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ORIGINAL REPORT

Adoptive Transfer of Epstein-Barr Virus (EBV) Nuclear Antigen 1–Specific T Cells As Treatment for EBV Reactivation and Lymphoproliferative Disorders After Allogeneic Stem-Cell Transplantation

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ABSTRACT

Purpose
Reactivation of Epstein-Barr virus (EBV) after allogeneic stem-cell transplantation (SCT) can lead to severe life-threatening infections and trigger post-transplantation lymphoproliferative disease (PTLD). Since EBV-specific T cells could prevent PTLD, cellular immunotherapy has been a promising treatment option. However, generation of antigen-specific T-cell populations has been difficult within a short time frame.

Patients and Methods
To improve availability in urgent clinical conditions, we developed a rapid protocol for isolation of polyclonal EBV nuclear antigen 1 (EBNA-1) –specific T cells by using an interferon gamma (IFN-γ) capture technique.

Results
We report on the use of adoptive transfer of EBNA-1–specific T cells in 10 pediatric and adult patients with EBV viremia and/or PTLD after SCT. No acute toxicity or graft-versus-host disease (GVHD) of more than grade 2 occurred as a result of adoptive T-cell transfer. In vivo expansion of transferred EBNA-1–specific T cells was observed in eight of 10 patients after a median of 16 days following adoptive transfer that was associated with clinical and virologic response in seven of them (70%). None of the responders had EBV-associated mortality. Within clinical responders, three patients were disease free by the day of last follow-up (2 to 36 months), three patients died of other infectious complications, and one patient died as a result of relapse of malignancy. EBV-related mortality was observed in two of 10 patients, and another patient had ongoing viremia without clinical symptoms at last follow-up.

Conclusion
Adoptive ex vivo transfer of EBNA-1–specific T cells is a feasible and well-tolerated therapeutic option, representing a fast and efficient procedure to achieve reconstitution of antiviral T-cell immunity after SCT.

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INTRODUCTION

Epstein-Barr virus (EBV) is a human gamma herpesvirus that transforms B-cell growth with the potential to induce malignancies. EBV reactivation and post-transplantation lymphoproliferative disease (PTLD) are an important cause of morbidity and mortality after allogeneic stem-cell transplantation (SCT).[1-3] T-cell responses are essential for the control of EBV-infected B cells.[4-8] Reconstitution of the new, donor-derived immune system can take several months after SCT,[9,10] implying deficiency of host T-cell immunity over a long time. In 10% to 50% of patients who have had SCT, EBV reactivation can occur followed by PTLD.[4,11] This is a severe and life-threatening condition in association with insufficient EBV-specific T-cell responses.[6,7] Both EBV infection and PTLD are difficult to treat, because existing antiviral agents have poor efficacy against EBV, and they have no evident impact on the course of lymphoproliferative disease.[12,13] Treatment options include reducing immunosuppressive therapy and targeting B cells with monoclonal anti-CD20 antibodies or chemotherapy. Currently, there

is no standard treatment for patients with EBV-related conditions refractory to these therapeutic steps. Several studies have shown that adoptive transfer of donor-derived EBV-specific T cells is an effective and safe treatment option in transplantation recipients with EBV-related complications.[14-25] Most of the methods described are logistically and technically demanding, lasting 4 to 12 weeks because of repetitive antigen stimulation of T cells and expansion in vitro. Such protocols are difficult to apply in urgent clinical cases. Previous studies[26] indicated that in vitro acquisition of full effector function of adoptively transferred $CD8^+$ T cells paradoxically impairs their in vivo efficacy. Analogous to our recently published protocols for production of adenovirus- and cytomegalovirus-specific T cells,[27-30] we used EBV nuclear antigen 1 (EBNA-1) as an antigen to generate EBV-specific $CD4^+$ and $CD8^+$ T cells from EBV-seropositive donors in a time-saving and simple procedure without any in vitro expansion steps. This approach is based on the infusion of small amounts of donor T cells and their subsequent in vivo expansion to mount an antiviral immune response in the recipient. The EBNA-1 protein is involved in the replication of viral episomes and is therefore crucial for the persistence of EBV infection. It is the only viral protein required for replication of EBV in its latent form, and importantly it is an EBV antigen that is universally expressed in EBV^+ PTLD.[31,32] EBNA-1 has also been shown to contain immunodominant T-cell epitopes that induce T-cell responses ($CD4^+$ and $CD8^+$) in the healthy population.[31] In this article, we report our experience with 10 patients after SCT who had chemorefractory EBV-related conditions that were treated with transfusion of low numbers of EBNA-1–specific T cells to restore their protective T-cell immunity against EBV and thereby prevent EBV-related complications.

PATIENTS AND METHODS

Ex Vivo Generation of EBNA-1–Specific T Cells

The procedure is based on the ability of T cells to secrete interferon gamma (IFN-γ) after ex vivo stimulation with viral antigens.[28-30] EBNA-1–specific T cells were isolated from whole blood or unstimulated apheresis of the same donor used for SCT (blood or apheresis was decided by the donor). All donors were tested for the presence of T cells against EBNA-1. They were eligible for generation of EBNA-1–specific lymphocytes if the number of EBV-specific T cells was more than 0.01% of the $CD3^+$ lymphocytes. Before donor blood collection, the patients and/or their parents gave written informed consent for adoptive T-cell therapy to their treating physician. The adoptive T-cell transfer was done as a single-case treatment in accordance with the regulations of the institutional review board. EBNA-1–specific T cells were given on the basis of an off-label use, according to the indication set by their individual treating physician.

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque (Biochrome, Berlin, Germany) density gradient centrifugation, diluted to 1×10^7 cells/mL with culture medium (RPMI 1640l [Biochrome] plus 10% human AB-serum) and stimulated with 10 μL/mL recombinant EBNA-1 protein (tebu-bio; Le-Perray-en-Yvelines, France) in patients 1 through 7 or with EBNA-1 Peptivator (Miltenyi Biotec; Bergisch-Gladbach, Germany) in patients 8 through 10. Good manufacturing process (GMP) –grade EBNA-1 overlapping peptides became commercially available after patient 7, and the protocol change was required by the regulatory authority. Appropriate change control experiments were performed (Appendix, online only). Stimulation was done in a humidified incubator overnight at 37°C. Enrichment of cytokine-secreting cells was performed by using the cytokine secretion system and the CliniMACS device for immunomagnetic separation (both Miltenyi Biotec). EBV-specific T cells were either transfused directly after the isolation procedure without any further in vitro expansion or they were cryopreserved. Microbiologic analysis of the cells was performed before transfusion. The cell processing was done in a central GMP laboratory at the University Children's Hospital Tübingen. The cells were brought to the treating center and infused on the same day as the cell isolation procedure.

Detection of EBV-Specific T Cells

T cells were analyzed in a central laboratory and stimulated ex vivo at 37°C with 10 μL/mL recombinant EBNA-1 antigen or latent membrane protein 2 (LMP-2) Pepmix (JPT, Berlin, Germany). After addition of BrefeldinA (Sigma, Taufkirchen, Germany) for 4 hours, intracellular cytokine IFN-γ staining was performed by using saturating conditions of the following antibodies: anti-CD4 or anti-CD8 (clones SK3 or SK1), anti–IFN-γ (clone 25723.11), and anti-CD3 (clone SK7; all from Becton Dickinson, Heidelberg, Germany). At least 1×10^6 lymphoid cells were analyzed on an FACSCalibur flow cytometer using Cellquest software (Becton Dickinson).

Epitope specificity against major histocompatibility complex (MHC) class II binding EBNA-1 peptides was analyzed among EBNA-1–specific T cells. A total of 5×10^4 cells was cultivated in Iscove's Modified Dulbecco's Media (Lonza, Basel, Switzerland) with 5% AB-serum, 50 μmol/L β-mercaptoethanol (Roth, Karlsruhe, Germany), 1% penicillin/streptomycin, 25 μg/mL gentamicin (Lonza), 1.5×10^5 irradiated allogeneic PBMCs, 1.5×10^4 irradiated LG2 cells, 1 μg/mL phythemagglutinine-L (Sigma), and 150 U/mL interleukin 2 (IL-2; Proleukin, Novartis, Basel, Switzerland). After 4 days of in vitro expansion and then every other day, 150 U/mL IL-2 was added. After 2 weeks, cells were challenged overnight with 10 μg/mL of specific peptide and were used in intracellular cytokine staining. Phorbol 12-myristate 13-acetate 150 ng/mL with ionomycin 1 μmol/L (both Sigma) was used as a positive control, and the self-peptide filamin A was used as a negative control. Antibodies include live/dead-aqua (Invitrogen Life Technologies, Darmstadt, Germany), anti-CD4 APC/Cy7 (BD Biosciences, Heidelberg, Germany), anti-CD8 PerCP (BioLegend, San Diego, CA); intracellular staining with anti–tumor necrosis factor Pacific Blue (BioLegend); anti-CD154 fluorescein isothiocyanate and N-hydroxysuccinimide-fluorescein are amine-reactive derivatives, anti–IFN-γ PE-Cy7 and anti–IL-2 PE (BD). Cells were analyzed by using an FACSCanto II flow cytometer (BD) and FlowJo (Tree Star, Ashland, OR). MHC class II binding EBNA-1 peptides have been described previously[33] and were synthesized as described.[34]

Patient Characteristics

Seventeen EBNA-1–specific T-cell isolation procedures were performed between 2007 and 2010 (Appendix Table A1, online only). Since some of the patients received repetitive infusions and some received none (sufficient disease control was reached by conventional therapy), a total of 10 patients received EBV-specific T cells, and they represent all cases with EBNA-1–specific T-cell transfer for EBV-related complications after SCT (Table 1). All patients previously underwent allogeneic SCT (Table 1) and were between 2 and 51 years of age (mean, 18 years). Viremia and/or PTLD occurred in the first year after SCT. In patients 3, 5, and 8, a second adoptive T-cell transfer was performed within 7 to 54 days after the first transfer.

Eligibility/indication for EBNA-1–specific T cells was refractory viremia and/or PTLD and occurred on days 59 to 362 (mean, day 148) after SCT. Refractory viremia was defined as a persistent or increasing number of EBV copies in blood (detected by polymerase chain reaction [PCR]) under antiviral treatment of more than 14 days (changes in copy numbers were defined as ≥ 1 log change). Refractory PTLD was defined as persistence or deterioration of bulky disease under treatment. Seven patients (1 through 3 and 5 through 8) suffered from refractory PTLD and viremia, and two patients (4 and 9) presented with chronic viremia only. One patient (10) had a persistent cervical lymphadenopathy (histologic B-cell non-Hodgkin lymphoma with preexisting viremia).

We defined safety thresholds for the first T-cell dose as $\leq$ 25,000 cells per kilogram in HLA-matched donors and $\leq$ 5,000 cells per kilogram in HLA-mismatched donors. Otherwise all EBNA-1–specific T cells that could be isolated from 1×10^9 PBMCs were infused. In case the number of T cells exceeded the safety thresholds, we cryopreserved cells for a second dose. Criteria for infusion of a second cell dose were nonresponse to the first dose.

Table 1. Patient Characteristics and EBV Disease Status Prior to T-Cell Transfer

Patient No.	Age (Years)	Primary Condition	Indication For Adoptive T-Cell Transfer	Graft	EBV IgG Donor/ Recipient	Conditioning Regimen	GVHD Prophylaxis	Coinfection at T-Cell Transfer	Immunosuppression at T-Cell Transfer	Antiviral Drugs at T-Cell Transfer	Rituximab Before T-Cell Transfer	Cytostatic Therapy For PTLD
1	51	Secondary AML (history of mamma carcinoma); graft dysfunction	PTLD, EBV pneumonia, viremia, lymphadenopathy	MMUD (9 of 10 matched)	+/N/A	Total body irradiation, cyclophosphamide, antithymocyte globulin	Mycophenolate mofetil, cyclosporin, methotrexate	CMV	Prednisone	Aciclovir, ganciclovir, foscarnet, valganciclovir	Yes	No
2	14	T-ALL relapse, MAS with cerebral HLH	Monoclonal CNS PTLD, viremia, lymphadenopathy	MMUD (9 of 10 matched)	+/+	Total body irradiation, etopside, antithymocyte globulin	Cyclosporin, methotrexate	None	Prednisone	Cidofovir	Yes	Cytarabin/dexamethasone intrathecal and etopside single doses (HLH therapy)
3	9	SAA, graft dysfunction	PTLD viremia, tonsillitis, cervical lymphadenopathy	Haplo (6 of 10 matched)	+/+	Fludarabine, thiotepa, melphalan, muronomab	Cyclosporin, mycophenolate mofetil, prednisone	None	Not given	Aciclovir, cidofovir, valganciclovir	Yes	No
4	2	HLH	Chronic EBV viremia and fever for 6 months	MSD	+/−	Busulfan, etopside, cyclophosphamide	Mycophenolate mofetil, cyclosporin, methotrexate	None	Not given	Valacyclovir	No	No
5	25	AML relapse	PTLD, viremia, lymphadenopathy, tonsilitis	Haplo (second graft)	+/+	Clofarabin, thiotepa, melphalan, muronomab	Not given	None	Prednisone, tacrolimus	Aciclovir	Yes	No
6	16	SAA, graft dysfunction	PTLD, viremia, EBV pneumonia	MUD	+/+	Fludarabine, cyclophosphamide, antithymocyte globulin	Cyclosporin, methotrexate	BKV	Not given	Aciclovir, cidofovir	Yes	Lenalidomide
7	8	Neuroblastoma stage IV relapse, MAS with HLH	EBV septicemia, monoclonal PTLD/B-cell lymphoma	Haplo	+/+	Fludarabine, thiotepa, melphalan, muronomab	Mycophenolate mofetil	None	Not given	Aciclovir, cidofovir, ribavirin	Yes	Cyclophosphamide, vincristine, methotrexate 5 days after T-cell transfer; infliximab and basiliximab 2 days after T-cell transfer
8	19	T-ALL relapse, graft dysfunction	Malignant PTLD/B-cell lymphoma (CNS, liver, spleen, kidney), viremia	MUD	+/+	Total body irradiation, cyclophosphamide, antithymocyte globulin	Cyclosporin, mycophenolate mofetil, prednisone	ADV, BKV	Hydrocortisone	Cidofovir, aciclovir	Yes	Cyclophosphamide, prednisone, one cycle CHOP 2 days before first T-cell transfer
9	2	AML	Persistent EBV viremia for 29 days	MUD	+/−	Busulfan, etopside, cyclophosphamide, antithymocyte globulin	Cyclosporin, mycophenolate mofetil	*Aspergillus*	Rapamycin, tacrolimus	Cidofovir, ribavirin	No	No
10	35	AML	PTLD (B-cell NHL), persistent cervical lymphadenopathy for 90 days	MMUD (9 of 10 matched)	+/N/A	Total body irradiation, cyclophosphamide, antithymocyte globulin	Cyclosporin, methotrexate	None	Prednisone	Cidofovir, aciclovir	Yes	No

Abbreviations: ADV, adenovirus; AML, acute myeloid leukemia; BKV, BK virus [polyoma virus]; CHOP, cyclophosphamide, doxorubicin, vincristine, prednisone; CMV, cytomegalovirus; EBV, Epstein-Barr virus; GVHD, graft-versus-host disease; Haplo, haploidentical stem-cell transplantation from parents or siblings; HLH, hemophagocytic lymphohistiocytosis; IgG, immunoglobulin G; MAS, macrophage activation syndrome; MMUD, mismatched unrelated donor; MSD, matched sibling donor; MUD, matched unrelated donor; N/A, not applicable; NHL, non-Hodgkin lymphoma; PTLD, post-transplantation lymphoproliferative disease; SAA, severe aplastic anemia; T-ALL, T-cell acute lymphoblastic leukemia.

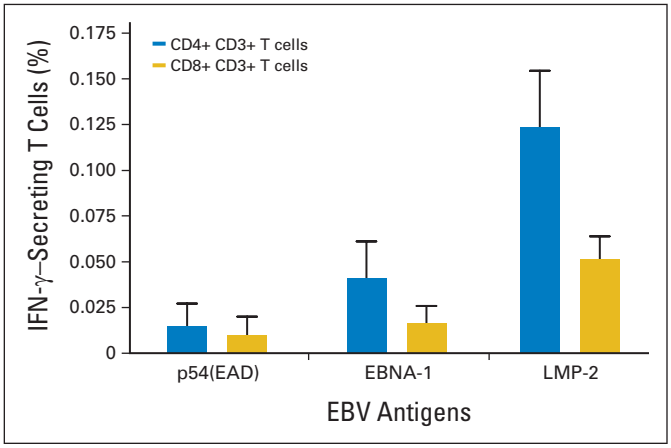


Fig 1. T-cell responses against Epstein-Barr virus nuclear antigen 1 (EBNA-1), latent membrane protein 2 (LMP-2), and p54 in healthy donors. Mean frequency of antigen-specific T cells against EBNA-1 is compared with early antigen p54 and LMP-2 (n = 8). Antigen-specific T lymphocytes were found in all seropositive patients. Although LMP-2 induced the strongest response among healthy donors, we had to select EBNA-1 for adoptive T-cell transfer, because LMP-2 failed to meet good manufacturing practice criteria. In future trials, the inclusion of LMP-2 antigens that meet good manufacturing practice criteria will enable stronger immunogenicity for T cells. EAD, early antigen D; EBV, Epstein-Barr virus; IFN-γ, interferon gamma.

For toxicity evaluation, institutional standard criteria for donor lymphocyte infusion were applied. Toxicity criteria were acute allergic reaction or any change in vital signs during and after adoptive transfer, impairment of blood count, liver and kidney function without another obvious cause, EBV PCR at least weekly, as well as signs of graft-versus-host disease (GVHD) for up to 6 weeks after transfer. Antiviral chemotherapy remained unchanged after adoptive T-cell transfer. Heart rate, blood pressure, and oxygen saturation were monitored and physical examinations were performed during and for 2 hours after adoptive T-cell transfer. T-cell response was evaluated for all patients in a central laboratory with uniform detection threshold. Quantitative PCR of the EBV load was done in the treating centers. Response criteria were reduction of viral load in copy numbers ≥ 1log and clinical and radiographic involution of PTLD foci and lymphadenopathy.

RESULTS

EBV-Derived Antigens and Frequency of Specific T Cells in Healthy Individuals

We compared IFN-γ^+ T-cell responses in healthy donors against early antigen p54, LMP-2, and EBNA-1. The analysis showed the highest number of specific T cells in peripheral blood for LMP-2, followed by EBNA-1; the lowest T-cell response was detected against early antigen p54 (Fig 1). Since available LMP-2 failed to meet GMP criteria in 2007, EBNA-1 was chosen as a target antigen for the production of EBV-specific T cells.

Large-Scale Generation of EBNA-1–Specific T Cells

We isolated EBNA-1–specific T cells for patients with EBV reactivation after SCT from their healthy stem-cell donors. All donors were EBV seropositive with EBNA-1–specific T-cell responses. HLA profiles were not relevant for the manufacturing process. Antigen-specific T lymphocytes were isolated under GMP conditions. The whole procedure took about 30 hours and was successfully performed in 16 (94%) of 17 samples. Frequency of EBV-specific T cells in donor samples was 0.02% to 1.51% of total T cells. Isolation and enrichment of antigen-specific T cells was possible to a purity of 57% ± 22.6% (mean ± standard deviation). The isolated EBNA-1–specific T cells contained both CD4$^+$IFN-γ^+ (54.5% ± 30%) and CD8$^+$IFN-γ^+ (35.8% ± 30%) T cells detected in percent of CD3$^+$ cells. Microbiologic contamination could be excluded in all preparations (Appendix Table A1). Specificity of transferred T cells to known HLA-binding peptide motifs was confirmed in 80% of CD4$^+$ T cells with three known peptides. Polyfunctional responses on restimulation with MHC class II binding peptides showed simultaneous secretion of IFN-γ, tumor necrosis factor α (TNF-α), and IL-2 (Fig 2).

Adoptive Transfer of EBNA-1–Specific T Cells

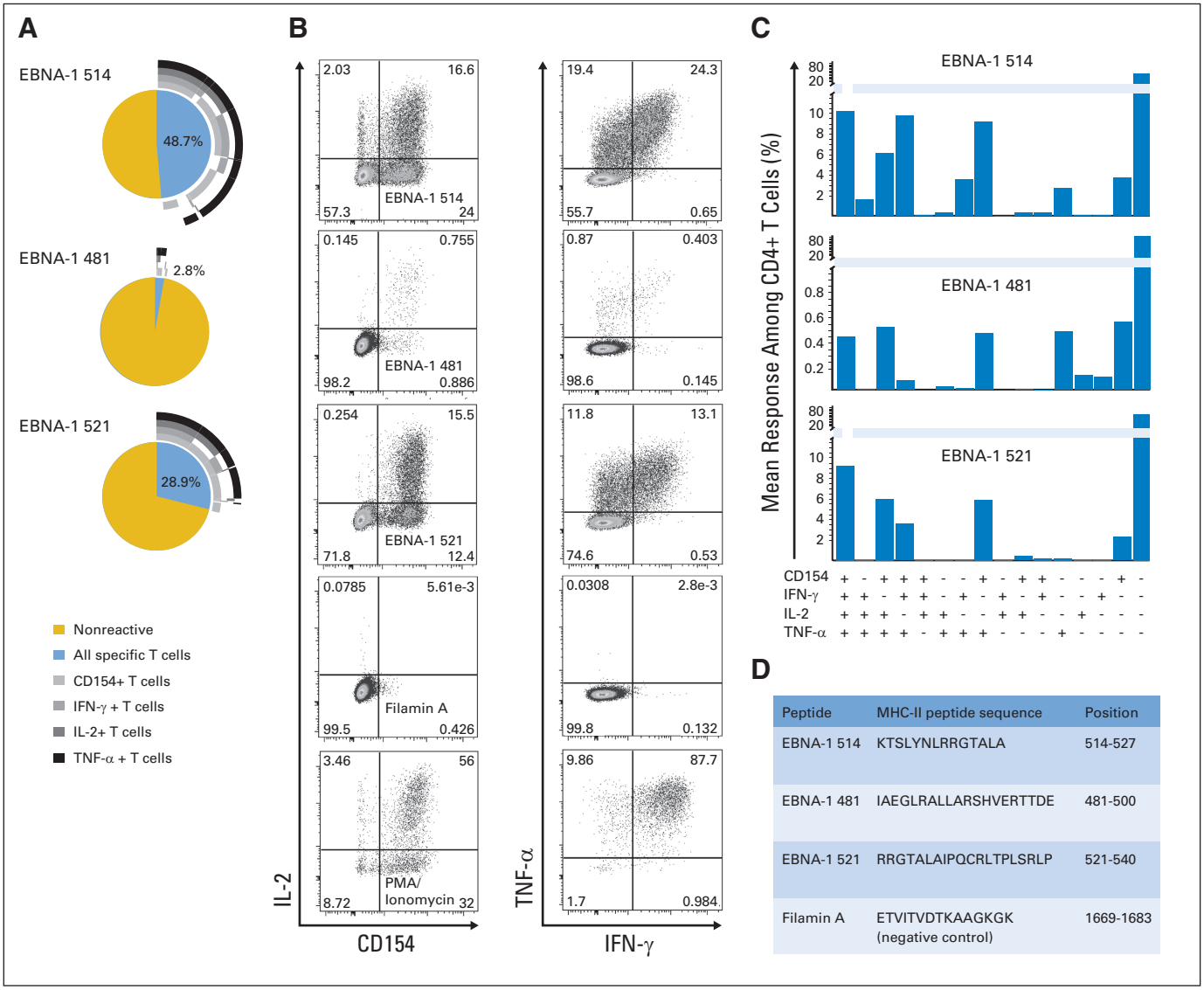
Antigen-specific T cells were isolated from the transplantation donors by using the previously described protocol and were concentrated to a small volume of approximately 5 to 10 mL. They were then immediately infused intravenously to the transplantation recipients. The procedure took 5 to 10 minutes, and vital signs were monitored. In three of the recipients, a second T-cell administration after 7 to 54 days was required to achieve a sustained immunologic response. The mean T-cell dose was 5,794 CD3$^+$ cells per kilogram of body weight (range, 150 to 53,796 CD3$^+$cells per kilogram) and was determined by the yield of cells recovered from the donor's whole blood or leucapheresis starting fraction (Appendix Table A1). No acute adverse reactions were observed in any of the patients. GVHD was considered to be a consequence of EBNA-1–specific T-cell transfer in case of new onset or worsening less than 6 weeks after adoptive T-cell transfer (Appendix Table A2, online only). Patient 5 developed transient grade 1 to 2 acute skin GVHD 15 days after the first donor lymphocyte infusion, which was thought to be related to adoptive transfer; symptoms responded well to treatment and resolved within 3 to 4 weeks.

Induction of Protective T-Cell Immunity Through Transfusion of EBNA-1–Specific T Cells

Before adoptive T-cell transfer, seven of 10 evaluable patients with refractory disease had T lymphocytes but no EBV-specific T lymphocytes, and two patients had no T lymphocytes, demonstrating the correlation between the presence of T-cell immunity and disease control.

EBNA-1–specific T-cell levels were monitored for up to 10 months after adoptive transfer (Fig 3). Eight (80%; 1, 3, and 5 through 10) of 10 patients developed a detectable in vivo expansion of EBNA-1–specific T cells (0.02% to 0.5% among CD3$^+$) 3 to 43 days after adoptive transfer, resulting in clinical and virologic responses in seven (70%; 1, 3, 5, 6, and 8 through 10) of 10 recipients (Table 2). In vivo expansion of T lymphocytes was found within the CD8$^+$ T-cell compartment in all positive patients as well as in the CD4$^+$ T-cell compartment in six patients (exclusive expansion of CD8$^+$IFN-γ^+ T-lymphocytes could be detected in patients 7 and 10). EBNA-1–specific T cells were detected in recipients for up to 9 months after adoptive T-cell transfer.

Three patients (3, 7, and 8) also developed significant amounts of LMP-2–specific T cells simultaneously with expansion of EBNA-1–specific T cells. No analysis of EBNA-1–specific T-cell expansion could be performed in patient 2 because of fulminant PTLD; the patient died only 2 days after adoptive transfer.



Peptide	MHC-II peptide sequence	Position
EBNA-1 514	KTSLYNLRRGTALA	514-527
EBNA-1 481	IAEGLRALLARSHVERTTDE	481-500
EBNA-1 521	RRGTALAIPQCRLTPLSRLP	521-540
Filamin A	ETVITVDTKAAGKGK (negative control)	1669-1683

Fig 2. (A) Peptide specificity of the Epstein-Barr virus nuclear antigen 1 (EBNA-1) -specific T-cell graft against major histocompatibility complex (MHC) class II binding motifs of the EBNA-1 protein. Because generation of EBNA-1-specific T cells was performed with an antigen that is used independent of the HLA type, we confirmed the specificity with known defined HLA class II binding peptide epitopes. Since the majority of T cells were $CD4^+$ T cells, we selected three peptides (D) that are supposed to bind to HLA class II according to the SYFPEITHI database and synthesized these peptides. Before analysis, the EBNA-1-specific T cells were expanded with irradiated allogeneic peripheral blood mononuclear cells, interleukin 2 (IL-2), and phytohemagglutinin without the addition of antigen. (B) Then the EBNA-1-specific T cells were restimulated with the synthesized MHC-II peptides, and the specific response was analyzed through CD154 expression and an intracellular stain of interferon gamma (IFN-γ), IL2, and tumor necrosis factor α (TNF-α). (A) We confirmed that 80.4% of $CD4^+$ T cells responded to one of the three selected peptides, although initial isolation of T cells was performed with an overlapping peptide mix. In (A) and (C), the mean (n = 2; negative control was subtracted) response of $CD4^+$ T cells is shown with an analysis of the overlapping expression of the four markers for specificity (CD154, IFN-γ, IL-2, and TNF-α). This analysis confirmed a high frequency of polyfunctional T cells that secrete multiple cytokines in response to Epstein-Barr virus. Visualization of multicolor flow cytometric data was done with Spice software (http://exon.niaid.nih.gov/spice/). PMA, phorbol 12-myristate 13-acetate.

Clinical Response and Follow-Up After Adoptive T-Cell Transfer

Eight of ten patients showed in vivo expansion of EBNA-1–specific T cells. This was associated with a clinical and virologic response in seven (70%) of them, defined as decrease of viral load more than 1log and resolution of PTLD. Patient 7 showed T-cell expansion 6 days after transfer but succumbed as a result of hemophagocytic lymphohistiocytosis and multiorgan failure only 11 days after transfer. In the two cases with absence of in vivo T-cell expansion, no clinical improvement was observed.

Within clinical responders, three patients were disease-free at last follow-up (2 to 36 months), three patients died of other infectious complications, and one patient died as a result of relapse of malignancy. Among evaluable patients, eight suffered from refractory PTLD. In this subgroup, six patients (75%) responded to adoptive T-cell transfer. In two patients (2 and 8), malignant degeneration of monoclonal PTLD into B-cell lymphoma with cerebral involvement was present at adoptive transfer. Even at that advanced stage of disease, adoptive immunotherapy was successful in patient 8. EBV viremia was moderate in most patients, and

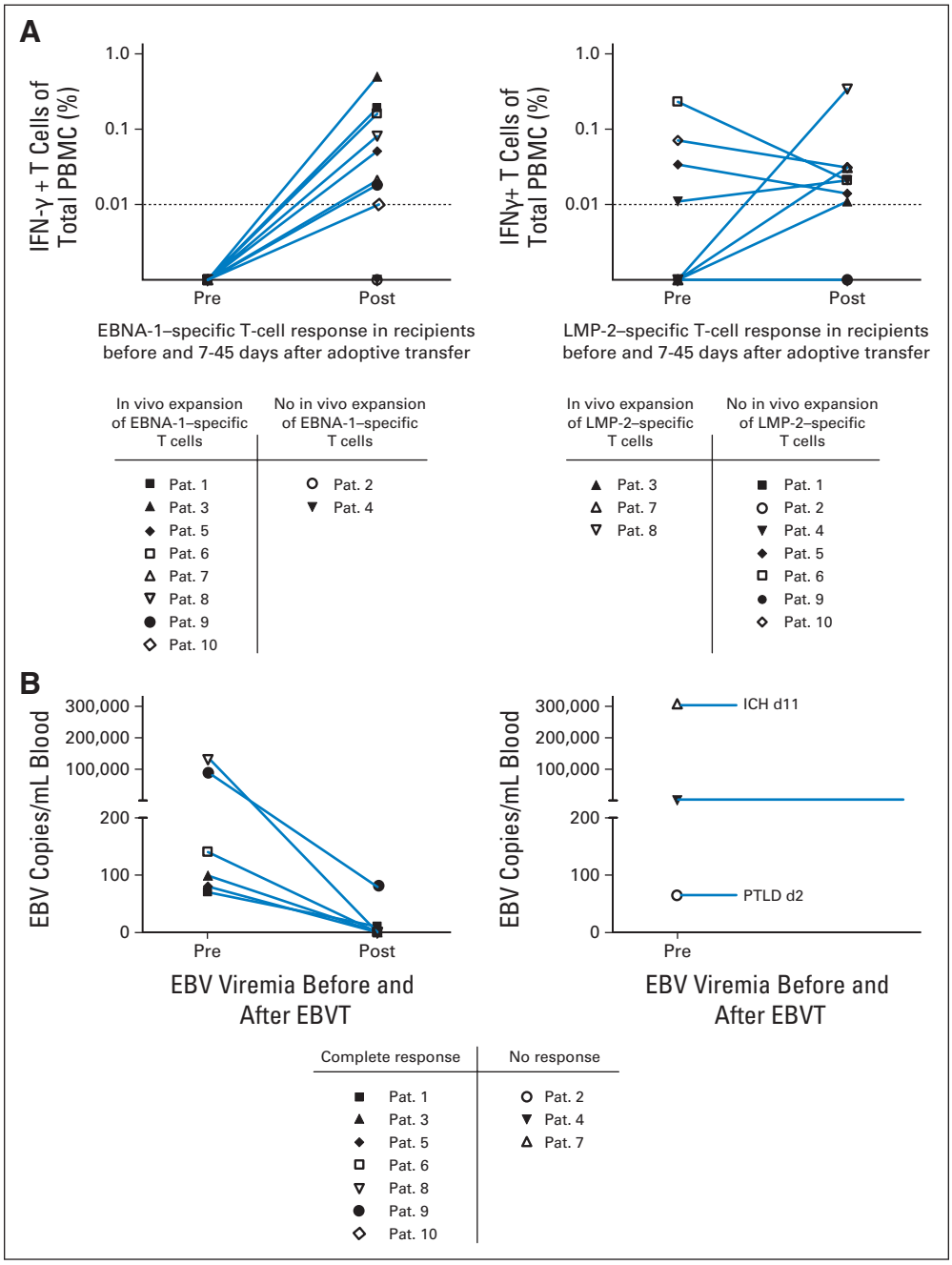


Fig 3. In vivo T-cell response and virologic response after adoptive transfer of Epstein-Barr virus nuclear antigen 1 (EBNA-1) –specific T cells in 10 patients after post allogeneic stem-cell transplantation who had refractory Epstein-Barr virus (EBV) –related conditions. (A) In vivo expansion of the transferred T cells within 4 to 6 weeks after adoptive immunotherapy. Antigen-specific T cells were detected by stimulation of blood samples with EBNA-1 antigen, followed by intracellular cytokine staining by flow cytometry. Eight patients had a successful T-cell response after adoptive transfer. Six of them showed expansion of both $CD4^+$ and $CD8^+$ T cells in vivo. Only patients 7 and 10 developed $CD8^+$ T cells exclusively. Patient 2 is not shown because blood samples for evaluation of response were not available. The threshold of a positive antigen-specific T-cell response was 0.01% of viable T cells. T-cell response to EBNA-1 after adoptive T-cell transfer was compared with the response against latent membrane protein 2 (LMP-2). Three patients also developed an LMP-2–specific T-cell response after adoptive T-cell transfer of EBNA-1–specific T cells. This could be explained either by a coincidence of an endogenous response to LMP-2 or by potential epitope spreading through the T-cell response against EBV-infected cells. (B) Virologic response of 10 patients to adoptive T-cell transfer in terms of viral copies in peripheral blood. Before the T-cell transfer, patients showed viremia, lymphadenopathy, and post-transplantation lymphoproliferative disease (PTLD) unresponsive to treatment with antivirals and/or rituximab. Lymphadenopathy and PTLD resolved in the responders with decreasing viral load. In seven patients, PTLD and viremia resolved. In patient 1, PTLD resolved completely, but viremia was recurrent (positive). In patient 4, EBV levels were not influenced by adoptive transfer. In patient 7, viremia decreased. This patient died from intracerebral hemorrhage (ICH) 11 days after adoptive transfer. In patients 1, 3, and 5, only qualitative polymerase chain reaction results were available. In Patient 10, no viremia was present at T-cell transfer and therefore the trend of viremia is not shown in the graph; in that case, refractory lymphoproliferative disease resolved after adoptive immunotherapy. EBVT, Epstein-Barr virus T-cell transfer; ICH d11, died as a result of ICH 11 days after T-cell transfer; IFN-γ, interferon gamma; Pat., patient; PBMC, peripheral blood mononuclear cell; PTLD d2, died as a result of PTLD on day 2 after T-cell transfer.

Table 2. Response to Adoptive Transfer of EBNA-1–Specific T Cells

Patient No.	Adoptive T-Cell Transfer (days after SCT)	$CD3^+$ Cells Per Kilogram Administered	Immunologic Response/In Vivo Expansion of Transferred EBV-Specific T Cells	Virologic Response/Course of EBV Infection 4 Weeks After T-Cell Transfer	Clinical Outcome Until Date of Last Observation/ Cause of Death	Patient Status	Evaluation of Response
1	72	2,169	In vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific T-cells 15 days after transfer	EBV PCR negative on day 8 after T-cell transfer and PCR positive again on day 15	Death 26 days after T-cell transfer (idiopathic pneumonia, respiratory failure)	Responder	PTLD cleared completely, transient clearance of viremia
2	151	1,675	Patient died 2 days after T-cell transfer; no data available	N/A	Patient died 2 days after T-cell transfer as a result of progression of cerebral HLH and PTLD	Nonresponder	PTLD and HLH were cause of death
3	362	1,140	In vivo expansion of $CD4^+$ EBNA-1–specific T-cells 37 days after transfer	EBV PCR negative on day 7 after transfer and then PCR positive again in leucocytes on day 28 after T-cell transfer	Transient clearance of viremia, transient amelioration of PTLD, progress to a second EBVT	Responder	Transient clearance of PTLD and viremia
3	413	7,753	In vivo expansion of $CD4^+$ 43 days after second transfer and persistence of $CD4^+$ and $CD8^+$ EBNA-1–specific T-cells up to 9 months after T-cell transfer	EBV PCR negative in blood	PTLD resolved, no viremia on day 14 after second transfer, CR until date	Responder	Complete clearance of PTLD and viremia
4	224	1,143	EBNA-1–specific and LMP-2–specific T-cells detectable before transfer; no further expansion after transfer	EBV PCR positive in blood	No reduction of viral load	Nonresponder	Chronic persistent viremia until date
5	97	288	LMP-2–specific T-cells detectable before transfer; in vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific T-cells 14 days after transfer	EBV positive below detection threshold in leucocytes	Persistence of PTLD and viremia; progress to a second adoptive T-cell transfer	Nonresponder	Persistence of PTLD and viremia
5	151	674	In vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific T-cells 20 days after second transfer	EBV PCR negative in blood	Clearance of EBV infection and PTLD; death as a result of AML relapse	Responder	Clearance of PTLD and viremia
6	80	148	LMP-2–specific T-cells detectable before transfer; in vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific and LMP-2–specific T-cells 3 days after transfer	EBV PCR negative in blood on day 26 after T-cell transfer	Clearance of PTLD and viremia, CR until date	Responder	Clearance of PTLD and viremia
7	188	382	In vivo expansion of $CD8^+$ EBNA-1–specific and LMP-2–specific T-cells 7 days after transfer	1log decrease of EBV load 11 days after T-cell transfer	Death 11 days after T-cell transfer (massive intracerebral hemorrhage due to HLH)	Nonresponder	Persistence of viremia and PTLD
8	114	1,088	In vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific and LMP-2–specific T-cells 27 days after first transfer	EBV PCR negative in blood 34 days after first T-cell transfer	Death 220 days after SCT and 106 days after T-cell transfer (grade 4 GVHD, ADV infection). Viremia cleared 34 days after T-cell transfer, PTLD cleared; only necrotic lesions in the affected organs at autopsy	Responder	Viremia and peripheral PTLD cleared; histologically almost complete necrosis of B-cell NHL lesions
8	121	53,796	In vivo expansion of $CD4^+$ and $CD8^+$ EBNA-1–specific and LMP-2–specific T-cells 20 days after second transfer	EBV PCR negative in blood 27 days after second T-cell transfer	Death 220 days after SCT and 99 days after second T-cell transfer (multiorgan failure; grade 4 GVHD; ADV, *Candida*, and enterococci infection)	Responder	Viremia and peripheral PTLD cleared; histologically almost complete necrosis of B-cell NHL lesions
9	59	1,160	In vivo expansion of $CD8^+$ EBNA-1–specific T-cells 26 days after transfer and of $CD4^+$ EBNA-1–specific T-cells 39 days after transfer	3log decrease of EBV load by 24 days after T-cell transfer	Death 140 days after SCT and 81 days after T-cell transfer (multiorgan failure, Aspergillosis)	Responder	3log decrease of viremia
10	131	9,756	In vivo expansion of $CD8^+$ EBNA-1–specific T-cells 16 days after transfer	EBV PCR negative in blood before and after T-cell transfer	Clearance of PTLD and lymphadenopathy; CR until date	Responder	Clearance of PTLD

Abbreviations: ADV, adenovirus; AML, acute myeloid leukemia; CR, complete remission; EBNA-1, Epstein-Barr virus nuclear antigen 1; EBV, Epstein-Barr virus; EBVT, EBV T-cell transfer; GVHD, graft-versus-host disease; HLH, hemophagocytic lymphohistiocytosis; LMP-2, latent membrane protein 2; N/A, not applicable; NHL, non-Hodgkin lymphoma; PCR, polymerase chain reaction; PTLD, post-transplantation lymphoproliferative disease; SCT, stem-cell transplantation.

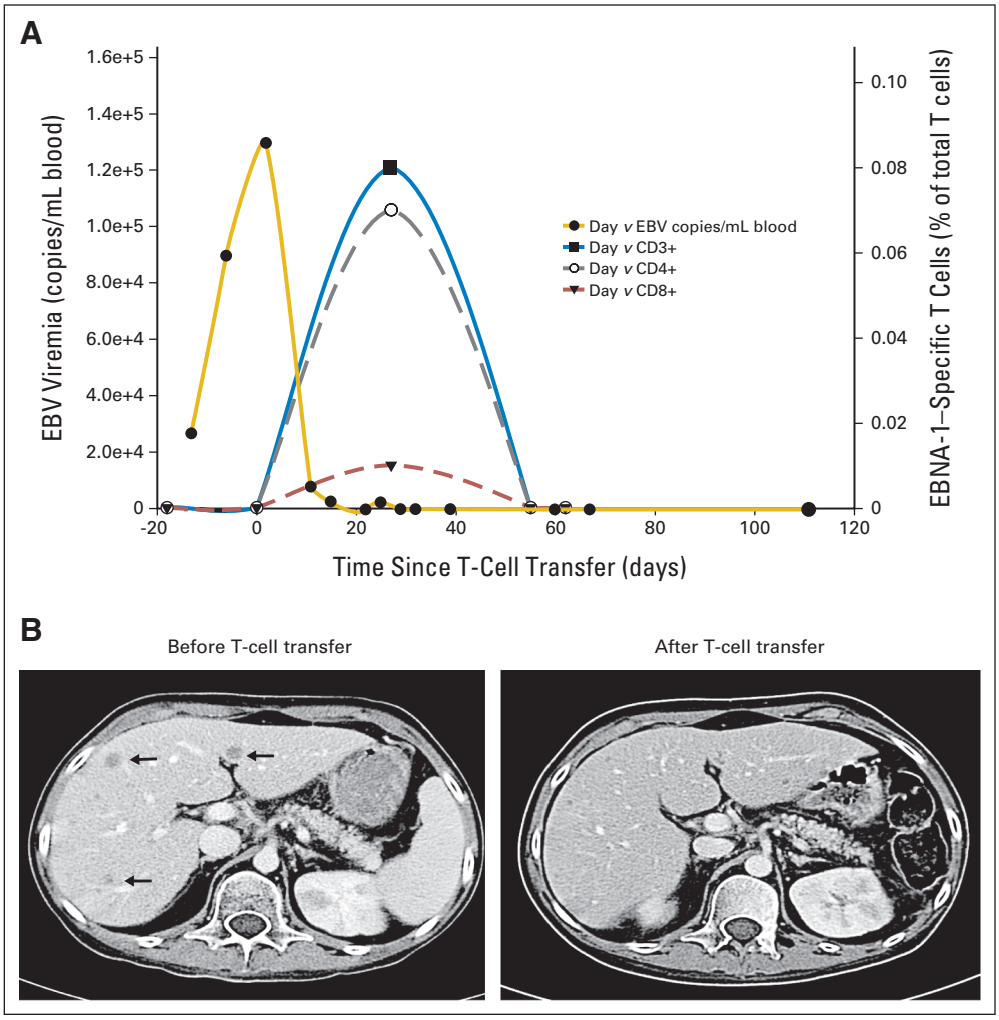


Fig 4. Clinical course of in vivo T-cell response and virologic response in patient 8: expansion of Epstein-Barr virus nuclear antigen 1 (EBNA-1) -specific T cells after adoptive transfer was associated with fast decrease of viral load and clearance of post-transplantation lymphoproliferative disease. EBV, Epstein-Barr virus.

resolution of PTLD was associated with decrease of viral load in responders (Figs 3 and 4).

DISCUSSION

Adoptive transfer of T-cell immunity is a promising approach, but it has been restricted to only a few centers in the past. Now, logistic matters present an obstacle for application of adoptive T-cell transfer in an urgent clinical setting, and availability is delayed through an extensive and laborious production period. Therefore, in the future, application of adoptive T-cell transfer will depend on the availability of adequate transfer protocols. We describe a fast and simple method that takes 30 hours to isolate polyclonal EBV-specific T cells from donor lymphocytes ex vivo by using their EBNA-1–specific IFN-γ secretion properties as a selection marker. This enables the direct transfusion of functionally active IFN-γ–secreting T cells. Isolation and stimulation is not dependent on donors' HLA profiles. No in vitro expansion steps were included in the protocol, since even a single antigen-specific T cell can repopulate distinct T-cell subsets in vivo,[35] and in vitro expanded cell lines showed reduced efficacy in vivo.[26] Thus, loss of expansion potential because of terminal differentiation during in vitro culture could be avoided. Even a T-cell dose of 150 cells per kilogram of a recipient's body weight (patient 6) was shown to be sufficient for a successful T-cell expansion. The isolation of IFN-γ–secreting cells enables the generation of $CD4^+$ and $CD8^+$ T-cell responses to multiple epitopes.[36] The provision of $CD4^+$ T-cell help is essential for a physiologic and sustained immune response, whereas $CD8^+$ T cells are considered to exert rapid but potentially transient antiviral effects.[37,38] Therefore, a combination of $CD4^+$ and $CD8^+$ T cells for adoptive transfer is beneficial for restoring a protective and sustained immunity.

In this article, we summarize our clinical experience in 10 patients with refractory EBV infection or PTLD after SCT. Transfusion of low numbers of EBNA-1–specific T cells could restore protective T-cell immunity against EBV and treat chemorefractory disease in seven of 10 patients with PTLD. None of the patients with an in vivo expansion of EBNA-1–specific T cells died of EBV-related complications. This suggests a strong correlation between in vivo expansion of EBNA-1–specific T cells and clinical response. Interestingly, the success of adoptive T-cell transfer was not related to the T-cell dose. A tendency toward improved outcome and sustained antiviral response in patients who received repetitive EBV-specific T-cell transfers was observed, suggesting that iterative transfusions could be more advantageous and of longer-lasting effect.

Furthermore, patients suffering from PTLD with CNS involvement showed a fulminant course of disease, indicating that in these cases, a small window of opportunity is available to reverse the process. Since the time needed for virus-specific T cells to expand in vivo is 3 to 28 days, early measures should be taken to perform a T-cell transfer before progression of PTLD. This is underlined by the clinical course of patient 8, who had a complete response with clearance of peripheral PTLD lesions and almost complete necrosis and involution of cerebral lesions assigned to repetitive adoptive T-cell transfer in combination with local irradiation. CNS response was delayed compared with peripheral response in that patient.

Compared with unselected T-cell boosts, adoptive T-cell transfer offers the advantage of a well-targeted treatment with lower rates of GVHD because of the low alloreactivity of virus-specific T lymphocytes[10,30,39] and the low number of potentially alloreactive T cells.

Three of the patients also developed significant amounts of LMP-2–specific T cells simultaneously with expansion of EBNA-1–specific T-cells. This could be explained by either a coincidence of an endogenous response to LMP-2 or by potential epitope spreading through the T-cell response against EBV-infected cells.

Our method has some limitations. For example, it is not suitable for EBV-seronegative donors or for seropositive donors with an extremely low frequency of $CD3^{+}IFN\text{-}\gamma^{+}$ cells and IFN-γ low-expressing T cells. In vivo expansion of EBV-specific T cells was achieved in 80% of patients, which is in accordance with the clinical response (70%). For more successful T-cell expansion in vivo, optimal recipient conditions need to be investigated, as well as extension to other EBV antigens and improved purity and viability of T-cell preparations in the future. Despite encouraging results, controlled and randomized clinical trials with a larger number of patients are needed to analyze the benefit of EBV-specific T-cell transfer when applied in addition to standard therapy. The optimal time point and frequency of adoptive immunotherapy still remains to be determined. Adoptive transfer of EBV-specific T cells has the potential to become a valuable clinical extension to existing treatment in the initial phase of EBV reactivation and for EBV positive malignancies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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REFERENCES

1. Ocheni S, Kroeger N, Zabelina T, et al: EBV reactivation and post transplant lymphoproliferative disorders following allogeneic SCT. Bone Marrow Transplant 42:181-186, 2008
2. Handgretinger R, Lang P, Schumm M, et al: Immunological aspects of haploidentical stem cell transplantation in children. Ann N Y Acad Sci 938: 340-357, 2001; discussion 357-358
3. Mohty M, Jacot W, Faucher C, et al: Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin-based reduced intensity preparative regimen. Leukemia 17:2168-2177, 2003
4. Chiusolo P, Metafuni E, Cattani P, et al: Prospective evaluation of Epstein-Barr virus reactivation after stem cell transplantation: Association with monoclonal gammopathy. J Clin Immunol 30:894-902, 2010
5. Tran H, Nourse J, Hall S, et al: Immunodeficiency-associated lymphomas. Blood Rev 22:261-281, 2008
6. Meij P, van Esser JW, Niesters HG, et al: Impaired recovery of Epstein-Barr virus (EBV)-specific CD8+ T lymphocytes after partially T-depleted allogeneic stem cell transplantation may identify patients at very high risk for progressive EBV reactivation and lymphoproliferative disease. Blood 101:4290-4297, 2003
7. van Esser JW, van der Holt B, Meijer E, et al: Epstein-Barr virus (EBV) reactivation is a frequent event after allogeneic stem cell transplantation (SCT) and quantitatively predicts EBV-lymphoproliferative disease following T-cell-depleted SCT. Blood 98:972-978, 2001
8. Buyck HC, Ball S, Junagade P, et al: Prior immunosuppressive therapy with antithymocyte globulin increases the risk of EBV-related lymphoproliferative disorder following allo-SCT for acquired aplastic anaemia. Bone Marrow Transplant 43:813-816, 2009
9. Lewin SR, Heller G, Zhang L, et al: Direct evidence for new T-cell generation by patients after either T-cell-depleted or unmodified allogeneic hematopoietic stem cell transplantations. Blood 100: 2235-2242, 2002
10. Bahceci E, Epperson D, Douek DC, et al: Early reconstitution of the T-cell repertoire after non-myeloablative peripheral blood stem cell transplantation is from post-thymic T-cell expansion and is unaffected by graft-versus-host disease or mixed chimaerism. Br J Haematol 122:934-943, 2003
11. Wagner HJ, Cheng YC, Huls MH, et al: Prompt versus preemptive intervention for EBV lymphoproliferative disease. Blood 103:3979-3981, 2004
12. Heslop HE: How I treat EBV lymphoproliferation. Blood 114:4002-4008, 2009
13. Heslop HE, Li C, Krance RA, et al: Epstein-Barr infection after bone marrow transplantation. Blood 83:1706-1708, 1994
14. Comoli P, Basso S, Zecca M, et al: Preemptive therapy of EBV-related lymphoproliferative disease after pediatric haploidentical stem cell transplantation. Am J Transplant 7:1648-1655, 2007
15. O'Reilly RJ, Doubrovina E, Trivedi D, et al: Adoptive transfer of antigen-specific T-cells of donor type for immunotherapy of viral infections following allogeneic hematopoietic cell transplants. Immunol Res 38:237-250, 2007
16. Slobod KS, Benaim E, Woodruff L, et al: T cell immunotherapeutic populations control viral infections in bone marrow transplant recipients. Immunol Res 24:289-301, 2001
17. Gustafsson A, Levitsky V, Zou JZ, et al: Epstein-Barr virus (EBV) load in bone marrow transplant recipients at risk to develop posttransplant lymphoproliferative disease: Prophylactic infusion of EBV-specific cytotoxic T cells. Blood 95:807-814, 2000
18. Moosmann A, Bigalke I, Tischer J, et al: Effective and long-term control of EBV PTLD after transfer of peptide-selected T cells. Blood 115:2960-2970, 2010
19. Dong L, Gao ZY, Chang LJ, et al: Adoptive transfer of cytomegalovirus/Epstein-Barr virus-specific immune effector cells for therapeutic and preventive/preemptive treatment of pediatric allogeneic cell transplant recipients. J Pediatr Hematol Oncol 32:e31-e37, 2010
20. Uhlin M, Okas M, Gertow J, et al: A novel haplo-identical adoptive CTL therapy as a treatment for EBV-associated lymphoma after stem cell transplantation. Cancer Immunol Immunother 59:473-477, 2010
21. Heslop HE, Slobod KS, Pule MA, et al: Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. Blood 115:925-935, 2010
22. Rooney CM, Smith CA, Ng CY, et al: Infusion of cytotoxic T cells for the prevention and treatment

of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. Blood 92:1549-1555, 1998

23. Riddell SR, Watanabe KS, Goodrich JM, et al: Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. Science 257:238-241, 1992

24. Leen AM, Myers GD, Sili U, et al: Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. Nat Med 12:1160-1166, 2006

25. Merlo A, Turrini R, Dolcetti R, et al: Adoptive cell therapy against EBV-related malignancies: A survey of clinical results. Expert Opin Biol Ther 8:1265-1294, 2008

26. Gattinoni L, Klebanoff CA, Palmer DC, et al: Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest 115:1616-1626, 2005

27. Feuchtinger T, Lang P, Hamprecht K, et al: Isolation and expansion of human adenovirus-specific CD4+ and CD8+ T cells according to IFN-gamma secretion for adjuvant immunotherapy. Exp Hematol 32:282-289, 2004

28. Feuchtinger T, Matthes-Martin S, Richard C, et al: Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. Br J Haematol 134:64-76, 2006

29. Feuchtinger T, Richard C, Joachim S, et al: Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. J Immunother 31:199-206, 2008

30. Feuchtinger T, Opherk K, Bethge WA, et al: Adoptive transfer of pp65 specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. Blood 116:4360-4367, 2010

31. Jones K, Nourse JP, Morrison L, et al: Expansion of EBNA1-specific effector T cells in posttransplantation lymphoproliferative disorders. Blood 116: 2245-2252, 2010

32. Young LS, Rickinson AB: Epstein-Barr virus: 40 years on. Nat Rev Cancer 4:757-768, 2004

33. Demachi-Okamura A, Ito Y, Akatsuka Y, et al: Epstein-Barr virus nuclear antigen 1-specific CD4+ T cells directly kill Epstein-Barr virus-carrying natural killer and T cells. Cancer Sci 99:1633-1642, 2008

34. Meyer VS, Drews O, Günder M, et al: Identification of natural MHC class II presented phosphopeptides and tumor-derived MHC class I phospholigands. J Proteome Res 8:3666-3674, 2009

35. Stemberger C, Huster KM, Koffler M, et al: A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. Immunity 27:985-997, 2007

36. Fujita Y, Leen AM, Sun J, et al: Exploiting cytokine secretion to rapidly produce multivirus-specific T cells for adoptive immunotherapy. J Immunother 31:665-674, 2008

37. Sun JC, Williams MA, Bevan MJ: CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. Nat Immunol 5:927-933, 2004

38. Bevan MJ: Helping the CD8(+) T-cell response. Nat Rev Immunol 4:595-602, 2004

39. Melenhorst JJ, Leen AM, Bollard CM, et al: Allogeneic virus-specific T cells with HLA alloreactivity do not produce GVHD in human subjects. Blood 116:4700-4702. 2010

