Immune reconstitution of HLA-A*0201/BMLF1- and HLA-A*1101/LMP2-specific Epstein Barr virus cytotoxic T lymphocytes within 90 days after haploidentical hematopoietic stem cell transplantation

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Abstract

Background: Haploidentical hematopoietic stem cell transplant (haplo-HSCT) recipients are at high risk for Epstein Barr virus (EBV)-related diseases. EBV-specific CD8+ cytotoxic T cells can control EBV-infected B cell expansion; however, no studies have investigated EBV-specific immune reconstitution after HSCT, particularly haplo-HSCT. Therefore, in this study, we aimed to characterize EBV-specific immune cell reconstitution after haplo-HSCT.

Methods: HLA-A*1101 and HLA-A*0201 pentamers folded with immunodominant EBV peptides were used to detect EBV-specific CD8+ T cells by flow cytometry in peripheral blood mononuclear cells from 19 haplo-HSCT recipients and the results were compared with those in controls. We also compared the EBV-specific pentamer-binding cell frequencies in patients with or without EBV-related diseases by flow cytometry.

Results: Pentamer-binding EBV-specific CD8+ T cells were detected at + 30, + 60 and + 90 days after haplo-HSCT in EBV-seropositive patients subjected to haplo-HSCT from an EBV-seropositive donor. The frequencies of the HLA-A*0201/BMLF1-GLC pentamer in haplo-HSCT patients at + 30 days were significantly lower than those in HLA-A*0201-positive healthy controls ($p = 0.019$) and patients at + 60 days ($p = 0.003$). The frequencies of the HLA-A*1101/LMP2-SSC pentamer at + 30, + 60, and + 90 days were significantly decreased compared with those in healthy controls ($p = 0.009, 0.019, 0.039$, respectively); however, the frequencies of the HLA-A*1101/LMP2-SSC pentamer did not differ significantly among patients at + 30, + 60, and + 90 days ($p = 0.886$). There was a significant difference in the frequency of the HLA-A*0201/BMLF1-GLC pentamer at + 60 days between patients with and without EBV-related diseases ($p = 0.024$). Patients with EBV-related diseases showed lower percentages of HLA-A*0201/BMLF1-GLC specific CD8+ T cells.

Conclusions: Haplo-HSCT recipients could generate EBV-specific CD8+ T cells within + 30 days after transplantation. The HLA-A*0201/BMLF1-GLC pentamer cell frequency at + 60 days may be a useful indicator for monitoring EBV-related diseases in patients after haplo-HSCT. Transfusion with EBV-CTLs within 60 days after haplo-HSCT may have prophylactic effects against EBV-related diseases.

Keywords: Haploidentical hematopoietic stem cell transplantation, Epstein-Barr virus, CD8 cells, Pentamers
Background
Epstein-Barr virus (EBV) reactivation frequently occurs after hematopoietic stem cell transplantation (HSCT) [1, 2]; however, EBV-related diseases, including EBV-related post-transplant lymphoproliferative disorders (PTLDs), can cause significant morbidity and mortality in HSCT recipients. Before the year 2000, mortality from PTLDs after HSCT reached 84.6% [3]. Considerable improvements in outcomes have been achieved since the introduction of new approaches for EBV-related diseases/PTLDs, including monitoring of EBV-DNA by polymerase chain reaction (PCR), pre-emptive therapy, and timely treatment with rituximab; however, mortality remains high, and approximately one-third of patients diagnosed with EBV-PTLDs die [4].

Factors associated with a high risk of EBV-PTLDs include HLA mismatch, EBV serology donor/recipient mismatch (donor+/recipient–), grade II–IV acute graft-versus-host disease (aGVHD), pre-transplant splenectomy, and treatment with mesenchymal stem cells [5]. Furthermore, a high incidence of PTLDs has been reported in pediatric patients who have undergone reduced intensity conditioning (RIC) regimens including antithymocyte globulin (ATG) or alemtuzumab (Campath) [6, 7]. Thus, compared with other types of HSCT recipients, those undergoing haploidentical HSCT (haplo-HSCT) may be at a higher risk of EBV-related diseases, including PTLDs. HLA disparity and ATG use are important components of EBV reactification after haplo-HSCT.

The primary aim of treatment in patients with EBV-related diseases/PTLDs is to recover EBV-specific immunity [8], as indicated by numerous clinical trials using EBV-specific cytotoxic T lymphocytes (EBV-CTLs) derived from stem cell donors or third-party banks [9–11]; however, little is known about EBV-specific immune reconstitution after HSCT, particularly in haplo-HSCT recipients. Thus, there is no generally accepted time point for transfusion of EBV-CTLs as prophylaxis against the occurrence of EBV-related diseases/PTLDs during the initial period after HSCT.

In this study, we used class I major histocompatibility complex pentamers, HLA-A*0201/BMLF1-GLC and HLA-A*1101/LMP2-SSC, to investigate the dynamics of EBV-CTL immune reconstitution in 19 patients who underwent haplo-HSCT.

Methods and materials
Patients and collection of blood samples
Twenty-eight patients undergoing haplo-HSCT using RIC regimens at Shanghai Dao-pei Hospital between November 2012 and January 2015 were prospectively included in this study. All patients underwent single haplo-HSCT from donors who were HLA-A*0201- or HLA-A*1101-positive. Nine patients were excluded because they relapsed (three cases) or were treated with donor lymphocyte infusion (six cases) within 100 days after transplantation; finally, 19 evaluable patients were enrolled in this study.

The donors for the 19 patients enrolled were positive for HLA-A*0201 (10 cases) or HLA-A*1101 (9 cases), the 28 healthy volunteers (all of them were long-term EBV carriers) carried HLA-A*0201 (9 cases) or HLA-A*1101 (19 cases), and the 49 negative controls were both HLA-A*0201- and HLA-A*1101-negative. Patients were divided into HLA-A*0201 and HLA-A*1101 groups according to the tissue type of their donors. The patients, their donors, healthy long-term EBV carriers, and negative controls all showed negative reactions in an EBV-IgM serum array and positive reactions in an EBV-IgG serum array. All patients were 100% donor types by bone punctures and chimerism examinations at +30, +60, and +90 days after transplantation during their lifetimes.

Peripheral blood samples (5 mL) were collected from patients during routine laboratory blood draws. Blood samples from 28 healthy volunteers who were HLA-A*0201- or HLA-A*1101-positive were obtained to identify the baseline level of EBV-CTLs in healthy long-term EBV carriers. Blood samples from HLA-A*0201- and HLA-A*1101-negative volunteers were obtained and used as negative controls. Forty-eight blood samples were obtained from the 19 patients. In the HLA-A*0201 group, blood samples were obtained from five patients gave blood samples at +30, +60, and +90 days; from four patients at +30 and +60 days; and from one patient at +30 days after haplo-HSCT. One patient died at +43 days because of invasive pulmonary aspergillosis, two patients died at +74 and +87 days after haplo-HSCT because of EBV-related diseases/PTLDs, and two patients were lost to follow-up at +90 days (they did not die, and returned to follow-up at +120 days). We found that the patient who died at +43 days was a 100% donor type by bone puncture and short tandem repeat (STR) examination at +30 days after transplantation. However, at +33 days, this patient developed hyperthermia, pulmonary infection, and decreased blood cells and was found to have decreased chimerism to 23% of that of the donor recipient at +37 days. Secondary graft failure was observed, which was thought to be related to the serious pulmonary fungal infection. In the HLA-A*1101 group, blood samples were obtained from six patients at +30, +60, and +90 days and from three patients at +30 and +60 days; the other three patients died because of invasive pulmonary aspergillosis (one case), cytomegalovirus (CMV) pneumonia (one case), and EBV-related gastroenteritis (one case).

Assay to detect serum EBV-specific antibody status
The serum EBV-IgM and IgG statuses before HSCT in patients, their donors, the healthy long-term EBV carriers,
and negative controls were detected using an anti-Epstein-Barr virus (EBV-VCA) IgM human in vitro Enzyme-Linked Immunosorbent Assay (ELISA) kit and an EBV-VCA-IgG human in vitro ELISA kit (EK-Bioscience, Shanghai, China) according to the manufacturer’s instructions.

**HLA disparity**

HLA-A, HLA-B, HLA-Cw, and HLA-DRB1 typing was performed using high-resolution DNA techniques according to the manufacturer’s instructions. The reagents (Special Monoclonal Tray-Asian HLA Class I and Micro SSP HLA Class I and II ABDR DNA Typing Tray; One Lambda, Canoga Park, CA) were commercially imported.

**Chimerism analyses**

Bone marrow chimerism was detected every month for six months after haplo-HSCT. Chimerism was determined by one of two methods: DNA fingerprinting of STR, and chromosomal fluorescence in situ hybridization (FISH). Chimerism was evaluated by DNA fingerprinting of STR in recipient BM cells in sex-matched donor-recipient pairs; however, in sex-mismatched donor-recipient pairs, chimerism was analyzed by FISH.

**Haplo-HSCT process**

Conditioning regimens were as follows: intravenous administration of cytarabine (2.0 g/m²) once daily on days − 4 to day 02) were harvested on day 01 and 02, respectively.

Supportive care was similar for all patients. All blood products were irradiated and leukocyte-depleted. Antifungal prophylaxis was routinely administered with caspofungin during the conditioning process and voriconazole was administered after transplantation. Pneumocystis prophylaxis, typically trimethoprim-sulfamethoxazole, was administered until 6 months post-transplantation. Acyclovir, typically 400 mg orally twice per day, was administered until 24 months post-transplantation.

**Monitoring of EBV reactivation in peripheral blood and definition of EBV-related diseases**

The EBV-DNA copy number was determined by quantitative PCR (qPCR) using peripheral blood samples or biopsy specimens. Briefly, genomic DNA was isolated from 250 μL whole blood or digestive tissues with an Axyprep Mag tissue-blood genomic DNA kit (Axygen, Union City, CA, USA) according to the manufacturer’s instructions. qPCR was performed on an ABI 7300 thermal cycler using commercially available PCR kits for EBV (Daan Gene Technology, Guangzhou, China) and the results were shown as DNA copies/mL of whole blood, with a detection threshold of 500 copies/mL for EBV.

EBV-DNA copy numbers were monitored weekly during the first 30 days after haplo-HSCT and subsequently every other week until 3 months after transplantation or until EBV-DNA became undetectable in the peripheral blood.

EBV-DNAemia was defined as EBV-DNA loads of more than 500 copies/mL at two consecutive time points, without any signs or symptoms of EBV-related diseases, including probable and proven PTLDs. Probable PTLDs were defined as significant lymphadenopathy, hepatosplenomegaly, or other end-organ manifestations, accompanied by a high EBV-DNA blood load and the absence of tissue biopsy and other documented causes [12]. Proven PTLDs were diagnosed as detection of EBV nucleic acids or EBV-encoded proteins in a tissue specimen, together with symptoms and/or signs from the affected organ [12].

**MHC class I-peptide pentameric complexes (pentamers) and reagents**

Two EBV peptide epitopes were used: the HLA-A*0201-restricted epitope, GLCTLVAML, derived from the lytic cycle protein BMLF1 (amino acids 259–267), and the HLA-A*1101-restricted epitope SSCSSCPLSK, derived from the latent cycle protein, LMP2 (amino acids 340–349). The pentamers, HLA-A*0201/BMLF1-GLC and HLA-A*1101/LMP2-SSC, were purchased from ProImmune, Ltd. (Oxford, UK). Pentamers were labeled with phycoerythrin (PE). Monoclonal antibodies, including anti-CD3 (peridinin chlorophyll protein-[PerCP]), anti-CD8 (fluorescein isothiocyanate-[FITC]), and anti-CD19
(allophycocyanin-[APC]), were purchased from BD Biosciences (Franklin Lakes, NJ, USA). The experimental protocol and operation were recommended by the Pentamer Handbook from ProImmune, Ltd. (Oxford, UK). According to the protocol that cells obtained from a mismatched pentamer (irrelevant MHC allele and/or irrelevant peptide) can be used to control non-specific staining, and that exclusion of B cells is likely to reduce most of the non-specific background, we used both HLA-A*0201- and 1101-negative adult volunteers as negative controls and added anti-CD19 monoclonal antibody to remove B lymphocytes. Briefly, forward and side scatters were used to gate viable populations of cells, and CD3+CD19− cells were then gained as target cells for FACS analysis. The percentage of pentamer-stained CD3+CD8+CD19− cells were gained as CD8+ T lymphocytes. Next, CD3+CD19− CD8+ cells were gained as CD8+ T lymphocytes, and finally, pentamer-stained CD3+CD8+CD19− cells were gained as target cells for FACS analysis. The percentage of EBV-CTLs was calculated as follows: percentage EBV-CTLs = (percentage EBV-CTLs in patients or percentage EBV-CTLs in healthy long-term EBV carriers (healthy controls) – percentage EBV-CTLs in negative controls) × 100%.

Blood samples were collected from patients at + 30, + 60, and + 90 days after haplo-HSCT, whereas blood samples were collected only once from healthy volunteers as HLA-A*0201- or HLA-A*1101-positive and negative controls. We evaluated the performance of HLA-A*0201/BMLF1-GLC and HLA-A*1101/LMP2-SSC pentamers based on the percentages of EBV-specific CD8+ T cells by four-color flow cytometry. All experiments were performed in triplicate.

**Pentamer staining assays**

FACS lysis solution (BD Biosciences) was added to the blood samples to lyse the erythrocytes. Cells were washed twice and resuspended in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 0.1% sodium azide. The number of nucleated cells was counted under a microscope and adjusted such that each tube contained 1–2 × 106 nucleated cells. Titrated pentamers were then added to the samples. After incubation for 10 min at room temperature (25 °C) without light, titrated monoclonal antibodies (PerCP-, FITC-, or APC-conjugated CD3, CD8, or CD19, respectively) were added for surface staining. After incubation for 20 min at 4 °C without light, the cells were washed twice with PBS containing 1% FCS and 0.1% sodium azide and then stored in 1% paraformaldehyde at 4 °C. Samples were analyzed on a FACS Calibur instrument (BD Biosciences) within 6 h. At least 5000 live CD3+ lymphocytes were counted by flow cytometry. CellQuest (BD Biosciences) was used for analysis. The frequencies of pentamer-binding CD8+ T cells were calculated based on the percentages of CD3+CD8+CD19− T cells that bound to the pentamers. Blood samples from HLA-A*0201- and HLA-A*1101-negative patients were used as negative controls.

**Statistics**

Statistical analysis was performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL, USA). Quantitative data with normal distributions were expressed as the mean ± standard deviation. Continuous variables and quantitative data without normal distributions were expressed as the median and range. The percentages of pentamers between two groups were compared using independent sample t tests. Wilcoxon rank-sum tests were used to compare the frequencies of EBV-A*0201/BMLF1-GLC pentamers at + 60 days between patients with and without EBV-related diseases. Analysis of variance was used to compare the percentages of pentamer-reactive cells among multiple groups. All analyses were two-tailed and p < 0.05 was considered significant.

**Results**

**Patient characteristics**

The characteristics of the 19 patients included in this study are presented in Table 1. The general data including sex (p = 0.770), age (p = 0.967), diseases (p = 0.669), and conditioning regimens (p = 0.849) were not significantly different between the HLA-A*0201 and 1101 groups.

All the 19 patients showed 100% chimerism at + 30 days after haplo-HSCT. One patient in the HLA-A*0201 group lost the 100% chimerism at + 37 days after HSCT because of severe invasive pulmonary aspergillosis and died at + 43 days. The other patients sustained the 100% chimerism during their lifetimes.

EBV-DNA was detected in blood or tissue samples from 10 patients, including five with EBV-DNAemia and five with EBV-related diseases. All five cases with EBV-related diseases were probable PTLDs based on positron emission tomography-computed tomography (PET-CT) imaging or tissue biopsy combined with EBV-DNA copy number assays by qPCR, and histological examination by in situ hybridization to detect EBV-encoded small RNA transcripts or viral antigens was not performed because of the small sample size. In the five patients with EBV-related diseases, three had fever, tonsillitis, adenopathy, and high peripheral blood EBV-DNA loads; EBV-DNA was not detected in the peripheral blood of two patients. However, EBV-DNA was found in intestinal biopsy specimens obtained by colonoscopy in one of these two patients and in cerebrospinal fluid collected by lumbar puncture in the other one.

**Percentages of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs in healthy controls and patients after haplo-HSCT**

The negative controls were from EBV-seropositive, HLA-A*0201 and 1101-negative patients (Fig. 1).
The mean percentages of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs were 2.222% ± 1.665% in healthy controls, 0.587% ± 0.365% in patients at + 30 days after haplo-HSCT, 2.101% ± 1.328% in patients at + 60 days post-haplo-HSCT, and 1.244% ± 0.157% in patients at + 90 days after haplo-HSCT. The mean percentage of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs at + 30 days was significantly lower than that of HLA-A*0201-positive healthy controls ($p = 0.019$), whereas the mean percentages at + 60 and + 90 days were not significantly different ($p = 0.867$ and 0.117, respectively). A comparison of the three time points (+ 30, + 60, and + 90 days) after haplo-HSCT indicated significant differences between the mean percentages of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs at + 30 and + 60 days ($p = 0.003$), whereas the differences between + 30 and + 90 days ($p = 0.529$) and between + 60 and + 90 days ($p = 0.262$) were not significant (Fig. 2).

The mean percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs were 2.508% ± 1.766% for healthy controls, and 0.774% ± 0.747% for patients at + 30 days post-haplo-HSCT, 0.945% ± 0.828% for patients at + 60 days post-haplo-HSCT, and 0.886% ± 0.572% for patients at + 90 days after haplo-HSCT. The mean percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs at + 30, + 60, and + 90 days were significantly decreased compared with those in healthy controls, ($p = 0.009$, 0.019, and 0.039 respectively). A comparison of the three-time points (+ 30, + 60, and + 90 days) after haplo-HSCT revealed no significant differences among the three groups ($p = 0.886$) (Fig. 3).

The mean percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs at + 30, + 60, and + 90 days after haplo-HSCT in patients with and without EBV-related diseases were not significantly different ($p = 0.500$, 0.889, and 1.000, respectively). A comparison of the three-time points (+ 30, + 60, and + 90 days) after haplo-HSCT revealed no significant differences among the three groups ($p = 0.886$) (Fig. 3).

% The percentages of HLA-A*0201/EBV-BMLF1-GLC-specific and HLA-A*1101/EBV-LMP2-SSC-specific CTLs in healthy controls and patients after haplo-HSCT were similar to those of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs in EBV-seropositive healthy controls (2.508% ± 1.766% vs 2.222% ± 1.665%, $p = 0.688$). The percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs at + 60 days were significantly lower than those of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs (0.945% ± 0.828% vs 2.101% ± 1.328%, $p = 0.042$). A comparison of the mean percentages of the two EBV-specific CTLs at + 30 and + 90 days revealed no significant differences (0.774% ± 0.747% vs 0.886% ± 0.572% vs 1.244% ± 0.157%, $p = 0.491$ and 0.211, respectively) (Fig. 4 and Table 2).

The mean percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs at + 30, + 60, and + 90 days after haplo-HSCT in patients with and without EBV-related diseases were not significantly different ($p = 0.500$, 0.889, and 1.000, respectively). A comparison of the three-time points (+ 30, + 60, and + 90 days) after haplo-HSCT revealed no significant differences among the three groups ($p = 0.886$) (Fig. 3).

### Table 1 Patient characteristics

| Characteristics                              | Cases (n = 19) |
|---------------------------------------------|----------------|
| Patient age, median (range), years          | 33 (3–57)      |
| Sex                                         |                |
| Male                                         | 13             |
| Female                                       | 6              |
| Diagnosis                                   |                |
| AML                                         | 3              |
| ALL                                         | 7              |
| MDS                                         | 7              |
| PID                                         | 2              |
| Pre-HSCT EBV status, donor/recipient        | 19             |
| Positive/positive                           |                |
| MNC, median (range), $\times 10^9$/kg        | 5.17 (2.53–9.59) |
| CD34+ cell absolute count, median (range), $\times 10^6$/kg | 6.37 (1.61–11.13) |
| CD3+ cell absolute count, median (range), $\times 10^8$/kg | 2.76 (0.82–7.83) |
| Engraftment                                 |                |
| White cells, median (range), days           | 12 (10–18)     |
| Platelets, median (range), days             | 13 (9–18)      |
| Acute graft-versus-host disease, n           |                |
| No                                          | 7              |
| I–II degree                                 | 10             |
| III–IV degree                               | 2              |
| EBV infection after HSCT within 100 days    |                |
| No                                          | 9              |
| EBV-DNAemia                                  | 5              |
| EBV disease                                 | 5              |
| CSA stop                                     |                |
| No                                          | 4              |
| Yes                                         | 15             |
| CSA stopping time, median (range), days      | 32.5 (4–88)    |

The mean percentages of HLA-A*1101/EBV-LMP2-SSC-specific CTLs in patients with and without EBV-related diseases were not significantly different ($p = 0.500$, 0.889, and 1.000, respectively).

### Comparison of HLA-A*1101/EBV-LMP2-SSC-specific CTLs in patients with and without EBV-related diseases

Among the nine patients with HLA-A*1101/EBV-LMP2-SSC-specific CTL data, five showed no evidence of EBV reactivation, two had EBV-DNAemia, and two had EBV-related diseases; one patient who suffered from EBV-related gastroenteritis died.

EBV-DNA copies were not simultaneously detected in the peripheral blood of the patient with EBV-related gastroenteritis. We speculated that the detection of high EBV-DNA copies in blood was not always synchronous with EBV-related diseases. Therefore, we did not associate EBV-DNA copies with EBV-CTLs.
Comparison of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs in patients with and without EBV-related diseases

Among the nine patients with HLA-A*0201/EBV-BMLF1-GLC-specific CTL data, three showed no evidence of EBV reactivation, three had EBV-DNAemia, and three had EBV-related diseases; two patients suffering from EBV-related diseases died. EBV-DNA copies were not simultaneously detected in the peripheral blood of the patient with EBV-related encephalitis. The characteristics of these nine patients are presented in Table 3.

The mean percentages of HLA-A*0201/BMLF1-GLC-specific CTLs at +30 days after haplo-HSCT in patients...
with and without EBV-related diseases were not significantly different ($p = 0.909$). However, there was a significant difference in the mean percentages of HLA-A*0201/BMLF1-GLC-specific CD8$^+$ T cells at +60 days between patients with and without EBV-related diseases ($p = 0.024$), with patients with EBV-related diseases showing a lower percentage than those without EBV-related disease (Fig. 5). Five patients had EBV-CTL percentage data at +90 days in the HLA-A*0201 group, but none suffered from EBV-related diseases. Thus, we did not analyze the difference in HLA-A*0201/EBV-BMLF1-GLC-specific CTLs at +90 days.

**Discussion**

Multiple studies have suggested the importance of EBV-specific T cells in controlling EBV infections, particularly after HSCT [13, 14]. However, little is known about EBV-specific immune reconstitution after HSCT. The results of our current study provided insight into the early reconstitution of EBV-specific CD8$^+$ T cells after haplo-HSCT with the RIC protocol including ATG. Our study of the dynamics of EBV-specific immune reconstitution in the early stage after haplo-HSCT will assist in determining the appropriate time point of EBV-specific immune therapy to prevent or treat EBV infections.

HLA-A*0201/BMLF1-GLC tetramers or pentamers can be used to detect EBV-CTLs recognizing the BMLF1 lytic cycle protein in healthy long-term EBV carriers, patients with infectious mononucleosis, and those who have undergone solid organ transplants or HSCT [15–18]. HLA-A*1101/LMP2-SSC tetramers are often used to detect EBV-CTLs against the LMP2 latent cycle protein in healthy controls were 2.508% ± 1.766%, and the percentages of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs in healthy controls were 2.222% ± 1.665%.
Table 2: Percentages of EBV specific CTLs in 19 patients at + 30, + 60, and + 90 days after haplo-HSCT and in healthy controls

| Groups        | Patients/healthy controls (NO.) | Percentages at + 30 days | Percentages at + 60 days | Percentages at + 90 days |
|---------------|---------------------------------|--------------------------|--------------------------|--------------------------|
| HLA-A*0201 group |                                 |                          |                          |                          |
| P1            | 0.1982                          | 2.9100                   | 1.0950                   |
| P2            | 0.8337                          | 1.9200                   | 1.3644                   |
| P3            | 0.7296                          | 3.0000                   | 1.0119                   |
| P4            | 0.3252                          | 3.1700                   | 1.4483                   |
| P5            | 0.0179                          | 1.9000                   | 1.1990                   |
| P6            | 1.0790                          | 4.2400                   | –                        |
| P7            | 0.5619                          | 0.6900                   | –                        |
| P8            | 0.2727                          | 0.5300                   | –                        |
| P9            | 0.9600                          | 0.5500                   | –                        |
| P10           | 0.8924                          | –                        | –                        |
| H1            | 2.1000                          |                          |                          |
| H2            | 0.6200                          |                          |                          |
| H3            | 1.5900                          |                          |                          |
| H4            | 5.7900                          |                          |                          |
| H5            | 1.7700                          |                          |                          |
| H6            | 0.3000                          |                          |                          |
| H7            | 2.8800                          |                          |                          |
| H8            | 1.4700                          |                          |                          |
| H9            | 3.4800                          |                          |                          |
| HLA-A*1101 group |                                 |                          |                          |                          |
| P1            | 2.4667                          | 0.0672                   | –                        |
| P2            | 0.3413                          | 0.5073                   | 0.4926                   |
| P3            | 0.1585                          | 0.3339                   | 0.3434                   |
| P4            | 1.2534                          | 1.5635                   | 1.0086                   |
| P5            | 1.0931                          | 2.8223                   | 1.1053                   |
| P6            | 0.2601                          | 0.5272                   | –                        |
| P7            | 0.6569                          | 0.7229                   | 0.4940                   |
| P8            | 0.5787                          | 1.0769                   | –                        |
| P9            | 0.1537                          | 0.8800                   | 1.8715                   |
| H1            | 3.5392                          |                          |                          |
| H2            | 0.7454                          |                          |                          |
| H3            | 2.1402                          |                          |                          |
| H4            | 3.5406                          |                          |                          |
| H5            | 2.0589                          |                          |                          |
| H6            | 2.7509                          |                          |                          |
| H7            | 6.1721                          |                          |                          |
| H8            | 0.6712                          |                          |                          |
| H9            | 2.7146                          |                          |                          |
| H10           | 2.6164                          |                          |                          |
| H11           | 0.7193                          |                          |                          |
| H12           | 1.4663                          |                          |                          |
| H13           | 0.9191                          |                          |                          |
| H14           | 6.6107                          |                          |                          |
patients with nasopharyngeal carcinoma or lymphoma [19, 20], but are not frequently employed to screen transplant recipients. We detected EBV-CTLs using both GLC- and SSC-pentamers specific to the lytic cycle protein BMLF1 and the latent cycle protein LMP2, respectively, in all patients after haplo-HSCT and in healthy volunteers. The results indicate that the two pentamers were also useful for detecting EBV-CTLs in the peripheral blood of patients suffering from HSCT.

Samples from healthy adult volunteers produced negative and positive reactions in EBV-IgM and EBV-IgG serum arrays, respectively. Hence, the frequencies of EBV-CTLs may reflect the levels of EBV-CTLs in healthy long-term EBV carriers. In our study, the frequencies of EBV lytic antigen BMLF1-GLC and latent antigen LMP2-SSC associated with EBV-CTLs in healthy long-term EBV carriers were 2.222% ± 1.665 and 2.508% ± 1.766%, respectively. These results demonstrated that the frequencies of EBV-CTLs against the immediate early lytic cycle protein and latent cycle protein were comparable in healthy long-term EBV carriers. Another study also showed that EBV-specific CD8+ T cells against lytic protein epitopes HLA*A2/GLC and HLA*B8/RAK were found to be higher than those against latent protein epitopes HLA*A2/SVR and HLA*B8/FLR, respectively [16]. The difference in the two studies indicates that larger sample sizes and a greater number of pentamers of different antigen epitopes are needed to detect statistical significance. Additionally, our results should be further verified using other methods, such as ELISPOT.

This study was a preliminary investigation of the dynamics of GLC-specific and SSC-specific EBV-CTL reconstitution after haplo-HSCT. Compared with healthy EBV carriers, GLC-specific EBV-CTLs appeared at +30 days after haplo-HSCT, although at a significantly lower level, subsequently recovering to normal levels at +60 days and decreasing slightly at +90 days after haplo-HSCT. SSC-specific EBV-CTLs were detected in all nine patients at +30, +60, and +90 days after haplo-HSCT; however, the frequencies were significantly lower than those in healthy EBV carriers, indicating that SSC-specific EBV-CTLs recognizing the latent protein, LMP2, did not recover to normal levels within 90 days after haplo-HSCT. Frequencies of HLA-A*1101/LMP2-SSC-specific CTLs at +60 days were significantly lower than those of HLA-A*0201/EBV-BMLF1-GLC-specific CTLs. The frequencies of the two types of EBV-CTLs reached a balance at +90 days after HSCT. The dynamics of

| Table 2 | Percentages of EBV specific CTLs in 19 patients at +30, +60, and +90 days after haplo-HSCT and in healthy controls (Continued) |
|----------|----------------------------------------------------------------------------------------------------------------------------------|
| Groups   | Patients/Healthy controls (NO.) | Percentages at +30 days | Percentages at +60 days | Percentages at +90 days |
| H15      | 3.5822                                                                    | 3.5822                                                                   | 3.5822                                                                   |
| H16      | 3.5711                                                                    | 3.5711                                                                   | 3.5711                                                                   |
| H17      | 2.2981                                                                    | 2.2981                                                                   | 2.2981                                                                   |
| H18      | 2.6966                                                                    | 2.6966                                                                   | 2.6966                                                                   |
| H19      | 2.8326                                                                    | 2.8326                                                                   | 2.8326                                                                   |

Abbreviations: P patient, H healthy controls. The percentages of EBV specific CTLs of healthy controls were detected at only one time point during our study.

| Table 3 | Characteristics of nine patients with HLA-A*0201 |
|----------|---------------------------------------------------|
| Patient No | EBV state          | Percentages of EBV-CTLs at +30 days | Percentages of EBV-CTLs at +60 days | Percentages of EBV-CTLs at +90 days | Time of CSA withdrawal | aGvHD | Result | Cause of death |
| 1         | No                 | 0.1982                             | 2.9100                               | 1.0950                               | –                      | 20    | Grade I | alive          |
| 2         | EBV-DNAemia        | 0.8337                             | 1.9200                               | 1.3644                               | 1.0119                 | 19    | Grade I | alive          |
| 3         | No                 | 0.7296                             | 3.0000                               | 1.4483                               | –                      | Grade I | alive |
| 4         | EBV-DNAemia        | 0.3252                             | 1.7000                               | 3.1900                               | 1.4483                 | –     | Grade I | alive          |
| 5         | EBV-DNAemia        | 0.0179                             | 1.9000                               | 1.1990                               | 1.0119                 | 16    | Grade I | alive          |
| 6         | No                 | 1.0790                             | 4.2400                               | –                                    | 11                     | Grade I | dead  | Aspergillus pneumonia |
| 7         | EBV-related disease| 0.5619                             | 0.6900                               | –                                    | 5                      | Grade I | dead  | EBV encephalitis |
| 8         | EBV-related disease| 0.2727                             | 0.5300                               | –                                    | –                      | no alive |       |
| 9         | EBV-related disease| 0.9600                             | 0.5500                               | –                                    | 58                     | Grade I | dead  | EBV enteritis    |

Note: The patient who died at +43 days was a 100% donor type by bone puncture and STR examination at +30 days after transplantation. However, at +33 days, this patient developed hyperthermia, pulmonary infection, and decreased blood cells and was found to have decreased chimerism to 23% of that of the donor recipient at +37 days. Secondary graft failure was observed, which was thought to be related to the serious pulmonary fungal infection. We did not include this patient in this Table.
EBV-specific immune reconstitution were similar to those of immune constitution observed in patients with infectious mononucleosis who exhibited abundant reactive EBV-CTLs against lytic cycle proteins, whereas EBV-CTLs against latent cycle peptides were detectable, but at low levels. From 3 to 8 months after acute infection, EBV-CTLs against lytic antigens gradually decreased, whereas those recognizing latent antigen began to increase until homeostasis between the two groups of EBV-CTLs was reached. Marshall et al. [21] reported similar findings in one post-transplant patient; at +30 days after transplantation, the levels of EBV-CTLs against lytic and latent phase antigenic peptides were significantly lower in the patient. However, the EBV-CTLs against lytic or latent antigen were clearly increased at +60 days, and the EBV-CTLs recognizing the lytic antigen were predominant. They also found that the EBV-specific immune response was recovered at +30 days after HSCT.

These results regarding the kinetics of EBV-specific immune reconstitution can facilitate EBV-specific immunotherapy. Thus, we compared the levels of EBV-CTLs in patients with or without EBV-related diseases. Those with EBV-related diseases showed lower levels of GLC-specific EBV-CTLs at +60 days after haplo-HSCT than those without EBV-related diseases. This indicated that patients with a lower response against the lytic protein BMLF1-GLC at +60 days were at a higher risk of developing EBV-related diseases. Annels et al. [18] simultaneously analyzed viral loads and T cell reconstitution and arrived at a similar conclusion, demonstrating that the effectiveness of preemptive intervention may be limited in patients who lack EBV-specific T cell expansion during the initial phase of EBV reactivation. Our findings were also in accordance with the results reported by D’Aveni et al. [22], who monitored EBV-specific T cell quantification by Elispot assay at +60, +100, +180, and +360 days after HSCT. For cases in which EBV DNAemia occurred and cleared spontaneously, their ELISPOT results indicated the presence of more than >1000 spot-forming cells per 10^6 PBMCs.

This finding suggested that the low frequency of EBV-CTLs recognizing BMLF1-GLC at +60 days after haplo-HSCT could be used as a marker for the occurrence of EBV-related diseases. Transfusion with EBV-CTLs within 60 days after haplo-HSCT may have prophylactic effects against EBV-related diseases. However, we analyzed a small number of cases which may have resulted in large deviations. Further studies with larger sample sizes are needed to verify our findings.

**Conclusion**

In contrast to similar studies, the patients in our study were relatively homogeneous with regard to the type of donor, source of graft, conditioning, source and dose of ATG, and type of immunosuppressive prophylaxis. However, our study had a small number of patients, and thus the findings should be interpreted with caution. Overall, our data indicated that EBV-specific immune reconstitution after haplo-HSCT significantly affected the control of EBV-related diseases. Thus, transfusion with EBV-CTLs within 60 days after haplo-HSCT may have prophylactic effects against EBV-related diseases.

**Abbreviations**

aGVHD: Acute graft-versus-host disease; APC: Allophycocyanin; ATG: Antithymocyte globulin; CSA: Cyclosporine A; CTLs: Cytotoxic (CD8+) T lymphocytes; EBV: Epstein Barr virus; FCS: Fetal calf serum; FITC: Fluorescein isothiocyanate; Haplo-HSCT: Haploidentical hematopoietic stem cell transplant; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PE: Phycoerythrin; PerCP: Peridinin chlorophyll protein; PTLDs: Post-transplant lymphoproliferative disorders; RIC: Reduced intensity conditioning

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**Authors’ contributions**

LZ performed this study and contributed to isolate collection and experimentation, data collection, data analysis, and writing of the
manuscript; DL was the supervisor of the study. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
The study protocol was approved by the ethics committee of Shanghai Daon-Pei Hospital. Informed consent was obtained from the study participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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