Strategic use of an adenoviral vector for rapid and efficient 
ex vivo-generation of cytomegalovirus pp65-reactive cytolytic 
and helper T cells

Human cytomegalovirus (CMV) is a widely spread herpes 
virus that resides dormant after infection. Patients that have 
undergone haematopoietic stem cell transplantation (HSCT) 
or solid organ transplantation are at high risk for development 
of CMV-associated diseases (Meyers et al, 1986; Ljungman 
et al, 1992). The introduction of prophylactic or pre-emptive 
antiviral therapy has been a successful treatment for early onset 
of CMV disease (Meyers, 1991; Ljungman, 2002). However, 
this has led to the identification of CMV strains that are 
resistant to antiviral therapy and an increase in late onset of 
CMV disease (Li et al, 1994; Krause et al, 1997; Einsele et al, 

In healthy CMV-seropositive individuals, the cell-mediated 
immune system, *i.e.* CD8*⁺* cytolytic and CD4*⁺* helper T cells, 
repress viral reactivation and protect against disease (Gilles-
pie et al, 2000; Kern et al, 2002). Therefore, adoptive transfer 
of donor-derived CMV-specific T cells may be an attractive 
approach to treat transplant patients with CMV disease. It 
was shown more than a decade ago that infusion of donor-
derived CMV-specific CD8*⁺* T cells can reconstitute CMV 
immunity after HSCT (Riddell et al, 1992, 1994; Walter 
et al, 1995). They were able to reconstitute and restore 
CMV-specific CD8*⁺* T-cell immunity. However, the magni-
tude of the CMV-specific CD8*⁺* T-cell response decreased 
with time in patients with no reconstitution of endogenous 
transferred donor-derived CMV-specific CD4*⁺* T cells which 
also suppressed the virus, but only if the patient reconsti-
tuted an endogenous CMV-specific CD8*⁺* T-cell response. 
Jointly, this implies that the optimal way to treat HSCT

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Summary

Cytomegalovirus (CMV) reactivation can cause severe complications for 
transplant patients. Such patients can be protected against CMV-associated 
diseases through reconstitution of donor-derived CMV-reactive cytolytic and 
helper T cells. We have developed a strategic protocol for efficient 
simultaneous generation of CMV-reactive CD8⁺ and CD4⁺ T cells \textit{ex vivo}. 
The protocol uses peripheral blood lymphocytes (PBLs), antigen-modified 
mature dendritic cells (DCs) generated in only 3 d and an adenoviral vector 
encoding the CMV pp65 antigen (Adpp65) both as an endogenous and 
exogenous source of antigen. PBLs stimulated once with Adpp65-transduced 
DCs (endogenously expressed pp65) resulted in preferential activation and 
expansion of pp65-specific CD8⁺ T cells while PBLs stimulated with DCs 
pulsed with cell lysate from Adpp65-transduced autologous monocytes 
(exogenously expressed pp65) yielded pp65-specific CD4⁺ T cells. 
Stimulation with double-modified DCs efficiently activated and expanded 
cytolytic and helper T cells simultaneously. The frequency of T cells 
producing interferon-γ in response to pp65 increased after one stimulation 
on average 9.6-fold to 4.3% for CD8⁺ T cells and 25.8-fold to 6.5% for CD4⁺ 
T cells. This implies that sufficient number of pp65-specific cytolytic and 
helper T cells for adoptive transfer may be obtained in only 2 weeks.

Keywords: cytomegalovirus, pp65, adenoviral vector, CD8⁺ T cells, CD4⁺ 
T cells.
patients with CMV disease would be to transfer donor-derived CMV antigen-specific T cells of both CD4+ and CD8+ subsets.

Dendritic cells (DCs) are professional antigen-presenting cells that prime and induce T-cell responses. Several methods have been described for the generation of monocyte-derived DC using various media, serum, cytokines and maturation stimuli (Jonuleit et al., 1997; Reddy et al., 1997; Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Cella et al., 1999; Lee et al., 2002; Dauer et al., 2003). Most protocols include 7 d of cell culture with granulocyte-macrophage colony-stimulation factor (GM-CSF) and interleukin (IL)-4 followed by 2 d of culture with a cocktail of cytokines and maturation stimuli. Therefore, it normally takes 7–9 d to produce ‘conventional’ DCs for T-cell stimulation studies. Recent studies have implied the effectiveness of ‘fast’ DCs which are generated in only 48–72 h (Dauer et al., 2003; Obermaier et al., 2003; Xu et al., 2003; Ho et al., 2006).

Dendritic cells efficiently present peptides derived from endogenously expressed proteins on major histocompatibility complex (MHC) class I molecules for activation of CD8+ T cells and peptides derived from endocytosed exogenous proteins on MHC class II molecules for activation of CD4+ T cells. They also have the capacity to present peptides from endocytosed proteins on class I molecules, a process known as cross-presentation (Albert et al., 1998; Rock, 2003; Burgdorf et al., 2007). We and others have shown that DCs transduced with an adenoviral vector expressing the full length CMV pp65 protein (Adpp65) or transfected with mRNA encoding CMV pp65 primarily induce pp65-specific CD8+ T cells while DCs pulsed with CMV lysate primarily induce CD4+ T cells (Carlsson et al., 2003, 2005; Foster et al., 2004; Heine et al., 2006). We have also shown that DCs transfected with pp65 mRNA and pulsed with recombinant pp65 protein activates pp65-specific CD8+ and CD4+ T cells simultaneously (Carlsson et al., 2005). For clinical use it may be complicated to combine several sources of clinical grade CMV antigen. We reasoned that simultaneous CD8+ and CD4+ T-cell activation should be obtained if DCs were both transduced with Adpp65 (endogenous pp65 expression) and pulsed with cell lysate from autologous Adpp65-transduced monocytes (exogenous pp65 expression).

Herein we present a rapid and straightforward protocol for simultaneous activation and expansion of CMV pp65-specific CD8+ and CD4+ T cells. The protocol uses fast DCs and only one source of pp65 antigen in the form of Adpp65 that can easily be produced at clinical grade (Leen et al., 2006). It only requires a single stimulation of peripheral blood lymphocytes (PBLs) with Adpp65-modified fast-matured DCs, i.e., sufficient number of cytolytic and helper T cells can be obtained in 2 weeks. The rapid and unspecific expansion protocol of T cells has been omitted. We believe that this protocol is applicable in a clinical setting because of its robustness and effectiveness.

Materials and methods

Reagents

Culture medium. All primary cell cultures were maintained in RPMI 1640 medium supplemented with 1% pooled normal human serum (Uppsala University Hospital Blood Centre, Uppsala, Sweden), 10 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1 mmol/l l-glutamine, 20 μmol/l β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in a 37°C, 5% CO2, humidified incubator.

Adenoviral vectors. The replication-deficient serotype 5 adenoviral vectors coding for the full length CMV pp65 transgene (Adpp65) and the vector without transgene (AdMock) have been described previously (Carlsson et al., 2003). Viruses were produced in 911 cells and the virus titres were determined using fluorescent-forming unit (FFU) assay, as described previously (Dzojic et al., 2007).

Peptides and tetramer. The HLA-A*0201-restricted peptide from the CMV pp65 antigen (aa 495-503, NLYPMATV) and the negative control antigen VMAT-1 (aa 31-39, LLLDNMLFT) were purchased from Sigma Genosys, Haverhill, Suffolk, UK). The phycoerythrin (PE)-labelled HLA-A*0201/pp65 tetramer was purchased from Beckman Coulter (San Diego, CA, USA).

Antibodies and cytokines. Fluorescence-labelled monoclonal antibodies against human interferon gamma (IFN-γ)–fluorescein isothiocyanate (FITC), CD3-allophycocyanin (APC), CD8-peridinin chlorophyll protein (PerCP), CD8-PE, CD4-PE, CD14-FITC, CD25-FITC, CD27-FITC, CD28-FITC, CD40-FITC and -PE, CD45RA-FITC, CD45RA-APC, CD62L-FITC, CD80-PE, CD83-PE, CD86-APC, CCR7-PE, HLA-ABC-FITC and HLA-DR-FITC (BD Biosciences, San Diego, CA, USA), CCR7-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD45RO-FITC and Multimix Dual-Colour Control Mouse IgG1-FITC/IgG1-PE (γ1/γ2) (Dako Cytomation, Glostrup, Denmark). Recombinant human IL-4 and GM-CSF (Gentaur Molecular Products, Brussels, Belgium), IL-2 and IL-7 (Biosource, Camarillo, CA, USA). Polyinosinic-Polyrictidylic acid potassium [Poly(I:C)] and propidium iodide (Sigma-Aldrich, St Louis, MO, USA).

Isolation of peripheral blood mononuclear cells, rapid generation of antigen-presenting DCs, and stimulation of antigen-specific CD8+ and CD4+ T cells

Buffy coats from approximately 420 ml peripheral blood were obtained from healthy CMV-seropositive, HLA-A*0201-positive blood donors. Informed consent was obtained from each
Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation according to the manufacturer’s instruction. PBMCs were resuspended in 120 ml culture medium, divided into six T-75 flasks (Corning, New York, NY, USA) and incubated for 90 min. Non-adherent cells, mainly PBLs, were collected, counted and cultured for 72 h in fresh T-75 flasks (1 × 10^6 cells/ml culture medium) with low-dose IL-2 (10 IU/ml) (Fig 1). Adherent cells, mainly monocytes, were divided into three portions.

**CD8 arm.** One portion of adherent cells (1) was collected, centrifuged, resuspended in 300 µl culture medium and transduced for 2 h with Adpp65 or AdMock at a multiplicity of infection (MOI) of 400 FFU/cell. The cells were then cultured in fresh T-75 flasks (1 × 10^6 cells/ml culture medium) and the transduced monocytes were differentiated into immature DCs by addition of IL-4 (25 ng/ml) and GM-CSF (50 ng/ml) at 0, 24 and 48 h. They were matured by addition of TNF-α (40 ng/ml) and Poly(I:C) (30 µg/ml) at 48 h and cultured for another 24 h. These DCs were used as MHC class I peptide-presenting cells of endogenously expressed pp65. This part of the protocol is referred to as the CD8 arm.

**CD4 arm.** A second portion of adherent cells (2) (Fig 1) was differentiated into immature DCs with IL-4 (25 ng/ml) and GM-CSF (50 ng/ml) added at 0, 24 and 48 h. After 48 h the cells were pulsed with exogenously expressed pp65 in the form of total cell lysate or stressed cell debris from the third portion (3) of Adpp65-transduced monocytes. These DCs were then matured and used as MHC class II peptide-presenting cells of exogenously expressed pp65. This part of the protocol is referred to as the CD4 arm.

The pp65 peptide-presenting DCs from the CD8 and CD4 arms were either pooled or kept separately and used for stimulation of autologous PBLs. T cell proliferation is measured for 5 d following stimulation. T-cell specificity and activity are analysed by tetramer-recognition and IFN-γ production before and 11 d after stimulation. Cytolytic activity is evaluated 11 d after stimulation.

**Annexin V apoptosis assay**

An Annexin V apoptosis assay (BD Biosciences) was used to evaluate the extent of apoptosis in heat/irradiated stressed monocytes. Briefly, after heat/irradiation treatment cells were cultured for 24 h, washed twice in cold phosphate-buffered saline and resuspended in binding buffer. Cells were incubated with Annexin V-FITC and PI for 15 min at room temperature.
Cells were washed and analysed on a FACSCalibur flow cytometer (BD Biosciences).

**Carboxyfluorescein succinimidyl ester proliferation assay**

The CellTrace carboxyfluorescein succinimidyl ester (CFSE) cell proliferation kit (Invitrogen, Eugene, OR, USA) was used to evaluate CD8+ and CD4+ T-cell proliferation. Briefly, 2 x 10^6 PBLs were dyed with 10 µmol/l CFSE solution and co-cultured in 12-well plates with 2 x 10^5 autologous DCs irradiated at 40 Gy. The DCs were unmodified, pulsed with cell lysate from Adpp65 or AdMock-transduced monocytes, or transduced with Adpp65 or AdMock. After 5 d cells were incubated with anti-human CD3-APC and either CD8-PE or CD4-PE antibodies for 30 min at 4°C, washed and analysed by flow cytometry.

**Intracellular interferon gamma staining**

Modified DCs from the CD8 and CD4 arms were mixed with autologous unstimulated PBLs for prestimulation analysis or stimulated PBLs for poststimulation analysis at a stimulator to responder (DC/PBL) ratio of 1:2. After 2 h of incubation at 37°C, protein secretion was blocked by the addition of 8 µg/ml Brefeldin-A (Sigma) and the incubation was continued for an additional 5 h. Cells were then permeabilized with BD-perm (BD Biosciences) and incubated with anti-human CD3-APC, IFN-γ-FITC and either CD8-PE or CD4-PE antibodies for 30 min at 4°C, washed and analysed by flow cytometry.

**Tetramer analysis**

The PE-labelled HLA-A*0201/pp65495-503 tetramer was used together with anti-human CD8-PerCP and CD3-APC antibodies to determine the percentage of tetramer-positive cells before and after stimulation. Cells were incubated for 30 min at 4°C, washed and analysed by flow cytometry.

**Cytotoxicity assay**

The human Epstein–Barr virus (EBV)-transformed B lymphoblastoid cell line C1R-A2 was cultured in RPMI-1640 method (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen) and 200 µg/ml of geneticin (Sigma). C1R-A2 (5 x 10^6 cells in 300 µl medium) were left unmodified or transduced with Adpp65 or AdMock (400 MOI) for 2 h and then cultured for 48 h. The cells were then labelled with chromium 51 (51Cr) (GE Healthcare) for 1 h at 37°C, washed three times and unmodified cells were pulsed with pp65495-503 peptide (5 µg/ml) or VMAT-131-39 peptide (5 µg/ml) for 2 h. Stimulated PBLs were added to 2500 51Cr-labelled C1R-A2 target cells in V-bottomed 96-well plates at effector to target ratio (E:T) from 0:1:1 to 50:1 and incubated for 5 h at 37°C. The released 51Cr was counted in a Microbeta Trilux 1450-024 (Helsinki, Finland). Triplate wells were averaged and the percentage of specific lysis was calculated as follows: Percentage lysis = 100 \times \left[\frac{\text{sample } 51\text{Cr release} - \text{spontaneous } 51\text{Cr release}}{\text{maximum } 51\text{Cr release} - \text{spontaneous } 51\text{Cr release}}\right].

**DC phenotype**

Monocyte-derived mature DCs obtained in either 3 or 9 d were compared regarding cell surface markers and cytokine secretion. DCs obtained in 3 d were supplemented with fresh IL-4 (25 ng/ml) and GM-CSF (50 ng/ml) at 0, 24 and 48 h and TNF-α (40 ng/ml) and Poly(I:C) (30 µg/ml) at 48 h. DCs matured in 9 d were supplemented with fresh IL-4 and GM-CSF every second day and TNF-α and Poly(I:C) after 7 and 8 d. Cells were harvested, washed, divided and incubated with antibodies against various cell surface markers for 30 min at 4°C, washed and analysed by flow cytometry.

Cytometric bead array (Human inflammatory kit; BD Biosciences) was used to measure cytokine production from 3 to 9 d DCs according to the manufacturer’s instructions for supernatant arrays. Samples were run in triplicate and analysed by flow cytometry.

**T-cell phenotype**

Unstimulated and stimulated A*0201/pp65495-503 tetramer-binding T cells were analysed for activation markers. Cells were washed and incubated with antibodies for 30 min at 4°C washed and analysed by flow cytometry.

**Statistical analysis**

The phenotype of DCs analysed by cyometric bead array and the phenotype of HLA-A*0201/pp65495-503 tetramer-binding T lymphocytes, before and after stimulation, were compared using the Wilcoxon signed rank test with paired data (p < 0.05). Frequencies of HLA-A*0201/pp65495-503 tetramer-binding CD8+ T cells and IFN-γ production by CD8+ or CD4+ T cells were compared before and after stimulation using the Wilcoxon rank sum test with unpaired data (p = 0.01-0.001).

**Results**

**Phenotypic similarity between mature DCs generated in 3 or 9 d**

We generated mature fast DCs in 3 d and conventional DCs in 9 d to compare their phenotype and stimulatory capacity. Conventional DCs were larger and more granular than fast DCs when examined by light microscopy (data not shown) and flow cytometry (Fig 2A). Conventional and fast DCs had similar expression levels of HLA-ABC, HLA-DR, CD40, and CD86 and no expression of the monocyte marker CD14. Conventional DCs had a somewhat higher expression of CD54, CD80 and CD83 than fast DCs. Histograms from one
representative experiment out of five is shown in Fig 2B. There were no differences in IL-12, IL-6 or IL-8 production as assessed by cytometric bead arrays (Fig 2C). The high levels of IL-6 and IL-8 were significantly ($\alpha = 0.05$) reduced when Poly(I:C) was omitted from the maturation stimuli (data not shown). Fast DCs produced significantly ($\alpha = 0.05$) lower amounts of IL-10 and significantly ($\alpha = 0.05$) higher amounts of IL-1b than conventional DCs, indicating that fast DCs may be preferable for T-cell stimulation considering the inhibiting effect of IL-10 on T-cell proliferation.

Efficient generation of CMV pp65-specific CD8$^+$ T cells using Adpp65-transduced fast DCs; CD8 arm of the protocol

We have previously shown that conventional DCs transduced with Adpp65 were efficient in generating pp65-specific CD8$^+$ T cells (Carlsson et al., 2003). We now examined whether Adpp65-transduced fast DCs were able to induce T-cell proliferation, increase specificity and mediate cytolytic capacity in a similar manner. Irradiated Adpp65- or AdMock-transduced fast DCs from CMV-seropositive HLA-A$^*$0201-positive donors were cultured with autologous CFSE-labelled PBLs for 5 d. T-cell proliferation was measured by loss of CFSE signal intensity. One example out of three is shown in Fig 3A. Adpp65-transduced DCs were able to efficiently induce proliferation of CD8$^+$ T cells (range: 27–32%), as illustrated by six distinct peaks in Fig 3A. They also induced proliferation of CD4$^+$ T cells to a lesser degree (4–16%). AdMock-transduced DCs induced minimal T-cell proliferation (0–5–1%).

The increase in CMV pp65-specific CD8$^+$ T cells was evaluated by HLA-A$^*$0201/pp65459-503 tetramer staining before (pre-stim) and 11 d after (post-stim) stimulation. One example is shown in Fig 3B where the frequency of tetramer-binding CD8$^+$ T cells increased from 1.2% to 38% when Adpp65–transduced DCs were used as stimulators. As expected, AdMock–transduced DCs did not increase the frequency of pp65-specific CD8$^+$ T cells. Overall, the frequency of tetramer-binding CD8$^+$ T cells after Adpp65/DC stimulation had a mean score of 23% (median: 15.5%), which was significantly ($\alpha = 0.001$) higher than the mean score of 3.1% (median: 2.4%) before stimulation and 2.3% (median: 1.8%) after AdMock stimulation. There was no significant difference in frequency before and after AdMock stimulation (Fig 3C).

We next measured IFN-\(\gamma\) production by PBLs before (pre-stim) and 11 d after (post-stim) stimulation. One example is
shown in Fig 3D where the frequency of IFN-γ-producing CD8+ T cells increased from 0% to 8% upon Adpp65 stimulation. Overall, the IFN-γ production of CD8+ T cells had a mean score of 3.5% (median: 2.6%) after a single Adpp65/DC stimulation (Fig 3E). This frequency was significantly (α = 0.001) higher than both for prestimulated CD8+ T cells (mean: 1.0%, median: 0.6%), and for AdMock-stimulated CD8+ T cells (mean: 0.8%, median: 0.5%). The frequency of IFN-γ producing CD3+CD8+ (i.e. CD4+) T cells was not significantly increased when Adpp65-transduced DCs were used as stimulators compared to AdMock-transduced DCs (data not shown).

The cytolytic capacity of Adpp65/DC-stimulated T cells was examined by chromium release assay using C1R-A2 as target cells in order circumvent the use of autologous CMV-infected fibroblasts. Cytotoxic T-lymphocyte generated through stimulation with Adpp65-transduced DCs were able to efficiently lyse Adpp65-transduced or pp65495-503 peptide-pulsed C1R-A2 but had no cytolytic effect on AdMock-transduced C1R-A2 or C1R-A2 pulsed with VMAT-131-39, an irrelevant HLA-A*0201-binding peptide used as negative control (Fig 3F). This indicates that the recombinant adenoviral vector by itself did not induce an immune response.

To further investigate the phenotype of the HLA-A*0201/pp65495-503-specific CD8+ T cells, we analysed this T-cell population for various activation markers before and after stimulation (Table I). The frequencies of CD45RA+R0+, CD25+, CD27+ and CD28+ T cells within the HLA-A*0201/pp65495-503 population is given in Table I. The frequencies of CD45RA+/R0+ and CD27+ T cells were not significantly increased when Adpp65-transduced DCs were used as stimulators compared to AdMock-transduced DCs (data not shown).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Prestimulation (%)</th>
<th>Poststimulation (%)</th>
<th>P-value</th>
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<tr>
<td>CD45RA+/R0+</td>
<td>28 (11–57)</td>
<td>0 (0–0)</td>
<td>≤0.05</td>
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<tr>
<td>CD45RA+/R0+</td>
<td>60 (31–83)</td>
<td>97 (93–99)</td>
<td>≤0.05</td>
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<tr>
<td>CD25+</td>
<td>0.5 (0.4–1)</td>
<td>19 (11–36)</td>
<td>≤0.05</td>
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<tr>
<td>CD27+</td>
<td>46 (38–50)</td>
<td>44 (34–60)</td>
<td>ns</td>
</tr>
<tr>
<td>CD28+</td>
<td>10 (6–14)</td>
<td>49 (47–58)</td>
<td>≤0.05</td>
</tr>
<tr>
<td>CD62L+</td>
<td>7 (4–19)</td>
<td>11 (5–12)</td>
<td>ns</td>
</tr>
<tr>
<td>CCR7+</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>ns</td>
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*Median (range); ns, not significant. n = 4.
tetramer-binding population were significantly higher after stimulation than before stimulation. The frequencies of CD45RA+/RO− T cells were significantly lower after stimulation than before stimulation while the frequencies of CD27+, CD62L+ and CCR7+ T cells were not significantly altered. Taken together, the data indicate that the tetramer-binding population is shifted from a memory phase to an effector phase upon stimulation.

**Generation of CMV pp65-specific CD4+ T cells using fast DCs pulsed with cell lysate from autologous Adpp65-transduced monocytes; CD4 arm of the protocol**

As the frequency of IFN-γ-producing CD4+ T cells was not significantly increased when PBLs were stimulated with Adpp65-transduced DCs, we hypothesized that pp65-specific CD4+ T cells should be activated if the pp65 protein was taken up by DCs as an exogenous source of antigen. We therefore designed the CD4 arm of the protocol, where monocytes were transduced with Adpp65, harvested 2 d later and fed to autologous immature DCs (Fig 1). Two methods were evaluated for extraction of pp65 antigen from transduced monocytes. DCs were either pulsed with cell lysate from Adpp65-transduced monocytes obtained by four freeze–thaw cycles, which mainly generated necrotic cell death, or by Adpp65-transduced monocytes exposed to heat and irradiation, which mainly generated apoptotic cell death. Heat/irradiated monocytes (stressed monocytes) had an Annexin V staining (apoptotic cell death) of 44–66% while Annexin V staining of normal monocytes was 10–14%. One example out of three is shown in Fig 4A. pp65-specific CD4+ T cells were activated when PBLs were stimulated with Adpp65-modified DCs (Fig 4B). However, there was no significant difference when comparing cell lysate-modified DCs and stressed cell-modified DCs. One representative experiment out of three is shown in Fig 4B. The convenience of preparing cell lysate by four freeze–thaw cycles persuaded us continue with this method as a source of exogenously expressed antigens in all subsequent experiments.

To demonstrate proliferation capacity of stimulated CD4+ T cells the CFSE proliferation assay was performed. One example out of three is shown in Fig 5A. Efficient proliferation of CD4+ T cells was observed (11–24%) after stimulation with Adpp65-modified DCs. The CD4 arm of the protocol was able to induce substantial proliferation of the CD8+ T-cell subset as well (11–19%). AdMock-modified DCs did not induce or induced only minimal CD4+ T-cell proliferation (1.4–1.6%).

We next investigated the activity of the pp65-directed helper T cells obtained in the CD4 arm of the protocol. Figure 5B shows that the frequency of IFN-γ-producing CD4+ T cells was 13% after Adpp65 lysate/DC stimulation compared to 1.2% before stimulation. Furthermore, the frequency of IFN-γ-producing CD3+/CD4+ cells increased from less than 0.1% to 3.5%, indicating that the CD4 arm of the protocol also increases the frequency of pp65-specific CD8+ T cells. Overall, the IFN-γ production by CD4+ T cells had a mean score of 3.8% (median: 2.8%) after Adpp65 lysate/DC stimulation (Fig 5C). This was significantly (α = 0.001) higher than both before stimulation (mean: 0.5%, median: 0.2%) and after AdMock lysate/DC stimulation (mean: 1.3%, median: 1.2%).

**Simultaneous generation of activated CMV-specific CD8+ and CD4+ T cells by strategic use of Adpp65 and fast DCs**

In order to maintain an effective cell-mediated immune response against CMV it is important to have CMV antigen-specific cytolytic and helper T cells. We therefore stimulated PBLs from individual CMV-seropositive blood donors with Adpp65-modified DCs using the CD8 and CD4 arms (Fig 1) of the protocol simultaneously. One example of T-cell activation is shown in Fig 6A where 5.2% of CD8+ T cells and 6.7% of CD4+ T cells produced IFN-γ after Adpp65/DC stimulation. The IFN-γ response is mainly directed against pp65 since stimulation with AdMock/DC generated only 1.1% IFN-γ-producing CD8+ T cells and 1.1%
IFN-γ-producing CD4+ T cells. The average frequency of CD8+ T cells producing IFN-γ after Adpp65/DC stimulation was 4.3% (median: 4.1%), which was significantly higher than the average frequency of 1.1% (median: 0.9%) before stimulation and 0.8% (median: 0.7%) after AdMock/DC stimulation (Fig 6B). Furthermore, simultaneous CD8 arm and CD4 arm stimulation was significantly more efficient in generating IFN-γ-producing CD8+ T cells than if only the CD4 arm was used (mean: 1.3%, median: 0.8%), showing that even if the CD4 arm yielded CD8+ T cells it was not as efficient as the CD8 arm. The average frequency of CD4+ T cells producing IFN-γ after Adpp65/DC stimulation was 6.5% (median: 4.7%), which is significantly higher than the average frequency of 0.7% (median: 0.6%) before stimulation and 0.9% (median: 0.5%) after AdMock/DC stimulation (Fig 6C). Induction of IFN-γ-producing T cells from 10 individual donors before and after Adpp65/DC stimulation is illustrated in Fig 6D. All samples showed, to various degrees, an increase of IFN-γ-producing CD8+ and CD4+ T cells after stimulation. The mean fold induction for CD8+ T cells was 9.6-fold (range: 1.3–35) while it was 25.8-fold (range: 1.1–128) for the CD4+ T cells.

**Discussion**

The new protocol presented herein is specifically designed to activate CMV pp65-reactive CD8+ and CD4+ T cells simultaneously. DCs strategically modified with Adpp65 were used as stimulators to present peptides from endogenously expressed pp65 on MHC class I molecules and peptides from exogenously expressed and endocytosed pp65 on MHC class II molecules. There are several advantages to this protocol. First, a clinical product can be obtained in only 2 weeks. Second, only one clinical grade vector, Adpp65, is needed and through strategic usage it becomes the source of both endogenously and exogenously expressed pp65 antigen. Third, peripheral blood cells are used and at no stage in the protocol is there a need to freeze and store either DCs or T cells. Fourth, the protocol uses only autologous cells and cytokines that can be obtained at clinical grade. A single stimulation with Adpp65-modified DCs yielded on average a 9.6-fold increase of IFN-γ producing CD8+ T cells and a 25.8-fold increase of IFN-γ producing CD4+ T cells. The fact that the CD4 arm of the protocol, besides activating CD4+ T cells, also activates CD8+ T cells is interesting. It indicates that part of the endocytosed pp65 antigen from freeze–thawed transduced monocytes was processed and cross-presented on MHC class I molecules. The protocol has so far only been used with blood from healthy CMV-seropositive individuals. Therefore, it can be used to activate pp65-specific cytolytic and helper T cells from CMV-seropositive donors for prevention or treatment of CMV reactivation in HSCT patients. We next plan to examine whether it can be used to expand pp65-specific T cells from immunosuppressed patients suffering from CMV disease following solid organ transplantation.

Strategies to specifically expand CMV-specific CD4+ and CD8+ T cells have been described previously. Rauser *et al* (2004) combined CMV lysate for activation of CD4+ T cells with HLA-A*A0201- and HLA-B*B0702-restricted peptides from the pp65 and IE antigens for activation of CD8+ T cells.
Following an overnight stimulation of $2-4 \times 10^8$ PBMCs with CMV lysate and peptides, on average $3 \times 10^6$ IFN-γ-producing cells were isolated and expanded over 10–11 d to an average of $4 \times 10^8$ cells using high-dose IL-2 and irradiated autologous PBMCs as feeder cells. Their protocol also appeared highly efficient in generating CMV-specific cytolytic and helper T cells. One advantage with our protocol is that it avoids expansion with high-dose IL-2, which may exhaust T cells. A second advantage is that one clinical grade adenoviral vector is used instead of both CMV lysate and various synthetic peptides. Furthermore, we have previously shown that Adpp65 can be used to generate CD8+ T cells against multiple epitopes simultaneously in accordance with the individuals HLA-ABC molecules (Carlsson et al., 2003).

We established the protocol starting with $15 \times 10^6$ PBLs and after stimulation we always recovered between $13$ and $17 \times 10^6$ cells. We do not know the exact number of pp65-specific T cells in each preparation after stimulation but we know that, on average, 4.3% of all CD8+ T cells and 6.5% of all CD4+ T cells produced IFN-γ in response to pp65. The presented protocol may yield sufficient numbers of pp65-specific CD8+ and CD4+ T cells for adoptive transfer as if it is and if needed, it can easily be scaled up 10-fold from a single buffy coat of blood. This implies that sufficient numbers of T cells can be obtained without the rapid non-specific T-cell expansion protocol that utilizes anti-CD3 antibody, irradiated feeder cells and high-dose IL-2. The number of adoptively transferred T cells needed for an efficient control of CMV disease is not fully known. Walter et al. (1995) used CD8+ T-cell clones specific for CMV and as many as $3-3-100 \times 10^7$ cells/m2 were transferred. In later studies of adoptive transfer, far less T cells have generally been used. Einsele et al. (2002) used four stimulations with CMV lysate to obtain CMV-specific CD4+ T cells. The transferred T-cell dosage, including both CD8+ and CD4+ T cells, was $1 \times 10^7$ cells/kg and the frequency of IFN-γ-producing CD4+ T cells was in some case as high as 7-4% (Einsele et al., 2002). Peggs et al. (2001) also used CMV lysate to expand donor-derived T cells. They later infused $1 \times 10^5$ cells/kg (Peggs et al., 2003). Donor-derived CMV-specific CD8+ T cells have also been isolated and adoptively transferred using HLA-peptide tetramers (Cobbold et al., 2005). In that case CD4+ T cells were not included and the median cell dosage was in this case $8-6 \times 10^5$ cells/kg. It appears that the number of transferred T cells needed for sustained clinical responses depends on the quality and differentiation status of the T cells.

Fig 6. Simultaneous activation of pp65-specific CD8+ and CD4+ T cells by concurrent use of the CD8 and CD4 arms of the protocol. Fast DCs, generated from CMV-seropositive donors, were transduced with either Adpp65 or AdMock and subsequently pulsed with cell lysate from either Adpp65- or AdMock-transduced monocytes. The double-modified DCs were then used to stimulate autologous PBLs. (A) PBLs were stained for IFN-γ, CD3 and CD8 or CD4 before (pre-stim) and 11 d after (post-stim) Adpp65/DC or AdMock/DC stimulation. The number in the upper right quadrant represents the frequency of IFN-γ-producing CD3+ T cells within the CD8+ or CD4+ population, respectively. (B) Summary of IFN-γ-producing CD8+ T cells before and after stimulation from 10 individual donors. The efficacy in obtaining IFN-γ-producing CD8+ T cells using the CD8 and CD4 arms simultaneously is also compared to the efficacy of using the CD4 arm (12 donors). (C) Summary of IFN-γ-producing CD4+ T cells before and after stimulation from 10 individual donors. The efficacy in obtaining IFN-γ-producing CD4+ T cells using the CD8 and CD4 arms simultaneously is also compared to the efficacy of using the CD8 arm (11 donors). The bars include the data range, the box represents the 25th to 75th percentile and the line in the box represents the median. (D) Fold induction of IFN-γ-producing T cells from 10 individual donors after Adpp65/DC stimulation compared to before stimulation. Bars illustrate mean values.

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It also appears that both CD8\(^+\) and CD4\(^+\) T cells specific for CMV should be transferred for optimal results (Leen et al., 2006). Although it still needs to be proven, we expect that our protocol will yield long-lasting pp65-specific cytolytic and helper T cells as only one antigen-specific stimulation with low-dose cytokines is used.

Several researchers have tried to dissect whether apoptotic bodies or necrotic cell lysate is the better source of antigen for DCs when it comes to T-cell activation (Sauter et al., 2000; Kotera et al., 2001; Schnurr et al., 2002; Prasad et al., 2005). Adpp65-transduced monocytes subjected either to four freeze–thaw cycles, to harvest cell lysate, or to heat and 2005). Adpp65-transduced monocytes subjected either to four freeze–thaw cycles, to harvest cell lysate, or to heat and irradiation, to induce stressed cells going through apoptosis, were equally good sources of pp65 antigen for DCs regarding the activation of pp65-specific CD4\(^+\) T cells. Our data indicate that fast DCs are phenotypically similar to conventional DCs. They are also highly efficient to activate pp65-specific T cells in blood from CMV-seropositive individuals. We did not assess whether fast DCs can be used to activate pp65-specific T cells from a naïve precursor population in CMV-seronegative individuals. However, Ho et al. (2006) recently demonstrated that fast DCs can be successfully used to activate tumour antigen-associated T cells from naïve precursor populations.

In conclusion, we present a rapid and straightforward strategic approach for efficient simultaneous ex vivo-generation of CD8\(^+\) and CD4\(^+\) CMV pp65-reactive T cells that may be used to adoptively transfer donor-derived T cells to treat and/or prevent CMV reactivation in HSCT patients.

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Conflict of interest

The authors have no conflicting financial interests.

Author contribution

O.F. designed and performed research, collected and analysed data, performed statistical analysis and wrote the manuscript; B.C. and T.H.T. assisted in research design and revised the manuscript; M.E. designed research, evaluated data and wrote the manuscript.

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