Simultaneous Generation of Cytomegalovirus-Specific CD8⁺ and CD4⁺ T Lymphocytes by Use of Dendritic Cells Comodified with pp65 mRNA and pp65 Protein

Björn Carlsson, Mingyan Hou, Valeria Giandomenico, Berith Nilsson, Thomas H. Tötterman, and Magnus Essand
Division of Clinical Immunology, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden

Cytomegalovirus (CMV) disease remains a severe complication in patients who have undergone transplantation. Viremia can be prevented and treated by the adoptive transfer of donor-derived CMV-directed T cells. To ensure long-term protection against CMV disease, it is important to transfer CMV antigen–specific T cells that represent both the CD8⁺ and the CD4⁺ subsets. In the present study, we used as stimulators dendritic cells (DCs) that were electroporated with in vitro–transcribed 5’-capped polyadenylated messenger RNA (mRNA) that encoded the CMV pp65 protein (i.e., pp65 mRNA). These DCs could efficiently activate CMV-directed CD8⁺ T cells, as assayed by tetramer staining, interferon-γ production, and cytolytic activity. We also used DCs that were pulsed with a recombinant pp65 protein to activate CMV-directed CD4⁺ T cells. When DCs were comodified with pp65 mRNA and pp65 protein, large numbers of CMV-directed CD8⁺ and CD4⁺ T cells were generated simultaneously. The approach outlined in the present study can be adapted for a clinical protocol that circumvents potential virus-related biohazards and is available to all patients independently of their human leukocyte antigen haplotype.

Received 28 June 2005; accepted 7 September 2005; electronically published 21 October 2005.

Potential conflicts of interest: none reported.

Financial support: Swedish Cancer Society (grant 4419-B03-04XBB); Swedish National Gene Therapy Program (grant K2002-89XG-13288-04A); Swedish Research Council (grant K2004-16X-15008-01A).

Reprints or correspondence: Dr. Magnus Essand, Div of Clinical Immunology, Rudbeck Laboratory, Uppsala University, S-751 85 Uppsala, Sweden (magnus.essand@klinimm.uu.se).

The Journal of Infectious Diseases 2005;192:1912–20
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0022-1899/2005/19211-0009$15.00

Human cytomegalovirus (CMV) is a herpesvirus that lays dormant in the myeloid cells of 50%–100% of the adult population. In healthy seropositive individuals, CMV is effectively suppressed by virus-specific T cells directed against a limited number of immunodominant proteins, such as pp65, the major intermediate-early protein exon 4, glycoprotein B, and pp150 [1–4]. In patients who have undergone transplantation, CMV-specific T cells are suppressed or depleted, and the patients are at high risk for CMV reactivation or primary infection [5]. The introduction of ganciclovir or foscarnet prophylaxis has reduced the frequency of early-onset CMV disease, but many patients who undergo transplantation are still at risk for late-onset CMV disease [6–8]. Prolonged treatment with antiviral drugs leads to nephrotoxicity and myelosuppression, followed by the development of bacterial and fungal infections [9–13]. In addition, clinical CMV strains that are resistant to standard antiviral treatments are emerging [14, 15].

CMV reactivation can be prevented by adoptive transfer of ex vivo–generated, donor-derived, CMV-specific T cells [16–18]. In the initial study by Walter et al. [16], CMV-specific CD8⁺ T cells were transferred, and survival of these cells was dependent on the reconstitution of endogenous CMV-specific CD8⁺ T cells in the patient. In a later study, Einsele et al. [18] transferred donor-derived CMV-specific CD4⁺ T cells, which could also suppress the virus, but only if the patient reconstituted an endogenous CMV-specific CD8⁺ T cell response. Together, these findings imply that the optimal way of treating patients with CMV disease who undergo hematopoietic stem cell transplantation would be to transfer donor-derived CMV antigen–specific T cells of both the CD4⁺ and the CD8⁺ subsets.

We and other investigators have described different
ways of generating CMV-specific CD4+ and CD8+ T cells for adoptive transfer. T cells have been activated and expanded using dendritic cells (DCs), autologous fibroblasts, or Epstein-Barr virus-infected B cells that were either transduced with antigen-coding viral vectors or infected with CMV [19–26]. These approaches have proven very effective in generating CMV-directed T cells. However, the use of intact CMV or recombinant viral vectors represents a potential biohazard, because there is an associated risk of transmitting replication-competent viruses to the immunosuppressed patient. DCs have also been successfully pulsed with CMV lysate for subsequent use as stimulators for T cell expansion [18, 27, 28]. However, crude CMV lysate might influence the efficacy of ex vivo generation of T cells as a result of the blocking effects on antigen-presenting machinery that are inflicted by viral immune evasion proteins [28–33], although these proteins have been shown not to prevent a diverse CD8+ T cell response in natural infection [34]. Alternatively, T cells have been generated by stimulation with CMV peptide-pulsed DCs or artificial antigen-presenting cells [3, 19, 23, 28, 35, 36]. This approach is efficient, safe, and easily adaptable to a clinical setting. However, the use of peptides for stimulation of either CD8+ or CD4+ T cells implies that the immunodominant peptides restricted by the HLA-ABC or HLA-DR/DQ haplotype of the donor are known; this is not the case for all HLA molecules [37]. Thus, there is a need for an alternative strategy to stimulate both CD4+ and CD8+ CMV-specific T cells without the use of recombinant viral vectors or live or attenuated virus.

In the present study, we investigated whether DCs electroporated with CMV pp65 mRNA and pulsed with recombinant pp65 protein are able to stimulate the expansion of CMV pp65-directed CD8+ and CD4+ T cells. We show that simultaneous activation of CD8+ and CD4+ T cells against CMV can readily be achieved.

MATERIALS AND METHODS

Plasmid construction and in vitro mRNA transcription. The sequence encoding enhanced green fluorescent protein (EGFP) was obtained from pEGFP-C2 (BD Clontech), and the CMV pp65 sequence was obtained from pBSpp65-18 (gift from J. A. Zaia, City of Hope, Duarte, CA); both sequences were amplified by polymerase chain reaction (PCR) and were inserted in pVAX1 (Invitrogen). The constructs were sequenced (Big Dye Terminator Cycle Sequencing Ready Reaction Kit; PerkinElmer). The plasmids were linearized, and 7-methylguanosine 5′-capped mRNAs were transcribed using T7 RNA polymerase (mMESSAGE mMACHINE; Ambion). The reactions were treated with DNaseI, and the synthesized mRNAs were polyadenylated (poly[A]) using Escherichia coli poly(A) polymerase (Ambion). The reactions were purified by LiCl precipitation, and mRNA quality was analyzed by denatured agarose gel electrophoresis.

In vitro transcription-coupled translation. 35S-Met-labeled EGFP and pp65 proteins were produced by in vitro transcription-coupled translation from the plasmid constructs described above, by use of T7 RNA polymerase and rabbit reticulocyte lysate (Promega). Samples were resolved by electrophoresis performed on a 10% SDS-polyacrylamide gel. The gel was dried and was subjected to autoradiography.

Production of recombinant fusion proteins. A recombinant CMV pp65 protein was constructed with the κ mouse immunoglobulin light chain (Igκ) leader sequence at the N terminal and the constant domain of a human IgG, antibody (Fcγ) at the C terminal. The Igκ leader sequence was included for secretion of the recombinant protein into the supernatant of the producer cells. The leader sequence is cleaved off at the time of secretion. The Fcγ domain was included to facilitate purification of the secreted recombinant protein. The 63-nucleotide-long Igκ leader sequence was obtained by annealing 2 complementary primers, the sequences of which were derived from the pSecTag/FRT/V5-His-TOPO plasmid (BD Clontech). By use of PCR, the CMV pp65 coding sequence was amplified from the pBSpp65-18 plasmid (gift from J. A. Zaia), and, also by use of PCR, the Fcγ coding sequence was amplified from the pEFc, plasmid (gift from P.-Å. Nygren, Royal Institute of Technology, Stockholm, Sweden) [38]. The Igκ, pp65, and Fcγ sequences were subcloned into the pcDNA3 plasmid (Invitrogen), yielding pcDNA3(Igκ-pp65-Fcγ). The insert was sequenced and subcloned into the pFastBac1 donor plasmid (Invitrogen).

For recombinant protein expression, the Bac-to-Bac (Invitrogen) baculoviral expression system was used. In brief, recombinant baculovirus DNA was constructed by transposition of the pFastBac1(Igκ-pp65-Fcγ) donor plasmid into an E. coli strain that contains baculovirus backbone (DH10Bac; Invitrogen). Recombinant baculovirus DNA was then isolated and transfected into Sf9 insect cells by use of Cellfectin (Invitrogen). The virus supernatant was harvested, and the baculoviral stock was amplified by 3 rounds of infections with increasing volumes of Sf9 cells (with use of 0.1 pfu/cell). For protein production, Sf9 cells were infected with 5 pfu of amplified baculovirus/cell. The recombinant pp65-Fcγ protein was harvested from the supernatant, and the protein was purified on a Protein G Sepharose column (Amersham Biosciences). Recombinant EGFP with an Igκ N-terminal tag and an Fcγ C-terminal tag was produced for controls by use of the same protocol.

Production of recombinant adenoviruses. Replication-deficient recombinant human serotype 5 adenoviral vectors coding for EGFP (AdEGFP) and without transgene (AdMock) were produced from pAdTrack-CMV and pShuttle-CMV, respectively, by use of the AdEasy vector system [39] (Q-Biogene and
Cell line and adenovirus transduction. The C1R.A2 cells (gift from J. Berzofsky, National Cancer Institute, National Institutes of Health, Bethesda, MD) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and, at regular intervals, with 200 ng of Genetin (Invitrogen) per mL, to guarantee HLA-A*0201 expression. The surface HLA-A*0201 expression of C1R.A2 was analyzed by flow cytometry (FACS-Calibur; Becton Dickinson) performed using a fluo escein isothiocyanate–labeled anti–HLA-A2 antibody (One Lambda). C1R.A2 cells were transduced with Adpp65, AdEGFP, or AdMock at an MOI of 100. Four h later, the cells were washed once and then were cultured for 48 h under standard conditions. EGFP expression was analyzed by flow cytometry.

Western blot analysis and Coomassie blue staining. Purified recombinant pp65-Fc, protein (2 mg) or total protein extract from Adpp65- or AdMock-transduced C1R.A2 cells (25 mg) was resolved by electrophoresis performed on 8% SDS-polyacrylamide gels and was transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed with a mouse monoclonal anti-pp65 antibody (Abcam) and a goat polyclonal anti-β-actin antibody (Santa Cruz Biotechnology). The secondary antibodies were horseradish peroxidase–conjugated anti–mouse/anti–rabbit (Roche) and horseradish peroxidase–conjugated donkey anti–goat (Santa Cruz Biotechnology) antibodies. The blots were visualized using the BM Chemiluminescence Western blotting kit (Roche) and the Western Blotting Luminol Reagent (Santa Cruz Biotechnology), respectively. To assess the purity of the recombinant pp65-Fc, protein, 2 μg of purified protein was resolved on a 6% SDS-polyacrylamide gel and was visualized using Gelcode blue staining reagent (Pierce).

Generation of monocyte-derived DCs. Monocyte-derived DCs were generated from peripheral blood mononuclear cells (PBMCs) from the buffy coats of healthy CMV-seropositive blood donors, as described elsewhere [19]. Informed consent was obtained from each subject.

Preparation of mRNA-modifie DCs. Immature DCs (4 × 10^6 cells) were mixed with 20 μg of mRNA in a final volume of 200 μL and were electroporated in a 4-mm cuvette by use of a GenePulsar II electroporator (Bio-Rad). The optimized physical parameters were as follows: 300 V, 150 μF, and infinite resistance (Ω), giving a time constant of 4–12 ms. After electroporation, the cells were transferred to fresh culture medium containing granulocyte macrophage–colony–stimulating factor, interleukin-4, and tumor necrosis factor–α, at concentrations stated elsewhere [19], and they were cultured for 48 h to allow the DCs to fully mature.

Preparation of protein or lysate-modifie DCs. Immature DCs (1 × 10^6 cells) were pulsed either with 1 μg of recombinant pp65-Fc, or EGFP-Fc, protein or with 1 μg of CMV lysate (Advanced Biotechnologies) in a final volume of 1 mL. After 8 h, granulocyte macrophage–colony–stimulating factor, interleukin-4, and tumor necrosis factor–α were added at concentrations stated elsewhere [19], and the cells were cultured for another 48 h to allow the DCs to fully mature.

T cell stimulation. DCs were modifie by mRNA, protein, or CMV lysate, as described above. Alternatively, they were modifie by adenovirus transduction or pp65495–503 peptide pulsing, as described elsewhere [19]. The modified fully matured DCs were subsequently used to stimulate autologous T cells, as described elsewhere [19].

Tetramer analysis. The HLA-A*0201/pp65495–503, HLA-A*0101/pp65353–363, and HLA-B*0702/pp65407–416 tetramers (Beckman Coulter Immunomics Operations) were used to detect CMV pp65–specific T cells before stimulation and 12 days after stimulation with antigen-modifie DCs, as described elsewhere [19].

Intracellular interferon (IFN)–γ staining. T cells were analyzed for IFN-γ production by intracellular staining done directly after the initial stimulation or directly after restimulation with antigen-modifie or unmodifie DCs, PBMCs, or C1R.A2 cells, as described elsewhere [19].

Cytotoxicity analysis. Twelve days after stimulation, T cells stimulated by autologous pp65 mRNA–modifie DCs were analyzed for cytotoxic activity against ^3^Cr-labeled C1R.A2 cells that were either transduced with Adpp65 or AdMock or pulsed with the pp65495–503 peptides or an irrelevant HLA-A*0201–binding peptide. The chromium release assay that was used has been described elsewhere [19].

RESULTS

Optimization of mRNA synthesis and DC electroporation. To evaluate the stimulatory capacity of mRNA-modifie DCs, 5’-capped poly(A) mRNAs encoding EGFP and pp65 were synthesized in vitro (fig. e1A). The ability of the mRNA molecules to produce full-length proteins was verifie by in vitro translation analysis performed using rabbit reticulocyte lysate. EGFP appears at ∼30 kDa and pp65 appears at ∼68 kDa, as expected on a 10% SDS-polyacrylamide gel (fig. e1B). We next optimized the conditions for mRNA electroporation of immature DCs by use of in vitro–synthesized 5’-capped poly(A) mRNA coding for EGFP. Under optimal conditions (see Materials and Methods), 50%–70% of the mRNA-electroporated DCs typically expressed EGFP, as assessed by fluo escence microscopy and flow cytometry. A representative flow-cytometri example of EGFP mRNA–electroporated DCs is shown in fig. e1C.

CMV pp65 mRNA–modifie DCs activate pp65–specific CD8+ T cells. Because mRNA-electroporated DCs were found to efficiently express the translated protein, we next evaluated the stimulatory capacity of antigen-modifie DCs by compar-
Figure 1. In vitro transcription of mRNA and electroporation of dendritic cells (DCs). A, Schematic illustration of the enhanced green fluorescent protein (EGFP) and pp65 mRNA constructs. A 7-methylguanosine “cap” is located at the 5′ end, and a polyadenylated (poly(A)) tail is located at the 3′ end of the coding mRNA sequences. B, In vitro transcription-coupled translation analysis of EGFP and pp65. Molecular mass markers, expressed in kilodaltons, are indicated. C, Immature DCs were electroporated with 5′-capped poly(A) mRNA coding for EGFP. After 48 h, the cells were analyzed for green fluorescence by flow cytometry. The unshaded curve denotes DCs electroporated with EGFP mRNA, whereas the shaded curve denotes negative control DCs electroporated with pp65 mRNA.

Figure 2. Generation of pp65-specific CD8+ T cells. A, Stimulation of autologous lymphocytes by use of modified dendritic cells (DCs). DCs from a cytomegalovirus (CMV)-seropositive HLA-A*0201–positive donor with preexisting CD8+ T cells against pp65495–503 were modified with either pp65 mRNA electroporation, pp65 adenoviral vector (Adpp65) transduction, or pp65495–503 peptide pulsing, and they then were used to stimulate autologous lymphocytes. T cells were analyzed before and 12 days after stimulation. The percentage in the upper right corner of each box is the percentage of tetramer-positive cells in the CD8+ T cell population. B, Stimulation of autologous lymphocytes by use of pp65 RNA–electroporated DCs. DCs from a CMV-seropositive, HLA-A*0201/HLA-B*0702–positive but HLA-A*0101–negative donor were electroporated with pp65 mRNA and were used to stimulate autologous lymphocytes. Twelve days after stimulation, we observed a 5-fold increase in the percentage of tetramer-positive cells in the CD8+ T cell population.

This particular case, Adpp65-transduced DCs yielded 18% tetramer-positive T cells in the CD8+ T cell population. However, the overall frequency of tetramer-positive CD8+ T cells in the entire T cell population (including both CD8+ and CD8− T cells) was similar when pp65 mRNA–electroporated DCs and Adpp65-transduced DCs were used as stimulators. This finding indicates that Adpp65-transduced DCs activate CD8+ T cells not only against the CMV pp65 transgene protein but also against adenoviral proteins from the vector.

We next evaluated whether DCs modified with full-length mRNA are able to activate T cells against multiple HLA-specific peptide epitopes from pp65. Immature DCs obtained from a CMV-seropositive, HLA-A*0201/HLA-B*0702–positive but HLA-A*0101–negative donor were electroporated with pp65 mRNA and were used to stimulate autologous lymphocytes. Twelve days after stimulation, we observed a 5-fold increase in the percentage of tetramer-positive cells in the CD8+ T cell population.
Figure 3. Specific cytolytic activity exhibited by T cells stimulated by pp65 mRNA electroporation. A–C, Validations of target cells. A, Cell surface expression of HLA-A*0201 molecules (unshaded curve) on C1R.A2 cells analyzed by flow cytometry. The shaded curve denotes background control. B, C1R.A2 cells were transduced with an adenoviral vector coding for enhanced green fluorescent protein (EGFP) (MOI, 100) and were analyzed by flow cytometry 48 h later. The unshaded curve denotes EGFP expression, whereas the shaded curve denotes C1R.A2 cells transduced by adenoviral vectors without transgene (AdMock). C, C1R.A2 cells transduced with pp65 adenoviral vector (Adpp65) or AdMock (MOI, 100). After 48 h, the cells were harvested, and total protein lysates were separated by SDS-PAGE, blotted to a nitrocellulose membrane, and probed with an anti-pp65 antibody. An anti–β-actin antibody was used as protein loading control. Molecular mass markers, expressed in kilodaltons, are indicated. D, T cells obtained from a cytomegalovirus (CMV)–seropositive HLA-A*0201–positive blood donor and stimulated with autologous pp65 mRNA–electroporated dendritic cells (DCs). The frequency of pp65495–503–specific CD8+ T cells was analyzed using HLA-A*0201/pp65495–503 tetramer before and 12 days after stimulation. The percentage in the upper right corner of each box denotes the percentage of tetramer-positive cells in the CD8+ T cell population. E, Twelve days after stimulation with pp65 mRNA–electroporated DCs, the T cells were analyzed for interferon (IFN)–γ production, by use of intracellular IFN-γ staining, in response to stimulation with Adpp65-transduced (MOI, 100), AdMock-transduced (MOI, 100), pp65495–503–pulsed, or irrelevant peptide–pulsed C1R.A2 cells. The percentage in the upper right corner of each box is the percentage of cells in the CD8+ T cell population that produced IFN-γ. F, Analysis of T cells stimulated by pp65 mRNA–modified DCs for cytotoxic activity against antigen-modified C1R.A2 cells by use of a standardized chromium release assay. The percentage of specific lysis is expressed as the mean value of the triplicate samples.

number of both HLA-A*0201/pp65495–503 tetramer– and HLA-B*0702/pp65353–363 tetramer–positive CD8+ T cells (figure 2B). As a control, HLA-A*0101/pp65353–363 tetramer–positive CD8+ T cells were not detected either before or after stimulation.

Overall, in 8 of 8 cases, there was an increase in the frequency of pp65-specific CD8+ T cells observed after stimulation of T cells by pp65 mRNA–modifie DCs. When considered with other findings this will demonstrate that pp65 mRNA and pp65 peptides produce similar frequencies of pp65-specific CD8+ T cells but that mRNA has the advantage of simultaneously yielding CD8+ T cells against multiple pp65 epitopes that are presented in accordance with the HLA-ABC molecules of the individuals.

T cells stimulated by pp65 mRNA–modifie DCs exhibit specific cytotoxicity against target cells that express pp65. Next, we analyzed whether T cells stimulated by pp65 mRNA–modifie DCs are able to respond by secreting IFN-γ and exerting cytotoxicity against target cells that display the relevant pp65495–503 peptide. To circumvent the use of autologous CMV-infected fibroblasts, we used C1R.A2 cells as T cell targets. Expression of HLA-A*0201 by C1R.A2 cells is high (figure e 3A), and C1R.A2 cells are easily transduced with adenoviral vectors at an MOI of 100, with AdEGFP transduction yielding ~70% EGFP-positive cells after 48 h, as seen by flow cytometry (figure e 3B), and with Adpp65 yielding strong expression of pp65 protein after 48 h, as seen by Western blot analysis (figure e 3C). T cells obtained from an HLA-A*0201–positive blood donor with 1.6% of preexisting CD8+ T cells specific for the HLA-A*0201/pp65495–503 tetramer were stimulated with pp65 mRNA–electroporated DCs. Twelve days later, the frequency of HLA-A*0201/pp65495–503 tetramer–binding CD8+ T cells had increased to 18.7% (figure e 3D). As seen in figure e 3E, 2% and 5% of the CD8+ T
cell population obtained by mRNA stimulation produced IFN-γ when mixed with Adpp65-transduced or pp65_{495-503} peptide-pulsed C1R.A2 target cells, respectively. T cells did not produce IFN-γ when mixed with AdMock-transduced or irrelevant peptide-pulsed C1R.A2 cells (figure 3E). When the T cells were tested for cytotoxic activity against Adpp65-transduced and pp65_{495-503} peptide-pulsed C1R.A2 target cells, ~55% and ~86% of the target cells were lysed at a ratio of 50:1 (figure 3F). AdMock-transduced C1R.A2 cells and irrelevant peptide-pulsed C1R.A2 target cells were not lysed.

Production of recombinant pp65 protein. To efficiently activate CD4+ T helper cells, we produced a recombinant pp65-Fc3 fusion protein in a baculovirus expression system. The protein was purified by a protein G affinity column and was analyzed by SDS-PAGE and Western blot analysis. As seen in figure 4A, Coomassie blue staining of the gel revealed a protein band of the expected size (~100 kDa). When the protein was blotted to a membrane, the ~100-kDa band was specifically recognized by an anti-pp65 antibody (figure 4B).

Activation of pp65-specific CD4+ and CD8+ T cells by use of pp65 protein–pulsed and pp65 mRNA–electroporated DCs. We next analyzed whether DCs pulsed with the baculovirus-produced recombinant pp65-Fc3 fusion protein were able to activate CD4+ T cells. We repeatedly found that the frequencies of IFN-γ-producing CD4+ T cells were increased. Importantly, there was no frequency increase when DCs where pulsed with the baculovirus-produced recombinant EGFP-Fc3 fusion protein, indicating that the CD4+ T cells responded to the CMV pp65 portion and not to the human Fcportion or the potential contaminants in the recombinant protein preparation (data not shown).

We next wanted to analyze whether DCs modified with both the pp65-Fc3 protein and pp65 mRNA could activate CMV-specific CD4+ and CD8+ T cells simultaneously. We used CMV lysate as a positive control of CD4+ T cell expansion. T cells obtained from a CMV-seropositive blood donor who had pre-existing T cells against pp65_{495-503} were used. DCs pulsed with pp65 protein or CMV lysate yielded IFN-γ secretion by 0.07% and 0.3% of the CD4+ T cells, respectively (figure 4C). DCs comodified with pp65 protein and pp65 mRNA induced IFN-γ secretion in 0.13% of the CD4+ T cells and in 0.23% of the CD3+CD4+ T cells. T cells stimulated with EGFP-Fc3 protein–pulsed DCs did not secrete IFN-γ (data not shown). Twelve days after the initial stimulation, the T cells were restimulated with antigen-modified DCs. The IFN-γ response of the CD4+ T cells stimulated with pp65 protein had increased from 0.07% to 5.9%, and the frequency of IFN-γ secretion by CD4+ T cells stimulated with CMV lysate had increased from 0.3% to 25.6% (figure 4C). IFN-γ secretion from CD3+CD4+ T cells had not increased substantially. On the contrary, T cells stimulated with pp65 protein plus pp65 mRNA–modifed DCs induced secretion of IFN-γ by 4.8% of the CD4+ T cell population and by 3.5% of the CD3+CD4+ T cell population. Nonmodifed DCs were used to measure background secretion of IFN-γ from the T cell cultures, and the values for background secretions have been subtracted from the values shown in figure 4C. These finding demonstrate that the recombinant pp65 protein was able to activate pp65-specific CD4+ T cells in 6 of 6 experiments.
Simultaneous expansion of the number of CD4+ and CD8+ T cells by use of DCs comodified by pp65 protein and pp65 mRNA were observed in 4 of 4 cases.

**DISCUSSION**

In the present study, we comodified DCs with CMV pp65 mRNA electroporation and pp65 protein pulsing to simultaneously stimulate virus-specific T cells of both the CD8+ and CD4+ subsets. When T cells were stimulated with pp65 mRNA–electroporated DCs, we observed an increase in the frequency of pp65-specific CD8+ T cells in all donors who had detectable pp65495–503-specific T cells before stimulation. The increases were similar to the increase observed when pp65 peptides were used. The T cells also displayed specific IFN-γ secretion and cytotoxicity against the pp65-modified target cell line CIR.A2. In addition, pp65 mRNA–electroporated DCs were able to increase the frequency of T cells directed against multiple HLA-restricted peptide epitopes of pp65 simultaneously, in accordance with the HLA-ABC molecules of the individuals. The use of pp65 mRNA–electroporated DCs would be a safe way of generating CD8+ T cells from all stem cell donors who had preexisting pp65-specific CD8+ T cells, without the need for recombinant viral vectors or live or attenuated CMV. Furthermore, the use of mRNA-modified DCs has previously been demonstrated to have a good safety profile and therapeutic potential in the clinic [40].

It has become apparent that, to ensure long-term protection against CMV disease in patients who undergo transplantation, the infused T cells need to comprise both CMV-specific CD8+ and CD4+ T cells. This conclusion can be drawn from the clinical trials conducted by Riddell et al. [20] and Einsele et al. [18], but it can also be drawn from the studies of infusion of EBV-specific CD4+ and CD8+ T cells by Heslop et al. [41]. We intended to activate CMV-specific CD4+ T cells by means that did not involve the use of live or attenuated CMV or CMV lysate and that therefore produced a recombinant pp65 protein that was subsequently pulsed onto DCs. When using pp65 protein–pulsed DCs, we were able to stimulate specific CD4+ T cells from all blood donors tested (i.e., 6 of 6 donors). In theory, the recombinant pp65 protein is able to activate pp65-specific CD4+ T cells from any CMV-seropositive hematopoietic stem cell donor or recipient who has preexisting pp65-specific precursors of any HLA-DR/DQ haplotype. As a positive control to pp65-pulsed DCs, we used CMV lysate–pulsed cells. In these experiments, the CMV lysate–pulsed DCs generated higher frequencies of activated CD4+ T cells. One likely reason for this is that the CMV lysate contains several CMV antigens, including pp65, allowing for activation of additional families of CMV-directed T cells. Another reason is that the lysate might contain adjuvant-like factors. When pp65 mRNA electroporation of DCs was combined with pp65 protein pulsing, we observed joint activation and expansion of pp65-specific CD8+ and CD4+ T cells in 4 of 4 experiments. Exogenous antigens—that is, lysate or protein—are capable of generating antigen-specific CD8+ T cells only by means of cross-priming. This pathway to activate CD8+ T cells is inferior to the use of HLA-restricted peptides or to the introduction of antigen-coding sequences for subsequent protein translation, processing, and presentation [42, 43].

The number of CMV-specific CD8+ and CD4+ T cells that need to be infused for clinical effects is not stringently defined at this time. In an early T cell transfer study, 3.3 × 10^2–1 × 10^4 clonal CD8+ CMV-specific T cells/m^2 were transferred [16]. In later studies, fewer T cells have been transferred with apparent therapeutic success. For example, Peggs et al. [17] transferred 1 × 10^3 CMV lysate–stimulated T cells/kg and stated that the number of HLA-A*0201/pp65495–503 tetramer–positive T cells was 350–400 cells/kg. Einsele et al. [18] transferred 1 × 10^7 CMV-specific T cells/m^2. In the present study, we isolated PBMCs from buffy coats originating from 420 mL of blood, and readily obtained 2 × 10^2–2 × 10^4 viable T cells typically were recovered. At that point, the frequency of HLA-A*0201/pp65495–503 tetramer–positive CD8+ T cells was 10%–56% (i.e., 1 × 10^2–1 × 10^3 cells), with an average of 31% in 6 independent experiments. Hypothetically, if the entire buffy coat would have been used, the number of pp65495–503-specific CD8+ T cells would have been 5 × 10^3–2 × 10^4 cells. For the generation of pp65-specific CD4+ T cells, we measured the frequency of IFN-γ–secreting cells. When we started with 4 × 10^4 pp65 protein–pulsed DCs, we typically obtained 3 × 10^2–2 × 10^3 pp65-specific IFN-γ–secreting CD4+ T cells. From an entire buffy coat, the number of pp65-specific CD4+ T cells obtained would hypothetically be 1.5 × 10^3–4 × 10^3 T cells. Therefore, we believe that, through one stimulation of lymphocytes with pp65 mRNA– and pp65 protein–modified DCs, one should be able to obtain a clinically relevant number of CMV-specific CD8+ and CD4+ T cells for adoptive transfer from as little as 420 mL of blood.

In conclusion, our results demonstrate that DCs comodified with pp65 mRNA and pp65 protein efficiently stimulate T cells to generate a multiclonal CD8+ and CD4+ T cell population that encompasses a broad repertoire of CMV-specific effector T cells. Transfer of such T cells would likely, and in a safe manner, confer protection against CMV disease during the period of immunodeficiency that follows hematopoietic stem cell transplantation. In addition, the current protocol utilizing full-length CMV pp65 mRNA and protein can be offered to patients with all possible HLA haplotypes.
Acknowledgment

We thank Lindsay Stanbridge and Jared Cartwright (University of York, York, United Kingdom) for technical support and assistance in baculovirus construction and recombinant protein production.

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