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ORIGINAL ARTICLE

Transfer of minimally manipulated CMV-specific T cells from stem cell or third-party donors to treat CMV infection after allo-HSCT

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Cytomegalovirus (CMV) infection is a common, potentially life-threatening complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT). We assessed prospectively the safety and efficacy of stem cell-donor- or third-party-donor-derived CMV-specific T cells for the treatment of persistent CMV infections after allo-HSCT in a phase I/IIa trial. Allo-HSCT patients with drug-refractory CMV infection and lacking virus-specific T cells were treated with a single dose of *ex vivo* major histocompatibility complex-Streptamer-isolated CMV epitope-specific donor T cells. Forty-four allo-HSCT patients receiving a T-cell-replete (D^+ repl; $n = 28$) or T-cell-depleted (D^+ depl; $n = 16$) graft from a CMV-seropositive donor were screened for CMV-specific T-cell immunity. Eight D^+ depl recipients received adoptive T-cell therapy from their stem cell donor. CMV epitope-specific T cells were well supported and became detectable in all treated patients. Complete and partial virological response rates were 62.5% and 25%, respectively. Owing to longsome third-party donor (TPD) identification, only 8 of the 57 CMV patients transplanted from CMV-seronegative donors (D^-) received antigen-specific T cells from partially human leukocyte antigen (HLA)-matched TPDs. In all but one, TPD-derived CMV-specific T cells remained undetectable. In summary, adoptive transfer correlated with functional virus-specific T-cell reconstitution in D^+ depl patients. Suboptimal HLA match may counteract expansion of TPD-derived virus-specific T cells in D^- patients.

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INTRODUCTION

Annual numbers of allogeneic hematopoietic stem cell transplantations (allo-HSCTs), a potentially curative treatment for malignant hematological diseases, have steadily increased over recent years.[1] However, allo-HSCT remains fraught with complications, including graft-versus-host disease (GVHD) or opportunistic infections. Donor-derived T cells herein have a central, though ambiguous role: while mediating GVHD, they contribute at the same time crucially to protection against pathogens as well as relapse. Consequently, transplantation protocols using T-cell-depleted HSC grafts show diminished GVHD but augmented relapse and infection rates, with the latter often caused by reactivation of latent herpes viruses such as cytomegalovirus (CMV) or Epstein–Barr virus. In healthy individuals, these life-long, chronic infections are held in check by virus-specific cytotoxic T cells but will recur with life-threatening replication rates in allo-HSCT patients with abrogated T-cell immunity. Donor-derived virus-specific T cells can be instrumental to bridge the vulnerable period of 3–12 months following transplantation until a new T-cell compartment reconstitutes spontaneously. Another high-risk group for unchecked viral reactivation are seropositive recipients of grafts from donors lacking the specific antiviral immunity. As the seroprevalence for

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CMV infections in the western hemisphere is only approximately 50%, latently infected allo-HSCT recipients of HSC grafts from CMV-seronegative donors, whether T cell-replete or not, face a substantial risk for CMV reactivation.[2]

Although the pharmacological armamentarium against CMV has expanded, available antiviral treatment regimens are unsatisfactory owing to toxicity, limited efficacy and risk of developing resistance. In recent years, adoptive transfer of HSC donor-derived CMV-specific T cells has been explored as an alternative.[3–8] Even though evidence of reconstitution of virus-specific immunity by adoptively transferred T cells has been provided, its adoption into routine clinical practice is hampered by the paucity of fast, practical and GMP-compatible selection technology. We have previously reported purification of minimally manipulated CMV-specific $CD8^+$ T cells using major histocompatibility complex (MHC)–peptide complexes reversibly multimerized with magnetic beads (MHC-Streptamers).[9,10] The purity and yield of MHC-Streptamer-isolated clinical cell products, which generally depends on the precursor frequency of the selected T-cell population, could be substantially optimized before and during this study.[11]

As predicted by single T-cell transfer experiments in mice,[12–14] lowest amounts of human MHC-Streptamer-purified T cells were found to expand vigorously in compassionate use treatments,[7] illustrating the therapeutic potential of virus-specific memory T cells that have been isolated directly *ex vivo*.[15]

Based on our own and other promising results in retrospective case series, we have tested the use of *ex vivo* MHC-Streptamer-isolated CMV-specific T cells for the first time in a prospective phase I/IIa clinical trial. Allo-HSCT patients with refractory CMV infection as defined by persistence of CMV viremia after at least 14 days of antiviral chemotherapy and lacking CMV-specific T cells were eligible for adoptive T-cell transfer. Patients with CMV-seropositive HSC grafts (D^+ patients, either treated with T-cell-replete or T-cell-depleted grafts) received MHC-Streptamer-selected CMV epitope-specific T cells from the original stem cell donor. If the original stem cell donor was CMV seronegative (D^- patients), a best human leukocyte antigen (HLA)-matched CMV-seropositive third-party donor (TPD) was selected, where available. Following its first use in solid organ transplant patients with Epstein–Barr virus complications,[16] adoptive T-cell transfer from TPDs has been studied in small case series[17,18] and was recently used in a larger trial to treat Epstein–Barr virus, CMV or adenovirus manifestations in allo-HSCT patients.[19] In this study, we compare patient recruitment efficiency, feasibility of donor selection, safety of adoptive transfer and viremic response rates in D^+ and D^- patients.

MATERIALS AND METHODS

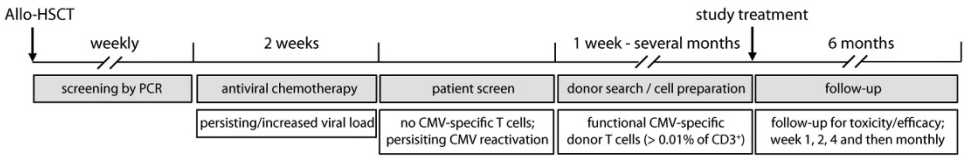
Patients

In this multicenter phase I/IIa trial, allo-HSCT recipients who were either themselves CMV (sero)positive and/or had received a graft from a CMV-seropositive donor were potentially eligible. Patients were recruited from 11 German transplant centers to receive MHC-Streptamer-selected CMV-specific T cells if they fulfilled the following inclusion and exclusion criteria: they had persistent CMV viremia after at least 14 days of antiviral chemotherapy (see Supplementary Table S1 for details) and did not have detectable functional CMV-specific $CD8^+$ T cells (tested by MHC-Streptamer staining and intracellular cytokine staining (ICS)) in peripheral blood screening. Additional criteria included absence of grade III/IV acute or extensive chronic GVHD (cGVHD), steroid treatment of < 1 mg/kg bodyweight prednisolone equivalent and informed consent.

Subsequently, peripheral blood of the CMV-seropositive stem cell donor or, in case of a seronegative stem cell donor, of an acceptably matched TPD was screened. Adoptive T-cell isolation was performed if a sufficiently large CMV-specific $CD8^+$ donor T-cell population ($> 0.01\%$ of total T cells) could be identified (Figure 1). TPDs were either selected from unrelated donors registered in the 'Deutsche Stammzellspenderdatei' registry in Frankfurt, Germany or within the patient's family. Patient and TPD were required to match in high resolution for at least the one allele in the HLA class I molecule that restricted the targeting CMV-specific T-cell response; what constituted an 'acceptable match' was left to the discretion of the treating physician.

CMV-specific T cells were isolated from an unstimulated donor leukapheresis and were infused within 24 h of the end of the selection or 72 h of the end of apheresis. Isolation of patient peripheral blood mononuclear cells (PBMCs), characterization of functional CMV-specific T cells (by MHC-Streptamer staining and ICS) and MHC-Streptamers used are described in Supplementary Material.

The primary objective of the study was the assessment of safety, evaluated by documentation of toxicity (acute allergic reaction or any change in vital signs during and after transfer) and of induction/aggravation of acute GVHD or cGVHD. The secondary objectives were reduction or elimination of viremia (assessed by quantitative PCR or CMV pp65 assay during a 6-month follow-up period) and induction of a CMV-specific T-cell response (evaluated by MHC-Streptamer staining and ICS). Patients did not receive additional cellular therapies during this 6-month follow-up period. Virostatic treatment after adoptive T-cell transfer was left to the discretion of the treating physician.



follow-up:

toxicity	
acute allergic reaction or any change in vital signs	
signs of GVHD	
efficacy	assay
virus clearance / reduction	PCR
T cell induction	flow cytometry (MHC multimer staining / ICS)
T cell tracking	single cell RACE PCR

Figure 1. Study design. Allo-HSCT patients with persistent or progressive CMV viremia refractory to antiviral chemotherapy (at least 2 weeks) were screened for functional CMV-specific T cells. Patients with no detectable virus-specific T cells were recruited into the study. Corresponding donors (stem cell donors or TPDs) were similarly screened. Donors with at least 0.01% CMV-specific T cells of total T cells were subjected to leukapheresis and CMV-specific T-cells were isolated using the GMP-grade Streptamer technology. T-cell products were transferred to the patients who were subsequently monitored for toxicity and efficacy up to 6 months (follow-up). The primary objective of the study was safety (acute infusional toxicity, GVHD), the secondary end point was efficacy as assessed by the clearance/reduction of viremia and the induction of CMV-specific T-cell responses.

Isolation of CMV-specific T cells

For the immunomagnetic separation of CMV-specific T cells, the Clinical Streptamer Isolation Kit (Juno Cell Therapeutics, formerly Stage Cell Therapeutics, Goettingen, Germany) was used as previously described[7,10,11] (see Supplementary Material for details).

Tracking of donor-derived MHC-Streptamer$^+$ CD8$^+$ T cells

Donor-specific CDR3 sequences were identified by performing single-cell Rapid Amplification of cDNA Ends PCR[20] from FACS (fluorescence-activated cell sorter)-sorted (MoFlo XDP, Beckman-Coulter, Krefeld, Germany) CMV epitope-specific T cells. Based on these sequences, CDR3 region-specific primers were designed and used in conjunction with Vα- and Vβ-specific primers. cDNA generated from patient and donor (as control) PBMCs were analyzed in parallel with the clonotypic PCR and amplicons were subsequently sequenced to confirm CDR3 identity (Sanger sequencing by GATC, Constance, Germany).

Calculations and statistical analyses

Safety and potential side effects of the adoptive T-cell transfer were evaluated immediately after injection (acute side effects and extravasation) or for up to 6 months by monitoring for GVHD (according to the grading of Glucksberg). The level of viremia was evaluated by quantitative PCR (copies per ml blood or copies per µg DNA) or by CMV pp65 assay (antigen-positive cells) per participating centers' policy. A complete remission was specified by clearance (negative PCR) on two consecutive study dates (weeks 1, 2, 3, 4 and months 2, 3, 4, 5, 6 after transfer) and a partial remission by a consecutively confirmed viral load reduction by >80% with regard to the measurements before.

To compare groups for statistically significant differences, data were analyzed with the appropriate Mann–Whitney *U*-test using GraphPad Prism version 5.00 for Windows (GraphPad Software Inc., La Jolla, CA, USA).

Study approval

The study protocol (EudraCT-No. 2006-006146-34, registered in the European Union Clinical Trials Register) was performed in accordance with the Declaration of Helsinki and approved by the federal authority (Paul-Ehrlich-Institute, protocol number 100505) and the local ethics committees. All human participants gave written informed consent.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

Between 2008 and 2014, we screened 101 allo-HSCT recipients with CMV infection from 11 clinical centers in Germany. Seventeen patients (16.8%) fulfilled the strict inclusion and exclusion criteria, had a suitable donor (see Figure 1 and Methods) and thus were eligible for treatment with MHC-Streptamer-purified CMV-specific T cells. Inclusion rates for patients with CMV-seropositive grafts (D$^+$) and CMV-seronegative grafts (D$^-$) were 20.5% (9/44 D$^+$ screenings) and 14% (8/57 D$^-$ screenings), respectively. However, reasons for the rather low inclusion rates in both groups differed substantially between D$^+$ and D$^-$ patients (Figure 2 and Supplementary Table S2).

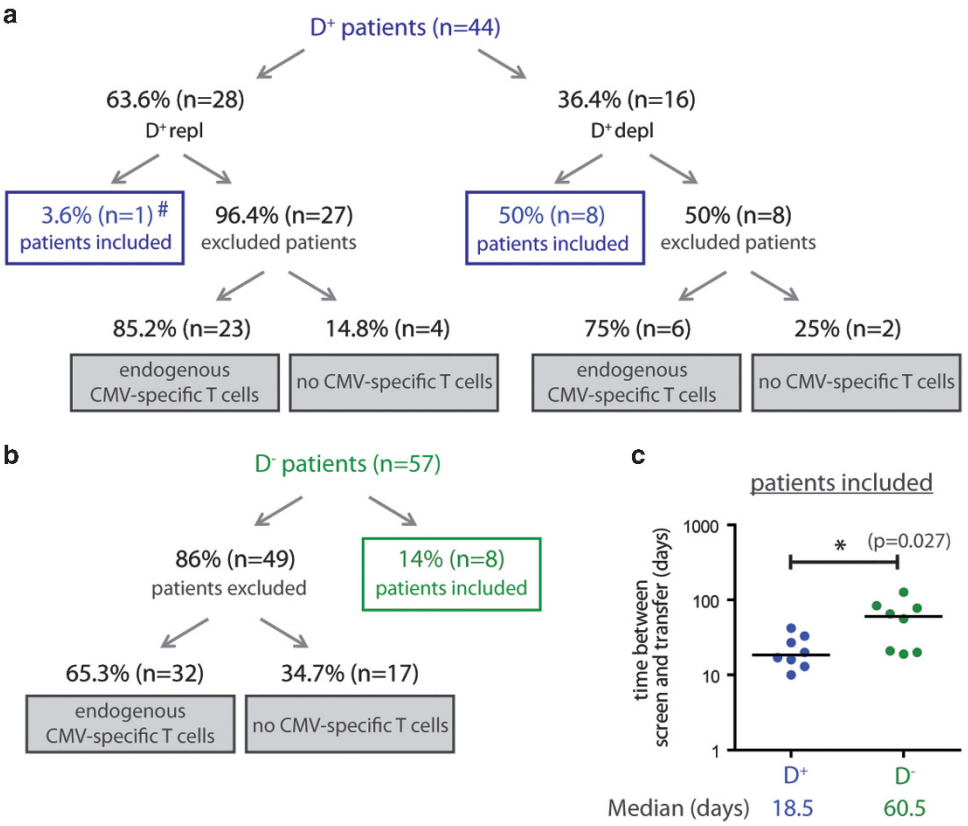


Figure 2. T-cell screening and donor selection procedure for D$^+$ and D$^-$ patients. (**a**) Recruitment efficiency of D$^+$ patients (CMV-seropositive HSC donor) transplanted with T-cell depleted (D$^+$ depl) or unmodified (D$^+$ repl) HSC grafts is shown (left). D$^+$ patients with negative CMV-specific T-cell screening and selectable CMV-specific MHC multimer$^+$ HSC donor T cells were included. Excluded D$^+$ patients had either endogenous CMV-specific T cells or donor T cells were technically or logistically not available. #Insufficient donor T-cell purification; patient died before a secondary purification procedure. (**b**) Recruitment efficiency of D$^-$ patients (CMV-seronegative HSC donor) is demonstrated in analogy to panel (**a**). (**c**) Comparison of time intervals between T-cell screen and adoptive T-cell transfer in D$^+$ and D$^-$ patients.

Table 1. Clinical parameters of D^+ (white) and D^- (gray shaded) patients receiving adoptive transfer of CMV-specific $CD8^+$ T cells

Patient	*Gender*	*Indication for therapy*	*Conditioning*	*T-cell depletion*	*Allo-HSC donor*	*CMV-specific T-cell donor*	*CMV serostatus of patient before Tx*	*Transfer after Tx (days)*	*Target*	*Viral outcome*
#01	Male	AML	Thiotepa, Fludarabin, TBI, OKT 3	CD34+ selection	Haplo	HSC donor	CMV+	55	CMV A1pp50	CR
#02	Female	ALL	RIT-anti-CD66, Cyclophosphamid, TBI	Campath i.v.	Haplo	HSC donor	CMV+	138	CMV A24pp65	PR
#03	Female	AML	Fludarabin, Thiotepa, Melphalan, OKT 3	CD34+ selection, CD56+ selection	Haplo	HSC donor	CMV+	52	CMV A2pp65	CR
#04	Male	Immunocytoma	Melphalan, Cyclophosphamid, Fludarabin, TBI	No	MMUD (cord blood) (1) 4/6; (2) 5/6	MMUD 4/8	CMV+	85	CMV A2 IE-1	CR
#05	Male	prä T-ALL	Fludarabin,Amsacrin, AraC, ATG, TBI	No	MUD 8/8	MMUD 5/8	CMV−	274	CMV A2pp65	PR
#06	Male	AML	RIT-anti-CD66, Thiotepa, Fludarabin, TBI	CD34+ selection	Haplo 5/8	Haplo	CMV+	300	CMV A2pp65	NR
#07	Male	AML	Cyclophosphamid, Fludarabin, TBI	No	MMU(cord blood) (1) 3/5; (2) 3/5D	MMUD 7/8	CMV+	86	CMV B7pp65	NR
#08	Male	CMML	Fludarabin, Melphalan	No	MUD 8/8	MMUD 5/8	CMV+	69	CMV B7pp65	CR
#09	Female	AML	Thiotepa, Fludarabin, Muromonomab, TBI	CD34+ selection, CD56+ selection	Haplo	HSC donor	CMV+	78	CMV A2pp65	CR
#10	Male	Multiple myeloma	Fludarabin, Thiotepa, Melphalan	α/β-depletion	Haplo	HSC donor	CMV+	75	CMV A1pp50	NR
#11[a]	Male	AML	Fludarabin, Cytarabin Amsacrin, Cyclophosphamid, ATG, TBI	No (but secondary GVHD treatment with Campath)	MRD 8/8	HSC donor	CMV+	No transfer	CMV A2IE-1	-
#12	Male	AML	Fludarabin, Cytarabin Amsacrin, Cyclophosphamid, ATG, TBI	No	MMUD 7/8	MMUD 5/8	CMV+	232	CMV A2pp65	NR
#13	Male	B-NHL	Fludarabin, Melphalan, Thiotepa	CD34+ selection	Haplo	HSC donor	CMV+	84	CMV A2pp65	PR
#14	Male	NHL	Fludarabin, Thiotepa, TBI	Campath in the bag	MRD 8/8	HSC donor	CMV−	150	CMV B7pp65	CR
#15	Female	AML	Fludarabin, Cytarabin Amsacrin, Cyclophosphamid, ATG, TBI	No	MUD 8/8	MRD 5/8	CMV+	139	CMV B7pp65	CR
#16	Male	MDS	Fludarabin, Melphalan	No	MUD 8/8	MUD 8/8	CMV+	112	CMV B7pp65	CR
#17	Male	T-ALL	Clofarabin, Etopophos, Cyclophosphamid, Fludarabin, Melphalan, Thiotepa, ATG	CD3/CD19 depletion	Haplo	HSC donor	CMV+	125	CMV B7pp65	CR

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; ATG, antithymocyte globulin; CMML, chronic myelomonocytic leukemia; CMV, cytomegalovirus; CR, complete remission; GVHD, graft-versus-host disease; Haplo, mismatched related haploidentical donor; HSC, hematopoietic stem cell; i.v., intravenous; MDS, myelodysplastic syndrome; MMUD, mismatched unrelated donor; MRD, matched related donor; MUD, matched unrelated donor; NHL, non-Hodgkin lymphoma; NR, no response; PR, partial remission; RIT, radioimmunotherapy; TBI, total body irradiation. [a]CMV reactivation after Campath treatment of acute GVHD; isolation of antigen-specific T cells failed.

Table 2. Summary of all severe adverse events (SAE) reported by the local investigators

Patient	*Description of SAE*	*Days between study intervention SAE*	*Outcome of SAE*	*Causative relationship to investigational drug*
#3	aGVHD	31	Recovery	Possible
#6	Aggravation of preexisting CMV pneumonia	9	Death	Uncommon
#7	Relapse of underlying disease (AML)	66	Death	Not related
#10	Pneumonia with septical disease	110	Death	Not related
#12	Systemic aspergillosis (CNS and lung)	43	Death	Not related
#13	Adenovirus hepatitis	55	Death	Not related
#14	cGVHD	50	Recovery	Not assessable
#15	Fever and dysuria (1 day after adoptive T-cell transfer)	1	Recovery	Uncommon
	Fever and CMV reactivation	27	Recovery	Uncommon

Abbreviations: aGVHD, acute graft-versus-host disease; AML, acute myeloid leukemia; cGVHD, chronic graft-versus-host disease; CMV, cytomegalovirus; CNS, central nervous system.

Eight of the nine included D^+ patients had received T-cell-depleted stem cell products (D^+ depl patients; Table 1). Therefore, we compared screened D^+ depl patients with D^+ patients with unmodified grafts (D^+ repl patients) in more detail (Figure 2a). Only one of the D^+ repl patients (3.6%) fulfilled the inclusion criteria, and he had received a T cell-depleting GVHD treatment before CMV manifestation (see Table 1). The low inclusion rate in D^+ repl patients was mainly due to the presence of endogenous (presumably graft derived) CMV-specific T cells (85.2%, Figure 2a left). In contrast, inclusion rates in the D^+ depl group were much higher (50.0%). D^+ depl patients excluded because of the presence of CMV-specific T cells were mostly screened long after allo-HSCT ($\mu = 176$ days) and had presumably undergone endogenous T-cell reconstitution. Thus T-cell depletion is a risk factor for delayed T-cell reconstitution and a need for adoptive transfer in recipients of D^+ grafts.

In D^- patients, high exclusion rates were also partly due to the presence of endogenous CMV-specific T cells (Figure 2b), again explainable by rather late T-cell screenings in this subgroup ($\mu = 196$ days). A substantial number of patients without endogenous T cells (34.7%, $n = 17$; screening at $\mu = 143$ days after transplantation) who would have been eligible for T-cell transfer could not be included because a suitable donor was not found within an acceptable time frame. In more than half of these cases, patients died during the donor search. The overall prolonged duration of TPD selection was reflected by a significantly longer interval between T-cell screen and transfer in D^- patients (median 60.5 days; 21–127 days) in comparison with D^+ patients (median 18.5 days; 10–42 days; Figure 2c, $P = 0.027$). In summary, delays in TPD identification contributed substantially to a high exclusion rate of D^- patients.

Feasibility of adoptive T-cell transfer

MHC-Streptamer purification of CMV epitope-specific T cells[11] was successful for 16 of the 17 included patients (Table 1, Supplementary Material and Supplementary Table S3). One purification procedure (patient #11) failed, presumably owing to suboptimal MHC multimer binding possibly caused by low T-cell receptor expression on targeted donor cells.

Patient follow-up

Primary objective of this phase I/II trial was estimation of safety in terms of acute transfusion-related reactions and the induction or aggravation of acute GVHD and cGVHD. Heart rate, blood pressure, oxygen saturation, temperature and clinical condition of the patient were monitored and documented during and up to 4 h after T-cell transfer. In none of the 16 patients, an acute transfusion-related reaction occurred during this period. For assessment of safety and efficacy, patients underwent additional physical examinations and blood testing before study treatment; 1, 2 and 4 weeks after transfer; and then monthly for the duration of the 6-month follow-up period. For assessment of CMV-specific immune reconstitution, 20–50 ml heparin blood was taken at the respective time points.

Severe adverse events

Only one patient developed fever 1 day after T-cell transfer (patient #15) leading to hospitalization (Table 2). However, the responsible physician diagnosed a cystitis and estimated a causative relationship as uncommon. During the follow-up period of 6 months, nine severe adverse events of eight patients were reported. Two of these severe adverse events were related to GVHD. In patient #3, an acute gut GVHD II–III occurred 31 days after T-cell transfer. However, this patient had received 21 and 8 days before the study treatment a natural killer cell transfusion and an unselected donor lymphocyte infusion, respectively. Content of non-CMV-specific CD3+ T cells was 10-fold higher in both lymphocyte infusions compared with the study treatment. GVHD disappeared after steroid therapy. In patient #14, a preexisting cGVHD (skin, liver, wasting syndrome) became worse during reduction of immunosuppression 50 days after study treatment. After adoption of the immunosuppressive medication, cGVHD improved and eventually resolved completely. In both cases, a causal relationship to the study medication is unlikely. The other severe adverse events were classified as uncommon or not related to study medication (Table 2). In conclusion, transfer of MHC-Streptamer-selected CMV-specific T cells in allo-HSCT patients was safe with no transfusion-related reaction and a low acute and cGVHD rate of 2/16 during a 6-month observation period.

Reconstitution of CMV-specific T-cell immunity after transfer of MHC-Streptamer-selected $CD8^+$ T cells

In vivo persistence/expansion of transferred T cells was monitored using CMV epitope-specific MHC multimers (Figure 3) and ICS (Supplementary Figures S1 and S2). In all treated D^+ patients, CMV-specific T cells of the transferred specificity became detectable around 2–3 weeks after transfer (Figure 3a) and all but one identified T-cell populations proliferated extensively. Treatment resulted in five complete (62.5%) and two partial virus load responses (25%; Table 1 and Supplementary Table S4). Patient #10 died soon after T-cell transfer of a multiple myeloma relapse and could therefore not reach sustained virus control (formally classified as 'no response') despite expanding functional T cells and declining viremia (< 500 copies on day 112). In summary, we detected in all treated D^+ patients emerging CMV epitope-specific T-cell responses after transfer, which were in most cases followed by strong expansion and associated with a reduced or cleared virus load and establishment of long-term adoptive immunity.

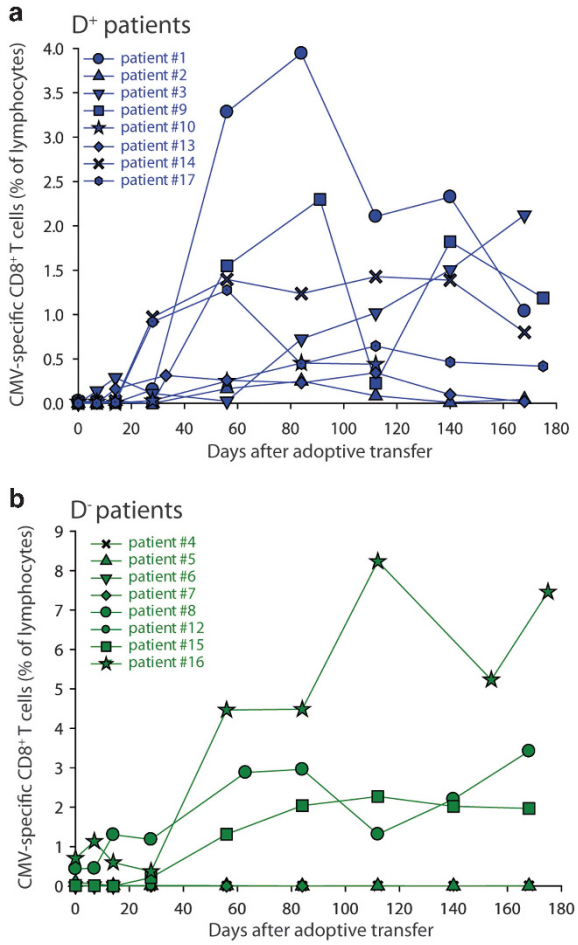


Figure 3. Expansion of CMV-specific $CD8^+$ T cells after adoptive T-cell transfer into D^+ and D^- patients. Monitoring of adoptively transferred CMV-specific $CD8^+$ T cells in D^+ (**a**) and D^- (**b**) patients using suitable CMV epitope-specific MHC multimers, (HLA-A*0201/pp65, HLA-B*0702/pp65, HLA-A*2402/pp65, HLA-A*0101/pp50 or HLA-A*0201/IE). PBMCs were analyzed before transfer (day 0), weekly (days 7–14) and then monthly (up to 6 months) after T-cell transfer.

In sharp contrast, five of the eight D^- patients had no detectable TPD-derived CMV-specific T cells at any point after transfer, even though they received higher total numbers of antigen-specific T cells than the D^+ patients (D^- $\mu = 14.16 \times 10^6$; D^+ $\mu = 6.34 \times 10^6$ CMV-specific T cells). Furthermore, in all three remaining D^- patients (patients #8, #15, #16), the detection of CMV epitope-specific T cells already at transfer implied that the consecutive strong expansion of CMV-MHC-multimer$^+$ cells in this minority was driven by endogenous T cells that had appeared in the interval between patient inclusion and cell transfer (Figure 3b). All three D^- patients with CMV-specific T-cell proliferation had a complete virus load response, while only one of the five D^- patients with undetectable CMV-specific immunity reached complete virus control (Table 1 and Supplementary Table S4). Thus the absence of demonstrable CMV-specific T-cell proliferation in the majority of D^- patients correlated with insufficient virus control.

Expansion of functional CMV epitope-specific T cells is associated with reduced viremia

In order to learn more about T-cell characteristics predicting protective immunity after adoptive transfer, we analyzed in detail the phenotypic characteristics and kinetics of T-cell immunity in treated D^+ patients (Figure 4 and Supplementary Figure S2). CMV-specific T cells proliferated strongly after transfer in all but one D^+ patient (#13), who exceptionally did also not reach virus control (see Supplementary Material and Supplementary Figure S3 for details). Interestingly, T-cell expansion was nearly always triggered in the others by a secondary viremic episode after adoptive transfer, which led to a reconstituted T-cell compartment and partial (patient #2) or complete (patients #1, #3, #9, #14, #17) virus load responses. Expanding cells were found to be interferon-gamma-, tumor necrosis factor- and partially IL-2-producing poly-functional T cells (Supplementary Figure S2 and data not shown).

Finally, concomitantly developing endogenous T cells, which might have contributed to the observed effects, were detectable in some D^+ patients (see Supplementary Material and Supplementary Figure S5 for details).

In summary, adequate proliferation and functional differentiation of adoptively transferred virus-specific T cells was associated with viral clearance.

Lack of expansion of adoptively transferred CMV-epitope-specific T cells in D^- patients

Monitoring T-cell responses and viral loads of D^- patients was challenging (Figure 5). Patient #6 died of CMV-related pneumonia within 2 weeks of transfer, reflecting our selection of CMV high-risk patients. CMV epitope-specific T cells were undetectable at that time. Patients #7 and #12 could be observed for 1 and 3 months, respectively, but CMV-specific T cells also remained undetectable in those patients. The interpretation of viremic response rates was difficult owing to the limited observation period before death, but none of the three patients fulfilled the definition of a partial or complete virus load response.

In contrast, D^- patients #8, #15 and #16 showed functional proliferation of CMV epitope-specific T cells (Figure 5 and Supplementary Figure S4) and correlating control of the viral infection (Table 1). In order to test whether the expansion of virus-specific T cells after TPD transfer in that minority of D^- patients was driven by endogenous T cells, adoptively transferred TPD-derived cells or both, we used CDR3 T-cell tracking to identify TPD-derived T cells after adoptive transfer. We FACS-sorted CMV epitope-specific donor T cells and identified donor-specific CDR3 sequences using a recently described single-cell PCR approach.[20] We employed sequence-specific primers to amplify the identified CDR3 sequences in PBMCs of the adoptively transferred patients (Figure 6). Although we found CDR3 sequences identical to the donor in PBMC samples of all available D^+ patients (patients #9, #13 and #17, Supplementary Figure S7) as previously described,[7,10] we were not able to identify matching sequences in reconstituting D^- patients #8 and #15 (Figures 6a and b). For patient #15, we confirmed the absence of transferred TPD cells among the expanding CMV B7 pp65-specific T-cell population also by available donor/patient-discriminating HLA-A2-specific antibodies (see Supplementary Table S5 and Supplementary Figure S8). A donor-specific CDR3 sequence was found only in D^- patient #16 (Figure 6c), likely indicating that TPD-derived T cells had contributed to CMV-specific T-cell reconstitution. In searching for an explanation for this nonhomogeneous T-cell tracking result among the three reconstituted D^- patients, we analyzed the degree of HLA matching between TPDs and D^- patients in more detail. Of potential relevance to this observation, patient #16, the only one with detection of a donor-identical CDR3 sequence, had a complete match in HLA-A, -B, -C and -DR (two-digit level of resolution), whereas the other two patients had only five out of the eight matching HLA alleles (Supplementary Table S5). Possibly underscoring the relevance of a good HLA-match for proper function of an adoptive T-cell graft, all remaining D^- patients with absent CMV-specific T-cell immunity (#4, #5, #6, #7 and #12;

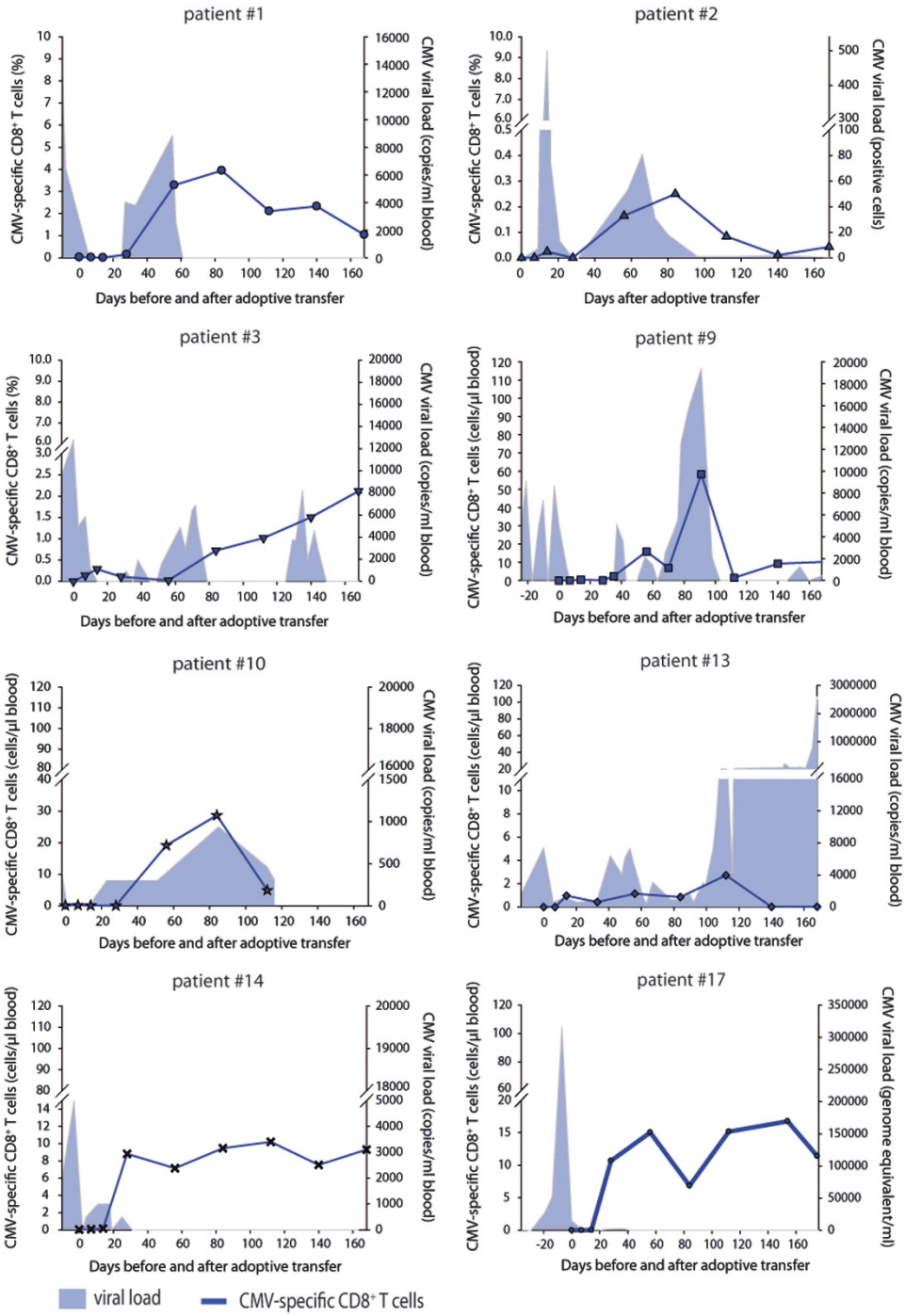


Figure 4. Proliferation of functional CMV-specific T cells in D^+ patients correlates with sustained virus control. Monitoring of adoptively transferred CMV-specific T cells by MHC multimer staining (blue line) and viral load (filled area) in D^+ patients. Where available, concentrations, otherwise frequencies, of antigen-specific T cells among living lymphocytes are shown. Viral load was determined by PCR (patient #1, #3, #9, #10, #13, #14: copies/ml blood; patient #17: genome equivalent/ml) or CMV pp65 antigen detection (patient #2: pp65-positive cells).

Figure 5) had low HLA matches to their TPDs (2/8–5/8) (Supplementary Table S6).

In summary, even though included D^- patients received TPD T cells that were quantitatively and qualitatively at least comparable to D^+ patients (Supplementary Table S3), we were unable to detect transferred TPD-derived CMV epitope-specific T cells in most (7/8) D^- patients. In line with previous reports,[16] our observations indicate that high HLA concordance between patient and TPD is an important requirement for successful virus-specific adoptive T-cell therapy in D^- patients.

DISCUSSION

In this study, treatment of allo-HSCT recipients for refractory viremia with *ex vivo* isolated, minimally manipulated CMV-specific $CD8^+$ donor T cells was found to be feasible and safe, specifically

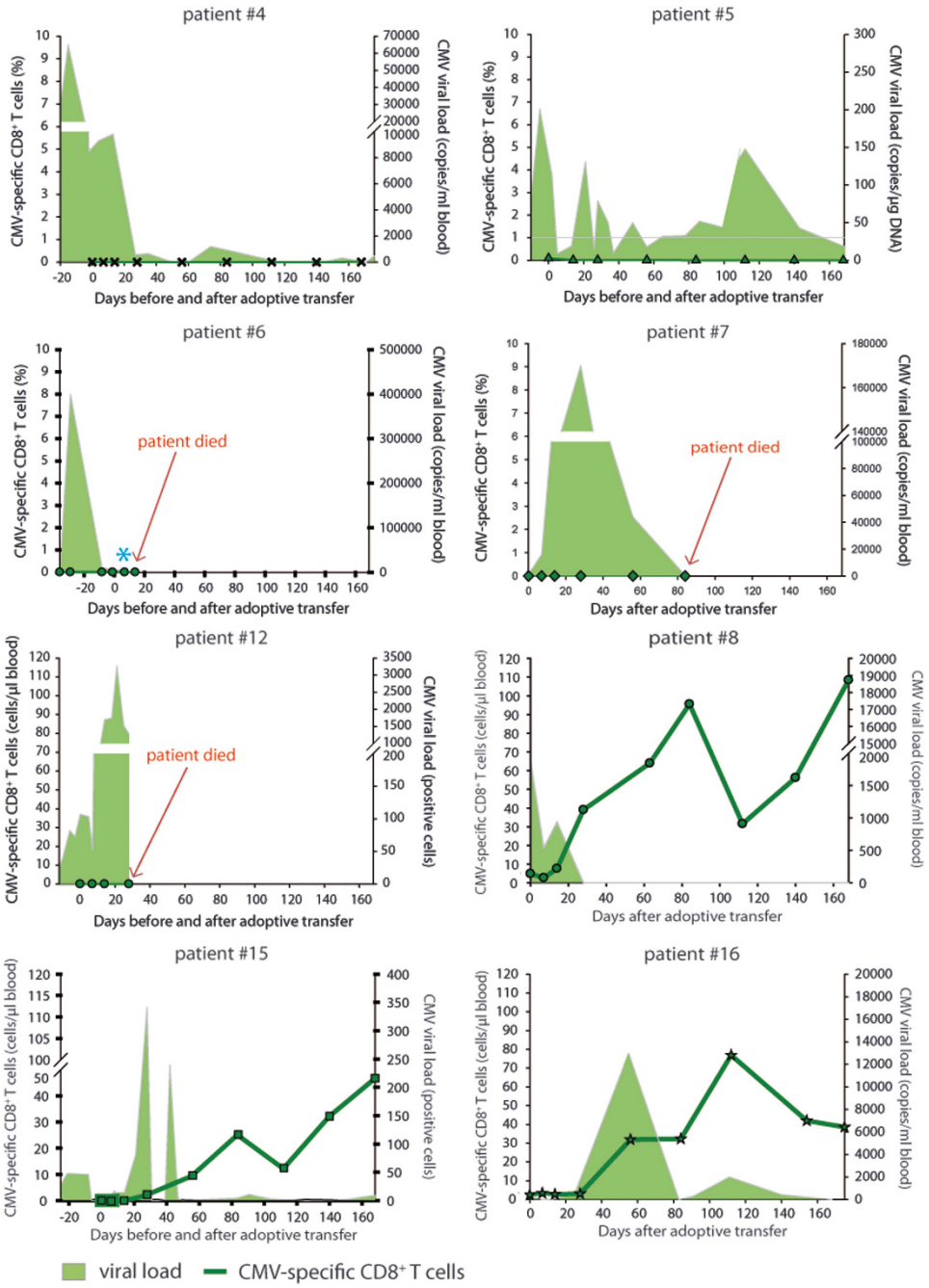


Figure 5. Overlay of CMV viral load and CMV-specific CD8$^+$ T cells in D$^-$ patients. D$^-$ patients receiving TPD T-cell products were analyzed for CMV-specific T cells by MHC multimer staining (solid green line). Where available, concentrations, otherwise frequencies, of antigen-specific T cells among living lymphocytes are shown. Viral loads (filled area) were determined by PCR (patient #4, #6, #7, #8, #16: copies/ml blood; patient #5: copies/µg DNA) or CMV pp65 antigen detection (patient #12, #15: pp65-positive cells). *5×10^3 CMV copies/ml were detected in the bronchoalveolar lavage of patient #6, 9 days after adoptive T-cell transfer.

showing low GVHD rates. To the best of our knowledge, our study is the first registered clinical phase 1/2a study with *ex vivo*-isolated CMV-specific T cells showing its safety and feasibility prospectively while using an innovative, practically relevant technological approach. Our study confirms the excellent safety profile of virus-specific T cells suggested in earlier reports[3,8,17,19,21–24] in combination with the better availability and regulatory advantages of directly *ex vivo* MHC-Streptamer-isolated T cells.

Furthermore, we could extend this experience in this study to D$^-$ patients treated from partially HLA-matched TPDs and made the important observation that T-cell expansion remained undetectable after transfer of suboptimally HLA-matched TPD T cells. Purification of CMV epitope-specific CD8$^+$ T cells by reversible MHC-Streptamers,[11] provided suitable donors were identified, was fast and reliable and led to clinical T-cell products for 16 of the 17 included patients. MHC-Streptamer-purified virus-specific T cells have been classified by the CAT advisory body to the European Medicines Agency as modified lymphocyte products and hence, as non-advanced therapy medicinal products (ATMPs), by virtue of which these minimally manipulated cells might finally become a source for more widespread use of antiviral adoptive T-cell therapy. We observed substantial epitope-specific T-cell

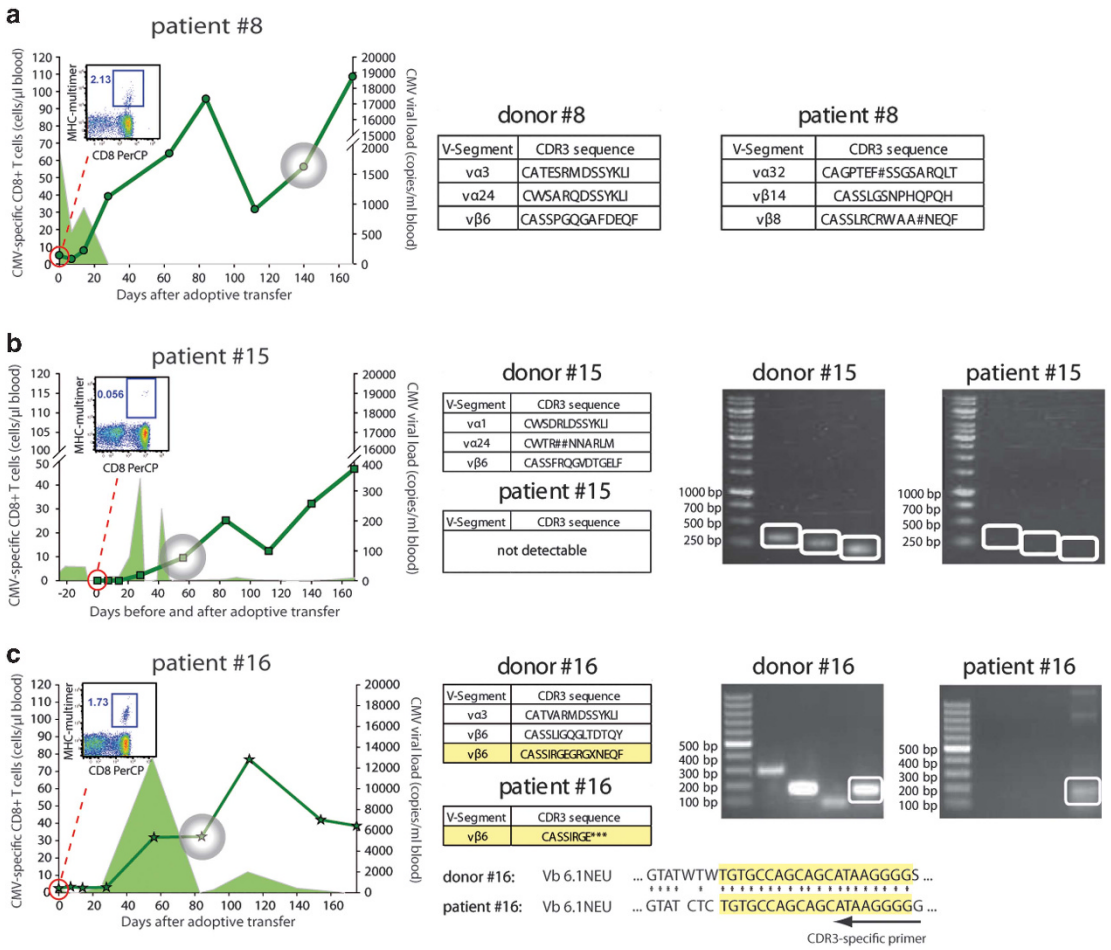


Figure 6. CDR3-Tracking of TPD-derived CMV-specific T cells in D^- patients. TPD-specific CDR3 sequences from FACS-sorted CMV epitope-specific T cells were identified by single-cell Rapid Amplification of cDNA Ends PCR. The CDR3-derived primers were then used for T-cell tracking at the indicated time points (gray circle, left). (**a**) Patient #8: T-cell tracking revealed no matched CDR3 sequences of alpha and beta chain in MHC-Streptamer-sorted antigen-specific T-cell populations of TPD and D^- patient (5 months after adoptive transfer). (**b**) Patient #15: no detection of donor-derived CDR3 sequences in patient PBMCs 2 months after transfer. (**c**) Patient #16: a donor-specific CDR3 sequence could be amplified in donor and patient PBMCs (3 months after transfer), and amplificates were subsequently sequenced confirming CDR3 identity. Inserts show CMV-HLA-multimer stainings directly before transfer. The identical segments are indicated (highlighted in yellow). CMV load (filled area) was measured by PCR (copies/ml blood).

expansion in D^+ patients, even after transfer of very low numbers of MHC-Streptamer-purified CMV-specific $CD8^+$ T cells. We detected donor-specific CDR3 sequences in all evaluable D^+ patients, which suggests that circulating cells are the result of *in vivo* expansion of the adoptively transferred T cells. However, a potential additional contribution of endogenous CMV-specific T cells, which we found in some D^+ patients (Supplementary Figure S5), is difficult to exclude. *In vivo* T-cell expansion was mostly correlated with sustained decreases (five complete responses and two partial responses) in viremia. A limitation was, however, that not all centers used PCR to measure viremia. CMV pp65 antigenemia was used instead in a minority of patients, which has been described as less sensitive.[25] However, single available PCR results in patients followed by pp65 antigenemia were found to be mainly compatible (data not shown). Furthermore, the infection-related mortality of treated D^+ patients within the 6-month observation period was very low (12.5%). Even though these are very promising results, the interpretation of effects of adoptive T-cell transfer on mortality will require a placebo-controlled study. That said, we have already compared retrospectively the observed mortality in treated D^+ patients with the outcome of screened D^+ patients that were not included (Figure 2 and Supplementary Table S7). During an equivalent 6-month observation period, the all-cause mortality (50%, $n=28$) and particularly the infection-related mortality (35.7%, $n=28$) of untreated D^+ patients was high, which is in line with recent observations in allo-HSCT recipients refractory to antiviral chemotherapy.[3,19] Importantly, mortality was most pronounced among excluded D^+ depl patients (all-cause mortality 66.7% and infection-related mortality 50%, $n=6$). These data strongly suggest that control of treatment-refractory CMV reactivations by adoptive T-cell transfer substantially lowers the incidence of fatal infections either caused directly by CMV or indirectly by bacterial and fungal pathogens, which are known to be unleashed by uncontrolled CMV infections.[26] In contrast, the infection-related mortality of excluded D^+ patients who had received T cell-replete HSC grafts and had detectable CMV-specific T cells at patient screening was relatively low (22.2%, $n=18$), supporting our strategy to exclude CMV reactivating patients with endogenous virus-specific T cells. Altogether, the vigorous expansion of functional virus-specific $CD8^+$ T cells, the associated virus clearance and the low mortality in comparison to untreated patients in aggregate indicate that virus-specific T-cell therapy with *ex vivo* MHC-Streptamer-selected cells from a virus-seropositive stem cell donor is a promising

strategy for allo-HSCT patients. Noticeably, co-transfer of virus-specific $CD4^+$ T cells was obviously dispensable to the activity of MHC-Streptamer-purified CMV-specific $CD8^+$ T cells. Furthermore, the enormous potential of primary *ex vivo*-isolated T cells to proliferate and reconstitute permanent pathogen-specific immunity makes them attractive candidates for prophylactic interventions,[7] particularly if long-lived memory T cells will be transferred.[27] Our data suggest that patients with T-cell-depleting allo-HSCT protocols might profit especially from stem cell donor-derived adoptive T-cell prophylaxis avoiding a prolonged absence of protective T-cell immunity. Clinical trials using prophylactic T-cell transfer have been recently designed with the aim of preventing opportunistic infections after T-cell-depleted allo-HSCT transplantation. A poly-specific collection of combined MHC-multimer-isolated $CD8^+$ T cells (T-CONTROL, EudraCT-No. 2014-003171-39) or low doses of Fab-Streptamer-selected central memory T cells[7,13,14,28] containing the complete $CD4^+$ and $CD8^+$ donor T-cell repertoire (PACT, EudraCT-No. 2015-001522-41), respectively, will be used to test the safety and efficacy of such an approach.

By contrast, 95.5% of the screened D^+ patients without T-cell depletion had functional endogenous CMV-specific T cells. The overall favorable outcome of the D^+ patients with evidence of adaptive CMV immunity seems to justify their exclusion. We cannot exclude that some of them might have still profited from adoptive T-cell therapy, but better predictive markers for (lack of) protection have not been identified. Future identification of such markers might also allow recognition of D^+ recipients of a T cell-replete graft who might nevertheless benefit from adoptive T-cell therapy.

Complete absence of CMV-specific T cells in CMV-seronegative donors puts CMV-seropositive allo-HSCT recipients at a high risk for reactivation. In consequence, TPD-derived CMV-specific T-cell products have been discussed intensively during the past years as an option to gain transient control over T-cell therapy-refractory viremia.[16,18,19,29] TPD registries and banks for 'off-the-shelf' adoptive T-cell technology platforms have been recently established.

However, in this study we could only treat 14% of the 57 screened D^- patients, because identification of appropriate TPDs proved to be very difficult. Many patients died before TPD identification. Much more important, we did not find evidence for systemic expansion of the transferred CMV-specific T cells except in one of the eight treated D^- patients, even though we used very sensitive techniques such as epitope-specific multimer and ICS flow cytometry, HLA-surface expression and molecular CDR3 T-cell tracking. This was in sharp contrast to D^+ patients, in whom all recipients had engraftment, and seven of the eight showed *in vivo* expansion of the transferred T cells.

Based on these observations, the following aspects should be taken into account if one considers use of TPD-derived virus-specific T cells in future. Direct availability of TPDs seems to be crucial to avoid fatal outcomes during or shortly after prolonged TPD searches. Banks of well-characterized, cryopreserved donor T cells[19,29] or CMV-donor registries could solve this issue, but broad availability of prestored CMV T-cell products will be probably limited by high costs and challenging logistics. Second, a high concordance of HLA molecules between TPD cells and stem cell donor/recipient seems to be an important prerequisite for systemic survival of adoptively transferred cells. Rejection of incompletely HLA-matched T cells would ideally be prevented by use of HLA-identical TPDs. However, their identification in HSCT donor registries is unlikely, because an available CMV-seropositive HLA-identical donor would have been directly used as stem cell donor avoiding a D^-R^+ high-risk constellation in the first place. Broad access to alternative registries (thrombocyte or blood donors) would probably be needed to raise the prospects of finding the apparently required best HLA-matched TPDs. As illustrated in D^+ patients, this quality seems to be indispensable to contain virus replication until hematopoietic stem cell-derived T-cell immunity eventually recovers.

In summary, our clinical multicenter trial allows, for the first time, a direct comparison of CMV-infected D^+ and D^- allo-HSCT patients receiving adoptive T-cell transfer from their CMV-seropositive stem cell donors or a TPD, respectively. D^+ patients receiving T-cell-depleted grafts have a high likelihood that an adoptive T-cell graft of CMV-specific cells can be generated and is clinically efficacious, with high safety and none of the toxic side effects of CMV drugs. We found CMV-specific T cells in almost all recipients of T-cell-replete grafts from CMV-seropositive donors; so their treatment with adoptive transfer is thus likely seldom justified. Partially HLA-matched, TPD-derived, CMV-specific T cells are safe but of questionable efficacy. The only D^- recipient in whom a TPD-derived CMV-specific T-cell population could be identified had the highest HLA-match (8/8) to the TPD. Transfer of less well-matched T cells may not be justified.

CONFLICT OF INTEREST

LG is an employee of and holds shares in Stage Cell Therapeutics, Göttingen, Germany; now Juno Therapeutics GmbH, Munich, Germany. DHB invented the Streptamer technology and holds shares of Juno Cell Therapeutics Inc. The other authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MN, JA, MO, GD, FS, SL, HB, TT, KM and MS performed purification or monitoring analyses; JA, MN and GUG analyzed the data; UG, DHB, HE and LG conceived the study; MN, MO, JA and DHB planned the monitoring experiments; GUG, DHB, SH, EMW, HM, MV, LU, NK, EW, GK, MS and GH were responsibly involved in patient treatment; DHB, HE, TT, HB and LG performed and supervised the clinical cell selection; MN, JA, HB, HE, GUG and DHB wrote the paper.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)