bjh research paper

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

Ole Forsberg, Björn Carlsson, Thomas H. Tötterman and Magnus Essand
Clinical Immunology Division, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden

Received 28 September 2007; accepted for publication 21 November 2007
Correspondence: Magnus Essand, Clinical Immunology Division, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden.
E-mail: magnus.essand@klinimm.uu.se

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 *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.

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*, 2000; Zaia *et al*, 2001).

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 (Gillespie *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 magnitude of the CMV-specific $CD8^+$ T-cell response decreased with time in patients with no reconstitution of endogenous CMV-specific helper T-cell response. Einsele *et al* (2002) transferred donor-derived CMV-specific $CD4^+$ T cells which also suppressed the virus, but only if the patient reconstituted an endogenous CMV-specific $CD8^+$ T-cell response. Jointly, this implies that the optimal way to treat HSCT

First published online 26 February 2008
doi:10.1111/j.1365-2141.2008.07026.x

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 β2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured in a 37°C, 5% CO_2 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, NLVPMVATV) 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/$pp65_{459\text{-}503}$ tetramer was purchased from Beckman Coulter (San Diego, CA, USA).

Antibodies and cytokines. Fluorescence-labelled monoclonal antibodies against human interferon gamma (IFN-γ)-fluoroscein 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-PE (BD Biosciences, San Diego, CA, USA), CKR-7-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-12 and IL-7 (Nordic Biosite, Taby, Sweden), IL-2 (Chiron Corporation, Emeryville, CA, USA) and tumour necrosis factor (TNF)-α (Biosource, Camarilo, CA, USA). Polyinosinic-Polycytidylic 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

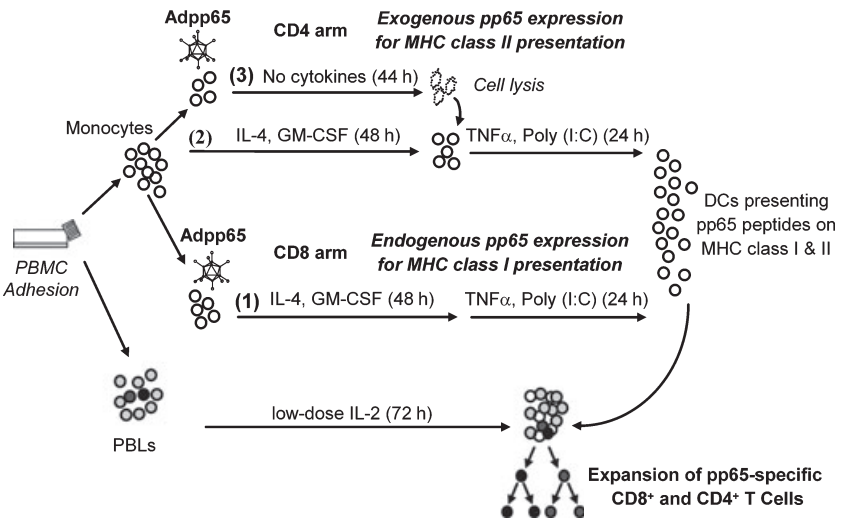

Fig 1. Schematic illustration of the protocol for rapid and simultaneous generation of pp65-specific $CD8^+$ and $CD4^+$ T cells. PBMCs are subjected to plastic adhesion for 90 min. Non-adherent cells (PBL fraction) are collected and maintained with low-dose interleukin (IL)-2 for 72 h. Adherent cells (monocyte fraction) are divided into three portions. One portion (1) is transduced with Adpp65 and differentiated into immature dendritic cells (DCs) with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) for 48 h and further matured with tumour necrosis factor (TNF)-α and Poly(I:C) for 24 h. These DCs are used as MHC class I peptide-presenting cells of endogenously expressed pp65. This part of the protocol is referred to as the CD8 arm. A second portion of monocytes (2) is differentiated into immature DCs and pulsed with the cell lysate from the third portion (3) of Adpp65-transduced monocytes. These DCs are 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 are 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.

subject. 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 ($1{\cdot}5 \times 10^6$ 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- or AdMock-transduced adherent cells. Total cell lysate was obtained by four freeze–thaw cycles while stressed cell debris was obtained by heat (42°C, 1 h) followed by irradiation (100 Gy). The pulsed DCs were matured by addition of TNF-α (40 ng/ml) and Poly(I:C) (30 μg/ml) and cultured for another 24 h. These DCs were 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 for activation of pp65-specific $CD8^+$ and $CD4^+$ T cells (Fig 1). PBLs (15×10^6) were then mixed with autologous antigen-modified DCs at a responder to stimulator (PBL/DC) ratio of 10:1 and cultured for 11 d with 0·1 ng/ml IL-12 and 20 ng/ml IL-7. Fresh medium with 20 ng/ml IL-7 was added after 7 d.

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).

Carboxyfluoroscein succinimidyl ester proliferation assay

The CellTrace carboxyfluoroscein succinimidyl ester (CFSE) cell proliferation kit (Invitrogen, Eugene, OR, USA) was used to evaluate $CD8^+$ and $CD4^+$ T-cell proliferation. Briefly, 2×10^6 PBLs were dyed with 10 μmol/l CFSE solution and co-cultured in 12-well plates with 2×10^5 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/$pp65_{459\text{-}503}$ 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 (5×10^6 cells in 500 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 (^{51}Cr) (GE Healthcare) for 1 h at 37°C, washed three times and unmodified cells were pulsed with $pp65_{495\text{-}503}$ peptide (5 μg/ml) or $VMAT\text{-}1_{31\text{-}39}$ peptide (5 μg/ml) for 2 h. Stimulated PBLs were added to 2500 ^{51}Cr-labelled C1R-A2 target cells in V-bottomed 96-well plates at effector to target ratio (E:T) from 0·4:1 to 50:1 and incubated for 5 h at 37°C. The released ^{51}Cr was counted in a Microbeta Trilux 1450-024 (Helsinki, Finland). Triplicate wells were averaged and the percentage of specific lysis was calculated as follows: Percentage lysis = 100 × [(sample ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)].

DC phenotype

Monocyte-derived matured 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/$pp65_{495\text{-}503}$ 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 cytometric bead array and the phenotype of HLA-A*0201/$pp65_{495\text{-}503}$ tetramer-binding T lymphocytes, before and after stimulation, were compared using the Wilcoxon signed rank test with paired data ($\alpha = 0{\cdot}05$). Frequencies of HLA-A*0201/$pp65_{495\text{-}503}$ 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 ($\alpha = 0{\cdot}01$–$0{\cdot}001$).

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

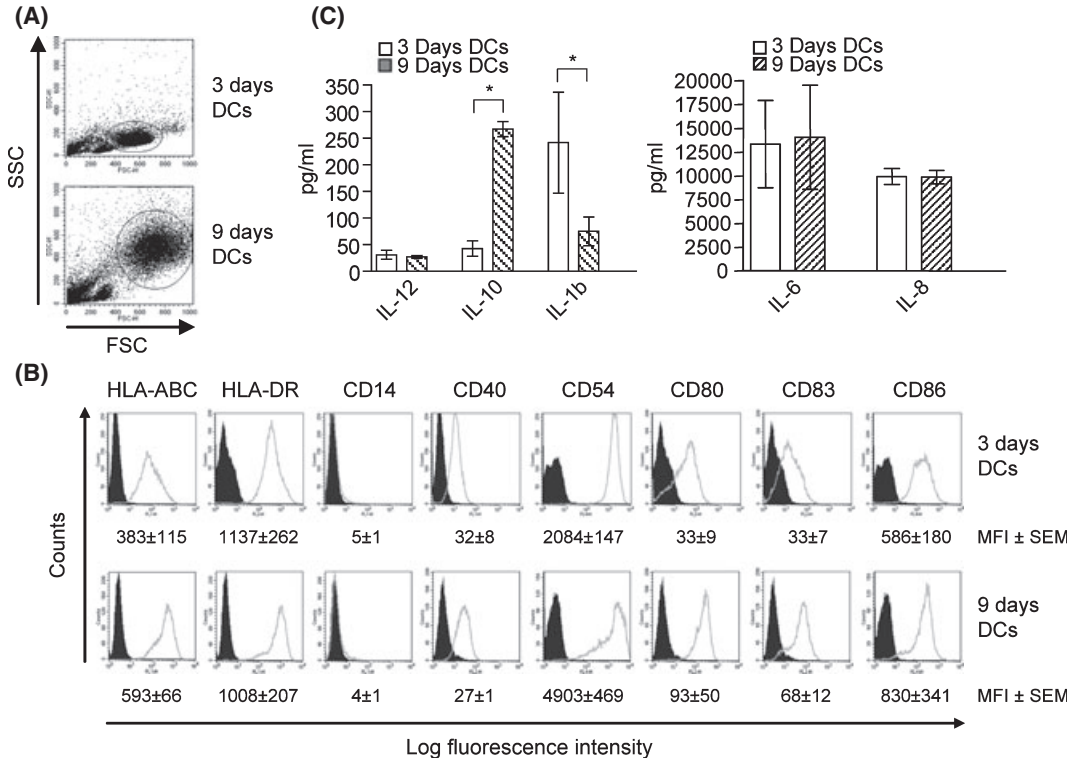

Fig 2. Phenotypic similarities between mature monocyte-derived DCs generated in 3 d (fast DCs) and 9 d (conventional DCs). The adherent cell fraction of PBMCs were maintained in culture media with interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) for differentiation of monocytes into immature DCs. Tumour necrosis factor (TNF)-α and Poly(I:C) was added for maturation the last 24 h (3 d DCs) or 48 h (9 d DCs). The DCs were then compared by flow cytometric analysis for: (A) cell size and granularity using forward scattering (FSC) *versus* side scattering (SSC). DCs are circled; (B) expression of the cell surface markers HLA-ABC, HLA-DR, CD14, CD40, CD54, CD80, CD83, CD86. Mean fluorescence intensity (MFI) are presented as mean values ± SEM from five experiments; (C) production of the cytokines IL-12, IL-10, IL-1b, IL-6, IL-8. The mean value with standard deviation is shown from four to six experiments with triplicate samples.

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 ($\alpha = 0{\cdot}05$) reduced when Poly(I:C) was omitted from the maturation stimuli (data not shown). Fast DCs produced significantly ($\alpha = 0{\cdot}05$) lower amounts of IL-10 and significantly ($\alpha = 0{\cdot}05$) 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/pp65$_{459\text{-}503}$ 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 ($\alpha = 0{\cdot}001$) 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-γ production by PBLs before (pre-stim) and 11 d after (post-stim) stimulation. One example is

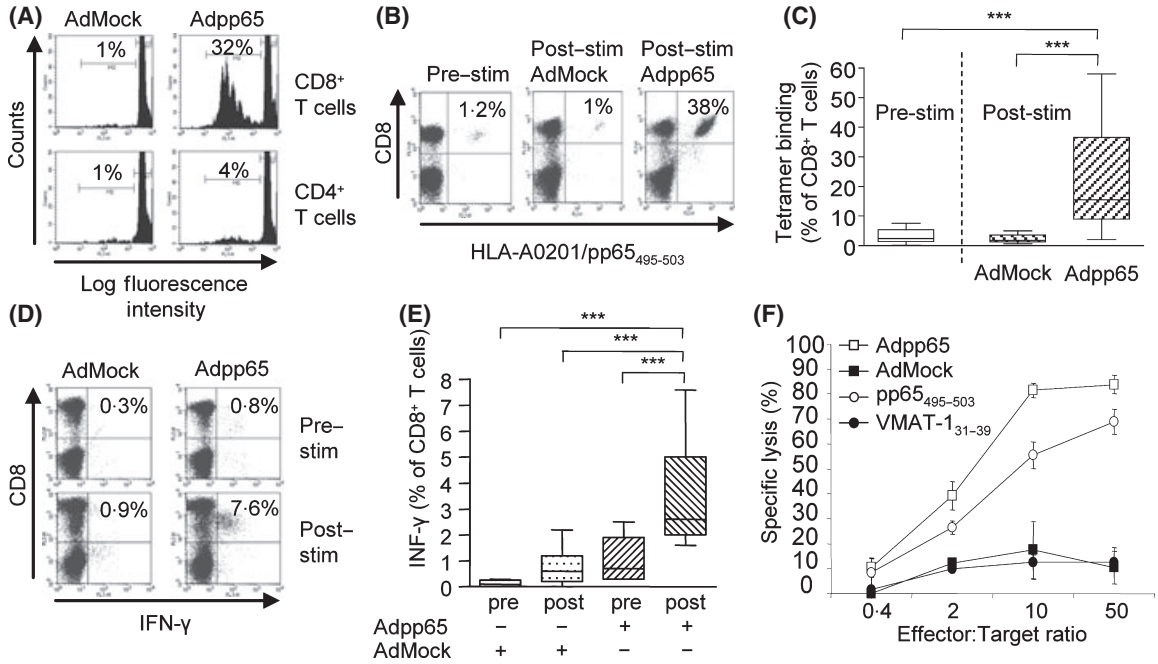

Fig 3. Fast DCs transduced with Adpp65 efficiently expand pp65-specific effector CTLs. Fast DCs, generated from CMV-seropositive HLA-A*0201 positive donors, were transduced with Adpp65 or AdMock and used to stimulate autologous PBLs. (A) T-cell proliferation was analysed by using CFSE-stained PBLs that were co-cultured with transduced DCs for 5 d. Cells were stained for CD3 and either CD8 or CD4 expression and analysed by flow cytometry. Cell division was visualized through dilution of the CFSE dye. (B) PBLs were stained with HLA-A*0201/pp65$_{459-503}$ tetramer, anti-CD3 and anti-CD8 antibodies before (pre-stim) and 11 d after (post-stim) stimulation. The number in the upper right quadrant represents the frequency of tetramer-positive cells within the CD3$^+$, CD8$^+$ population. (C) Summary of tetramer staining of PBLs before (pre-stim) and 11 d after (post-stim) stimulation from 20 individual 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) PBLs were stained with anti-IFN-γ, anti-CD3 and anti-CD8 antibodies before (pre-stim) and 11 d after (post-stim) stimulation. The number in the upper right quadrant represents the frequency of IFN-γ-producing cells within the CD3$^+$, CD8$^+$ population. (E) Summary of IFN-γ-producing CD8$^+$ T cells before (pre-stim) and after (post-stim) stimulation from 11 individual donors. The bars include the data range, the box represents the 25th to 75th percentile and the line in the box represents the median. (F) PBLs, stimulated by Adpp65-transduced DCs, were after 11 d mixed with ^{51}Cr-labelled, antigen-modified C1R-A2 target cells (Adpp65- or AdMock-transduced, pp65$_{459-503}$ or VMAT-1$_{31-39}$ peptide-pulsed). The percentage of specific lysis is expressed as the mean value of the triplicate sample with standard deviation.

shown in Fig 3D where the frequency of IFN-γ-producing CD8$^+$ T cells increased from 0·8% to 7·6% upon Adpp65/DC 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 ($\alpha = 0{\cdot}001$) 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 pp65$_{495-503}$ peptide-pulsed C1R-A2 but had no cytolytic effect on AdMock-transduced C1R-A2 or C1R-A2 pulsed with VMAT-1$_{31-39}$, 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/pp65$_{495-503}$-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$^+$ and CD28$^+$ T cells within the HLA-A*0201/pp65$_{495-503}$

Table I. Phenotype of HLA-A*0201 pp65/$_{495-503}$ tetramer binding T lymphocytes.

Phenotype	Prestimulation (%)*	Poststimulation (%)*	*P*-value
CD45RA$^+$/RO$^-$	28 (11–57)	0·1 (0·1–0·5)	≤0·05
CD45RA$^-$/RO$^+$	60 (31–83)	97 (93–99)	≤0·05
CD25$^+$	0·5 (0·4–1)	19 (11–36)	≤0·05
CD27$^+$	46 (38–50)	44 (34–60)	ns
CD28$^+$	10 (6–14)	49 (47–58)	≤0·05
CD62L$^+$	7 (4–19)	11 (5–12)	ns
CCR7$^+$	0 (0)	0 (0)	ns

*Median (range); ns, not significant. $n = 4$.

tetramer-binding population were significantly higher after stimulation than before stimulation. The frequencies of $CD45RA^+/R0^-$ 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,

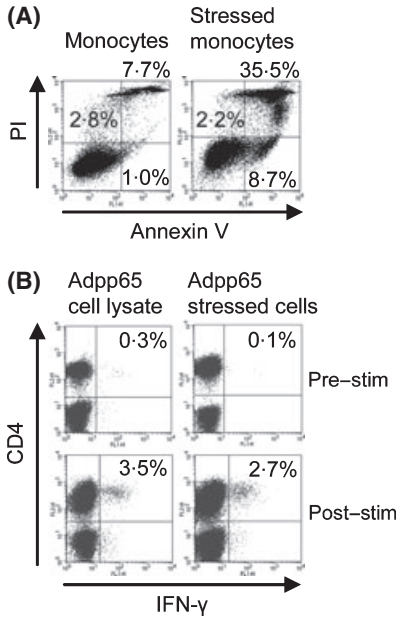

Fig 4. DCs pulsed with pp65 antigen retrieved through apoptotic and necrotic cell death of Adpp65-transduced monocytes works equally well to induce pp65-specific $CD4^+$ T-cell activation. Adpp65-transduced monocytes were heated and irradiated to induce stressed monocytes undergoing mainly apoptotic cell death. Other Adpp65-transduced monocytes were subjected to four freeze–thaw cycles to induce necrotic cell death followed by harvesting of cell lysate. (A) Propidium iodide (PI) and Annexin V staining of normal monocytes and heat/irradiated (stressed) monocytes. (B) DCs were pulsed with cell lysate from autologous Adpp65-transduced monocytes or with stressed autologous Adpp65-transduced monocytes and subsequently used to stimulate autologous PBLs. PBLs were stained with anti-IFN-γ, anti-CD3 and anti-CD4 antibodies before (pre-stim) and 11 d after (post-stim) stimulation. The number in the upper right quadrant represents the frequency of IFN-γ-producing cells within the $CD3^+$, $CD4^+$ population.

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 ($\alpha = 0{\cdot}001$) 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%

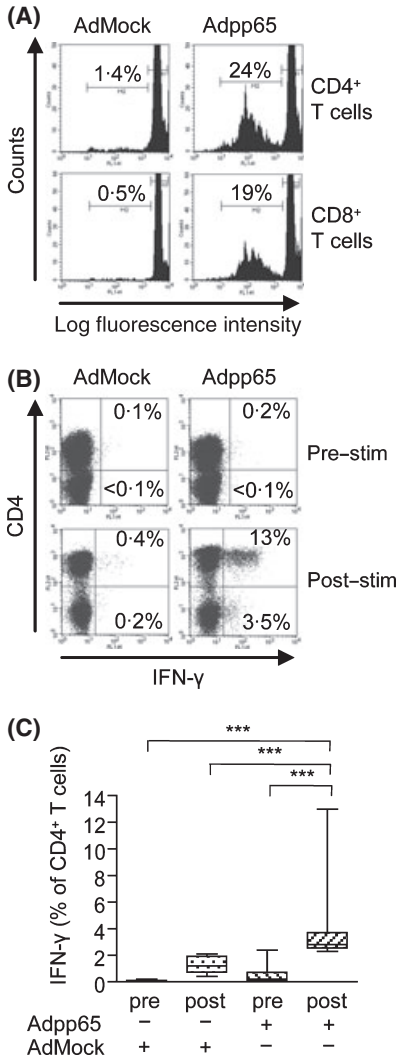

Fig 5. DCs pulsed with cell lysate from Adpp65-transduced monocytes efficiently expand pp65-specific $CD4^+$ T cells and, to a lesser degree, $CD8^+$ T cells. Fast DCs, generated from CMV-seropositive donors, were pulsed with cell lysate from either Adpp65- or AdMock-transduced monocytes and used to stimulate autologous PBLs. (A) T-cell proliferation was analysed by using CFSE-stained PBLs that were co-cultured with cell lysate-pulsed DCs for 5 d. Cells were stained for CD3 and either CD4 or CD8 and analysed by flow cytometry. (B) PBLs were stained for IFN-γ, CD3 and CD4 before (pre-stim) and 11 d after (post-stim) stimulation. The number in the upper right quadrant represents the frequency of IFN-γ-producing cells within the $CD3^+$, $CD4^+$ population while the number in the lower right quadrant represents the frequency of IFN-γ-producing cells within the $CD3^+$, $CD4^-$, *i.e.* $CD8^+$, population. (C) Summary of IFN-γ-producing $CD4^+$ T cells before (pre-stim) and after (post-stim) stimulation from 12 individual donors. The bars include the data range, the box represents the 25th to 75th percentile and the line in the box represents the median.

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*0201- and HLA-B*0702-restricted peptides from the pp65 and IE antigens for activation of $CD8^+$ T cells.

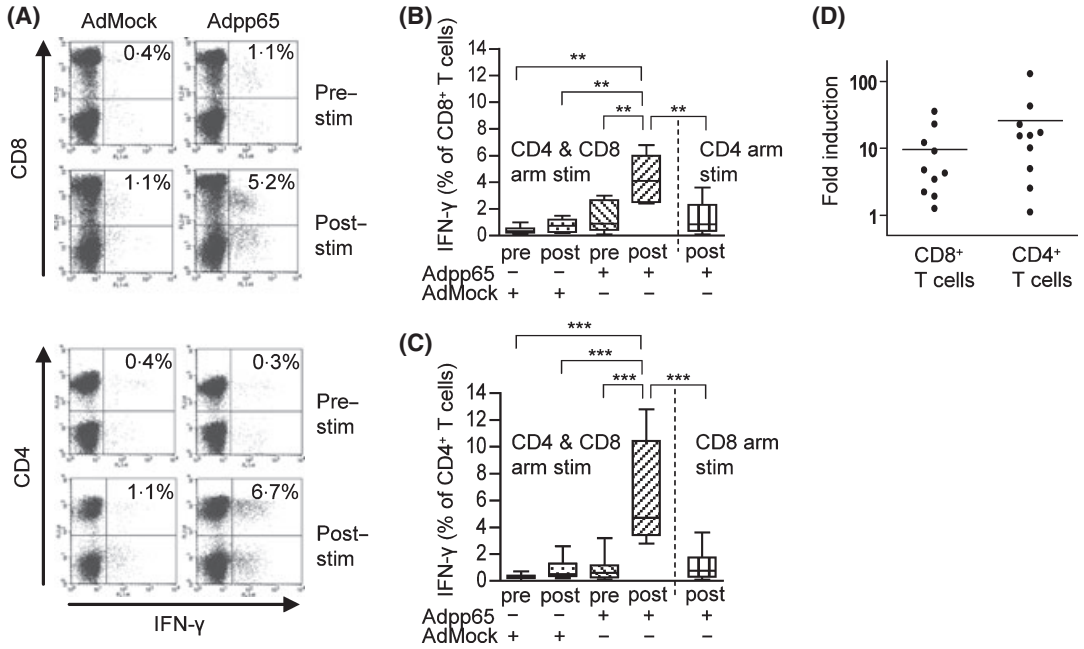

Fig 6. Simultaneous activation of pp65-specific $CD8^+$ and $CD4^+$ T cells by concurrent use of the CD8 and CD4 arms of the protocol. Fast DCs, generated from CMV-seropositive donors, were transduced with either Adpp65 or AdMock and subsequently pulsed with cell lysate from either Adpp65- or AdMock-transduced 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.

Following an overnight stimulation of $2–4 \times 10^8$ PBMCs with CMV lysate and peptides, on average 3×10^6 IFN-γ-producing cells were isolated and expanded over 10–11 d to an average of $4{\cdot}6 \times 10^8$ 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 15×10^6 PBLs and after stimulation we always recovered between 13 and 17×10^6 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 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 $3{\cdot}3–100 \times 10^7$ cells/m^2 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 1×10^7 cells/m^2 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 1×10^5 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 $8{\cdot}6 \times 10^3$ 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.

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 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.

Acknowledgements

The authors wish to thank Berith Nilsson for technical assistance during virus production. The Swedish Cancer Society (Grant 4419-B05-06XBC) supported the work. M.E. is a recipient of the Göran Gustafsson's Award.

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.

References

Albert, M.L., Pearce, S.F., Francisco, L.M., Sauter, B., Roy, P., Silverstein, R.L. & Bhardwaj, N. (1998) Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *Journal of Experimental Medicine*, **188,** 1359–1368.

Bennett, S.R., Carbone, F.R., Karamalis, F., Flavell, R.A., Miller, J.F. & Heath, W.R. (1998) Help for cytotoxic-T-cell responses is mediated by CD40 signalling. *Nature*, **393,** 478–480.

Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P.A. & Kurts, C. (2007) Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science*, **316,** 612–616.

Carlsson, B., Cheng, W.S., Totterman, T.H. & Essand, M. (2003) Ex vivo stimulation of cytomegalovirus (CMV)-specific T cells using CMV pp65-modified dendritic cells as stimulators. *British Journal of Haematology*, **121,** 428–438.

Carlsson, B., Hou, M., Giandomenico, V., Nilsson, B., Totterman, T.H. & Essand, M. (2005) Simultaneous generation of cytomegalovirus-specific CD8+ and CD4+ T lymphocytes by use of dendritic cells comodified with pp65 mRNA and pp65 protein. *Journal of Infectious Diseases*, **192,** 1912–1920.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I. & Lanzavecchia, A. (1999) Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *Journal of Experimental Medicine*, **189,** 821–829.

Cobbold, M., Khan, N., Pourgheysari, B., Tauro, S., McDonald, D., Osman, H., Assenmacher, M., Billingham, L., Steward, C., Crawley, C., Olavarria, E., Goldman, J., Chakraverty, R., Mahendra, P., Craddock, C. & Moss, P.A. (2005) Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *Journal of Experimental Medicine*, **202,** 379–386.

Dauer, M., Obermaier, B., Herten, J., Haerle, C., Pohl, K., Rothenfusser, S., Schnurr, M., Endres, S. & Eigler, A. (2003) Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. *Journal of Immunology*, **170,** 4069–4076.

Dzojic, H., Cheng, W.S. & Essand, M. (2007) Two-step amplification of the human PPT sequence provides specific gene expression in an immunocompetent murine prostate cancer model. *Cancer Gene Therapy*, **14,** 233–240.

Einsele, H., Hebart, H., Kauffmann-Schneider, C., Sinzger, C., Jahn, G., Bader, P., Klingebiel, T., Dietz, K., Loffler, J., Bokemeyer, C., Muller, C.A. & Kanz, L. (2000) Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. *Bone Marrow Transplantation*, **25,** 757–763.

Einsele, H., Roosnek, E., Rufer, N., Sinzger, C., Riegler, S., Loffler, J., Grigoleit, U., Moris, A., Rammensee, H.G., Kanz, L., Kleihauer, A., Frank, F., Jahn, G. & Hebart, H. (2002) Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood*, **99,** 3916–3922.

Foster, A.E., Bradstock, K.F., Sili, U., Marangolo, M., Rooney, C.M. & Gottlieb, D.J. (2004) A comparison of gene transfer and antigen-loaded dendritic cells for the generation of CD4+ and CD8+ cytomegalovirus-specific T cells in HLA-A2+ and HLA-A2- donors. *Biology of Blood and Marrow Transplantation*, **10,** 761–771.

Gillespie, G.M., Wills, M.R., Appay, V., O'Callaghan, C., Murphy, M., Smith, N., Sissons, P., Rowland-Jones, S., Bell, J.I. & Moss, P.A. (2000) Functional heterogeneity and high frequencies of cytomegalovirus-specific CD8(+) T lymphocytes in healthy seropositive donors. *Journal of Virology*, **74,** 8140–8150.

Heine, A., Grunebach, F., Holderried, T., Appel, S., Weck, M.M., Dorfel, D., Sinzger, C. & Brossart, P. (2006) Transfection of dendritic cells with *in vitro*-transcribed CMV RNA induces polyclonal CD8+- and CD4+-mediated CMV-specific T cell responses. *Molecular Therapy*, **13,** 280–288.

Ho, W.Y., Nguyen, H.N., Wolfl, M., Kuball, J. & Greenberg, P.D. (2006) *In vitro* methods for generating CD8+ T-cell clones for immunotherapy from the naive repertoire. *Journal of Immunological Methods*, **310,** 40–52.

Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J. & Enk, A.H. (1997) Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *European Journal of Immunology*, **27,** 3135–3142.

Kern, F., Bunde, T., Faulhaber, N., Kiecker, F., Khatamzas, E., Rudawski, I.M., Pruss, A., Gratama, J.W., Volkmer-Engert, R., Ewert, R., Reinke, P., Volk, H.D. & Picker, L.J. (2002) Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. *Journal of Infectious Diseases*, **185,** 1709–1716.

Kotera, Y., Shimizu, K. & Mule, J.J. (2001) Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. *Cancer Research*, **61,** 8105–8109.

Krause, H., Hebart, H., Jahn, G., Muller, C.A. & Einsele, H. (1997) Screening for CMV-specific T cell proliferation to identify patients at risk of developing late onset CMV disease. *Bone Marrow Transplantation*, **19,** 1111–1116.

Lee, A.W., Truong, T., Bickham, K., Fonteneau, J.F., Larsson, M., Da Silva, I., Somersan, S., Thomas, E.K. & Bhardwaj, N. (2002) A clinical grade cocktail of cytokines and PGE2 results in uniform maturation of human monocyte-derived dendritic cells: implications for immunotherapy. *Vaccine*, **20**(Suppl. 4)**,** A8–A22.

Leen, A.M., Myers, G.D., Sili, U., Huls, M.H., Weiss, H., Leung, K.S., Carrum, G., Krance, R.A., Chang, C.C., Molldrem, J.J., Gee, A.P., Brenner, M.K., Heslop, H.E., Rooney, C.M. & Bollard, C.M. (2006) Monoculture-derived T lymphocytes specific for multiple viruses expand and produce clinically relevant effects in immunocompromised individuals. *Nature Medicine*, **12,** 1160–1166.

Li, C.R., Greenberg, P.D., Gilbert, M.J., Goodrich, J.M. & Riddell, S.R. (1994) Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. *Blood*, **83,** 1971–1979.

Ljungman, P. (2002) Prevention and treatment of viral infections in stem cell transplant recipients. *British Journal of Haematology*, **118,** 44–57.

Ljungman, P., Engelhard, D., Link, H., Biron, P., Brandt, L., Brunet, S., Cordonnier, C., Debusscher, L., de Laurenzi, A., Kolb, H.J., Messina, C., Newland, A.C., Prentice, H.G., Richard, C., Ruutu, T., Tilg, H. & Verdonck, L. (1992) Treatment of interstitial pneumonitis due to cytomegalovirus with ganciclovir and intravenous immune globulin: experience of European Bone Marrow Transplant Group. *Clinical Infectious Diseases*, **14,** 831–835.

Meyers, J.D. (1991) Prevention and treatment of cytomegalovirus infection. *Annual Review of Medicine*, **42,** 179–187.

Meyers, J.D., Flournoy, N. & Thomas, E.D. (1986) Risk factors for cytomegalovirus infection after human marrow transplantation. *Journal of Infectious Diseases*, **153,** 478–488.

Obermaier, B., Dauer, M., Herten, J., Schad, K., Endres, S. & Eigler, A. (2003) Development of a new protocol for 2-day generation of mature dendritic cells from human monocytes. *Biological Procedures Online*, **5,** 197–203.

Peggs, K., Verfuerth, S. & Mackinnon, S. (2001) Induction of cytomegalovirus (CMV)-specific T-cell responses using dendritic cells pulsed with CMV antigen: a novel culture system free of live CMV virions. *Blood*, **97,** 994–1000.

Peggs, K.S., Verfuerth, S., Pizzey, A., Khan, N., Guiver, M., Moss, P.A. & Mackinnon, S. (2003) Adoptive cellular therapy for early cytomegalovirus infection after allogeneic stem-cell transplantation with virus-specific T-cell lines. *Lancet*, **362,** 1375–1377.

Prasad, S.J., Farrand, K.J., Matthews, S.A., Chang, J.H., McHugh, R.S. & Ronchese, F. (2005) Dendritic cells loaded with stressed tumor cells elicit long-lasting protective tumor immunity in mice depleted of CD4+CD25+ regulatory T cells. *Journal of Immunology*, **174,** 90–98.

Rauser, G., Einsele, H., Sinzger, C., Wernet, D., Kuntz, G., Assenmacher, M., Campbell, J.D. & Topp, M.S. (2004) Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood*, **103,** 3565–3572.

Reddy, A., Sapp, M., Feldman, M., Subklewe, M. & Bhardwaj, N. (1997) A monocyte conditioned medium is more effective than defined cytokines in mediating the terminal maturation of human dendritic cells. *Blood*, **90,** 3640–3646.

Riddell, S.R., Watanabe, K.S., Goodrich, J.M., Li, C.R., Agha, M.E. & Greenberg, P.D. (1992) Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science*, **257,** 238–241.

Riddell, S.R., Walter, B.A., Gilbert, M.J. & Greenberg, P.D. (1994) Selective reconstitution of CD8+ cytotoxic T lymphocyte responses in immunodeficient bone marrow transplant recipients by the adoptive transfer of T cell clones. *Bone Marrow Transplantation*, **14**(Suppl. 4)**,** S78–84.

Ridge, J.P., Di Rosa, F. & Matzinger, P. (1998) A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature*, **393,** 474–478.

Rock, K.L. (2003) The ins and outs of cross-presentation. *Nature Immunology*, **4,** 941–943.

Sauter, B., Albert, M.L., Francisco, L., Larsson, M., Somersan, S. & Bhardwaj, N. (2000) Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *Journal of Experimental Medicine*, **191,** 423–434.

Schnurr, M., Scholz, C., Rothenfusser, S., Galambos, P., Dauer, M., Robe, J., Endres, S. & Eigler, A. (2002) Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells. *Cancer Research*, **62,** 2347–2352.

Schoenberger, S.P., Toes, R.E., van der Voort, E.I., Offringa, R. & Melief, C.J. (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature*, **393,** 480–483.

Walter, E.A., Greenberg, P.D., Gilbert, M.J., Finch, R.J., Watanabe, K.S., Thomas, E.D. & Riddell, S.R. (1995) Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *New England Journal of Medicine*, **333,** 1038–1044.

Xu, S., Koski, G.K., Faries, M., Bedrosian, I., Mick, R., Maeurer, M., Cheever, M.A., Cohen, P.A. & Czerniecki, B.J. (2003) Rapid high efficiency sensitization of CD8+ T cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12-dependent mechanism. *Journal of Immunology*, **171,** 2251–2261.

Zaia, J.A., Gallez-Hawkins, G., Li, X., Yao, Z.Q., Lomeli, N., Molinder, K., La Rosa, C. & Diamond, D.J. (2001) Infrequent occurrence of natural mutations in the pp65(495-503) epitope sequence presented by the HLA A*0201 allele among human cytomegalovirus isolates. *Journal of Virology*, **75,** 2472–2474.

