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Phase I Clinical Trial of 4-1BB-based Adoptive T-Cell Therapy for Epstein-Barr Virus (EBV)-positive Tumors

Hyeon-Seok Eom,† Beom K. Choi,‡ Youngjoo Lee,† Hyewon Lee,* Tak Yun,* Young H. Kim,§ Je-Jung Lee,‖ and Byoung S. Kwon‡¶*

Summary: Although adoptive cell therapy using Ag-specific T cells has been tested successfully in the clinic, the production of these T cells has been challenging. By applying our simple and practical 4-1BB-based method for the generation of Ag-specific $CD8^+$ T cells, here we determined the maximum tolerated dose, toxicity profile, immunologic responses, and clinical efficacy of autologous Epstein-Barr virus (EBV)/LMP2A-specific $CD8^+$ T cells (EBV-induced Natural T cell; EBViNT) in patients with relapsed/refractory EBV-positive tumors. This was a single-center, phase I, dose-escalation trial study evaluating 4 escalating dosing schedules of single injected EBViNT. $CD8^+$ T-cell responses against different LMP2A peptides in each patient were determined, and the most effective peptides were used to produce EBViNT. The produced autologous EBViNTs were single infused to patients with EBV-associated malignancy who had failed to standard treatments and were of HLA-A02 or A24 type. Of 11 patients enrolled, 8 patients received a single infusion of EBViNT: 4 with nasopharyngeal carcinomas, 1 with Hodgkin lymphoma, 2 with extranodal NK/T lymphomas, and 1 with diffuse large B-cell lymphoma. Single infusion of EBViNT was well tolerated by all the patients and generated objective antitumor responses in 3 of them. EBViNT infusion induced 2 waves of interferon-γ response: 1 approximately 1 week and the other 4–8 weeks after the treatment. The strength of the second wave was related to the efficacy of the treatment. The current trial shows that EBViNT therapy is safe and may provide a new option for treating EBV-positive recurrent cancer patients resistant to conventional therapy.

Key Words: 4-1BB (CD137), $CD8^+$ T cells, IFN-γ, adoptive therapy, EBViNT

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Epstein-Barr virus (EBV) is a member of the herpes virus family and has been implicated in the pathogenesis of Burkitt lymphoma, Hodgkin lymphoma (HL), non-HL, nasopharyngeal carcinoma, lymphoproliferative disease, and other epithelial malignancies arising in the gastric region and breast.[1–4] EBV-specific cytotoxic T lymphocytes (CTLs) can be produced because of the strong antigenicity of EBV antigens (Ags).[5] Ag-specific T cells targeting immunodominant viral Ags of cytomegalovirus and EBV have been used with dramatic success to treat viral reactivation after bone marrow transplantation.[6] EBV-specific CTLs have been tried in patients with EBV-positive HL with multiple relapses or with minimal residual disease postautologous hematopoietic stem cell transplantation.[7] Although adoptive immunotherapy using EBV-specific CTLs has been tested successfully against EBV-associated malignancies,[8] the production of these CTLs has been challenging.

There is an increasing need for a simple and effective method for isolating Ag-specific T cells. 4-1BB (CD137) is an inducible costimulatory receptor on T cells that preferentially activates $CD8^+$ T cells in vitro and in vivo; it also prevents activation-induced cell death of $CD8^+$ T cells, and selectively induces Th1-type cytokines such as interferon (IFN)-γ and TNF-α.[9,10] On the basis of the unique characteristic of 4-1BB, namely that it is expressed specifically on activated T cells, we have previously designed a simple and practical protocol for producing Ag-specific $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs).[11] Isolating peptide-stimulated T cells with agonistic anti-4-1BB monoclonal antibody (mAb) not only provides a general and convenient method for preparing Ag-specific T cells, but is also expected to enhance the potential of the latter for proliferation, survival, and memory formation.

Latent EBV infection is associated with several malignancies, which falls into 3 types. Type I latency tumors such as Burkitt lymphoma are poorly immunogenic and only express EBNA-1; type II comprise Hodgkin and NK/T lymphomas, which are immunogenic and express LMP1/2 and EBNA-1; and type III, such as lymphoblastoid cell lines and lymphoproliferative disorders, are highly immunogenic and express EBNA-1/2/3 and LMP1/2.[12] As type II and III latency tumors are immunogenic,

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From the *Hematologic Oncology Clinic, Center for Specific Organs Cancer, National Cancer Center, Korea; †Center for Clinical Trial, National Cancer Center, Korea; ‡Cancer Immunology Branch, Division of Cancer Biology; §Immune Cell Production Unit, Program for Immunotherapeutic Research, Research Institute and Hospital, National Cancer Center, Ilsandong-gu, Goyang, Gyeonggi; ‖Department of Hematology-Oncology, Chonnam National University Medical School, Gwangju, Korea; and ¶Department of Medicine, Tulane University Health Sciences Center, New Orleans, LA.

H.S.E., Y.J.L., B.K.C., and B.S.K.: conceived and designed the study. B.K.C., Y.H.K., and B.S.K.: developed the methodology. H.S.E., Y.J.L., H.W.L., and T.Y.: acquired the data, managed patients, and provided facilities. H.S.E., Y.J.L., Y.H.K., and B.S.K.: analyzed and interpreted the data. H.S.E., Y.J.L., B.K.C., Y.H.K., and B.S.K.: wrote and reviewed the manuscript. H.S.E., Y.J.L., and B.S.K.: supervised the study. All authors read and approved the final manuscript.

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Reprints: Byoung S. Kwon, Eutilex, Co., Ltd., Suite 1310 Songeui Multiplex Hall, Catholic University School of Medicine, 222 Banpo-daero Seocho-gu Seoul Korea 06591 (e-mail: bskwon@eutilex.com).

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they are expected to be susceptible to T-cell therapy targeting EBNA-1 or LMP1/2. Expression of LMP2A in HL and nasopharyngeal carcinoma is known to play a role in the maintenance of EBV latency in the bone marrow and may be associated with oncogenesis.[13] If patients have detectable amounts of LMP2A-reactive $CD8^+$ T cells in their blood, it should be possible to isolate and expand these $CD8^+$ T cells by the procedure that we have previously developed.[11] In the present work, therefore, we first evaluated the $CD8^+$ T-cell responses against a number of different LMP2A peptides in each of the patients and used the most effective peptides in each case for producing EBV/LMP2A-specific $CD8^+$ T cells (EBV-induced Natural T cell; EBViNT) in a good manufacturing practice (GMP) facility.[11] EBViNTs were infused once in escalating doses—0.5, 1.0, 2.0, and 4.0×10^8 cells/m^2 into each of the 8 patients with EBV-expressing tumors. We describe the clinical responses to the treatment and the immunologic profiles of each patient as well as evidence for epitope spreading.

MATERIALS AND METHODS

Epitope Screening

PBMCs were isolated from each cancer patient, aliquoted into 8 tubes, and cultured with 8 different EBV/LMP2A peptides (CLGGLLTMV, LTAGFLIFL, FLYALALLI, TVCGGIMFL, TYGPVFMSL, TYGPVFMCL, PYLFWLAAI, IYVLVMLVL) for 14 days, replacing half of the medium on days 7, 9, 11, and 13. On day 14, the PBMCs were washed and restimulated with the same peptide (2 μg/mL). After 24 hours, the cells were stained with anti-4-1BB-PE and anti-CD8α-PE-Cy5, and analyzed by FACSCalibur (BD Bioscience).

Isolation and Expansion of EBV/LMP2A-specific $CD8^+$ T Cells (EBViNT)

EBViNTs were routinely produced from EBV-related cancer patients as described in Figure 1. The best LMP2A peptide for $CD8^+$ T-cell production was selected for each patient based on the epitope screening (Fig. 1A), and EBV LMP2A-specific $CD8^+$ T cells were produced as previously described.[11] In brief, PBMCs were isolated from 50 mL of blood and suspended in RPMI1640 medium (WelGENE) supplemented with 4 mM L-glutamine, 12.5 mM HEPES, 50 μM 2-mercaptoethanol, and 3% autologous serum (CTL medium; CM) at 1×10^6 cells/mL density. The PBMCs were cultured in 14 mL round-bottomed tubes (BD Biosciences) and EBV/LMP2A-specific $CD8^+$ T cells were amplified by incubating them with 2 μg/mL EBV/LMP2A peptide for 14 days (Fig. 1B). On day 14, the cells were harvested, washed, and restimulated with 5 μg/mL of EBV/LMP2A peptide and 100 IU/mL interleukin (IL)-2. After 24 hours, 4-$1BB^+$ $CD8^+$ T cells were sorted by placing the cells in anti-4-1BB mAb-coated 6-well plates for 10 minutes in a 37°C CO_2 incubator (Fig. 1C). The plate-bound cells were maintained in CM containing 1000 IU/mL IL-2 for 2 days and then rapidly expanded as follows: the sorted cells (5×10^5 cells) were mixed with 30 ng/mL anti-CD3 mAb, 1000 IU/mL IL-2, and a >200-fold of irradiated allogeneic PBMCs in 50 mL ALyS505N medium (CSTI). The mixture was injected into a 1 L culture bag (Nipro Inc.) and cultured in a CO_2 incubator for 14 days with routine addition of fresh CM containing 1000 IU/mL IL-2 (Fig. 1D). As in-process control, 4-1BB expression by the $CD8^+$ T cells was monitored by flow cytometry on days 9, 14, and 15. IFN-γ and LAMP1 assays were performed on day 28 and all the cells were recovered from the culture bag on day 31. Some were used in quality control tests.

Characterization of EBViNT for Infusion

The manufactured batches of EBViNT were suspended in sterile saline solution (DAI HAN PHARM. Co.) containing 5% human albumin (SK Chemicals) at concentrations of 3.5×10^6 cells/mL, and 100 mL aliquots of the suspensions were placed in infusion bags (GREEN CROSS CORP). Each batch was validated in compliance with the specifications defined in the clinical trial including: (1) absence of bacterial, fungal, mycoplasma, and viral contamination; (2) >65% of $CD8^+$ T cells; (3) >10% of IFN-γ^+ and LAMP-1^+ cells among the $CD8^+$ T cells; (4) phenotypic characterization of the $CD8^+$ T cells using mAbs specific for CD57 (< 35%), PD-1 (< 20%), CD45RA (< 20%), and CD45RO (> 80%); (5) dominant TCRvβ type of $CD8^+$ T cells (> 20%) as analyzed using TCRvβ panel mAbs. TCRvβ typing of $CD8^+$ T cells using PBMCs of each patient and the produced EBViNT Cells indicate that certain types of $CD8^+$ T cells were predominantly enriched in each batch of EBViNT (Supplementary Fig. S1 and Fig. 2, Supplemental Digital Content 1, http://links.lww.com/JIT/A413).

The Clinical Trial

This study was a single-center phase I trial conducted at the National Cancer Center in Goyang, Korea from May 2011 to January 2013. Patients with any EBV-associated malignancy who had failed to respond to standard treatments and were of the HLA-A02 or A24 type were enrolled in the study. EBV-positivity was assessed by in situ hybridization or quantitative polymerase chain reaction (qPCR) for EBV DNA. Patients were excluded who had a brain metastasis, inadequate bone marrow or organ function, active infection, or a previous history of autoimmune or inflammatory disease. No anticancer and immunosuppressive agents including systemic steroids were allowed within 3 weeks of enrollment. EBV/LMP2A-specific $CD8^+$ T cells were isolated from each patient who was positive in the EBV/LMP2A epitope screening, and expanded as described. The cells were then administered to the patient once at a given dose. None of enrolled patients received any transfusion or cytokines including G-CSF and erythropoietin during the study period, except the patient NCC-04 who was transfused 2 U of packed red blood cell. Tumor responses were evaluated for the first time 4 weeks after treatment and then every 8 weeks. They were assessed in accordance with the guidelines established by the International Working Group response criteria committee[14] for malignant lymphoma and by the Response Evaluation Criteria in Solid Tumor (RECIST) committee[15] for solid cancer.

The primary aim of the study was to define the maximum tolerated dose of EBViNT. Secondary objectives included toxicity profile, immunologic responses, and clinical efficacy. Cell dosage was doubled from the starting dose of 0.5×10^8 cells/m^2 (first dose level) to a maximum of 4.0×10^8 cells/m^2 (fourth dose level). Dose escalation was based on the Accelerated Titration Design of Simon et al.[16] According to this design, a single patient was enrolled at each dose level but if any dose-limiting toxicity (DLT) developed or if 2 patients experienced ≥2 grade toxicity, it was planned that the study would be converted to the standard 3 + 3 design at the particular dose level attained. DLT was monitored for 4 weeks after T-cell infusion. If no DLT was observed throughout the trial, the maximum

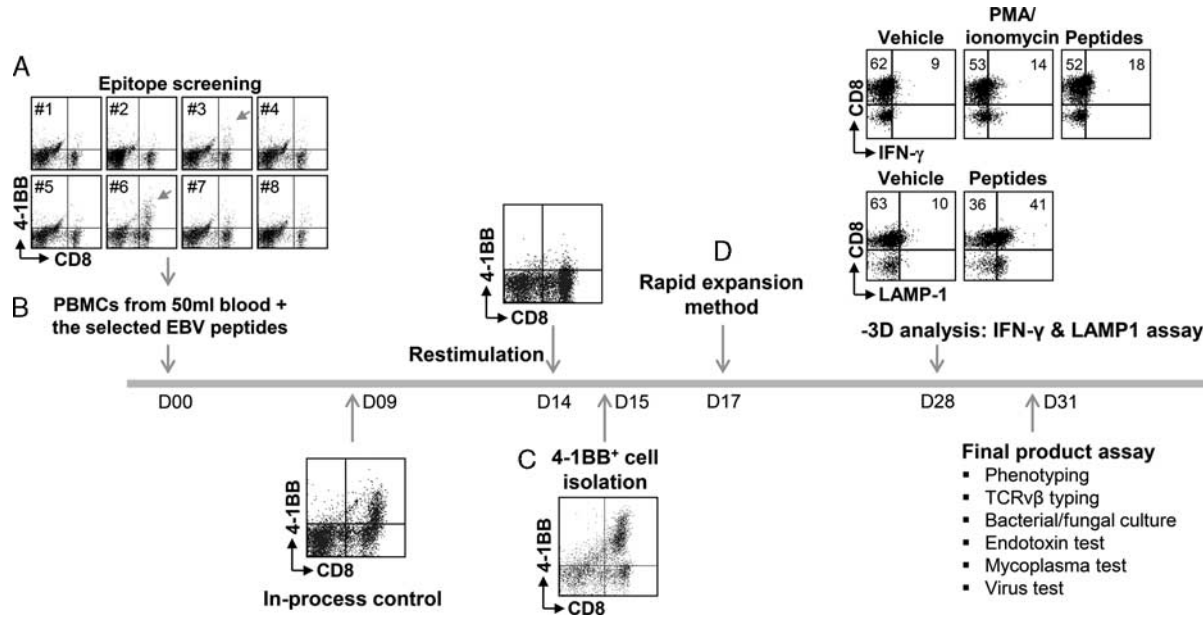

FIGURE 1. Manufacturing of EBV-induced Natural T cell (EBViNT). A, Epitope screening to select Epstein Barr virus (EBV)/LMP2A peptides. B, Primary amplification of LMP2A-specific $CD8^+$ T cells from peripheral blood mononuclear cells (PBMCs) of cancer patient using the selected LMP2A peptides for 14 days. 4-1BB-expressing $CD8^+$ T cells were assessed on day 9 as in-process control. C, The cultured PBMCs were restimulated with the same LMP2A peptides for 24 hours to induce 4-1BB on $CD8^+$ T cells. 4-1BB-expressing $CD8^+$ T cells were sorted using anti-4-1BB mAb-coated plate on day 15. D, Rapid expansion method is as follows: the selected 4-1BB-expressing $CD8^+$ T cells (5×10^5 cells) were mixed with 30 ng/mL anti-CD3 mAb, 1000 IU/mL IL-2, and a >200-fold of irradiated allogeneic PBMCs in 50 mL medium, injected into a 1 L culture bag (Nipro Inc.) and cultured for 14 days with routine addition of fresh medium containing 1000 IU/mL interleukin (IL)-2. LAMP1 assay and intracellular interferon (IFN)-γ staining were performed on day 28 (−3D analysis), and phenotyping, TCRvβ typing, and quality control tests were performed on day 31 (final product assay).

tolerated dose would be 4.0×10^8 cells/m^2. This study was approved by the Institutional Review Board of the National Cancer Center (Goyang, Korea, NCC-CTS-10-503), and all patients provided written informed consent.

Immunologic Monitoring Following Infusion of EBViNT

Peripheral blood was collected from each patient on days 1, 3, 7, 14, 28, 56, 112, and 168. PBMCs were isolated from the freshly isolated blood using Ficoll gradients. The freshly isolated PBMCs were counted using an ADAM MC Automated Mammalian Cell Counter (Bulldog Bio Inc., Rochester, NY). To measure inflammatory cytokines, 1×10^6 PBMCs were plated in 48-well culture plates and treated with DMSO, LMP2A peptides, or PMA/ionomycin for 48 h. Cytokines in the culture supernatants, IL-1β, IL-6, IL-10, IL-4, TNF and IFN-γ were measured using a CBA Kit (BD Bioscience). To characterize the cells, the freshly isolated PBMCs were stained with anti-CD8, anti-CD56, and anti-CD14 mAbs. To assess the activation status of the $CD8^+$ T cells, PBMCs were stained with anti-CD8 and anti-CD62L mAbs. All samples were subsequently analyzed by FACSCalibur (BD Bioscience).

RESULTS

Patient Characteristics and Clinical Responses

A total of 22 patients were screened, 11 of whom satisfied the inclusion criteria. The 8 patients shown in Table 1 received a single infusion of EBViNT. Three other patients did not receive the treatment because of premature death in the case of 2 patients and failure of T-cell production in the third. Patient age at enrollment ranged from 41 to 70 years. Underlying malignancies were as follows: 4 nasopharyngeal cancer (50%), 2 extranodal NK/T cell lymphoma of nasal type (25%), HL (12.5%), and 1 diffuse large B-cell lymphoma (12.5%). Prior treatments include surgical resection, radiation, and chemotherapy. Tumor biopsies from the enrolled patients showed positive for EBERs by in situ hybridization. Baseline plasma EBV DNA titer measured by real-time PCR was higher than detection limit (5300 copies/mL) in 5 patients (62.5%) and their median EBV DNA titer was 33,500 copies/mL (8900–123,400).

EBViNT was well tolerated by all patients. No DLT or treatment-related toxicities were observed. Clinical responses are shown in Table 2. At week 4 after infusion, tumor shrinkage was observed in 2 patients (NCC-03 and NCC-18) and their responses lasted for 8 weeks. Patient NCC-03 had 2 types of cancers: EBV-negative thyroid cancer and EBV-positive HL. Infusion of EBViNT resulted in the shrinkage of EBV^+ HL at week 4, but HL was found in other lymph node regions from week 8 (Fig. 3A). However, EBV-negative thyroid cancer had no tumor regression during the follow-up examinations; standardized uptake value of PET image was not changed (4.4 before EBViNT infusion, 3.0 at week 4, 3.9 at week 8, and 4.2 at week 24). The results indicate that the infused EBViNT selectively recognized and lysed EBV^+ cancer cells in vivo.

In another 2 patients (NCC-06 and NCC-17) computed tomography examinations at week 4 showed that the disease was stable, but it had progressed by week 8. It is interesting to note that another patient, NCC-04, experienced disease progression at week 4, but thereafter her tumor remained stable for some time followed by a further

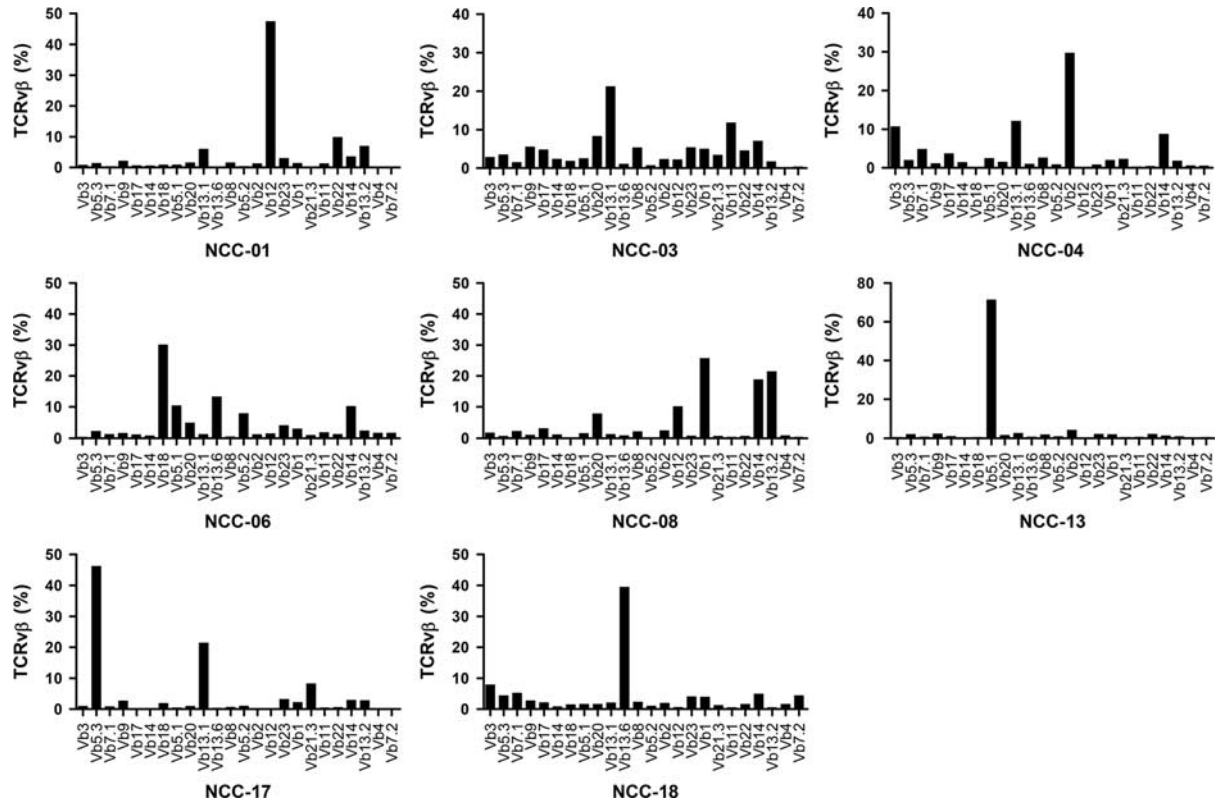

FIGURE 2. TCRvβ typing of EBV-induced Natural T cell (EBViNT). TCRvβ typing was performed with the final products of EBViNT Cells using IOTest Beta Mark TCR V beta Repertoire Kit (Beckman Coulter). In brief, the cultured T cells were stained with anti-CD8-PE-Cy5 along with FITC-conjugated or PE-conjugated anti-TCRvβ mAbs. $CD8^+$ T cells were gated and the percentages of each TCRvβ type were analyzed according to manufacturer's instructions.

decrease in size after 24 weeks (Fig. 3B), and the patient achieved complete response (CR) 1 year after EBViNT infusion.

Two Waves of Post-EBViNT IFN-γ Responses

We collected heparinized whole blood from patients on days 1, 3, 7, 14, 28, 56, 112, and 168, and monitored serum cytokines (IL-1β, IL-6, IL-10, IL-4, TNF, and IFN-γ), total PBMCs (numbers/mL), percentages of myeloid and lymphoid cells ($CD3^+$, $CD4^+$, $CD8^+$, $CD56^+$, $CD14^+$, $CD19^+$, $CD14^+CD25^+$, and $CD3^+CD56^+$), and the ratios of $CD62L^{low}$ to each TCRvβ-specific cell type among the $CD8^+$ T cells. The cytokine production patterns of the PBMCs were measured in culture supernatants following stimulation with EBV/LMP2A peptides or with PMA/ionomycin. Plasma levels of EBV DNA were also determined.

As the manufactured batches of EBViNT contained 20%–80% of a given TCRvβ subtype among the infused $CD8^+$ T cells, we expected to be able to monitor the infused EBViNT in the blood, but the infusion did not alter the overall TCRvβ composition (data not shown). There were temporal changes in the numbers and proportions of $CD8^+$ T cells, as well as in the proportion of $CD62L^{low}$ effector $CD8^+$ T cells (Supplementary Fig. S2, Supplemental Digital Content 1, http://links.lww.com/JIT/A413) in some patients. However, we found no correlation between the changes in $CD8^+$ T-cell numbers and the clinical outcomes. We also failed to find any correlation between changes of lymphoid and myeloid cell composition and clinical outcomes (Supplementary Fig. S3, Supplemental Digital Content 1, http://links.lww.com/JIT/A413).

We measured cytokine production in PBMCs that were stimulated with EBV/LMP2A peptides or PMA/ionomycin for 48 hours. Stimulation of PBMCs with the LMP2A peptides that were used to produce EBViNT did not increase the levels of any of the cytokines tested, namely IL-1β, IL-6, IL-10, IL-4, TNF, and IFN-γ, whereas pan-T-cell activation with PMA/ionomycin did induce cytokine production (Figs. 4A–F). It was notable that the pan-T-cell activation resulted in 2 waves of IFN-γ production: the first at 1 week postinfusion, and the second at around 4 weeks postinfusion. As IFN-γ production was induced by pan-T cell activation but not by the EBV/LMP2A peptides, the secondary IFN-γ expression was probably due to T cells that were reactive with various other tumor Ags, not the LMP2A peptides. It is interesting to note that as shown in patients NCC-04 and NCC-18, prognoses and clinical outcomes were improved when the secondary IFN-γ expression was strong and extended (Fig. 4F) and there was less production of inflammatory cytokines such as IL-1β, IL-6, IL-10, and IL-4 (Figs. 4A–D). Two waves of IFN-γ production were also seen in patients NCC-03 and NCC-13, but the second IFN-γ peak was not strong. These patients appeared not to have benefited from the EBViNT therapy (Fig. 4 and Table 2).

Taken together, these data suggest that the infused EBViNT induces primary antitumor responses within 1–2

TABLE 1. Patient Characteristics

Patient No.	Sex/Age	Diagnosis	Initial Stage	Previous Treatments	Involved Sites Before EBViNT Cell Therapy
NCC-01	F/64	Nasopharyngeal cancer	Unknown	Resection of tumor, cervical lymph nodes dissection, tonsilectomyconcurrent chemoradiation Cisplatin, 5-fluorouracil, docetaxel, TS-1, irrinotecan, gemcitabine	Lymph nodes (aortocaval, para-aortic, and external iliac), spleen, liver, and bone (multiple spines, both scapulae, humeri, pelvis, and femurs)
NCC-03	M/63	Hodgkin lymphoma	IIA	Splenectomy, excision of para-aortic lymph node, wedge resection of lung radiation Doxorubicin, bleomycin, vinblastine, dacarbazine, dexamethasone, cytarabine, cisplatin	Lymph nodes (mediastinal, supraclavicular) and thyroid gland*
NCC-04	F/64	Extranodal NK/T-cell lymphoma, nasal type	IV	Ifosfamide, methotrexate, etoposide, prednisolone	Nasal cavity, skin, and renal mass
NCC-06	M/51	Nasopharyngeal cancer	IV	Concurrent chemoradiation 5-fluorouracil, cisplatin, docetaxel	Lung and pleura
NCC-08	M/49	Nasopharyngeal cancer	III	Concurrent chemoradiation Docetaxel, cisplatin	Lymph nodes (supraclavicular and axillary) and bone (rib and vertebra)
NCC-13	M/41	Nasopharyngeal cancer	Unknown	Concurrent chemoradiation Metastasectomy of lung, cisplatin, docetaxel, TS-1	Lymph nodes (mediastinal), lung, and pleura
NCC-17	F/67	Diffuse large B-cell lymphoma†	III	Doxorubicin, bleomycin, vinblastine, dacarbazine	Lymph nodes (cervical, mediastinal, axillary, retroperitoneal, and inguinal) and spleen
NCC-18	F/70	Extranodal NK/T-cell lymphoma, nasal type	IIE	Concurrent chemoradiation Etoposide, dexamethasone, L-asparaginase, methotrexate, ifosfamide, cytarabine, cisplatin	Nasal cavity

*This lesion did not respond to EBViNT cell, and was proven to papillary thyroid carcinoma by subsequent aspiration cytology after study treatment.
†This was a second malignancy. She was diagnosed to Hodgkin lymphoma at first, but biopsy during the study confirmed diffuse large B-cell lymphoma.
EBViNT indicates EBV-induced natural T cells.

weeks, and that these induce subsequent rounds of anti-tumor activity that are reflected in IFN-γ responses. Strong secondary antitumor responses resulted in better clinical outcomes.

EBV/LMP2A-specific CD8$^+$ T-Cell Responses and Plasma Levels of EBV DNA

EBV DNA copy number in a patient's peripheral blood provides a measure of the tumor burden in EBV$^+$ cancers. Although we enrolled patients with EBV$^+$ cancers, additional tumor biopsies of the relapsed lesions were not available immediately before the EBViNT treatment in some of the patients due to the locations of their tumors. Moreover, we expected that strong CD8$^+$ T cell responses against the EBV/LMP2A peptides in vitro would indicate that the tumor cells actively processed and presented the selected LMP2A epitopes. We were able to monitor CD8$^+$ T-cell responses against LMP2A peptides on day 15 of

TABLE 2. Assessment of Clinical Response

Patient No.	HLA Type	CD8$^+$ T (%)	Infused Cell Number ($\times 10^8/m^2$)	4 wk	8 wk	16 wk	24 wk	Last Status	Toxicity (Any Grade)
NCC-01	A02/A26	78.18	0.5	PD	—	—	—	Death	None
NCC-03	A02/A11	85.34	1.0	PR	PR	PD	—	Death	None
NCC-04	A02/A24	80.97	2.0	PD*	SD*	SD	SD	Alive PD after 11 mo	None
NCC-06	A02/A26	72.34	4.0	SD	PD	—	—	Death	None
NCC-08	A02/A33	86.80	4.0	PD	—	—	—	Death	None
NCC-13	A02/A26	71.62	4.0	PD	PD	—	—	Alive response after salvage chemotherapy	None
NCC-17	A24/A26	82.12	4.0	SD	PD	—	—	Alive with disease	None
NCC-18	A11/A24	72.39	4.0	CR	CR	PD	SD†	Alive with disease	None

*Tumor of NCC-04 had increased during the first 4 weeks. However, it stopped to progress and started to decrease in 8 weeks.
†Although this patient experienced disease progression in 16 weeks, she did not show rapid increase of tumor after progression.
CR indicates complete response; HLA, human leukocyte antigen; PD, progressive disease; PR, partial response; SD, stable disease.

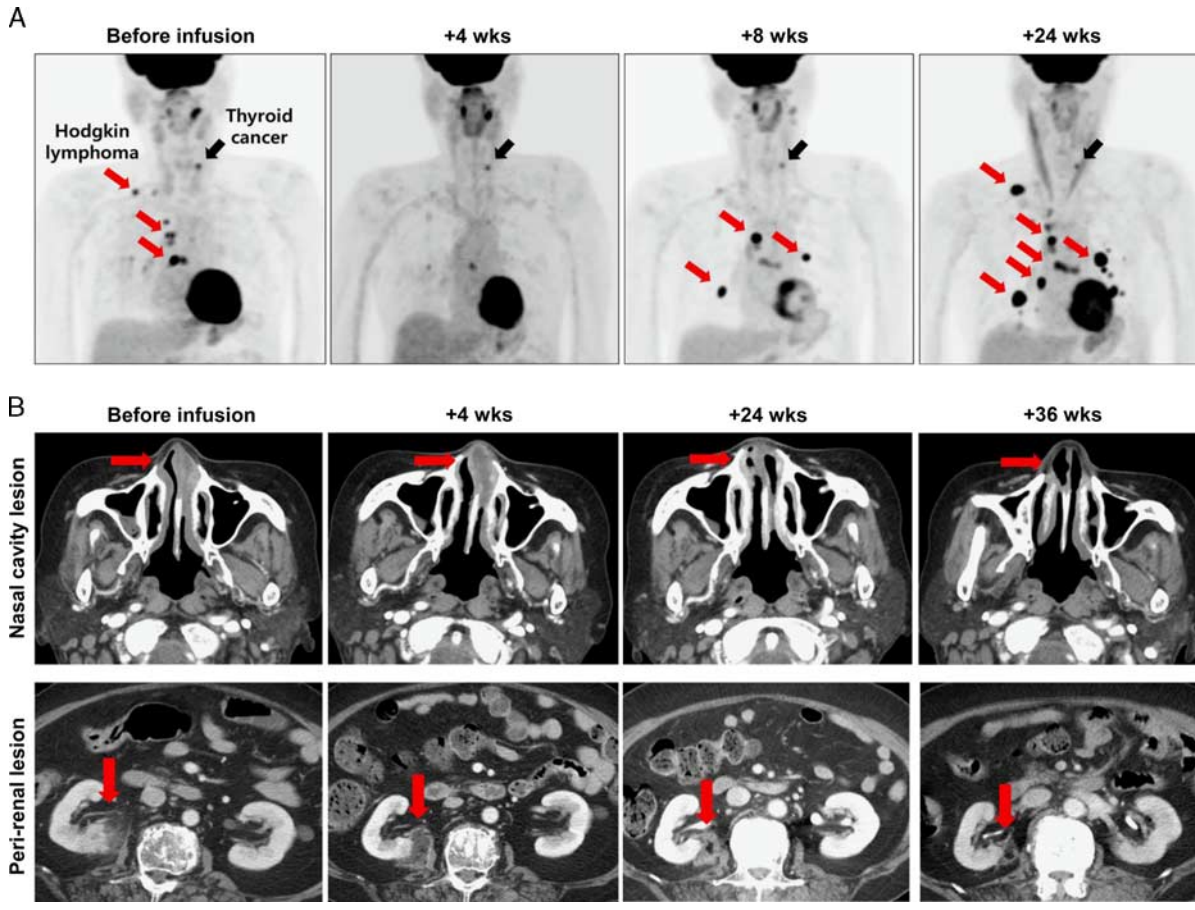

FIGURE 3. Cancer regression in patients. A, Positron emission tomography (PET) images of EBV$^+$ Hodgkin lymphoma (red arrow) and EBV$^-$ thyroid cancer (black arrow) in patient NCC-03 taken before treatment and 4, 8, and 24 weeks after infusion of EBV-specific CD8$^+$ T cells. B, Computed tomography (CT) images of cancer tissues in the right lower leg, nasal cavity, and perirenal lesion in patient NCC-04 before treatment and 4, 24, and 36 weeks after infusion of EBV-specific CD8$^+$ T cells. EBV$^+$ cancer regions are indicated by red arrows.

in vitro culture by assessing 4-1BB$^+$ CD8$^+$ T-cell numbers. Flow cytometric analysis indicated that the numbers of 4-1BB$^+$ CD8$^+$ T cells reactive with LMP2A peptides differed greatly from patient to patient (Fig. 5A). Robust CD8$^+$ T-cell responses were found in NCC-08 and NCC-17 although their clinical outcomes were poor, whereas patients NCC-03, NCC-04, and NCC-18 gave only moderate responses but underwent objective regression (Fig. 5A and Table 2).

When the numbers of EBV DNA copies in patients' blood were determined following the infusion of EBViNT, EBV DNA was found to increase continuously in patients NCC-01 and NCC-06, was undetectable in patients NCC-08, NCC-13, and NCC-17, and tended to decrease in patients NCC-03, NCC-04, and NCC-18 (Fig. 5B). From the levels of 4-1BB$^+$ CD8$^+$ T cells on day 15 of culture (Fig. 5A), it seems likely that the EBV-specific CD8$^+$ T cells were ineffective in patients NCC-01 and NCC-06 because the selected LMP2A epitopes were not strongly presented by the tumor cells in those patients. Although patients NCC-08 and NCC-17 gave robust CD8$^+$ T-cell responses to LMP2A peptides, their EBV DNA titers were very low. In those patients the expanded CD8$^+$ T cells may have been tumor-unrelated LMP2A-specific memory T cells (Figs. 5A, B). However, in patients NCC-03, NCC-04, and NCC-18, the EBV-specific CD8$^+$ T cells were effective in inducing antitumor responses by targeting substantial numbers of EBV$^+$ cancer cells that presumably presented the selected LMP2A epitopes.

DISCUSSION

On the basis of the unique property of 4-1BB—its expression specifically on activated T cells—we designed a simple and practical protocol to produce Ag-specific CD8$^+$ T cells, and have now applied it to produce EBV/LMP2A-specific CD8$^+$ T cells from the PBMCs of EBV-positive tumor patients. Infusion of EBViNT resulted in objective responses in 3 of 8 patients. We noted that IFN-γ was produced in a "2 wave" manner by the PBMCs in the patients with positive clinical outcomes; presumably the tumor cells of those patients presented substantial amounts of the target Ag.

Comoli et al[17] obtained 2 partial responses (PRs) and 4 cases of disease stabilization in patients with relapsed/refractory stage IV nasopharyngeal cancer using a treatment consisting of a first dose of 2×10^7 CTL/m^2 of body surface area (BSA), followed by subsequent doses of 4×10^7 CTL/m^2 BSA.

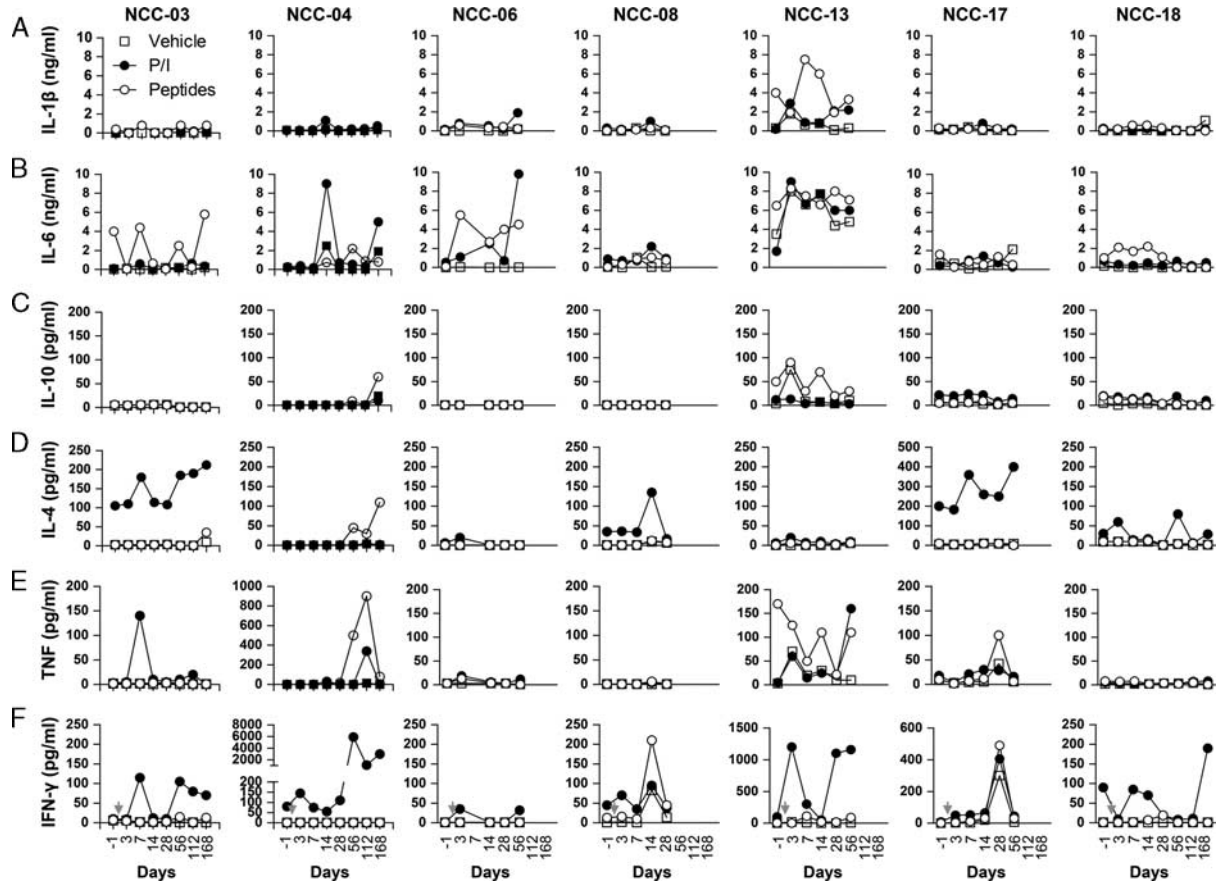

FIGURE 4. Changes in cytokine levels of peripheral blood mononuclear cells (PBMCs) following infusion of Epstein Barr virus (EBV)-specific $CD8^+$ T cells. Heparinized blood samples were collected from each patient at the indicated days before and after EBV-induced Natural T cell (EBViNT) infusion. The isolated PBMCs were plated on 48-well culture plate (5×10^5 cells/well) and simultaneously treated with DMSO (vehicle), PMA, and ionomycine (P/I) or 5 μg/mL of EBV/LMP2A peptides (peptides) for 48 hours. Cytokines in the culture supernatant were assessed using a CBA Kit (BD Bioscience) (A–F). Gray arrows represent infusion days.

Heslop and colleagues infused 3 dose levels of autologous EBV-specific CTL, 2.0×10^7, 1.0×10^8, and 2.0×10^8 CTL/m^2 in 6 patients with relapsed/refractory nasopharyngeal cancer, and reported 2 CR, 1 PR, 1 case of stable disease, and 2 no responses.[18] In a clinical trial in patients with relapsed HL expressing LMP1 and LMP2 Ags, 14 patients received 4.0×10^7 CTL/m^2 or 1.2×10^8 CTL/m^2. After EBV-specific CTL infusion, 5 CRs, 1 PR, and 5 SDs were recorded, with good tolerability.[19] Moreover, lymphodepletion can enhance the expansion of infused EBV-specific CTL; 8 patients with relapsed nasopharyngeal cancer received anti-CD45 mAb followed by escalating doses of autologous EBV-specific CTL. Six of the 8 showed transient lymphopenia and an increase in IL-15 level, and 3 clinical benefits, 1 CR, and 2 SDs, were noted without significant toxicity even at a dose of 3 of 1.0×10^8 CTL/m^2.[20] These studies clearly demonstrate that simple infusion of EBV-specific CTLs alone can be a good option for treating EBV-expressing tumor patients resistant to conventional treatments. However, CTL therapy targeting EBV is still in phase I or II clinical trials because EBV-specific CTLs have been generally produced by repeatedly stimulating PBMCs with irradiated autologous lymphoblastoid cell lines, which means that the production process is lengthy and not well standardized for cGMP translation. We suggest that our procedure overcomes the major obstacles preventing CTL therapy targeting EBV from entering phase III clinical trials.

EBV/LMP2 Ag-specific CTLs have been effective in treating HL and nasopharyngeal cancer.[17–20] However, EBViNTs were not effective in treating the nasopharyngeal cancer patients recruited in this study (Table 2). We suspect that the EBViNTs may not have been effective because the selected CTL epitopes were not actively presented in the nasopharyngeal cancer patients NCC-01 and NCC-06 (Fig. 5A), and EBV titers were too low in patients NCC-08 and NCC-13 (Fig. 5B). In both cases the infused EBViNTs would not have been able to find and kill their target cells efficiently. As we used epitope-specific CTLs rather than polyclonal EBV-specific CTLs, expression of the target Ag and presentation of the selected epitope by the tumor cells are pivotal to controlling disease progression.

It is notable that when the PBMCs from patients who received EBViNT were stimulated with PMA/ionomycin, IFN-γ production proceeded in a "2 wave" manner in some patients (Fig. 4F). As stimulation with LMP2A peptides did not induce detectable levels of IFN-γ from the PBMCs (Fig. 4F), the infused EBViNT may have acted as driver $CD8^+$ T cells, inducing primary EBV-specific antitumor responses and promoting the induction of secondary antitumor responses against other tumor-associated Ags. Indeed,

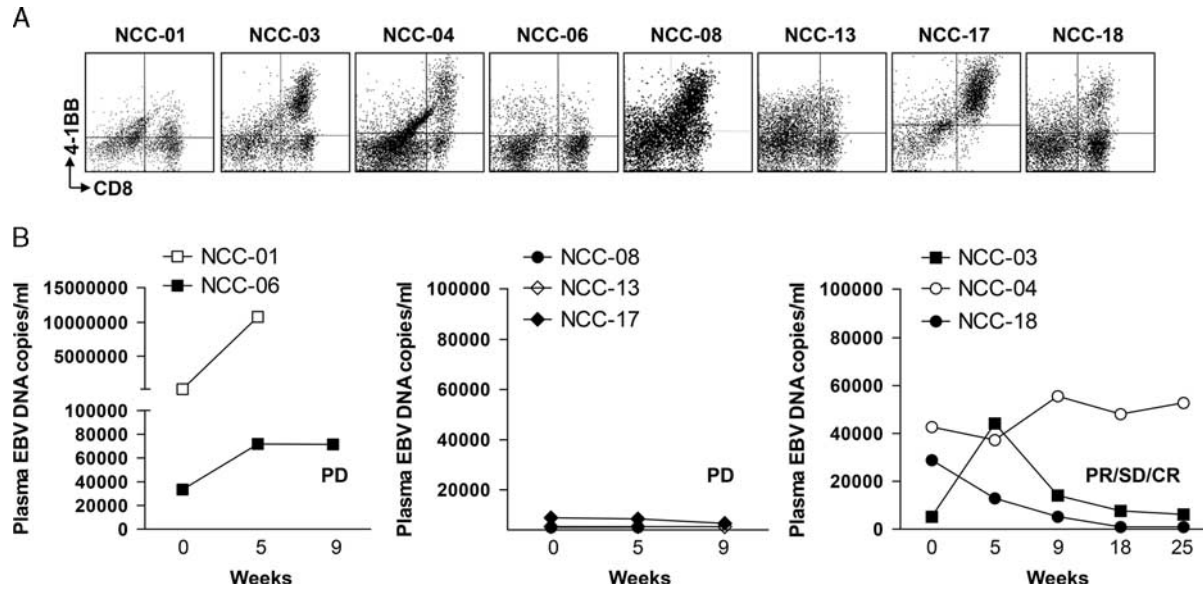

FIGURE 5. CD8$^+$ T-cell responses toward EBV/LMP2A peptides and changes of plasma Epstein Barr virus (EBV) DNA copies following EBV-induced Natural T cell (EBViNT) therapy. A, 4-1BB-expressing CD8$^+$ T cells at day 15 in each manufactured batch of EBViNT. During the manufacturing process of EBViNT, peripheral blood mononuclear cells (PBMCs) cultured with LMP2A peptides for 14 days were restimulated with the same peptides for another 24 hours and stained with anti-4-1BB and anti-CD8 Abs at day 15. B, EBV DNA copies in patient plasma before and after EBViNT infusion.

the broadening of epitope recognition by a patient's T cells, which is called "epitope spreading," is considered crucial for inducing durable complete regression of tumors.[21–23] Robbins et al[24] reported that mutated Ag-reactive T cells were the dominant populations of T cells present among therapeutically effective TILs for melanoma patients, and that the expansion of TILs recognizing mutated Ags was pivotal to successful treatment of melanoma patients.

Taken together, these results indicate that EBViNTs are safe and offer a new option for treating EBV$^+$ tumor patients resistant to conventional treatments, whereas for preparing standardized EBV/LMP2A-specific CD8$^+$ T cells for clinical use, the 4-1BB-based procedure to isolate and expand Ag-specific CD8$^+$ T cells serves as a simple and practical method.

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CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

All authors have declared there are no financial conflicts of interest with regard to this work.

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