Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation

Tobias Feuchtinger,[1] Kathrin Opherk,[1] Wolfgang A. Bethge,[2] Max S. Topp,[3] Friedhelm R. Schuster,[4] Eva M. Weissinger,[5] Mohamad Mohty,[6] Reuven Or,[7] Michael Maschan,[8] Michael Schumm,[1] Klaus Hamprecht,[9] Rupert Handgretinger,[1] *Peter Lang,[1] and *Hermann Einsele[3]

[1]Department of Pediatric Hematology/Oncology, University Children's Hospital, Tübingen, Germany; [2]Department of Hematology/Oncology, Eberhard-Karls University, Tübingen, Germany; [3]Department of Hematology/Oncology, University Hospital Würzburg, Würzburg, Germany; [4]Clinic of Pediatric Oncology, Hematology and Immunology, Heinrich-Heine-University, Düsseldorf, Germany; [5]Hannover Medical School, Clinic for Hematology, Hemostasis, Oncology and Stem Cell Transplantation, Hannover, Germany; [6]Hematologie Clinique, Universite de Nantes, Nantes, France; [7]Department of Bone Marrow Transplantation, Hadassah University Hospital, Jerusalem, Israel; [8]Russian Children's Hospital, Moscow, Russia; and [9]Institute of Virology, Eberhard-Karls University, Tübingen, Germany

Cytomegalovirus (CMV) disease and infection refractory to antiviral treatment after allogeneic stem cell transplantation (allo-SCT) is associated with a high mortality. Adoptive transfer of CMV-specific T cells could reconstitute viral immunity after SCT and could protect from CMV-related complications. However, logistics of producing virus-specific T-cell grafts limited the clinical application. We treated 18 patients after allo-SCT from human leukocyte antigen–mismatched/haploidentical or human leukocyte antigen–matched unrelated donors with polyclonal CMV-specific T cells generated by ex vivo stimulation with pp65, followed by isolation of interferon-γ–producing cells. Patients with CMV disease or viremia refractory to antiviral chemotherapy or both were eligible for adoptive T-cell transfer and received a mean of 21×10^3/kg pp65-specific T cells. In 83% of cases CMV infection was cleared or viral burden was significantly reduced, even in cases of CMV encephalitis (n = 2). Viral control was associated with in vivo expansion of CMV-specific T lymphocytes in 12 of 16 evaluable cases, resulting in reconstitution of antiviral T-cell responses, without graft-versus-host disease induction or acute side effects. Our findings indicate that the infusion of low numbers of CMV-specific T cells is safe, feasible, and effective as a treatment on demand for refractory CMV infection and CMV disease after allo-SCT. (*Blood*. 2010;116(20): 4360-4367)

Introduction

Viral infections remain important causes of morbidity and mortality after allogeneic stem cell transplantation (SCT), especially in recipients of stem cell transplants from unrelated or mismatched donors.[1-3] Reconstitution of the new, donor-derived immune system can take several months,[4,5] which leaves the host with deficient T-cell immunity. Despite antiviral drug treatment, including ganciclovir, foscarnet, or cidofovir, a considerable number of patients are facing an insufficient control of cytomegalovirus (CMV) reactivation after SCT.[6] Because reconstitution of CMV-specific T cells confer protection against the development of CMV disease after SCT,[7] attempts have been made to restore antiviral immunity. The procedure known as adoptive transfer involves induction of virus-specific T-cell immunity in the patient by direct infusion of antigen (Ag)–specific T cells. So far, cellular immunotherapy against CMV has been performed with CMV-specific cytotoxic T-cell lines generated by repetitive stimulation in vitro over several weeks.[8-13] Despite this success, use of cellular therapy in the clinic has been limited by logistical difficulties, because the approach is time and labor consuming and difficult to establish under current regulations of good manufacturing practices (GMP). In addition, no sustained response was seen after adoptive transfer that involved only $CD8^+$ T cells. This phenomenon is supported by the fact that recall responses to latent infections depend on the presence of $CD4^+$ T cells to help cytotoxic T cells.[14,15] An alternative approach for the transfer of T-cell immunity is the isolation of Ag-specific T cells from the blood of CMV seropositive donors, based on the interferon γ (IFN-γ) secretion of T cells after ex vivo stimulation with viral Ag.[16,17] Therefore, to enable cellular therapy on demand, a simple short-term ex vivo protocol was developed with the pp65-specific IFN-γ secretion of T cells to isolate a combination of $CD4^+$ and $CD8^+$ CMV-reactive T cells.[18] In this report we summarize the clinical experience of several centers with infusion of low numbers of pp65-specific

Submitted January 5, 2010; accepted May 17, 2010. Prepublished online as *Blood* First Edition paper, July 12, 2010; DOI 10.1182/blood-2010-01-262089.

*P.L. and H.E. contributed equally to this study.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. T-cell graft of pp65-specific T cells

Patient no.	Donor CMV-specific T cells, %	Purity IFN-γ^+, %	CD3/kg	CD4/kg	CD8/kg
1	0.17	78	13 950	3906	9486
2	0.51	86	14 873	3420	10 709
3	0.57	83	5000	4300	650
4	0.46	42	2677	1900	590
5	1.18	10	2500	1100	1225
6	2.8	11	2500	1250	1175
7	0.07	95	7529	6118	1117
8	0.22	85	6058	4777	1454
9	0.06	79	4464	2723	1740
10	0.19	94	16 650	13 150	3500
11	0.32	58	27 807	18 800	7800
12	+	38	42 945	24 756	18 188
13	+	49	166 394	125 373	41 020
14	0.26	97	4540	2895	1125
15	1.28	10	50 000	28 175	19 460
16	0.14	85	5724	4141	1116
17	0.11	82	1221	846	356
18	0.2	83	10 000	3276	724
Mean	0.56	65	21 380	13 939	6746
SD	0.72	31	38 793		

T cells to restore protective T-cell immunity against CMV in a posttransplantation setting. Adoptive T-cell transfer was performed as a salvage treatment in patients with chemorefractory CMV disease, or chemorefractory viremia defined as increasing viral load under antiviral chemotherapy. Chemorefractory CMV infection and disease is affected by an extremely high mortality rate and therefore require the development of new treatment approaches.

Table 2. Patient characteristics and CMV disease/infection after allogeneic SCT

Patient no.	Age, y*	Diagnosis	Graft	CMV IgG donor/recipient	Body weight, kg	Conditioning regimen	GVHD prophylaxis	GVHD status before T-cell transfer	GVHD prophylaxis at T-cell transfer	GVHD status at T-cell transfer
1	16	AML	Haplo	+/+	45	Flu, TT, Mel, OKT3	CSA. MMF	Skin III, gut I°	CSA. MMF	Skin I
2	5	AML	MUD	+/+	15	Flu, TT, Treo, ATG	CSA, MMF	Skin II°	—	—
3	9	ALL	Haplo	+/+	19	Flu, TT, Mel, OKT3	MMF	—	—	—
4	36	NHL	MUD	+/−	60	Flu, Mel, Alemtuzumab	CSA	Skin I	CSA	—
5	17	ALL	Haplo	+/+	34	Clo, TT, Mel, OKT3	MMF	—	MMF	—
6	0.4	Tay-Sachs	Haplo	+/+	5	Bu, Cy, TT, OKT3	MMF	—	—	—
7	59	AML	Haplo	+/+	80	Flu, Mel, TT, OKT3	MMF	Gut III	MMF	—
8	13	RMS	Haplo	+/+	60	Flu, Cy Treo	CSA, MTX	—	CSA	—
9	6	ALL	Haplo	+/+	18	Clo, TT, Mel, OKT3	MMF	Skin I, gut II, BOOP	MMF, Etanercept	Gut I
10	52	AML	Haplo	+/+	65	Flu, TT, TLI, OKT3	—	—	—	—
11	20	ALL	Third-party MMUD after cord blood SCT	−/+	52	Flu, Cy, OKT3	CSA, MMF	Skin II	—	—
12	37	MDS	MUD	+/+	63	Bu, Cy, Alemtuzumab	CSA, MMF	Skin I-II (suspected)	CSA	—
13	48	ALL	MMUD	+/+	62	TBI, Cy, ATG	CSA, MTX	—	CSA, Prednisolone	—
14	7	JMML	Haplo	+/+	17	Bu, Cy, Mel, OKT3	MMF	—	—	—
15	42	AML	MRD	+/+	55	Flu, Bu, ATG	CSA	Skin II	—	—
16	7	ALL	Haplo	+/+	18	Flu, TT, Mel, OKT3	CSA, MMF	Skin II and gut III	Tacrolimus, MMF	Skin I
17	46	AML	Third-party MMUD after cord blood SCT	−/+	84	Flu, Cy	CSA; MMF	Gut I-II	MMF	Gut I
18	26	AML	Haplo	+/+		Flu, TT, Mel, OKT3	—	—	—	—

IgG indicates immunoglobulin G; GVHD, graft-versus-host disease; AML, acute myelocytic leukemia; Flu, fludarabin; TT, thiotepa; Mel, melphalan; CSA, cyclosporine A; MMF, mycophenolate mofetil; MUD, matched unrelated donor; Treo, treosulfan; ATG, antithymocyte globulin; ALL, acute lymphoblastic leukemia; NHL, non-Hodgkin lymphoma; Clo, clofarabin; Bu, busulfan; Cy, cyclophosphamide; RMS, rhabdomyosarcoma; MTX, methotrexate; BOOP, bronchiolitis obliterans-organizing pneumonia; TLI, total lymphoid irradiation; MMUD, mismatched unrelated donor; MDS, myelodysplastic syndrome; TBI, total body irradiation; JMML, juvenile myelomonocytic leukemia; and MRD, matched related donor.

*Mean age, 25 ± 19 years.

†Mean body weight, 47 ± 24 kg.

Table 3. Follow-up after adoptive transfer of pp65-specific T cells

Patient no.	Antiviral drugs before T-cell transfer	Site and symptoms of CMV infection before T-cell transfer	Day after SCT*	In vivo expansion of transferred CMV-specific T cells
1	Ganciclovir, foscarnet	Pneumonia, viremia	158	In vivo expansion 14 d after T-cell transfer
2	ganciclovir, foscarnet, cidofovir	Meningoencephalitis, cerebro spinal fluid, viremia	308	Viability of tested samples too low after transport
3	Foscarnet, ganciclovir, cidofovir	Fatigue, viremia	111	In vivo expansion 28 d after T-cell transfer
4	Ganciclovir	Viremia	93	Specific T cells undetectable after T-cell transfer
5	Ganciclovir, foscarnet	Viremia	50	In vivo expansion 28 d after T-cell transfer
6	Ganciclovir, foscarnet	Pneumonia, viremia	146	Specific T cells undetectable after T-cell transfer
7	valganciclovir	Viremia	102	In vivo expansion 28 d after T-cell transfer
8	Ganciclovir	Viremia	60	In vivo expansion 14 d after T-cell transfer
9	Valganciclovir	Dyspnea, diarrhea, fatigue, viremia	212	In vivo expansion 14 d after T-cell transfer
10	Ganciclovir, cidofovir	Pneumonia, viremia	19	In vivo expansion 8 d after T-cell transfer
11	Foscarnet, ganciclovir, cidofovir	Encephalitis, cerebrospinal fluid, viremia	137	Transient in vivo expansion 10 d after T-cell transfer
12	Ganciclovir, foscarnet, cidofovir	Reactivation since d 76 after SCT	116	Specific T cells undetectable after T-cell transfer
13	Ganciclovir, foscarnet	3× CMV reactivation under treatment	64	In vivo expansion d 7 after T-cell transfer up to 3.6% at d 60
14	Ganciclovir, foscarnet, cidofovir	Blood	156	In vivo expansion 10 d after T-cell transfer
15	Ganciclovir, foscarnet	Blood, urine	70	Viability of tested samples too low after transport
16	Ganciclovir, foscarnet, cidofovir	Blood, fatigue, diarrhea	199	In vivo expansion d 11 after T-cell transfer
17	Ganciclovir, foscarnet, cidofovir	Pneumonia, colitis, viremia	60	Transient in vivo expansion d 16 after T-cell transfer
18	Valganciclovir, foscavir, cidofovir	Blood	70	In vivo expansion d 28 after T-cell transfer

PCR indicates polymerase chain reaction; CSF, cerebrospinal fluid; cGVHD, chronic graft-versus-host disease; and GIT, gastrointestinal tract.
*Mean day after SCT, 118 ± 71.
†Mean last observation day after T-cell transfer, 173.

Methods

Generation of pp65-specific T cells

Generation of CMV-specific T cells was performed for all patients in a central GMP facility located in the University Children's Hospital Tübingen, as described recently.[16,18] Leukapheresis with 1×10^9 cells or 500 mL of whole blood was obtained from the donor, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll/Paque (Biochrome) density gradient centrifugation, diluted at 1×10^7 cells/mL culture medium (RPMI1640 [Biochrome] + 10% human AB serum) and stimulated with 10 μL/mL pp65protein (Miltenyi Biotec) for 16 hours at 37°C. Magnetic enrichment of cytokine-secreting cells was performed with the Cytokine-Secretion-System and the CliniMACS device (Miltenyi Biotec). The T-cell dose was restricted to ≤ 50 000/kg of body weight and was usually determined by the yield of 500 mL of whole blood starting fraction. CMV-specific T cells were infused directly after the isolation procedure without any further in vitro expansion. In patients 12 and 13 a short-term expansion in vitro was performed, as described,[18] to perform functional in vitro assays before infusion. Purity of the isolated pp65-specific T cells was assessed by detection of IFN-γ^+ cells in flow cytometry (Table 1), with release criteria of ≥ 10% IFN-γ^+ T cells. The T-cell response in related donors (parents or siblings of patients 5, 6, and 16) was above the average of unrelated donors. The German regulatory authorities have approved the GMP generation of pp65-specific T cells and their use in humans. The pp65-specific T cells were given on the basis of an off-label use in the different centers.

Patient characteristics and follow-up after adoptive T-cell transfer

We treated 18 patients between 2005 and 2009 after allogeneic SCT from a CMV-seropositive donor with a CMV infection refractory to treatment with intravenous ganciclovir or foscarnet or both (Table 2). The patients were treated according to a common management plan. Definition of "refractory" was a persistent positive CMV polymerase chain reaction with > 14 days of treatment or an increasing/unchanged copy number in quantitative polymerase chain reaction. Changes in copy numbers are defined as ≥ 1 log change. Antiviral chemotherapy was continued during and after adoptive T-cell transfer. Some patients received additional treatment with cidofovir because of adenovirus coinfection. Except patients 11 and 17, who received T cells from a third-party donor, all other patients received adoptive T-cell transfer from the stem cell donor. Data from patient 11 have been published in part previously in a case report.[19] Patients and donors gave written informed consent, and the adoptive T-cell transfer was performed in accordance with the regulations of the institutional ethics committee. Monitoring during and after adoptive T-cell transfer included heart rate, blood pressure, oxygen saturation, physical examination, blood count, C-reactive protein, and liver and kidney function tests.

Detection of CMV-specific T-cell responses after adoptive T-cell transfer

Analysis of CMV-specific T cells was done for all patients in a central laboratory as described recently.[17] In brief, PBMCs were stimulated ex vivo with 10 μL/mL pp65 protein (Miltenyi Biotec). T cells with Ag-specific secretion of IFN-γ were detected on the following day. Flow cytometric assessment of IFN-γ secretion of viable T cells was carried out by intracellular cytokine staining after addition of Brefeldin A (Sigma) for

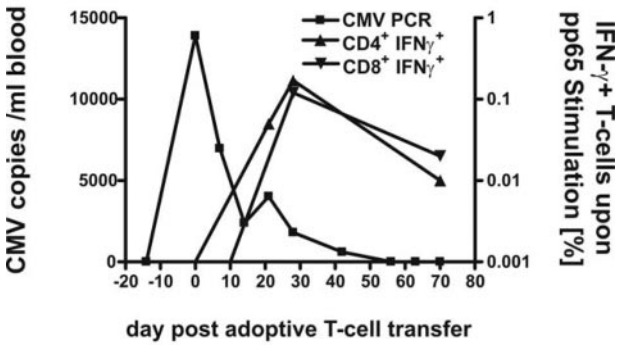

Figure 1. Time course of in vivo T-cells response and virologic response in 1 case. Adoptive T-cell transfer of pp65-specific T cells was associated with clearance of viremia.

Table 3. Follow-up after adoptive transfer of pp65-specific T cells (continued)

Patient No.	Course of CMV infection 4 wk after T-cell transfer	Clinical outcome/cause of death until last observation	Last observation day after T-cell transfer†
1	2 log decrease of viral copies in peripheral blood	Heart failure d 188 after SCT	Succumbed d 30
2	Negative PCR in CSF and blood	Renal failure, d 425 after SCT	Succumbed d 117
3	Negative CMV PCR in blood	Clearance of CMV infection	Follow-up until d 357
4	Negative CMV PCR in blood	Clearance of CMV infection	Last observation d 177
5	600 CMV copies/mL blood	Clearance of CMV from urine and throat until d 64, viremia until d 106 after SCT	Follow-up until d 650
6	2 log decrease of CMV viremia	delayed pulmonary toxicity syndrome d 172 after SCT	Succumbed d 26
7	Negative CMV PCR in blood	Clearance of CMV infection	Follow-up until d 56
8	Viremia	Sepsis, heart failure d 79 after SCT	Succumbed d 19
9	Negative CMV PCR in blood, throat, and stool	CMV reactivation after high-dose methylprednisolone at d 251 after SCT	Last observation d 409
10	pp65 negative in peripheral blood; < 600 CMV DNA copies/mL blood	Renal failure and respiratory failure d 45 after SCT	Succumbed d 26
11	Negative CMV PCR in blood	Clearance of CMV infection	Follow-up until d 145
12	Transient reduction of pp65 viremia	cGVHD, upper GIT hemorrhage	Follow-up until d 21
13	Clearance of pp65 viremia d 26 after T-cell transfer	Clearance of CMV infection, no further ganciclovir treatment	Follow-up until d 130
14	2 log decrease of CMV viremia	Clearance of CMV infection	Last observation d 112
15	Clearance of viremia d 14 after T-cell transfer	No further CMV reactivation for 3 mo after T-cell transfer	Last observation d 201
16	Clearance of CMV infection	No further CMV reactivation for 2 mo after T-cell transfer	Follow-up until d 390
17	Transient reduction of viral load 1 log d 16 after T-cell transfer	Died in multiorgan failure, mainly pneumonia 4 wk later	Succumbed d 28
18	2 log decrease of CMV viremia	Clearance of CMV below threshold 6 wk after T-cell transfer	Follow-up until d 220

PCR indicates polymerase chain reaction; CSF, cerebrospinal fluid; cGVHD, chronic graft-versus-host disease; and GIT, gastrointestinal tract.
*Mean day after SCT, 118 ± 71.
†Mean last observation day after T-cell transfer, 173.

4 hours. Surface staining was performed with saturating conditions of the following antibodies: anti-CD4 or anti-CD8 (clones SK3 or SK1), anti–IFN-γ (clone 25723.11), anti-CD3 (clone SK7), all from Becton Dickinson. At least 100 000 lymphoid cells were analyzed on a FACSCalibur with CellQuest software Version 3.3 or LSR-II using FACSDIVA software Version 6.1.2 (Becton Dickinson).

Analysis of T-cell subpopulations and T-cell function

Surface staining was performed with saturating conditions of the following antibodies: anti-CD27, CD28, CD62L, CD45RO, all from Becton Dickinson. Expression of CD62L and CD45RO or CD27 and CD28 was analyzed in gated populations of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells. Naive T cells were defined as $CD45RO^-$ $CD62L^+$, central memory T cells as $CD45RO^+$ $CD62L^+$, and effector memory T cells as $CD45RO^+$ $CD62L^-$. Proliferation was detected with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) according to a recently published protocol.[20] PBMCs were enriched by Ficoll separation and washed with phosphate-buffered saline. CFSE was then added to the cells to a final concentration of 1.6μM in phosphate-buffered saline, and cells were incubated for 9 minutes at 37°C. CFSE was then neutralized by fetal bovine serum and washed off with RPMI 1640. PBMCs were resuspended in RPMI 1640 with 10% AB serum to a final concentration of 2.5×10^6 cells/mL. After stimulation by adding pp65, the stained cells were incubated in a 96-well plate, and cell proliferation was finally assessed by flow cytometry. A positive response was defined as > 10% above background proliferation in the unstimulated negative controls. Positive controls were carried out with staphylococcal enterotoxin B (Sigma Chemical).

Results

Generation and in vitro analysis of CMV-specific T cells

We isolated pp65-specific T cells in 18 healthy, seropositive donors for adoptive T-cell transfer. Recipients were patients after allogeneic SCT. Patient characteristics are summarized in Table 2. A detection and isolation of pp65-specific T cells was possible in 100% of seropositive donors. The generation of a pp65-specific T-cell infusion was independent of the donor and recipient human leukocyte Ag (HLA) type. The mean (± SD) CMV-specific T-cell response was 0.56% ± 0.7% of total donor T cells before the isolation of specific T cells. To obtain CMV-specific T cells, we stimulated mononuclear cells isolated from 200 to 500 mL of peripheral blood of the stem cell donor with pp65 Ag. In all 18 cases this procedure efficiently increased the percentage of CMV-specific T cells to 65% ± 31% as determined by flow cytometry (see Table 1). In 3 cases of haploidentical SCT (patients 5, 6, and 15) the precursor frequency was too high for the isolation technique, and false, IFN-γ^{low+} cells contaminated the T-cell graft, leading to a purity of ~ 10% pp65-specific T cells. The isolated specific T cells contained a mean of 65% $CD4^+$ and a mean of 32% $CD8^+$ T cells.

In vitro analysis of CMV-specific T cells grafts

After isolation of IFN-γ^+ T cells, in vitro analysis of the T-cell product was done. Isolated T cells showed a good proliferation in CFSE assays (data not shown). However, the proliferation potential has been described to be inversely correlated with the T-cell maturation.[21] Therefore, markers of T-cell maturation, CD27, CD28, CD62L, and CD45RO, were analyzed among $CD3^+CD4^+$ and $CD3^+CD8^+$ double-positive gated cells after the isolation procedure. The analysis of T-cell subpopulations showed a heterogeneous expression of CD27 and CD28 in the Ag-specific T-cell graft. Both, early, double-positive, and late double-negative T cells were among the CD4 and CD8 subgroups. Because the isolated T cells are memory T cells, the late effector stages were the largest subpopulation. Analysis of alloreactivity and functional analysis of infused cells was only done in 2 patients (patients 12 and 13) to rule out alloreactivity in a mixed lymphocyte reaction as described recently.[18] In all other patients functional in vitro analysis of directly infused cells was

not possible, because absolute cell numbers would have required in vitro expansion steps for functional assays. However, specificity and alloreactivity have been analyzed in our previous preclinical study.[18]

Feasibility and side effects of cellular therapy

We treated patients for documented CMV infection between day 19 and 308 after haploidentical/mismatched related (n = 12), HLA-matched unrelated donor (n = 3), and HLA-mismatched unrelated (n = 1) transplantation. In 2 cases the original stem cell transplant donor was not available. Therefore, pp65-specific T cells derived in these 2 cases were from a partially HLA-matched third-party donor. The mean T-cell dose was low with 21.3×10^3 CD3/kg of body weight, relying on an in vivo expansion in the presence of Ag and avoidance of long-term in vitro expansion steps. The isolated T cells were immediately infused within a small volume ~ 5 mL on the day of isolation. The infusion was well tolerated, without any acute side effects. Although 15 patients received HLA-mismatched or haploidentical grafts, pp65-specific T cells were infused without induction of graft-versus-host disease in 17 of 18 patients. One of the 2 patients receiving expanded cells (patient 12) additionally received interleukin-2 (IL-2) subcutaneously after adoptive T-cell transfer. Patient 12 had upper gastrointestinal bleeding 2 days after T-cell transfer. We cannot exclude a treatment-related event in this patient, induced by invasion of pp65-specific T cells to a subclinical CMV gastritis (Tables 2-3).

Outcome and follow-up of CMV infection after adoptive T-cell transfer

Before adoptive transfer patients had a refractory CMV infection, defined as increasing viral load in peripheral blood, refractory to antiviral chemotherapy with ganciclovir or foscarnet or both (Table 1). In 15 of 18 cases clearance of CMV viremia or a significant reduction (> 1 log) of viral load was associated with adoptive T-cell transfer (Figures 1-2; Table 3). The effect of adoptive T-cell transfer was associated with the in vivo expansion of transferred T cells and took between 3 and 6 weeks and 8 weeks in 1 case. Only 3 patients did not respond to adoptive T-cell transfer of pp65-specific T cells. In 2 of the nonresponders (patients 12 and 17) the transferred T cells did not expand in vivo; hence, no clinical or virologic effect occurred. The third nonresponder (patient 8) died of bacterial sepsis 19 days after adoptive T-cell transfer, which was not related to the T-cell transfer, and no effect of the T-cell transfer on viremia could be detected in this patient within this short time frame.

The analysis of the CMV-associated mortality shows that 4 patients died because of reasons potentially associated with CMV, although all of them had a transient response of their infection after T-cell transfer. Only 1 of 13 patients with T-cell expansion in vivo died of CMV disease (patient 10), whereas 3 of 4 patients without CMV-specific T-cell responses died of CMV disease (patients 6, 12, and 17). Two patients even cleared infection after adoptive T-cell transfer from CMV encephalitis with high numbers of CMV DNA in cerebrospinal fluid. In patient 10 adoptive T-cell transfer was associated with a transient clinical improvement and reduction of the viral load, but reactivation of CMV and pneumonitis finally lead to CMV-associated mortality.

Reconstitution of CMV-specific T cells after adoptive T-cell transfer

In all patients who received a pp65-specific T-cell transfer, no CMV-specific T-cell response was detectable before adoptive transfer (Figure 2). In all patients who developed a detectable in vivo expansion of pp65-specific T cells, viremia was cleared or significantly reduced (12 of 12 evaluable cases; Figure 2). Figure 1 shows the time course of viremia and pp65-specific T-cell response in these patients. A successful in vivo expansion of CMV-specific T lymphocytes was detectable in 12 of 16 cases, resulting in reconstitution of viral T-cell immunity (Table 2; Figure 2). In vivo expansion was within the $CD4^+$ as well as in the $CD8^+$ T-cell compartment in all cases. The pp65-specific T-cell response was detected until 6 months after adoptive T-cell transfer in single patients. In 4 patients (Figure 2A) an in vivo detection of pp65-specific T cells could not be documented in peripheral blood after adoptive T-cell transfer. For logistic reasons determination of CMV-specific T cells failed in 2 patients (patients 2 and 15).

Discussion

CMV disease after allogeneic SCT is associated with a high mortality. Especially patients with a CMV infection refractory to ganciclovir and foscarnet, have an extremely high case fatality rate. Adoptive T-cell transfer has been considered a promising option for these patients.[11,22] Application of adoptive cellular therapy for CMV reactivation after SCT has been limited by the labor-intensive nature of the in vitro culture methods to select and expand specific T cells from donor T cells. Providing a fast and relatively simple method for the generation of a functional Ag-specific T-cell product will enable clinicians to use adoptive T-cell transfer in patients with CMV reactivations after allogeneic SCT. We present a short-term ex vivo protocol with the use of the pp65-specific IFN-γ secretion of T cells to isolate $CD4^+$ and $CD8^+$ CMV-reactive T cells. Because even a single Ag-specific T cell can repopulate distinct T-cell subsets in vivo[23] and because in vitro culture was shown to reduce antiviral efficacy in vivo,[24] in vitro expansion steps were not included in the protocol to avoid loss of expansion potential due to terminal differentiation during in vitro expansion. In this report we summarize our clinical experience with infusion of low numbers of pp65-specific T cells and investigate their ability to restore protective T-cell immunity against CMV and to treat chemorefractory disease and reactivation in a posttransplantation setting. The treatment was done as a salvage therapy for refractory CMV infections and CMV disease after SCT with extremely high viral load in some of the patients. We infused small numbers of IFN-γ–secreting T cells after stimulation with pp65 Ag. This approach has a number of potential advantages, including the obvious safety (no acute side effects) and the fast availability (30 hours). The fast availability of an adoptive T-cell product is clinically relevant, because it enables the clinician to tailor the treatment to the individual disease and immune status in the posttransplantation situation.

Interestingly, the success of adoptive T-cell transfer was not related to the T-cell dose. Furthermore, we describe a strong correlation of in vivo expansion of pp65-specific T cells and the clinical response. Only 1 of 12 patients with an in vivo expansion of pp65-specific T-cells died of CMV-related complications, compared with high mortality in patients without a protective T-cell response. However, the conditions for a

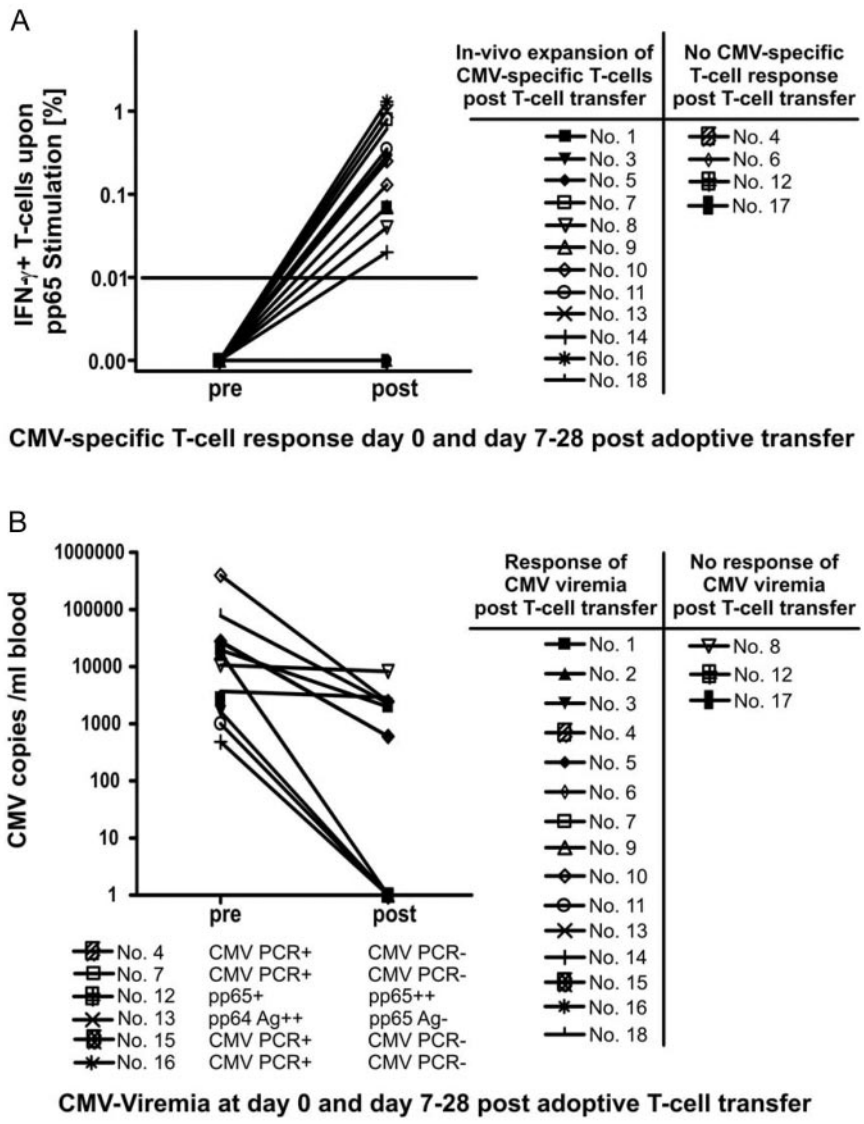

Figure 2. In vivo T-cell response and virologic response after adoptive transfer of pp65-specific T cells for the treatment of refractory CMV infection post allogeneic stem cell transplantation. Adoptive T-cell transfer of pp65-specific T cells was performed in 18 patients after allogeneic SCT. All recipients had no detectable T-cell response before adoptive T-cell transfer. (A) Shown is the in vivo expansion of the transferred T cells within 4 weeks after adoptive T-cell transfer. Twelve of 16 patients had a successful T-cell response after adoptive T-cell transfer. Detection of Ag-specific T cells was done by stimulation of blood samples with recombinant pp65, followed by intracellular cytokine staining in flow cytometry after 16 hours. Although control Ag's did not usually stimulate any IFN-γ production, the percentage of specific T cells was calculated by subtraction of the frequency obtained by the respective negative control. The threshold of a positive Ag-specific T-cell response was 0.01% of viable T cells. (B) Shown is the virologic response to adoptive T-cell transfer in terms of viral copies in peripheral blood. Before the T-cell transfer, all patients had increasing viral load unresponsive to treatment with ganciclovir or foscarnet or both. In patients 4, 7, 15, and 16 quantitative polymerase chain reaction of the virologic response was not available, but qualitative polymerase chain reaction results changed from positive to negative. In patient 13 only pp65 antigenemia was available, which turned to zero 26 days after adoptive T-cell transfer. Patients 2 and 15 are missing in panel A, because blood samples were not available for analysis.

successful T-cell response in vivo remain to be investigated in future studies. The short-term in vitro expansion in patients 12 and 13 was performed because of low cell numbers after the isolation procedure and was done to achieve a higher T-cell dose. However, in vitro expansion and higher T-cell doses did not result in higher efficacy. Even a T-cell dose of 360/kg was shown to be sufficient for a successful T-cell transfer. The provision of $CD4^+$ T-cell help is essential for a physiologic and sustained immune response, whereas $CD8^+$ T cells are considered to exert rapid antiviral effects. Therefore, a combination of $CD4^+$ and $CD8^+$ T cells for adoptive transfer is supposed to be beneficial to restore a sustained and protective immunity. Although purity is theoretically higher in T cells sorted by peptide major histocompatibility complex multimers,[25] compared with cytokine-capture systems, the isolation of IFN-γ–secreting cells enables the generation of $CD4^+$ and $CD8^+$ T-cell responses to multiple epitopes,[26] the application of functionally active (IFN-γ secreting) T cells, and applicability to all patients, independent of the HLA type.

Because detection of a specific T-cell response has been shown to be protective against CMV-related complications, the expansion potential of transferred T cells is essential for the success of adoptive T-cell transfer. Furthermore, the transfer of small T-cell populations could only result in a sustained T-cell immunity, if some transferred T cells have a repopulating capacity. This feature has been attributed to naive or central memory T cells according to the expression of the homing molecule CD62L as a marker for in vivo proliferation potential, which is described to be inversely correlated with the T-cell maturation.[21] In the IFN-γ–selected T-cell population we identified a mixture of naive, central memory, and effector memory stages (Figure 3). Early, CD27/CD28 double-positive and late double-negative T cells were detected. Because the isolated T cells are memory T cells, the late effector stages formed the largest subpopulation. The pp65-specific T-cell immunity could be detected for > 6 months in patients after adoptive T-cell transfer.

In conclusion, our approach results in a rapid production of a safe and effective therapeutic T-cell product with limited ex vivo manipulation and has the potential to be applied to a range of other

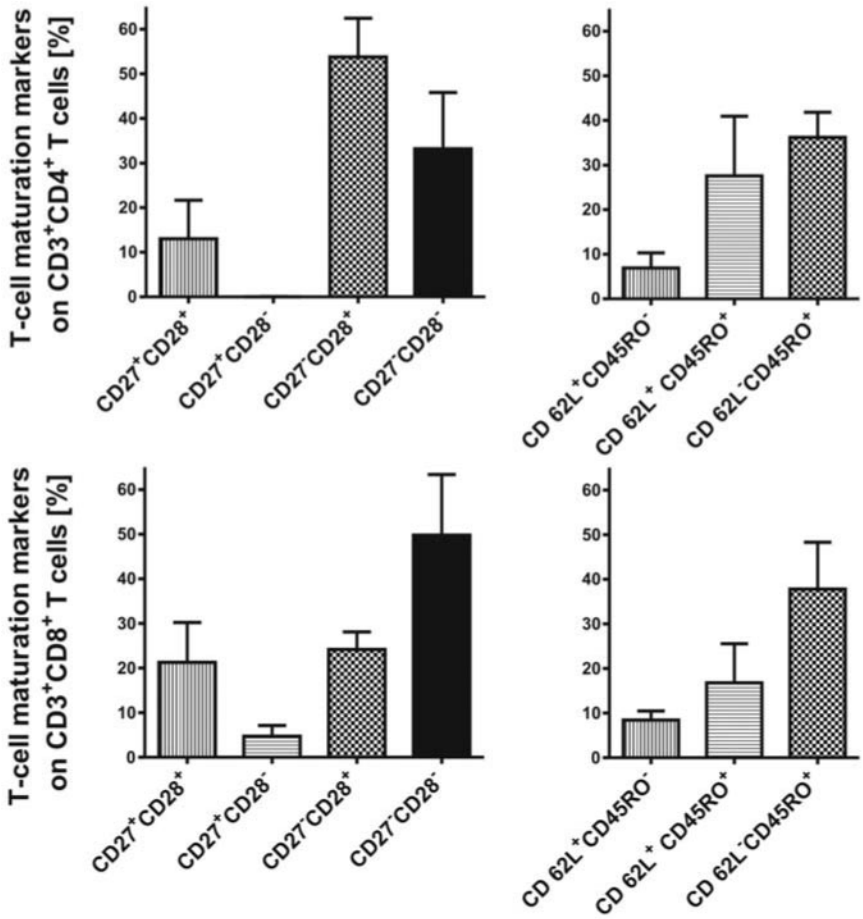

Figure 3. T-cell phenotype of pp65-specific T cells. Markers of T-cell maturation, CD27, CD28, CD62L, and CD45RO, were analyzed among $CD3^+CD4^+$ and $CD3^+CD8^+$ double-positive gated cells after the isolation procedure. The analysis of T-cell subpopulations showed a mixed Ag-specific T-cell graft, according to the expression of CD27 and CD28. Both early double-positive and late double-negative T cells were among the $CD4^+$ and $CD8^+$ subgroups. Naive T cells were defined as $CD45RO^-$ $CD62L^+$, central memory T cells as $CD45RO^+$ $CD62L^+$, and effector memory T cells as $CD45RO^+$ $CD62L^-$.

targets, including tumor Ag's, for which the secretion of IFN-γ by the transferred T cells has been emphasized.[27] This approach was used as salvage therapy and could become a clinical valuable extension of the treatment options for patients at extremely high risk of CMV-related mortality. In the future, controlled clinical trials have to investigate the optimal conditions for a successful in vivo expansion after adoptive T-cell transfer.

Acknowledgments

We thank Christiane Braun, Michael H. Scheible, Sylvia Borchers, Judith Feucht, Joseph Leibold, and Desiree Schelling for their excellent technical assistance; and D. Wernet for donor issues and human AB Serum.

This work was supported by the Deutsche Forschungsgemeinschaft (grant SFB 685) and the Fortuen/AKF Program (University Tuebingen; T.F.).

Authorship

Contribution: The design of this scientific work was done by T.F., P.L., R.H., M.S.T., and H.E.; generation of pp65-specific T cells was done by T.F., M.S., and M.S.T.; patients 1, 3, 5, 6, 9, 14, and 16 was treated by T.F., R.H., K.O., and P.L.; patient 2 was treated by M. Maschan; patients 4, 7, 10, and 18 were treated by W.A.B.; patient 8 was treated by F.R.S.; patient 11 was treated by M.S.T. and H.E.; patients 12 and 13 were treated by M.S.T., E.M.W., and H.E.; patient 15 was treated by R.O.; patient 17 was treated by M. Mohty; data analysis was done by T.F., K.O., P.L., and H.E.; and the manuscript was written by T.F. and reviewed by all authors.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Tobias Feuchtinger, Department of Pediatric Hematology/Oncology, University Children's Hospital, Eberhard-Karls University, Hoppe-Seyler Str 1, D-72076 Tübingen, Germany; e-mail: tobias.feuchtinger@med.uni-tuebingen.de.

References

1. Handgretinger R, Lang P, Schumm M, et al. Immunological aspects of haploidentical stem cell transplantation in children. *Ann N Y Acad Sci.* 2001;938:340-357.
2. Lang P, Handgretinger R, Niethammer D, et al. Transplantation of highly purified CD34+ progenitor cells from unrelated donors in pediatric leukemia. *Blood.* 2003;101(4):1630-1636.
3. Mohty M, Jacot W, Faucher C, et al. Infectious complications following allogeneic HLA-identical sibling transplantation with antithymocyte globulin-based reduced intensity preparative regimen. *Leukemia.* 2003;17(11):2168-2177.
4. Lewin SR, Heller G, Zhang L, et al. Direct evidence for new T-cell generation by patients after either T-cell-depleted or unmodified allogeneic hematopoietic stem cell transplantations. *Blood.* 2002;100(6):2235-2242.
5. Bahceci E, Epperson D, Douek DC, Melenhorst JJ, Childs RC, Barrett AJ. Early reconstitution of the T-cell repertoire after non-myeloablative peripheral blood stem cell transplantation is from post-thymic T-cell expansion and is unaffected by

graft-versus-host disease or mixed chimaerism. *Br J Haematol*. 2003;122(6):934-943.

6. Ljungman P, Brand R, Einsele H, Frassoni F, Niederwieser D, Cordonnier C. Donor CMV serologic status and outcome of CMV-seropositive recipients after unrelated donor stem cell transplantation: an EBMT megafile analysis. *Blood*. 2003;102(13):4255-4260.
7. Boeckh M, Nichols WG. The impact of cytomegalovirus serostatus of donor and recipient before hematopoietic stem cell transplantation in the era of antiviral prophylaxis and preemptive therapy. *Blood*. 2004;103(6):2003-2008.
8. Rooney CM, Smith CA, Ng CY, et al. Infusion of cytotoxic T cells for the prevention and treatment of Epstein-Barr virus-induced lymphoma in allogeneic transplant recipients. *Blood*. 1998;92(5): 1549-1555.
9. Walter EA, Greenberg PD, Gilbert MJ, et al. Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med*. 1995;333(16):1038-1044.
10. Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood*. 2002;99(11):3916-3922.
11. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science*. 1992; 257(5067):238-241.
12. Bollard CM, Straathof KC, Huls MH, et al. The generation and characterization of LMP2-specific CTLs for use as adoptive transfer from patients with relapsed EBV-positive Hodgkin disease. *J Immunother*. 2004;27(4):317-327.
13. Peggs KS, Verfuerth S, Pizzey A, et al. Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet*. 2003; 362(9393):1375-1377.
14. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol*. 2004;5(9):927-933.
15. Bevan MJ. Helping the CD8(+) T-cell response. *Nat Rev Immunol*. 2004;4(8):595-602.
16. Feuchtinger T, Lang P, Hamprecht K, et al. Isolation and expansion of human adenovirus specific CD4+ and CD8+ T-cells according to IFN-gamma secretion for adjuvant immunotherapy. *Exp Hematol*. 2004;32(3):282-289.
17. Feuchtinger T, Matthes-Martin S, Richard C, et al. Safe adoptive transfer of virus-specific T-cell immunity for the treatment of systemic adenovirus infection after allogeneic stem cell transplantation. *Br J Haematol*. 2006;134(1):64-76.
18. Rauser G, Einsele H, Sinzger C, et al. Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood*. 2004;103(9):3565-3572.
19. Schottker B, Feuchtinger T, Schumm M, et al. Five donors-one recipient: modeling a mosaic of granulocytes, natural killer and T cells from cord-blood and third-party donors. *Nat Clin Pract Oncol*. 2008;5(5):291-295.
20. Feuchtinger T, Richard C, Joachim S, et al. Clinical grade generation of hexon-specific T cells for adoptive T-cell transfer as a treatment of adenovirus infection after allogeneic stem cell transplantation. *J Immunother*. 2008;31(2):199-206.
21. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function in vitro paradoxically impairs the in vivo antitumor efficacy of adoptively transferred CD8+ T cells. *J Clin Invest*. 2005;115(6):1616-1626.
22. Leen AM, Myers GD, Sili U, et al. Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nat Med*. 2006;12(10):1160-1166.
23. Stemberger C, Huster KM, Koffler M, et al. A single naive CD8+ T cell precursor can develop into diverse effector and memory subsets. *Immunity*. 2007;27(6):985-997.
24. Reddehase MJ. Antigens and immunoevasins: opponents in cytomegalovirus immune surveillance. *Nat Rev Immunol*. 2002;2(11):831-844.
25. Yao J, Bechter C, Wiesneth M, et al. Multimer staining of cytomegalovirus phosphoprotein 65-specific T cells for diagnosis and therapeutic purposes: a comparative study. *Clin Infect Dis*. 2008;46(10):e96-e105.
26. Fujita Y, Leen AM, Sun J, et al. Exploiting cytokine secretion to rapidly produce multivirus-specific T cells for adoptive immunotherapy. *J Immunother*. 2008;31(7):665-674.
27. Hunder NN, Wallen H, Cao J, et al. Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med*. 2008; 358(25):2698-2703.