Treatment of refractory cytomegalovirus reactivation after allogeneic stem cell transplantation with in vitro generated cytomegalovirus pp65-specific CD8+ T cell lines


Submitted
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 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 and/or HLA-B*0702 restricted pp65 peptides (NLV/TPR) and one day after stimulation interferon-gamma (IFNγ) producing cells were enriched using the IFNγ capture assay, and cultured with autologous feeders and low dose IL-2. After 8-14 days of culture quality controls were performed and the CMV-specific T cell lines were administered or cryopreserved. The T cell lines generated contained 1-17x10^6 cells, comprising 54-96% CMV pp65-specific CD8+ T cells, and showed CMV-specific lysis of target cells. To date 14 CMV-specific T cell lines were generated of which 7 were administered to patients with refractory CMV reactivation. After administration no adverse events were observed and CMV load decreased and remained negative. 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 efficacy of administered CMV pp65-specific CD8+ T cell lines was 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 also be performed to prevent the development of severe GvHD after alloSCT. However, T cell depletion or immune suppression significantly delays immune reconstitution in patients after alloSCT, which is associated with an increased risk of opportunistic infections including CMV infection [1-3]. Reactivation of CMV can cause serious morbidity and mortality during the prolonged period of immune deficiency following alloSCT. Reactivation of CMV following 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 following 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, like CMV pp65, 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 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 [10-16]. 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. Since it has been demonstrated that adoptive transfer of interferon-gamma (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 based on their IFNγ production is an attractive strategy for generating effective CMV-specific CD8+ T cell lines [4,6,7,9,15,18,19]. Although clinical studies have shown that adoptive transfer of CMV-specific T cells is safe and effective, in
these studies the infusion of CMV specific CD8+ T cells could not always be directly correlated to a clinical effect.

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 a IFNγ-based isolation technique and cultured for 1 to 2 weeks. Five patients with CMV reactivation failing antiviral therapy were treated with these CMV pp65-specific CD8+ T cell lines. In all five patients CMV DNA load turned negative and CMV-specific T cells could be detected in the peripheral blood. In two patients a direct relation was shown 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 alloSCT were eligible. Patients were weekly monitored for CMV reactivation after receiving a stem cell transplant. Refractory CMV reactivation was defined as high CMV load for more than two weeks while receiving pharmacotherapy or as early relapse after therapy. Since only peptides binding HLA-A*0201 or HLA-B*0702 were available for clinical use and therefore only T cells specific for these epitopes could be isolated, only HLA-A*0201 and/or HLA-B*0702 positive patients were eligible. When the donor was CMV seropositive a donor-derived T cell line was generated, when the donor was CMV seronegative a patient-derived T cell line was generated. When the CMV pp65-specific T cell line met the release criteria and CMV load was still positive the T cell line was administered to the patient. The procedure could be repeated two times in case of stable or progressive disease and no severe toxicity.

Collection of peripheral blood cells

After informed consent at least 2x10^8 peripheral blood mononuclear cells (PBMC) were harvested by leukopheresis from each donor or patient. Red blood cells were lysed using a NH4Cl (8.4 g/l) and KHCO3 (1 g/l) buffer (pH=7.4) (Leiden University Medical Center, Department of Clinical Pharmacy, Leiden, The Netherlands). PBMC were directly used or cryopreserved in the vapor phase of liquid nitrogen until further use. Cells were cryopreserved in Iscove's modified Dulbecco's medium (IMDM) (Lonza, Basel, Switzerland) containing 20 g/L human albumin (CeAlb, Sanquin, Amsterdam, The Netherlands) and 10% dimethyl sulfoxide (DMSO) (Leiden University Medical Center, Department of Clinical Pharmacy, Leiden, The Netherlands).

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

Donor or patient PBMC were resuspended in medium consisting of IMDM supplemented with 3 mmol/L L-glutamine (Lonza) and 100 U/ml penicillin and 100 µg/ml streptomycin (Lonza) plus 10% heat-inactivated pooled prescreened AB serum from healthy blood bank donors. Cells were cultured at a cell concentration of 10x10^6 cells/ml and 1 µg/ml of peptide was added. Peptides used were the HLA-A*0201 binding peptide NLVPVMATV (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 (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer’s instructions. After washing the positive fraction was resuspended in culture medium containing 10 IU/ml IL-2 (Proleukin, Novartis Pharma B.V., Arnhem, The Netherlands). Cells were cultured at a 1:10 ratio with irradiated (30 kGy) feeder cells, which were obtained from the negative fraction after isolation. Cultures were refreshed every 3 to 4 days and after 8 to 14 days the T cell lines were analyzed. Cultures were evaluated for the presence of CMV NLV- 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 within the CD3+ population, (4) the presence of maximally 10% CD19+ B-cells and (5) functionality in a 51Cr release assay 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 Cryocryte Freezing Container (Baxter, Deerfield, IL).

Phenotypic analysis

To determine the composition and phenotype of the starting material, the fractions after isolation and the T cell line, cells were stained using FITC-labeled TCRαβ, CD14, CD4 and HLA-DR (BD Biosciences, San Jose, CA), PE-labeled TCRγδ, CD56, CD25 (BD) and IFNγ (Miltenyi Biotec), PerCP-labeled CD8 and CD3 (BD), APC labeled CD19, CD33 (BD) and CD4 (Beckman Coulter, Fullerton, CA) and PE- or APC-labeled tetramers of the NLVPVMATV binding peptide to HLA-A*0201 or TPRVTGGGAM binding peptide to HLA-B*0702, which
were prepared as described previously [20]. After labeling cells were analyzed on a FACS Calibur (BD).

**Cytotoxicity assay**

To determine the cytotoxicity of the T cell lines standard ⁵¹Chromium (⁵¹Cr) release assays were performed as described previously [21]. Target cells (donor and patient phytohemagglutinin (PHA) blasts) were loaded with 100 µCi of Na²⁹Cr₂O₄ (Amersham, Roosendaal, The Netherlands) for 1 hour and when necessary 1µM of peptide (NLV or TPR) was also added. Effector/Target (E/T) ratios ranged from 30:1 to 1:1 and after 4 hours of incubation ⁵¹Cr release was determined.

**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, PBMC from the patient were stained using HLA class I tetramers specific for NLV or TPR. Subsequently, the-HLA tetramer-positive CD8+ T cells were selected by flow cytometric sorting using a FACS Diva and CellQuest software (BD). The DNA profile of the sorted cells was established by chimera analysis based on short tandem repeats polymorphism as previously described [22].

**Results**

**Generation of clinical grade CMV specific CD8+ T cell lines**

A total of 11 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 both donor and patient were 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 (1A - 1D) and patient-derived (1E – 1H) CMV specific T cell line. The precursor frequencies of the NLV-specific T cells in the PBMC starting material were 0.4% in the donor material (Figure 1A) and 1.45% in the patient material (Figure 1E). After overnight stimulation with the HLA-A*0201 binding NLV peptide, IFNγ producing cells were isolated using the Cytokine Capture System. The positive fraction contained between 20 – 25% IFNγ positive CD8+ T cells within the T cell fraction (Figure 1B and Figure 1F). 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 (Figure 1C and 1G), specific proliferation of the NLV-specific CD8+ T cells resulted in the donor-derived T cell line containing 83% NLV-specific CD8+ T cells and the patient-derived T cell line containing 66% NLV-specific CD8+ T cells.

**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 NLV (A) and TPR (E) -specific T cells in the starting material (day 0); (B, F) the percentage of IFNγ positive cells in the positive fraction directly after isolation; (C, G) the percentage of NLV (C) or TPR (G) in the T cell line after culture (day 7); (D, H) functionality of the T cell lines in a ⁵¹Cr release assay. Dot plots are shown and events shown are gated on lymphocyte and PI-negative gate. Percentage lysis after 4 hours in a ⁵¹Cr release assay at an E:T ratio of 3:1 is depicted.
Less than 1% of CD19+ B-cells were detected in the T cell lines (data not shown). As is shown in Figure 1D and 1H both T cell lines showed reactivity against NLV- or TPR peptide-loaded target cells and no reactivity against donor or patient cells. Furthermore, both T cell lines also showed recognition of endogenously presented antigen using pp65 transduced EBV-LCL.

Table 1 shows the results of all the CMV-specific T cell lines generated. Precursor frequencies of NLV- 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 – 1.45% of total PBMC. Cell numbers obtained direct after isolation ranged from 1.0 to 14.4x10^6 cells and contained between 3 to 45% IFN\(\gamma\) positive CD8+ T cells within the T cell population. During culture no increase in total cell numbers was observed, however, enrichment of CMV-specific T cells was observed, since the T cell lines contained between 54-96% of NLV-and/or TPR-specific T cells.

All 14 donor 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. All T cell lines contained more than 50% of NLV- and/or TPR-specific T cells within the CD3+ population and all T cell lines showed recognition of peptide-loaded PHA blasts and no reactivity against donor or patient derived PHA blasts.

**Administration of CMV-specific CD8+ T cell lines**

In a phase I/II study the toxicity and the potential antiviral effect of treatment with CMV pp65-specific T cell lines for refractory CMV reactivation following allogeneic SCT was investigated. Five patients with refractory CMV reactivation following alloSCT received donor derived CMV pp65-specific T cell line(s). Three patients (patient 2, 3 and 4) received one CMV pp65-specific T cell line and two patients (patient 1 and 9) received two CMV pp65-specific T cell lines. The other T cell lines generated were not administrated, since patients were CMV load negative (n=4), had relapsed (n=1) or deceased due to CMV disease (n=2) at the time the production of the CMV pp65-specific T cell line was completed. Despite treatment with antiviral agents all five patients who received a CMV pp65-specific T cell line had positive CMV DNA loads (log 2.5 to log 3.2) at the time of administration of the CMV specific T cell lines. The patients received a total cell dose ranging from 1.0 to 7.5x10^6 cells, corresponding to 0.6 to 6.1x10^6 NLV- and/or TPR-specific T cells. None of the five patients developed any 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- and/or TPR-specific CD8+ T cells could be detected after administration of the T cell lines. To determine the 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. Prior to infusion of the CMV-specific T cell lines in 3 of the 5 patients CMV pp65-specific T cells with the same specificity as the T cell line could already be detected in peripheral blood. In two of these patients a rise in CMV-specific T cells in peripheral blood was observed after infusion of the T cell line. In the other patient the numbers of CMV-specific T cells remained stable after infusion, however, in this
patient a very high level of CMV-specific T cells was already observed prior to infusion of the T cell line. In 2 of the 5 patients (patient 4 and 9) no CMV-specific T cells were detected prior to 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 peripheral blood, and the clinical effect. Patient 4 developed CMV reactivation within the first month after alloSCT which was treated with antiviral drugs (Figure 2). The rise in CMV coincided with a rise in CMV TPR-specific T cells. Four months after alloSCT the patient received unmanipulated donor lymphocyte infusion (DLI), and six 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 (1x10^6 T cells/kg) and within 2 weeks TPR-specific CD8+ T cells appeared in peripheral blood and the CMV load turned negative and remained negative (follow up >4 years). To assess why no persistence of the TPR-specific T cells from the first peak response was observed and to determine whether the 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 TPR-specific T cells were isolated from both peak responses by FACS sorting and DNA profiling showed that the CMV-specific T cells during the first response (day 33) were patient derived, 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 TPR-specific T cell line and the in vivo appearance of the donor derived TPR-specific T cells.

Patient 9 developed refractory CMV reactivation post alloSCT in the absence of CMV-specific T cells (Figure 3). A CMV NLV-specific T cell line was administrated (day 72), but no CMV NLV-specific T cells appeared in peripheral blood and the CMV load remained positive. The quality of this T cell line was very poor due to poor quality of the starting material and this might explain that there was no in vivo persistence of the infused T cells. A second CMV NLV-specific T cell line was generated, from new starting material, that was administered at day 116 after alloSCT (3.1x10^6 T cells/kg) and after two weeks CMV NLV-specific T cells appeared in peripheral blood, which was coincided with a disappearance of CMV DNA load (follow up >2 years).

Figure 2. 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). , TPR-specific T cells; , NLV-specific T cells; , 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.

Figure 3. 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+/tetramer+) are expressed as absolute numbers per liter in peripheral blood (left axis). , TPR-specific T cells; , NLV-specific T cells; , 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.
Discussion

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. 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.

Both donor- and patient-derived CMV pp65-specific CD8+ T cell lines could be reproducible generated, even when precursor frequencies of CMV pp65-specific CD8+ T cells were very low (<0.01%). Directly after isolation the purity of the positive fraction (% CD8+/IFNγ+ T cells) appeared to be low for some isolations, which was also observed in other studies using the IFNγ based isolation method [18, 23]. This can be due to nonspecific co-isolation of other cells and may also be due to the fact that the antibody on the microbeads used for isolation and the antibody used for analysis of the population recognize the same epitope. 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 five patients with refractory CMV reactivation received one or two 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 three patients, T cells with the same reactivity as the T cell line could already be detected in peripheral blood of the patients prior to 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 can not be provided. However, in two of the five patients a direct 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. No CMV-specific T cells were present prior to the administration of the T cell lines, and within two weeks after administration of the T cell line increasing numbers of NLV- or 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 co-incident 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 [10-17].

References


Two patients eligible for the study deceased due to 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 is effective and does not lead to GvHD induction [18].

In the current protocol T cell lines only could be generated for patients who were HLA-A*0201 and HLA-B*0702 positive. 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 [24-26]. 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 show better persistence in the presence of CMV-specific CD4+ T cells [10, 12, 14-16, 26].

In conclusion, we have shown that the clinical grade generation of donor 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.


