTLD remains unsatisfactory.[3,9,10]

Based on our five cases (Table 1), we focus mainly on current perspectives and challenges in the management of EBV-PTLD after allo-HSCT.

Case 1

A 60-year-old woman was diagnosed with type 2 chronic myelomonocytic leukemia (CMML-2). She underwent three cycles of decitabine (DAC) with sorafenib and then haploidentical HSCT (haplo-HSCT) in September 2017, following DAC + busulfan (Bu) + cyclophosphamide (Cy) + fludarabine (Flu) + cytarabine (Ara-C) conditioning. Cyclosporine (CsA) + mycophenolate mofetil (MMF) + methotrexate (MTX) + rabbit anti-thymocyte globulin (r-ATG) (10 mg/kg) was used to prevent graft *versus* host disease (GvHD). Neutrophils were more than 0.5×10^9/l on day +13. EBV-DNA in her blood reached 1096 copies/ml on day +99, and kept increasing while she had grade III gastrointestinal acute GvHD (treated with CsA + ruxolitinib + methylprednisolone) and active cytomegalovirus (CMV) infection (treated with intravenous CMV neutralizing immunoglobulin + ganciclovir). Without any symptoms or signs, she received three doses of preemptive rituximab (375 mg/m², weekly). However, her EBV-DNA loads continued to increase (60,969 copies/ml on day +127). Donor-derived EBV-CTLs were then employed for the management of EBV-DNAemia. Finally, EBV-DNA returned to normal levels after two further courses of rituximab and four doses of EBV-CTL infusion (2.5×10^7, 2×10^7, 3.8×10^7, and 3×10^7) without the exac-

erbation of GvHD. The patient is currently surviving with normal EBV-DNA levels.

For EBV-DNAemia after allo-HSCT, rituximab ± RI would eliminate the reactivated viruses for most patients. However, when patients respond poorly to rituximab ± RI, EBV-CTLs or DLI should be considered as early as possible, as in the targeted treatment.

Case 2

A 23-year-old woman was diagnosed with severe aplastic anemia in March 2008. She underwent matched related allo-HSCT following Bu + Flu + CTX + r-ATG (10 mg/kg) conditioning in March 2016. GvHD prevention comprised CsA and MTX. Neutrophil recovery was achieved on day +12. From day +34, the patient complained of fever that did not respond to cefoperazone-tazobactam and oseltamivir. Physical examination revealed enlarged and tender lymph nodes on both sides of her retroauricular, submandibular, and neck region on day +40. Computed tomography (CT) showed enlarged lymph nodes in the axillae, mediastinum, retroperitoneum, pelvic cavity, and groins. The EBV-DNA load was 117,532 copies/ml on day +40. A diagnosis of probable EBV-PTLD was made, and rituximab (375 mg/m², weekly) + reduction of CsA was initiated immediately. Subsequent biopsy of the enlarged lymph node indicated polymorphic PTLD [EBV-encoded RNA positive (EBER⁺)]. After three doses of rituximab, the patient's EBV-DNA levels returned to normal, with symptoms resolved and lymph nodes shrunken. The patient has subsequently survived free from EBV-PTLD.

The first-line treatment, rituximab ± RI, should be initiated as soon as the probable diagnosis is made, even though histopathologic confirmation would need more time. During treatment, imaging examinations and biopsy should be completed whenever feasible to confirm the diagnosis.

Case 3

The patient was a 36-year-old man diagnosed with acute myelogenous leukemia (AML). He reached complete remission (CR) after one cycle of induction chemotherapy, and accepted two cycles of consolidation chemotherapy. Afterwards,
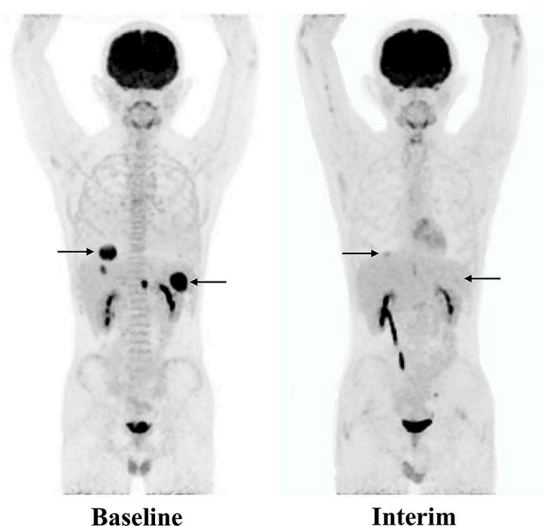
Baseline　　　　**Interim**

Figure 1. ^{18}F-FDG–PET/CT images (maximum-intensity projection).
Baseline images showed extranodal lesions in the liver (SUVmax, 17.6) and the spleen (SUVmax, 19.6) (left, arrows); ^{18}F-FDG–PET/CT after three cycles of R-COP and four doses of EBV-CTLs showed limited residual hypermetabolic lesions in the liver (SUVmax, 3.7) and the spleen (SUVmax, 3.4) (right, arrows).
^{18}F, radionuclide fluorine 18; FDG, fluorodeoxyglucose; PET, positron emission tomography; CT, computed tomography; R, rituximab; COP, regimen including cyclophosphamide, vincristine and prednisone; EBV-CTLs, Epstein–Barr virus specific cytotoxic T lymphocytes; SUVmax, standard uptake value maximum.

he underwent haplo-HSCT (conditioned with Bu + Flu) in June 2018 and received MTX + CsA + MMF + r-ATG (7.5 mg/kg) for GvHD prevention. Hematopoiesis was reconstituted on day +10. Acute GvHD (grade III) occurred on day +18, involving predominantly the skin and gastrointestinal tract. Diarrhea was alleviated following methylprednisolone, oral budesonide, and ruxolitinib but worsened with bloody stools on day +46 when the patient was on oral budesonide and ruxolitinib. EBV-DNA loads were positive in stools from day +61 but negative in peripheral blood. Enteroscopy revealed erosion and anabrosis involving the ileocaecum and ileocaecal valve, the biopsy results of which turned out to be EBER^{+} polymorphic PTLD. After four cycles of rituximab, ^{18}F-FDG-PET/CT confirmed the status of CR. The patient has been in continual CR to the latest follow-up.

Clinicians must never forget EBV-PTLD in differential diagnosis, especially when patients suffer from changes during the process of improvement. Although it is certainly helpful to monitor EBV-DNA in the peripheral blood of patients after allo-HSCT, there are still a few cases of proven EBV-PTLD without EBV-DNAemia. In that condition, other detection methods, such as exfoliative cytology, imaging examinations, enteroscopy, and biopsy, may provide complementary proof.

Case 4

A 21-year-old man with AML reached CR after the first cycle of chemotherapy. Then, he received two cycles of consolidation chemotherapy and underwent haplo-HSCT in January 2018 with Ara-C + Bu + Cy + semustine in condition and MTX + CsA + MMF + r-ATG (7.5 mg/kg) for GvHD prevention. Hematopoiesis was reconstituted on day +10. He suffered from acute GvHD (skin, grade II), which faded after CsA and MTX. The regimen was then adjusted into tacrolimus, methylprednisolone, and ruxolitinib due to hemorrhagic cystitis with recurrent fever and cutaneous chronic GvHD. The EBV-DNA load in the blood was 7490 copies/ml on day +157 and continued to increase thereafter. After four cycles of rituximab, EBV-DNA became negative. Accidentally, CT revealed multiple low-density nodules in the patient's liver on day +191, among which the largest nodule was 3.5×3.2 cm. ^{18}F-FDG-PET/CT showed extranodal lesions in the liver [standard uptake value maximum (SUVmax), 17.6] and the spleen (SUVmax, 19.6) (Figure 1, left). Biopsy of the liver revealed an EBER^{+} post-transplant diffuse large B-cell lymphoma (DLBCL). Donor-derived EBV-CTLs (2×10^{7}/kg, weekly) and rituximab (R) + cyclophosphamide, vincristine and prednisone (COP) regimen (every 21 days) was applied. After four doses of EBV-CTLs and three courses of R-COP, ^{18}F-FDG-PET/CT showed limited residual hypermetabolic lesions in the liver (SUVmax 3.7) and the spleen (SUVmax 3.4) (Figure 1, right). The patient finally reached CR and has since been free of EBV-PTLD.

The patient was not misdiagnosed thanks to the accidental CT scan. It has been stressed that CT or 18F-FDG-PET/CT should play a role in the diagnosis of probable or proven EBV-PTLD. Additionally, clinicians sometimes need to evaluate the necessity of CT or 18F-FDG-PET/CT for asymptomatic patients with positive blood tests for EBV-DNA, since clinical manifestations of
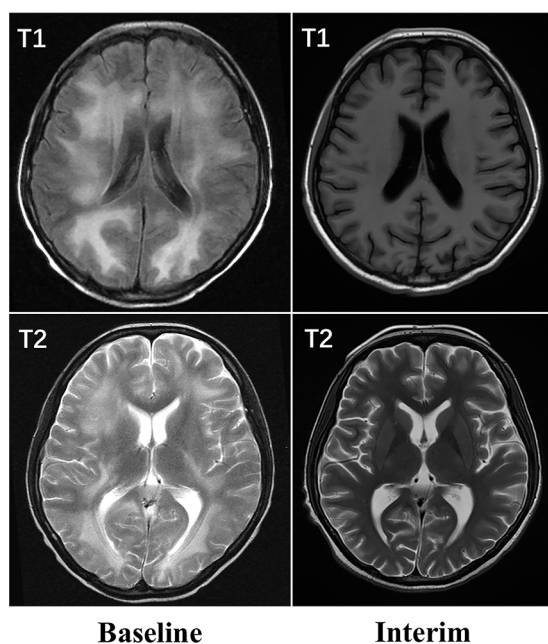
Figure 2. MRI images. Baseline images showed symmetrical, extensive, supra- and infra-tentorium lesions (left); after three doses of intrathecal rituximab, MRI showed a completely normal image (right).
MRI, magnetic resonance imaging.

EBV-PTLD are heterogeneous and nonspecific. Furthermore, response evaluation should accompany the whole treatment process. Well-timed initiation of second-line treatment would improve the therapeutic outcome when patients respond poorly to rituximab ± RI.

Case 5
A 20-year-old woman, diagnosed with acute lymphocytic leukemia (ALL), reached CR after one cycle of induction chemotherapy. She underwent matched unrelated HSCT in December 2010 after two cycles of consolidation chemotherapy, and, then, with total body irradiation (TBI) + Cy + etoposide (VP-16) in condition and MTX + CsA + r-ATG (7.5 mg/kg) for GvHD prevention. Hematopoiesis was reconstituted on day +16. On day +49, the patient had fever, headache, severe emesis, and positive EBV-DNA (8.4×10^4 copies/ml) in the blood while on CsA. Cerebrospinal fluid (CSF) was also EBV-DNA positive. The patient also demonstrated lymphadenopathy of the bilateral cervical lymph nodes, and the excisional biopsy confirmed post-transplant DLBCL. Two cycles

of intravenous rituximab (375 mg/m², weekly) attenuated her clinical symptoms. However, the patient later manifested a lack of alertness, cognitive impairment, and gatism, and brain magnetic resonance imaging (MRI) showed symmetrical, extensive, supra- and infra-tentorium lesions (Figure 2, left). Rituximab (10 mg) was thus administered intrathecally with dexamethasone and relieved the neurological symptoms and signs. After two more cycles (20 mg and 30 mg, respectively), EBV-DNA in her CSF was cleared and MRI returned to normalization (Figure 2, right). The patient has been free of PTLD to date.

The central nervous system (CNS) is one of the potential sites affected by EBV-PTLD. Measurement of EBV-DNA in the CSF and MRI are conducive to the diagnosis. For these patients, intrathecal administration of rituximab is a successful exploration.

What is the pathogenesis of EBV-PTLD?
EBV, initially described by Michael Anthony Epstein and Yvonne Barr in 1964, is a double-stranded DNA gamma herpesvirus. More than 90% of the world's adult population have been infected by EBV, usually during childhood through oral transmission *via* saliva. EBV enters the body *via* epithelial cells and naïve B cells in the nasopharyngeal tract, and induces a lytic infection with the production of new viral particles.[8] EBV then accompanies B cells to migrate, and finally establishes a latent infection by integrating its genome into the host nucleus. In immunocompetent individuals, the T-cell response is directly against EBV-specific epitopes and limits the infection.[8,11] In addition, natural killer (NK) cells also play a notable role.[8] However, immunodeficiency after allo-HSCT, particularly impaired T-cell immunity, loses control of EBV primary infection or reactivation in recipients' bodies.[8,12] Proteins such as LMP1, LMP2A, LMP2B, EBNA1, EBNA2, and others then mediate suppression or subversion of the host immune response and the immortalization of infected lymphocytes that ultimately progresses to EBV-PTLD.[11] EBV-PTLD following allo-HSCT is almost exclusively of donor origin and generally develops during the second to fourth month after transplantation.[4,6,13–17] However, no characteristic signature of oncogene expression or mutation in EBV-PTLD has been identified.[8]

Pre-transplant risk factors:

- T-cell depletion (either in vivo or ex vivo)
- EBV serology donor/recipient mismatch
- HLA mismatch
- MUD or MMUD
- CBT
- Splenectomy
- Second HSCT

Post-transplant risk factors:

- Severe acute (especially steroid-refractory) or chronic GvHD requiring intensive immunosuppressive therapy
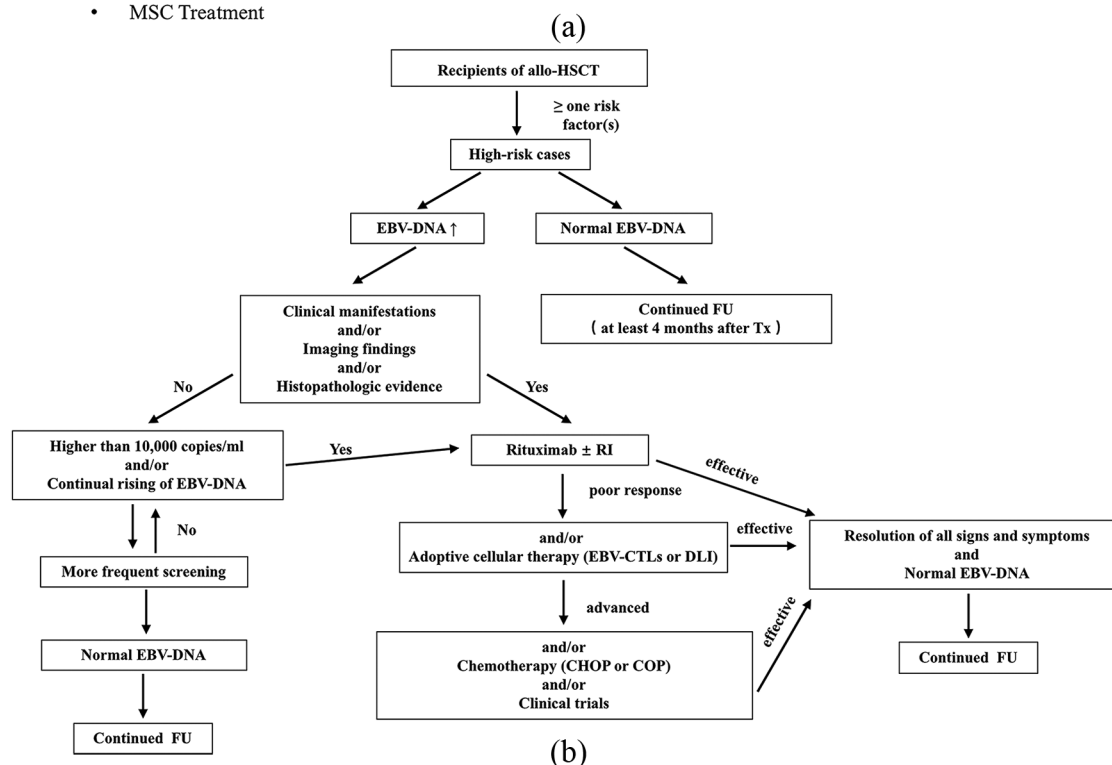- MSC Treatment

(a)

(b)

Figure 3. (a) Risk factors for EBV-PTLD after HSCT listed in ECIL-6 guidelines. Recipients are defined as a high-risk group for EBV-PTLD if they undergo MFD allo-HSCT with at least one risk factor or alternative-donor HSCT, including MUD/MMUD allo-HSCT and CBT.(b) Proposed management algorithm for high-risk recipients of allo-HSCT.
CBT, cord blood transplantation; CHOP, regimen including cyclophosphamide, doxorubicin, vincristine and prednisone; COP, regimen including cyclophosphamide, vincristine and prednisone; DLI, donor lymphocyte infusion; EBV-CTLs, Epstein–Barr virus-specific cytotoxic T lymphocytes; EBV-PTLD, Epstein–Barr virus-related post-transplant lymphoproliferative disorder; ECIL-6, the sixth edition guideline published by the European Conference on Infections in Leukaemia; FU, follow-up; GvHD, graft-versus-host disease; HLA, human leukocyte antigen; haplo-PTCy-HSCT, haploidentical hematopoietic stem cell transplantation incorporating post-transplant cyclophosphamide; HSCT, hematopoietic stem cell transplantation; MFD, matched family donor; MMUD, mismatched unrelated donor; MUD, matched unrelated donor; MSC, mesenchymal stem cell; RI, reduction of immunosuppression; Tx, transplantation.

Sporadically, EBV invades T or NK cells and leads to T-/NK- EBV-PTLD.[11] EBV-negative PTLD cases are more likely *de novo* lymphomas in post-transplant recipients.[18] Furthermore, studies focusing on gene expression profiling propose EBV-negative cases to be 'classical' lymphomas coincidentally occurring in post-transplant recipients rather than real PTLD.[19]

How are high-risk recipients identified?

Several factors increase the risk of EBV-PTLD after allo-HSCT by dampening immunity or enhancing EBV primary infection/reactivation. The degree of immunodeficiency, especially T-cell immunodeficiency, is the most important factor in the development of PTLD. T-cell depleting agents (ATG, anti-CD3 monoclonal antibodies, or alemtuzumab) or methods are associated with an increased risk of PTLD.[5,20–23] The EBV serostatus of recipients is another important risk factor for the development of EBV-PTLD.[2,4] Kalra and colleagues confirmed that EBV seromismatch [recipient (–)/donor (+)] increased the incidence of EBV-PTLD after HSCT with ATG-included conditioning.[9] Other risk factors include human leukocyte antigen (HLA)-mismatch, unrelated donor, cord blood transplantation (CBT), use of reduced intensity conditioning (RIC), GvHD, older recipients (age ⩾ 50 years), splenectomy prior to HSCT, second transplantation, and mesenchymal stem cell (MSC) treatment.[2–5,23–25] Moreover, with a higher susceptibility of primary EBV infection, pediatric recipients are more likely to develop EBV-PTLD after allo-HSCT.[26] The sixth edition guideline published by European Conference on Infections in Leukaemia (ECIL-6) classifies risk factors for EBV-PTLD into existing pre-transplant and developing post-transplant (Figure 3a).[2] In individuals without or with one risk factor (ATG included), the cumulative incidence of PTLD was 0.4%, while for those with two, three, four, and five risk factors, the incidence increased to 3.0%, 10.4%, 26.5%, and 40%, respectively.[4] In ECIL-6 guidelines, risk stratification includes low risk (i.e. auto-HSCT), standard risk (i.e. MFD allo-HSCT without risk factors; haplo-PTCy-HSCT), and high risk (i.e. MFD allo-HSCT with at least one risk factor; alternative-donor HSCT, including MUD/MMUD allo-HSCT and CBT).[2] All of our cases used ATG; Cases 1, 3 and 4 underwent haplo-HSCT without PTCy, and Case 5 accepted matched unrelated HSCT; Cases 1 and 3 experienced acute GvHD (Table 1). According to ECIL-6 guidelines, all of them were at high risk.

Are prophylactic strategies needed?

Prophylaxis may benefit patients at high risk. Depletion of both T and B cells, rather than T cells alone, is accompanied by a relatively lower incidence of EBV-related diseases.[5,25] GvHD prevention containing sirolimus or cyclophosphamide is also followed by a lower incidence of EBV-related diseases.[27,28] In a large single-center retrospective study, prophylactic rituximab reduced the incidence of EBV-DNAemia and EBV-PTLD, and attenuated grade II–IV acute GvHD (20% *versus* 38%; *p* = 0.02).[29] However, rituximab apparently delays the reconstitution of B-cell immunity and leads to fatal complications such as cytopenia and infections.[9,30,31] Therefore, prophylactic rituximab should be accompanied by immunoglobulin replacement and other supportive methods.[2] Adoptive cellular therapy (EBV-CTLs or DLI) has shown efficacy in preventing EBV-PTLD in a multi-center phase I trial,[32] but preparation of EBV-CTLs costs money and time, and DLI may exacerbate GvHD. Antiviral drugs (e.g. acyclovir and ganciclovir) are not effective for latent EBV due to the lack of EBV thymidine kinase. Thus, antiviral drugs are not recommended to prevent EBV-PTLD after allo-HSCT.[2,28] Given the unavoidable adverse effects, prophylactic strategies should be administered prudently to recipients of allo-HSCT. Instead, close monitoring of EBV-DNA and clinical manifestations was carried out in our cases.

How should EBV-DNA be monitored and EBV-DNAemia be managed?

Regular monitoring of EBV-DNA by quantitative polymerase chain reaction (PCR) is recommended for recipients after allo-HSCT. EBV-DNAemia, defined as an abnormal increase in EBV-DNA in the peripheral blood, usually occurs prior to EBV-PTLD, although the data are somewhat conflicting.[2,7] Whole blood, plasma and serum are all suitable materials.[2] Abnormal EBV-DNA is generally detected earlier in whole blood,[33,34] and plasma samples have higher specificity for higher loads.[7] It is preferable to start screening EBV-DNA within the first month after HSCT, and to screen weekly for at least 4 months. Longer and more frequent monitoring is considered for those with poor T-cell reconstitution, namely, when receiving treatment for severe GvHD, after haplo-HSCT, with T-cell depletion, or when experiencing an early EBV reactivation (Figure 3b).[2] Regarding the cut-off values, the data vary and are related to local experience, but 1000 copies/ml is generally accepted.[4,12,20,22,27,29] Notably, discrepant results of EBV-DNA quantification for the same sample

may occur in different laboratories.[35,36] The continual increase in EBV-DNA quantities in the same laboratory is more informative and valuable.[35,36] Quantification of EBV-DNA in the CSF is informative for CNS involvement.[16] Quantification of EBV-CTLs, using HLA tetramers, enzyme-linked immune-spot, or flow-cytometry-based intracellular cytokine staining, are also promising methods,[37–39] but current assays are costly, complex, time-consuming and nonstandardized.

Furthermore, there is no consensus on the threshold of EBV-DNA load that predicts progression to EBV-PTLD. Rare proven cases have been reported to be EBV-DNAemia negative,[17,40] such as Case 3. Recently, Wareham and colleagues proposed a model that improves the identification of recipients at high risk of developing PTLD.[41] In addition to EBV-DNA screening, laboratory parameters [hemoglobin, thrombocytes, and C-reactive protein (CRP)] and clinical information (gender, age, year of transplantation, transplant type, number of transplants, and high-risk EBV serostatus) should be taken into consideration. However, this model still needs confirmation by more studies. In our cases, EBV-DNA in the plasma was measured weekly for the first 4 months after transplantation, then once every 2 weeks for the fifth and sixth months, and once per month for the seventh to twelfth months. Once positive, EBV-DNA was measured at least twice a week.

EBV-DNAemia without clinical symptoms/diseases in high-risk recipients is the indication for preemptive therapy.[2] The incidence of EBV-DNAemia varies from 18.6% to 81.7% depending on the transplantation type, risk factors, assay sensitivity, cut-off values, and so on.[7,10,14,17,30,41] However, it is difficult to define an EBV-DNA load for the initiation of preemptive therapy that produces maximal benefits and limited toxicities. The velocity of rising EBV-DNA seems better for discriminating between recipients who are more likely or unlikely to develop PTLD. EBV-DNA loads indeed increased significantly faster in patients who developed PTLD than those who did not ($p < 0.0001$) in a large retrospective study, but static EBV-DNAemia appeared to be a better and simpler biomarker for PTLD development.[14] Preemptive rituximab was reported to result in a survival advantage for allo-HSCT recipients with higher EBV-DNA loads (50,000 copies/ml) in a single-center retrospective study.[42] Consistently, Kalra and colleagues also suggested a threshold

between 100,000 and 1,000,000 copies/ml.[14] Thus, rapidly increased or higher EBV-DNA loads and local experience can be referenced. In our center, preemptive rituximab will be initiated for high-risk patients when their EBV-DNA loads are higher than 10,000 copies/ml (plasma) or, in a continual rise, as in Case 1 (Figure 3b). EBV-DNAemia in recipients at low or standard risk tends to be self-limited, and more frequent screening is preferred.

Rituximab (375 mg/m², weekly), as preemptive therapy, is documented to have a response rate of >70%.[14,15,17,27,30] Preemptive rituximab cleared high EBV-DNAemia (EBV-DNA ≥20,000 copies/ml) in a single-center retrospective study,[21] and reduced the incidence of EBV-PTLD (1.4% *versus* 21.7%; $p = 0.003$) compared with that of historical controls in another prospective observation, with a more striking benefit for patients with higher EBV-DNAemia (EBV-DNA ≥ 40,000 copies/ml) (2.7% *versus* 62.5%; $p < 0.0001$).[20] However, such treatment did not improve overall survival (OS) or mortality.[20,21] Recently, low-dose rituximab (100 mg/m², weekly) for preemptive treatment has been investigated retrospectively, and resulted a comparative success rate (93.4%, 15/16) but a relatively higher relapse rate (37.5%, 6/16) than the standard dose.[43] Of course, prospective, randomized, multicentric trials with larger number of patients are needed to determine the best rituximab dose. RI, the first step for the management of EBV-PTLD after SOT, is defined as a sustained reduction of at least 20% of the daily dose of immunosuppressive drugs, with the exception of low-dose corticosteroids.[26,44] However, RI has limitations such as slow response, relatively low efficacy,[44] and the possibility of GvHD aggravation. Given that rituximab may reduce the risk of GvHD, RI is applied mostly in combination with rituximab if applicable.[2,3] Donor- or third-party-derived EBV-CTLs are highly efficacious but not widely available. If available, EBV-CTLs can be added to obliterate EBV-DNAemia in cases of poor response to rituximab ± RI, as in Case 1. Preemptive therapy is to obtain negative EBV-DNA without progression or relapse.[2] Furthermore, clinicians should keep an eye on symptoms or signs of patients.

How is EBV-PTLD diagnosed?
Clinical manifestations of EBV-PTLD are heterogeneous, nonspecific, and highly variable with

localized or disseminated lesions.[1] Extranodal involvement is common,[45,46] and lesions may invade the liver, gastrointestinal tract, spleen, lung, and others.[6,13,24,40] Fever and lymphadenopathy are the most common symptoms and signs.[2,3,24,40,47] If not treated promptly, this rapidly progressive disease may soon lead to multiple organ dysfunction and death.[2,8]

Imaging examinations are helpful for providing information on lesions and instructing the subsequent biopsy. ^{18}F-FDG-PET/CT has already shown high sensitivity and specificity in detecting nodal and extranodal involvements in three single-center retrospective studies,[45,46,48] as well as occult lesions not identified by other imaging methods.[45,48] Generally, more aggressive lesions have a significantly higher SUVmax.[45,46] Therefore, ^{18}F-FDG-PET/CT has an excellent ability to differentiate PTLD from nonmalignant diseases.[46] Moreover, ^{18}F-FDG-PET/CT is also recommended in treatment evaluation and follow up.[1,49] However, it is necessary to exclude false positive results of infection or inflammation and identify false negative results of high background fluorodeoxyglucose (FDG) uptake, CNS involvement, and T-cell PTLD. Additionally, CT or MRI is complementary to ^{18}F-FDG–PET/CT.[2] For probable cases with respiratory or gastrointestinal symptoms, endoscopy should also be included.[2] In Case 3, enteroscopy played a vital role in the differentiation between EBV-PTLD and GvHD. In Case 4, CT revealed PTLD lesions in the liver by accident, while ^{18}F-FDG–PET/CT helped to show nodal and extranodal involvement and to evaluate the therapeutic response. MRI, with its advantage in showing CNS lesions, was irreplaceable in Case 5.

Patients are diagnosed with probable EBV-PTLD if they have EBV-DNAemia and suspicious symptoms or signs but no histopathologic evidence. Proven EBV-PTLD is defined by the detection of EBV nucleic acids or EBV-encoded proteins in a tissue specimen, together with symptoms and signs. If biopsy cannot be obtained, EBV-DNAemia combined with PET-CT/CT scan can be considered.[2] However, clinicians need to exclude other infections, relapse of primary disease, cyclosporine-related encephalopathy, epilepsy, and cerebrovascular diseases. Considering the rapid progression and high fatality, we suggest that targeted rituximab ± RI be initiated immediately once a probable diagnosis of EBV-PTLD is made, and that imaging and histopathologic confirmation be accomplished without delay. In Case 2, we immediately initiated rituximab monotherapy when the patient was diagnosed with probable EBV-PTLD. A biopsy of enlarged lymph nodes followed. Of note, clinicians must be careful if they are to make a diagnosis of PTLD for recipients at low or standard risk. As the staging system specific for PTLD is currently not formed, the Ann Arbor classification and Lugano classification based on PET/CT are usually referenced.[2]

Histopathologic examination with EBV detection is requisite for the establishment of proven EBV-PTLD.[2] An excisional biopsy is usually preferred over a core biopsy, which should be done only if an excisional operation is unfeasible. EBER *in situ* hybridization is recommended to determine the existence of EBV.[50] Immunohistochemistry for EBV proteins (e.g. LMPs and EBNAs) offers information about the infectious stage of the virus.[1] WHO revised the classification of PTLD in 2016, with six main types recognized: plasmacytic hyperplasia PTLD, infectious mononucleosis PTLD, florid follicular hyperplasia PTLD, polymorphic PTLD, monomorphic PTLD (B- and T-/NK-cell types), and classical Hodgkin lymphoma PTLD.[51] The former three types are nondestructive lymphoplasmacytic proliferations. The wide spectrum of PTLD causes difficulties in pathological diagnosis. Different morphologic subtypes may be present within different locations in the same body or even within a single biopsy sample,[1] whereupon a new biopsy should be considered if ^{18}F-FDG-PET-CT suggests a more malignant disease (especially after core needle biopsy).[45] Generally, biological behavior is assumed to depend on the most malignant subtype, although well-defined criteria are lacking.[1] It is critical to exclude specific and nonspecific lymphoplasmacytic infiltrations associated with infection, GvHD, or recurrence from a known lymphoma.[1] Monomorphic PTLD is the most common diagnosis after allo-HSCT, among which DLBCL is the most predominant subtype.[6,17] Distinct genetic differences have been explored and have shown promising results between post-transplant DLBCL and DLBCL arising in immunocompetent patients and between EBV-positive and EBV-negative post-transplant DLBCL, which may be useful for more accurate diagnosis and treatment.[18,52,53] Exfoliative cytology sometimes provides diagnostic information, especially when it is difficult to perform a biopsy.

Specimens can be obtained from CSF, peritoneal fluid, pleural fluid, and bronchoalveolar lavage fluid.[54]

How should probable or proven EBV-PTLD be treated?

Targeted treatment is applied upon the diagnosis of probable or proven EBV-PTLD. Rituximab + RI is recommended as the first-line treatment, if patients are free of severe GvHD. Second-line options include adoptive cellular therapy (EBV-CTLs or DLI) and chemotherapy ± rituximab.[2] Clinical trials, for example, novel EBV-CTLs or monoclonal antibodies, small molecule inhibitors, proteasome inhibitors, and new chemotherapy agents, should also be considered.[55] The treatment goal is the resolution of all signs and symptoms and negativity of EBV-DNA (Figure 3b).

First-line treatment

Rituximab monotherapy ($375 \, mg/m^2$, weekly), with a positive response rate higher than 60%, is a reliable choice for EBV-PTLD.[3,12,13,40,56] Generally, no more than four doses are appropriate due to the downregulation of CD20 expression and subsequent decrease in efficacy of additional doses.[2] Combination strategies of rituximab and RI or chemotherapy are also applicable and highly efficient. In a single-center case-control study, rituximab + (RI or chemotherapy) achieved a better CR rate (80.6% *versus* 44.4%; $p = 0.043$) and 2-year OS (48.2% *versus* 13.2%; $p = 0.020$) than alternative therapies.[47] In a multi-center retrospective study from Europe, rituximab-based therapy resulted in a response rate of 69.45%, while rituximab with RI significantly lowered the mortality (16.19% *versus* 38.71%; $p = 0.006$), improved the 3-year OS (59.86% *versus* 40.89%; $p = 0.024$) and did not exacerbate acute GvHD compared with the corresponding results without reducing immunosuppression.[3] Therefore, rituximab should be combined with RI for PTLD patients whenever possible. Histopathologic confirmation is not required for the initiation of first-line treatment, as in Case 2. During rituximab administration, imaging examinations and biopsy should be completed wherever feasible. In Case 2, EBV-PTLD was localized to the lymph nodes and remitted after three doses of rituximab.

Rituximab also plays an important role in treating CNS involvement of EBV-PTLD.[57] Intrathecal administration is required due to the low penetrance of rituximab across the blood–brain barrier.[16] Intrathecal rituximab (on a sequential dose-escalation schedule (10, 20, 30, 40, and 50 mg, weekly), starting from 7–15 days after intravenous rituximab-based treatment) resulted in a good response for CNS involvement after the failure of intravenous rituximab-based treatment.[16,56] Intravenous rituximab failed in Case 5, while intrathecal administration finally rescued the patient.

Response to rituximab is defined as at least one log10 decrease in EBV-DNA load within the first week of treatment.[2] Rebound or persistently detectable EBV-DNAemia after rituximab administration is correlated with a poor outcome.[3,14] Factors associated with poor response to rituximab include age $\geqslant 30$ years, extranodal disease, grade II–IV acute GvHD and without RI, and the mortality in patients with 0/1, 2, and 3 factors were 7%, 37%, and 72%, respectively ($p < 0.001$).[3] Clinicians must carefully evaluate patients' responses to rituximab ± RI. In cases with the above factors or of poor response to rituximab, it is preferred to combine EBV-CTLs or DLI as early as possible.

Second-line treatment

EBV-CTLs or DLI, defined as adoptive cellular therapy, mainly assist the reconstitution of T-cell immunity for the control of EBV primary infection/reactivation, which may still be effective in rituximab ± RI refractory cases. Generally, three methods are in use clinically:[58] multimer selection that selects T cells against specific viral peptides in the context of specific HLA class I molecule; IFN-γ capture, in which T cells are selected based on their secretion of IFN-γ under the stimulation of viral antigens;[59,60] and faster CTL culture methods, using dendritic cells expressing viral antigens to induce T cells in the presence of cytokines. Of 13 recipients with biopsy-proven or probable EBV-PTLD, 11 achieved sustained CR after infusion of EBV-CTLs.[32] In a single-center experiment, DLI achieved rates of durable CR and partial remission (PR) comparable with those of EBV-CTL infusion (73% *versus* 68%), but the higher risk of reversible acute GvHD (17% *versus* 0%) in patients using DLI could not be ignored.[61]

Sequential administration of EBV-CTLs or DLI and rituximab-based treatment may elevate the CR rate and reduce the relapse of PTLD after allo-HSCT. In a multi-center prospective open-label phase II study, EBV-CTLs (1×10^6/kg, biweekly $\times 8$ doses) or DLI (CD3^{+} T cells, 2×10^7/kg, monthly $\times 4$ doses) following rituximab-based treatment significantly increased the CR rate from 62% to 91% and resulted in a 5-year cumulative incidence of PTLD relapse as low as $4.5\% \pm 3.3\%$. The sequential therapeutic strategy achieved a 5-year OS and progression-free survival (PFS) after PTLD of $70.7\% \pm 5.2\%$ and $68.9\% \pm 5.3\%$, respectively.[56] Furthermore, the early start of concurrent or sequential strategies might benefit patients with factors of poor response to rituximab. Given the GvHD-inducing potential of DLI, specific cellular therapy is preferred.[2,62] However, DLI should be considered when EBV-CTLs are unobtainable. In recent years, various kinds of EBV-CTLs have been reported, such as EBV-CTLs resistant to calcineurin inhibitors or EBNA-1 specific CTLs,[59,60] LMP1/LMP2-specific CTLs,[63] genetically manipulated EBV-CTLs,[64] banked third-party EBV-CTLs,[61,65] and multi-virus (BK virus, adenovirus, CMV, and EBV)-specific T cells.[66] In a phase II multicenter clinical trial, Haque and colleagues treated 33 EBV-PTLD patients with *in vitro* stimulated and expanded EBV-CTLs (2×10^6/kg weekly, for 4 weeks-doses) generated from partially HLA-matched unrelated donors, of which the response rate (complete or partial) was 52% at 6 months, and none of the patients developed adverse effects.[65] The persistence *in vivo* and long-term efficacy of third-party T-cells remains to be investigated,[62] although related adverse effects such as aggravation of GvHD and cytokine release syndrome (CRS) have been rarely documented. Overall, EBV-CTLs may become widely available and be recognized as another important first-line treatment for EBV-PTLD in the near future.

Chemotherapy has shown excellent control of EBV-PTLD in recipients after SOT. It was reported that rituximab (375 mg/m², weekly) + chemotherapy (CHOP or COP, regimens including cyclophosphamide, doxorubicin, vincristine and prednisone, or cyclophosphamide, vincristine and prednisone, respectively) generated a better PFS ($p = 0.005$) and OS ($p = 0.013$) than rituximab monotherapy in allo-HSCT recipients with EBV-PTLD, although with no difference in CR rate (59% *versus* 66%;

$p = 0.505$).[56] However, recipients after allo-HSCT are usually too weak to bear chemotherapeutic regimens such as CHOP or COP; thus, chemotherapy is recommended as second-line therapy.[2] It is more suitable for refractory or relapsing EBV-PTLD after allo-HSCT, and appropriate regimens can also be applied to EBV-negative PTLD and T-/NK-PTLD, which are rare and commonly resemble de novo lymphomas rather than PTLD.[2] If progression after rituximab $\pm$ RI and EBV-CTLs/DLI occurs, it is advisable to proceed to chemotherapy $\pm$ rituximab immediately.[67] In the treatment of all CD20-positive subtypes, rituximab should be combined.[67] In Case 4, rituximab monotherapy failed to remove lesions in the liver and spleen; thereafter, EBV-CTLs and R-COP were applied and turned the situation around at last.

Ongoing clinical trials should be considered for patients with PTLD, if feasible. (https://clinical-trials.gov)

Conclusion

EBV-PTLD is one of the most serious complications after allo-HSCT. It is crucial to identify recipients at high risk of EBV-PTLD and to monitor EBV-DNA loads continuously. Earlier diagnosis and treatment would improve clinical outcomes. Timely initiation of rituximab + RI is the most important step in the treatment of EBV-PTLD, and EBV-CTLs or DLI can complement this when patients response is unsatisfactory. However, further investigations are still warranted to optimize the management strategies.

Conflict of interest statement
The author(s) declare that there is no conflict of interest.

Ethics and consent statements
This work has been approved by the Ethics Committee of Blood Diseases Hospital, Chinese Academy of Medical Sciences (Approval No. NI2019010-EC-1). Consent has been waived, as no private patient information was released, and patients were not exposed to any harm.

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