Rituximab-based treatments followed by adoptive cellular immunotherapy for biopsy-proven EBV-associated post-transplant lymphoproliferative disease in recipients of allogeneic hematopoietic stem cell transplantation

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ABSTRACT
To improve prognosis of post-transplant lymphoproliferative disease (PTLD), a sequential therapeutic strategy that rituximab-based treatments followed by donor lymphocyte infusion (DLI) or autologous EBV-specific cytotoxic T lymphocytes (EBV-CTL) for biopsy-proven EBV-associated PTLD in recipients of allogeneic hematopoietic stem cell transplantation was designed. 84 patients with EBV-PTLD were enrolled in this prospective study. After two cycles of the rituximab-based treatments, 68 of 84 patients (81% [95% CI 71–88]) responded and 52 (62% [51–72]) had CRs. This increased to 73 of 77 patients (95% [87–98]) with completion of sequential cell infusions, and 70 of 77 (91% [82–96]) achieved CRs after DLI or autologous EBV-CTL infusion. 22 patients experienced acute GVHD (aGVHD) (grade I in 5 and grade II in 13, grade III in 4) and 13 chronic GVHD (limited cGVHD in 7 and extensive cGVHD in 6) in 62 patients undergoing a median of three doses of DLI. The incidences of GVHD were similar between DLI and EBV-CTL group (aGVHD 35% vs. 33%, p = 0.876; cGVHD 21% vs. 13%; p = 0.503). EBV-CTL activity after the rituximab-based treatments did not change, while increased after cell infusions and reached its maximum in the 3rd or 6th month after EBV-CTL or DLI treatment, respectively. The 5-y cumulative incidence of PTLD relapse was 4.5% ± 3.3%. The 5-y overall survival (OS) and progression-free survival (PFS) after PTLD were 70.7% ± 5.2% and 68.9% ± 5.3%, respectively. Rituximab-based treatments combined with adoptive cellular immunotherapy might elevate CR rates and reduce relapse of PTLD after allo-HSCT.

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; BMT, bone marrow stem cell transplantation; CNS, central nervous system; CR, complete remission/response; DCs, dendritic cells; DLI, granulocyte colony-stimulating factor-mobilized donor lymphocyte infusion; EBV-CTL, Epstein–Barr virus-specific cytotoxic T lymphocytes; EBV-LCL, EBV-transformed lymphoblastoid cell line; GVHD, graft versus host disease; MODS, multiple-organ disfunction syndrome; NR, no remission/response; OS, overall survival; PBMC, peripheral blood mononuclear cells; PBSCT, peripheral blood stem cell transplantation; PD, progression of disease; PFS, progression-free survival; PGE2, prostaglandin E2; PR, partial remission/response; PTLD, post-transplant lymphoproliferative disease; R, rituximab; R-COP, rituximab + cyclophosphamide + vincristine + prednisolone; R-CHOP, R-COP + epirubicin; rhGM-CSF, recominant human granulocyte-macrophage colony stimulating factor; rhIL-4, recominant human interleukin-4; rhTNF-α, recominant human tumor necrosis factor-α; RI, reduction of immunosuppression.

Introduction
Epstein–Barr virus (EBV)-associated PTLD is a life-threatening complication in the recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT). With rituximab introduced as preemptive therapy in high-risk patients and treatment for PTLD, the morbidity and mortality of PTLD have been reduced. Rituximab administered preemptively can induce sustained reversal of EBV-emia in up to 90% of patients, but only about 50% of established PTLD achieve complete remission (CR) with the treatment of rituximab. Meanwhile, rituximab has side effects, such as an increase in immunocompromise, and not restoring EBV-specific immunity, making PTLD at risk of relapse. In the recipients of allo-HSCT, we observed that two of nine PTLD patients treated with rituximab-based treatment experienced PTLD relapse within two years. Adoptive cellular immunotherapies, including EBV-CTL and DLI, may induce durable remissions of PTLD, with a response rate of up to 90%. With adoptive cellular immunotherapies, no documented cases of PTLD relapse have been reported. Despite high CR rates, the disadvantages of adoptive...
cellular immunotherapies should be taken into account that ex vivo generation of EBV-CTL requires time and facilities, leading to treatment delay which is associated with a mortality of > 90%.\textsuperscript{6,11} \textsuperscript{6,11} DLI may increase the risk of graft vs. host disease (GVHD)\textsuperscript{12} and contraindicated in patients with pre-existing GVHD, compared with rituximab. Thus, rituximab is recommended as the highest priority for PTLD, and EBV-CTL or DLI should be taken into account when available or as a second-line therapy according to the Second European Conference on Infections in Leukemia.\textsuperscript{11}

Based on the aforementioned problems, in this prospective study, a sequential therapeutic strategy of rituximab-based treatments followed by adoptive cellular immunotherapy was devised for biopsy-proven PTLD. The aim of this study was to investigate whether the strategy can elevate CR rate and reduce relapse of PTLD, and overcome the drawbacks of the adoptive cellular immunotherapy.

**Results**

**Patient, transplant and clinical characteristics**

84 patients with EBV-associated PTLD were enrolled in this trial. Thirty patients were females and 54 males. The median age was 23.5 (range, 9–49) y old. Primary diseases included acute myeloblastic leukemia (n = 39), acute lymphoblastic leukemia (n = 30), myelodysplastic syndrome (n = 4), chronic myeloid leukemia (n = 1), non-Hodgkin lymphoma (n = 1), and severe aplastic anemia (n = 9). Sixty-five patients received related and nineteen unrelated donor transplants. Seventeen patients received HLA-matched and 67 HLA-mismatched transplants. Fifty-three patients developed nodal disease, twenty-eight had lymph nodes accompanying extranodal tissues involved, and three developed primary extranodal PTLD. The involved extranodal sites included lung (n = 1), Waldeyer ring (n = 8), liver (n = 5), spleen (n = 6), nasal cavity (n = 2) and stomach (n = 3). The histopathology included diffuse large B-cell lymphoma (n = 54), polymorphy PTLD (n = 21), early lesions (infectious mononucleosis-like lesion, n = 7; plasmacytic hyperplasia, n = 1), and Hodgkin lymphoma (n = 1).

**Treatment and response**

At the time of rituximab-based treatments initiated, 59 patients were receiving single-agent and 7 multiple immunosuppressants as prophylaxis or treatment for GVHD, including ciclosporin (n = 48), tacrolimus (n = 11) and meprednisone plus tacrolimus (n = 7). At the time of the adoptive cellular immunotherapies, 24 patients were receiving single-agent and 3 multiple immunosuppressants, including ciclosporin (n = 19), tacrolimus (n = 5) and meprednisone plus tacrolimus (n = 3). There were no differences in immunosuppressant treatment between patients receiving rituximab and rituximab plus chemotherapy or DLI and EBV-CTL (data not shown).

Forty (48%) patients accepted reduction of immunosuppression (RI), and 46 rituximab monotherapy and 38 rituximab combined with chemotherapy. Seventy-seven received DLI (n = 62) or autologous EBV-CTL (n = 15), including 27 for inducing CR after rituximab-based treatment failure and preventing relapse and 50 for only relapse prophylaxis after the planned rituximab-based treatments. In 4 of 7 patients with PD during or after rituximab-based treatment, DLI or autologous EBV-CTL was applied ahead of schedule by a median of 15 d. Seven (8%) of 84 patients did not receive the planned DLI or EBV-CTL treatment: five (6%) because of death from disease progression (PD) during rituximab-based treatments, two CR patients died from infections and multiple-organ dysfunction syndrome (MODS), respectively.

After two cycles of rituximab-based treatment, the donor lymphocytes or EBV-CTL was available and no patient experienced EBV-CTL production failure. A total of 256 doses of DLI or EBV-CTL were administered to prevent PTLD relapse, including 170 doses of DLI in 52 cases, with a median of 3 (range, 1–4) doses per patient and 86 doses of EBV-CTL in 14 cases, with a median of 6 (range, 3–8) doses per patient. The initial administration of DLI or EBV-CTL for inducing CR or preventing relapse was performed on day 121 (range, 47–1172) post-transplantation.

After the rituximab-based treatments, 68 of 84 patients (81% [95% CI 71–88]) had either a complete or partial response (PR) after rituximab-based treatment. This increased to 73 of 77 patients (95% [87–98]) with completion of sequential cell infusions. Fifty-two of 84 (62% [51–72]) reached CR after rituximab-based treatments and 70 of 77 (91% [82–96]) obtained CR following rituximab-based treatments and DLI or EBV-CTL. Fourteen (88%) of sixteen patients in PR after rituximab-based treatments achieved CR with cellular immunotherapy. Eleven of sixteen rituximab-based treatment non-responders received DLI or EBV-CTL and seven responded (six CR and one PR), and four cell therapy non-responders died of PD.

In addition, intrathecal rituximab (sequential dose-escalation schedule [10 mg, 20 mg, 30 mg, 40 mg and 50 mg/time] weekly) was used in six patients with CNS involvement, who had failed intravenous rituximab-based treatments, from day 7–15 after the intravenous rituximab-based treatments, and finally they all obtained CR. The efficacy of rituximab alone was similar to that of rituximab plus chemotherapy (CR rate: 27 [59%] of 46 vs. 25 [66%] of 38, p = 0.505). Patients with isolated lymph node involved were more responsive to the rituximab-based treatments than those with extranodal involvement (CR rate: 38 [72%] of 53 vs. 14 [45%] of 31, p = 0.016). The CR rates were not different between the patients with early lesion/polymorphic PTLD and those with monomorphic PTLD (21 [72%] of 29 vs. 31 [56%] of 55, p = 0.097). The DLI efficacy was similar to that of autologous EBV-CTL (CR rate 13[68%] of 19 vs. 7[88%] of 8, p = 0.302) (Tables 1, 2).

**GVHD**

During the RI and rituximab-based treatments, 13 patients developed de novo aGVHD (grade I, n = 3; grade II, n = 5; grade III, n = 4; grad IV, n = 1), 8 were controlled with GVHD treatments. Of the 62 patients undergoing DLI,
Characteristics of patients receiving rituximab-monotherapy or rituximab
in both groups (Grade III/IV, 4 [6%] of 62 vs. 1[7%] of 15; EBV-CTL, and the severity of aGVHD and cGVHD was similar
no difference in the incidence of aGVHD (grade III in 1) and 2 (13% [2
in 5) and grade III in 4) and cGVHD in 6). Of the 15 patients undergoing EBV-CTL, 5 patients (33%
[15–59]) developed aGVHD (grade I in 1, grade II in 3 and grade III in 1) and 2 (13% [2–39]) limited cGVHD. There were
no difference in the incidence of aGVHD (p = 0.876) and
cGVHD (p = 0.503) between the patients received DLI and
EBV-CTL, and the severity of aGVHD and cGVHD was similar
in both groups (Grade III/IV, 4 [6%] of 62 vs. 1[7%] of 15;
aGVHD occurred in 22 patients (35% [95% CI 25–48]; grade I
in 5 and grade II in 13, grade III in 4) and cGVHD occurred in
13 (21% [13–33]; limited cGVHD in 7 and extensive cGVHD
in 6). Of the 15 patients undergoing EBV-CTL, 5 patients (33%
[15–59]) developed aGVHD (grade I in 1, grade II in 3 and grade III in 1) and 2 (13% [2–39]) limited cGVHD. There were
no difference in the incidence of aGVHD (p = 0.876) and
cGVHD (p = 0.503) between the patients received DLI and
EBV-CTL, and the severity of aGVHD and cGVHD was similar
in both groups (Grade III/IV, 4 [6%] of 62 vs. 1[7%] of 15;
extensive cGVHD 6 [10%] of 62 vs. 0 of 15). No patient died of
GVHD, while two patients who developed grade II aGVHD
after DLI died of CMV pneumonia during GVHD treatments.

Lymphocyte subsets and EBV-CTL activity
Lymphocyte subset analyses showed that the percentages of
CD3+ T cells, and CD16+CD56+ NK cells after the rituxi-
numab-based treatments were not different from those before
the treatment, but began to increase consistently following
cell infusions and reached their maximal at the 3rd and 6th
month after cellular immunotherapies, respectively, and no
difference among those of the 6th, 9th, 12th was evident.
The CD3+CD4+:CD3+CD8+ T cell ratio increase consist-
tently after the 3rd month and reached its maximal at the 6th
month after cell therapy. The percentage of CD19+ B
cells decreased rapidly after the rituximab-based treatments,
while increased after cell infusions till the 6th month and
did not change significantly thereafter. The percentages of T
lymphocyte subsets, NK cells and B cells were not different
between patients undergoing DLI and EBV-CTL at every
time point. EBV-CTL activity after the rituximab-based
treatments was not different from those before the treat-
ments, while increased after cell infusions. In the EBV-CTL
group, the spot numbers in the Elispot assay reached their
maximum in the 3rd month since the first infusion and
which did not change significantly thereafter. In the DLI
group, the spot numbers reached their maximum in the 6th month
and stabilized thereafter. The EBV-CTL activity was signifi-
cantly higher in the EBV-CTL treatment group than that in
the DLI group at every time point. The details are shown in
Fig. 2. In one relapsed PTLD case, CD4+CD8+ reverse and
a steady decrease in percentage of NK cells were observed
since the 9th month after DLI (Fig. 3) and EBV-CTL activity
was not detectable when relapse occurred.

Table 2. Characteristics of patients receiving DLI or EBV-CTL for inducing CR

|                | CTL | DLI |
|----------------|-----|-----|
| n             | 8   | 19  |
| Age median(range) | 23(14–41) | 19(13–45) |
| Sex male       | 6   | 72  |
| Stage I/II     | 7   | 88  |
| Nodal only     | 6   | 75  |
| ≤2 extranodal sites | 0   | 2   |
| Histology      | 0   | 1   |
| Polymorphic monomorphic | 6   | 13  |
| HLA-matched+   | 1   | 13  |
| Donor related+ | 7   | 88  |
| PBSC+          | 1   | 13  |
| RI             | 2   | 25  |
| R-CHOP+COP     | 4   | 50  |

|                | n | %  |
|----------------|---|----|
| Age median(range) | 21.5 (14–46) | 25 (9–49) |
| Sex male         | 21 | 61 |
| Stage I/II       | 29 | 76 |
| Nodal only       | 21 | 55 |
| ≤2 extranodal sites | 3 | 8  |
| Histology        | 0  | 8  |
| Polymorphic monomorphic | 38 | 17  |
| HLA-matched+     | 6  | 16 |
| Donor related+   | 27 | 71 |
| PBSC+           | 12 | 32 |
| RI             | 21 | 55 |
| DLI for inducing CR and relapse-prophaxis | 7 | 18 |
| CTL for inducing CR and relapse-prophaxis | 4 | 11 |
| DLI for only relapse-prophaxis | 17 | 45 |
| CTL for only relapse-prophaxis | 6 | 16 |

Table 1. Characteristics of patients receiving rituximab-monotherapy or rituximab-chemotherapy

|                | n-COP/R-CHOP | Rituximab monotherapy |
|----------------|--------------|-----------------------|
| n             | %  | n | %  | p |
| Age median(range) | 38 | 46 |
| Sex male       | 21.5 (14–46) | 25 (9–49) |
| Stage I/II     | 29 | 76 |
| Nodal only     | 21 | 55 |
| Histology      | 0  | 8  |
| Polymorphic monomorphic | 38 | 17  |
| HLA-matched+   | 6  | 16 |
| Donor related+ | 27 | 71 |
| PBSC+          | 12 | 32 |
| RI             | 21 | 55 |
| DLI for inducing CR and relapse-prophaxis | 7 | 18 |
| CTL for inducing CR and relapse-prophaxis | 4 | 11 |
| DLI for only relapse-prophaxis | 17 | 45 |
| CTL for only relapse-prophaxis | 6 | 16 |

1Patients receiving HLA-matched grafts.
2Patients transplanted from related donors.
3Patients receiving only PBSCT, relative to the patients receiving PBSCT+BMT.
4RL, reduction of immunosuppression; R, rituximab; R-COP, rituximab (375 mg/m2, day 0) + cyclophosphamide (600 mg/m2, day 1) + vincristine (2 mg, day 1) + prednisolone (60 mg, day 1-5); R-CHOP, R-COP + epirubicin (75 mg/m2, day 1); PBSC, peripheral blood stem cell transplantation; BMT, bone marrow stem cell transplantation; DLI, Granulocyte colony-stimulating factor-mobilized donor lymphocyte infusion; EBV-CTL, Epstein-Barr virus-specific cytotoxic T lymphocytes.
Survival and relapse

Median response duration had not been reached (>75.7 mo) at the study cut-off date (Fig. 4A). Within a median of 24.0 (0.9–75.8) mo, two of the 72 CR patients had a relapse at 4.5 mo and 2.1 y after treatment, respectively, and died despite second-line chemotherapy. The 5-y cumulative incidence of PTLD relapse was 4.3% ± 3.3%.

Median time to progression had not been reached (>79.2 mo) (Fig. 4B). Cox regression analysis of time to progression was performed taking into account the following covariates: age, sex, presence of extranodal disease, stage of disease, monomorphic disease, rituximab plus chemotherapy, lung involvement and CNS involvement. Rituximab plus chemotherapy were associated with extended time to progression intervals (HR 0.059 [95% CI 0.010–0.361], p = 0.002), a higher risk of disease progression was observed in patients with lung involvement (HR 13.157 [95% CI 1.040–166.484], p = 0.047).

Within a median follow-up of 23.1 (range, 0.7 to 79.5) mo after treatment, median PFS and median OS had not been reached (Fig 4C, D). 61 patients survived and 23 died. Cox regression analysis of survival were performed taking into account the following factors: age, sex, presence of extranodal disease, stage of disease, monomorphic disease, rituximab plus chemotherapy, lung involvement and CNS involvement, rituximab combined with chemotherapy was associated with superior PFS and OS compared with rituximab monotherapy (HR 0.208 [95% CI 0.070–0.615], p = 0.005; HR 0.238 [95% CI 0.076–0.743], p = 0.013), and shorter PFS and OS were evident in patients with lung involvement (HR 6.502 [95% CI 1.271–33.254], p = 0.025; HR 5.941 [95% CI 1.128–31.299], p = 0.036).

Five-year PFS was 68.9% ± 5.3%, and OS was 70.7% ± 5.2%. The causes of death included PTLD progression (n = 9), infection (n = 9; CMV pneumonia in 2; viral myocarditis in 1, bacterial infection in 4, fungal infection in 2), MODS (n = 1), PTLD relapse (n = 2) and primary malignancy relapse (n = 2). Up till now, two experienced primary malignancy relapse. The 5-y cumulative incidence of primary malignancy relapse post-transplantation was 5.9±4.0%. 
The EBV-CTL activity in EBV-CTL treatment group was higher than that in the DLI group (\( p < 0.001 \)). The percentages of CD3^+ T cells, and CD16^+ CD56^+ NK cells before cellular immunotherapies were not different from those before rituximab-based treatment (\( p = 0.471 \) and \( p = 0.603 \), respectively), while the CD19^+ B cells before the cell infusions were less than those before the rituximab-based treatments (\( p < 0.001 \)). The percentages of CD3^+ T cells, CD16^+ CD56^+ NK cells, CD19^+ B cells were increased in the 1st month after cellular immunotherapies than those before cell infusions (\( p = 0.001, p < 0.001, p < 0.001 \), respectively), higher in the 3rd month than those in the 1st month after DLI or EBV-CTL (\( p < 0.001, p = 0.009, p < 0.001 \), respectively), and reached their maximal in the 3rd, 6th, 6th month, respectively. The CD4^+ T cell to CD8^+ T cell ratio increased since the 3rd month (\( p < 0.001 \)), no differences among the 6th, 9th and 12th month. (D) In the spot assay, the IFN\(_\gamma\) spot numbers before the cell infusion were not different from those before rituximab-based treatments (\( p = 0.296 \)), while more in the 1st month than those before cell infusion (\( p < 0.001 \)), more in the 3rd month than those in the 1st month after cellular immunotherapies (\( p < 0.001 \)) and more in the 6th month than those in the 3rd month (\( p < 0.001 \)), no differences among the 6th, 9th and 12th month after cellular immunotherapies. The EBV-CTL activity in EBV-CTL treatment group was higher than that in the DLI group (\( p = 0.001 \)). Notes: Arrows denote the time of rituximab-based treatments.

**Figure 2.** Lymphocyte percentages in circulation and EBV-specific cytotoxic lymphocyte activity of patients who received DLI and EBV-CTL infusion. No significant difference was observed between patients received DLI and EBV-CTL in lymphocytes (CD3^+ T cells, \( p = 0.552 \); CD16^+ CD56^+ NK cells, \( p = 0.549 \); CD19^+ B cells, \( p = 0.704 \)). (A), (B) and (C): The percentages of CD3^+ T cells, and CD16^+ CD56^+ NK cells before cellular immunotherapies were not different from those before rituximab-based treatment (\( p = 0.471 \) and \( p = 0.603 \), respectively), while the CD19^+ B cells before the cell infusions were less than those before the rituximab-based treatments (\( p < 0.001 \)). The percentages of CD3^+ T cells, CD16^+ CD56^+ NK cells, CD19^+ B cells were increased in the 1st month after cellular immunotherapies than those before cell infusions (\( p = 0.001, p < 0.001, p < 0.001 \), respectively), higher in the 3rd month than those in the 1st month after DLI or EBV-CTL (\( p < 0.001, p = 0.009, p < 0.001 \), respectively), and reached their maximal in the 3rd, 6th, 6th month, respectively. The CD4^+ T cell to CD8^+ T cell ratio increased since the 3rd month (\( p < 0.001 \)), no differences among the 6th, 9th and 12th month. (D) In the spot assay, the IFN\(_\gamma\) spot numbers before the cell infusion were not different from those before rituximab-based treatments (\( p = 0.296 \)), while more in the 1st month than those before cell infusion (\( p < 0.001 \)), more in the 3rd month than those in the 1st month after cellular immunotherapies (\( p < 0.001 \)) and more in the 6th month than those in the 3rd month (\( p < 0.001 \)), no differences among the 6th, 9th and 12th month after cellular immunotherapies. The EBV-CTL activity in EBV-CTL treatment group was higher than that in the DLI group (\( p = 0.001 \)). Notes: Arrows denote the time of rituximab-based treatments.

**Discussion**

The introduction of rituximab has improved the prognosis of PTLD, and it is readily available and favorably tolerated,\(^7\) but the efficacy is inferior to cellular immunotherapy and cannot restore EBV-specific immunity. The efficacy of rituximab-based treatment for PTLD depends on the early diagnosis and prompt administration, and the location and degree of organ involvement.\(^9,13-15\) PTLD with extranodal involvement carries worse prognosis than those with isolated lymph node involvement, which might result from the difficulty in early diagnosis and low concentrations of medication distributed in affected tissues,\(^16,17\) in this cohort, lung involvement associated with a higher risk of disease progression and poorer survival. Our results also revealed that the patients with extranodal involvement were less responsive to rituximab than those with isolated lymph node involvement, but had a higher response rate than those with extranodal involvement in reports,\(^14,18\) which might be attributed to early diagnosis and prompt treatment as well as special medication approach. In our centers, EBV-DNA monitoring is routinely performed, which is helpful to early diagnose PTLD.\(^19\) Six patients with CNS involvement who had failed intravenous rituximab-based treatments all obtained CR after intrathecal rituximab combination. In addition, some novel B cell-targeted agents has been proved to mediate potent antitumor activity in B cell malignancy such as ibrutinib in chronic lymphocytic leukemia, blinatumomab in relapsed/refractory precursor B cell acute lymphoid leukemia, and it is readily available and favorably tolerated,\(^2\) but the introduction of rituximab has improved the prognosis of PTLD.\(^7,9,10\) Moreover, they are also effective for rituximab-resistant PTLD. However, as aforementioned, they also have disadvantages. For example, the long time required for EBV-CTL production limits its prompt application, and DLI result in high risk of GVHD. The efficacy of cellular immunotherapy depends on effector cell to targeted tumor cell ratio.\(^9\) There is a lack of randomized controlled trials for comparing efficacy between rituximab and cellular immunotherapy. There is also no comparison between rituximab combined with cellular immunotherapy and rituximab alone or cellular immunotherapy alone. Doubrovina et al observed equivalent efficacy of DLI and EBV-CTL for PTLD. In this study, we designed a sequential therapeutic strategy of the rituximab-based treatments followed by adoptive cellular immunotherapy. In the patients who had not achieved CR after rituximab-based treatments, the cellular immunotherapies were
is an ideal method to restore EBV-specific immunity to reduce PTLD relapse. Immunosuppressant discontinuation was well-tolerated with the literature.9

Based treatments combined with EBV-CTL, which was consistent with the literature.23-25 In this study, rituximab-based treatments upgraded to CR following cellular immunotherapy might increase the effector cell to targeted tumor cell ratio, the former decrease the tumor loads so that the effector cells can fully exert anti-residual tumor effect. Our results showed that 20 of the 27 non-CR patients after rituximab-based treatments upgraded to CR following cellular immunotherapy, resulted in an increase of 29% (from 62% to 91%) in CR rate as expected. The efficacy was not different between rituximab-based treatments combined with DLI and rituximab-based treatments combined with EBV-CTL, which was consistent with the literature.9

The introduction of rituximab has improved PTLD remission, thus PTLD relapse might become a crucial factor on long-term survival. The incidence of PTLD relapse varies from 12% to 50% in recipients of solid organ transplant (SOT) treated with rituximab,5,26,27 while there is absence of large sample data in recipients of HSCT. The development of PTLD is closely related to immune status.28 Reconstituting EBV-specific immunity is essential to reduce PTLD relapse. Immunosuppressant discontinuation is an ideal method to restore EBV-specific immunity, but is not feasible in the recipients of allo-HSCT because of high morbidity and mortality of GVHD resulted from immunosuppressant withdrawal. The complete reconstitution of immune function needs generally 3–5 y in recipients of HSCT.29 EBV-specific T-cell recoveries can be influenced by many factors, such as global T-cell recovery.30 Some studies indicated that the majority of EBV-CTL generated ex vivo show an effector memory phenotype,31,32 and adoptive cell infusion can result in sustained expansion of EBV-CTL at high frequency in vivo and durable remission of PTLD.7,9,28 In this study, to eradicate minimal residual tumor, the adoptive cellular immunotherapies were administrated as prophylaxis in patients with CR. Our study demonstrated that the EBV-specific CD8+ T cell reactivity improved significantly after autologous EBV-CTL or DLI treatment, additionally, NK cells and CD4+ to CD8+ T cell ratio showed remarkable increases in these patients after cell infusions. Within a median follow-up of 24.0 mo, only two of the 72 CR patients experienced relapse. The incidence of PTLD relapse was lower than those of PTLD patients after SOT and that of historical PTLD patients in our center.6,26,27 The late-relapsed case had steady decreases in lymphocytes from the 9th month after DLI and the EBV-CTL activity was undetectable when PTLD relapsed, which might be attributable to the long-term immunosuppressive therapies because of extensive cGVHD. These data suggested that adoptive cell infusion can restore EBV-specific immunity against minimal residual tumor. As far as we know, this is the first report to employ the adoptive cellular immunotherapy for preventing PTLD relapse.

The main adverse effect of DLI is associated with the risk at increasing the morbidity and mortality of GVHD. Our previous studies and other reports indicated that G-CSF-mobilized DLI had a lower risk of GVHD compared with steady DLI.33-35 In this study, G-CSF-mobilized DLI instead of steady DLI was performed, the incidences of aGVHD and cGVHD were 35% and 21%, respectively, after DLI, which were similar to those of the general recipients in our institution.36 No patient died of GVHD. There were no differences in incidence and severity of GVHD between the patients received DLI and EBV-CTL. These results suggested that rituximab-based treatment followed by G-CSF-mobilized DLI was safe without increasing morbidity and mortality of GVHD.

Interestingly, we observed that only two patients experienced primary malignancy relapse. The incidence of primary malignancy relapse was lower than other population in our transplant centers and literatures.36,37 A reasonable interpretation of our findings is that adoptive cells mediate graft-versus-tumor (GVT) response. It is recognized that DLI induce GVT effect.38,39 The antitumor activity of EBV-CTL may be ascribed to that the lymphocytes used to generate EBV-CTL might contain tumoricidal effecter T cells targeting primary malignancy and the high levels of interferon-γ secreted by EBV-CTL also induce antitumor response.40 In addition, the rapid reconstitution of immunity attributable to RI might also be one cause of lower primary malignancy relapse.

In summary, rituximab-based treatments combined with adoptive cellular immunotherapy might elevate CR rates for PTLD, and rituximab-based treatments followed by adoptive cellular immunotherapy might decrease PTLD relapse and improve survival. Considering the limitation of the small number of patients studied, our results need to be further confirmed in more patients and prospective, two-arm trials.

**Patients and methods**

**Ethics statement**

The prospective, open-labeled, phase 2 study was conducted in four centers. The study was performed in accordance with the modified Helsinki Declaration, and the protocol was approved by the Ethics
Boards of our centers. All recipients, donors and/or guardians provided written informed consent to participate in the study.

**Eligibility and PTLD diagnosis**

Treatment-naive EBV-associated PTLD Patients after allo-HSCT were enrolled from March 2008 to June 2014 if confirmed by biopsy, and patients with the following criteria were excluded: irreversible organ failure not related to PTLD or active visceral hemorrhage or Eastern Cooperative Oncology Group (ECOG) performance status higher than 2. EBV-associated PTLD was diagnosed according to the WHO criteria.\(^41\) EBV-DNA in blood and secretions was detected by Real-time quantitative polymerase chain reaction assay (RQ-PCR) and EBV in tissues was verified by in situ hybridization for EBV-encoded small nuclear RNA (EBV-EBER) in lymphocytes of biopsy specimens.\(^42\)

**Study treatment**

The study treatments included reducing immunosuppression (RI) and rituximab-based treatments followed by adoptive cellular immunotherapies. Immunosuppressants were withdrawn in a stepwise fashion (i.e., total dose reduced by 20%/week) if tolerated. Rituximab alone (375 mg/m\(^2\) intravenously once a week, 4 doses as one cycle with a cycle interval of 2 weeks, for a total of 2 cycles) or rituximab combined with chemotherapy (two 21-day cycles of COP consisting of rituximab 375 mg/m\(^2\) intravenously on day 0, cyclophosphamide 600 mg/m\(^2\) intravenously on day 1, vincristine 1.4 mg/m\(^2\) intravenously on day 1,

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![Figure 4. Response duration, time to progression, progression-free survival and overall survival.](image-url)
and limited to 2 mg absolute per cycle, and prednisolone 60 mg orally on days 1–5 or two 21-day cycles of CHOP consisting of COP regimen and epirubicin 75 mg/m² intravenously on day 1) were given based on PTLD histopathology and blood counts. Generally, rituximab monotherapy was administered in the patients with early lesions or polymorphic PTLD or white blood cells less than 4 × 10⁹/L and/or platelets less than 50 × 10⁹/L and R-CHOP/R-COP in those with monomorphic PTLD and white blood cells no less than 4 × 10⁹/L and platelets no less than 50 × 10⁹/L. After two cycles of rituximab-based treatments (starting 4 weeks after the last dose of rituximab or R-COP/R-CHOP), the adoptive cellular immunotherapies would be administered for improving response and preventing relapse, including donor lymphocytes given at a dose of 2.0 × 10⁷/kg CD3⁺ T cells/kg for the patients with availability of the original donor and no pre-existing GVHD, and autologous EBV-CTL given at a dose of 1 × 10⁶ cells/kg for the patients had pre-existing GVHD or lack of original donor access. In those presenting clinical signs of disease progression during rituximab-based treatments or interval, DLI or autologous EBV-CTL treatment was started immediately if the adoptive cells were prepared and disease progression was verified. DLI was performed once monthly for a total of four doses and EBV-CTL every two weeks for a total of eight doses. When GVHD occurred, DLI or EBV-CTL infusion would be discontinued. The Trial profile is shown in Fig. 1.

**Donor lymphocytes and EBV-CTL preparation**

Donor lymphocytes were obtained from the original donor. Collections were begun on day 5 after Granulocyte colony-stimulating factor mobilized, and consecutive daily collections were performed until CD3⁺ T cell yields were more than 8.0 × 10⁶/kg (recipient body weight). A dose of 2.0 × 10⁷/kg CD3⁺ T cells was infused every time. Because of unavailability of the original donor, EBV-CTL were generated from autologous peripheral blood mononuclear cells (PBMC) which were isolated from recipients themselves by blood cell separator. To isolated adequate PBMC, we waited enough times using blood cell separator. For generating dendritic cells (DCs), PBMC were washed 4 to 5 times in RPMI 1640 and incubated in serum-free medium AIM-V (Gibco, Life Technologies, Grand Island, NY) in 75-cm² tissue culture flasks (37°C, 5% CO₂). After 2 h of incubation, plastic adherent cells were cultured (37°C, 5% CO₂) in 10 mL of DC medium (AIM-V medium supplemented with 1000 U/mL granulocyte-macrophage colony stimulating factor [GM-CSF] and 1000 U/mL recombinant human interleukin-4 [rhIL-4], both from Schering-Plough) after removal of non-adherent cells. After 5 d, non-adherent cells were rinsed off the flasks and cultured in six-well plates (Costar, Corning, NY) at a final concentration of 5 × 10⁵ cells per well in 3 mL of DC medium and a cytokine cocktail consisting of 10 ng/mL recombinant human tumor necrosis factor-α (rhTNF-α, Sigma), 10 ng/mL interferon-α (Genzyme, Cambridge, MA), 1000 U/mL rhIL-6 (Genzyme), and 1 mg/mL prostaglandin E₂ (PGE₂, Sigma) were added to promote DC maturation, and 24 h later, 3 ug/mL lysates (freeze–thaw lysates) of EBV-transformed lymphoblastoid cell line (EBV-LCL) were added and incubated for 4 h. The EBV-CTL were generated by stimulating the recipient’s lymphocytes with autologous EBV-LCL-pulsed DCs at a responder-to-stimulator ratio of 30:1 in AIM-V medium containing 10 IU/mL rhIL-2, 10 ng/mL rhIL-7 and 10 ng/mL rhIL-15 (Genzyme), then harvested after 10 d, and administered to the recipient. A dose of 1.0 × 10⁶ autologous EBV-CTL/kg was infused every time. EBV-CTL was defined as CD4⁺ or CD8⁺ T-cell responsive to EBV-LCL stimulation that secretes interferon-gamma observed in Elispot assays.  

**EBV-DNA monitoring in blood**

EBV-DNA monitoring of blood was based on our previous description in the recipients post-transplantation. During study treatments, EBV-DNA was monitored twice weekly till CRs achieved. EBV-DNA monitoring was performed once weekly for 3 mo after CRs; the frequency was once every 2 weeks in the 4th to the 9th month, once monthly from the 10th to the 24th month and once every 2 mo from the 25th to the 36th month after CRs.

**Detection of lymphocyte subsets and EBV-CTL activity**

T lymphocyte subsets (CD3⁺, CD3⁺CD4⁺, CD3⁺CD8⁺), CD19⁺ B cells and CD16⁺CD56⁺ NK cells in peripheral blood were analyzed by flow cytometry and EBV-CTL activity was monitored by Elispot assay before study treatments, and at different time points after treatment initiated.

**Evaluation points and statistical analysis**

Our data were analyzed on June 15, 2015. The primary endpoints included efficacy of sequential treatment measured as CR rates and relapse rates. Secondary endpoints included the incidence and severity of GVHD attributable to cellular immunotherapy and survival. To determine the CR rate and the duration at 2 y with a CI of 10% on a 95% confidence level, 62-97 patients were necessary. Our assumptions were that rituximab-based treatment would result in a CR rate of 50%, and that sequential application of CHOP would increase the CR rate by 30%.

Interim response assessments were scheduled 3–4 weeks after rituximab-based treatment, 4 weeks after the second dose of DLI and 2 weeks after the fourth dose of EBV-CTL, final response was assessed 1 mo after the last dose of DLI/EBV-CTL. The responses and relapse of PTLD were according to the revised response criteria for malignant lymphoma, the responses were classified into CR, partial remission (PR) and no remission (NR). Stable disease and progressive disease (PD) were classified as NR. In addition, EBV-DNA negativity in blood and secretions of affected tissues were required in the designation of CR. Isolated EBV-DNA viremia was not regarded as an evidence of relapse. GVHD diagnosis was according to the literatures. The data were analyzed on SPSS 19.0. We calculated CIs and best point estimate for observed response rates using the adjusted Wald method. The
Categorical values were compared by the Chi-squared test. Lymphocyte percentages and EBV-CTL activity at different times were compared using Repeated-Measures Analysis of Variance. Kaplan–Meier analysis was used to estimate relapse incidence and survival. Cox proportional hazard model with stepwise backward selection was applied to survival to identify potential prognostic factors. A p value < 0.05 was considered statistically significant, tests are two-tailed.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**Ethics statement**

The study was performed in accordance with the modified Helsinki Declaration, and the protocol was approved by the Ethics Boards of our centers.

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