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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

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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.

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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.

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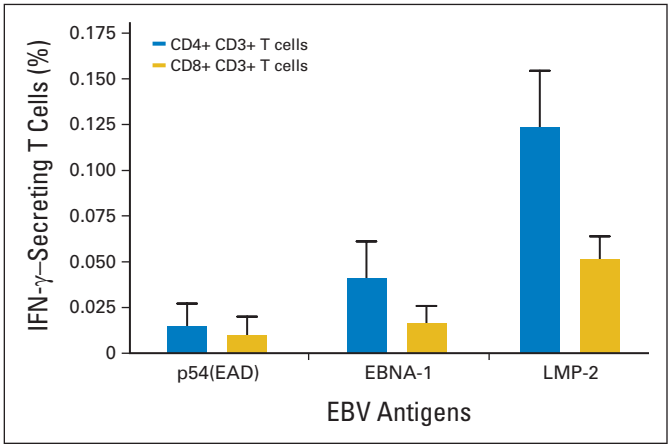

Fig 1. T-cell responses against Epstein-Barr virus nuclear antigen 1 (EBNA-1), latent membrane protein 2 (LMP-2), and p54 in healthy donors. Mean frequency of antigen-specific T cells against EBNA-1 is compared with early antigen p54 and LMP-2 (n = 8). Antigen-specific T lymphocytes were found in all 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.

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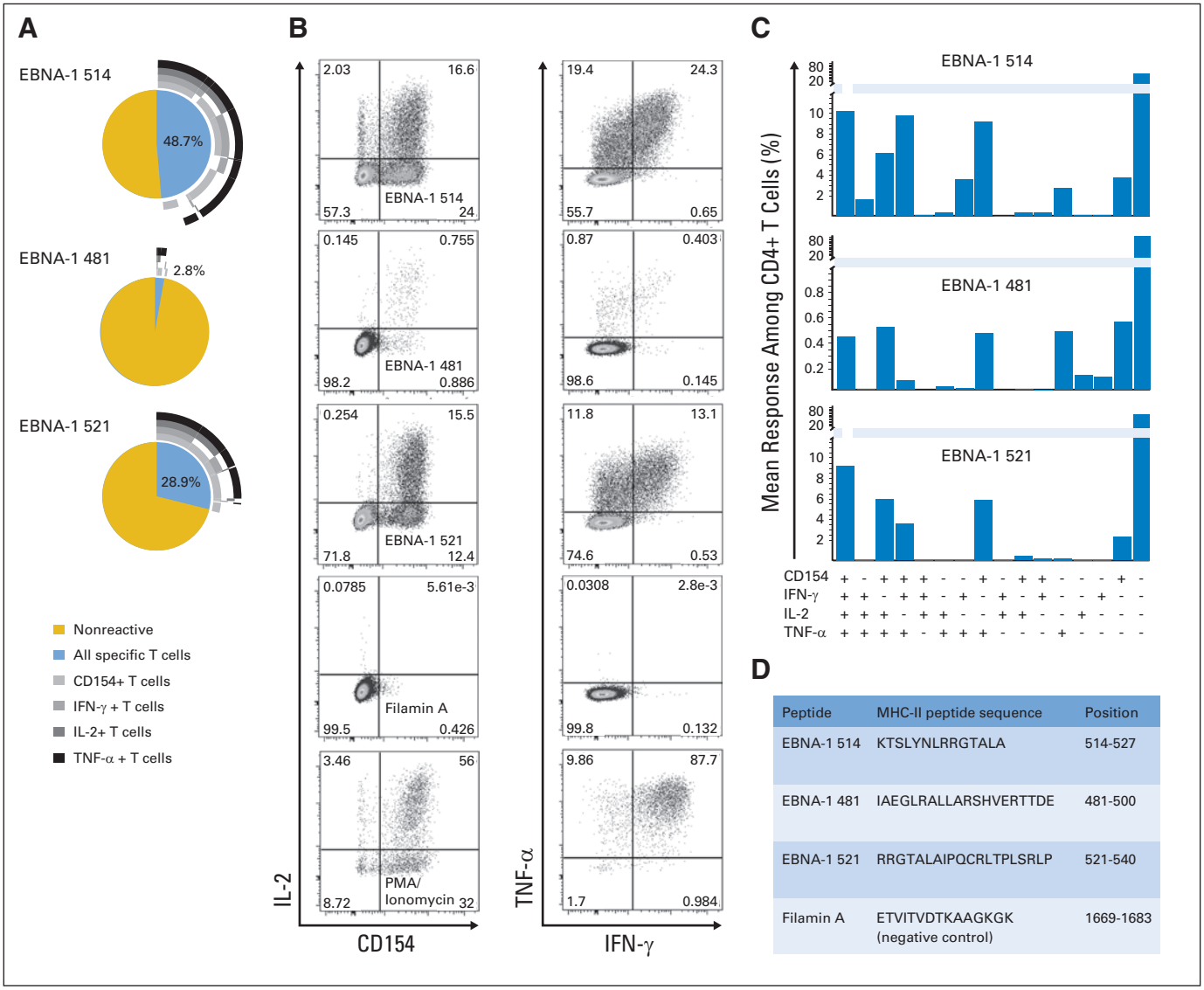

Peptide	MHC-II peptide sequence	Position
EBNA-1 514	KTSLYNLRRGTALA	514-527
EBNA-1 481	IAEGLRALLARSHVERTTDE	481-500
EBNA-1 521	RRGTALAIPQCRLTPLSRLP	521-540
Filamin A	ETVITVDTKAAGKGK (negative control)	1669-1683

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

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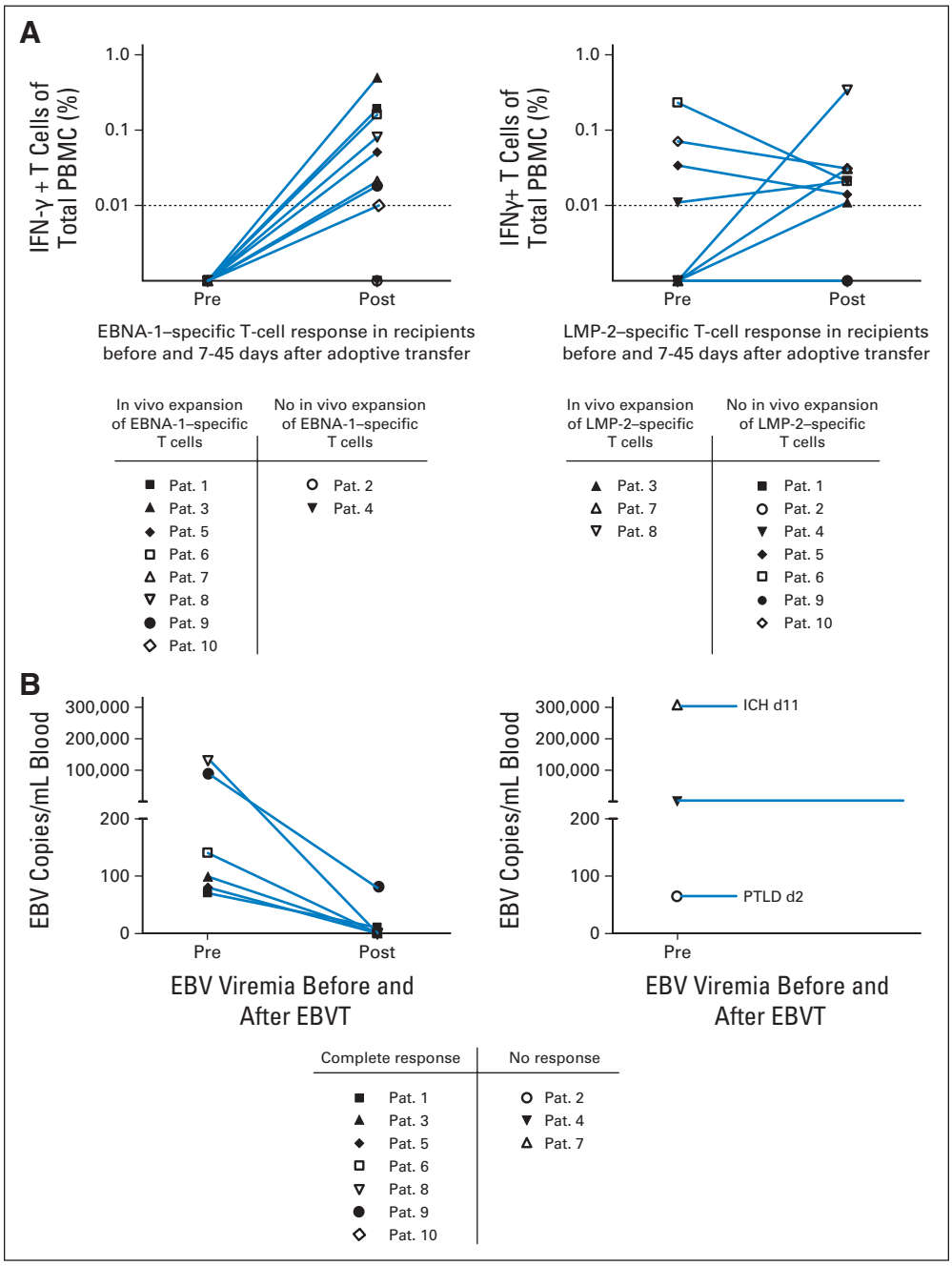

Fig 3. In vivo T-cell response and virologic response after adoptive transfer of Epstein-Barr virus nuclear antigen 1 (EBNA-1) –specific T cells in 10 patients after post allogeneic stem-cell transplantation who had refractory Epstein-Barr virus (EBV) –related conditions. (A) In vivo expansion of the 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.

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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.

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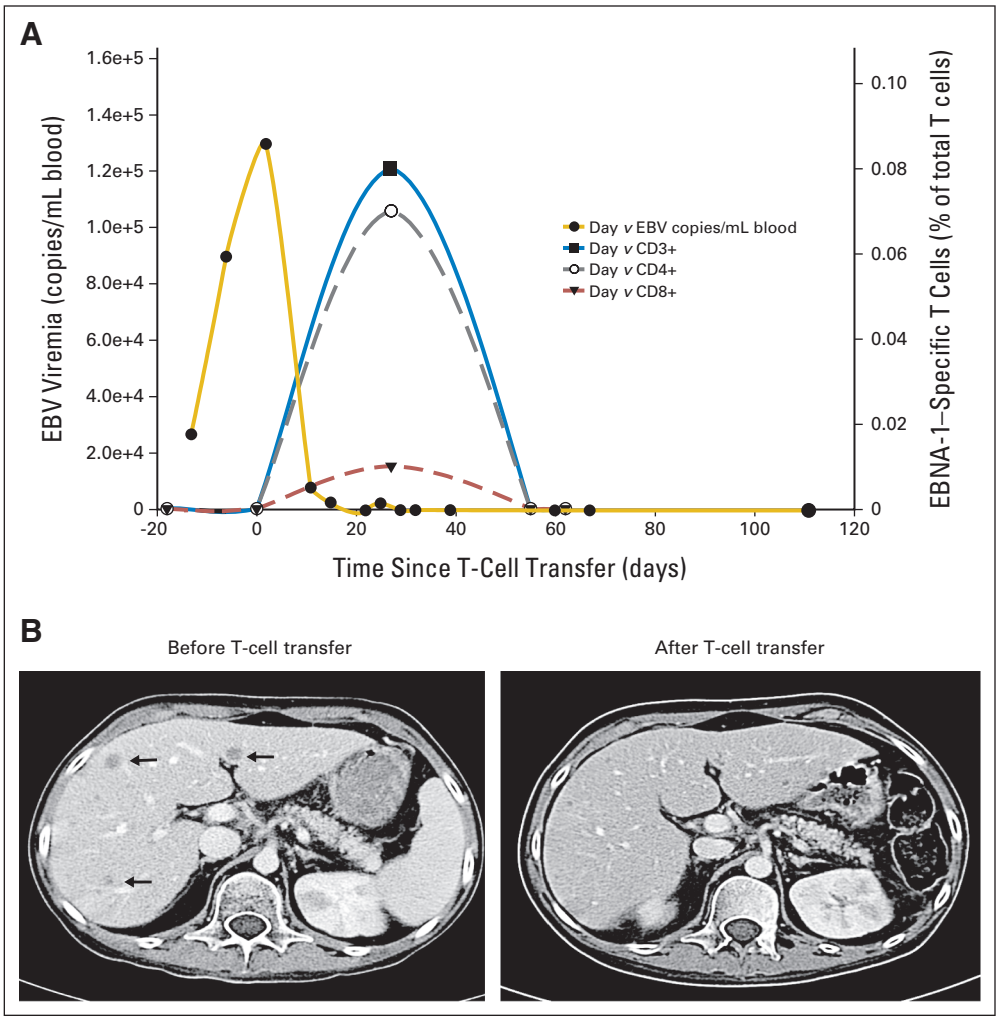

Fig 4. Clinical course of in vivo T-cell response and virologic response in patient 8: expansion of Epstein-Barr virus nuclear antigen 1 (EBNA-1) -specific T cells after adoptive transfer was associated with fast decrease of viral load and clearance of post-transplantation lymphoproliferative disease.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

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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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Appendix

Individual Outcome

Seven patients (1, 3, 5, 6, 8, 9, and 10) responded to adoptive T-cell transfer with clearance of post-transplantation lymphoproliferative disease (PTLD) and/or viremia. In three of them (3, 5, and 8), adoptive transfer was performed twice. Patient 1 reacted with complete resolution of lymphadenopathy and PTLD, but subsequently developed pneumonia and died of respiratory failure 26 days after adoptive transfer. Patient 3 recovered from refractory viremia and PTLD after the second adoptive T-cell transfer and has been disease-free for 2 years. Patient 5 died of relapse of acute myelocytic leukemia 90 days after stem-cell transplantation in complete remission of PTLD and absence of Epstein-Barr virus (EBV) viremia. In patient 6, lymphoproliferative disease and monoclonal gammopathy resolved after adoptive transfer. That patient has been free of EBV-related disease for 3 years. Patient 8 showed a fast and complete remission of peripheral PTLD manifestation, whereas the cerebral PTLD lesions involuted with slower kinetics and received additional local radiotherapy. As is often observed, a measurable expansion of Epstein-Barr virus nuclear antigen 1 (EBNA-1) –specific T cells was seen after decrease of viral load (Fig 2). This patient died of severe adenovirus infection combined with gut graft-versus-host disease (GVHD) 106 days after transfer of EBV-specific T cells. Postmortem EBV polymerase chain reaction of the cerebral lesion was negative, and histologically, a mixed picture of necrotic tissue and B-cell lymphoma areas was present. Adoptive T-cell transfer in patient 9 was indicated for persisting EBV viremia. The EBV-specific T-cell product was first cryopreserved and administered to the patient 4 weeks later. Viremia regressed by 3log after transfer. The patient died 81 days after adoptive immunotherapy for *Aspergillus* septicemia and multiorgan failure. In patient 10, EBV reactivation presented with initial viremia, which resolved as a result of expansion of patient's intrinsic EBV-specific T cells. During the course of disease, the intrinsic EBV-specific T-cell response disappeared and cervical lymphadenopathy (histologically B-cell non-Hodgkin lymphoma) occurred, which was refractory to chemotherapy. Adoptive immunotherapy was performed at that point. This resulted in clearance of PTLD; the patient has been disease-free for 3 months. Three patients (2, 4, and 7) did not respond to adoptive T-cell transfer. In patient 2, the transfer was performed in a multimorbid situation with distinct symptoms of high intracranial pressure due to a large intracerebral lesion. The patient died 2 days after donor leukocyte infusion due to a fulminant monoclonal PTLD and hemophagocytic lymphohistiocytosis (HLH) with cerebral involvement and multiorgan dysfunction. Death was not attributed to the T-cell transfer by the treating physician. In this patient, T cells could not expand and become effective in the short period of time. In patient 4, neither in vivo expansion nor clinical response was observed. To date, chronic viremia is still present in that patient. Patient 7 showed a partial response with an initial increase of viremia as an expression of intensified lysis of EBV-infected cells by the transferred T lymphocytes. Expansion of EBV-specific T cells was associated with more than 1log decrease of viral load. However, the patient died 11 days after adoptive transfer as a result of HLH progression manifesting as cerebral mass bleeding originating from an HLH lesion and hemorrhagic enteritis.

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

In patient 4, purity analysis of the isolated EBNA-1–specific T cells failed because of technical problems with the flow cytometer. Because of the small starting volume of donor blood (95 mL) and low number of isolated T cells, the treating physician abstained from further dispensing of cells for analysis and decided to transfuse the T-cell preparation without further analysis. In patient 1, isolation of EBNA-1–specific T cells resulted in relatively low purity of interferon gamma (IFN-γ^+) lymphocytes. This was related to a rather small antigen-specific T-cell population, with weak expression of IFN-γ^+. On the basis of this experience, the preisolation test panel was expanded to several EBNA-1 antigens, and the threshold was increased to IFN-γ^{high+} lymphocytes. Preparations of enriched EBNA-1–specific T cells had an end volume of 5 to 10 mL and could either be cryopreserved for later use or be directly transfused to the patient. Nine patients received the T-lymphocyte preparation immediately after isolation; in patient 9, donor lymphocytes were first cryopreserved to be administered at a later point for persisting viremia. In patient 8, repetitive adoptive transfer was possible from one donor's whole blood sample because of the high yield of EBNA-1–specific T cells. Donor peripheral blood mononuclear cells were split and one donor leukocyte infusion was performed immediately with a dose of 1,088 $CD3^+$ cells per kilogram, while the second portion of peripheral blood mononuclear cells was cultured in vitro in the presence of EBNA-1 antigen for 6 days. EBNA-1–specific T lymphocytes were then isolated via immunomagnetic separation again and a dose of 5.37×10^4 $CD3^+$ cells per kilogram was transfused to the patient.

Tolerability and Adverse Effects

Patient 2 suffered from grade 1 cutaneous GVHD before EBV-specific T-cell transfer, which did not deteriorate in the follow-up period. Patient 6 developed transient grade 1 cutaneous GVHD 5 weeks after transfer, but this was chronologically closely related to a $CD3^+/CD133^+$ lymphocyte boost with 5×10^4 $CD3^+$cells per kilogram performed because of a mixed chimerism and was not likely to be caused by the EBV-specific T cells. Patient 8 suffered from grade 3 liver GVHD and grade 2 intestinal GVHD before Epstein-Barr virus T-cell transfer. Because of the combined adenovirus and EBV infection associated with graft deficiency, immunosuppression was discontinued in that patient despite high-grade GVHD resulting in a subsequent progression of GVHD to grade 4, which was most likely related to the absence of immunosuppressive medication. Still, a GVH effect of adoptive T-cell transfer cannot be completely excluded in that case. Patient 9 developed grade 4 intestinal GVHD 8 weeks after adoptive transfer, which was related to tapering of immunosuppression.

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Table A1. Generation of EBNA-1–Specific T Cells From Seropositive Stem-Cell Donors (n = 17)

Isolation Procedure No.	EBV-Specific T Cells Administered to Patient No.	Material	Volume (mL)	Patient Body Weight (kg)	$CD3^+IFN\text{-}\gamma^+$ T Cells in Donor Sample Before Isolation (%)	Purity of EBV-Specific $CD3^+IFN\text{-}\gamma^+$ T Cells After Isolation (%)	$CD3^+$ Cells Per Kilogram After Isolation	$CD3^+IFN\text{-}\gamma^+$ Cells Per Kilogram	$CD4^+IFN\text{-}\gamma^+$ Cells Per Kilogram	$CD8^+IFN\text{-}\gamma^+$ Cells Per Kilogram
1	Cryo	Leucapheresis	145	65	0.18	26	2,817	732	549	349
2	Cryo	Whole blood	540	21	1.51	72.8	2,468	1,797	227	1,535
3	1	Leucapheresis	55	84	0.03	2.72	2,169	59	23	26
4	2	Whole blood	447	58	0.08	68.5	1,675	1,147	802	216
5	3	Whole blood	444	30	0.03	77	1,140	880	40	800
6	4	Whole blood	95	12.4	0.84	N/A	1,143	N/A	N/A	N/A
7	3	Whole blood	625	29	0.07	44.2	7,753	3,427	643	2,954
8	5	Whole blood	515	45	0.03	60	288	173	86	89
9	5	Whole blood	650	47	0.02	63.3	674	427	297	81
10	N/A	Whole blood	206	54	0.08	52.5	828	434	261	94
11	6	Leucapheresis	10	73	0.09	63	148	93	83	22
12	7	Whole blood	555	25	0.06	48	382	183	96	80
13	9 (Cryo)	Whole blood	520	14	0.16	66.2	1,160	768	132	227
14	Cryo	Leucapheresis	8	24	0.21	29.3	11,215	3,656	3,285	11
15	8	Leucapheresis	318	53	0.14	67.5	1,088	735	300	23
16	8	Leucapheresis	318	53	0.57	83	53,796	44,650	43,037	915
17	10	Whole blood	585	49	0.38	88.7	9,756	8,653	7,639	576
Mean				43.3	0.26	57.05	5,794	4,215	3,613	500
SD				20.8	0.39	22.63	12,826	10,997	10,698	780

Abbreviations: Cryo, cryopreservation of Epstein-Barr virus–specific T cells for later use; EBNA-1, Epstein-Barr virus nuclear antigen 1; EBV, Epstein-Barr virus; IFN-γ, interferon gamma; N/A, not applicable; SD, standard deviation.

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Table A2. GVHD and Adoptive Transfer of EBNA-1–Specific T Cells

Patient No.	Primary Condition	Adoptive T-Cell Transfer (days after SCT)	Stem-Cell Boost (days after SCT)	Graft	Conditioning Regimen	GVHD Prophylaxis	Immunosuppression at T-Cell Transfer	GVHD Status Prior to T-Cell Transfer	GVHD Status After T-Cell Transfer
1	Secondary AML (history of mamma carcinoma); incomplete engraftment	72	–	MMUD (9 of 10 matched)	Total body irradiation, cyclophosphamide, antithymocyte globulin	Mycophenolate mofetil, cyclosporin, methotrexate	Prednisone	–	–
2	T-ALL relapse, MAS with cerebral HLH	151	–	MMUD (9 of 10 matched)	Total body irradiation, etopside, antithymocyte globulin	Cyclosporin, methotrexate	Prednisone	Grade 1 skin GVHD	Grade 1 skin GVHD
3	SAA, incomplete engraftment	362 and 413	358	Haplo (6 of 10 matched)	Fludarabine, thiotepa, melphalan, muronomab	Cyclosporin, mycophenolate mofetil, prednisone	–	–	–
4	HLH	224	–	MSD	Busulfan, etopside, cyclophosphamide	Mycophenolate mofetil, cyclosporin, methotrexate	–	–	–
5	AML relapse	97 and 151	–	Haplo (second graft)	Clofarabin, thiotepa, melphalan, muronomab	—	Prednisone, tacrolimus	–	Grade 1 skin GVHD 15 days after first T-cell transfer; no GVHD after second T-cell transfer
6	SAA, incomplete engraftment	80	79 and 100 ($CD3^+$ boost)	MUD	Fludarabine, cyclophosphamide, antithymocyte globulin	Cyclosporin, methotrexate	–	–	Grade 1 skin GVHD 2 weeks after T-cell boost and 5 weeks after T-cell transfer
7	Grade 4 neuroblastoma relapse, MAS with HLH	188	175	Haplo	Fludarabine, thiotepa, melphalan, muronomab	Mycophenolate mofetil	–	–	–
8	T-ALL relapse, incomplete engraftment	114 and 121	–	MUD	Total body irradiation, cyclophosphamide, antithymocyte globulin	Cyclosporin, mycophenolate mofetil, prednisone	Hydrocortisone	Grade 2 gut GVHD, grade 3 liver GVHD	Grade 4 gut GVHD, grade 4 liver GVHD (due to discontinuation of immunosuppression)
9	AML	59	–	MUD	Busulfan, etopside, cyclophosphamide, antithymocyte globulin	Cyclosporin, mycophenolate mofetil	Rapamycin, tacrolimus	Grade 1 skin GVHD	No GVHD 28 days after T-cell transfer; grade 3 to 4 gut GVHD 8 weeks after T-cell transfer
10	AML	131	–	MMUD (9 of 10 matched)	Total body irradiation, cyclophosphamide, antithymocyte globulin	Cyclosporin, methotrexate	Prednisone	Grade 2 gut GVHD	–

Abbreviations: AML, acute myeloid leukemia; EBNA-1, Epstein-Barr virus nuclear antigen 1; GVHD, graft-versus-host disease; Haplo, haploidentical stem-cell transplantation from parents or siblings; HLH, hemophagocytic lymphohistiocytosis; MAS, macrophage activation syndrome; MMUD, mismatched unrelated donor; MSD, matched sibling donor; MUD, matched unrelated donor; SAA, severe aplastic anemia; SCT, stem-cell transplantation; T-ALL, T-cell acute lymphoblastic leukemia.

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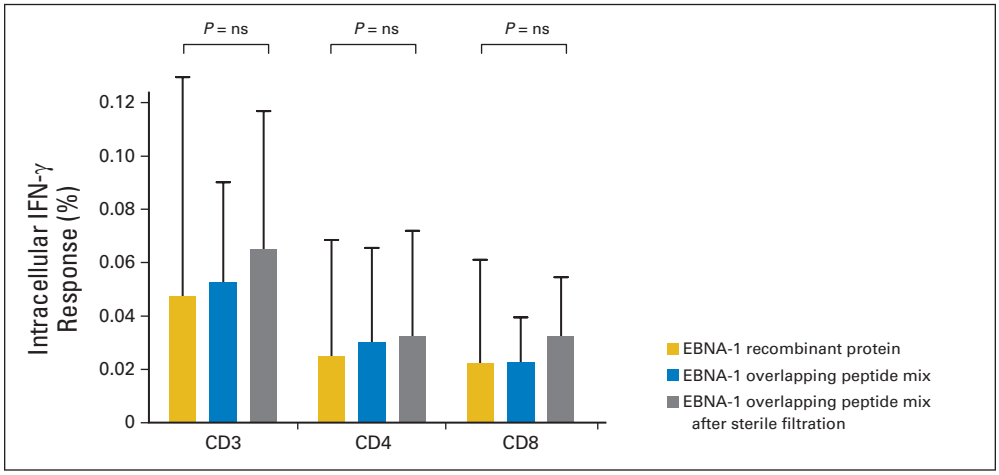

Fig A1. T-cell responses against Epstein-Barr virus nuclear antigen 1 (EBNA-1) recombinant protein and EBNA-1 overlapping peptides in four healthy seropositive donors. Mean frequency of antigen-specific T cells against the EBNA-1 protein compared with the EBNA-1 overlapping peptide mix before and after sterile filtration. There was no significant difference in frequencies of $CD4^+$ and $CD8^+$ antigen-specific T lymphocytes between recombinant protein and peptide mix. IFN-γ, interferon gamma; ns, not significant.

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