Clinical-scale isolation of ‘minimally manipulated’ cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease

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Abstract

Background aims. Reactivation of cytomegalovirus (CMV) after hematopoietic stem cell transplantation remains a major cause of morbidity despite improved antiviral drug therapies. Selective restoration of CMV immunity by adoptive transfer of CMV-specific T cells is the only alternative approach that has been shown to be effective and non-toxic. We describe the results of clinical-scale isolations of CMV-specific donor lymphocytes with the use of a major histocompatibility (MHC) class I peptide streptamer-based isolation method that yields minimally manipulated cytotoxic T cells of high purity. Methods. Enrichment of CMV-specific cytotoxic T lymphocytes (CTLs) was performed by labeling 1 × 10^9 leukocytes from a non-mobilized mononuclear cell (MNC) apheresis with MHC class I streptamers and magnetic beads. Thereafter, positively labeled CMV-specific CTLs were isolated through the use of CliniMACS (magnetic-activated cell sorting), and MHC streptamers were released through the use of d-biotin. The purity of enriched CMV-specific CTLs was determined on the basis of MHC streptamer staining and fluorescence-activated cell sorting. Results. A total of 22 processes were performed with the use of five different MHC class I streptamers. The purity of enriched CMV-specific CTLs was determined on the basis of MHC streptamer staining and fluorescence-activated cell sorting. Results. A total of 22 processes were performed with the use of five different MHC class I streptamers. The median frequency of CMV-specific CTLs in the starting apheresis product was 0.41% among CD3+ T cells. The isolation process yielded a total of 7.77 × 10^6 CMV-specific CTLs, with a median purity of 90.2%. Selection reagents were effectively removed from the final cell product; the CMV-specific CTLs displayed excellent viability and cytotoxicity and were stable for at least 72 h at 4°C after MNC collection. Conclusions. Clinical-scale isolation of “minimally manipulated” CMV-specific donor CTLs through the use of MHC class I streptamers is feasible and yields functional CTLs at clinically relevant dosages.

Key Words: adoptive T-cell transfer, CliniMACS, CMV-specific T cells, MHC streptamer technology

Introduction

Despite the advent of new antiviral drugs and improvements in immune surveillance protocols, cytomegalovirus (CMV) reactivation in immunocompromised patients remains associated with grave clinical complications and considerable morbidity.

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and mortality (1–3). Administration of new antiviral drugs diminishes the risk of early-onset CMV disease after chemotherapy and hematopoietic stem cell transplantation (HSCT) but is frequently associated with substantial organ toxicity, for example, nephrotoxicity, the development of late-onset CMV disease (4–8), impaired immune reconstitution leading to fungal infections and bacterial sepsis (2,9) and, as a result of viral resistance, is often non-effective (4,10).

Because CMV reactivation arises from impaired CMV-specific T-cell immunity, various adoptive transfer protocols have been developed to restore cellular immunity against CMV (11–13).

The potential of adoptive transfer of CMV-specific cytotoxic T lymphocyte (CTL) clones to restore antiviral immunity after allogeneic stem cell transplantation was first shown by Ridell et al. (11). This approach was based on selective propagation of human CMV-specific CTL clones from bulk donor lymphocytes in vitro, thus exploiting the benefits of donor lymphocyte infusion by transferring CMV-specific T cells contained within the graft while minimizing the risk of an acute graft-versus-host disease (aGvHD). Although this approach is effective, its application has several drawbacks. It is very time-consuming and laborious to prepare suitable cell numbers necessary for an effective restoration of CMV immunity. Patients with an acute CMV infection need immediate medical treatment within days, which does not comply with a protocol of in vitro propagation of T-cell clones for several weeks. Hence, such an in vitro approach is only effective when performed pre-emptively.

Because only a minority of immunocompromised patients have resistant CMV disease, and thus the majority of cell products will never be used, such an approach is by far too costly and labor-intensive to be broadly introduced into the clinic. Moreover, the potential risks for patients, including cross-contamination or introduction of infectious agents into the product during processing, must be considered, as well as the manufacturing process that qualifies the cell product as an advanced therapy medicinal product (ATMP) with all the regulatory complexities associated with this product rating.

To overcome these difficulties regarding the generation of antigen-specific T cells and its clinical use, various approaches have been established aiming for the direct ex vivo selection of virus-specific T cells on the basis of magnetic-activated cell sorting (MACS) technology.

The cytokine secretion assay (CSA) is based on the capture—by means of bi-specific antibodies—of cytokines, such as interferonγ, on the surface of the memory/effector T cells that are induced to produce and secrete cytokines on in vitro stimulation with the respective virus-peptides (14). Thus, after stimulation (4–6 h), virus-reactive T cells can be labeled selectively according to the bound cytokines and subsequently enriched by MACS (15,16). With the use of recombinant protein or peptide-pools consisting of overlapping peptides spanning an entire immunodominant protein, the CSA had already been successfully used to simultaneously enrich CMV-, Epstein-Barr virus—specific and adeno-virus-specific CD4+ T-helper cells and CD8+ CTLs irrespective of human leukocyte antigen (HLA) restriction for therapeutic approaches. Yet, these enrichment protocols were typically followed by further in vitro culture (12,17–20).

The second approach for the enrichment of virus-specific T cells uses the direct labeling of antigen-specific T cells by means of multimeric peptide class I major histocompatibility (MHC) complex staining and subsequent sorting.

Tetrameric MHC class I peptide complexes are soluble complexes of four synthetic biotinylated β2-microglobulin/MHC monomers loaded with an antigenic peptide and streptavidin. By use of multimerization, an exponential increase in avidity is created to generate stable binding of MHC tetramer complexes of four synthetic Strep-tag labeled β2-microglobulin/MHC monomers loaded with an antigenic peptide and streptavidin. By use of multimerization, an exponential increase in avidity is created to generate stable binding of MHC tetramer complexes.
complexes to its cognate T-cell receptors (TCRs). Labeling of streptavidin, which functions as “backbone” for the tetramer complex with fluorochromes or magnetic microparticles, allows specific visualization and prospective enrichment of antigen-specific T cells in combination with fluorescence-activated cell sorting (FACS) or MACS.

Reversible MHC class I multimer staining is performed with the use of the high affinity of d-biotin as a competitor for the binding sites of Strept-Tactin, which can be applied as an analogue of streptavidin and is therefore referred to as MHC streptamer. Thus, multimeric peptide class I MHC complexes, which use the binding of Strep-tag II to Strept-Tactin to form complexes, can be easily disassembled by the addition of d-biotin. Because of their low affinity, peptide MHC class I monomers dissociate from their TCR spontaneously within seconds (Figure 1) (21).

On account of this and in contrast to conventional non-reversible MHC tetramer stainings, reversible MHC streptamer staining does not noticeably alter T-cell function or activate T cells through cross-linking TCRs—which, in the absence of co-stimulatory signals—can lead to induction of apoptosis and in addition excludes possible toxic or immunogenic side effects owing to conferred multimer complexes on the cell surface (22).

However, both multimeric MHC class I peptide staining techniques have been successfully used for enrichment of CMV-specific CTLs for clinical use (23,24).

This study aimed at transferring the laboratory-scale MHC streptamer-based isolation of CMV-specific donor CTLs into a clinical-scale, Good Manufacturing Practice (GMP)-compliant process in preparation of a phase I/II clinical trial in patients with recurrent CMV viremia after stem cell transplantation.

Methods

Donor inclusion

Mononuclear cells (MNC) were collected by means of apheresis from healthy donors who were tested for the presence of CMV-specific CTLs (>0.02% CMV-specific CTLs among CD3+ T cells). CMV-specific CTLs were stained with the use of MHC streptamers as described later, and the frequency of CMV-specific CTLs was determined according the gating strategy shown in Figure 2.

The study protocol was approved by the local ethics committee, and donors gave written informed consent.

Preparation of magnetic MHC streptamer beads

All manufacturing steps were performed under full GMP conditions in the GMP facility of the German Red Cross Blood Donor Service Baden-Württemberg-Hessen, Frankfurt am Main, with a manufacturing authorization from the municipal government within a federally approved Paul Ehrlich

Figure 2. Gating strategy used for the determination of the frequency of viable CMV-specific CD8+ CTLs among CD3+ T cells. Dead cells were excluded from the analysis by gating out 7-AAD—positive cells. The frequency (purity) of CMV-specific CTLs within each fraction (prior enrichment, negative fraction and the final enriched product) are shown for 7-AAD—negative CD3+ lymphocytes.
Institute clinical trial or as part of a process validation in the GMP facility of the Department of Medicine I, University Hospital of Dresden.

All starting materials for the large-scale magnetic enrichment of CMV-specific CTLs, including MHC streptamers and magnetic beads, were manufactured for the purpose of clinical application in GMP-like manner. For release of the starting materials, the batches were further analyzed for identity, sterility and purity and formally released for clinical use by the Department of Quality Management of the German Red Cross Blood Donation Service Baden-Württemberg-Hessen, Frankfurt am Main, or by the Quality Management Department of Medicine I, University Hospital of Dresden. For the magnetic enrichment of CMV-specific CTLs from non-mobilized MNC apheresis, 0.750 mg of magnetic beads and 1 mg of MHC class I peptide (both Stage Cell Therapeutics, Göttingen, Germany) were mixed in a 500-mL transfer bag (Fresenius, Bad Homburg, Germany). CliniMACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) was used.

CliniMACS buffer (340 g for 20 min at 4°C) was added to yield a final volume of 100 mL and incubated for at least 1 h at 4°C. To remove free unbound MHC class I peptide monomers, the transfer bag was connected to a CliniMACS Tubing Set TS (Miltenyi Biotec), and enriched magnetic MHC streptamer beads were collected with the use of Depletion Program 1.2.

Isolation of CMV-specific CTLs

For each magnetic enrichment process, 1.04 × 10^10 leukocytes (median, range; 7.0 × 10^8 to 2.6 × 10^10) from a non-mobilized MNC apheresis were transferred into transfer bags (Fresenius) and CliniMACS buffer (Miltenyi Biotec) containing 20% human serum albumin (HSA) (Baxter, Unterschleißheim, Germany) was added to yield a final volume of approximately 500 mL. Cells were washed twice (210 g for 20 min without brake at 4°C) to remove platelets. After resuspension in CliniMACS buffer yielding a cell concentration of 2 × 10^6 cells/mL, cells were incubated with magnetic MHC streptamer beads for 20 min at 4°C under constant agitation. Unbound magnetic beads were removed by washing the cells twice with CliniMACS buffer/20% HSA (centrifugation, 340 g for 20 min with brake at 4°C). Cells were resuspended in 200 mL of CliniMACS buffer, and magnetically labeled cells were isolated with the use of the CliniMACS, CliniMACS Tubing Set LS and CD34 selection II program (all Miltenyi Biotec). CliniMACS buffer was added to the enriched cell fraction to a final volume of 100 mL. To obtain untouched antigen-specific CTLs, the MHC magnetic streptamers were released by incubation of the isolated cells twice in d-biotin solution (Stage Cell Therapeutics) (final concentration of 1 mmol/L) for 20 min at 4°C under constant shaking. Released MHC streptamers were removed by washing the cells with CliniMACS buffer (340 g for 20 min at 4°C). The release of MHC streptamers by addition of d-biotin was repeated, followed by washing the enriched cells twice with 500 mL of CliniMACS buffer (340 g for 20 min at 4°C). The cells were resuspended in 5–10 mL of HSA. From the positive fraction, 2 mL was taken to determine the number of CMV-specific CTLs and of residual non-CMV-specific, potentially allo-reactive T cells within the sample as well as product sterility. The complete enrichment process was performed under a laminar flow bench (class A) in a clean room (class B).

Calculations and statistical analyses

To enumerate the cell number of white blood cells (WBC), lymphocytes, monocytes, granulocytes and platelets, a Sysmex XT-1800i (Sysmex, Norderstedt, Germany) was used.

For the determination of the frequency of CD3+, CD4+, CD8+, CD14+, CD19+, CD45+ and CD56+ cells by flow cytometry, CD3/CD8/CD45/CD4, CD14/CD45 BD Multitest kit, anti-CD19 fluorescein isothiocyanate (FITC) and CD56 antigen-presenting cells (APC), as well as Calibur and Cellquest software or, as an alternative, a FacsCanto II in combination with FacsCanto Software (all reagents and software BD Biosciences, Heidelberg, Germany), were used.

Purity of CMV-specific CTLs in the final cell product was defined as the frequency of CMV-specific CD8+ T cells among living CD3+ lymphocytes (see gating strategy, Figure 1). Absolute cell number of CMV-specific CTLs was calculated as follows: number of WBC × frequency of CMV-specific CTLs among CD3+ T cells × volume. Data are expressed as mean, standard deviation, median and range. To compare groups for statistically significant differences, the GraphPad prism program (GraphPad Software, La Jolla, CA, USA) and a two-sided Student's t-test for unpaired data were used. A result was considered significant at the 5% level of significance. Linear regression analyses were used when mentioned.

MHC streptamers

The following Strep-tagged MHC class I peptides were used for the multimerization with either phycoerythrin (PE)-conjugated or magnetic bead–conjugated Strep-Tactin complexes: MHC class I HLA-A*0101 CMV pp50_245–253 (VTEHDTLLY), HLA-A*0201 CMV pp65_495–503 (NLVPVMATV), HLA-A*0201 CMV IE_1316–324 (VLEETSVML),
MHC class I HLA-B*0702 CMV pp65<sub>417-426</sub> (TPRVTGGGAM) and HLA-A*2402 CMV pp65<sub>341-349</sub> (QYDPVAALF). All reagents were obtained from IBA Stage Cell Therapeutics. MHC streptamer-PE were multimerized within 2–4 h before staining by incubation of Strep-Tactin PE and MHC class I peptide (both IBA Stage) in cell wash (BD Biosciences) for 1 h at 4°C in the dark.

**Staining of CMV-specific CTLs by MHC streptamer PE**

The purity of enriched CMV-specific CTLs was calculated on the basis of MHC streptamer PE staining and flow cytometric analysis with the use of a FACS Calibur and CellQuest Pro software or FacsCanto II and FacsCanto software (all BD Biosciences).

In brief, approximately 0.2–1 x 10<sup>6</sup> peripheral blood mononuclear cells of each fraction were stained with relevant MHC streptamer PE for 10 min at 4°C, followed by incubation with anti-CD3 FITC and anti-CD8 APC (both BD Biosciences) for 20 min at 4°C in the dark. Cells were washed 2 x with phamrylase buffer and resuspended with cell wash buffer (both BD Biosciences). To exclude dead cells from analysis and determine the viability of cells, either 7-actinomycin D (7-AAD) (BD Biosciences) or propidium iodide (PI) (Sigma Aldrich) was added before FACS analysis to each sample. 7-AAD–positive or PI-positive events were regarded as dead cells.

**Testing of MHC streptamer release by Western blot**

The presence of MHC class I peptides on cells of a CMV HLA-A<sup>*</sup>0201-specific human T-cell line was tested by means of Western blot analysis; 1 x 10<sup>5</sup> cells of a CMV HLA-A<sup>*</sup>0201-specific T-cell line were lysed with a high salt buffer (200 mmol/L NaCl) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cells lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis under non-reducing conditions. Cell lysates were run over a 10% polyacrylamide gel and blotted in the cold room (4°C) for 15 min on ice and used for Western blot analysis. The membrane was then blocked with blocking buffer (phosphate-buffered saline, 5% Tween and 5% milk powder) for 1 h at room temperature. The detection of Strep-tag MHC class I peptides were carried out with the use of Strep-Tactin–horseradish peroxidase (1:4000) in blotting buffer followed by incubation with Western lightning chemiluminescence reagent plus (PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany) according to the manufacturer’s specifications.

**Cytotoxicity**

Cytotoxic activity of the streptamer-isolated CTLs was tested against unloaded or peptide-pulsed HLA-A<sup>*</sup>0201-positive T2 cells in a 4-h standard <sup>51</sup>Cr-release assay. Briefly, T2 cells were loaded with the HLA-A<sup>*</sup>0201-restricted CMV pp65 peptide at a concentration of 50 μg/mL for 3 h and labeled with 100 μCi<sup>51</sup>Cr (sodium chromate, Perkin-Elmer Life Sciences) for 1 h at 37°C in 1 mL of Roswell Park Memorial Institute 1640 medium. <sup>51</sup>Cr-labeled target cells were washed three times and plated in round-bottomed 96-well plates. CTLs that were stored approximately 16–18 h at 4°C after MHC streptamer isolation were added in triplicates to 5 x 10<sup>3</sup> CMV peptide-pulsed, <sup>51</sup>Cr-labeled T2 target cells in wells of a 96-well plate at effector cell–to–target cell ratios of 10:1, 20:1 or 40:1. As negative control, isolated T cells were added to unloaded and T2 target cells loaded with an irrelevant human immunodeficiency virus (HIV) peptide incubated under identical conditions. After 4 h of incubation, 100 μL of supernatant was collected from each well, and the released <sup>51</sup>Cr was determined in a beta-counter (PerkinElmer Life Sciences). Maximal and spontaneous release was measured by treating labeled cells with 2% Triton X-100 (Ferak, Berlin, Germany) or medium alone, respectively. The depicted results represent the mean values of triplicate determinations for three different T-cell enrichments (Figure 5); bars indicate standard error of the mean.

The specific cytotoxicity was calculated according to the formula:

\[
\text{Percent specific lysis} = 100 \times \frac{(\text{counts per minute (cpm) experimental release minus cpm spontaneous release})}{(\text{cpm maximal release minus cpm spontaneous release})}
\]

**Sterility testing**

Growth of aerobic and anaerobic bacteria was tested by inoculating 0.5 mL of the final cell product and 4.5 mL of supernatant from the last washing step BacT/Alert flasks (bioMerieux, Nürtingen, Germany), respectively. The BacT/Alert flasks were then incubated for 7 days according to the manufacturer’s instructions, and growth of bacteria was recorded with the use of an automated microbial detection system (bioMerieux). Positive readings were sent to the Department of Microbiology of Goethe University Medical Centre, Frankfurt am Main, for confirmation and bacteria identification. Only confirmed positive samples were considered contaminated.

**Results**

**Magnetic enrichments with the use of five different MHC streptamers**

A total of 22 processes from 21 MNC aphereses were performed with the use of five different MHC
Details of leukapheresis material (before enrichment) and the enrichment factor 4868 lymphocytes present within the final number of 7.77 accounted for 9.95% (range: 0.2–47.1%) contained in the MNC apheresis, the isolation process yielded a total number of 7.77 × 10^6 (range: 0.11–31.00 × 10^6) CMV-specific CTLs, representing an overall median enrichment factor of 2541 (range: 105–27,440) for CMV-specific CTLs (Table I).

The composition of the final cell product with regard to different lymphocytes population varied and is depicted in Table II. Briefly, the majority of lymphocytes comprised CD3+ T cells (median: 66.0%, range: 23.0–95.0%), of which CD8+ T cells accounted for 47.1% (range: 19.8–91.5%), CD19+ B cells accounted for 9.95% (range: 0.2–77.5%) of total lymphocytes present within the final product exceeding CD4+ T cells (median: 7.7%, range: 1.3–41.0%) and CD56+ natural killer (NK) cells (median: 1.6%, range: 0.0–20.4%), respectively.

Among leukocytes (WBC), the median frequency of lymphocytes within the final cell product was 74.9% (range: 23.3–96.9%). The median frequency of monocytes and granulocytes accounted for 12.0% (range: 1.0–64.7%) and 6.7% (range: 0.2–57.7%) of leukocytes, respectively.

No correlation was found between the frequency of CMV-specific CTLs within the MNC apheresis and in the final cell product (P = 0.214) or between the purity of CMV-specific CTLs and other cell populations within the final cell product (B cells, NK cells, monocytes, granulocytes or platelets; data not shown), which implies that the presence of these cell populations in general does not predict or impair the efficiency of the enrichment process itself. Most importantly, the usage of the two most frequently used MHC streptamers (n > 5) showed no significant differences with regard to the purity of CMV-specific CTLs within the final cell product (Table III). The obtained median purity of 95.4% of CMV-specific CTLs in the final cell product with the use of HLA-B*0702 CMV pp65 streptamers (range: 82.6–98.6%) was not significantly higher (P = 0.08) compared with the median purity of 90.1% when HLA-A*0201 CMV pp65 streptamers (range: 17.7–99.5%) were used. Also, no significant difference was detected between the purity of enriched CMV-specific CTLs with the use of HLA-A*2401 CMV pp65 (median purity: 30.7%, range: 29.9–88.7%) and HLA-A*0201 CMV pp65 (P = 0.16). Significant differences were observed

Table I. Efficiency of MHC streptamer selections.

<table>
<thead>
<tr>
<th>Before enrichment</th>
<th>Mean ± standard deviation</th>
<th>Median (range)</th>
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<tbody>
<tr>
<td>WBCs [1 × 10^9 cells]</td>
<td>1.10 ± 0.48</td>
<td>1.04 (0.07–2.59)</td>
</tr>
<tr>
<td>CD3+ T cells [1 × 10^9 cells]</td>
<td>5.42 ± 3.49</td>
<td>5.64 (0.03–1.61)</td>
</tr>
<tr>
<td>CMV-specific T cells [1 × 10^7 cells]</td>
<td>5.05 ± 11.36</td>
<td>1.14 (0.16–53.28)</td>
</tr>
<tr>
<td>CMV-specific T cells [%]</td>
<td>1.21 ± 3.11</td>
<td>0.41 (0.03–14.8)</td>
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<table>
<thead>
<tr>
<th>After enrichment</th>
<th>Mean ± standard deviation</th>
<th>Median (range)</th>
</tr>
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<tbody>
<tr>
<td>WBCs [1 × 10^7 cells]</td>
<td>3.18 ± 3.08</td>
<td>1.93 (0.11–13.1)</td>
</tr>
<tr>
<td>CD3+ T cells [1 × 10^6 cells]</td>
<td>1.72 ± 2.04</td>
<td>1.25 (0.04–9.03)</td>
</tr>
<tr>
<td>CMV-specific T cells [1 × 10^6 cells]</td>
<td>12.59 ± 18.01</td>
<td>7.77 (0.11–31.00)</td>
</tr>
<tr>
<td>CMV-specific T cells [%]</td>
<td>75.1 ± 27.5</td>
<td>90.2 (17.7–99.5)</td>
</tr>
<tr>
<td>Viability [%]</td>
<td>92.0 ± 8.11</td>
<td>91.5 (75.0–100.0)</td>
</tr>
<tr>
<td>Recovery [%]</td>
<td>100.2 ± 153.7</td>
<td>67.2 (0.9–139.2)</td>
</tr>
<tr>
<td>Enrichment factor</td>
<td>4868 ± 7168</td>
<td>2541 (105–27,440)</td>
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The frequency and absolute cell count of leukocytes subpopulation and platelets within the final product were determined by Sysmex 1800i. The frequency of CD3+, CD4+ and CD8+ T cell subpopulations, CD19+ B cells and CD56+ NK cells was determined by FACS and is given as percentage among living lymphocytes.
when comparing the yielded purity with the use of HLA-B*0702 CMV pp65 streptamers and HLA-A*2401 CMV pp65 ($P = 0.048$). The purities after the enrichment with the use of HLA-A*0101 CMV pp50 (36.7%) and HLA-A*0201 CMV IE-1 (97.8%) streptamers were not considered for statistical analysis because of their infrequent usage (Table III).

However, most importantly, highly significant differences ($P = 0.003$) with regard to the obtained purities of the MHC streptamer-enriched CMV-specific CTLs in the final cell product could be observed when comparing MHC streptamer enrichments before 2010 and thereafter (Figure 3). The median purity of CMV-specific CTLs from 14 MHC streptamer selection processes before the year 2010 was 70.7% (ranging from 17.7% to 97.8%). In contrast, median purity of eight MHC streptamer selections thereafter was 95.2% (ranging from 90.3% to 99.5%; Table IV), which clearly indicates an improvement with regard to process efficiency and reproducibility over time.

**Dissociation of MHC streptamers from T cells**

To obtain fully functional antigen-specific T cells for therapeutic use, MHC streptamers were designed to dissociate from their cognitive TCRs after the addition of d-biotin (22). The dissociation of MHC streptamers was demonstrated by means of staining a human CMV pp65-specific CTL line generated from an MHC class I/HLA-A*0201/CMV pp65-positive donor with MHC streptamer PE (Figure 4A,B). After addition of d-biotin, the staining intensity was reduced to the level of unstained cells (Figure 4A), which demonstrates loss of Strep-Tactin PE bound to the CTLs. The presence of MHC monomers after dissociation was further analyzed by Western blotting. MHC streptamers were detected within the cell lysate of $1 \times 10^5$ cells stained with MHC streptamer PE. After dissociation with d-biotin, MHC streptamers could not be detected within a cell lysate of stained cells, which demonstrates dissociation of MHC monomers from the TCRs caused by the low affinity when not complexed as multimers (Figure 4B).

**Cytotoxic potential of MHC streptamer-enriched CMV-specific CTLs**

The potency displayed by the antigen-specific cytotoxicity is the most crucial functional parameter for the success of this cell therapy. To confirm the cytotoxic potential of MHC, streptamer-enriched CMV pp65-specific CTLs isolated from three healthy donors were tested against unloaded or peptide-pulsed HLA-A*0201 T2 cells in a 4-h standard $^{51}$Cr-release assay (25) at various effector–target cell ratios. CMV pp65-specific CTLs efficiently lysed T2 cells (mean: 77.5% ± 15.5%) loaded with the CMV pp65 peptide at an effector–target cell ratio of 40:1. In contrast, unloaded T2 cells and T2 cells loaded with an irrelevant HLA-A*0201-restricted HIV peptide (pol) were only lysed to a marginal extent (mean: 8.6% ± 3.6% or 9.1% ± 0.5%, respectively) (Figure 5).

**Viability of MHC streptamer-enriched T cells after magnetic enrichment**

To test viability of T cells contained within the final cell product, PI incorporation into the DNA of cells

<table>
<thead>
<tr>
<th>Mean ± standard deviation [%]</th>
<th>Median (range) [%]</th>
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<tr>
<td>Before 2010</td>
<td>63.5 ± 29.1</td>
</tr>
<tr>
<td>After 2010 and later</td>
<td>90.3 ± 3.2</td>
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Table IV. Purity of CMV-specific CTLs among CD3+ T cells in the final cell product manufactured before 2010 and thereafter.
with compromised plasma membrane integrity was determined by means of flow cytometry as described. T cells enriched by means of MHC streptamer technology and CliniMACS were stored for up to 72 h at 4°C, and the cell viability was determined immediately after enrichment as well as after 24 h, 48 h and 72 h of storage at 4°C. Immediately after enrichment, viability of T cells exceeded 95%. After 24 h, the cell viability still amounted to 90.0% and after storage for 48 and 72 h was 81.6% and 68.0%, respectively (Figure 6).

Discussion

CMV reactivation is a significant clinical problem after allogeneic HSCT; in particular, patients with CMV infections refractory to antiviral drugs have a high mortality rate. Antiviral drugs are also associated with substantial toxicity (responsible for severe neutropenia) and the development of late-onset CMV disease. CMV disease is strongly correlated with the impairment of CMV-specific T cell immunity after the conditioning treatment. Thus, the reconstitution of CMV-specific T cell immunity by adoptive T cell transfer has been proposed for the treatment of chemotherapy-refractory CMV disease (11), and several clinical studies have shown its efficacy in patients requiring minimal or no immunosuppression (11,23,24). Adoptive transfer of non-manipulated donor lymphocytes also proved beneficial for the management of CMV and Epstein-Barr virus infections yet is often associated with an aGvHD caused by the presence of excessive doses of alloreactive T cells (26,27). Minimizing the risk of an aGvHD achieved by the generation of CMV-specific T cell clones by repeated stimulation of donor lymphocytes with the respective antigen showed the full potential of adoptive transfer of CMV-specific T cells without concomitant aGvHD (11,28).
MHC streptamers are based on multimer technology and allow fast isolation of fully functional antigen-specific T cells but, in contrast to MHC tetramers, can be easily detached from T cells by addition of d-biotin (22). The complete release of the MHC streptamers from the T cell surface is a crucial requirement for the isolation of “minimally manipulated” and functionally unaltered T cells for clinical use. MHC streptamer-isolated T cells do not enter apoptosis or tolerance as reported for the conventional MHC class I tetramers through their remaining stimulus via their TCRs in the absence of co-stimulatory factors presented by professional APC (29–32). Additionally, the risk of possible toxic or immunogenic side effects owing to conferred multimer complexes on the cell surface is minimized. The Paul Ehrlich Institute and the European Medicines Agency have therefore confirmed these advantages and granted HLA class I streptamer-purified T cells the status of “minimally manipulated” cell products, which are not classified as ATMPs. This classification substantially facilitates the broader application of MHC streptamer-purified T cells for clinical use.

However, the use of MHC streptamer technology requires the knowledge of immunodominant epitopes of the antigen of interest and the availability of the respective MHC class I molecule used for the production of MHC streptamers. Because both knowledge and availability have considerably increased within the last decade, the number of various MHC streptamers that can be used for the isolation of CTLs with various antigen specificities has also continuously grown and with that, the number of eligible patients.

It remains worth mentioning that concerns about the efficacy of the adoptive transfer of CMV-specific CTLs alone, without the co-transfer of CD4+ T-helper cells, have repeatedly been refuted by demonstration that adoptively transferred CMV-specific CTLs are able to mediate sustained protective immunity to CMV (23,24). Whether or not the survival and functionality of the adoptively transferred antigen-specific CTLs depend on the presence of T-helper cells remains to be determined.

The purity, recovery and number of CMV-specific cytotoxic T cells within the final product are essential because these parameters define the dose of transferable, presumably non-alloreactive T cells with known specificity and therefore the potential benefit and risk for the patient. The yielded purity, recovery and viability of CMV-specific T cells are comparable to studies in which MHC class I tetramer technology for the isolation of CMV-specific CTLs was used (23,33). The purity exceeded the yielded purities of CMV-specific T cells through the use of the CSA technology (17,18). Overall, the achieved purity in this work allows the safe administration of a substantial number of CMV-specific CTLs while at the same time minimizing the risk of co-transferring significant numbers of potentially alloreactive T cells. However, a number of MHC streptamer selections resulted in poor purity (frequency <50%). Similar purities of CMV-specific CTLs yielded from magnetic selection had been reported from other authors who used conventional non-reversible MHC class I tetramer staining methods followed by enrichment with the use of anti-PE microbeads. Keenan et al. (33) report purities of 91% of enriched HLA-A*0201 CMV pp65-specific T cells among CD8+ T cells after two subsequent magnetic enrichment processes (range:13.0–99.8%) (33). Slightly higher purities were reported by Cobbold et al. (23) (median: 98.8%; range: 10–99.5%). Both authors also report on individual selections with poor purities of CMV-specific T cells within the final cell product.

To improve the quality of the enrichment process, we sought to identify the pivotal elements that are likely to have an impact on purity.

First, we searched for the seemingly most obvious reason for an effective enrichment process, that is, the frequency of CMV-specific CTLs within the apheresis product.

We were not able to find a clear correlation of significance between the frequency of CMV-specific CTL prior enrichment (contained within the apheresis product) and within the final cell product ($r^2 = 0.06$). Yet, considering the variation regarding the detected initial frequency of HLA-A*0201 CMV pp65, HLA-B*0702 and HLA-A*2401 CMV pp65-specific CTLs, the initial frequency of CMV-specific T cells within MNCs of the apheresis product is likely to have contributed to the relative poor purity observed in individual cases.

In comparing the yielded purities with regard to the MHC streptamer usage, we observed enrichments through the use of HLA-B*0702CMV pp65 exceeding the performance of the remaining MHC streptamers. Whereas the performance of HLA-A*2402 CMV pp65 was significantly reduced

Results on enrichment efficacy

In the present study, we demonstrated the successful transfer of the laboratory-scale MHC streptamer isolation of CMV-specific donor CTLs into a clinical-scale GMP-compliant process for a phase I/II clinical trial in patients with recurrent CMV viremia after HSCT. In total, we performed 22 MHC streptamer-based magnetic enrichments.
compared with HLA-B*0702 CMV pp65 (P = 0.048), no significant differences were observed between HLA-B*0702 CMV pp65 and HLA-A*0201 CMV pp65 (P = 0.079).

The purity after the enrichment with the use of HLA-A*0101 CMV pp50 and HLA-A*0201 CMV IE-1 streptamers was not considered for statistical analysis because of their rare/infrequent usage (Table III). Furthermore, the impact of the presence of CD19+ B cells, CD56+ NK cells, monocytes, granulocytes or platelets with regard to the purity of CMV-specific T cells within the final product was investigated. Their presence showed no correlation with the purity of CMV-specific CTLs, which indicates that the efficiency of the enrichment process is not affected by their presence.

The most striking significant difference with regard to the purities of CMV-specific CTLs within the final cell product was observed when comparing the efficiency of MHC streptamer selection processes before 2010 and thereafter. Taking into account that there were no changes with regard to the MHC streptamer selection process itself, it must be assumed that stability and shelf life of MHC streptamers have had a direct impact on the efficiency of the enrichment processes. From 2010 on, no peptide MHC class I monomers that had been stored more than 3 months at −80°C after shipment were used for multimerization.

Therefore, although the stability and shelf life of various MHC streptamers used for the enrichment of CMV-specific CTLs has not been analyzed in detail, we assume that the stability and shelf life of peptide MHC class I monomers contributed significantly to the efficiency of the MHC streptamer selection processes.

However, in the present report, we discuss complex enrichment processes performed over a period of more than 7 years. Therefore, many factors, such as gaining experience with the enrichment process, the development and improvements of standardized operation procedures required for the production and quality control and eventually the stability of MHC streptamers, must be considered when establishing an efficient enrichment process of antigen-specific CTLs.

**Potency and viability of the enriched CMV-specific CTLs within the final cell product**

The cytotoxic potential of the CMV-specific CTLs for up to 24 h after enrichment was confirmed by the specific lysis of CMV peptide-loaded T2 cells after 4-ht co-incubation with CMV pp65-specific CTLs from three different enrichment processes used for validating the process. MHC streptamer-isolated CTLs contained within the tested final cell products showed superior functional properties with regard to specific killing of CMV pp65-loaded target cells, showing the potency of the CMV-specific CTLs within the final cell product for therapeutic approaches. To test stability during storage, cell viability of the final cell product was tested up to 72 h after enrichment or 96 h after MNC collection, respectively. After 24-h storage at 2–6°C after enrichment, cell viability within all tested final products exceeded 90%, meeting the predefined minimal specification requirements of 80%. The observed mean viability of 81.6% after 48-h storage also fulfilled these minimal requirements, although viability of individual cell products might slightly vary, resulting in slightly poorer cell viability and thus failing the minimal specification requirement. In conclusion, the cell stability appeared to be given for at least up to 48 h, which will provide enough time to ship the final cell product to any transplant center. However, this would require the validation of the potency, which was in our case only validated for a storage time of 24 h.

**Safety**

The sterility of the final products was tested after the selection process. No growth of aerobic or anaerobic bacteria with the use of an automated microbial detection system testing was reported.

**Conclusions**

The MHC streptamer technology-based approach to isolate CMV-specific CTLs results, owing to the reversibility of MHC streptamer binding in “minimally manipulated,” functional T cells, are—according to the verdict of the Committee for Advanced Therapies at the European Medicines Agency—not considered as an ATMP. The major advantage of the MHC streptamer technology is its full GMP compliance. The T cells are stable for up to 48 h after purification (72 h after MNC collection), thus allowing enough time to transfer cells to the respective transplant center.

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Disclosure of interests: D. Busch is holder of the Streptamer patent (US Patent 7,776,562, EP 01 986 941.1). L. Germeroth is CEO of IBA GmbH and Stage Cell Therapeutics, which are commercially distributing streptamer reagents for basic research as well as clinical applications. The other authors declare no competing financial interests.

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**Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcyt.2014.05.023.