Rapid generation of combined CMV-specific CD4\(^+\) and CD8\(^+\) T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants

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Adoptive transfer of cytomegalovirus (CMV)-specific T cells can restore long-lasting, virus-specific immunity and clear CMV viremia in recipients of allogeneic stem cell transplants if CD4\(^+\) and CD8\(^+\) CMV-specific T cells are detected in the recipient after transfer. Current protocols for generating virus-specific T cells use live virus, require leukapheresis of the donor, and are time consuming. To circumvent these limitations, a clinical-scale protocol was developed to generate CMV-specific T cells by using autologous cellular and serum components derived from a single 500-mL blood draw. CMV-specific T cells were stimulated simultaneously with CMV-specific major histocompatibility complex class I (MHC I)-restricted peptides and CMV antigen. Activated T cells were isolated with the interferon-\(\gamma\) (IFN-\(\gamma\)) secretion assay and expanded for 10 days. In 8 randomly selected, CMV-seropositive donors, 1.34 x 10\(^8\) combined CD4\(^+\) and CD8\(^+\) CMV-specific T cells, on average, were generated, as determined by antigen-triggered IFN-\(\gamma\) production. CMV-infected fibroblasts were efficiently lysed by the generated T cells, and CMV-specific CD4\(^+\) and CD8\(^+\) T cells expanded if they were stimulated with natural processed antigen. On the other hand, CD4\(^+\) and CD8\(^+\) T cell-mediated alloreactivity of generated CMV-specific T-cell lines was reduced compared with that of the starting population. In conclusion, the culture system developed allowed the rapid generation of allodepleted, highly enriched, combined CD4\(^+\) and CD8\(^+\) CMV-specific T cells under conditions mimicking good manufacturing practice. (Blood. 2004; 103:3565-3572)

Introduction

Patients undergoing allogeneic stem cell transplantation (SCT) are still at high risk for cytomegalovirus (CMV)-associated disease despite the introduction of new antiviral prevention and treatment strategies.\(^1\)\(^-\)\(^5\) Intensified immunosuppression or T-cell depletion as increasingly performed for unrelated and mismatched or haploidentical SCT further increases the incidence of and mortality from CMV infection.\(^6\)\(^,\)\(^7\) Prophylactic administration of ganciclovir or foscarnet to patients at high risk may reduce the risk and protect them from fatal CMV disease. However, these prophylactic strategies are complicated by myelosuppression, nephrotoxicity, or both, resulting in a heightened risk for bacterial or fungal infection.\(^1\)\(^,\)\(^4\) In addition, increasing numbers of reports identify clinical CMV strains resistant to current standard antiviral therapy,\(^8\) underscoring the need for new modalities to prevent and treat CMV infection in recipients of allogeneic SCT.

In healthy CMV-seropositive individuals, a high frequency of CMV-specific CD4\(^+\) and CD8\(^+\) T cells mediate control of viral reactivation so that episodes do not become clinically symptomatic.\(^9\)\(^,\)\(^10\) The reconstitution of CMV-specific immune responses after allogeneic SCT is protective against the development of CMV disease.\(^11\)\(^,\)\(^12\) CMV-specific T-cell immunity can be transferred to recipients of allogeneic SCT through the infusion of ex vivo-generated, donor-derived, CMV-specific CD8\(^+\) T cells.\(^13\)\(^,\)\(^14\) The magnitude of the CMV-specific CD8\(^+\) T-cell response decreased with time in patients with no reconstitution of endogenous CMV-specific CD4\(^+\) T\(_H\) cell response.\(^13\)\(^,\)\(^14\) By contrast, the detection of endogenous CMV-specific CD4\(^+\) T\(_H\) cells was associated with sustained CMV-specific CD8\(^+\) T-cell responses.\(^13\)\(^,\)\(^14\) Transferring donor-derived, CMV-specific CD4\(^+\) T cells into recipients of allogeneic SCT can clear CMV viremia, but only if patients demonstrate reconstitution of an endogenous CMV-specific CD8\(^+\) T-cell response.\(^15\) In extension of these data, the concurrent transfer of CD4\(^+\) and CD8\(^+\) EBV-specific T cells results in the long-term persistence of transferred T cells and in protection from EBV-associated posttransplantation lymphoproliferative diseases (PTLDs) in patients at high risk,\(^16\) stressing the importance of transferring both CD4\(^+\) and CD8\(^+\) virus-specific T cells to establish long-lasting viral immunity in recipients of allogeneic SCT.

Different strategies to generate virus-specific T-lymphocytes have been described. Ex vivo induction of CMV-specific T cells using CMV-infected autologous fibroblasts, EBV-infected B cells, or antigen-presenting cells (APCs) transduced with genes of interest as stimulator cells\(^13\)\(^,\)\(^17\)\(^-\)\(^19\) is an effective approach, but the potential biohazard resulting from the presence of live viruses may...
not be applicable to current good manufacturing practice (GMP) standards. Generating CMV-specific T cells using CMV peptide-pulsed dendritic cells (DCs)\textsuperscript{20,21} or CMV antigen-pulsed DCs\textsuperscript{17,22} or using genetically modified APCs\textsuperscript{18,19} is also an effective approach, but each is labor intensive and requires several weeks for the generation of a clinical product. Thus, alternative strategies are warranted to avoid the application of replicative virus during the stimulation procedure and to reduce the time required to generate sufficient CMV-specific T cells for adoptive transfer.

In this study we investigated whether a new isolation strategy for antigen-specific T cells can be translated to current GMP standards for the generation of combined CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cell lines for adoptive transfer into recipients of allogeneic SCT. Our results suggest that sufficient numbers of functional CD4\textsuperscript{+} and CD8\textsuperscript{+} CMV-specific T cells for adoptive transfer can be generated from 8 of 8 CMV seropositive donors from a single 500-ml blood donation in 10 days using only autologous cellular and humoral components for T-cell expansion.

**Patients, materials, and methods**

**Study subjects**

After obtaining informed consent, 500 ml peripheral blood from 8 HLA-A*0201\textsuperscript{+} and HLA-B*0702\textsuperscript{+} healthy, randomly selected, CMV-seropositive blood donors was obtained for isolation of peripheral blood mononuclear cells (PBMCs) and autologous serum.

**Peptides and CMV antigen**

The following peptides were obtained more than 80% pure using high-performance liquid chromatography (HPLC) (Varian star; Zinsser, Munich, Germany): HLA-A*0201 binding peptides NLVPMTAVT (AA 495-503, referred to as NLV) from the CMVpp65 phosphoprotein and VLEETSVML (AA 316-324, referred to as VLE) from the CMVIE antigen, and the HLA-B*0702 binding peptide TPRVTGGGAM (AA 417-425, referred to as TPR) from the CMVpp65 phosphoprotein. CMV lysate was purchased from Biodesign (Saco, ME) or was kindly provided by Viral Antigens (Memphis, TN).

**Isolation of virus-specific T cells**

PBMCs were isolated by Ficoll-Hypaque (BioWhittaker, Walkersville, MD) density-gradient centrifugation of 360 ml heparinized blood. Then PBMCs (2\times10^8) were diluted at 1\times10^7 cells/ml cytotoxic T lymphocyte (CTL) medium (RPMI 1640 [Gibco BRL, Eggenstein, Germany]) plus 10% autologous heat-inactivated serum, 500 IU/ml penicillin G, and 500 \(\mu\)g/ml streptomycin. HLA-restricted CMV synthetic peptides were added to a final concentration of 1 \(\mu\)g/ml, CMV lysate was added to a final concentration of 10 \(\mu\)g/ml, and cells were incubated overnight in a 37°C humidified incubator. Magnetic enrichment of cytokine-secreting cells was performed as described previously\textsuperscript{23-25} using the CliniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were labeled with anti-IFN-\(\gamma\) monoclonal antibody conjugated to leukemia-specific (CD45) antibody (Miltenyi Biotec), diluted at 1\times10^6 cells/ml in CTL medium, and incubated for the IFN-\(\gamma\)-capturing period (45 minutes). Thereafter cells were magnetically labeled with anti-IFN-\(\gamma\) microbeads (Miltenyi Biotec), incubated for 15 minutes at 4°C, and washed with buffer (phosphate-buffered saline [PBS; BioWhittaker] containing 1% autologous serum and 2 mM EDTA [ethylenediaminetetraacetic acid]). Cells were resuspended in 30 ml buffer and then selected with the fully automated CliniMACS device (Miltenyi Biotec) equipped with clinical grade separation columns using the enrichment program 3.1 according to the manufacturer’s instructions (Figure 1).

**Expansion of cells**

Isolated cells were expanded for up to 11 days. Selected cells (5 \times 10^6) were placed in a 24-well plate (Becton Dickinson, Heidelberg, Germany) in the presence of 3500 cGy-irradiated autologous PBMCs (2.5 \times 10^7) in CTL medium containing 50 IU recombinant human interleukin-2 (rIL-2) per milliliter (Proleukin; Chiron, Ratingen, Germany). Cultures were supplemented with fresh medium and 50 U/ml rIL-2 on days 4, 7, and 10.

**Generation of monocyte-derived dendritic cells**

DCs were generated from isolated PBMCs as previously described,\textsuperscript{26} with some modifications. In brief, 1 \times 10^7 PBMCs were plated in each well of a 6-well plate (Becton Dickinson) in RPMI 1640 medium [Gibco] supplemented with 10% heat-inactivated fetal calf serum [FCS; Biochrom, Berlin, Germany] penicillin, and streptomycin). After 2 hours of incubation at 37°C, nonadherent cells were removed by repeated washing with prewarmed PBS, and the remaining adherent cells were cultured in RPMI 1640 medium supplemented with 100 ng/ml human recombinant granulocyte macrophage colony-stimulating factor (GM-CSF) (Leukomax; Novartis, Basel, Switzerland) and 800 IU/ml IL-4 (R&D Systems GmbH, Wiesbaden, Germany). GM-CSF (100 ng/ml) and IL-4 (800 IU/ml) were added again on day 3 in 1 ml fresh medium per well. On day 6, nonadherent cells were collected, counted, transferred into a 24-well plate at a density of 2 \times 10^7 cells/ml, and matured for 24 hours with GM-CSF (100 ng/ml), IL-4 (800 U/ml), IL-1\beta (5 ng/ml; R&D Systems GmbH), IL-6 (10 ng/ml; R&D Systems GmbH), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (25 ng/ml; Boehringer Ingelheim, Vienna, Austria), and prostaglandin E\(_2\) (PGE\(_2\)) (1 ng/ml; Cayman Chemicals, Ann Arbor, MI) in RPMI 10 medium. Further maturation of DCs was confirmed in selected samples by surface staining for CD83 and CD40. For major histocompatibility complex class I (MHC I) presentation, mature DCs were loaded for 4 hours with 10 \(\mu\)g/ml CMV peptide corresponding to the HLA type of the donor. For...
MHC II presentation, 10 μg/mL CMV lysate was added to immature DCs simultaneously with the maturation cocktail for the last 24 hours.

**Cell staining using MHC peptide tetrameric complexes**

Frequency of peptide-specific CD3⁺/CD8⁺ T cells was assessed by staining with phycoerythrin (PE)–labeled tetrameric HLA-A*0201 and HLA-B*0702 peptide complexes (Proimmune, Oxford, United Kingdom), fluoroescein isothiocyanate (FITC)–conjugated anti-CD8, and peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 (Becton Dickinson). Samples were analyzed using FACSCalibur (Becton Dickinson).

**Intracellular IFN-γ staining**

Intracellular cytokine staining was performed as recently described, with the following modifications. PBMCs (2 × 10⁶/mL) were stimulated for 6 hours with 10 μg/mL CMV peptide or 10 μg/mL CMV lysate, T-cell lines were stimulated with autologous lysate/peptide–loaded DCs (effector/stimulator cell ratio, 5:1), both in the presence of the costimulatory monoclonal antibodies CD28 and CD49d (Becton Dickinson; 2 μg/mL each). Brefeldin A (10 μg/mL; Sigma, Deisenhofen, Germany) was added for the last 5 hours of incubation. Positive controls were performed by stimulating the cells with 0.5 μg/mL phorbol 12-myristate 13-acetate (PMA) and 1 μg/mL ionomycin (both Sigma). Samples were permeabilized and stained with fluorochrome-labeled anti-CD8, anti-CD4, or antihuman IFN-γ antibodies (all Becton Dickinson) and were analyzed using FACS-Calibur (Becton Dickinson).

**CFSE staining**

Fluorescence labeling of PBMCs and generated T-cell lines was achieved as described with some modifications. Briefly, cells were washed and labeled with 0.6125 μM carboxy-fluorescein diacetate succinimidyl ester (CFDAS; Molecular Probes, Eugene, OR), unbound CFDASE, or the deacetylated form, CFSE, was quenched by the addition of RPMI 1640 medium containing 15% human serum. Labeled cells were washed twice and plated at 1.0 × 10⁷ cells per milliliter in a round-bottomed, 6-well plate with 2 × 10⁵ mature, autologous DCs preincubated with 10 μg/mL CMV lysate. On day 1 of culture, 5 IU/mL rhIL-2 was added. After 7 days, cells were harvested, counted, and stained with anti-CD8 PE and anti-CD4 PerCP (both Becton Dickinson). Three-color flow cytometry was performed on the flow cytometer (FACSCalibur; Becton Dickinson).

**Cytotoxicity assay**

The capacity of lysing CMV-infected target cells was evaluated with a standard 4-hour chromium Cr 51 (⁵¹Cr) release assay with HLA-A*0201+ CMV-infected and noninfected fibroblasts. Fibroblasts were infected for 2 hours with the CMV strain AD169 or were left uninfected, labeled with CrO₂ for 1 hour at 37°C, washed twice, and added to varying ratios of T-cell lines. After 4 hours of incubation at 37°C and 5% CO₂, supernatants were harvested, and time-resolved chromium release was counted in a gamma counter. For evaluation of peptide specificity, HLA-A*0201+ T cells (174 × CEM.T2 hybridoma, transporter associated with antigen processing-1 [TAP1] and TAP2 deficient) were used as targets. T cells were labeled simultaneously for 1 hour with CrO₂ and the HLA-A*0201 peptides NLY, VLE, and —as a control—an irrelevant MHC 1 peptide, washed twice, and added to T-cell lines. Specific lysis was determined using the formula: % specific lysis = ([experimental release − spontaneous release]/[maximum release − spontaneous release]) × 100.

**Mixed lymphocyte reaction**

Purified CD4⁺ T cells (1 × 10^⁵) isolated with CD4⁺ MicroBeads (Miltenyi Biotech), derived from the starting fraction or after selection and expansion, were cultured with 1 × 10^⁶ third-party mature DCs as stimulator cells for 5 days in 200 μL RPMI 1640 (Gibco) supplemented with 10% FCS in round-bottomed, 96-well microculture plates. To determine unspecific incorporation, DCs and selected CD4⁺ T cells from the starting fraction and after selection and expansion were cultured alone. One μCi (0.037 MBq) [³²P]-thymidine (Amersham, Braunschweig, Germany) per well was added for the last 18 hours of the culture. DNA synthesis was assayed by adding 1 μCi (0.037 MBq) [³¹P]-thymidine (Amersham, Braunschweig, Germany) per well during the last 18 hours of culture. Thereafter the cells were harvested on glass filter paper, and the counts per minute (cpm) were determined using a liquid scintillation counter. Proliferative responses are expressed as the median [³¹P]-thymidine incorporation (cpm) of the stimulated cells minus [³¹P]-thymidine incorporation of the unstimulated control. CD8⁺ T cell–mediated alloreactivity was assessed by in vitro stimulation of purified CD8⁺ T cells (isolated with CD8⁺ MicroBeads; Miltenyi Biotech), derived from untouched PBMCs or from isolated and expanded T cells from the same donor. In brief, 1.75 × 10⁸ CD8⁺ T cells were plated with 0.35 × 10⁶ mature DCs in 1 well of 24-well plate. Cultures were supplemented on days 1 and 3 with 5 IU/mL rhIL-2. On day 7 the total number of viable CD8⁺ T cells per in vitro stimulation (IVS) was calculated by cell counting (exclusion of dead cells by trypan blue dye uptake) and surface staining for CD8⁺.

**Results**

**Generation of combined CD4⁺ and CD8⁺ CMV-specific T-cell lines**

CMV-specific CD4⁺ and CD8⁺ T cells were enriched from HLA-A*0201+ or HLA-B*0702+ PBMCs of 8 randomly selected, healthy, CMV-seropositive blood donors (Table 1) using the IFN-γ secretion assay after stimulation with CMV lysate and the respective CMV peptides. On average, 3.0 × 10⁶ (2 × 10⁵-1 × 10⁷; n = 8) cells were isolated from 2 × 10⁶ to 4 × 10⁸ PBMCs and could be expanded ex vivo after 7 days to 4.6 × 10⁷ cells (7 × 10⁶-2.4 × 10⁷; n = 8) and after 10 or 11 days to 4.6 × 10⁸ cells (8.4 × 10⁷-2.2 × 10⁸; n = 7) (Table 1).

### Table 1. HLA-A and HLA-B of blood donors, with cell numbers before and after enrichment and after 7 and 10 to 11 days of expansion

<table>
<thead>
<tr>
<th>Donor</th>
<th>HLA-A/HLA-B</th>
<th>No. cells before enrichment</th>
<th>No. cells after enrichment</th>
<th>No. cells after 7 days of expansion</th>
<th>No. cells after 10 to 11 days of expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSA2</td>
<td>A2/A68/B51/B65</td>
<td>40 × 10⁶</td>
<td>0.3 × 10⁷</td>
<td>6.5 × 10⁷</td>
<td>ND</td>
</tr>
<tr>
<td>LSA4</td>
<td>A2/A33/B44</td>
<td>30 × 10⁶</td>
<td>0.5 × 10⁷</td>
<td>4.2 × 10⁷</td>
<td>21 × 10⁷</td>
</tr>
<tr>
<td>LSA5</td>
<td>A2/B44/B18</td>
<td>40 × 10⁶</td>
<td>0.1 × 10⁷</td>
<td>2.3 × 10⁷</td>
<td>9.8 × 10⁷</td>
</tr>
<tr>
<td>LSA6</td>
<td>A2/A23/B8/B44</td>
<td>30 × 10⁶</td>
<td>0.09 × 10⁷</td>
<td>0.7 × 10⁷</td>
<td>8.4 × 10⁷</td>
</tr>
<tr>
<td>LSA7</td>
<td>A2/A11/B51/B62</td>
<td>30 × 10⁶</td>
<td>0.13 × 10⁷</td>
<td>4.5 × 10⁷</td>
<td>10 × 10⁷</td>
</tr>
<tr>
<td>LSA8</td>
<td>A2/A3/B7/B8</td>
<td>40 × 10⁶</td>
<td>0.25 × 10⁷</td>
<td>3.6 × 10⁷</td>
<td>102 × 10⁷</td>
</tr>
<tr>
<td>LSA9</td>
<td>A2/A3/A7/B27</td>
<td>30 × 10⁶</td>
<td>1.0 × 10⁷</td>
<td>24 × 10⁷</td>
<td>216 × 10⁷</td>
</tr>
<tr>
<td>LSA10</td>
<td>A2/A7</td>
<td>20 × 10⁷</td>
<td>0.02 × 10⁷</td>
<td>1.0 × 10⁷</td>
<td>10 × 10⁷</td>
</tr>
</tbody>
</table>

* Number of cells after 13 days of expansion.
ND indicates not determined.
Frequency of CMV-specific CD8+ T cells as determined by tetramer staining

Tetramer staining was performed to determine the percentage of CMV peptide-binding CD8+ T cells in the starting fraction and after 7 days of culturing the isolated cells. In the 7 HLA-A*0201+ donors, 0.82% (range, < 0.1%-2.85%; n = 7) of the CD3+/CD8+ lymphocytes bind the HLA-A*0201/pp65495-503 tetramer, and 1.65% (range, < 0.1%-6.45%) bind the HLA-A*0201/IE316-324 tetramer. After 7 days of expansion, 35.7% (range, 3.78%-92.39%; n = 7) and 18.2% (range, < 0.1%-66.61%; n = 7) of the CD3+/CD8+ lymphocytes stained positive for either the CMV pp65 or the CMV IE cognitive tetramer, respectively (Figure 2). The percentage of HLA-B*0702/pp65417-426 tetramer-positive CD3+/CD8+ lymphocytes from the HLA-B*0702+ donors (n = 3) varied between less than 0.1% and 3.70% in the starting fraction and, after 7 days of expansion, increased to 30.37% (7.38%–59.13%) (Figure 2). In 3 donors, tetramer-positive T cells could not be detected for some of the evaluated CMV-epitopes (frequency less than 0.1%) in the starting fraction but could be readily visualized after enrichment and expansion of the T cells. In 4 of 5 donors who demonstrated frequency of more than 0.1% for at least 2 of the tested epitopes in the starting fraction, the relative proportional frequency between the epitope-specific T cells remained stable after enrichment and expansion (Figure 2).

Functional enumeration of CMV-specific CD4+ and CD8+ T cells by intracellular IFN-γ staining

Antigen-triggered production of cytokines in T cells can be used to determine the frequency and functionality of antigen-specific T cells. Therefore, IFN-γ was detected using intracellular cytokine staining (ICC) after activation with either CMV-antigen (CMV-Ag) or CMV-MHC I peptides on the starting cell fraction or after enrichment and 7 days of expansion. The percentage of IFN-γ-producing CD4+ T cells after stimulation with the CMV-Ag was on average 0.57% in the starting PBMC fraction (range, 0.21%-2.03%; n = 8) and increased after enrichment and 7 days of culturing to 27.35% (15.08%-50.91%; n = 8) (shown for one representative donor in Figure 3A). Flow cytometry analysis of the PBMC fraction from the HLA-A*0201+ donors shows on average 0.59% (less than 0.1%-3.04%; n = 7) of the CD8+ T cells producing IFN-γ when stimulated with the NLV peptide and 0.91% (less than 0.1%-5.32%; n = 7) when stimulated with the VLE peptide (results shown for one representative donor in Figure 3A). After enrichment and 7 days of expansion, 26.9% (3.10%-60.39%; n = 7) and 17.7% (less than 0.1%-66.74%; n = 7) of the CD8+ T cells secreted IFN-γ after activation with the NLV peptide or the VLE peptide, respectively (results shown for one representative donor in Figure 3A). The percentage of the CD8+ T cells from the 3 HLA-B*0702+ donors producing IFN-γ after stimulation with the TPR peptide was between 0.16% and 1.53% (n = 3) in the starting PBMC fraction and between less than 0.1% and 16.53% (n = 3) after enrichment plus 7 days of expansion. In all donors (starting fraction and cells after enrichment and expansion), the percentage of IFN-γ-producing CD8+ T cells on stimulation was always lower than the percentage of CD8+ T cells staining positive with the corresponding tetramer (n = 8; data not shown).

In contrast to these results, using peptide-pulsed DCs as stimulators only, 1.31% (range, 0.36%-45.37%; n = 8) of all CD8+ T cells could be activated by CMV-Ag-pulsed mature DCs (data not shown). To exclude the possibility that DCs pulsed with CMV-Ag might have not matured appropriately when compared with DCs incubated with maturation cocktail, CD83 expression was verified 24 hours later in DCs pulsed with peptide or with CMV-Ag. No significant difference was detected by peptide-pulsed or CMV-Ag–pulsed DCs (data not shown).
Based on the percentage of IFN-γ-producing T cells and on cell counts, the absolute numbers of CMV-specific T cells was calculated in the starting fraction and after isolation plus 10-day expansion. As shown for 4 representative donors in Figure 3B-C, the absolute numbers of CMV-specific CD4+ T cells increased on average from 7.3 × 10^5 to 9.0 × 10^5 (n = 8), and the absolute numbers of CMV-specific CD8+ T cells increased on average from 2.1 × 10^6 to 4.4 × 10^6 (n = 8). The observed difference in absolute numbers of combined CD4+ and CD8+ CMV-specific T cells generated from the different donors after 10 days correlates with the absolute numbers of CD4+ and CD8+ CMV-specific T cells in the starting fraction.

**Generated CD4+ and CD8+ CMV-specific T-cell lines expand after restimulation**

To assess whether the generated CMV-specific CD4+ and CD8+ T cells can appropriately divide after stimulation with endogenously processed antigen, T cells were labeled with CFSE and were cocultured for 7 days with mature autologous DCs preincubated with CMV antigen. The intensity of CFSE staining was controlled before stimulation of the CFSE-marked cells with CMV-Ag–loaded DCs. All expanded T cells stained 0.5 log brighter in the FL-1 channel when compared with the uncultured T cells (data not shown). Analysis of CFSE staining on day 7 demonstrated, as depicted for one representative donor in Figure 4, that more than 95% of the isolated and expanded CD4+ and CD8+ T cells underwent at least one cell division, as measured by loss of intensity in the CFSE signal after activation with CMV-Ag–loaded DCs.

**Ex vivo–generated CMV-specific T-cell lines specifically lyse CMV-infected targets**

In vitro cultures of virus-specific T cells can impair the cytolytic capacity of virus-specific CD8+ T cells. To address this question, standard chromium release assays were performed with HLA A*0201+ CMV-infected fibroblasts as targets. CMV-infected fibroblasts were lysed specifically by 3 of 3 CMV-specific T-cell lines derived from HLA-A*0201+ donors, whereas the uninfected fibroblasts were not lysed (Figure 5A). To assess whether CMV pp65- and CMV IE–specific T cells derived from HLA-A*0201+ donors participated in mediating specific lysis of the CMV-infected fibroblasts, T2 cells were pulsed with the respective peptide and used as targets. NLV- and VLE-peptide–pulsed HLA-A*0201+ T2
cells were killed efficiently by donors displaying high frequencies of CD8+ T cells for both epitopes (Figure 5B). In contrast, donors who did not display CMV-specific CD8+ T cells did not lyse specifically CMV-pulsed HLA-A*0201+ T2 cells.

Reduced alloreactivity of the generated CMV-specific T cells

Adoptive transfer of donor-derived T cells into recipients of allogeneic SCT may induce graft-versus-host disease. To investigate, if the generated CMV-specific T-cell lines had reduced alloreactivity when compared with the starting cell fraction, purified CD4+ T cells before and after enrichment and expansion were exposed to mature third-party DCs. In 3 of 3 donors, purified CD4+ T cells from the starting fraction elicited a strong proliferative response against third-party DCs. In contrast, CD4+ T cells derived from the enriched and expanded cultures showed a reduction of more than 95% in alloreactivity when compared with the CD4+ T cells selected from the starting cell fraction (Figure 6A). To assess whether the isolated and expanded virus-specific CD8+ T-cell lines have lost their allogeneic potential, IVS with allogeneic mature DCs was performed and compared with IVS with freshly isolated CD8+ T cells as effectors from the same donor. IVS performed with freshly isolated CD8+ T cells resulted, on average, in a 3.4-fold expansion of allostimulated CD8+ T cells. In contrast, the IVS cultures of the CMV-specific CD8+ T-cell lines stimulated with allogeneic DCs showed a reduction in cell numbers of more than 70% (Figure 6B).

Discussion

Infectious complications remain a serious adverse effect for the success of allogeneic SCT. Among all infectious complications, reactivation or de novo infection with herpes viruses is highly prevalent among SCT recipients and is still associated with high morbidity and mortality. In patients who undergo matched unrelated donor (MUD) SCT, mismatched SCT, or even haploidentical SCT, the risk for herpesvirus viremia and progression to disease rises dramatically, despite novel antiviral prevention and treatment strategies.1-5 Graft manipulation by adoptively transferring virus-specific T cells to the SCT recipients may prevent such infectious complications. Current approaches are time consuming, require leukapheresis of donor for the generation of virus-specific T cells, use replicative competent virus, which is prohibited under current GMP regulations, or isolate CD4+ or CD8+ virus-specific T cells for adoptive transfer.17-22

This study was conducted to circumvent limitations in the generation of virus-specific T-cell lines for adoptive transfer into allogeneic SCT recipients without impairing the functionality of the generated T-cell lines. To facilitate the enrichment of virus-specific T cells, the novel selection approach of the IFN-γ secretion assay was applied to current GMP regulations, and a methodology was developed for the rapid enrichment and expansion of combined CD4+ and CD8+ CMV-specific T cells. In preliminary experiments, using CMV-Ag only to elicit a combined CMV-specific CD4+ and CD8+ T-cell response was compared with simultaneous use of HLA-matched, CMV-specific MHC I epitopes and CMV-Ag. Responses were quantified by 6-hour ICC and demonstrated a clear advantage of using epitopes and CMV-Ag simultaneously (data not shown). These results are in contrast to recently published data22 and may be explained by the shorter incubation period in our assay and the potential difference in the integrity of the CMV-Ag. Incorporation of the preliminary data into the developed protocol resulted, on average, in the generation of 1.3 × 10^6 CMV-specific T cells from 8 of 8 randomly selected CMV-seropositive donors in 10 days from a single 500-mL blood draw using only autologous cellular and humoral components. The generated T-cell lines are enriched for CD4+ and CD8+ CMV-specific T cells, as demonstrated by ICC. Furthermore, CMV-infected targets are killed by generated T-cell lines, mediated potentially by different specificities of CMV-directed CTLs. More important, the generated CMV-specific T cells do not represent terminally differentiated CD4+ and CD8+ T cells; restimulation experiments led to several cell divisions, as demonstrated by the dilution of CFSE dye and a corresponding cell expansion. Thus, adoptive transfer of the generated T cells into SCT recipients may allow further expansion if T cells are stimulated by CMV-Ag–presenting cells in vivo.

The number of CMV-specific T cells and their composition with respect to the CD4+/CD8+ ratio required for prevention or treatment of CMV viremia after allogeneic SCT is not yet defined. Transfer of CMV immunity mediated by CD8+ T cells was established by adoptive transfer of up to 5 × 10^6/m2 T-cell
clones. Nevertheless, persistent specific cellular immunity was only detected in patients with endogenous reconstitution of CMV-specific CD4⁺ T cells. On the other hand, as demonstrated recently, the adoptive transfer of $1 \times 10^9$ to $5 \times 10^9$ CMV-specific CD4⁺ T cells was sufficient in some patients to clear CMV viremia, but only if an endogenous CD8⁺ T-cell response was detected after adoptive transfer of CMV-specific CD4⁺ T-cell lines. In addition, the adoptive transfer of $1 \times 10^7$ to $10^8$ combined CD4⁺ and CD8⁺ EBV-specific T cells into patients receiving T cell-depleted stem cell grafts resulted in long-term persistence and protection from PTLD in these patients. In light of these data, recent experimental observations in mice have implied that adoptively transferred antigen-specific T cells may also undergo homeostatic proliferation independent of antigen and, therefore, replenish the compartment devoid of T cells after allogeneic transplantation. This strategy has been applied recently in a clinical trial by transferring ex vivo expanded autologous tumor-infiltrating lymphocytes into melanoma patients who received, before transfer, a T cell–depleting conditioning regimen consisting of fludarabine and cyclophosphamide. The transferred T cells in these lymphopenic patients expanded rapidly in vivo and represented, in some of them, as much as 80% of all measured CD8⁺ T cells in the peripheral blood. Therefore, early transfer of virus-specific T cells into SCT recipients who underwent transplantation with a T cell–depleted graft could potentially lead to rapid immune reconstitution.

Infusion of donor-derived PBMCs, even in small numbers, may also transfer alloreactive T cells in numbers sufficient to cause severe or even lethal graft-versus-host disease (GVHD), particularly if the donor and the host differ in one or more HLA alleles. On the other hand, extensive culturing of T cells or even establishing T-cell clones may eradicate alloreactive T cells but may also result in replicative senescence of the ex vivo–manipulated virus-specific T cells. In this present study, allogeneic was assessed by stimulating isolated CD4⁺ and CD8⁺ T cells derived from the starting cell fraction and the enriched/expanded cell fraction. In two thirds of donors, the CD4⁺ T cell–mediated allogeneic was completely abolished in the enriched/expanded CMV-specific CD4⁺ T cells and was reduced by more than 95% in the T-cell line from the remaining donor when compared with the purified CD4⁺ T cells from the starting cell fraction. Similarly, no expansion of purified CD8⁺ T cells derived from the enriched/expanded cell fraction could be observed; rather, a reduction of viable CD8⁺ T cells after stimulation with allogeneic DCs was observed despite the presence of IL-2 as a growth and survival factor for T cells. Other groups have also quantified the alloreactivity of their generated virus-specific T-cell lines either by using intracellular cytokine staining or by using third-party phytohemagglutinin (PHA) blasts as targets in a chromium release assay after one round of stimulating their effector cells. Both groups could report on a marked reduction in alloreactivity of the virus-specific T-cell lines by the applied assays. Nevertheless it remains unclear which of the described assays may represent the criterion for assessing the loss of alloreactivity in the generated virus-specific T cells before adoptive transfer and whether the generated data do inversely correlate with the incidence of GVHD after adoptive transfer.

As reported recently by 2 groups and confirmed in 2 donors in this study, some CMV-seropositive patients have a higher frequency of HLA-A*0201–restricted CMV IE-specific CD8⁺ T cells than of HLA-A*0201–restricted CMV pp65–specific CD8⁺ T cells. Considering that CMV IE–specific CD8⁺ T-cell clones do not efficiently kill human CMV (HCMV)–infected fibroblasts and that CMV IE antigen processing and presentation are selectively inhibited by CMV IE65 in HCMV-infected fibroblasts, the question becomes how such high frequencies of CMV IE–specific CD8⁺ T cells are maintained. Intermittent CMV reactivation resulting in the expression of immediate early protein in previously latently infected cells, such as DCs or endothelial cells, before the expression of the late gene CMV IE55, shuts down CMV IE antigen processing. It is conceivable then that CMV IE–specific CD8⁺ T cells contribute significantly to controlling CMV reactivation. Thus, transferring such CMV IE–specific T cells in combination with CMV pp65 into SCT patients without CMV viremia might be advantageous and potentially more potent in preventing reactivation of the CMV virus in recipients of allogeneic SCT than transferring CMV pp65–specific T cells alone.

Apart from the CMV IE peptide and the 2 CMV pp65 peptides used in this study, an increasing number of CMV epitopes are characterized for either CMV IE or CMV pp65, covering 96% of the current, relevant HLA types in the white population. This information will encompass almost every future allogeneic SCT patient with a CMV-seropositive donor. Finally, the newly developed technology is not restricted to patients with CMV infection but may be applied to patients with Epstein-Barr virus and adenovirus infection after allogeneic SCT.

References

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Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants

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