Control of cytomegalovirus viremia after T cell depleted allogeneic stem cell transplantation

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Chapter 5

Effective treatment of refractory CMV reactivation after allogeneic stem cell transplantation with in vitro-generated CMV pp65-specific $CD8^+$ T cell lines

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Abstract

To treat patients with refractory cytomegalovirus (CMV) reactivation after allogeneic stem cell transplantation, a phase I/II clinical study on adoptive transfer of in vitro-generated donor-derived or patient-derived CMV pp65-specific $CD8^+$ T cell lines was performed. Peripheral blood mononuclear cells from CMV seropositive donors or patients were stimulated with HLA-A*0201-restricted and/ or HLA-B*0702-restricted CMV pp65 peptides (NLV/TPR) and 1 day after stimulation interferon-γ)-producing cells were enriched using the CliniMACS Cytokine Capture System (interferon-γ), and cultured with autologous feeders and low-dose interleukin-2. After 7–14 days of culture, quality controls were performed and the CMV-specific T cell lines were administered or cryopreserved. The T cell lines generated contained 0.6–17 x 10^6 cells, comprising 54%–96% CMV pp65-specific $CD8^+$ T cells, and showed CMV-specific lysis of target cells. Fifteen CMV-specific T cell lines were generated of which 8 were administered to patients with refractory CMV reactivation. After administration, no acute adverse events and no graft versus host disease were observed and CMV load disappeared. In several patients, a direct relation between administration of the T cell line and the in vivo appearance of CMV pp65-specific T cells could be documented. In conclusion, administration of CMV pp65-specific $CD8^+$ T cell lines was found to be feasible and safe, and enduring efficacy of administered CMV pp65-specific $CD8^+$ T cell lines could be demonstrated.

Introduction

Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for a variety of hematological malignancies and inherited hematopoietic disorders. A significant complication of alloSCT is the development of severe graft versus host disease (GvHD), which can be treated by immune suppression. Depletion of T cells from the stem cell graft can be performed to prevent the development of severe GvHD. However, T cell depletion and immune suppression significantly delay immune reconstitution in patients after alloSCT, which is associated with an increased risk of opportunistic infections including cytomegalovirus (CMV) infection.[1-3] Reactivation of CMV can cause serious morbidity and mortality during the prolonged period of immune deficiency after alloSCT, which is also illustrated by increased CMV-related morbidity in patients receiving a graft from a CMV seronegative donor.[4-5] The availability of antiviral agents like ganciclovir and foscarnet has contributed to a significant reduction of CMV-related morbidity and mortality after alloSCT. However, if appropriate T cell responses against CMV do not develop after alloSCT, subsequent viral reactivations and refractory disease are commonly observed. Reconstitution of the CMV-specific T cell repertoire directed against immunodominant proteins in the first year after alloSCT has been demonstrated to confer sustained protection from CMV disease. Furthermore, for the long-term protection against CMV, the development of CMV-specific T cell immunity has been found to be essential.[6-9] Therefore, adoptive transfer of donor-derived virus-specific T cells is an attractive strategy for treatment and prevention of viral disease in alloSCT recipients. Clinical studies have demonstrated that the adoptive transfer of donor-derived CMV-specific memory T cells can be a safe and effective treatment for patients with refractory CMV reactivation.[10-18]

In most clinical studies, CMV-specific $CD8^+$ T cell lines were generated by repetitive stimulation of the T cells in vitro for several weeks to obtain highly pure T cell lines to minimize the risk of the induction of GvHD.[11,13-18] However, isolation of CMV-specific T cells from peripheral blood also allows the generation of highly specific T cell lines without prolonged culture and repetitive stimulation. As it has been demonstrated that adoptive transfer of interferon-γ (IFNγ)-producing memory T cells can provide long-lived functional memory T cell reconstitution and that the presence of IFNγ-producing CMV-specific T cells is associated with protection from CMV reactivation, isolation of CMV-specific memory $CD8^+$ T cells based on their IFNγ production is an attractive strategy for generating effective CMV-specific $CD8^+$ T cell lines.[4,6,8,9,12,13,19] Although clinical studies have shown that adoptive transfer of CMV-specific T cells is safe and effective, in these studies the clinical effect could not always be directly correlated to the infusion of CMV-specific $CD8^+$ T cells.

In this study, a phase I/II clinical protocol for the adoptive transfer of donor or patient-derived CMV pp65- specific $CD8^+$ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. CMV- specific T cells were isolated using an IFNγ-based isolation technique and cultured for 1–2 weeks. Six patients with CMV reactivation failing antiviral therapy were treated with these CMV pp65-specific $CD8^+$ T cell lines. In all the 6 patients,

CMV DNA load turned negative and CMV- specific T cells could be detected in the peripheral blood. In 2 patients, a direct relation was demonstrated between the administration of CMV-specific T cells, the appearance of CMV-specific T cells in the peripheral blood, and the clearance of CMV reactivation.

Materials and methods

Study design

Adult and pediatric patients with refractory CMV reactivation after HLA-matched alloSCT were eligible. Patients were weekly monitored for CMV reactivation after receiving a stem cell transplant by measuring CMV DNA copy numbers with quantitative polymerase chain reaction. Refractory CMV reactivation was defined as persisting CMV viremia for >2 weeks while receiving pharmacotherapy or as early relapse (within 2 wk) after therapy. As only peptides binding in HLA-A*0201 or HLA-B*0702 were available for clinical use and therefore only T cells specific for these epitopes could be isolated, HLA-A*0201 and/or HLA-B*0702^{+} patients were eligible. In case of a CMV seropositive donor, a donor-derived T cell line was generated. When the donor was CMV seronegative, a patient-derived T cell line was generated from cryopreserved peripheral blood mononuclear cells (PBMNCs) harvested before transplantation. When the CMV pp65-specific T cell line met the release criteria and CMV was still detectable, the T cell line was administered to the patient. Antiviral treatment could be continued conform standard protocol. The procedure was repeated in case of stable or progressive CMV reactivation and no severe toxicity. Patients or their parents (in case of children) gave written informed consent, and the study was performed in accordance with the regulations of the institutional ethics committee.

The aim of this phase I/II study was to investigate the feasibility of generating CMV-specific T cell lines for clinical application, of which at least half should be administrated. Endpoints of the study were feasibility and toxicity of the treatment. Feasibility was defined as ≥80% success rate in generating a T cell line and toxicity was defined as ≥30% of patients developing GvHD after administration of the T cell line. In addition, the efficacy of the treatment was analyzed; however, this was not a primary endpoint.

Collection of peripheral blood cells

After informed consent, at least 2 x 10^9 PBMNCs were harvested by leukapheresis from each donor or patient. Red blood cells were lysed using an NH_4Cl (8.4g/L) and $KHCO_3$ (1 g/L) buffer (pH = 7.4) (LUMC Pharmacy, Leiden, The Netherlands). PBMNC were directly used or cryopreserved in the vapor phase of liquid nitrogen until further use. Cells were cryopreserved in IMDM (Lonza, Basel, Switzerland) containing 20 g/L human albumin (CeAlb; Sanquin, Amsterdam, The Netherlands) and 10% dimethyl sulphoxide (LUMC Pharmacy).

Generation and administration of clinical grade CMV-specific CD8$^+$ T cell lines

PBMNC were resuspended in culture medium consisting of IMDM supplemented with 3mmol/L L-glutamine (Lonza) and 100 U/mL penicillin and 100 mg/mL streptomycin (Lonza) plus 10% heat-inactivated pooled prescreened AB serum from healthy blood bank donors (Sanquin). Cells were cultured at a cell concentration of 10 x 10^6 cells/mL and 1 mg/mL of peptide was added. Peptides used were the HLA- A*0201-binding peptide NLVPMVATV (NLV) and the HLA-B*0702-binding peptide TPRVTGGGAM (TPR) both purchased from Bachem (Bubendorf, Switzerland).

After overnight stimulation, IFNγ-secreting cells were isolated using the CliniMACS Cytokine Capture System (IFNγ) (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. After washing, the positive fraction was resuspended in culture medium containing 10 IU/mL interleukin (IL)-2 (Proleukin; Novartis Pharma B.V., Arnhem, The Netherlands). Cells were cultured at a 1:10 ratio with 30 Gy-irradiated feeder cells, which were obtained from the negative fraction after isolation. Cultures were refreshed every 3–4 days, and after 7–14 days the T cell lines were analyzed. Cultures were evaluated for the presence of CMV NLV-specific and/or TPR-specific CD8$^+$ T cells by tetramer staining, and quality control was performed. Quality assurance specifications included: (1) no microbiological contamination; (2) confirmation of the origin of the material (donor or patient); (3) the presence of minimally 50% of CMV-specific CD8$^+$ T cells as measured by tetramer staining; and (4) the presence of maximally 10% CD19$^+$ B cells. Functionality was tested in a ^{51}Cr release assay and defined as minimally 20% lysis of positive target cells and maximally 10% lysis of negative target cells. When released for administration, the CMV-specific T cell lines were resuspended in 100 mL NaCl 0.9% (LUMC Pharmacy) supplemented with 2% human albumin (Sanquin) in a 500 mL cryocyte freezing container (Baxter, Deerfield, IL).

Phenotypic analysis

To determine the composition and phenotype of the starting material, the fractions after isolation, the T cell line, and the PBMC fractions from the peripheral blood cells were stained using fluorescein isothiocyanate-labeled TCRab, CD14, CD4, HLA-DR, CD27 (BD Biosciences, San Jose, CA) and CD197 (R&D systems, Minneapolis, MN) antibodies, phycoerythrin-labeled TCRgd, CD56, CD25 (BD) and IFNγ (Miltenyi Biotec) antibodies, PerCP-labeled CD8$^+$, CD3 (BD) and CD45RO (Invitrogen, Frederick, MD) antibodies, allophycocyanin (APC)-labeled CD19, CD33, CD45RA, CD28 (BD), and CD4 (Beckman Coulter, Fullerton, CA) antibodies, and PE-labeled or APC-labeled tetramers of the NLVPMVATV peptide bound to HLA-A*0201 or the TPRVTGGGAM peptide bound to HLA-B*0702, which were prepared as described previously.[20] After labeling, cells were analyzed on a FACSCalibur (BD).

Cytotoxicity assay

To determine the cytotoxicity of the T cell lines, standard ^{51}Cr release assays were performed as described previously.[21] Target cells (donor and patient phytohemagglutinin (PHA) blasts,

HLA-A*0201 and HLA-B*0702^{+} EBV-LCL (JY), or pp65-transduced JY (JY-pp65)[22]) were loaded with 100 mCi of $Na^{51}Cr_2O_4$ (Amersham, Roosendaal, The Netherlands) and when necessary with 1mM of peptide (NLV or TPR) for 1 hour. Effector/target ratios ranged from 30:1 to 1:1 and after 4 hours of incubation ^{51}Cr release was determined.

Ex vivo analysis of cytokine production of CMV-specific T cells

After thawing, PBMNC from the patients were resuspended in culture medium and with 10^{-6} M NLV or TPR peptides for 5 hours at 371C and 5% CO_2. After 1 hour of peptide stimulation, 10mg/mL of brefeldin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) was added for the remaining 4 hours of incubation. After stimulation, cell-surface staining with monoclonal antibodies was performed, followed by intracellular staining as described previously.[23] Cells were stained using fluorescein isothiocyanate-labeled IFNγ (BD), PE-labeled tumor necrosis factor (TNFα) (BD), PerCP-labeled CD4 and CD8^{+} (BD), and APC-labeled IL-2 (BD).

Ex vivo identification of CMV-specific CD8^{+} T cells

To identify donor or recipient origin of CMV-specific T cells reconstituting in peripheral blood after infusion of the CMV pp65-specific CD8^{+} T cell lines, PBMNC from the patient were stained using HLA class I tetramers specific for NLV or TPR. Subsequently, the tetramer^{+} CD8^{+} T cells were selected by flow cytometric sorting using a FACSDiva and Cellquest software (BD). The DNA profile of the sorted cells was established by chimerism analysis based on short tandem repeats polymorphism as previously described.[24]

Results

Generation of clinical grade CMV-specific CD8^{+} T cell lines

A total of 12 donor-derived and 3 patient-derived CMV-specific T cell lines specific for the HLA-A*0201-restricted NLV epitope and/or for the HLA-B*0702-restricted TPR epitope were generated for patients failing antiviral therapy after alloSCT. Donor-derived T cell lines were generated when the donor was CMV seropositive, whereas patient-derived T cell lines were generated when the donor was CMV seronegative and the patient CMV seropositive. **Figure 1** shows the data of a representative donor-derived (Figs. 1A–D) and patient-derived (Figs. 1E–H) CMV-specific T cell line. The frequencies of the NLV-specific T cells in the PBMNC starting material were 0.4% in the donor (Figure 1A) and 1.45% in the patient (Figure 1E). After overnight stimulation with the HLA-A*0201-binding NLV peptide, IFNγ-producing cells were isolated using the CliniMACS Cytokine Capture System (IFNγ). The positive fraction contained between 20% and 25% IFNγ^{+} CD8^{+} T cells within the lymphocyte gate (Figure 1B, F), other T cells present in the positive fraction were CD4^{+} T cells and IFNγ-negative CD8^{+} T cells. After 10 days and 7 days of culture, respectively (Figs. 1C, G), specific proliferation of the NLV-specific CD8^{+} T cells resulted in a donor-derived T cell line containing 83% NLV-

specific $CD8^+$ T cells and in a patient-derived T cell line containing 66% NLV-specific $CD8^+$ T cells. Less than 1% of $CD19^+$ B cells were detected in the T cell lines (data not shown). As is shown in Figures 1D, H both T cell lines showed reactivity against NLV-loaded target cells and no reactivity against donor or patient cells. Furthermore, both T cell lines also showed recognition of endogenous presented antigen using CMV pp65-transduced EBV-LCL (JY-pp65).

Table 1 shows the results of all the CMV-specific T cell lines generated. Frequencies of NLV-specific and/or TPR- specific $CD8^+$ T cells in the donor or patient starting material used for the generation of the T cell lines ranged from 0.02% to 1.45% of total PBMNC. Cell numbers obtained directly after isolation ranged from 0.25 to 14.4 x 10^6 cells and contained between 3% and 45% $IFN\gamma^+$ $CD8^+$ T cells within the lymphocyte gate. Total cell numbers obtained after culture ranged from 0.6–17.1 x 10^6 cells. During culture, no increase in total cell numbers was observed, but further enrichment of CMV-specific T cells was observed resulting in T cell lines containing 54%–96% NLV-specific and/or TPR-specific T cells.

All donor-derived and patient-derived CMV-specific T cell lines generated met the quality control criteria. No microbiological contamination was detected in any of the T cell lines, and chimerism analysis showed the correct origin of the material. As is shown in Table 1, all T cell lines contained >50% of NLV-specific and/or TPR-specific T cells and showed recognition of peptide-loaded PHA blasts and no reactivity against donor-derived or patient- derived PHA blasts.

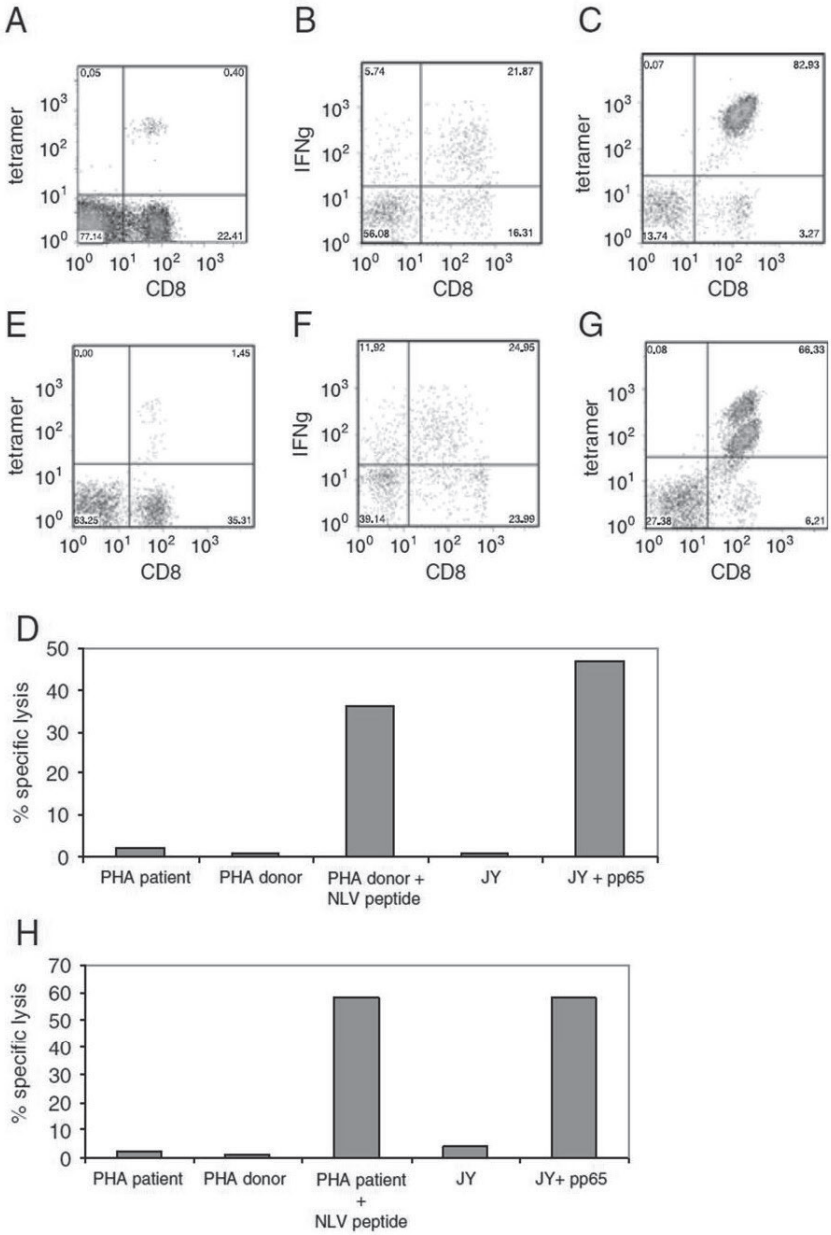

Figure 1
Composition and functionality of donor-derived (A-D) and patient-derived (E-H) CMV-specific T cell lines. **(A, E)** the percentage of CMV NLV (A) and CMV TPR (E) -specific T cells in the starting material (day 0); **(B, F)** the percentage of IFN positive cells in the positive fraction direct after isolation; **(C, G)** the percentage of CMV NLV (C) or CMV TPR (G) in the T cell line after culture (day 7); **(D, H)** functionality of the T cell lines in a ^{51}Cr release assay. Dot plots are shown and events shown are gated on the lymphocyte gate and PI-negative gate. Percentage lysis after 4 hours in a ^{51}Cr release assay at an E:T ratio of 3:1 is depicted. JY = EBV-LCL; JY-pp65 = CMV pp65 transduced JY.

Table 1. Characteristics of the CMV-specific T cell lines

CTL Line	Specificity	freq in PBMC	# cells (x 10^6)[2]	Phenotype (% positive cells)[2, 3]			# cells (x 10^6)[2]	Cytotoxicity[2,4]		
								PHA donor		PHA
				CD4	$CD8^+$	$CD8^+Tet^+$	$CD8^+Tet^+$	- peptide	+ peptide	patient
P1-a	TPR	0.28	3.4	26	71	68	2.3	1	53	4
P1-b	TPR	0.28	3.8	10	88	72	2.7	2	70	0
P2-a	TPR	0.3	7.5	9	91	81	6.1	2	56	2
P3-a	TPR	0.02	5.0	20	80	72	3.6	1	41	4
P4-a	NLV+TPR	<0.01 / 0.25	5.9	11	85	5 / 69	3.8	3	66	0
P4-b	NLV+TPR	<0.01 / 0.25	17.1	5	73	14 / 50	12.7	1	39	0
P5-a	TPR	0.14	4.8	4	95	95	3.1	0	26	1
P7-a	NLV+TPR	0.04 / 0.52	10	4	96	4 / 92	9.6	9	65	0
P8-a[1]	TPR	0.7	3.0	11	77	66	2.1	1	62	1
P9-a	NLV	0.48	1.0	22	77	65	0.7	1	80[5]	0
P9-b	NLV	0.98	3.3	7	93	83	2.7	2	30	1
P10-a[1]	NLV	1.45	6.0	8	90	79	4.7	1	58	2
P11-a[1]	NLV	1.1	6.7	27	61	54	3.6	2	25	4
P12-a	NLV	0.4	16.8	9	85	82	13.8	1	36	2
P14-a	NLV	0.17	0.6	n.d.	88	71	0.43	n.d.	n.d.	n.d.

[1]patient derived T cell line; [2] after 7-14 days culture; [3] within lymphocyte gate; [4] percentage lysis after 4 hours in a 51Cr release assay, E:T ratio of 3:1;
[5] measured in a CFSE-based cytotoxicity assay (29); Tet = tetramer; PHA = PHA blasts; TPR = TPRVTGGGAM peptide; NLV = NLVPMVATV; n.d. = not done.

Administration of CMV-specific CD8$^+$ T cell lines

In a phase I/II clinical study, the toxicity and the potential antiviral effect of treatment with CMV pp65- specific T cell lines for refractory CMV reactivation after alloSCT was investigated. Eight of the 15 T cell lines generated were administrated. Six patients with refractory CMV reactivation after alloSCT received donor-derived CMV pp65-specific T cell line(s). Four patients (patient 2, 3, 4, and 14) received 1 CMV pp65-specific T cell line and 2 patients (patient 1 and 9) received 2 CMV pp65-specific T cell lines. The other T cell lines generated were not administrated, as patients were CMV load negative (n = 4), had relapsing malignant disease (n = 1), or deceased because of CMV disease (n = 2) at the time the production of the CMV pp65-specific T cell line was completed.

Despite prior treatment with antiviral agents, all 6 patients who received a CMV pp65-specific T cell line had ongoing positive CMV DNA loads (log 2.5–log 3.2) at the time of administration of the CMV-specific T cell lines. The patients received a total cell dose ranging from 0.6 to 7.5 x 10^6 cells (analogous to 0.9 x 10^4–3.1 x 10^5 T cells/kg), corresponding to 0.4–6.1 x 10^6 NLV-specific and/or TPR- specific T cells. In 5 out of 6 patients, antiviral therapy was discontinued before infusion of the CMV-specific T cell line. Patient 9 was treated until 2 weeks after infusion of the second CMV-specific T cell line. None of the 6 patients developed GvHD or any other complications during infusion of the T cell lines, and all patients cleared CMV reactivation within weeks after administration of the CMV pp65-specific T cell line.

Reconstitution of CMV-specific T cells after administration of the CMV-specific CD8$^+$ T cell lines

In all patients CMV-specific, NLV-specific, and/or TPR-specific CD8$^+$ T cells could be detected after administration of the T cell lines. To determine a possible correlation between the infusion of the CMV-specific T cell lines and the appearance of CMV-specific T cells in peripheral blood of the patients, the presence of CMV- specific T cells was analyzed at different time points before and after administration of the T cell lines. Before infusion of the CMV-specific T cell lines in 4 of the 6 patients, CMV pp65-specific T cells with the same specificity as the T cell line could already be detected in the peripheral blood. In 3 of these patients, a rise in CMV-specific T cells in the peripheral blood was observed after infusion of the T cell line. In the other patient, the numbers of CMV-specific T cells was stably high after infusion. In 2 of the 6 patients (patient 4 and 9), no CMV-specific T cells were detected before infusion of the T cell line, and a direct relation was observed between infusion of the CMV pp65-specific T cell line, the appearance of CMV pp65-specific T cells in the peripheral blood.

From 3 patients showing CMV-specific T cells already before infusion, we compared the phenotype and functionality of the CMV-specific T cells in peripheral blood before and after infusion. As illustrated in **Figure 2A**, both before and after infusion the CMV-specific T cells in peripheral blood of the patients predominantly produced IFNγ and TNFα after antigenic stimulation; only a minority produced IL-2 after restimulation with the specific peptide. Both

effector memory (CD45RO$^+$, CD45RA$^-$, CD27$^-$, CD28$^-$) and effector (CD45RO$^-$, CD45RA$^+$, CD27$^-$, CD28$^-$) CMV-specific T cells were similarly found before and after infusion (data not shown). In addition, the tetramer-binding capacity of the CMV pp65 NLV-specific and/or TPR-specific T cells was not different before and after adoptive transfer, as is shown in **Figure 2B**. Thus, the contribution of the infused CMV-specific T cells to the clearance of CMV could not be determined in these patients.

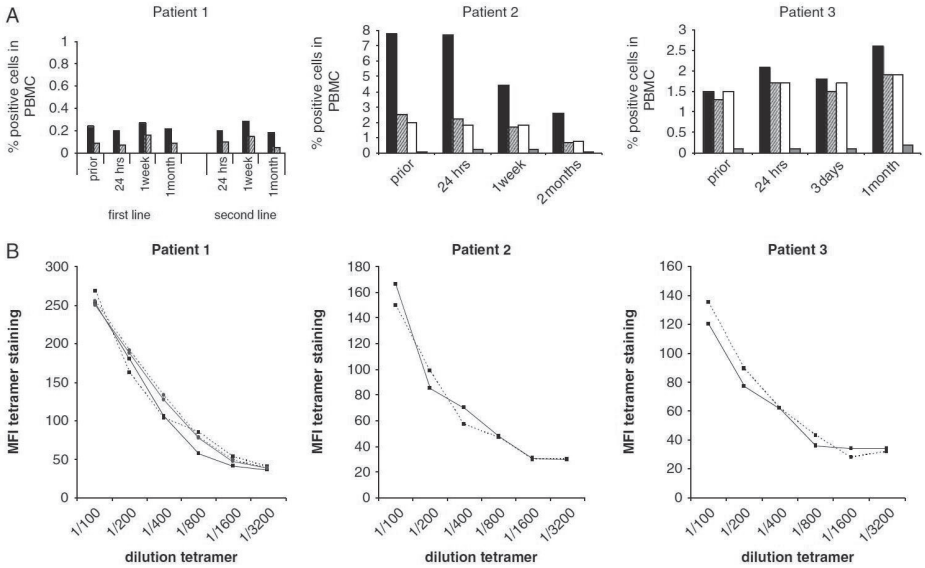

Figure 2.
A, Cytokine production after antigen-specific restimulation of cytomegalovirus (CMV)-specific T cells in PBMNC from patient 1 to 3 obtained prior and at different time points after infusion of the CMV-specific T cell line (depicted on the x-ax). The percentage of positive cells within the peripheral blood mononuclear cells (PBMNC) population is shown. Black bars represent tetramer positive cells; dashed bars represent interferon (IFN)γ-producing T cells; white bars represent TNFα-producing T cells; and gray bars represent interleukin-2- producing T cells. B, Tetramer-binding capacity of CMV TPR-specific T cells of patients 1, 2, and 3 in the peripheral blood before and after infusion of the CMV-specific T cell line. The mean fluorescence intensity of the tetramer staining and the dilution of the tetramer is depicted. Solid lines are before infusion and the broken lines are after infusion of the T cell lines; ■, first infusion; •, second infusion.

Patient 4 developed CMV reactivation within the first month after alloSCT, which was treated with antiviral drugs (**Figure 3**). The rise in CMV load coincided with a rise in CMV TPR-specific T cells. Four months after alloSCT, the patient received unmanipulated donor lymphocyte infusion for the treatment of progressive disease, and 6 months after alloSCT the patient developed GvHD for which he was treated. The CMV-specific T cells disappeared, and the patient developed refractory CMV reactivation. A CMV TPR-specific T cell line was administered 8 months after alloSCT (7.6 x 10^4 T cells/kg) and within 2 weeks CMV TPR-specific CD8$^+$ T cells appeared in peripheral blood, and the CMV load turned negative and remained negative (follow-up >4 y). To asses why no persistence of the CMV TPR-specific

T cells from the first peak response was observed and to determine whether the CMV TPR-specific T cells appearing in the second peak response were the same as the T cells in the first peak response, the patient or donor origin of the CMV-specific T cells from both peak responses (day 33 and day 289 after alloSCT, respectively) was determined. The CMV TPR-specific T cells were isolated from both peak responses by fluorescence-activated cell sorting, and DNA profiling showed that the CMV-specific T cells during the first response (day 33) were of patient origin, and that the CMV-specific T cells that appeared after administration of the T cell line (day 289) were completely donor-derived, illustrating the correlation between the infusion of the CMV TPR-specific T cell line and the in vivo appearance of the donor-derived CMV TPR-specific T cells. Ex vivo stimulation of PBMNC isolated from the patient after adoptive transfer of the T cell line with the specific CMV peptide illustrated high IFNγ and TNFα production by the CMV-specific T cells.

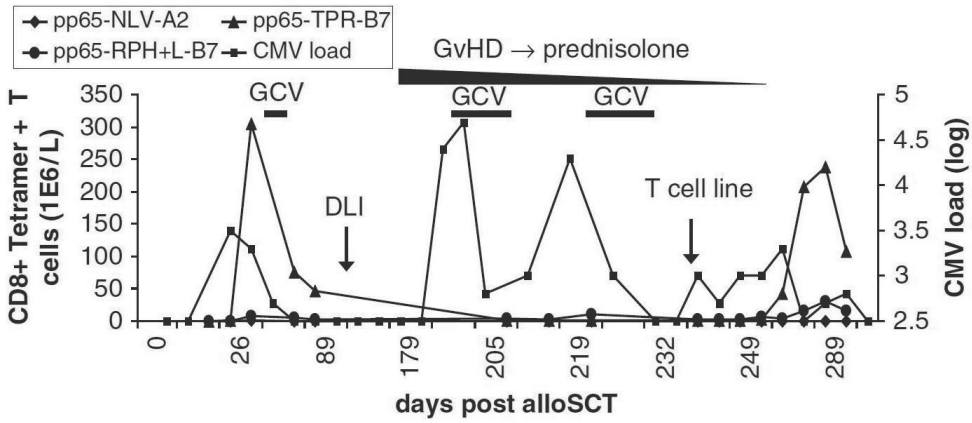

Figure 3
CMV pp65-specific T cells in peripheral blood from patient 4 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells ($CD8^+$/tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis). ▲, CMV TPR-specific T cells; ◆, CMV NLV-specific T cells; ●, CMV RPH-specific T cells. CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis).The lower level of detection is log 2.5. GCV = ganciclovir; GvHD = graft versus host disease.

Patient 9 developed refractory CMV reactivation post- alloSCT in the absence of CMV-specific T cells (**Figure 4**). A CMV NLV-specific T cell line was administrated at day 72 (1 x 10^5 T cells/kg), but no CMV NLV-specific T cells appeared in peripheral blood and the CMV load remained positive. The quality of the T cells in this T cell line was poor because of poor quality, as reflected by the low cell viability, of the starting material, explaining the absence of in vivo persistence of the infused T cells. A second CMV NLV-specific T cell line was generated from new starting material, which was administered at day 116 after alloSCT (3.1 x 10^5 T cells/kg). After 2 weeks, CMV NLV-specific T cells appeared in peripheral blood, which was coincided with a disappearance of CMV DNA load (follow-up >2 y).

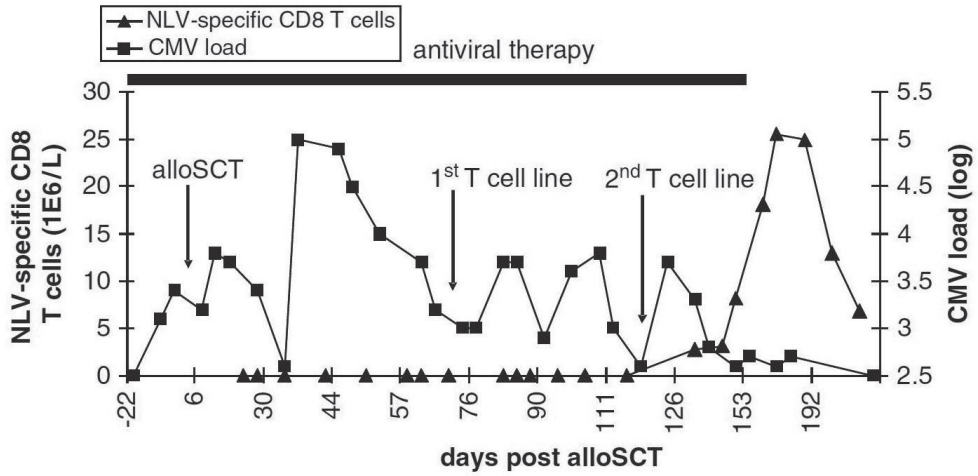

Figure 4.
CMV pp65 (NLV)-specific T cells in peripheral blood from patient 9 in relation to the CMV DNA load in peripheral blood. CMV-specific T cells (▲, $CD8^+$/CMV NLV-tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis) and CMV DNA load (■) is depicted as log CMV DNA load in peripheral blood (right axis). The lower level of detection is log 2.5.

Discussion

In this study, a phase I/II clinical protocol for the adoptive transfer of donor-derived or patient-derived CMV pp65-specific $CD8^+$ T cell lines for patients with refractory CMV reactivation after alloSCT was conducted. No toxicity was observed after administration of the T cell lines, and all patients who received a CMV-specific T cell line cleared CMV reactivation. A direct relation between administration of donor-derived CMV pp65-specific T cell lines and clinical effect could be demonstrated in 2 patients. Limited culture time in vitro using low doses of IL-2 apparently does not impair the ability of T cells to expand in vivo and to be effective. This study illustrates that clinical efficacy of adoptively transferred T cells can be achieved with low cell numbers.

Both donor-derived and patient-derived CMV pp65-specific $CD8^+$ T cell lines could be reproducibly generated, even when frequencies of CMV pp65-specific $CD8^+$ T cells were very low (< 0.01%). Directly after isolation, the purity of the positive fraction (percentage $CD8^+$ / $IFN\gamma^+$ T cells) appeared to be low for some isolation, which was also observed in other studies using the IFNγ-based isolation method.[12,26] This can be due to nonspecific co-isolation of other cells, what preponderates especially in those cases where the starting frequency of CMV-specific T cells is very low. Preferential expansion of the CMV-specific $CD8^+$ T cells during the subsequent culture period led to T cell lines with a high purity of CMV-specific $CD8^+$ T cells, reducing the risk of the induction of GvHD after administration.

In the current study, 6 patients with refractory CMV reactivation received 1 or 2 donor-derived CMV-specific CD8$^+$ T cell line(s). Administration was safe, no toxicity was observed after infusion, and all patients cleared CMV reactivation after treatment with the CMV-specific CD8$^+$ T cell lines. In 4 patients, T cells with the same reactivity as the T cell line could already be detected in peripheral blood of the patients before administration of the T cell lines. Although it is likely that the infused T cells contributed to the clearance of CMV reactivation in these patients, direct evidence for this cannot be provided. No differences in the phenotype, cytokine profile, or tetramer-binding capacity of the NLV-specific or TPR-specific T cells in the peripheral blood before and after adoptive transfer could be determined.

However, in 2 of the 6 patients a direct time correlation between the administration of donor-derived CMV pp65-specific T cell lines, the appearance of CMV-specific CD8$^+$ T cells, and clearance of CMV reactivation was demonstrated. In these patients, no CMV-specific T cells were present before the administration of the T cell lines, and within 2 weeks after administration of the T cell line increasing numbers of CMV NLV-specific or CMV TPR- specific CD8$^+$ T cells could be detected in peripheral blood of the patients, illustrating persistence and in vivo proliferation of the infused T cells. The appearance of the CMV-specific CD8$^+$ T cells coincided with the clearance of CMV reactivation. Although CMV-specific CD8$^+$ T cells have been detected in peripheral blood after the adoptive transfer of CD8$^+$ T cell lines in previous studies, this study shows a direct correlation between the administration of a CD8$^+$ T cell line and the clinical effect.[10,11,13-18]

Two patients eligible for the study deceased because of CMV disease. CMV-specific T cell lines could be generated for these patients, however, both patients deceased before the T cell line could be administered. Decreasing the time interval between the appearance of the clinical need and administration of the T cell product by direct administration of the isolated CMV pp65-specific T cells may improve the efficacy, although in vivo proliferation is also likely to be necessary in these patients to allow a clinical effect. It has been shown that direct administration of CMV-specific T cells isolated based on their IFNγ production can be effective and does not lead to GvHD induction[12]

In the current protocol, T cell lines could only be generated for patients who were HLA-A*0201 and HLA- B*0702$^+$. To increase the potential clinical use of CMV- specific T cells, a CMV pp65 whole protein overlapping 15-mer (11-mer overlapping) peptide pool may be used for the generation of CMV pp65-specific T cell lines. It has been shown by us and others that using this peptide pool CMV pp65-specific T cells can be isolated irrespective of HLA restriction of the donor and patient.[23,27-29] Furthermore, using these peptide pools also CMV-specific CD4$^+$ T cells will be isolated and studies have indicated that adoptively transferred CMV-specific CD4$^+$ T cells may promote development of a CMV-specific CD8$^+$ T cell response in vivo and that adoptively transferred CMV-specific CD8$^+$ T cells may show better persistence in the presence of CMV-specific CD4$^+$T cells.[12,13,15-18,27]

In conclusion, we have shown that the clinical grade generation of donor-derived and patient-derived CMV pp65-specific $CD8^+$ T cell lines is feasible, and that administration of CMV-specific T cell lines to patients with refractory CMV reactivation after alloSCT was safe. A direct relation between infusion of CMV-specific $CD8^+$ T cells and clinical effect could be demonstrated.

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