Robust Production of Cytomegalovirus pp65-Specific T Cells Using a Fully Automated IFN- γ Cytokine Capture System

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Keywords

Cell processing · Cell therapy · Cytomegalovirus · Cytotoxic T lymphocyte · Immunomagnetic separation · Interferon gamma

Summary

Background: Cytomegalovirus(CMV)-related diseases are a serious cause of morbidity and mortality following hematopoietic stem cell transplantation (HSCT). CMV-specific cytotoxic T lymphocytes (CMV-CTLs) have been reported as an alternative to antiviral drugs that provide long-term CMV-specific immunity without major side effects. However, their application has been limited by the prolonged manufacturing process required. **Methods:** In this study, we applied the IFN- γ cytokine capture system (CCS) using the fully automated CliniMACS Prodigy device for rapid production of CMV-CTLs, which may be applicable in clinically urgent CMV-related diseases. Five validation runs were performed using apheresis samples from randomly selected CMV-seropositive healthy blood donors. Successive processes, including antigen stimulation, anti-IFN- γ labeling, magnetic enrichment and elution, were then performed automatically using the CliniMACS Prodigy, which took approximately 13 h. **Results:** The original apheresis samples consisted mainly of CD45RA⁺ CD62L⁺ naïve T cells as well as 0.3% IFN- γ -secreting CD3⁺ T cells that showed a response to the CMV pp65 antigen (CD3⁺ IFN- γ ⁺ cells). Following IFN- γ enrichment, the target fraction contained 51.3% CD3⁺ IFN- γ ⁺ cells with a reduction in naïve T cells and selection of CD45RA[–] CD62L[–] and CD45RA⁺ CD62L[–] memory

T cells. Furthermore, extended culture of these isolated cells revealed functional activity, including efficient proliferation, sustained antigen-specific IFN- γ secretion, and cytotoxicity against pp65-pulsed target cells. **Conclusion:** The findings reported here suggest that the IFN- γ CCS by the CliniMACS Prodigy is a simple and robust approach to produce CMV-CTLs, which may be applicable for the treatment of clinically urgent CMV-related diseases.

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Introduction

There has been a great deal of research effort to enhance the success of allogeneic hematopoietic stem cell transplantation (HSCT) by improving pre-conditioning regimens and incorporating various immunosuppressants and graft manipulation methods. However, due to excessive immunosuppression, opportunistic pathogen infection has continued to be a serious cause of morbidity and mortality following HSCT. In particular, cytomegalovirus (CMV), a common herpes virus, is a major opportunistic pathogen that is frequently reactivated from latency during prolonged immunosuppression [1]. CMV causes diseases in different organs, including pneumonia, hepatitis, gastroenteritis, retinitis, and encephalitis. With the development of antiviral drugs, such as ganciclovir, foscarnet and cidofovir, the incidence of CMV-related diseases during the early post-transplantation period has decreased significantly. However, the broad use of antiviral drugs as pre-emptive and prophylactic therapies for CMV has produced new chal-

allenges, including severe drug toxicities, higher treatment costs, potential antiviral resistance, delayed CMV-specific T-cell responses, and late-onset CMV-related disease [2]. Currently, ganciclovir is considered the standard agent for prevention and treatment of CMV-related diseases but is associated with high costs and frequent side effects. The increasing use of and exposure to ganciclovir have promoted the occurrence of late CMV-related disease and drug resistance. While second-line treatment is currently in development, the prognosis of patients who do not respond to standard therapy remains poor. Therefore, new therapeutic approaches for CMV reactivation following HSCT are needed.

As adaptive immunity, specifically T-cell immune responses, plays a critical role in viral reactivation and disease, clinical application of cellular immune therapies for CMV have attracted a great deal of interest over the last two decades [3]. Adoptive transfer of CMV-specific T cells can provide prompt control of viral reactivation as well as reconstitution of long-term CMV-specific immunity without major side effects. Despite the consensus that adoptive T-cell therapy may provide potent therapeutic effects, its application has not yet been standardized in the clinical setting and has been restricted to a few academic centers. The main obstacle that arises with cellular therapies is that difficulties remain in efficiently generating sufficient amounts of functional antigen-specific T cells for clinical use. The development of methods for generation of these cells in accordance with current Good Manufacturing Practice (GMP) standards with satisfactory efficacy and cost may allow rapid translation of T-cell therapy into clinical practice.

Several strategies to produce CMV-specific cytotoxic T lymphocytes (CMV-CTLs) have been developed by different investigators and are available for clinical use [4]. Classically, antigen-specific CTLs are generated via extended culture protocols using antigen-presenting cells. Early studies on CMV-CTLs involved live CMV or lysate as the target antigen [5–7]; however, the availability of overlapping peptide pools now allows a simpler and safer manufacturing process [8]. Nonetheless, *ex vivo* culture involving antigen presentation requires a prolonged manufacturing time, which limits its use in emergency settings. Therefore, recent approaches have focused on rapid generation of CMV-CTLs by direct selection technologies, including the use of multimers [9, 10] and the IFN- γ cytokine capture system (CCS) [10–14]. Direct selection methods have allowed simplified manufacturing and quality control processes circumventing regulatory issues. In particular, the IFN- γ CCS has several advantages over the multimer-based selection method, as it is HLA-independent, selects for both cytotoxic and helper T cells, and pre-activates the T cells through antigen-specific stimulation. The IFN- γ CCS has been available for over a decade and has been accompanied by either research-grade MiniMACS [11, 14, 15] or clinical-grade CliniMACS Plus [10, 12–14, 16] (both Miltenyi Biotec, Bergisch Gladbach, Germany). Despite its clinical compliance, CliniMACS Plus still requires considerable hands-on activity by the operator, which introduces potential handling errors and manufacturing inconsistencies. To overcome these limitations, the CliniMACS Prodigy was developed as a technical upgrade over the CliniMACS Plus, allowing a fully automated system. The Clini-

MACS Prodigy (Miltenyi Biotec) was initially described for its automated density gradient separation, including red blood cell depletion, magnetic cell separation, and cell culture [17]. Recently, the integration of IFN- γ CCS software into the CliniMACS Prodigy has proven to be feasible for the automated production of CMV-specific T cells [18].

In this study, we applied the fully automated CliniMACS Prodigy for rapid production of CMV pp65-specific CTLs. Here, we report additional functional data on the final cell product isolated by the CliniMACS Prodigy, which may accelerate its translation into the clinic.

Material and Methods

Donors

Five randomly selected HLA-A*02:01+ or HLA-A*24:02+ CMV-seropositive blood donors were recruited. All donors signed an informed consent form approved by the Institutional Review Board of Seoul St. Mary's Hospital, and all procedures were performed in accordance with the Declaration of Helsinki Protocols (KC15TISI0494). Apheresis was performed using a process volume not exceeding 5 l and a target hematocrit level less than 4%. This study was registered and approved by Clinical Research Information Service, Republic of Korea, in the WHO Registry Network (KCT0001713).

Reagents

Prodigy TS500 tubing sets, MACS GMP CMV pp65 PepTivator, CCS Reagent, CliniMACS PBS/EDTA buffer, TexMACS GMP medium, and Transfer Set Couplers were all kindly provided by Miltenyi Biotec. Sodium chloride solution (0.9%; Dai Han Pharm, Seoul, South Korea) with 2.5% human serum albumin (HSA; Green Cross, Yongin, South Korea) was used as the elution buffer. Cell transfer bags were from Terumo (Tokyo, Japan), and luer/spike adaptors were from Baxter (Zurich, Switzerland).

Isolation of CMV pp65-Specific T Cells Using the CliniMACS Prodigy

The principle of the CCS has been described previously [19], and the CCS process using the CliniMACS Prodigy has also been described recently by Bunos and colleagues [18, 20, 21]. Briefly, PBS/EDTA buffer and elution buffer were first prepared by adding 0.5% and 2.5% HSA (w/v), respectively. The CMV pp65 PepTivator was reconstituted in distilled water and transferred to a transfer bag. The IFN- γ CCS protocol was selected in the Prodigy device, and the tubing set and appropriate buffers were installed manually. Following tubing set priming, PepTivator and the CCS were additionally attached to the tubing set. An aliquot of the apheresis product containing 10^9 cells was added to the application bag connected to the tubing set. The Prodigy device then automatically performed the successive processes, including sample washing, antigen stimulation, Catch-matrix labeling, anti-IFN- γ labeling, magnetic enrichment, and elution. Prior to magnetic enrichment, a quality control sample of the antigen-stimulated product was added manually. The applied sample was eluted into target cell and non-target cell bags following magnetic enrichment. Analyses were performed on samples taken from the quality control, target, and non-target cell bags. In addition, an aliquot of the target cells was used for extension culture.

Expansion of Cells

Isolated cells (1×10^5) were co-cultured with 3,500 cGy-irradiated autologous peripheral blood-derived mononuclear cells (PBMCs; 2.5×10^6) in a 24-well plate. Cultures were suspended in 5% human serum (Sigma-Aldrich, St. Louis, MO, USA) containing AIM-V medium (Life Technologies, Carlsbad, CA, USA) supplemented with 50 IU/ml recombinant human interleukin-2 (rhIL-2; Peprotech, Rocky Hill, NJ, USA). Fresh culture medium and rhIL-2 were added on day 4 and every 2 days up to day 14. Cultures were maintained at 37 °C under 5% (v/v) CO₂.

Table 1. Product properties

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5
<i>Apheresis product (original fraction)</i>					
WBC (total)	1×10^9	1×10^9	1×10^9	1×10^9	1×10^9
Viable leukocytes (viable among CD45+), %	90.5	99.8	94.0	78.4	99.2
CD3+ cells (among CD45+), %	89.3	71.9	66.1	81.7	55.6
IFN- γ -secreting T cells (IFN- γ + CD3+), %	0.30	0.057	0.11	0.31	2.51
<i>Isolated product (target fraction)</i>					
Target cells (CD3+IFN- γ +), %	84.9	63.7	36.1	8.74	51.3
Total target cells (IFN- γ + CD3+ viable cells)	4.7×10^5	3.5×10^5	1.4×10^5	2.7×10^3	4.7×10^5
Non-T target cells (IFN- γ + CD3- viable cells), %	8.11	2.90	3.67	2.58	12.5
Non-target cells (IFN- γ - among CD3+ viable), %	2.61	13.3	45.5	68.5	16.0
Total non-target T cells (IFN- γ - CD3+viable cells)	1.5×10^4	7.3×10^4	1.7×10^5	2.17×10^4	$1.4e \times 10^5$
CD3+ T cells (among CD45+), %	86.2	72.8	84.9	54.0	67.8
CD3- non-T cells (among CD45+), %	13.8	27.2	15.1	46.0	32.2
Viability (% viable among CD45+)	18.3	36.9	63.5	53.4	81.4
<i>Negative product (non-target fraction)</i>					
Target cells (CD3+ IFN- γ +), %	0.80	0.40	0.084	0.054	2.44
Non-target cells (IFN- γ - among CD3+ viable), %	85.2	72.8	72.3	84.2	37.2
CD3+ T cells (among CD45+), %	91.2	75.1	72.4	81.1	58.3
CD3- non-T cells (among CD45+), %	8.84	24.9	27.6	18.9	41.7
Viability (% viable among CD45+)	90.7	96.2	89.8	79.5	97.9

Flow Cytometric Analysis

Immunophenotyping of mononuclear cells was performed by staining for various combinations of surface markers using the following fluorescence-conjugated antibodies: CD45, CD3, CD19, CD56, CD8, CD4, CD45RA, CD45RO, CD62L, CCR7, and IFN- γ . The frequency of CMV pp65-specific CD3+/CD8+ T cells was assessed by staining with pentameric HLA-A*02:01 (pp65, NLVPM-VATV) and HLA-A*24:02 (pp65, QYDPVAALF) peptide complexes (Proimmune, Oxford, UK) and surface markers CD8, CD3, CD4, and CD45. The Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA, USA) was used to exclude dead cells. Flow cytometry was performed using a Fortessa cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star, San Carlos, CA, USA). The flow cytometric gating strategy used in the study is shown in figure 1A.

Intracellular IFN- γ Staining Following Peptide Stimulation

Expanded cells were stimulated for 6 h with CMV pp65 PepTivator (1 μ g/ml; Miltenyi Biotec). Brefeldin A (1 μ g/ml; Sigma-Aldrich) was added for the last 4 h of incubation at 37 °C under 5% (v/v) CO₂. Positive controls stimulated with phorbol 12-myristate 13-acetate (25 ng/ml; Sigma-Aldrich) and ionomycin (250 ng/ml; Sigma-Aldrich) and negative controls without peptide stimulation were included. Cells were first stained for surface markers, including CD45, CD19, CD56, CD3, CD8, and CD4. Intracellular staining of IFN- γ was performed using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. Flow cytometry was performed using the BD Biosciences Fortessa cytometer.

Preparation of Phytohemagglutinin(PHA)-Induced Blasts (PHA Blasts)

PHA -blasts were prepared from autologous PBMCs stimulated with PHA (3 μ g/ml; Sigma-Aldrich) and rhIL-2 (25 IU/ml) in AIM V medium containing 5% human serum for 3–4 days. For peptide pulsing, PHA blasts (1×10^7 /ml) were harvested and incubated with CMV pp65 PepTivator (Miltenyi Biotec) at 2 μ g/ml at 37 °C under 5% (v/v) CO₂ for 2 h.

Cytotoxicity Assay

Flow cytometry-based cytotoxicity assay was performed as described previously [22]. Briefly, target cells (1×10^6 /ml) were labeled with 3,3'-diiodoacetylloxycarbocyanine perchlorate (DIOC-18; Sigma-Aldrich) at 200 μ g/ml in complete culture medium at 37 °C under 5% (v/v) CO₂ for 45 min. Target cells ($1 \times$

10^5) were incubated with the effector cells at effector:target ratios of 6.25, 12.5, and 25 for 4 h. Prior to flow cytometric acquisition, cells were stained with propidium iodide (PI; Sigma-Aldrich) and cytotoxicity was evaluated using a FAC-SCalibur cytometer (BD Biosciences). Target cells were gated on DIOC-18+ cells and then examined for cell death by uptake of PI. The percentage of effector cell-mediated cytotoxicity was then calculated using the following equation:

$$1). \text{ Cytotoxicity (\%)} = \frac{(\text{Dead target cell (\%)} - \text{spontaneous death (\%)}) \times 100}{(100 - \text{spontaneous death (\%)})}$$

Statistical Analysis

Unless otherwise indicated, data are presented as the mean $\pm$ range.

Results

Final Product Properties of the CliniMACS Prodigy IFN- γ CCS

Five validation runs were performed using apheresis samples from healthy donors containing 10^9 cells. Each run took approximately 13 h including the initial priming process. All procedures were performed automatically by the CliniMACS Prodigy CCS software; however, the initial tubing set-up and buffer and antigen preparation prior to the CCS run and quality control sample removal were performed by the operator. Each run resulted in the collection of three fractions: the original (quality control sample) fraction, the non-target fraction, and the target fraction. The product properties of each fraction are listed in table 1 and figure 1B.

After stimulation with CMV pp65 PepTivator for 4 h, the original fraction following pp65 stimulation but prior to magnetic separation consisted of 0.3% CD3+ IFN- γ + T cells (range 0.057–2.51%) with 94% viability (range 78.4–99.8%). Following the IFN- γ CCS process, the target fraction contained 51.3% CD3+ IFN- γ + cells (range 8.74–84.9%) and 3.67% CD3- IFN- γ + cells (range 2.58–12.5%) with 53.4% viability (range 18.3–81.4%). The negative frac-

Fig. 1. Enrichment of CMV pp65-specific T cells by the CliniMACS Prodigy IFN-γ CCS. **A** Gating strategy for flow cytometric analysis. In this sample gating, cells were first gated for lymphocytes (SSC-A vs. FSC-A). The lymphocyte gate was then analyzed to determine lymphocyte expression of CD45 and the numbers of live versus dead cells (first row). Next, the cells were analyzed for IFN-γ expression (second row), pentamer-binding lymphocytes (third row), and memory T cell phenotype (third row). **B** Surface IFN-γ expression of the cell products produced by the CliniMACS Prodigy was assessed by flow cytometry. 'Original' indicates the apheresis sample prior to IFN-γ-based enrichment. 'Target' indicates the positive fraction labeled by the microbeads and eluted from the column. 'Non-target' indicates the negative fraction that is unlabeled and passes through the column during separation. The percentages of IFN-γ-positive T cells are indicated. **C** Pentamer staining of representative donors prior to and following IFN-γ-based enrichment is shown. Cells were co-stained with anti-CD3 and anti-CD8 antibodies. The percentages of pentamer-binding CD3+/CD8+ lymphocytes are indicated. **D** Diagrams show the percentages of pentamer-binding CD3+/CD8+ T cells from each donor (n = 5) prior to and following IFN-γ-based enrichment.

tion contained 0.4% CD3+ IFN- γ + cells (range 0.054–2.44%), similar to that of the original fraction; however, the negative fraction included significantly more IFN- γ - CD3+ cells (median 72%; range 37.2–85.2%) compared with the target fraction (median 16%; range 2.61–68.5%). The CMV pp65-specific CD3+, CD8+ and CD4+ IFN- γ + cells were enriched 120-fold (range 20.4–1,117-fold), 69-fold (range, 6.0–1,850-fold) and 84-fold (range, 6.1–1,957-fold), respectively.

In addition, the frequencies of CMV pp65-specific CD3+ and CD8+ T cells were analyzed by pentamer assays prior to and following enrichment. Interestingly, the ratio of CMV pp65 antigen-specific T cells within the target fraction was enriched compared with the original fraction (fig. 1C,D). Following the IFN- γ CCS, CMV pp65-specific CD3+ T cells were enriched 1.7-fold (range 1.0–14.3-fold), and CMV pp65-specific CD8+ T cells were enriched 2.7-fold (range 1.25–5.40-fold). These results suggest that the IFN- γ + T cells enriched by the IFN- γ CCS include CMV pp65 antigen-specific T cells.

While previous studies indicated that functional T cells are enriched by the IFN- γ CCS, the memory phenotype of the enriched cells was not discussed. In this study, we identified the T-cell subsets prior to and following IFN- γ -based enrichment (fig. 2). Prior to enrichment, the original apheresis sample consisted mainly of CD45RA+ CD62L+ naïve T cells among both CD4+ and CD8+ T cells (fig. 2A,C). Following enrichment, the target fraction showed decreased levels of naïve T cells and increased levels of CD45RA- CD62L- effector memory (TEM) and CD45RA+ CD62L- terminally differentiated effector memory (TEMRA) T cells (fig. 2B,C). CD4+ T cells consisted mostly of TEM (median 62.70%; range

17.30–89.60%) as well as TEMRA cells (median 22.40%; range 10.10–75.00%). Similarly, CD8+ T cells included TEM (median 59.70%; range 16.00–77.10%) and TEMRA cells (median 28.30%; range 2.25–77.60%). These results indicated that cells isolated by the IFN- γ -based CCS show selection of eTEM cells with minimal contamination of naïve cells.

Extended Culture Following Use of the IFN- γ CCS Results in Expansion of Functional CMV pp65-Specific T Cells

To further analyze the functional potential of the isolated CMV-specific T cells, we co-cultured cells from the target fraction with irradiated autologous mononuclear cells as feeder cells for 14 days. While the expansion rate was variable among donors, CMV-specific T cells proliferated efficiently, showing a 42–1,300-fold increase (median 178.5-fold) in the total number of cells (fig. 3A). Although the immunophenotype of the original and target fractions remained similar, expanded cells showed enrichment of CD3+ CD56+ natural killer T cells (median 44.9%; range 6.190–99.60%) and a reduction in CD19+ non-T cells (median 0.073%; range 0.01–1.460%). Furthermore, the ratio of CD4+ to CD8+ T cells decreased, as the CD8+ T-cell population became predominant (CD8+ T-cell median 80.40%; CD4+ T-cell median 10.90%) following expansion (fig. 3B).

In addition, pentamer assays confirmed that the frequency of CMV pp65-specific CD8+ T cells increased markedly during the extension culture period (fig. 3C). The absolute number of CD3+ CMV pp65-specific T cells showed a 431–6,588-fold increase (median 1,021-fold) following 14 days of expansion (table 2). The increase in CMV pp65-specific T cells was correlated with antigen-

Fig. 3. Robust expansion of functional CMV pp65-specific T cells following selection.

CMV pp65-specific T cells isolated by IFN- γ CCS were further expanded using an irradiated autologous feeder layer for 2 weeks. **A** Proliferation of CMV pp65-specific T cells from each donor was evaluated after 14 days of culture. **B** Immunophenotypic analysis of original apheresis products (white bar), target CMV pp65-specific T cells following enrichment (gray bar), and expanded CMV pp65-specific T cells (black bar) was performed by flow cytometry. Percentages are shown as median values, with error bars indicating the ranges.

C Representative FACS analysis of pentamer staining of CMV pp65-specific T cells prior to and following 14 days of expansion. The percentages of pentamer-binding CD3+/CD8+ lymphocytes are indicated on days 0, 7, and 14 of expansion. **D** Representative FACS analysis results of IFN- γ staining of CMV pp65-specific T cells following 14 days of expansion. Cells were stimulated with CMV pp65 peptide mix for 6 hrs. Percentages of IFN- γ + CD3+, CD4+, and CD8+ lymphocytes after stimulation are shown from four donors. Each symbol represents individual cell products from 5 donors, and the columns represent the median values and error bars the ranges.

Table 2. Expansion of CMV pp65 pentamer-specific CD3+ T cells

Donor	% of pp65 pentamer specific T cells after enrichment	No. pp65 pentamer specific T cells included after enrichment	% of pp65 pentamer specific T cells after 14 days of expansion	No. pp65 pentamer specific T cells included after 14 days of expansion	Fold expansion
Donor 1	0.71	1.42×10^3	5.64	7.05×10^6	4,965
Donor 2	0.17	3.40×10^2	0.86	2.25×10^6	6,588
Donor 3	4.3	4.30×10^3	12.5	4.38×10^6	1,021
Donor 4	1.15	17.44×10^1	2.26	7.49×10^3	43
Donor 5	20.5	7.26×10^4	32.4	1.04×10^7	143

specific IFN- γ secretion in response to CMV pp65 antigen (fig. 3D). Finally, expanded CMV pp65-specific T cells showed antigen-specific cytotoxicity against pp65-pulsed target cells, whereas autologous PHA blasts were not susceptible to lysis (fig. 4), in all four donors. Therefore, extended culture following use of the IFN- γ CCS indicated that the isolated cells had high proliferative potential with appropriate stimuli while maintaining their antigen-specific functions.

Expanded Cells Contained a Mixture of Both CD4+ and CD8+ T Cells Exhibiting a Memory Phenotype

To investigate the proliferative potential of naïve cells within the isolated CMV pp65-specific T cells, we examined the changes

in memory T-cell subsets following culture (fig. 5). After 14 days of culture, expanded CMV pp65-specific T cells contained minimal naïve T cells among both the CD4+ (median 0.017%; range 0.00–11.80%) and CD8+ (median 0.034; range 0.005–3.19%) T-cell populations. Interestingly, TEMRA cell numbers were decreased during culture among both CD4+ (median 0.087%; range 0.05–1.31%) and CD8+ (median 0.077%; range 0.02–8.94%) T cells. CD4+ T cells contained a mixture of CD45RA- CD62L+ central memory T cells (median 31.20%, range 14.80–92.00%) and TEM cells (median 68.70%; range 7.79–72.10%). Similarly, CD8+ T cells consisted predominantly of TEM cells (median 72.10%; range 1.62–84.90%) as well as central memory T cells (median 27.90%; range 0.43–97.90%).

Fig. 4. Cytotoxicity of expanded CMV pp65-specific T cells. Specific cytotoxic activity of expanded CMV pp65-specific T cells was evaluated on non-pulsed, CMV-pulsed PHA-induced blasts and lymphoblastoid cell lines by flow cytometry after 4 h of co-incubation. The measured cytotoxicity activities are shown as mean values, with error bars indicating the standard errors of the means from triplicate samples.

Fig. 5. Expanded CMV pp65-specific T cells consist of both central memory and effector memory phenotypes. Memory T-cell subsets of expanded CMV pp65-specific T cells were evaluated by flow cytometry. Subsets were categorized as naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory (T_{EMRA}) T cells based on the surface expression of CD45RA and CD62L. Each symbol represents individual cell products from five donors, and the columns represent the median values. Open circles represent the percentages of the CD4+ T-cell subsets, and closed squares represent the percentages of CD8+ T-cell subsets.

Discussion

In this study, we rapidly isolated CMV pp65-specific T cells by the IFN- γ CCS using the fully automated CliniMACS Prodigy device. We demonstrated through five validation runs that the IFN- γ CCS by the CliniMACS Prodigy provides a simple, robust, and feasible method to manufacture virus-specific T cells. Over the past few decades, most investigators have implemented the classical method, which involves use of antigen presenting cells, live virus, or viral vectors and prolonged culture that takes up to 12 weeks. This lengthy manufacturing time has limited the ready use of CTLs for urgent treatment of CMV reactivation. Advances in manufacturing technologies have allowed investigators to isolate CMV-

CTLs rapidly by immunomagnetic selection of HLA-A2-restricted peptide multimer-positive cells or IFN- γ -secreting T cells following stimulation by CMV peptides. In particular, the IFN- γ -based selection method rapidly selects for CMV-CTLs that secrete IFN- γ in response to stimulation by CMV pp65 peptides. IFN- γ CCS provides several advantages over both the classical and recent enrichment strategies by eliminating the need for antigen-presenting cells, live viruses, and viral vectors and by implementing HLA-unrestricted isolation that yields both CD4+ and CD8+ T cells in the final product.

A fully automated process has recently been made possible with the development of the CliniMACS Prodigy system, thus reducing overall labor intensity and improving standardization. Other investigators have also validated the feasibility of clinical-scale CMV-specific T-cell selection using the CliniMACS Prodigy [18, 20, 21]. However, full characterization of the final product has been insufficient due to the limited numbers of isolated cells. In the present study, we incorporated multicolor flow cytometry and extended cell culture to fully validate the functional and therapeutic potency of CMV-specific T cells enriched by the CliniMACS Prodigy IFN- γ CCS. As the selection is based on antigen-induced IFN- γ secretion, surface expression of IFN- γ has been used as the main marker to validate the isolation of antigen-specific T cells. Consistent with previous studies, we confirmed that the isolated target cell populations expressed IFN- γ (fig. 1B). However, the purity of the target cell population varied (median 51.3%; range 8.74–84.9%). In particular, the isolated product of donor 4 contained 8.74% CD3+ IFN- γ +, 2.58% CD3– IFN- γ +, and 68.5% CD3+ IFN- γ – cells despite completion of the entire procedure without any specific errors. These outlying results for donor 4 suggest that, while automation allows a high degree of standardization of the production process, the final cell product is still highly variable because of donor-intrinsic factors, such as a low initial frequency of IFN- γ -secreting cells.

While IFN- γ expression suggests functional capacity of the isolated cells, we also quantified T cells expressing CMV pp65-specific T-cell receptor by labeling peptide-MHC pentamers. Interestingly, we observed an increased proportion of pp65 pentamer-positive T cells following IFN- γ enrichment, providing direct evidence that the final product included CMV pp65 antigen-specific T cells (fig. 1B,C). Multimer selection is an alternative selection strategy that has been applied in clinical settings to isolate T cells reactive with viral peptides [4]. Despite the high purity and specificity of these cells, the need for HLA typing and donors with a high frequency of circulating antigen-specific T cells severely limited its clinical application. The total number of multimer-selected CMV-specific CD3+ T cells infused has varied from 1.23×10^3 to 2.2×10^5 cells/kg body weight in previous studies [9, 23]. Our results demonstrated that the final product following enrichment included a median of 1.42×10^3 (range 1.74×10^2 – 7.26×10^4) pp65-specific CD3+ T cells (table 2). Importantly, the proportions of IFN- γ + cells and multimer-positive T cells included within the final product showed some discrepancies. The majority of IFN- γ + CD3+ cells were expected to be pp65 TCR multimer-binding T cells, but in reality only a relatively small number of multimer-specific T cells was present following enrichment. A possible explanation for these results is that, while the CMV pp65 peptide mix (PepTivator) used to stimulate the T cells prior to enrichment represented the complete pp65 protein sequence, only one pentamer was used for each HLA-A allele including the most common epitopes (CMV pp65 NLVPMVATV/A*02:01 and QYDPVAALF/A*24:02). Therefore, CTLs specific for rare epitopes may not have been detected. In addition, we were unable to quantify antigen-specific CD4+ T cells using MHC class II multimers, which may also have resulted in underestimation of pp65 multimer-specific CTLs. Nonetheless, it is important that for all donors, enriched IFN- γ + T cells, regardless of the proportion of multimer-specific CTLs, show pp65 antigen-specific functional activity. In an early pre-clinical study, Rauser and colleagues [11] showed that CMV-CTLs isolated by an IFN- γ secretion assay do not necessarily exhibit a high frequency of multimer-binding T cells after enrichment and expansion of isolated cells. Similarly, we showed that extended culture can further expand pp65 pentamer-specific CD3+ T cells up to 6,588-fold (table 2; fig. 3C), suggesting that adequate stimulation, such as in vivo transfer, may stimulate rapid expansion of pp65-specific T cells without alterations in phenotype or function (fig. 3B, D). In addition, although we were unable to detect CMV pp65 pentamer-specific CD4+ T cells, the isolated cell populations included IFN- γ + CD4+ T cells (fig. 1B) at a ratio of approximately 1:1 with CD8+ T cells (fig. 1B, 3B). It has been reported that the involvement of CD4+ T cells may contribute to enhanced survival of CD8+ T cells in vivo, thereby providing better clinical responses [24]. However, whether the IFN- γ -selected CD4+ T cells directly provide antigen-specific cytotoxicity remains to be elucidated.

A potential concern of T-cell therapy is the transfer of naïve T cells that may induce graft-versus-host disease (GVHD) in allogeneic recipients. The risk of GVHD may be reduced by limiting the dose of nonspecific T cells; however, the final product produced by

the CliniMACS Prodigy IFN- γ included non-target IFN- γ - cells as well as small numbers of naïve T cells that may be potentially alloreactive. In a pre-clinical study, CMV-specific T cells selected by IFN- γ showed reduced alloreactivity against third-party dendritic cells [11]. However, the distinct populations of memory T cells were not characterized. We showed that CMV pp65-specific T cells enriched by the IFN- γ CCS have a marked reduction in the number of CD45RA+ CD62L+ naïve T cells (fig. 2) by approximately 7-fold compared with the original apheresis sample. The absolute number of naïve T cells within the final product, which ranged from 1,500 to 46,000 cells (data not shown), may be insufficient to induce GVHD. However, whether the naïve T cells included in the final product are associated with non-target IFN- γ - cells requires further analysis in future studies to fully define the composition and potential risk of contaminating cells. In addition, extended culture of the selected cells further eradicated potentially alloreactive naïve T cells, while the heterogeneity of both central memory and TEM cells was maintained (fig. 5). In addition, IFN- γ -enriched cells included mostly CD45RA- CD62L- TEM cells, which have homing abilities and induce effector functions following antigenic stimulation [25]. Interestingly, CD45RA- CD62L+ central memory T cells were expanded during culture, resulting in a 7:3 ratio of effector memory cells to central memory cells. It has been noted previously that central memory T cells persist longer in vivo following administration [26]. These results are important in that even small numbers of enriched cells may provide a durable response following infusion. Therefore, not only do the enriched/expanded CMV-specific T cells have reduced alloreactivity, but they also have the capacity for long-term survival and potent functional effects in vivo.

In the present study, we combined direct selection of IFN- γ -secreting cells with the T-cell expansion protocol. Although this additional step served mainly to characterize the CMV pp65 antigen-specific T cells, the results suggested that extended cell culture may provide a supplementary approach to antigen-specific T-cell therapy in future studies. Previous studies demonstrated that non-specific expansion of isolated IFN- γ + T cells using irradiated autologous feeder cells did not result in antigen specificity while showing the acquisition of large numbers of cells [11, 15, 16]. Similarly, we showed that expanded IFN- γ + T cells maintain high antigen-specific killing potential and functions (fig. 3, 4). While the IFN- γ CCS itself is promising, for broader application and successful treatment, it may be important to obtain greater numbers of cells. In the HSCT setting, even a cell dose of 360 cells/kg body weight was sufficient to elicit a clinical response [10]. However, patients receiving long-term immunosuppressants may require multiple doses of T cells for durable clinical response, and therefore the isolated final cell product could be allocated, expanded, and cryopreserved for multiple infusions, when needed. Furthermore, the optimal cell dose needed in an immunocompetent solid tumor patient may be different. For example, more than 2×10^7 CMV-specific T cells were infused for the treatment of CMV-associated glioblastoma, and the majority of the patients received up to four repeated infusions [27]. Therefore, expansion of highly enriched an-

tigen-specific T cells following use of the IFN- γ CCS may be useful for overcoming the limitations of solid tumors, such as the immunosuppressive tumor microenvironment. In addition, the CliniMACS Prodigy may be able to provide GMP-compliant and stable culture conditions via automated feeding and medium exchange. As all processes occur within a closed system with minimal manipulation, this may help to overcome some regulatory issues for manufacturing cell therapy products.

In addition to its clinical applicability, the CliniMACS Prodigy provides a more economically feasible therapy compared with current antiviral drugs. The toxicity of antiviral agents requires long-term hospitalization and additional costs for managing side effects. Furthermore, ganciclovir-induced myelosuppression limits its use for prophylaxis, resulting in overtreatment and continuous monitoring of CMV infection necessary for preemptive treatment. In contrast, adoptive CMV-CTL therapy is a safe and efficacious alternative that may successfully prevent CMV-related disease following HSCT. Our observations indicated that CMV-CTLs produced by the CliniMACS Prodigy have a high functional capacity and potential for long-term survival, providing evidence that reconstitution of CMV-specific immunity may be possible following administration. This will promote a significant reduction in treatment costs by eliminating treatment-related adverse events and avoiding unnecessary pharmacological management. Importantly, the reduced clean room requirements of the CliniMACS Prodigy allow treatment in outpatient clinics, thus reducing the need for hospital admission. Further studies and actual clinical trials using the CliniMACS Prodigy will be necessary to accurately characterize its benefits.

References

- Gandhi MK, Khanna R: Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis* 2004;4:725–738.
- Ljungman P: CMV infections after hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2008; 42(suppl 1):S70–S72.
- Peggs KS: Adoptive T cell immunotherapy for cytomegalovirus. *Expert Opin Biol Ther* 2009;9:725–736.
- Heslop HE, Leen AM: T-cell therapy for viral infections. *Hematology Am Soc Hematol Educ Program* 2013;2013:342–347.
- Einsele H, Roosnek E, Rufer N, Sinzger C, Riegler S, Löffler J, Grigoleit U, Moris A, Rammensee HG, Kanz L, et al: Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood* 2002;99:3916–3922.
- Peggs KS, Verfuert S, Pizzey A, Khan N, Guiver M, Moss PA, Mackinnon S: Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet* 2003;362:1375–1377.
- Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe KS, Thomas ED, Riddell SR: Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 1995;333:1038–1044.
- Micklethwaite K, Hansen A, Foster A, Snape E, Antonenas V, Sartor M, Shaw P, Bradstock K, Gottlieb D: Ex vivo expansion and prophylactic infusion of CMV-pp65 peptide-specific cytotoxic T-lymphocytes following allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2007;13:707–714.
- Cobbold M, Khan N, Pourghesari B, Tauro S, McDonald D, Osman H, Assenmacher M, Billingham L, Steward C, Crawley C, et al: Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med* 2005;202:379–386.
- Feuchtinger T, Opher K, Bethge WA, Topp MS, Schuster FR, Weissinger EM, Mohty M, Or R, Maschan M, Schumm M, et al: Adoptive transfer of pp65-specific T cells for the treatment of chemorefractory cytomegalovirus disease or reactivation after haploidentical and matched unrelated stem cell transplantation. *Blood* 2010;116:4360–4367.
- Rausser G, Einsele H, Sinzger C, Wernet D, Kuntz G, Assenmacher M, Campbell JD, Topp MS: Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* 2004;103:3565–3572.
- Meij P, Jedema I, Zandvliet ML, van der Heiden PL, van de Meent M, van Egmond HM, van Liempt E, Hoogstraten C, Kruithof S, Veld S, et al: Effective treatment of refractory CMV reactivation after allogeneic stem cell transplantation with in vitro-generated CMV pp65-specific CD8+ T-cell lines. *J Immunother* 2012;35:621–628.
- Peggs KS, Thomson K, Samuel E, Dyer G, Armoogum J, Chakraverty R, Pang K, Mackinnon S, Lowdell MW: Directly selected cytomegalovirus-reactive donor T cells confer rapid and safe systemic reconstitution of virus-specific immunity following stem cell transplantation. *Clin Infect Dis* 2011;52:49–57.
- Tischer S, Priesner C, Heuft HG, Goudeva L, Mende W, Barthold M, Kloess S, Arseniev L, Aleksandrova K, Maecker-Kolhoff B, et al: Rapid generation of clinical-grade antiviral T cells: selection of suitable T-cell donors and GMP-compliant manufacturing of antiviral T cells. *J Transl Med* 2014;12:336.
- Hammer MH, Meyer S, Brestrich G, Moosmann A, Kern F, Tesfa L, Babel N, Mittenzweig A, Rooney CM, Hammerschmidt W, et al: HLA type-independent generation of antigen-specific T cells for adoptive immunotherapy. *Eur J Immunol* 2005;35:2250–2258.
- Brestrich G, Zwinger S, Fischer A, Schmuck M, Rohm-hild A, Hammer MH, Kurtz A, Uharek L, Knosalla C, Lehmkuhl H, et al: Adoptive T-cell therapy of a lung transplanted patient with severe CMV disease and resistance to antiviral therapy. *Am J Transplant* 2009;9: 1679–1684.
- Sorg N, Poppe C, Bunos M, Wingenfeld E, Hummer C, Kramer A, Stock B, Seifried E, Bonig H: Red blood cell depletion from bone marrow and peripheral blood buffy coat: a comparison of two new and three established technologies. *Transfusion* 2015;55:1275–1282.

In conclusion, we demonstrated that the IFN- γ CCS by CliniMACS Prodigy provides robust production of functional CMV pp65-specific T cells, which may be a promising therapeutic approach for CMV-related diseases following HSCT. In Europe, the CliniMACS Prodigy and related components are currently available as CE-marked medical devices. However, the final cell product is regulated as an Advanced Therapy Medicinal Product under Regulation 1394/2007/EC. In the USA and Asia-Pacific region, the CliniMACS Prodigy has been approved for research use only and requires approved Investigational New Drug application or Investigational Device Exemption for human therapeutic use. We believe that the future outlook for the CliniMACS Prodigy is promising. Translation of the CliniMACS Prodigy system into the clinic may be accelerated worldwide as it has the potential to overcome the regulatory challenges of cellular therapy with its closed system, automation, and standardized materials and software.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (NRF-2016R1C1B2010800) and supported by research fund of Seoul St. Mary's Hospital, The Catholic University of Korea.

Disclosure Statement

The authors declare that they have no conflicting interests.

- 18 Bunos M, Hummer C, Wingenfeld E, Sorg N, Pfirmann V, Bader P, Seifried E, Bonig H: Automated isolation of primary antigen-specific T cells from donor lymphocyte concentrates: results of a feasibility exercise. *Vox Sang* 2015;109:387–393.
- 19 Saglio F, Hanley PJ, Bollard CM: The time is now: moving toward virus-specific T cells after allogeneic hematopoietic stem cell transplantation as the standard of care. *Cytotherapy* 2014;16:149–159.
- 20 Kumaresan P, Figliola M, Moyes JS, Huls MH, Tewari P, Shpall EJ, Champlin R, Cooper LJ: Automated Cell Enrichment of Cytomegalovirus-specific T cells for Clinical Applications using the Cytokine-capture System. *J Vis Exp* 2015;(104): doi: 10.3791/52808.
- 21 Priesner C, Esser R, Tischer S, Marburger M, Aleksandrova K, Maecker-Kolhoff B, Heuft HG, Goudeva L, Blasczyk R, Arseniev L, et al: Comparative analysis of clinical-scale IFN-gamma-positive T-cell enrichment using partially and fully integrated platforms. *Front Immunol* 2016;7:393.
- 22 Hoppner M, Luhm J, Schlenke P, Koritke P, Frohn C: A flow-cytometry based cytotoxicity assay using stained effector cells in combination with native target cells. *J Immunol Methods* 2002;267:157–163.
- 23 Schmitt A, Tonn T, Busch DH, Grigoleit GU, Einsele H, Odendahl M, Germeroth L, Ringhoffer M, Ringhoffer S, Wiesneth M, et al: Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* 2011;51:591–599.
- 24 Merlo A, Turrini R, Bobisse S, Zamarchi R, Alaggio R, Dolcetti R, Mautner J, Zanollo P, Amadori A, Rosato A: Virus-specific cytotoxic CD4+ T cells for the treatment of EBV-related tumors. *J Immunol* 2010;184: 5895–5902.
- 25 Sallusto F, Geginat J, Lanzavecchia A: Central memory and effector memory T cell subsets: function, generation, and maintenance. *Annu Rev Immunol* 2004;22: 745–763.
- 26 Berger C, Jensen MC, Lansdorp PM, Gough M, Elliott C, Riddell SR: Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. *J Clin Invest* 2008;118:294–305.
- 27 Schuessler A, Smith C, Beagley L, Boyle GM, Rehan S, Matthews K, Jones L, Crough T, Dasari V, Klein K, et al: Autologous T-cell therapy for cytomegalovirus as a consolidative treatment for recurrent glioblastoma. *Cancer Res* 2014;74:3466–3476.