



Epstein-Barr virus-specific cytokine-induced killer cells for treatment of Epstein-Barr virus-related malignant lymphoma

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Abstract

Background. Prolonged immunosuppression or delayed T-cell recovery may favor Epstein-Barr virus (EBV) infection or reactivation after allogeneic hematopoietic stem cell transplantation (HSCT), which can lead to post-transplant lymphoproliferative disease (PTLD) and high-grade malignant B-cell lymphoma. Cytokine-induced killer (CIK) cells with dual specific anti-tumor and virus-specific cellular immunity may be applied in this context. **Methods.** CIK cells with EBV-specificity were generated from peripheral blood mononuclear cells (PBMCs), expanded in the presence of interferon- γ , anti-CD3, interleukin (IL)-2 and IL-15 and were pulsed twice with EBV consensus peptide pool. CIK cells with EBV-specificity and conventional CIK cells were phenotypically and functionally analyzed. Additionally, CIK cells with EBV-specificity were applied to a patient with EBV-related PTLD rapidly progressing to highly aggressive B-cell lymphoma on a compassionate use basis after approval and agreement by the regulatory authorities. **Results.** Pre-clinical analysis showed that generation of CIK cells with EBV-specificity was feasible. *In vitro* cytotoxicity analyses showed increased lysis of EBV-positive target cells, enhanced proliferative capacity and increased secretion of cytolytic and proinflammatory cytokines in the presence of EBV peptide-displaying target cells. In addition, 1 week after infusion of CIK cells with EBV-specificity, the patient's highly aggressive B-cell lymphoma persistently disappeared. CIK cells with EBV-specificity remained detectable for up to 32 days after infusion and infusion did not result in acute toxicity. **Discussion.** The transfer of both anti-cancer potential and T-cell memory against EBV infection provided by EBV peptide-induced CIK cells might be considered a therapy for EBV-related PTLD.

Key Words: cytokine-induced killer cells, cytotoxic T cells, Epstein-Barr virus, immunotherapy, lymphoma, post-transplantation lymphoproliferative disease

Introduction

Epstein-Barr virus (EBV)-induced post-transplantation lymphoproliferative disease (PTLD) is a potentially life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT) [1]. The incidence of PTLD in stem cell transplant recipients is generally low, but the risk for development of PTLD increases due to compromised immune surveillance in the early post-transplantation period [2–6]. The disease sites, degree of tumorigenesis and therapeutic responses affect the course of the disease and

outcomes [7]. In most cases, PTLD is associated with EBV-driven abnormal lymphoid proliferation, either related to reactivation of the virus or to primary EBV infection after transplantation, which in immunocompetent individuals is controlled by cytotoxic T lymphocytes [8]. The presentation of PTLD varies from benign polyclonal B-cell hyperplasia to low- and high-grade malignant monoclonal B-cell lymphomas, which can progress to fulminant disease. A number of risk factors for PTLD development have been identified, including prolonged immunosuppression, delayed T-cell recovery, such as after profound T-cell depletion of the

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(Received 11 January 2018; accepted 22 April 2018)

graft or the use of anti-lymphocyte antibodies as part of the conditioning regimen, treatment or prophylaxis of graft-versus-host disease (GVHD), recipient-donor human leukocyte antigen (HLA) disparity and recipient-donor EBV-seromismatches [3].

Treatment options reflect the spectrum of clinical appearances and comprise anti-viral therapy [9], tapering of immunosuppression, local treatment (surgery or radiation), cytokines, chemotherapy, anti-B-cell antibodies such as rituximab [10–12] or donor-derived cellular immunotherapies, including unselected donor lymphocyte infusions (DLIs) or EBV-specific cytotoxic T cells (CTLs) [13]. The tapering of immunosuppression and rituximab treatment are effective when applied at an early stage of PTLD [9]. In more advanced disease, a diagnosis should be established for re-assessment and treatment planning, including local and systemic treatment [14]. Resistance to conventional anti-viral drugs and drug-induced (multi-) organ toxicity have increased the importance of application of virus-specific immune cells. Moreover, especially in the case of EBV-related PTLD, immunotherapy approaches with both anti-tumor and anti-infectious potential may be considered “more advanced”.

Especially in the HSCT setting, immune responses are important in the treatment of EBV-related PTLD. In addition to cessation of immunosuppressive agents, unselected DLIs and EBV-specific CTLs can provide additional and preservative immune responses [11,13,15,16]. Adoptive CTL therapy was also effectively applied in more advanced diseases, such as EBV-associated lymphomas after HSCT [17]. Various methods for the expansion or selection of virus-specific T lymphocytes for clinical use have been described [18].

Cytokine-induced killer (CIK) cells represent a novel immunotherapy because they target both virus-infected and transformed cells by T-cell receptor (TCR)-restricted and natural killer (NK) cell-like mechanisms. CIK cells are expanded from peripheral blood, bone marrow or cord blood mononuclear cells in the presence of interferon (IFN)- γ , anti-CD3 antibody, interleukin (IL)-2 and IL-15. CIK cells represent a heterogeneous, predominantly polyclonal T-cell population consisting of non-classical CD3⁺CD56⁻ T cells, CD3⁺CD56⁺ T-NK cells and a minor fraction of CD56⁺CD3⁻ NK cells. CIK cell-mediated cytotoxic activity is of both specific major histocompatibility complex (MHC)-restricted recognition and non-MHC-restricted, TCR potential [19–21]. Despite the high content of T cells, CIK cells display low alloreactivity and, therefore, pose a limited risk of inducing GVHD even in a MHC-mismatched transplantation setting. Phase 1/2 clinical trials yielded encouraging therapeutic effects against hematologic

malignancies and demonstrated the safety of CIK cell treatments even in haploidentical transplantation settings [22–25]. Recently, the possibility to generate dual-specific CIK cells, including both anti-leukemic and anti-viral activity, was described in pre-clinical analyses [20,26].

In this study, peripheral blood mononuclear cells (PBMCs) from EBV-seropositive donors were expanded in CIK cytokine cocktails with concurrent EBV-antigen pulsing to specifically expand EBV-reactive T cells among conventional CIK cells. This cell product was carefully characterized and further analyzed regarding its dual anti-malignant and anti-viral activity. Furthermore, a Good Manufacturing Practice (GMP)-compliant clinical-scale protocol was established, pre-clinically analyzed and finally administered on a compassionate use basis to a 17-year-old female patient *in extremis* for the treatment of EBV-associated PTLD, presented as an aggressive diffuse large B-cell lymphoma (DLBCL) during delayed T-cell recovery after an allogeneic HSCT for secondary myelodysplastic syndrome (MDS). To our knowledge, this is the first reported CIK cell treatment with augmented anti-EBV potential that was successfully applied for treatment of EBV-related high-grade malignant lymphoma after HSCT.

Material and methods

Patient characteristics

A 17-year-old female patient with secondary MDS after acute myeloid leukemia underwent an allogeneic HSCT from a matched unrelated donor. The conditioning regime included anti-thymocyte globulin (ATG). The patient was EBV-seronegative before transplantation. Sixty days after transplantation, she developed an infectious mononucleosis-like illness with cervical lymphadenopathy while being treated with cyclosporine A. T-cell recovery had not occurred. EBV viral load was detected in the peripheral blood. Due to the aggressive presentation, immune suppression was discontinued, and antibody therapy with rituximab was started. However, despite four cycles of rituximab treatment, progressive disease was rapidly observed. Histopathological examination of the cervical mass confirmed the presence of an EBV-positive DLBCL with low-level EBV viremia in the peripheral blood. Polychemotherapy was not possible because of weak hematopoiesis shortly after allogeneic transplantation. After a thorough discussion with the patient and her legal representatives, the decision was made to attempt CIK cell treatment with additional EBV-specificity because conventional donor-derived EBV-specific T cells ($0.1 \times 10^3/\text{kg}$) and conventional CIK cell treatment (T cells, $5 \times 10^6/\text{kg}$) did not show immediate responses. Therefore, the patient received CIK

cells with EBV-specificity (T cells, $10 \times 10^6/\text{kg}$; EBV-specific T cells, $9.57 \times 10^3/\text{kg}$) on a compassionate use basis after written informed consent was provided (Figure 4). Lymphocytes and T cells were $301/\mu\text{L}$ and $24/\mu\text{L}$, $263/\mu\text{L}$ and $23/\mu\text{L}$ and $255/\mu\text{L}$ and $41/\mu\text{L}$ at the time of conventional EBV-specific T cell, conventional CIK cell and CIK cell with EBV-specificity infusion, respectively. Therefore, we did not perform lymphodepletion chemotherapy prior to the respective infusions.

Functional analyses included cytokine secretion patterns in the serum of the patient as well as phenotypic and functional characterization of CIK cells with and without EBV-specificity *in vitro* and *in vivo*.

Generation of CIK cells with EBV-specificity

Donor PBMCs were obtained after written informed consent was given, with approval from the Ethical Review Board of the Medical Faculty of the University Hospital Frankfurt, Frankfurt/Main, Germany (Geschäfts No. 69/13). For *in vitro* experiments, conventional CIK cells and CIK cells with EBV-specificity were generated according to the protocol described previously [21]. In brief, CIK cells with EBV-specificity were generated from PBMCs and subsequently incubated with IFN- γ on day 0, and a monoclonal antibody against CD3 on day 1. IFN- γ was used for activation of monocytes offering antigen cross-presentation to T cells, while the monoclonal antibody against CD3 provided mitogenic signals for T lymphocytes. Further cell proliferation, survival and cytolytic effector function was achieved by stimulation with IL-2 on day 1 and sequential addition of IL-15 on days 4 and 8. In this study, $1 \mu\text{g}/\text{mL}$ of EBV-consensus peptide pool (content of EBV-proteins from latency or lytic replication of EBV: latent cycle EBNA1, BERF1 [EBNA3], BERF2 [EBNA4], BERF3 [EBNA6], LMP2a, BNLF1 [LMP1]; lytic cycle BRLF1, BMLF1 [EB2], BALF2 [DNBI], BMRF1, BZLF1, BNRF1 [MTP], BLLF1 [gp350]; Miltenyi Biotec) was added on days 0 and 2 for priming and expansion of T cells with EBV-specificity among CIK cells. Cells were harvested on days 10–15 of culture and immediately used for further analyses.

Clinical-scale generation of CIK cells with EBV-specificity

For generation of GMP-scale CIK cells with EBV-specificity, peripheral mononuclear cells from an unstimulated leukapheresis were collected from the original EBV-seropositive stem cell donor after written informed consent was provided. CIK cells were generated according to GMP conditions as described previously [22,27]. For specific enhancement of EBV-reactivity, cells were primed twice with $1 \mu\text{g}/\text{mL}$ of

human MACS GMP EBV-select peptide pool (Miltenyi Biotec) on days 0 and 2 of culture. Cells were cultured in VueLife culture bags (Medical Action Industries), harvested on day 10 of culture and infused immediately.

Phenotypic characterization of CIK cell products (Conventional and EBV-CIKs)

Cells were harvested and phenotypically characterized by 10-color flow cytometry (Navios, Beckman Coulter) using the following antibodies: anti-CD45 (J.33), anti-CD14 (RMO52), anti-CD3 (UCHT-1), anti-CD56 (N901), anti-CD19 (J3-119), anti-CD16 (3G8), anti-CD4 (13B8.2), anti-CD8 (B9.11), anti-CD25 (B1.49.9), anti-CD314 (ON72), anti- $\alpha\beta$ -TCR (BW242/412), anti- $\gamma\delta$ -TCR (IMMU510), anti-CD45RO (UCHL1), anti-CD45RA (2H4) and anti-CD62L (DREG56) (Beckman Coulter; except anti- $\alpha\beta$ -TCR, Miltenyi Biotec). For the assessment of viability, 7-aminoactinomycin D (Beckman Coulter) was used, and measurements were performed using a single-platform approach. EBV-specific lymphocytes were identified by staining with various MHC-I and MHC-II antibodies using multimer technology: LMP2A (A*02:01), EBNA1 (B*35:01), EBNA3A (B*07:02 and B*08:01), BMLF1 (A*02:01; Immudex) and EBNA1 (DRB1*04:01; ProImmune).

Immune monitoring

Monitoring EBV-specific CD4⁺ and CD8⁺ T cells in the peripheral blood was conducted by flow cytometry using EBV-MHC multimers. Blood samples were taken before as well as 7, 12, 15, 19, 26 and 32 days after infusion of CIK cells with EBV-specificity. Samples were stained with EBV LMP2A-specific dextramer (A*02:01; Immudex) for detection of EBV-specific CD8⁺ T cells and EBV EBNA1-specific tetramer (DRB1*04:01; ProImmune) for identification of EBV-specific CD4⁺ T cells.

Cytotoxicity

The cytotoxic potential of CIK cells with or without EBV-specificity against leukemia cell lines K562 (EBNA1-negative chronic myeloid leukemia [CML]; ECACC) and THP-1 (EBV-negative acute monocytic leukemia [AML]-M5; ECACC) and EBV peptide-loaded BJAB cells (EBV-negative Burkitt lymphoma; DSMZ) was analyzed using europium release assays. For EBV-peptide-loading, 1×10^6 BJAB cells were incubated with $5 \mu\text{g}$ of EBV-consensus peptide pool (Miltenyi Biotec) and $5 \mu\text{g}$ β 2-microglobulin (Sigma-Aldrich) for 45 min at room temperature. Next, 1×10^6 target cells were labeled with BATDA reagent

(PerkinElmer) and subsequently 5×10^3 target cells were co-cultured with CIK cells with or without EBV-specificity in triplicates at effector to target ratios (E:T) of 40:1 (2×10^5 CIK cells), 20:1 (1×10^5 CIK cells) and 5:1 (2.5×10^4 CIK cells). After 3-h co-incubation at 37°C and 5% CO₂, destroyed cells were measured as counts per minute (cpm) in a time-resolved fluorimeter (Victor 3 1420; PerkinElmer). Percent specific lysis was calculated using the formula (experimental cpm—spontaneous cpm)/(maximum cpm—spontaneous cpm) \times 100, where maximum cpm was determined by adding 4% Triton X-100 (Sigma-Aldrich).

The cytotoxicity of GMP-grade CIK cells with EBV-specificity was analyzed against T2 cells (EBV-positive fusion of the B-lymphoblastic cell line [B-LCL] 721.174 with a variant of the T-lymphoblastic cell line CEM [CEMR.3]) (kindly provided by the group of Thomas Lehrnbecher, Department of Children and Adolescents, University Hospital, Frankfurt; Goethe University, Frankfurt/Main, Germany) that were additionally loaded with an EBV-select peptide pool (Miltenyi Biotec) as described above.

Proliferation and phenotypic characterization of effector cells

The proliferative capacity of CIK cells with or without EBV-specificity in the presence of irradiated target cells was assessed using carboxyfluorescein succinimidyl ester (CFSE)-based proliferation assays as described by Quah *et al.* [28]. In brief, CIK cells were labeled with 5 μ mol/L CFSE solution (Invitrogen) for 5 min. After the cells were washed three times with phosphate-buffered saline (PBS) supplemented with 5% fetal calf serum (FCS), they were re-suspended in culture medium. Target cell lines were irradiated with 30 Gy. CIK cells with or without EBV-specificity and irradiated target cells were co-incubated at an E:T ratio of 5:1 (0.2×10^5 CIK cells in 0.2 mL) and analyzed after 3 h (incubation period of europium release assay) and 1, 2 and 5 days. Analysis was performed using FlowJo (FlowJo LLC).

As negative controls, EBV-negative K562 and THP-1 cells were used, whereas EBV-positive T2 cells were considered positive controls.

In parallel, CIK cells were labeled with anti-CD3 (UCHT-1), anti-CD56 (NCAM16.2), anti-CD8 (RPA-T8) and anti-CD4 (RPA-T4; BD Biosciences) and subsequently characterized by 10-color flow cytometry (FACSCanto, BD Biosciences).

Statistics

Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software). Differences between

data sets were calculated using the two-tailed, paired Student *t* test. *P* values were considered significant for a value < 0.05 and are indicated as **P* < 0.05 , ***P* < 0.01 and ****P* < 0.005 . The results are shown as the mean value \pm standard error of the mean (in all Figures) and mean value \pm standard deviation (data in the Results section).

Results

Pre-clinical data

Generation and expansion of CIK cells with EBV-specificity

The fold expansion during the generation of CIK cells with EBV-specificity (EBV-CIK) and conventional CIK cells (conv. CIK) of 12 individual donors revealed high expansion rates up to 140. Expanded cells consisted of T cells, increasing amounts of T-NK cells co-expressing CD3 and CD56 and a minor fraction of NK cells. In both cultures, the majority of the T cells were identified as CD8⁺ cytotoxic T cells. CIK cells with EBV-specificity, which were generated among classical CIK cells, also revealed a CD3⁺CD8⁺ cytotoxic T-cell phenotype. Further analysis showed that 89% of the EBV-positive fraction contained a T cell and 11% a CD3⁺CD56⁺T-NK cell phenotype (*n* = 4). Furthermore, about 90% of EBV⁺ T cells and 87% of EBV⁺T-NK cells within the EBV CIK cell culture expressed NKG2D on their surface. A more detailed analysis of EBV⁺ CD3⁺CD8⁺ cells showed a 10-fold increase in the frequency of LMP2A (*n* = 4), a 7.7-fold expansion of EBNA1 (*n* = 3) and a 3.6-fold increase of BMLF1- (*n* = 5) and EBNA3A- (*n* = 5) specific cells during culture. Conventional CIK cells showed low frequencies of EBV-specific cells during culture (Table I).

In vitro cytotoxicity of CIK cells with EBV-specificity

The cytotoxic capacity of CIK cells with and without EBV-specificity was analyzed against K562, THP-1 and EBV-consensus peptide-loaded BJAB cells on day 15 of culture. Specific lysis of peptide-loaded BJAB cells was significantly increased by CIK cells with EBV-specificity compared with that of conventional CIK cells (E:T ratio 5:1, 42.8% \pm 14.4% [EBV-CIK] to 22.7% \pm 8.9% [conv. CIK]; *P* < 0.05 ; *n* = 7; Figure 1A). There was no difference against the EBV-negative cell lines K562 and THP-1. The cytotoxic potential of CIK cells with EBV-specificity against K562 cells was 54.6% \pm 18.8%, 32.2% \pm 10.1% and 8.2% \pm 5.2% at E:T ratios of 40:1, 20:1 and 5:1, respectively (*n* = 10). Against THP-1 cells, CIK cells with EBV-specificity showed a mean specific lysis of 59.2% \pm 6.2%, 42.8% \pm 13.8% and 10.4% \pm 8.4% at E:T ratios of 40:1, 20:1 and 5:1, respectively (*n* = 10; Figure 1A).

Table I. Phenotypic characterization of CIK cells with and without EBV-specificity.

		EBV-CIK cells	Conv. CIK cells
Total cells (n = 12) (expansion rate)	Day 0	11 ± 4 × 10 ⁶	11 ± 4 × 10 ⁶
	Day 15	1010 ± 398 × 10 ⁶ (94 ± 28) ^c	831 ± 326 × 10 ⁶ (78 ± 27) ^c
CD3 ⁺ CD56 ⁻ (n = 12)	Day 0	59.7 ± 6.9%	59.7 ± 6.9%
	Day 15	76.3 ± 6.2% ^c	75.6 ± 6.8% ^c
CD3 ⁺ CD56 ⁺ (n = 12)	Day 0	2.7 ± 2.2%	2.7 ± 2.2%
	Day 15	22.6 ± 6.4% ^c	23.2 ± 7.0% ^c
CD3 ⁺ CD8 ⁺ (n = 12)	Day 0	32.4 ± 7.9%	32.4 ± 7.9%
	Day 15	81.7 ± 9.6% ^c	80.9 ± 8.8% ^c
LMP2A (n = 4)	Day 0	0.0 ± 0.0%	0.0 ± 0.0%
	Day 15	0.3 ± 0.2% ns	0.0 ± 0.0% ns
EBNA1 (n = 3)	Day 0	0.4 ± 0.2%	0.4 ± 0.2%
	Day 15	3.2 ± 2.2% ns	0.1 ± 0.1% ns
BMLF1 (n = 5)	Day 0	1.6 ± 0.7%	1.6 ± 0.7%
	Day 15	5.7 ± 1.9% ^b	1.7 ± 0.7% ns
EBNA3A (n = 5)	Day 0	0.6 ± 0.6%	0.6 ± 0.6%
	Day 15	2.2 ± 0.7% ^a	0.5 ± 0.2% ns
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Day 15		EBV CIK cells	Conv. CIK cells
EBV ⁺ CD45 ⁺ (n = 4)	CD3 ⁺ CD56 ⁻	2.6 ± 2.2%	0.9 ± 0.9%
	NKG2D ⁺	88.9 ± 5.3%	86.5 ± 10.3%
	CD3 ⁺ CD8 ⁺	90.6 ± 7.8%	95.0 ± 5.3%
	CD3 ⁺ CD56 ⁺	94.6 ± 3.1%	91.4 ± 6.9%
	NKG2D ⁺	11.1 ± 5.3%	12.7 ± 8.9%
	CD3 ⁺ CD56 ⁺	87.3 ± 13.6	87.5 ± 17.7%
	NKG2D ⁺	0.01 ± 0.03%	0.0 ± 0.0%
	NKG2D ⁺	0.0 ± 0.0	0.0 ± 0.0%
	CD19 ⁺	0.0 ± 0.0	0.0 ± 0.0%
	EBV ⁻ CD45 ⁺ (n = 4)	CD3 ⁺ CD56 ⁻	97.1 ± 1.9%
NKG2D ⁺		73.0 ± 5.4%	75.4 ± 9.2%
CD3 ⁺ CD8 ⁺		74.6 ± 10.3%	77.2 ± 10.5%
CD3 ⁺ CD56 ⁺		76.6 ± 12.6%	79.0 ± 14.1%
NKG2D ⁺		26.0 ± 4.9%	23.5 ± 9.5%
CD3 ⁺ CD56 ⁺		85.4 ± 17.3%	87.6 ± 15.1%
NKG2D ⁺		0.5 ± 0.2%	0.5 ± 0.3%
NKG2D ⁺		97.5 ± 2.8%	96.2 ± 2.3%
CD19 ⁺		0.0 ± 0.0	0.0 ± 0.0

Differences between data sets were calculated using the two-tailed, paired Student *t* test.

ns, not significant.

^a*P* < 0.05.

^b*P* < 0.01.

^c*P* < 0.005.

NKG2D ligand expression on target cells

All target cells were screened for the NKG2D ligands MIC A/B and ULBP1 as the killing of CIK cells can also function through the NKG2D receptor. THP-1 and K562 cells were positive for both ligands, whereas T2 cells showed no cell surface expression of NKG2D ligands. BJAB cells were negative for MIC A/B but positive for ULBP1 (data not shown).

Proliferation capacity of CIK cells with and without EBV-specificity in vitro

The proliferation capacity of CIK cells with and without EBV-specificity was analyzed in the pres-

ence of the irradiated EBV-negative target cell lines K562 and THP-1. Conventional and CIK cells with EBV-specificity both started to proliferate after a total of 5 days. In the presence of the EBV-positive target cell line T2, CIK cells with EBV-specificity revealed a proliferation rate of 2 in the CD3⁺ compartment, including the cytotoxic and T-NK cell subset (2.25 ± 0.58; n = 4; Figure 2).

Clinical data

Generation and expansion of clinical-scale CIK cells with EBV-specificity

Clinical-scale CIK cells with EBV-specificity were generated from 150.0 × 10⁶ PBMCs of the original EBV-seropositive stem cell donor. After 10 days, cells had expanded 10.7-fold, yielding a total of 1601.2 × 10⁶ cells. No EBV-specific T cells were found among PBMCs at the beginning of culture. After peptide stimulation, the frequency of EBV-specific T cells increased to 0.1% and 0.3% among the CD8⁺ and the CD4⁺ subsets, respectively (Figure 3). The applied cell dose contained a total of 10.0 × 10⁶ T cells/kg, including 9.57 × 10³/kg EBV-specific CD8⁺ T cells and 8.4 × 10³/kg EBV-specific CD4⁺ T cells. (The conventional EBV-specific T-cell treatment applied prior to the infusion of CIK cells with EBV-specificity contained 180-times lower numbers of EBV-specific T cells [0.1 × 10³/kg]).

In vitro cytotoxicity of clinical-scale CIK cells with EBV-specificity

The cytotoxic potential of clinical-scale CIK cells with EBV-specificity was tested against the leukemia cell line K562 and EBV-select peptide-loaded T2 cells using the europium release assay.

Specific lysis of EBV-peptide-loaded T2 cells was two-fold higher compared with that of wildtype T2 at an E:T ratio of 20:1 (72.33% ± 3.7% to 35% ± 3.6%; *P* < 0.05; n = 3). This effect was also observed at a higher E:T ratio of 40:1 (94% ± 5.5% to 54.6% ± 3.7%; *P* < 0.05; n = 3; Figure 1B). Specific lysis of EBV-negative K562 cells was retained when using CIK cells with EBV-specificity (82.3% ± 2.3%, 60.6% ± 6% and 22.3% ± 4.1% at E:T ratios of 40:1, 20:1 and 5:1, respectively; Figure 1B).

Clinical response after CIK cell treatment with EBV-specificity

Clinical-scale CIK cells with EBV-specificity were tested in a patient who presented with fulminating symptoms including high fever (>39°C), increasing lymphadenopathy including cervical lymph nodes and lymphatic tissue of the Waldeyer's tonsillar ring, loss of appetite, significant unintentional weight loss and

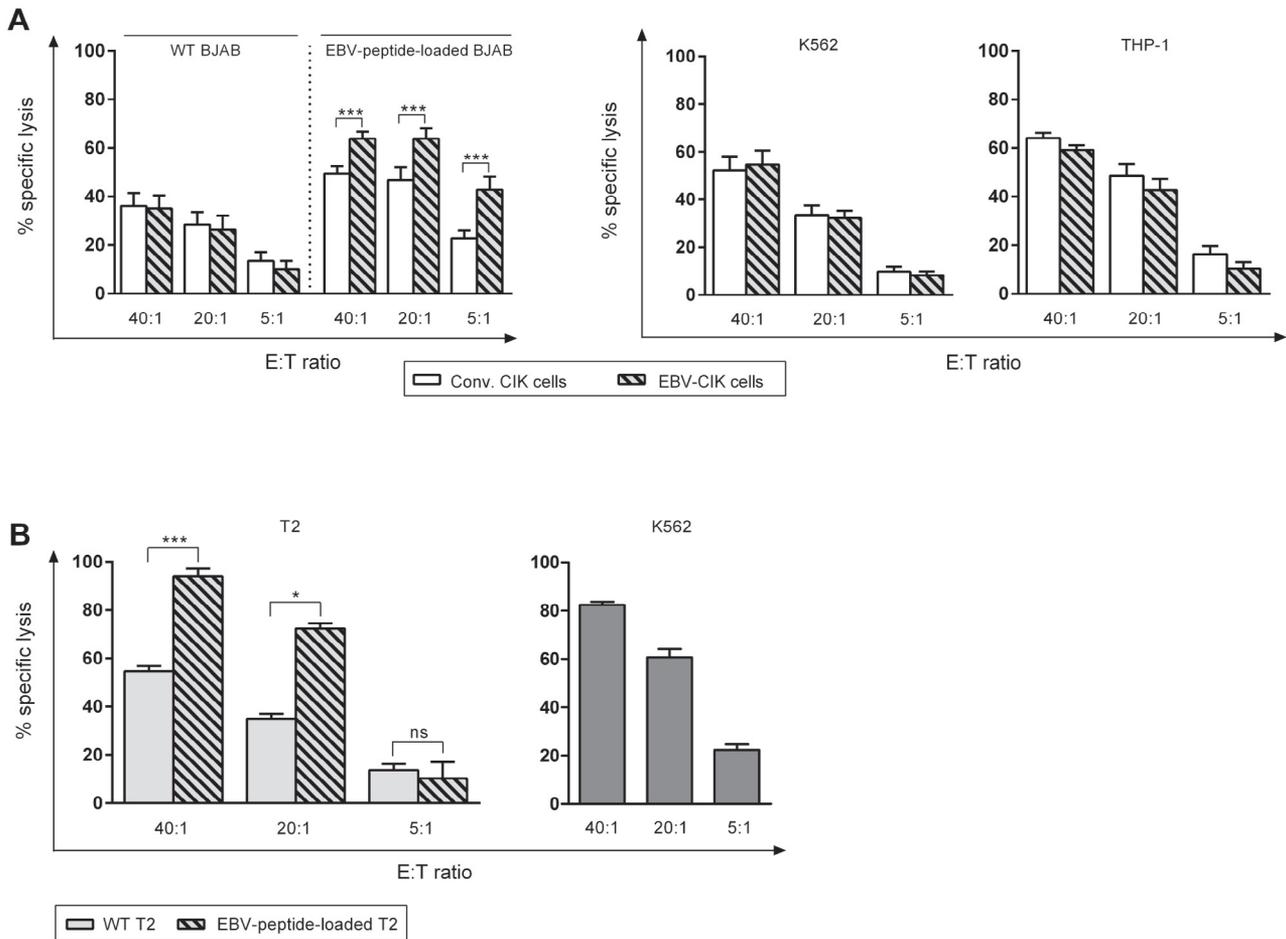


Figure 1. Cytotoxic potential of CIK cells with or without EBV-specificity. (A) Specific lysis was assessed using the europium release assay. Left figure: specific lysis of Wildtype and EBV-peptide-loaded BJAB cells by conventional (white bars) and CIK cells with EBV-specificity (hatched bars) at different E:T ratios ($n = 7$; $***P < 0.005$). Killing of peptide-loaded BJAB cells was significantly higher with CIK cells with EBV-specificity compared with that of conventional CIK cells. Right figure: K562 and THP-1 cells were both lysed to the same amount by CIK cells with EBV-specificity and conventional CIK cells ($n = 10$). (B) Left figure: specific lysis of wildtype T2 (WT, light gray bars) and EBV-peptide-loaded T2 (hatched bars) cells by clinical-scale CIK cells with EBV-specificity at the indicated E:T ratios ($n = 3$; $***P < 0.005$; $*P < 0.05$). Lysis of EBV-peptide-loaded T2 cells was significantly higher compared with WT T2 targets when using CIK cells with EBV-specificity. Right figure: $> 80\%$ of K562 cells were lysed by CIK cells with EBV-specificity at an E:T ratio of 40:1, and killing decreased with lower E:T ratios ($n = 3$). Data were analyzed using the two-tailed paired Student t test.

pronounced fatigue. Several courses of antibiotics had no effect on any of these symptoms. At day +40 after HSCT our patient showed a significant increase in B cells. We did not analyze them in detail but expected them to be EBV + lymphoproliferative B cells because EBV DNA became detectable in the peripheral blood and EBV-positive B cells were seen in a subsequent lymph node resection. Further staging included ultrasound of the neck and the abdomen, as well as x-ray of the chest, and confirmed localized lymphadenopathy. Rituximab treatment was given pre-emptively and immunosuppression was stopped, but both failed, despite clearance of B cells in the peripheral blood following four courses of rituximab treatment. Because of rapidly growing cervical lymphadenopathy, we conducted a resection of one of the patient's cervical lymph

nodes on day +70 after HSCT. Histopathology of the resected lymph node confirmed DLBCL, showing numbers of EBV-positive cells besides CD30-, CD79a-, PAX5-, MUM1- and Bcl2-positive infiltrating B cells with a proliferative index of 100%. Of note, the patient's lymphoma was negative for CD20 and, therefore, refractory to rituximab treatment (Figure 5). Lacking an initial biopsy, the patient may have also had monomorphic EBV+ PTLD presented as a DLBCL refractory to rituximab treatment. However, due to the poor clinical condition of the patient still lacking T-cell recovery after allogeneic HSCT, we considered that this patient was *in extremis*. Therefore, the patient was provided with different T-cell therapies until disease progression was stopped. EBV-specific CTL therapy from the seropositive stem cell donor was

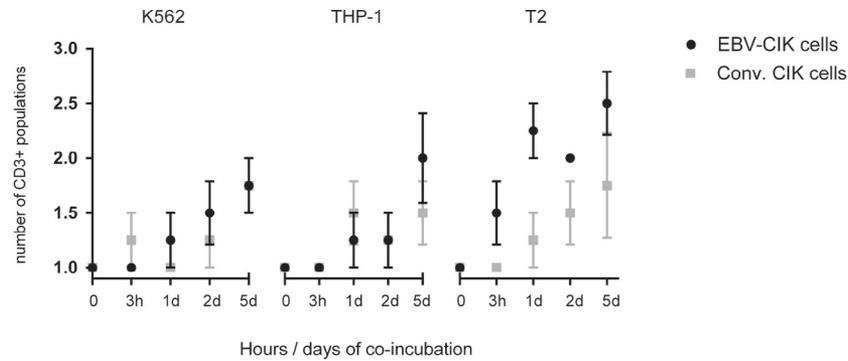


Figure 2. Proliferation capacity. CFSE-labeled CIK cells with and without EBV-specificity were co-incubated with irradiated EBV-negative target cell lines K562 and THP-1. The proliferative capacity of conventional CIK cells and CIK cells with EBV-specificity against leukemia cells was not statistically different ($n = 4$). In contrast, the EBV-specific proliferative capacity against EBV-positive T2 cells was enhanced and accelerated in CIK cells with EBV-specificity versus conventional CIK cells ($n = 4$).

considered at first. On day +70 after HSCT, a total of $0.1 \times 10^3/\text{kg}$ EBV-specific T cells were isolated in a conventional manner using the Cytokine Capture System and provided to the patient. But still, the lymphoma progressed. T-cell recovery was still lacking. Therefore, on day +80 unselected conventional CIK cell treatment was applied to augment T-cell recovery, but there was still no immediate clinical response. Due to this circumstance, we for the first time applied clinical-scale CIK cells with EBV-specificity on a compassionate use basis (day +90; Figure 4). With our protocol, we obtained $9.57 \times 10^3/\text{kg}$ CD8⁺ and $8.4 \times 10^3/\text{kg}$ CD4⁺ T-EBV-specific CTLs among $10 \times 10^6/\text{kg}$ CD3⁺ CIK cells, a 180-fold higher total number of EBV-specific T cells compared with the conventional EBV-specific CTL infusion previously offered to this patient. Lymphocytes and T cells were 301/ μL and 24/ μL , 263/ μL and 23/ μL and 255/ μL and 41/ μL in the peripheral blood of the patient at the time of EBV-specific T cell, conventional CIK cell and EBV-

enriched CIK cell infusion, respectively. Lymphopenia may have facilitated T-cell recovery and expansion, as reported for *in vivo* lymphodepletion chemotherapy, which is given with the aim to enhance immune cell expansion in several adoptive immunotherapy protocols. Therefore, we did not perform lymphodepletion chemotherapy prior to the respective infusions. CIK cell treatment with EBV-specificity resulted in long-term clearance of plasma EBV DNA and rapid and sustained disappearance of large DLBCL nodes within 7 days. No cytokine release syndrome (CRS) or acute infusion-related toxicities appeared after CIK cell treatment with EBV-specificity, confirmed by the absence of cytokines like IL-8, IL-2, IL-6 and IFN- γ (data not shown). T cells recovered, and, 2.5 years later, the patient is still in sustained remission. However, the patient developed *de novo* sclerotic-type chronic GVHD (cGVHD) of the skin 6 months after immune cell infusions. A correlation with the CIK cell treatment cannot be completely excluded. However, up to date

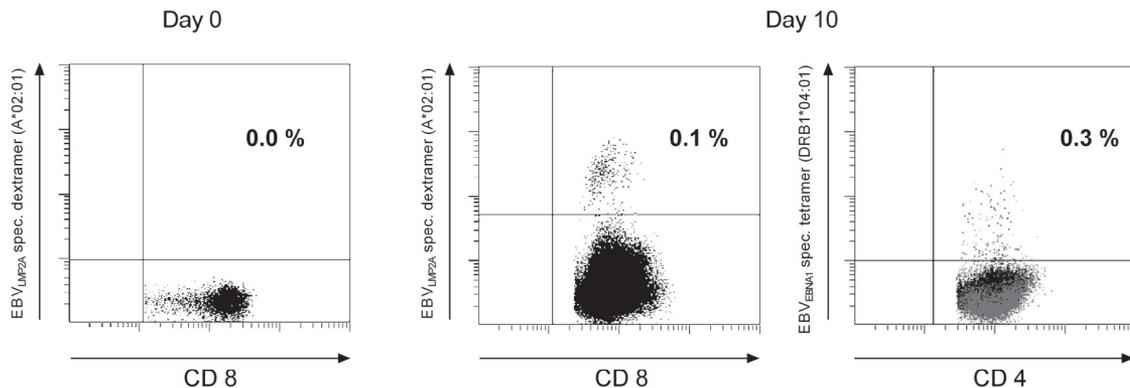


Figure 3. CIK cells with EBV-specificity. Dot plots of EBV-specific multimer staining (for CD3⁺CD8⁺/CD4⁺ T cells) on day 0 and day 10 of clinical-scale CIK cells with EBV-specificity manufactured under GMP conditions. CD3⁺CD8⁺ T cells were stained with an EBV LMP2A-specific dextramer (A*02:01) and CD3⁺CD4⁺ with an EBV EBNA1-specific tetramer (DRB1*04:01). The applied cell dose contained a total of 10.0×10^6 T cells/kg, including $9.57 \times 10^3/\text{kg}$ EBV-specific CD8⁺ T cells and $8.4 \times 10^3/\text{kg}$ EBV-specific CD4⁺ T cells.

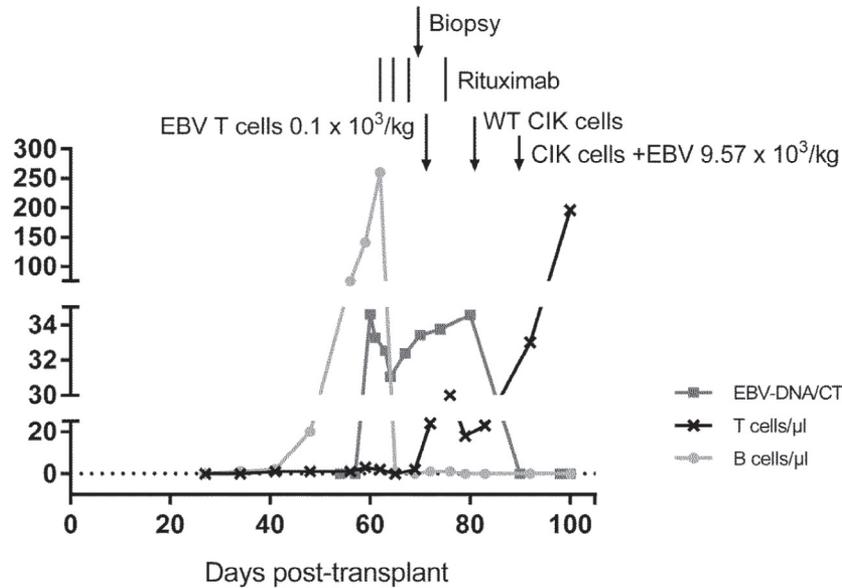


Figure 4. Treatment schedule. Monomorphic EBV⁺ PTLD presented as a DLBCL was observed during delayed T-cell recovery 2 months after HSCT. The patient received 4-times rituximab treatment (first infusion 60 days post-HSCT), EBV-specific T cells ($0.1 \times 10^3/\text{kg}$; 70 days post-HSCT), conventional CIK cells (T cells, $5.0 \times 10^6/\text{kg}$; 80 days post-HSCT) and CIK cells with EBV-specificity (T cells, $10.0 \times 10^6/\text{kg}$, including $9.57 \times 10^3/\text{kg}$ EBV-specific CD8⁺ T cells; 90 days post-HSCT). Treatment led to T-cell recovery, clearance of EBV viremia and clearance of DLBCL.

cGVHD has improved dramatically, only requiring low-dose immunosuppressive medication with cyclosporine and Methotrexat.

Detection of EBV-specific T cells and immune monitoring

Prior to CIK cell infusion with EBV-specificity, no EBV-specific cells were detectable in the peripheral blood of the patient. The frequency of EBV EBNA1-specific CD4⁺ T cells (5.5% of CD4⁺ T cells) peaked 7 days after infusion. Thereafter, the frequency of EBV-specific CD4⁺ T cells decreased but remained detectable until 32 days after infusion (1.2% of CD4⁺ T cells). In contrast, EBV LMP2A-specific CD8⁺ T cells were measurable only once, 15 days after infusion (4.1% of CD8⁺ T cells; Figure 6). Meanwhile the patient was cured from high-risk lymphoma. Therefore, *in vivo* immune monitoring of EBV-specific cells was not continued.

Discussion

In the present study, we describe the first successful generation of clinical-scale CIK cells with EBV-specificity for the treatment of a patient with EBV-related PTLD that presented as DLBCL early after allogeneic HLA-matched unrelated donor HSCT for secondary MDS.

In previous work, we used IL-15 instead of IL-2 for *in vitro* stimulation, showing that the heterogeneous CIK cell population can be generated more

rapidly and apparently with stronger cytotoxicity than the previously described IL-2-expanded CIK cells, despite lower CD56 expression [21]. Using immunomagnetic selection to both enhance cytotoxic activity and reduce alloreactive potential, previous data showed that cytotoxic potential of IL-15-activated CIK cells was negatively impacted by selection of CIK cell subpopulations [29]. Moreover, CIK cell manipulation is time-consuming and costly, and requires high numbers of PBMCs or CIK cells as starting material, which clearly limits clinical applicability, especially when considering sequential infusions of cells in rapidly progressing cancers. In addition, pre-clinical *in vivo* as well as clinical studies underlined the importance of originating T-cell progenitors among CIK cells for sustained cytotoxicity [20,22,27,30]. For priming and expansion of T cells with EBV-specificity among classical CIK cells, EBV peptide pool was added on days 0 and 2 of culture. With our newly established protocol, sufficient numbers of both anti-tumor and anti-EBV effector cells were generated rapidly and easily without laborious or expensive manipulation processes. This enabled the direct translation into the clinic.

Leen *et al.* generated their multi-virus-specific CTLs in previous work with the use of viral vectors (AdV) and live viruses (EBV) instead of viral peptides. Their manufacturing process was more complex and took more than 2 weeks [31]. Likewise, Heslop *et al.* [32] and Bollard *et al.* [33], who performed pioneering work

in the field of antiviral T cells, both used irradiated LCLs for stimulation of virus-specific CTLs with a main focus on cytotoxic T cells. Focusing more on infectious indications than on tumor toxicity, they did not report on the expression of CD56 or activating NK cell receptors like NKG2D or on the anti-tumor capacity in more detail.

Previous pre-clinical data of CIK cells with cytomegalovirus-specificity confirmed the NK-like anti-tumor activity provided by the activating NK cell receptor NKG2D and the acquired TCR-mediated anti-viral cytotoxicity [26]. Our actual study showed that generation of CIK cells with EBV-specificity was feasible by EBV-peptide stimulation performed twice

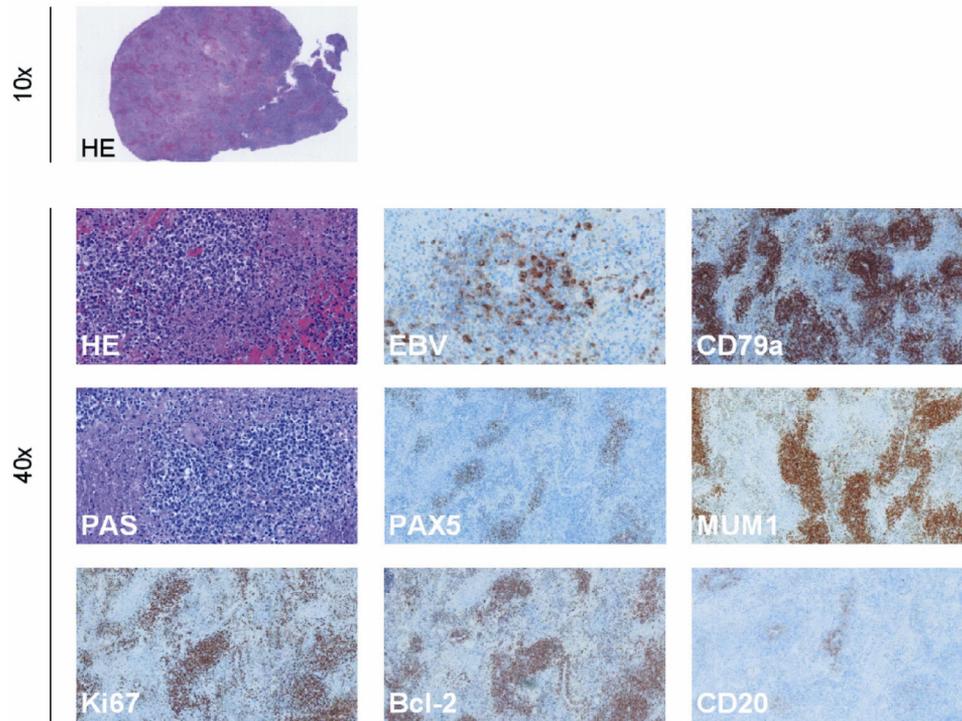


Figure 5. Pathological-anatomic assessment of the patient's lymph node. Microscopic analysis revealed necrobiotic modifications. The biopsy showed atypical populations with hyperchromatic, pleomorphic nuclei and mitoses. Immunohistochemical analyses were positive for EBV, Ki67 (proliferation rate up to 100%), PAX5, Bcl-2, CD79a and MUM1. Of therapeutic relevance, the material was negative for CD20. Investigation revealed a monomorphic PTLD with evidence of EBV. Morphology presented a diffuse large B cell Non-Hodgkin lymphoma with regressive-necrobiotic implications.

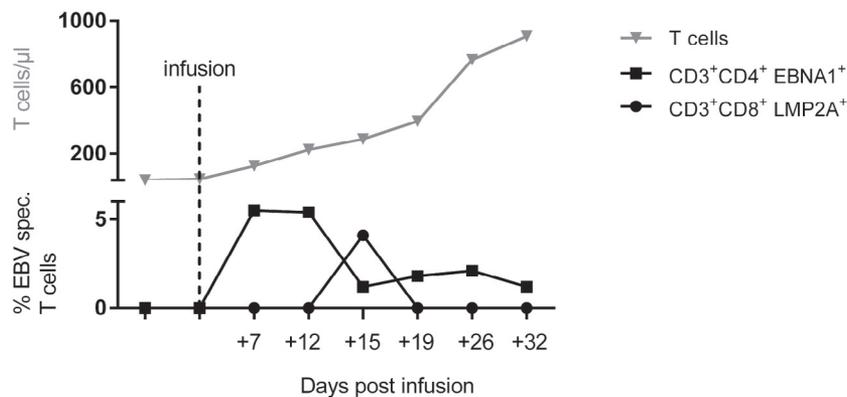


Figure 6. Peripheral blood monitoring. CIK cells with EBV-specificity were infused on day 90 after HSCT (dotted line). EBV-specific CD8⁺ (circled marks)/CD4⁺ (squared marks) T cells were monitored in the peripheral blood of the patient during clearance of lymphoma. Data prior to and up to 32 days after CIK cell infusion with EBV-specificity are given at the indicated time points. Specific cells were detected via flow cytometry (multimer technology). Numbers of absolute T cells/μL in peripheral blood are indicated by the gray line (triangular marks).

during culture of conventional CIK cells. *In vitro* cytotoxicity showed significantly increased lysis of EBV-positive target cells and an increased proliferative capacity of CD8⁺ T cells and T-NK cells, which are suggested to be the major effectors against EBV-positive target cells. In addition, NK-like cytotoxicity was retained against EBV-negative malignoma, such as THP-1 and K562 cells.

Based on the pre-clinical observations, we attempted for the first time to generate and apply clinical-scale CIK cells with EBV-specificity on a compassionate use basis after written informed consent for a patient who we would otherwise have lost. The patient's highly aggressive, EBV-positive DLBCL disappeared within 1 week after infusion. Because the patient received numerous therapies within a very short timeframe, it is difficult to say which therapy contributed most to the resolution of disease. Probably the combination of all therapies led to this result. Furthermore, it has to be mentioned that the increase of the patient's lymph node after EBV-specific CTL therapy should not be equated with treatment failure, as EBV-specific T cells can home to sites of disease and cause inflammation at that area [34]. Secondly, the fact that we could not measure EBV-specific cells in the patient's blood prior to infusion of CIK cells with EBV-specificity is no proof for their absence because they may have rather gathered at the sites of disease [35]. After clearance of low levels EBV viremia, EBV-specific CD8⁺ T cells were not persistently detected while EBV-specific CD4⁺ T cells remained detectable for a total of 32 days in the peripheral blood. Meanwhile the patient was cured from high-risk lymphoma. Therefore, *in vivo* immune monitoring of EBV-reactive cells was not continued routinely.

In vitro cytotoxicity analysis of clinical-scale CIK cells with EBV-specificity and CIK cells with EBV-specificity in general showed a significant increase in cytotoxic activity against EBV-positive targets compared with that of conventional CIK cells. Furthermore, killing of the EBV-negative leukemic cell line K562 was sustained by this novel treatment. However, culture conditions during *in vitro* cytotoxicity analysis are not completely comparable to the *in vivo* milieu. Antigen-presenting cells, contact time with target cells and inflammatory and activating cytokines may have also promoted the *in vivo* cytotoxicity of CIK cells with EBV-specificity. Furthermore, increased activity and EBV-specificity acquired by additional peptide stimulation as well as higher numbers of infused T cells among CIK cells with EBV-specificity may have augmented the anti-lymphoma toxicity of CIK cells with EBV-specificity as well. In addition, anti-tumor and anti-viral capacity of conventional EBV-specific T cells and conventional CIK cells that were given just 20 and 10 days before the infusion of CIK cells with EBV-

specificity may have also contributed to the extensive anti-tumor toxicity described here. Furthermore, we point out that no acute toxicities occurred during infusion, confirmed by the absence of cytokines involved in CRS, such as IL-8, IL-2, IL-6 and IFN- γ (data not shown). Of note, after 2.5 years the patient is still in remission.

The safety and to some extent the efficacy of conventional CIK cell therapy, including higher T-cell doses than those used in DLIs, were previously demonstrated in Phase 1/2 clinical trials [22–25]. The results showed limited occurrence of acute GVHD and cGVHD after unselected CIK cell treatments, even in the mismatched transplantation setting. However, if a donor was previously exposed to EBV, adoptive immunotherapy with EBV-specific donor T cells might be preferentially used because this approach is highly effective and less associated with GVHD than unselected allogeneic donor T-cell infusion [15,36]. Another advantage of EBV-specific T-cell approaches might be the lower T-cell dose needed for efficacy.

Based on our pre-clinical data, we showed that clinical-scale generation of CIK cells with EBV-specificity is feasible and provided sustained remission for now two and a half years even in a case of EBV-related DLBCL. Hence, CIK cells with additional EBV-specificity might be considered for upfront treatment of EBV-related PTLD given their anti-viral and anti-cancer potential.

Acknowledgments

The authors would like to thank the LOEWE Center for Cell and Gene Therapy Frankfurt, funded by the Hessen State Ministry of Higher Education, Research and the Arts (funding reference number: III L 4-518/17.004 [2013]), and the Else Kröner-Fresenius-Stiftung (P75/08//A62/08 and 2014_A305) for funding of this study. The authors would like to thank Olga Zimmermann and Sibylle Betz for the excellent technical support and their assistance.

Disclosure of interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

- [1] Rasche L, Kapp M, Einsele H, Mielke S. EBV-induced post transplant lymphoproliferative disorders: a persisting challenge in allogeneic hematopoietic SCT. *Bone Marrow Transplant* 2014;49:163–7. doi:10.1038/bmt.2013.96.
- [2] Lucas KG, Burton RL, Zimmerman SE, Wang J, Cornetta KG, Robertson KA, et al. Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. *Blood* 1998;91:3654–61.

- [3] Curtis RE, Travis LB, Rowlings PA, Socié G, Kingma DW, Banks PM, et al. Risk of lymphoproliferative disorders after bone marrow transplantation: a multi-institutional study. *Blood* 1999;94:2208–16.
- [4] van Esser JW, van der Holt B, Meijer E, Niesters HG, Trensche R, Thijssen SF, 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* 2001;98:972–8.
- [5] Zutter MM, Martin PJ, Sale GE, Shulman HM, Fisher L, Thomas ED, et al. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. *Blood* 1988;72:520–9.
- [6] Micallef IN, Chhanabhai M, Gascoyne RD, Shepherd JD, Fung HC, Nantel SH, et al. Lymphoproliferative disorders following allogeneic bone marrow transplantation: the Vancouver experience. *Bone Marrow Transplant* 1998;22:981–7. doi:10.1038/sj.bmt.1701468.
- [7] Styczynski J, Gil L, Tridello G, Ljungman P, Donnelly JP, van der Velden W, et al. Response to rituximab-based therapy and risk factor analysis in Epstein Barr Virus-related lymphoproliferative disorder after hematopoietic stem cell transplant in children and adults: a study from the Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. *Clin Infect Dis* 2013;57:794–802. doi:10.1093/cid/cit391.
- [8] Khanna R, Burrows SR. Role of cytotoxic T lymphocytes in Epstein-Barr virus-associated diseases. *Annu Rev Microbiol* 2000;54:19–48. doi:10.1146/annurev.micro.54.1.19.
- [9] Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000;343:481–92. doi:10.1056/NEJM200008173430707.
- [10] Gottschalk S, Rooney CM, Heslop HE. Post-transplant lymphoproliferative disorders. *Annu Rev Med* 2005;56:29–44. doi:10.1146/annurev.med.56.082103.104727.
- [11] Comoli P, Basso S, Zecca M, Pagliara D, Baldanti F, Bernardo ME, et al. Preemptive therapy of EBV-related lymphoproliferative disease after pediatric haploidentical stem cell transplantation. *Am J Transplant* 2007;7:1648–55. doi:10.1111/j.1600-6143.2007.01823.x.
- [12] van Esser JW, Niesters HG, van der Holt B, Meijer E, Osterhaus AD, Gratama JW, et al. Prevention of Epstein-Barr virus-lymphoproliferative disease by molecular monitoring and preemptive rituximab in high-risk patients after allogeneic stem cell transplantation. *Blood* 2002;99:4364–9.
- [13] Heslop HE. How I treat EBV lymphoproliferation. *Blood* 2009;114:4002–8. doi:10.1182/blood-2009-07-143545.
- [14] Gross TG, Steinbuch M, DeFor T, Shapiro RS, McGlave P, Ramsay NK, et al. B cell lymphoproliferative disorders following hematopoietic stem cell transplantation: risk factors, treatment and outcome. *Bone Marrow Transplant* 1999;23:251–8. doi:10.1038/sj.bmt.1701554.
- [15] Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulard F, Carabasi MH, et al. Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* 1994;330:1185–91. doi:10.1056/NEJM199404283301703.
- [16] De Pasquale MD, Mastronuzzi A, de Vito R, Cometa A, Inserra A, Russo C, et al. Unmanipulated donor lymphocytes for EBV-related PTLD after T-cell depleted HLA-haploidentical transplantation. *Pediatrics* 2012;129:e189–94. doi:10.1542/peds.2011-0636.
- [17] Rooney CM, Smith CA, Ng CY, Loftin SK, Sixbey JW, Gan Y, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood* 1998;92:1549–55.
- [18] Saglio F, Hanley PJ, Bollard CM. The time is now: moving toward virus-specific T cells after allogeneic hematopoietic stem cell transplantation as the standard of care. *Cytotherapy* 2014;16:149–59. doi:10.1016/j.jcyt.2013.11.010.
- [19] Sangiolo D, Martinuzzi E, Todorovic M, Vitaggio K, Vallario A, Jordaney N, et al. Alloreactivity and anti-tumor activity segregate within two distinct subsets of cytokine-induced killer (CIK) cells: implications for their infusion across major HLA barriers. *Int Immunol* 2008;20:841–8. doi:10.1093/intimm/dxn042.
- [20] Pievani A, Borleri G, Pende D, Moretta L, Rambaldi A, Golay J, et al. Dual-functional capability of CD3+CD56+ CIK cells, a T-cell subset that acquires NK function and retains TCR-mediated specific cytotoxicity. *Blood* 2011;118:3301–10. doi:10.1182/blood-2011-02-336321.
- [21] Rettinger E, Kuçi S, Naumann I, Becker P, Kreyenberg H, Anzaghe M, et al. The cytotoxic potential of interleukin-15-stimulated cytokine-induced killer cells against leukemia cells. *Cytotherapy* 2012;14:91–103. doi:10.3109/14653249.2011.613931.
- [22] Rettinger E, Huenecke S, Bonig H, Merker M, Jarisch A, Soerensen J, et al. Interleukin-15-activated cytokine-induced killer cells may sustain remission in leukemia patients after allogeneic stem cell transplantation: feasibility, safety and first insights on efficacy. *Haematologica* 2016;101:e153–6. doi:10.3324/haematol.2015.138016.
- [23] Linn YC, Niam M, Chu S, Choong A, Yong HX, Heng KK, et al. The anti-tumour activity of allogeneic cytokine-induced killer cells in patients who relapse after allogeneic transplant for haematological malignancies. *Bone Marrow Transplant* 2012;47:957–66. doi:10.1038/bmt.2011.202.
- [24] Laport GG, Sheehan K, Baker J, Armstrong R, Wong RM, Lowsky R, et al. Adoptive immunotherapy with cytokine-induced killer cells for patients with relapsed hematologic malignancies after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant* 2011;17:1679–87. doi:10.1016/j.bbmt.2011.05.012.
- [25] Introna M, Borleri G, Conti E, Franceschetti M, Barbui AM, Broady R, et al. Repeated infusions of donor-derived cytokine-induced killer cells in patients relapsing after allogeneic stem cell transplantation: a phase I study. *Haematologica* 2007;92:952–9.
- [26] Pfirrmann V, Oelsner S, Rettinger E, Huenecke S, Bonig H, Merker M, et al. Cytomegalovirus-specific cytokine-induced killer cells: concurrent targeting of leukemia and cytomegalovirus. *Cytotherapy* 2015;17:1139–51. doi:10.1016/j.jcyt.2015.04.011.
- [27] Rettinger E, Bonig H, Wehner S, Lucchini G, Willasch A, Jarisch A, et al. Feasibility of IL-15-activated cytokine-induced killer cell infusions after haploidentical stem cell transplantation. *Bone Marrow Transplant* 2013;48:1141–3. doi:10.1038/bmt.2013.19.
- [28] Quah BJ, Warren HS, Parish CR. Monitoring lymphocyte proliferation in vitro and in vivo with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nat Protoc* 2007;2:2049–56. doi:10.1038/nprot.2007.296.
- [29] Rettinger E, Kreyenberg H, Merker M, Kuçi S, Willasch A, Bug G, et al. Immunomagnetic selection or irradiation eliminates alloreactive cells but also reduces anti-tumor potential of cytokine-induced killer cells: implications for unmanipulated cytokine-induced killer cell infusion. *Cytotherapy* 2014;16:835–44. doi:10.1016/j.jcyt.2014.01.003.
- [30] Introna M, Pievani A, Borleri G, Capelli C, Algarotti A, Micò C, et al. Feasibility and safety of adoptive immunotherapy with

- CIK cells after cord blood transplantation. *Biol Blood Marrow Transplant* 2010;16:1603–7. doi:10.1016/j.bbmt.2010.05.015.
- [31] Leen AM, Myers GD, Sili U, Huls MH, Weiss H, Leung KS, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med* 2006;12:1160–6. doi:10.1038/nm1475.
- [32] Heslop HE, Slobod KS, Pule MA, Hale GA, Rousseau A, Smith CA, et al. Long-term outcome of EBV-specific T-cell infusions to prevent or treat EBV-related lymphoproliferative disease in transplant recipients. *Blood* 2010;115:925–35. doi:10.1182/blood-2009-08-239186.
- [33] Bollard CM, Gottschalk S, Leen AM, Weiss H, Straathof KC, Carrum G, et al. Complete responses of relapsed lymphoma following genetic modification of tumor-antigen presenting cells and T-lymphocyte transfer. *Blood* 2007;110:2838–45. doi:10.1182/blood-2007-05-091280.
- [34] Bollard CM, Aguilar L, Straathof KC, Gahn B, Huls MH, Rousseau A, et al. Cytotoxic T lymphocyte therapy for Epstein-Barr virus+ Hodgkin's disease. *J Exp Med* 2004;200:1623–33. doi:10.1084/jem.20040890.
- [35] Savoldo B, Goss JA, Hammer MM, Zhang L, Lopez T, Gee AP, et al. Treatment of solid organ transplant recipients with autologous Epstein Barr virus-specific cytotoxic T lymphocytes (CTLs). *Blood* 2006;108:2942–9. doi:10.1182/blood-2006-05-021782.
- [36] Porter DL, Orloff GJ, Antin JH. Donor mononuclear cell infusions as therapy for B-cell lymphoproliferative disorder following allogeneic bone marrow transplant. *Transplant Sci* 1994;4:12–14, discussion 14–16.