Coordinated isolation of CD8+ and CD4+ T cells recognizing a broad repertoire of cytomegalovirus pp65 and IE1 epitopes for highly specific adoptive immunotherapy

Maarten L. Zandvliet
Ellis van Liempt
Inge Jedema
Louise A. Veltrop-Duits
Roel Willemze
Henk-Jan Guchelaar
J.H. Frederik Falkenburg
Pauline Meij

Cytotherapy 2010, 12: 933-944

Comment in:
Cytotherapy 2010, 12: 857-858
Abstract

Background
Adoptive transfer of CMV-specific memory T cells can be used for treatment of CMV reactivation after allogeneic stem cell transplantation. Since coordinated CD8+ and CD4+ T cells specific for a broad repertoire of CMV epitopes may be most effective for adoptive immunotherapy, the aim of this study was to isolate these cells from peripheral blood of CMV seropositive donors, irrespective of their HLA type.

Methods
Activation of CMV-specific CD8+ and CD4+ T cells was compared after stimulation of donor peripheral blood with minimal epitope peptides, pools of overlapping 15-mer peptides, or full-length protein. Furthermore, the kinetics of IFNγ production after stimulation was analyzed to determine the optimal time-point for IFNγ-based isolation of CMV-specific T cells. The specificity, phenotype and functionality of generated T cell lines were analyzed.

Results
CMV protein-spanning 15-mer peptide pools induced simultaneous activation of both CD8+ and CD4+ CMV-specific T cells, while full-length CMV protein only efficiently activated CD4+ CMV-specific T cells. Isolation of IFNγ-secreting cells at the peak of the IFNγ response after 4 hours stimulation with CMV pp65 and IE1 peptide pools resulted in efficient enrichment of CMV-specific T cells. The T cell lines contained high frequencies of CD8+ and CD4+ T cells recognizing multiple CMV pp65 and IE1 epitopes, and produced IFNγ and TNFα upon specific restimulation.

Conclusion
This study provides a feasible strategy for the rapid generation of clinical grade CD8+ and CD4+ T cell lines with high specificity for multiple CMV pp65 and IE1 epitopes, which may be used for effective adoptive immunotherapy.

Introduction
In healthy individuals, recurrent reactivations of cytomegalovirus (CMV) are controlled by CMV-specific CD8+ and CD4+ T cells [1]. However, CMV reactivation can cause serious disease in the absence of an adequate CMV-specific T cell response in immunocompromised recipients of allogeneic stem cell transplantation (alloSCT) [2,3]. Pharmacological treatment of CMV reactivation is limited by toxicity and not sufficient for long-term anti-viral protection [4-6]. It has been shown that reconstitution of CD8+ and CD4+ T cells recognizing epitopes derived from immunodominant CMV proteins pp65 and IE1 in the first year after transplantation was associated with sustained protection from CMV disease [7-11]. Several clinical studies have demonstrated that reconstitution of donor-derived CMV-specific T cells by adoptive transfer can be a safe and effective treatment for CMV reactivation after alloSCT [12-21].

Clinical trials have indicated that adoptively transferred CMV-specific CD4+ T cells may promote development of a CMV-specific CD8+ T cell response in vivo [18-21]. Furthermore, adoptively transferred CMV-specific CD8+ T cells showed better persistence in the presence of CMV-specific CD4+ T cells [12]. These observations indicate the requirement of a CMV-specific CD4+ T cell response in the development of a sustained CMV-specific CD8+ T cell response and anti-viral protection, which has also been described after primary CMV infections in renal transplant recipients [22,23]. CMV-specific CD4+ T cells do not only support the development and function of CD8+ T cells, but can also exert direct cytolytic activity [24]. These data provide a rationale for the generation of CMV-specific T cell lines including both CD8+ and CD4+ T cells for adoptive immunotherapy.

Polyclonal CMV-specific T cell responses in healthy individuals are directed against a broad repertoire of epitopes from multiple CMV proteins [1,25]. Furthermore, restoration of immune protection against CMV in immunodeficient individuals has been shown to be associated with an enlarged repertoire of CMV-specific T cells [26]. Adoptive transfer of a coordinated CD8+ and CD4+ T cell response against multiple CMV epitopes may therefore be most effective for treatment of CMV disease in alloSCT recipients. Several strategies have been developed to isolate virus-specific memory T cells from donor peripheral blood for the generation of virus-specific T cell lines without the need for long-term in vitro culture. Clinical grade peptide-MHC class I multimers have become available, which can be used for isolation of epitope-specific CD8+ T cell populations due to the high affinity for a specific T cell receptor (TCR) [13,27,28]. However, to provide T cell therapy for every patient at risk of developing CMV disease and to generate T cell lines against multiple epitopes, this method requires knowledge of defined epitopes restricted by prevalent MHC
class I molecules, and production of numerous peptide-MHC multimers. Furthermore, antigen-specific CD4+ T cells cannot be isolated using this method due to the lack of functional peptide-MHC class II multimers. Alternatively, virus-specific memory T cells can be isolated based on activation-induced IFNγ production. Stimulation with minimal peptides corresponding to defined MHC class I restricted epitopes followed by IFNγ-based isolation has been demonstrated to result in efficient isolation of CMV-specific CD8+ T cells, but requires epitope knowledge as well [16,29]. Although this method may be extended to the isolation of CMV-specific CD4+ T cells, only a limited number of minimal peptides corresponding to defined MHC class II restricted epitopes has been characterized.

The use of full-length CMV proteins for stimulation circumvents the need of knowing the exact epitopes, and can provide CMV-specific T cell lines irrespective of the HLA type of the patient. However, the generation of a coordinated CD8+ and CD4+ CMV-specific T cell response from donor peripheral blood has been shown to be difficult. CMV antigen endogenously synthesized by transduction of specific cDNA into antigen-presenting cells was predominantly presented in MHC class I, while exogenously added CMV proteins or lysates resulted in selected presentation of CMV peptides in MHC class II [14,17-21,30]. Interestingly, 15-mer peptides have recently been described to simultaneously induce activation of both CD8+ and CD4+ T cells specific for CMV epitopes, and may be used for the generation of combined CD8+ and CD4+ T cell lines [31-33]. Synthetic protein-spanning pools of overlapping 15-mer peptides can be produced under good manufacturing practice (GMP) conditions more easily than recombinant proteins or vectors for genetic modification, and may therefore allow feasible and cost-effective production of CMV-specific T cells for adoptive immunotherapy.

The aim of this study was to develop a method for the generation of clinical grade coordinated CD8+ and CD4+ T cell lines with high specificity for immunodominant CMV proteins pp65 and IE1, irrespective of the HLA type of the patient. Full-length protein-spanning pools of overlapping 15-mer peptides induced simultaneous activation of both CMV-specific CD8+ and CD4+ T cells. To achieve high frequencies of CMV-specific T cells, activated T cells were isolated at the peak of the IFNγ response by the IFNγ capture assay. This strategy resulted in coordinated CD8+ and CD4+ T cell lines recognizing multiple CMV pp65 and IE1 epitopes, which can be generated irrespective of the patient HLA type, and may be used for treatment of CMV reactivation after alloSCT.

### Material and Methods

#### Donor cells

After informed consent, peripheral blood was obtained from CMV seropositive healthy individuals, and mononuclear cells (PBMC) were cryopreserved after Ficoll-Isopaque separation. Stable Epstein-Barr virus (EBV)-transformed B cell lines (EBV-LCL) were generated using standard procedures [34]. Monocytes were labeled with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and isolated from PBMC by immunomagnetic separation using the midi-MACS system (Miltenyi Biotec).

#### CMV antigens

Peptides used were derived from CMV pp65 protein (P06725, 561 amino acids) or CMV IE1 protein (P13202, 491 amino acids). Minimal peptides corresponding to previously described MHC I or MHC II restricted pp65 epitopes, and MHC I restricted IE1 epitopes were synthesized using standard solid-phase strategies (Department of Immunohematology, Leiden University Medical Center, Leiden, The Netherlands). The minimal MHC I restricted pp65 peptides used were YSEHPTFSQY (HLA-A*0101), NLVPMVATV (HLA-A*0201), QYDPVAALF (HLA-A*2402), TPRVTGCGAM (HLA-B*0702), RPHERNGFTVL (HLA-B*0702), IPSINVHHY (HLA-B*3501), minimal MHC I restricted IE1 peptides were VLEETSVML (HLA-A*0201), ELRRKMYIMM (HLA-B*0801), QIKVRVDVMV (HLA-B*0801), and minimal MHC II restricted pp65 peptides were LLQTGIHVRVSQPL, ALPLKMLNIPSINVH, IIKPGKISHMLDVA, PQYSEHPTFSQYIQ, PPWAGILARNLVPM, KYQEFFWHANDYRI [24,35,36]. Full pp65 or IE1 protein-spanning pools of 15-mer peptides overlapping with 11 amino acids (PepitivatorTM, Miltenyi Biotec, and PepMixTM, JPT Peptide Technologies, Berlin, Germany), and full-length recombinant pp65 protein (Miltenyi Biotec) were used.

#### Flow cytometric analyses

Cells were stained with FITC-labeled CD3, CD4, CD14, IFNγ (BD Biosciences, San Jose, CA, USA), PE-labeled CD56, TNFα (BD), CD40L (Beckman Coulter, Fullerton, CA, USA), PerCP-labeled CD3, CD8 (BD), APC-labeled CD4 (Beckman Coulter), CD19, IFNγ, and IL-2 (BD) monoclonal antibodies (mAbs). PE- and APC-labeled CMV peptide-MHC tetramers were produced as described previously [27]. Between 5x10⁴ and 5x10⁵ fluorescent events were analyzed for each sample using a FACS Calibur and Cellquest software (BD). The limit of detection was defined as a cluster of at least 10 specific events, or background events in negative control samples.
Intracellular cytokine staining

After thawing, PBMC were resuspended in culture medium, consisting of Iscove’s modified Dulbecco’s medium (IMDM, Lonza, Basel, Switzerland) supplemented with 10% pooled human serum, 100 U/ml penicillin/streptomycin (Lonza), and 3 mM L-glutamine (Lonza). For measurement of activated CMV-specific T cells in PBMC by cumulative intracellular cytokine staining, cells were stimulated in culture medium with 10^3 or 10^4 M CMV peptides or protein for 6 hours at 37°C and 5% CO2. During the last 5 hours of peptide stimulation, 10 μg/ml brefeldin A (BFA, Sigma-Aldrich, Zwijndrecht, The Netherlands) was added. For real-time kinetic measurement of intracellular IFNγ, cells were stimulated for 2 to 48 hours, and 10 μg/ml BFA was added only during the last 2 hours of stimulation. To allow detection of CMV-specific T cells in the T cell line, we used as stimulator cells autologous EBV-LCL or monocytes labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Leiden, The Netherlands) for distinction in FACS analysis as described before [37]. Stimulator cells were incubated overnight in culture medium with 10^{-6} M CMV peptides, washed, and incubated in culture medium with the T cell line in an effector/stimulator ratio of 1/5 for 4 hours in the presence of 10 μg/ml BFA. After stimulation, cells were harvested and washed in phosphate-buffered saline (PBS) and stained with PE-labeled tetramers for 15 minutes at 37°C. Subsequently, mAbs for cell-surface staining were added and incubated for 30 minutes at 4°C. Cells were washed in PBS and fixed with 1% paraformaldehyde for 8 minutes at 4°C. For permeabilization, samples were washed in PBS with 0.1% saponin (Sigma-Aldrich) and incubated for 30 minutes at 4°C. Cells were stained intracellularly with mAbs for 30 minutes at 4°C, washed, and fluorescent events were analyzed by flow cytometry.

Isolation and culture of IFNγ-secreting cells

For isolation of IFNγ-secreting cells, PBMC were stimulated in culture medium with 10^4 M CMV pp65 and IE1 protein-spanning peptide pools for 4 hours at 37°C and 5% CO2. After stimulation, cells were harvested and thoroughly washed in PBS, and IFNγ-secreting cells were stained by the IFNγ capture assay (Miltenyi Biotec) performed according to the manufacturer’s instructions. Briefly, cells were labeled with IFNγ-catch reagent and cultured for 45 minutes at 37°C. Cells were counterstained with PE-labeled IFNγ mAb. For subsequent isolation, the IFNγ-PE-labeled cells were bound to anti-PE microbeads, and isolated by immunomagnetic separation using the MidiMACS system (Miltenyi Biotec). The isolated IFNγ-enriched and IFNγ-depleted cell fractions were both cultured at 1 x 10^6 cells/ml in culture medium, containing 50 IU/ml IL-2 (Chiron, Amsterdam, The Netherlands) and 10 ng/ml IL-15 (Peprotech, Rocky Hill, NJ, USA) and 10 x 10^6 cells/ml 30 Gy-irradiated feeder cells derived from the IFNγ-depleted fraction. Cultures were supplemented with fresh medium, 50 IU/ml IL-2, and 10 ng/ml IL-15 every 3-4 days.

Results

Activation efficiency of CMV pp65-specific T cells in PBMC

To investigate whether a broad repertoire of CMV-specific T cells could be activated in PBMC from healthy CMV seropositive donors, irrespective of their HLA type, specific IFNγ production was determined following stimulation with different CMV pp65 antigens. Intracellular staining after stimulation with MHC class II binding immunodominant CMV pp65 peptides showed that CMV-specific CD4+ T cells produced IFNγ and concomitantly expressed CD40L, as shown for a representative example (Figure 1A). Stimulation with either full-length recombinant CMV pp65 protein or a CMV pp65 protein-spanning pool of overlapping 15-mer peptides induced IFNγ production by CMV-specific CD4+ T cells in PBMC from all 11 donors tested (Figure 1B). The HLA types of the donors were heterogeneous, containing 13 different HLA-DR and HLA-DQ alleles, indicating that CMV antigen was presented by various HLA alleles. Frequencies of activated CMV-specific CD4+ T cells were comparable using the protein or peptide pool, and ranged from 0.02% to 4.5% of CD4+ T cells. Addition of minimal peptides to the peptide pool and protein for stimulation did not further increase the frequency and magnitude of the CMV pp65-specific CD4+ T cell response. Since these paired measurements showed a similar pattern of frequencies of IFNγ-producing CD4+ T cells, it is likely that the CD4+ T cells activated with the 15-mer peptide pool were similar to the CD4+ T cells activated with the protein, and included the CD4+ T cells activated with the minimal peptides.

To determine whether stimulation with CMV pp65 protein or 15-mer peptide pool could simultaneously induce activation of a broad repertoire of CMV-specific CD8+ T cells irrespective of the HLA type, specific IFNγ production by CD8+ T cells was determined in PBMC from the same CMV seropositive donors. Their heterogeneous HLA types comprised 19 different HLA-A and HLA-B alleles. As expected, intracellular staining after stimulation with minimal MHC class I binding CMV pp65 peptides showed specific production of IFNγ by CMV-specific CD8+ T cells (Figure 1C). Stimulation with either the full-length recombinant CMV pp65 protein or the CMV pp65 protein-spanning pool of overlapping 15-mer peptides also induced IFNγ production by CMV-specific CD8+ T cells. However, paired measurements showed that frequencies of activated CMV-specific CD8+ T cells were higher after stimulation with the pp65 15-mer peptide pool compared to the recombinant pp65
protein, indicating that part of the CMV pp65-specific CD8+ T cells were not activated using full-length protein. Stimulation with the combination of minimal peptides, peptide pool, and protein induced similar frequencies of IFNγ-producing CD8+ T cells compared to stimulation with peptide pool only. These data suggest that stimulation with the pp65 15-mer peptide pool resulted in activation of CMV pp65-specific CD8+ T cells, including the CD8+ T cells activated with the minimal peptides or the protein.

Figure 1. Activation of CMV pp65-specific T cells in PBMC. (A) Detection of CMV pp65-specific CD4+ T cells by intracellular IFNγ and CD40L staining upon stimulation of PBMC from donor 9 with MHC class II restricted immunodominant pp65 peptide PQY. (B) Frequencies of IFNγ-producing CD40L+ cells among CD4+ T cells or (C) frequencies of IFNγ-producing cells among CD8+ T cells in PBMC of 11 healthy CMV seropositive donors after stimulation with minimal MHC class II or I restricted pp65 peptides, pp65 protein, 15-mer pp65 peptide pool, or these antigens combined. Paired measurements from each donor are connected with lines.

To analyze whether stimulation with the CMV pp65 peptide pool indeed induced activation of multiple individual CMV-specific CD8+ T cell populations, peripheral blood samples were selected from 10 donors containing high frequencies of CD8+ T cells specific for 5 known CMV pp65 epitopes. The CMV-specific CD8+ T cells were efficiently activated after stimulation with the corresponding minimal MHC class I binding CMV peptides, as illustrated by representative examples of peptide-MHC tetramer staining combined with intracellular IFNγ staining (Figure 2). Production of IFNγ co-incident with a decrease in tetramer staining, indicating TCR downregulation. After stimulation with the CMV pp65 peptide pool (10^{-6} M), CD8+ T cells specific for 4/5 CMV pp65 epitopes were efficiently activated as shown by IFNγ production, which was also found following stimulation of the CMV pp65-specific CD8+ T cells in the other donors. However, part of the CD8+ T cells specific for pp65-YSE/A1 did not produce IFNγ (range 20-83% IFNγ-producing tetramer+ T cells). Increasing the concentration of peptide pool to 10^{-5} M did not result in a higher frequency of pp65-YSE/A1-specific CD8+ T cells producing IFNγ. After stimulation with recombinant pp65 protein, only CD8+ T cells specific for pp65-NLV/A2 and pp65-TPR/B7 produced IFNγ and showed modest TCR downregulation, while CD8+ T cells specific for pp65-YSE/A1, pp65-RPH/B7, and pp65-IPS/B35 did not show any IFNγ production or TCR downregulation (Figure 2).

These results demonstrate that stimulation of donor PBMC with the CMV pp65 protein-spanning 15-mer peptide pool efficiently induced activation of CD8+ and CD4+ T cells specific for multiple CMV pp65 epitopes, irrespective of the donor HLA type. In contrast, stimulation with the full-length CMV pp65 protein resulted in efficient activation of pp65-specific CD4+ T cells, but only a fraction of the pp65-specific CD8+ T cells.

Figure 2. Activation of individual CMV pp65-specific CD8+ T cell populations in PBMC. Dot plots show representative examples of intracellular IFNγ staining of PBMC from different donors, containing CD8+ T cell populations specific for 5 immunodominant CMV pp65 epitopes, after stimulation with 10^{-6} M of the relevant minimal peptide, with 10^{-5} M 15-mer CMV pp65 peptide pool, or with 10^{-5} M full-length recombinant CMV pp65 protein. Samples were stained with peptide-MHC tetramers after stimulation, and dot plots were gated on total lymphocytes.
Figure 3. Activation of CMV IE1-specific T cells in PBMC. (A) Frequencies of IFNγ-producing CD4+ cells among CD4+ T cells or (B) frequencies of IFNγ-producing cells among CD8+ T cells in PBMC of 11 healthy CMV seropositive donors after stimulation with 15-mer CMV IE1 peptide pool. Filled symbols represent specific IFNγ-producing populations, and open symbols represent frequencies below the limit of detection. (C) Dot plots show representative examples of intracellular IFNγ staining of PBMC from different donors, containing CD8+ T cell populations specific for 3 immunodominant CMV IE1 epitopes, after stimulation with 10^-6 M of the relevant minimal peptide, or with 10^-6 M 15-mer CMV IE1 peptide pool. Samples were stained with peptide-MHC tetramers after stimulation, and dot plots were gated on total lymphocytes.

Chapter 3

Activation of CMV IE1-specific T cells in PBMC
To further extend the repertoire of CMV-specific T cells that could be activated in peripheral blood from CMV seropositive donors for IFNγ-based isolation, we next investigated whether stimulation of PBMC with a CMV IE1 protein-spanning pool of overlapping 15-mer peptides induced IFNγ production by CD8+ and CD4+ T cells specific for CMV IE1 epitopes. After CMV IE1 peptide pool stimulation, specific IFNγ-production by CD4+ T cells was detected in PBMC from 5/11 donors tested, with frequencies between 0.03% and 0.28% of CD4+ T cells (Figure 3A). CMV IE1-specific CD8+ T cells producing IFNγ upon stimulation with the CMV IE1 peptide pool were also detected in PBMC from 5/11 donors tested, with frequencies of CD8+ T cells ranging from 0.06% to 1.5% (Figure 3B). The efficiency of CMV IE1-specific T cell activation using the CMV IE1 peptide pool could not be properly evaluated due to the limited number of known MHC class I and II restricted CMV IE1 epitopes.

To analyze whether stimulation with the CMV IE1 peptide pool induced activation of multiple individual CMV-specific CD8+ T cell populations as well, peripheral blood samples were selected from 6 donors containing high frequencies of CD8+ T cells specific for 3 known CMV IE1 epitopes. As illustrated for representative examples, the CMV IE1-specific CD8+ T cells were efficiently activated after stimulation with the corresponding minimal MHC class I binding CMV IE1 peptides (Figure 3C). After stimulation with the CMV IE1 peptide pool, CD8+ T cells specific for 2/3 CMV IE1 epitopes were efficiently activated as shown by IFNγ production, while part of the CD8+ T cells specific for IE1-QIK/B8 did not produce IFNγ. Similar results were obtained for the CMV IE1-specific CD8+ T cell populations in PBMC from the other donors.

These results show that CMV IE1-specific T cells producing IFNγ after CMV IE1 peptide pool stimulation were observed in peripheral blood from 45% of healthy CMV seropositive donors. The efficiency of activation of CD8+ T cells specific for 3 CMV IE1 epitopes was consistent with the results obtained with the CMV pp65 peptide pool.

Kinetics of IFNγ response by CMV-specific T cells upon activation
To determine the optimal time-point for simultaneous isolation of a broad repertoire of CMV-specific CD8+ and CD4+ T cells by the IFNγ capture assay, the kinetics of IFNγ production following stimulation with CMV peptides was investigated. Real-time kinetic analysis after stimulation with the CMV pp65 or IE1 15-mer peptide pool showed a rapid and synchronized induction of IFNγ production by both CD8+ and CD4+ CMV-specific T cells, which was maximal after 4 hours and decreased thereafter, as depicted for
representative examples (Figure 4A-B). These data indicate that peptides derived from the 15-mer peptide pools were simultaneously presented in MHC class I and II following incubation. Similar kinetics of IFNγ production by CMV pp65-specific CD8+ T cells have previously been described after stimulation with minimal MHC I binding CMV pp65 peptides [29], and were shown to be similar after stimulation with minimal MHC I binding CMV IE1 peptides or minimal MHC II binding CMV pp65 peptides as well. These measurements show that kinetics of IFNγ production was similar for CMV-specific CD8+ and CD4+ T cells, with maximal IFNγ production 4 hours after stimulation with CMV peptides. Therefore, simultaneous isolation of CMV-specific CD8+ and CD4+ T cells by the IFNγ capture assay is likely to be most efficient after 4 hours of stimulation with CMV 15-mer peptide pools.

Figure 4. Kinetics of IFNγ response by CMV-specific T cells upon activation. Real-time intracellular IFNγ staining, with addition of BFA only during the last 2 hours of incubation, after stimulation of PBMC for 0, 2, 4, 8, 24, and 48 hours (A) with 15-mer pp65 peptide pool, or (B) with 15-mer IE1 peptide pool.

IFNγ-based enrichment and expansion of CMV pp65- and IE1-specific T cells

A clinical grade protocol was developed for the generation of T cell lines comprising a broad repertoire of CD8+ and CD4+ T cells specific for both CMV pp65 and IE1 epitopes. Isolation of IFNγ-secreting cells from PBMC was performed by IFNγ capture assay during the peak of IFNγ production after 4 hours of stimulation with CMV 15-mer peptide pools. Since low frequencies of IE1-specific T cells in PBMC were observed and IE1-specific T cell lines have not been described in detail before, T cell lines specific for IE1 were first generated from 3 CMV seropositive donors, resulting in high frequencies of CD8+ and CD4+ IE1-specific T cells after 14 days of culture (Table 1). Subsequently, T cell lines specific for both pp65 and IE1 were generated from 4 CMV seropositive donors, as shown for a representative example (Figure 5). Before enrichment, low frequencies of CD8+ T cells specific for CMV pp65 and IE1 epitopes and CD4+ T cells specific for CMV pp65 were detected in PBMC (Figure 5A). In the T cell line, high frequencies of CD8+ T cells specific for the same pp65 and IE1 epitopes were detected by peptide-MHC tetramer staining (Figure 5B). To determine the total frequency of CMV-specific T cells, the T cell lines was restimulated with autologous EBV-LCL loaded with CMV pp65 or IE1 peptide pools, which induced IFNγ production by high frequencies of CD8+ T cells specific for CMV pp65 and IE1 and CD4+ T cells specific for CMV pp65 (Figure 5B). The IFNγ-depleted fractions contained only low frequencies of CMV tetramer-positive CD8+ T cells, and CD8+ and CD4+ T cells producing IFNγ upon CMV-specific restimulation (Figure 5C). These data show efficient enrichment of CMV-specific T cells from PBMC, resulting in a T cell line containing CD8+ and CD4+ T cells specific for multiple pp65 and IE1 epitopes, with an overall specificity of 91% of T cells producing IFNγ upon CMV-specific restimulation.

Table 1. Generation of 7 coordinated CD8+ and CD4+ T cell lines with high specificity for CMV pp65 and IE1 epitopes derived from healthy CMV seropositive donors.
Using this method, T cell lines with high specificity for a broad repertoire of CMV CD8+ and CD4+ epitopes were reproducibly generated from all donors, irrespective of the HLA type (Table 1). The alloreactive recognition of third party donor PBMC by the CMV-specific T cell lines was reduced to <10% compared with the donor PBMC which had been used as starting material in a 3H-incorporation assay. Furthermore, the T cell lines retained the capacity to proliferate upon antigen-specific restimulation, and showed efficient and specific lysis of CMV peptide-loaded target cells (data not shown). The composition of the T cell lines correlated with the frequencies of pp65- and IE1-specific CD8+ and CD4+ T cells in donor PBMC, illustrating that the repertoire of CMV-specific T cells in donor PBMC was represented in the T cell lines, irrespective of the HLA type of the donor.

The phenotype and capacity to produce cytokines was compared between CMV-specific T cells in donor PBMC and in cultured T cell lines. CMV-specific T cells in donor PBMC predominantly showed a CD45RO+, CD27+ or CD27-, CD28+ or CD28-, CD62L-, and CCR7-effector memory T cell phenotype. After 6 days of culture following activation and isolation, the expression of CD27, CD28, CD62L, and CCR7 transiently increased, as we have recently described [29]. However, after 14 days of culture, the phenotype of CMV-specific T cells was similar to their original phenotype in donor PBMC again. Intracellular staining showed that CMV-specific CD8+ and CD4+ T cells in donor PBMC produced both IFN\(\gamma\) and TNF\(\alpha\) upon stimulation, and some CMV-specific T cell populations also produced IL-2, as shown for a representative CMV pp65-specific CD4+ T cell population (Figure 5D). Following restimulation of the cultured CMV-specific T cell lines with autologous EBV-LCL loaded with CMV peptide, CMV-specific T cells produced both IFN\(\gamma\) and TNF\(\alpha\), while no production of IL-2 was detected. To determine whether the capacity to produce IL-2 was downregulated by addition of exogenous IL-2 during culture, T cell lines were cultured for 14 days with IL-15 in the absence of IL-2. Thereafter, restimulation of the cultured CMV-specific T cell lines with autologous EBV-LCL loaded with CMV peptide suggested that IL-2 production by CMV-specific T cells was partly restored (Figure 5D).

These results demonstrate that coordinated CD8+ and CD4+ T cell lines with high specificity for multiple CMV pp65 and IE1 epitopes can be rapidly generated from all CMV seropositive donors using this method. The phenotype and function of CMV-specific T cells in generated T cell lines was comparable to CMV-specific T cells in donor PBMC.

**Discussion**

In this study, we developed a method for the simultaneous isolation of coordinated CD8+ and CD4+ T cell responses against multiple epitopes derived from both CMV pp65 and IE1
protein from donor peripheral blood, which may be used for treatment of CMV disease in alloSCT recipients. While peptide-MHC multimers can only be used for isolation of CD8+ T cell populations specific for single and defined epitopes, IFNγ-based isolation can be used for the simultaneous isolation of a broad repertoire of CMV-specific CD8+ and CD4+ T cells. We here demonstrate that CMV protein-spanning pools of overlapping 15-mer peptides can be used to efficiently activate a broad repertoire of CMV-specific CD8+ and CD4+ T cells, irrespective of the donor HLA type, while the use of CMV lysate or transduced antigen-presenting cells have not shown efficient simultaneous activation of both T cell subsets in previous studies. Subsequently, activated CMV-specific T cells were isolated at the peak of the IFNγ response by IFNγ capture assay. Using this method, highly specific T cell lines were generated from all CMV seropositive donors, and the composition of the T cell lines correlated with the frequencies of pp65- and IE1-specific CD8+ and CD4+ T cells in donor PBMC. The phenotype and function of CMV-specific T cells after culture was comparable to CMV-specific T cells in donor PBMC, although the capacity to produce IL-2 upon activation appeared to be downregulated during culture with exogenous IL-2. However, the IL-2 production capacity may be restored after adoptive transfer in vivo, since the negative feedback caused by addition of exogenous IL-2 during culture appeared to be reversible, which was consistent with previously described data [38].

The efficient activation of specific CD4+ T cells using 15-mer peptides or protein was anticipated, since processing of exogenous protein followed by presentation in MHC class II has been extensively described, and 15-mer peptides may also bind directly in MHC class II molecules at the cell surface [39]. The efficient activation of specific CD8+ T cells using 15-mer peptides, but not protein, can not easily be explained. Activation of CMV-specific CD8+ T cells may not only result from the presence of contaminating shorter peptide fragments, since purified 15-mer peptides have also been shown to induce CD8+ T cell activation [40].

Although the mechanism of processing and MHC class I presentation of exogenously added long synthetic peptides is not known, several models have been proposed, including entrance of MHC class I in the recycling endocytic MHC class II pathway where peptidase-trimmed exogenous antigens can exchange with peptides in the MHC class I molecules [41,42]. Alternatively, long peptides may reach the cytosol followed by the classical route of MHC class I loading [43].

Adoptive transfer of donor-derived CMV-specific T cell lines may be safe, since no adverse events associated with administration have been reported. However, total cell numbers infused were largely variable, and the frequency of CMV-specific T cells was often not determined or was limited. Identification of CMV CD8+ T cell epitopes and the availability of peptide-MHC tetramers have supported the development of efficient protocols for reproducible generation of T cell lines containing more than 70% CMV-specific CD8+ T cells [13,15,16,29]. In contrast, high specificity for CMV antigens was not demonstrated for the CD4+ T cells administered in clinical trials [14,18-20]. The risk of graft-versus-host disease may be determined or was limited. Identification of CMV CD8+ T cell epitopes and the availability of CMV-specific T cells, irrespective of the HLA type of the patient, which may be used for treatment of CMV infection after alloSCT. The applicability of this strategy may be extended by the isolation of T cells specific for other pathogens, provided that they produce IFNγ upon activation.

References
Chapter 3


Chapter 3


Chapter 3


