Adoptive Transfer of Cytomegalovirus/Epstein-Barr Virus-specific Immune Effector Cells for Therapeutic and Preventive/Preemptive Treatment of Pediatric Allogeneic Cell Transplant Recipients

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Summary: This report describes a safe and effective therapy through adoptive transfer of donor cytomegalovirus (CMV)/Epstein-Barr virus (EBV) immune effector cells. The patients, from 3 to 10 years of age, suffering from hematologic diseases received haploidentical transplantation. All 3 patients developed varying levels of viremia from days 13 to 31 and 2 patients developed CMV-interstitial pneumonitis or interstitial inflammation after transplantation. Tapering down the dose of immunosuppressives together with intensive antivirus therapy and escalated infusions of donor-derived CMV/EBV immune effector cells effectively controlled virus-related diseases. All 3 patients survived and remained CMV/EBV-free 14-16 months after transplantation.

Key Words: CMV, EBV, cytotoxic T cell, allogeneic hematopoietic cell transplant

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Cytomegalovirus (CMV)-related diseases are major causes of transplant-related morbidity and mortality in immunosuppressed patients, especially in the early post-transplant period.[1,2] Transplantation-related B-cell lymphoproliferative disease caused by the Epstein-Barr virus (EBV), with an incidence varying from 12% to 25%, can be life-threatening.[3–6] T-lymphocyte depletion of the graft or use of regimens containing antithymocyte globulins (ATG) and fludarabine can substantially increase the risk of activating CMV, EBV, adenovirus (ADV), BK virus (BKV) and other viruses after transplantation, which may lead to life-threatening infections.

Adoptive immunotherapy with cytotoxic T lymphocytes (CTLs) reactive with specific viral antigens has proven to be effective without stimulating acute graft-versus-host disease (GVHD) owing to the significantly reduced non-specific alloreactivity.[1–3] Unfortunately, the effect of the adoptive transfer of CMV/EBV-specific CTLs to pediatric patients is unknown because such case reports are rare. Here, we aim to evaluate the safety and efficacy of multiple infusions of therapeutic and preemptive CMV and EBV immune effector (IE) cells in young patients. The results of 3 case studies are presented and discussed.

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PATIENTS AND METHODS

Patient and Donor Details

This study was approved by the Institutional Ethics Committees of Fu Dan University, BMT Center, Shanghai Dao-Pei Hospital. Pediatric patients undergoing haploidentical hematopoietic stem cell transplant (HSCT) for hematologic disorders either considered high risk (diagnosis of severe aplastic anemia or less-than-standard body weight for age) or manifested a severe virus infection after transplantation were enrolled into the study. Written informed consent was obtained from the recipients' parents before enrollment. Three recipients were enrolled and each given haploidentical grafts [bone marrow (BM) + peripheral blood (PB)] from one of the parents. Conditioning regimens have been described earlier.[7,8] In brief, they were (1) Ara-c + BUCY + ATG: cytarabine (3 g/m^2/d) intravenously on days −10 to −9; busulfan (4 mg/kg/d) orally on days −8 to −6; cyclophosphamide (1.8 g/m^2/d) intravenously on days −5 to −4; Me-ccnu (250 mg/m^2) orally once on day −5, and ATG (Genzyme; 2.5 mg/kg/d) intravenously for 4 consecutive days from −5 to −2 and (2) CyFlu + ATG: Cy (50 mg/kg/d) intravenously on days −9 to −6, fludarabine (30 mg/m^2) on days −5 to −2; ATG, dose and schedule as above. GVHD prophylaxis included cyclosporine, short-term methotrexate, and 14 days mycophenolate mofetil.[7,8]

Quantitative Polymerase Chain Reaction of Viral DNA

Genomic DNA was isolated from 250 μL of whole blood with an Axyprep blood genomic DNA miniprep kit (Axygen, Union City, CA) according to the manufacturer's instruction. Quantitative polymerase chain reaction (PCR) was performed on an ABI 7300 thermal cycler using commercially available PCR kits for CMV, EBV, ADV (Daan Gene Technology, Guangzhou, China), and BKV (Zhijiang Biotech, Shanghai, China). Quantitative PCR

results were shown as DNA copies per milliliter of whole blood with a detection threshold of 10^3 copies/mL for CMV, ADV, and BKV or 500 copies/mL for EBV.

Immune Reconstitution and Antivirus Therapy

Lymphocyte immune reconstitution was monitored by an immunophenotype assay with a FACScan cytometer (Becton Dickinson, San Jose, CA). Ganciclovir (5 mg/kg twice daily) was routinely given intravenously, from days −9 to −2, as prophylaxis during conditioning therapy. After transplantation, qualitative CMV/EBV/ADV/BKV PCR was performed twice a week. If positive, CMV-DNAemia was treated preemptively with GCV (5 mg/kg intravenously twice daily for 21 d, and then 5 mg/kg intravenously once daily for 30 d) or with foscarnet (90 mg/kg twice a day) in the event of GCV toxicity. CMV/EBV-specific IE cells, which contained mainly CD3+ T cells with some CD3-CD56+ NK cells and less than 2% of CD3-CD56− cells, were infused directly into these patients within 12 hours of cell harvest. Median cell dosage was 0.6×10^6/kg/time (ranging from 0.31 to 6.2×10^6/kg/time) with >90% viability.

Preparation of 2-day Dendritic Cells and Generation of Antigen-specific IE Cells

PBMCs were plated into a 6-well plate at 1×10^7 cells/well and adhered for 2 hours in AIM-V (Gibco-BRL, CA). The nonadherent cells were removed gently and frozen as the source of lymphocytes for co-culture use. Adherent monocytes were cultured in AIM-V supplemented with 50 ng/mL of GM-CSF and 25 ng/mL interleukin (IL)-4 (eBiosource International, Inc., Camarillo, CA) for 24 hours and incubated for another 24 hours with tumor necrosis factor-α (50 ng/mL), IL-1-β (10 ng/mL), IL-6 (10 ng/mL, all from R&D Systems, MN) and PGE2 (1 μM, Sigma-Aldrich, MO) for maturation. Dendritic cell (DC)-activated antigen-specific IE cells were generated as described earlier.[9,10] In brief, mature 2-day DCs were loaded with pooled CMV or EBV pentadecapeptides (2.5 μg/mL) for 2 hours and irradiated (20 Gy or 2000 rads). The mixtures of 11 amino acid-overlapping pentadecapeptides spanning the entire protein of LMP2A of the EBV, pp65, and immediate-early 1 (IE-1) of the CMV, and Wilms tumor antigen (WT1) were purchased from JPT Peptide Technologies GmbH (Berlin, Germany). The antigen-pulsed DCs were co-cultured with autologous nonadherent PBMCs at a ratio of 1:20 in AIM-V with 5% human AB serum. On day 3, half of the medium was replaced with fresh medium supplemented with IL-2 (12.5 U/mL), IL-7 (5 ng/mL) and IL-15 (20 ng/mL, all from Gentaur, Aachen, Germany). Half of the medium was replaced with a fresh medium with cytokines every other day until harvest for analysis and infusion.

RESULTS

Case 1: Graft Failure Caused by Early CMV Primoinfection

A 5-year-old girl with acute myeloid leukemia in the first early relapse (6.5% blasts in BM) received her mother's G-CSF mobilized bone marrow and peripheral blood (G-BM/PB cell allograft on May 12, 2008. AraC + BU-CY + ATG were given as a conditioning regimen. CMV serologic examinations indicated that the girl was negative and her mother was positive. She developed fever with CMV primoinfection on day 13 after transplantation. Intravenous foscarnet (90 mg/kg twice a day) was given as preemptive therapy for 1 day only and was stopped because of obvious thrombocytopenia. The patient showed temporary hematopoietic reconstitution after day 22 and gradually developed pancytopenia on day 32 (Table 1 and Fig. 1), associated with a high CMV DNA levels (Fig. 2A). The patient also presented BK virus-related hemorrhagic cystitis on day 26. As the main treatment, cyclosporine was tapered down and donor-derived CMV/EBV-specific IE cells were prepared on day 25 and infused on day 35. The IE cells were activated with peptide-pulsed DCs and cultured for 17 days. Representative functional analyses of the CMV-specific and EBV-specific IE cells, including intracellular cytokine staining and CD107a degranulation (cytotoxicity function) are shown in Figures 1B and C; the IE cells showed antigen-specific release of interferon-γ and CD107a. In addition, there was a dominant CD4 or CD8 T-cell response in the IE cell population, which seemed to be donor- and antigen-dependent (additional data not shown). The patient received a combination of CMV/EBV-IE cells once a week from days 35 to 102 with a total of 8 infusions. Time points for the first 4 infusions are shown in Figure 2A; CMV-DNA copies sharply decreased within 3 days and became undetectable in 8 days after the first infusion. To assist in hematopoietic reconstitution, the patient received additional donor BM cells on days 39 and 40. She gradually achieved hematopoietic reconstitution on day 58 (day 19 after the additional donor BM infusion, Table 1, No. 1). She developed slight interstitial pneumonia after the clearance of CMV on day 68, evidenced by computed tomography (CT) imaging and arterial blood O_2 of 89% to 96% but recovered rapidly in 1 week with resolved clinical symptoms after tapering down the dose of immunosuppressives and ribavirin, aciclovir, and voriconazole therapy. She fully recovered from hemorrhagic cystitis by day 84, and has remained disease-free more than 16 months after transplantation.

Case 2: Preemptive Treatment of Early CMV/EBV Reactivations

A 10-year-old girl, weighing 27 kg, had severe aplastic anemia and more than 20 blood transfusions before transplant, received a G-BM/PB haploidentical graft from her father on July 18, 2008. Hematopoietic reconstitution was documented on day 13 but viral reactivations were also detected (EBV/BKV-DNA on day 13 and CMV-DNA on day 16) with increased copy numbers ($\geq 10^3$/mL for CMV, HSV, ADV, BKV and $\geq 5 \times 10^2$/mL for EBV) from days 20 to 31 (Fig. 2B). CMV pp65 and immediate-early 1 IE cells were prepared. She received preemptive infusions of CMV/EBV-IE cells on day 27 and continued once a week for 4 weeks (Fig. 2B, and Table 1, No. 2). The patient developed increased skin rash 8 days after IE cell infusion, which was higher than previously experienced with the IE cell infusions; therefore, corticosteroids (20 mg), were given intravenously every 12 hours, and FK506 sufficient to achieve a target blood level of 5 to 10 ng/mL. Virus DNA levels were significantly reduced on day 5 (CMV-DNA) and day 13 (EBV-DNA), and a total clearance of viremia was achieved on days 27 (CMV-DNA) and 35 (EBV-DNA) after the first IE cell infusion. As co-treatment, foscarnet (90 mg/kg) was given intravenously twice daily from days 20 to 41. The patient exhibited second-degree hemorrhagic cystitis on day 20 that was completely cleared on day 51

TABLE 1. Virus Surveillance and Antivirus Treatment After Transplantation

	Virus Infection/ Reactivation (Day)				Viral Diseases (Day)			IE Cells								DLI		Co-treatment
No.	CMV	EBV	ADV	BKV	CMV-IPn	EBV-LPD	HC	Types	First Infusion Day	Infusion Times	Cell Dose×106/kg Per Time	Total	T-cell Subtype (%) CD3	CD4	CD8	CD56	Start Times	Start day	MNC×10^8/ kg/Time	CD3+×10^6/ kg/Time	Medicine
1	13	—	—	26	68	—	26	CMV-IE	35	8	2.2-6.2	35.5	86.7	31.7	58.2	6.9	2(BM)	39	1	8.7	Foscarnet, 49-59 d
								EBV-IE	35	7	0.77-2	11.7	86	31.6	55.6	8.6					Early taper-down CsA
2	31	27	—	20	—	—	20	CMV-IE	27	4	0.33-0.45	1.498	91.3	61	28.9	5.7	—	—			Foscarnet, 20-41 d
								EBV-IE	27	4	0.31-1.11	3.418	91.3	59.2	30.4	5.9					Corticosteroid, CsA, FK506
3	30	—	—	Once	39	—	—	CMV-IE	24	3	0.6-0.84	2.23	96	44.8	44.9	9.5	2(BM)	38	0.36	1.63	Foscarnet, 39-62 d
								EBV-IE	24	3	0.4-0.78	1.61	97	52	37.6	6.7					GCV, 40-49 d

CMV indicates cytomegalovirus; DLI, donor leukocyte infusion; EBV, Epstein-Barr virus; HC, hemorrhagic cystitis; IE, immune effector; IPn, interstitial pneumonitis; MNC, mononuclear cell; LPD, lymphoproliferative disease.

without evidence of CMV-related or EBV-related disease. The patient was last documented in complete recovery from persistent virus reactivation with an acute grade 2 skin GVHD, and had been disease-free for more than 14 months.

Case 3: Adoptive Transfer of Donor CMV/EBV IE Cells as a Preventive Therapy

A boy, age 3 years and 10 months, with acute myeloid leukemia M5, received a G-BM/PB haploidentical graft from his mother. Considering his age and body weight (15.5 kg), donor-derived CMV/EBV-specific IE cells were prepared at donor PB harvest through leukapheresis on day 2 (BM harvest at day 1) and the IE cells became available after 21 days of expansion in vitro. Both of the CMV and EBV IE cells showed strong effector functions with higher content of CD8 effector T cells. The patient developed mild engraftment syndrome on day 11; complete engraftment was achieved on day 13 without acute GVHD (before donor IE cell infusion). First IE cell infusion was given on day 24 after transplantation, 6 days before the detection of CMV reactivation (CMV-DNA > 10^3/mL blood) on day 30. He developed lung infection on day 39 manifested with only a slight decrease in blood O_2 (95-98%) and chest CT showed slight interstitial inflammation. Treatment included adoptive transfer of donor IE cells once a week, and foscarnet (days 39-62) combined with GCV (days 40-49). The patient achieved recovery on day 41 (day 11 after the first IE cell infusion) with the disappearance of CMV-DNA, normalization of the chest CT image, and resolution of clinical symptoms. Meanwhile, he developed GCV-induced thrombocytopenia; therefore donor BM was infused on days 38 and 62. Thrombocytopenia was cleared (day 24 after donor BM infusion) (Table 1, No. 3). BKV-DNA was detected from days 34 to 61 without clinical symptoms. The patient received a total of 3 CMV/EBV-IE cell combined infusions without evidence of EBV reactivation. The patient remained EBV/CMV DNA-free with no detectable ADV/BKV DNA, and has been disease-free for more than 14 months.

DISCUSSION

Clinical reports of adoptive immune cell therapy in pediatric recipients are scarce. Micklethwaite et al recently reported the use of cytotoxic T cells as prophylactic therapy in 9 patients (age 4-65 years) on or after day 28 postallogeneic HSCT. The database included 2 pediatric patients but no detailed clinical outcomes.[11] Three recipients developed acute GVHD after infusion and 2 recipients died from thrombotic thrombocytopenic purpura secondary to cyclosporine and complications of GVHD.[11] In the cases reported here, infusions of donor IE cells helped establish an accelerated reconstitution of CMV/EBV-specific immunity without increasing the risk of GVHD. However, donor IE cell transfer did not completely prevent CMV-related diseases (cases 1 and 3). Nevertheless, it seemed that infusions of IE cells slowed down the course of CMV-related interstitial pneumonia and reduced its severity. The patients presented mild symptoms of interstitial inflammation, which was easily controlled and never developed into a life-threatening situation. These patients recovered quickly with a combination of treatment including donor BM and IE cell infusions, gradually tapering down immunosuppressive agents, and the use of foscarnet,

all of which might have contributed to disease control. Case 1 presented a grade 1 skin rash without a sign of gastrointestinal or liver injury after donor BM/IE cell therapy. Cases 2 and 3 were recipients of preemptive/preventive CMV/EBV IE cells. Both showed an intensive prophylaxis effect without EBV-related diseases and a rapid clearance of circulating CMV/EBV DNA. When retrospectively reviewed, a common phenomenon emerges that

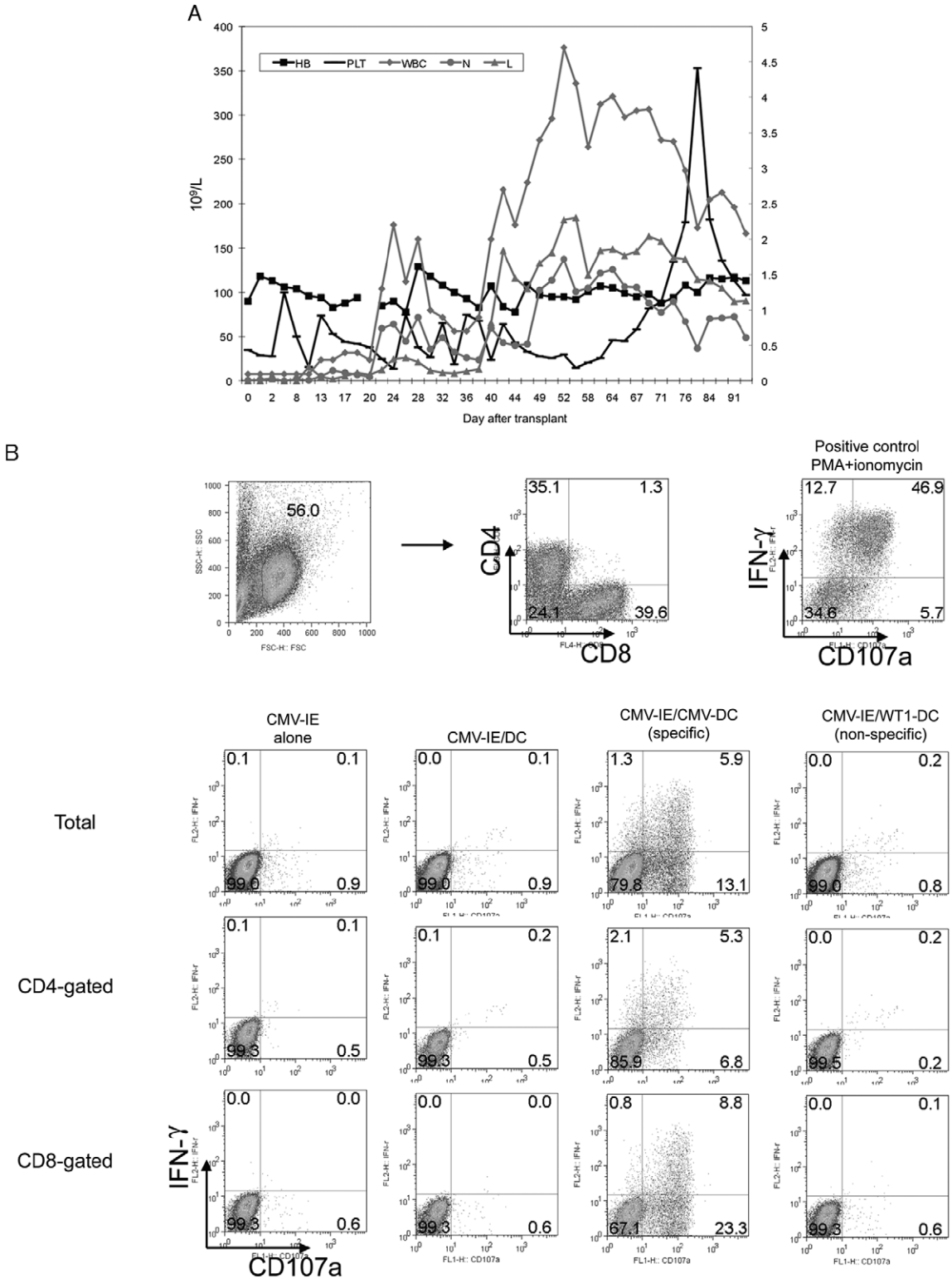

FIGURE 1. Continued.

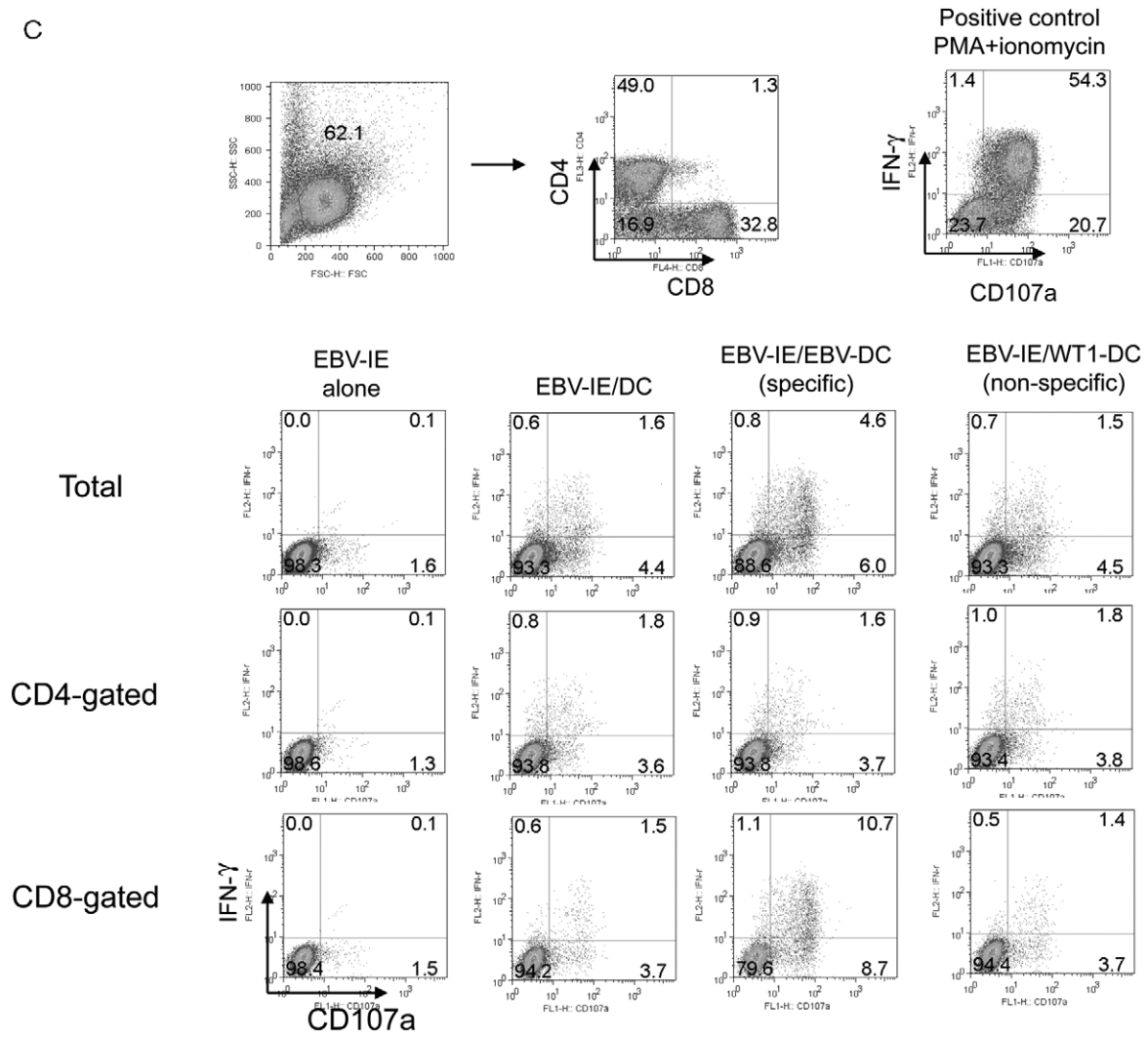

FIGURE 1. Analyses of reconstituted lymphocytes and infused immune effector (IE) cells. A, white blood cell (WBC), absolute neutrophil count, lymphocyte reconstitution after transplantation in case 1. The patient developed early cytomegalovirus (CMV) primoinfection on day 13. To establish antiviral immunity, the patient received CMV/Epstein-Barr virus (EBV) IE-cell infusions starting from day 35 continuously once a week for 8 times. Additional donor bone marrow infusions were given on days 39 and 40 to accelerate hematopoietic reconstitution. The scale to the left is for hemoglobin (HB), platelets (PLT) to the right for WBC, N (neutrophil), and L (lymphocyte). B, Flow cytometry functional analyses of CMV-specific and EBV-specific IE cells by antibody staining for intracellular cytokine interferon (IFN)-γ and degranulation of CD107a. The cells were restimulated with the specific peptide-pulsed autologous dendritic cell (DC), or control DC pulsed with Wilms tumor antigen pentadecapeptides (WT1). The IE cells treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin were included as positive control.

virus reactivation closely correlated with poor immune reconstitution and profound lymphocytopenia; this was readily controlled with improved immune reconstitution (Table 2).

How the CMV-specific CD8 and CD4 T-cell functions are restored after HSCT is still not well understood. Cwynarski et al examined CMV CTLs in recipients of allogeneic transplants from siblings (n = 13) or unrelated donors (n = 11) with fluorescent HLA-peptide tetramers specific for a dominant CMV epitope. Their study indicates that early reconstitution of CMV-specific immunity was not observed if either the donor or recipient was seronegative for CMV.[12] This may partially explain the status presented in case 1; the pediatric recipient was CMV seronegative and received a CMV seropositive haploidentical graft. She developed early CMV primoinfection and graft failure after transplantation. Adoptive transfer of donor virus-specific IE cells offered a successful immediate rescue for this girl.

CMV or EBV reactivation/infection is closely correlated with severe lymphocytopenia and the lack of CD3-positive cells. The recovery of CMV-specific CTLs to levels greater than 10^6/L has been associated with protection from CMV diseases.[9] Furthermore, it has been established that the recovery of CMV-specific T-cell response is essential for the control of viral replication and protection from severe disease in adults[13–15] and infants.[16] Thus, T lymphocytopenia is an important indicator that correlates closely with early virus activation/infection and must be monitored closely.

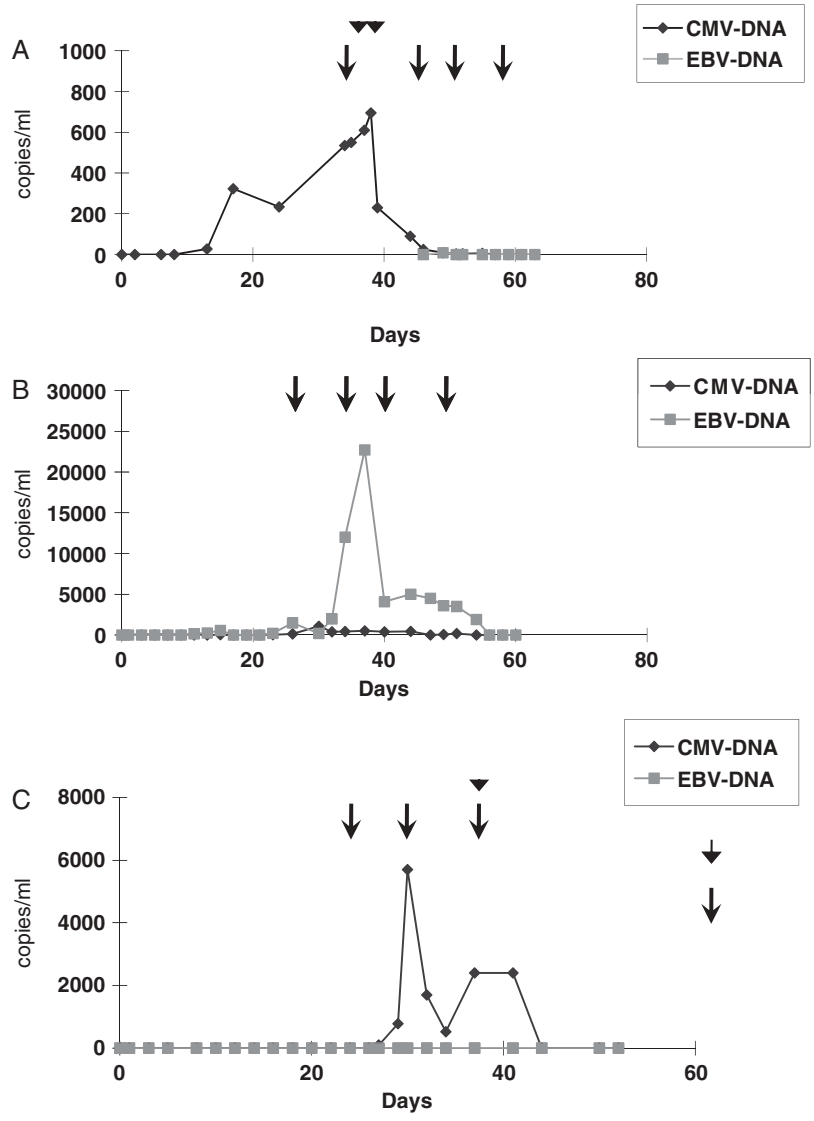

FIGURE 2. Correlation of cytomegalovirus (CMV)/Epstein-Barr virus (EBV)-DNA levels and time points of donor CMV/EBV immune effector (IE) cell infusions (arrows) and donor leukocyte infusion (DLI) or bone marrow-derived mononuclear cell (MNC) boost (arrowheads) in 3 cases. Data of detected virus DNA copies per milliliter of whole blood in case 1 were from Adicon Lab (Adicon Physical Examination Center, Hangzhou, China) with positive standard of CMV/EBV-DNA ≥ 25 copies/mL. Since July 2008, the data detected were all from our center's laboratory with a positive standard $\geq 10^3$ copies/mL for CMV, ADV, and BKV or ≥ 500 copies/mL for EBV.

In conclusion, immune cell therapy is relatively safe in pediatric patients and should be considered as supplemental therapy for conventional treatment. For young patients with rapidly progressing viral diseases, such as CMV/EBV-related infections, the immune cell therapy may offer an emergency rescue.

TABLE 2. Correlation Between CMV/EBV Reactivation and Period of Persistent Lymphopenia (≤ 300 and ≤ 1000 Lymphocytes/mm^3)

	CMV/EBV Reactivation		CMV/EBV-DNA Clearance		Days of Lymphopenia			
No.	Day	Lymphocyte×10^9/L	Day	Lymphocyte×10^9/L	$\leq 0.3\times10^9$/L	$\leq 1.0\times10^9$/L	Late CMV Diseases	Death
1	13	0.06	74	1.74	38	40	—	—
2	27	0.03	56	1.05	13	52	—	—
3	30	0.22	44	1.09	15	37	—	—

CMV indicates cytomegalovirus; EBV, Epstein-Barr virus.

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