

TRANSPLANTATION

Simultaneous Ex Vivo Expansion of Cytomegalovirus and Epstein-Barr Virus–Specific Cytotoxic T Lymphocytes Using B-Lymphoblastoid Cell Lines Expressing Cytomegalovirus pp65

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Cytomegalovirus (CMV) infection and Epstein-Barr virus (EBV)-induced lymphoproliferative disease are serious complications associated with allogeneic stem cell transplantation. Immunotherapy using ex vivo expanded, virus-specific cytotoxic T lymphocytes (CTL) has been explored and proven to be effective in therapeutic or prophylactic regimens for CMV and EBV infections. To generate CTL specific for both CMV and EBV, we engineered EBV-transformed B-lymphoblastoid cell lines (BLCL) to express CMV pp65 for use as antigen-presenting cells (APC). BLCL were transduced with a recombinant retrovirus encoding pp65, the immunodominant CMV polypeptide. Western blot analysis and immunocytochemistry confirmed the expression of pp65 in the transduced cells. Peripheral blood mononuclear cells (PBMC) from healthy CMV seropositive donors were stimulated with autologous pp65-expressing BLCL weekly for 3 weeks. Chro-

mium release assays showed that the resulting CTL cultures possessed specific cytotoxicity against EBV and CMV. Recombinant vaccinia viruses encoding individual CMV peptides were used to demonstrate that this CMV-specific cytotoxicity was specific for pp65. Assays on CD4- and CD8-depleted CTL fractions indicated that CD8⁺ CTL mediated the pp65-specific cytotoxicity. These CMV/EBV-specific CTL recognized CMV- and EBV-infected targets sharing HLA class I antigens, but not HLA mismatched targets. Our results demonstrate that BLCL can be used as APC to stimulate expansion of EBV- and CMV-specific CTL simultaneously. These findings have potential implications for posttransplant CMV and EBV immunotherapy in recipients of allogeneic stem cell transplants.

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HUMAN CYTOMEGALOVIRUS (CMV) and Epstein-Barr virus (EBV) are members of the herpesvirus family, and in the normal host these viruses cause self-limited disease.^{1,2} In immunocompromised individuals, especially recipients of allogeneic, T-cell-depleted (TCD) stem cell transplants (SCT), CMV and EBV are responsible for significant morbidity and mortality.³⁻⁸ The incidence of CMV reactivation has been reported to be between 70% to 80% after SCT.^{3,6,7,9} The likelihood of developing EBV-induced lymphoproliferative disease (EBV-LPD) can be as high as 20% after a TCD graft, depending on the patient's extent of immunosuppression.^{10,11} In these patients, cellular immunity, which plays a major role in preventing and controlling viral infections, is compromised as a result of immunosuppression aimed at preventing graft-versus-host disease (GVHD) and graft rejection.^{10,12,13} For example, more than 65% of patients at day 40 posttransplant are deficient in CD8⁺ T-cell responses to CMV.^{13,14} Similarly, there is a

correlation between the peak incidence of EBV-LPD and levels of EBV-specific cytotoxic T lymphocytes (CTL) in SCT patients, who are most susceptible to developing this complication between days 60 to 90 posttransplant.¹⁰ Thus, the restoration of cellular immunity is necessary to prevent viral reactivation.^{12,13,15,16} Although pharmacological agents exist to treat CMV infections, these medications have several side effects, the most serious of which is myelosuppression.^{7,17-19} Therefore, alternative therapies that restore host cellular immunity to CMV and EBV are of interest.

The infusion of ex vivo expanded, EBV-specific CTL has been proven to be safe and efficacious for the treatment and prophylaxis of EBV-LPD.^{15,16} To expand donor-derived, EBV-specific CTL, peripheral blood mononuclear cells (PBMC) are cocultivated ex vivo with autologous B-lymphoblastoid cell lines (BLCL), which are latently infected with EBV. Similar protocols have been developed to generate CMV-specific CTL cultures by cocultivating donor PBMC with CMV-infected autologous skin fibroblasts (SF), the only cell type supporting CMV replication in vitro.²⁰⁻²² CD8⁺, CMV-specific CTL used for patient infusion are cloned from bulk CTL cultures due to the high allogeneic cytotoxicity in the latter.¹⁴ Walter et al²³ and Riddell et al²⁴ demonstrated that the administration of CMV-specific CTL clones resulted in the restoration of CMV-specific cellular immunity in SCT patients at risk for CMV disease. Current strategies for posttransplant, antiviral adoptive immunotherapy may be limited by the need for cultivating separate CTL for each important target antigen.

BLCL can be readily established in the laboratory by EBV immortalization. These lines phenotypically resemble the transformed B cells in posttransplant EBV-LPD and express immunogenic EBV peptides,²⁵ making them ideal stimulators for expanding EBV-specific CTL. Among the more than 150 polypeptides encoded by CMV, several are known to be immunogenic.²⁶ Those targeted by CTL include the immediate-early protein, virion envelope glycoprotein B, and the internal matrix proteins pp65 and pp150.²⁷⁻³¹ CMV pp65 has been

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identified as the immunodominant antigen, targeted by 70% to 90% of CMV-specific CTL.^{29,32,33} Because SCT patients are at risk for both EBV and CMV disease, a CTL preparation specific for both EBV and CMV would be of potential benefit, especially if CMV/EBV-specific CTL could be expanded simultaneously in the same culture. To this end, we developed a novel system, using BLCL as antigen-presenting cells (APC) to present both EBV and CMV epitopes. BLCL were transduced with a recombinant retrovirus encoding CMV pp65 and were then used to prime autologous PBMC. We demonstrated that (1) CTL cultures stimulated with pp65-expressing BLCL had CMV- and EBV-specific cytotoxicity and (2) this cytotoxicity was mediated by CD8⁺ CTL.

MATERIALS AND METHODS

Donors. Four normal CMV-seropositive donors (AE, RB, SQ, and ST) and 2 seronegative donors (AD and SB) provided PBMC, SF, and serum for this study under protocols approved by the Institutional Review Board of the University of Alabama at Birmingham (UAB). CMV serostatus was determined by the UAB Core Immunology Laboratory with an IgG enzyme-linked immunosorbent assay (Abbott Laboratories, Chicago, IL). Major histocompatibility complex (MHC) class I and II typing was performed by the UAB Histocompatibility Laboratory.

Expression vectors and viruses. To construct the pp65-encoding recombinant retrovirus MSCVpp65, an 1,800-bp *EcoRI-BamHI* fragment containing the entire CMV pp65 coding sequence (a gift from J.A. Zaia,³⁴ City of Hope Medical Center, Duarte, CA) was subcloned into the *EcoRI-BglIII* linearized retroviral vector MSCV2.1 (a gift from R.G. Hawley,³⁵ University of Toronto, Toronto, Canada). CMV pp65 was placed under the control of the long terminal repeat (LTR), while the internal human phosphoglycerole kinase promoter drove the expression of the selection marker bacterial neomycin phosphotransferase.

To generate replication-defective recombinant retroviruses, CsCl-purified construct DNA was electroporated into psi-cre cells, an ecotropic packaging cell line.³⁶ Culture medium from the transfected psi-cre cells was used to infect the amphotropic producer cell line GP⁺envAM12.³⁷ The producer cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), and were selected with G418 (Geneticin, BRL-Life Technologies, Gaithersburg, MD) at 600 µg/mL for 7 days. The virus-containing media was collected after the producer cell line was grown to confluence, and this supernatant was used to infect either human SF or BLCL. Retrovirus titer was determined by G418-selectable colonies in human SF and ranged from 5×10^5 to 10^6 /mL.

Recombinant vaccinia viruses encoding CMV pp65 or pp150 were used as previously described.³³ Briefly, virus stocks were prepared in BSC-40 cells (American Type Culture Collection [ATCC], Rockville, MD). After infection at a multiplicity of infection (MOI) of 0.1, the cells were cultured for 48 hours and then harvested and underwent 3 cycles of freeze and thaw. Virus suspension was cleared of debris by centrifugation and stored in aliquots at -20°C. Vaccinia virus titers ranged from 1×10^7 to 10^8 /mL as determined by plaque assays in BSC-40 cells.

AD 169 human CMV was obtained from ATCC and virus was propagated in human SF. The initial infection was at an MOI of 0.1. Five days after cytopathological effects appeared in more than 90% cells, the virus-containing media were collected, passed through a 0.45-mm filter, and frozen in liquid nitrogen as aliquots. CMV titer was between 1×10^6 and 10^7 /mL by plaque assays in human SF.

Cell lines, retroviral transduction, and virus infection. PBMC were separated from heparinized whole blood by Histopaque (Sigma, St Louis, MO) gradient centrifugation and washed with RPMI 1640

(BRL-Life Technologies). BLCL were established from PBMC by EBV transformation and maintained in RPMI 1640 with 10% FBS, as described previously.¹⁰ BLCL were transduced with recombinant retroviruses by a 2-hour incubation with retrovirus-containing media. G418 selection was applied 48 hours after infection at 400 µg/mL for 14 days.

Primary human dermal fibroblast cell lines were established from skin biopsies from forearms. Human SF were grown in F12/DMEM (Mediatech) with 20% FBS and transduced with retroviruses by a 1-hour incubation with retrovirus-containing media. G418 selection was applied 24 hours after infection at 200 µg/mL for 5 days. When growth was sufficient, the cell lines were tested for mycoplasma contamination by a mycoplasma detection kit (BRL-Life Technologies). When used as targets in chromium-51 (⁵¹Cr) release assays, SF were treated with 100 IU/mL interferon-γ for 48 hours and were infected overnight with either recombinant vaccinia virus at an MOI of 5 or with CMV stock at an MOI of 10.

Protein analysis. For immunocytochemical detection, the cells were fixed in 96-well plates with a 50% mixture of acetone and methanol for 20 minutes. A monoclonal antibody against human CMV-pp65,³⁸ 205, was used to stain the cells for 60 minutes at a dilution of 1:25. Immunoperoxidase staining was performed with a kit as instructed by the manufacturer (Immupure Ultra-sensitive ABC Peroxidase Staining Kit; Pierce, Rockford, IL).

For immunoblotting, 0.2×10^6 cells were lysed in a buffer containing 4% sodium dodecyl sulfate (SDS), 0.1 mol/L Tris pH 6.8, 0.2% Brilliant Blue G250, 280 mmol/L 2-mercaptoethanol, and 20% glycerol. After boiling for 5 minutes, the samples were separated on an 8% SDS-polyacrylamide gel and transferred to a piece of Hybond-P membrane (Amersham Lifescience, Arlington Heights, IL). The blots were blocked with 5% nonfat milk in PBS-T (0.1 mol/L phosphate buffer, pH7.4, 200 mmol/L NaCl, 0.05% Tween 20), and probed with the monoclonal antibodies against pp65 (65-8)³⁹ at 1:100 dilution and/or EBNA2 (PE2)⁴⁰ at 1:500 dilution (Dako, Carpinteria, CA) for 60 minutes at room temperature. A horseradish peroxidase-labeled sheep antimouse IgG antibody (Amersham) was used as the secondary antibody at a dilution of 1:2,000. Enhanced chemiluminescence (ECL) detection was performed with ECL detection agents (Amersham) and recorded on Hyperfilm-ECL film (Amersham).

Ex vivo expansion of cytotoxic T lymphocytes. Both pp65-expressing BLCL and SF were used as stimulators for ex vivo CTL expansion. PBMC were cocultivated with autologous pp65-expressing BLCL in 24-well plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) in RPMI 1640 supplemented with 10% FBS and 50 µmol/L 2-mercaptoethanol (Sigma). BLCL were exposed to 100 Gy of gamma irradiation before use as stimulator cells. The CTL cultures were primed weekly following a regimen of decreasing responder:stimulator ratios from 40:1 at day 0, 20:1 at day 7, and 5:1 on day 14 over a period of 3 weeks. pp65-expressing SF were used as stimulators following conditions described previously using CMV-infected SF.^{13,20} Briefly, PBMC were dispensed at 10×10^6 cells/well in 6-well plates containing 0.5×10^6 pp65-expressing SF in RPMI 1640 with 10% human CMV seronegative AB serum and 50 µmol/L 2-mercaptoethanol (Sigma). Weekly the CTL cultures were collected, washed with RPMI 1640, and replated with the same number of fresh SF stimulators at a ratio of 20:1 using autologous gamma-irradiated PBMC as feeder cells. For both BLCL and SF stimulators, interleukin-2 (IL-2) (Collaborative Biomedical Products, Bedford, MA) was added 10 days after the initial stimulation to a final concentration of 2.5 IU/mL, and medium was then changed every 3 days by replacing one half of the supernatant with fresh medium.

Chromium release assays. Chromium release assays were performed as previously described.¹⁰ Target cells included the following: BLCL and BLCL transduced with MSCVpp65 or the backbone MSCV; SF, SF transduced with MSCVpp65 or the backbone MSCV, SF infected with CMV, and SF infected with recombinant vaccinia viruses encoding

either pp65 or pp150. Target cells were labeled with ^{51}Cr (New England Nuclear, Boston, MA) overnight ($100 \mu\text{Ci}/10^6$ cells) and labeled cells were harvested, either by trypsinizing for SF or by centrifugation for BLCL. Cells were then washed in phosphate-buffered saline (PBS) and dispensed in triplicates into 96-well V-bottom plates (ICN, Costa Mesa, CA) at 4×10^3 cells/well. The effector:target (E:T) ratios were 12.5:1, 5:1, and 2.5:1. Spontaneous and total release for each target were used to calculate percent specific release using the following formula:

$$\% \text{ Specific Release} = \frac{(\text{Experimental cpm} - \text{Spontaneous cpm})}{(\text{Total cpm} - \text{Spontaneous cpm})}$$

CD4⁺ and CD8⁺ cell isolation. To isolate CD4⁺ and CD8⁺ populations of polyclonal CMV/EBV-specific CTL, bulk culture CTL were depleted of either CD4⁺ or CD8⁺ cells and natural killer (NK) cells using monoclonal antibodies specific for CD4/CD56 or CD8/CD56 (Pharmingen, San Diego, CA). Briefly, cells were placed in RPMI 1640 at 4°C at a concentration of $5 \times 10^6/\text{mL}$, and the monoclonal antibody was added for a final concentration of 20 $\mu\text{g}/\text{mL}$. After a 30-minute incubation at 4°C, the cells were washed twice in RPMI 1640, and Dynabeads (DynaL, Lake Success, NY) were added following the manufacturer's instructions. After 30 minutes incubation at 4°C, the cell-bead suspension was placed on a magnet and the supernatant was removed, according to the manufacturer's guidelines.

Cytofluorometry. The cell-surface phenotype of EBV/CMV CTL was determined by staining with directly conjugated monoclonal antibodies and analyzing on a cytometer (FACScan; Becton Dickinson, San Jose, CA). These antibodies (Becton Dickinson) were specific for the following: CD3, CD4, CD8, and CD16/56 (simultest). Cells were washed with PBS supplemented with 3% FBS and 0.01% sodium azide. Dilutions and incubations for the antibodies followed the manufacturer's instructions.

RESULTS

APC presenting both EBV and CMV antigens. After selection with G418, BLCL and SF transduced with either the retrovirus MSCVpp65 (BLCLpp65 and SFpp65, respectively) or MSCV (BLCLmscv or SFmscv, respectively) continued to proliferate, whereas uninfected cells did not survive. Western blot analysis (Fig 1) of the transduced cells showed that the pp65-specific monoclonal antibody 65-8 detected a single band

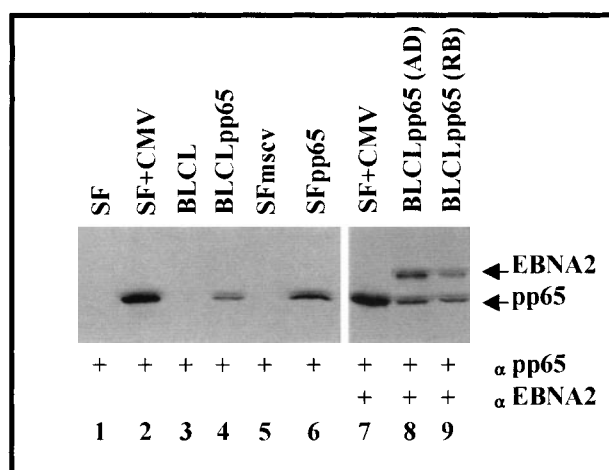


Fig 1. Immunoblot analysis for the expression of CMV pp65 and EBV EBNA2. Lanes 1 through 6, probed against CMV pp65; lanes 7 through 9, probed against both EBV EBNA2 and CMV pp65.

from BLCLpp65 and SFpp65 (lanes 4 and 6), but not in the MSCV backbone vector-transduced or the untransduced parental cells (lanes 1, 3, and 5). A band of similar size was also observed in the SF infected with CMV (lane 2), confirming the expression and the physical integrity of pp65 in the cells transduced with MSCVpp65. In addition, using a monoclonal antibody specific for EBV nuclear antigen 2 (EBNA2), a specific band was detected in the two BLCLpp65 tested (lane 8, from AD; lane 9, from RB), both of which also expressed pp65. Because EBNA2 is 1 of the immunogenic EBV polypeptides expressed in BLCL, the detection of both pp65 and EBNA2 from BLCLpp65 was consistent with our expectation that BLCLpp65 would present epitopes derived from both CMV and EBV.

To examine the expression of pp65 at the single cell level, a CMV pp65-specific monoclonal antibody (205)³⁸ was used to stain transduced cells immunocytochemically. The specific immunoperoxidase staining was localized to the nuclei in both SFpp65 and BLCLpp65 (Fig 2b and d). In addition to a diffuse staining pattern, some cells (most clearly in the SFpp65) displayed the characteristic inclusion body-like structure seen in CMV-infected cells.³⁹ While almost all of the SFpp65 showed positive staining, the percentage of G418-resistant BLCLpp65 cells positive for immunoperoxidase staining varied from approximately 50% to 90%. This may be related to intrinsic differences in these two types of cells and their level of differentiation. Contrary to the relatively homogeneous nature of SF cultures, BLCL are regarded as heterogeneous, proliferating B cells at different stages of differentiation. The expression of the pp65, which is controlled by MSCV LTR, might be subject to the differentiation status of BLCL, as it has been reported that the promoter activity of MSCV LTR is associated with the differentiation status of hematopoietic cells.⁴¹

Generation of CTL cultures. The transduced cells were cocultivated with autologous PBMC ex vivo to stimulate preferential expansion of EBV- and pp65-specific CTL. Before coculturing, the stimulators were confirmed for pp65 expression by immunoperoxidase staining. Pilot experiments included BLCLpp65 and SFpp65 as stimulators. Approximately 3 days after cocultivation, colonies composed of irregularly shaped cells were observed, most abundantly in the BLCLpp65-primed cultures. Although pp65-specific cytotoxicity was detected from CTL cultures using either cell line as an APC, SFpp65 were discontinued to be used as stimulators due to our limited access to human CMV seronegative AB serum, which the SF-based protocols called for.^{13,20,42}

Cytotoxic specificity of the BLCLpp65-stimulated CTL cultures. ^{51}Cr release assays were used to test CTL cultures against a panel of autologous and allogeneic target cells. Figure 3 shows that CTL cultures from all 6 donors lysed specific lysis of BLCL, as expected from their positive EBV-serology. In addition, CTL cultures from the 4 CMV-seropositive donors (AE, RB, SQ, and ST), but not the 2 CMV seronegative donors (AD and SB), also displayed specific cytotoxicity against MSCVpp65-transduced cells. Similar results were obtained for CTL cultures from day 14 and day 35 (data not shown). Although the stimulator BLCLpp65 should express the neomycin phosphotransferase, as indicated by the G418 selectibility, there was no specific killing against SFmscv in ^{51}Cr release

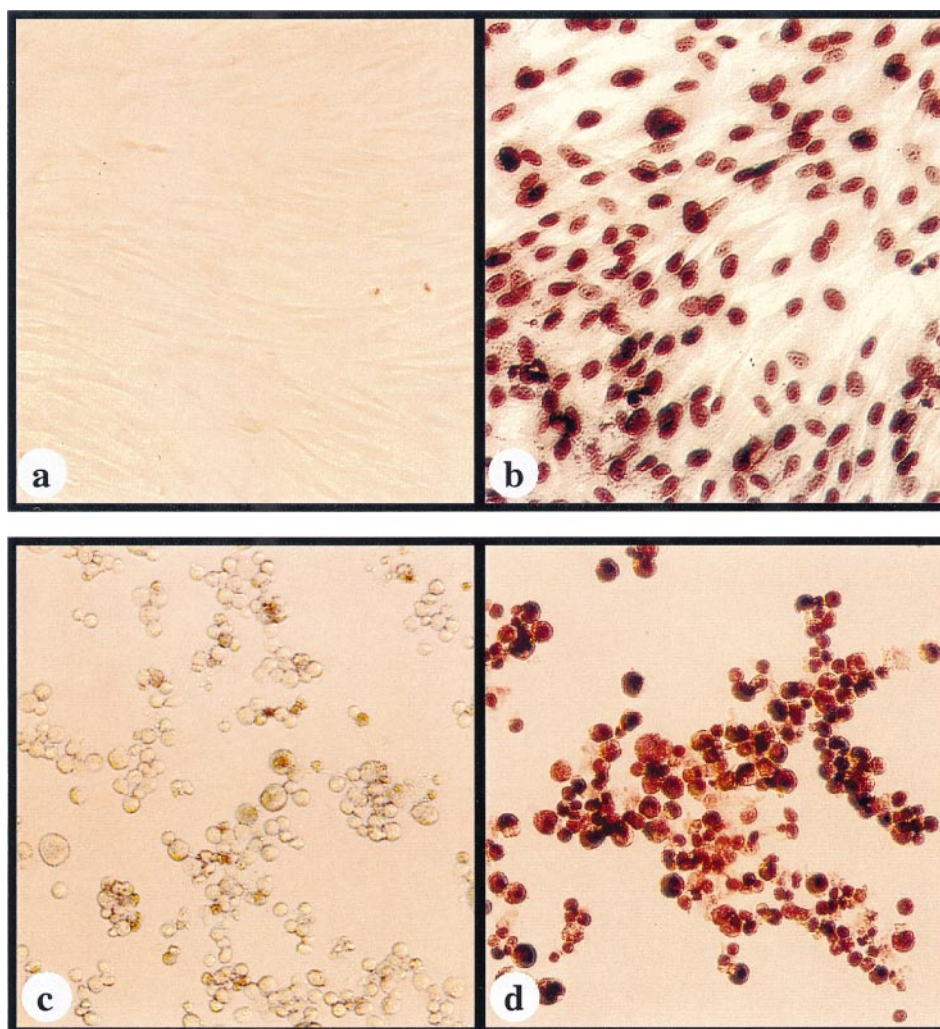


Fig 2. Immunoperoxidase staining using a pp65-specific monoclonal antibody with (a) SF transduced with MSCV; (b) SF transduced with MSCVpp65; (c) BLCL transduced with MSCV; and (d) BLCL transduced with MSCVpp65.

assays. The failure of *ex vivo* expansion of CTL specific for this bacterial protein, in contrast to the viral pp65, may be due to its weak immunogenicity, and/or a very low precursor frequency.

CTL cultures showing pp65-specific cytotoxicity also lysed autologous SF targets infected with CMV, suggesting that the pp65-specific cytotoxicity was effective in a setting close to natural CMV infection. However, a legitimate concern against this interpretation would be that residual CTL specific to other CMV antigens may be the cause of, or contribute to, the killing of the CMV-infected cells. To exclude this possibility, SF used as targets were infected with recombinant vaccinia viruses encoding either pp65 or pp150. CMV pp150 is another CMV matrix protein that is often targeted by specific CTL.³¹ Although all 3 CTL cultures lysed the pp65-vaccinia infected SF, none lysed pp150-vaccinia infected targets (Fig 4), indicating that the CMV-specific cytotoxicity was against pp65.

Effector cells of the CMV/EBV-specific cytotoxicity. To discern the nature of the EBV/CMV-specific cytotoxicity, bulk CTL cultures from donor ST were selectively depleted of either CD4⁺/CD56⁺ cells or CD8⁺/CD56⁺ cells. Resulting cells were greater than 96% positive for CD8⁺ and greater than 80% positive for CD4⁺ cells, respectively. Cell fractions were tested

in ⁵¹Cr release assays against autologous SFpp65, BLCL, or SFmscv (Fig 5). CD8-enriched cells lysed both SFpp65 and BLCL, but not SFmscv (Fig 5B). A similar cytotoxicity profile was observed from bulk culture CTL (Fig 5A). In contrast, the CD4-enriched fraction failed to lyse any of these targets (Fig 5C). Furthermore, bulk culture and CD8-enriched CTL sustained specific cytotoxicity against BLCL and SFpp65 at 3 E:T ratios (12.5:1, 5:1, and 2.5:1). The CD4-enriched fraction failed to show any lysis of the BLCL or SFpp65 at any E:T ratios. These data indicated that the CMV/EBV-specific cytotoxicity was mediated mostly, if not entirely, by CD8⁺ CTL.

The differential cytotoxicity towards the autologous and allogeneic pp65-expressing targets by BLCLpp65-stimulated CMV/EBV CTL was consistent with a pattern of HLA-restricted cytotoxicity (Fig 3). We confirmed this by comparing specific cytotoxicity against a panel of targets consisting of autologous, partially matched allogeneic, or completely mismatched allogeneic cells (Fig 6). CTL from donor ST were used, which shared A2,B44 with AD, a CMV seronegative donor of which the CTL culture showed no cytotoxicity against autologous pp65-expressing target cells. Bulk and CD8-enriched CTL from donor ST lysed all the 3 pp65-expressing

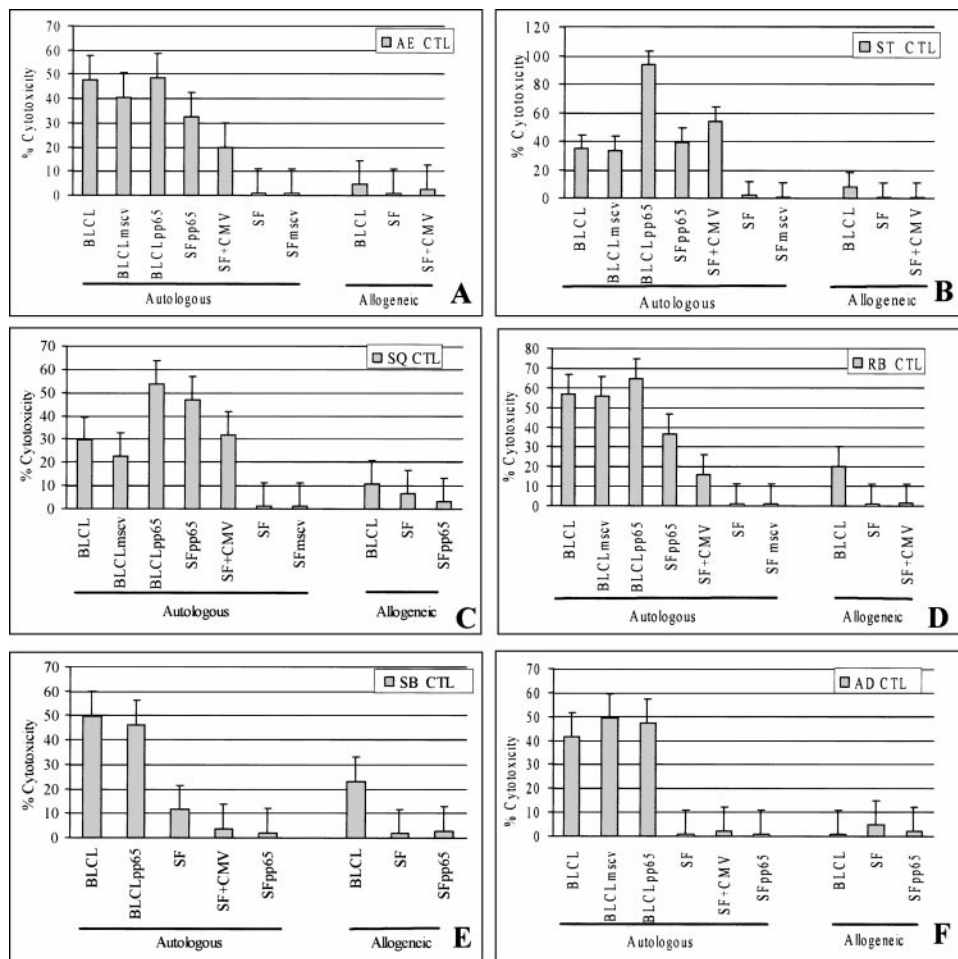


Fig 3. CTL cultures at 21 days after ex vivo stimulation with autologous BLCLpp65 were tested for specific cytotoxicity against EBV-transformed BLCL, CMV-infected SF, and MSCVpp65-transduced cells in chromium release assay. (A through D) CTL activity from 4 CMV seropositive/EBV seropositive individuals. (E through F) CTL activity from 2 CMV seronegative/EBV seropositive individuals. (E:T ratio, 12.5:1).

targets from AD almost as well as the autologous counterparts (ST). The same CTL cultures failed to lyse the completely mismatched targets from donor SQ (Fig 6) or targets from 2 other mismatched individuals (data not shown). Note the EBV

and CMV targets from SQ were lysed by the autologous CMV/EBV CTL (Fig 3D). This suggested that the pp65-specific cytotoxicity was HLA A2 and/or B44 restricted. The CMV/EBV-specific CTL from donor ST also showed cytotoxicity against the partially matched allogeneic BLCL, although the activity was lower than that for the autologous BLCL. This may be explained by the fact that BLCL express at least 8 EBV antigens, and it is possible that not all of the epitopes were presented by the 2 shared HLA alleles.

DISCUSSION

CMV disease and EBV-LPD are 2 of the most serious and potentially life-threatening viral complications after allogeneic, TCD-SCT.⁵⁻⁸ Previous studies have shown that adoptive immunotherapy with virus-specific CTL can be used to prevent complications related to these viruses.^{15,24} Current methodologies are limited by a requirement for developing CTL cultures using separate APC. This report demonstrated the feasibility of using retroviral technology to transduce EBV BLCL and achieve expression of CMV pp65. These BLCL present EBV peptides, as well as CMV pp65, and resulted in polyclonal, bispecific CTL cultures when cocultured with autologous

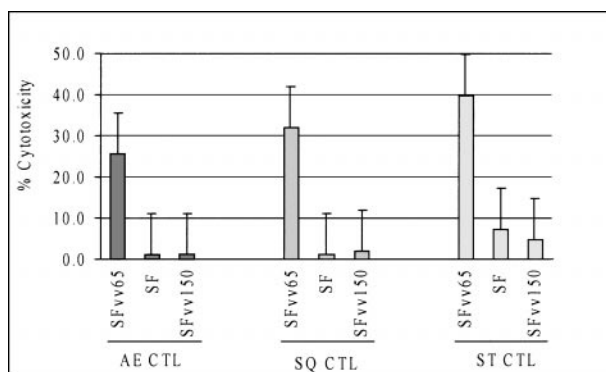


Fig 4. Cytotoxicity against CMV-infected cells by BLCLpp65-primed CTL cultures was mediated via pp65. CTL cultures lysed autologous fibroblasts infected with a recombinant vaccinia virus encoding CMV pp65, but not the one encoding CMV pp150. (E:T ratio, 12.5:1)

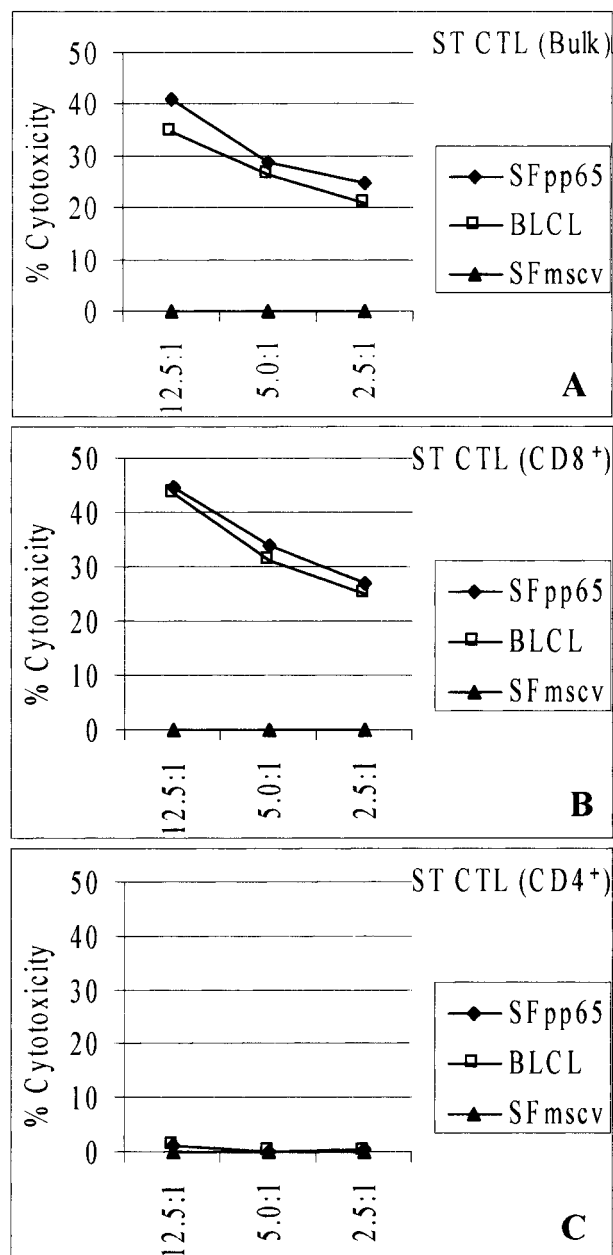


Fig 5. Cytotoxicity against EBV-transformed BLCL and MSCVpp65-transduced SF was mediated by CD8⁺ CTL. Bulk, CD8-enriched, or CD4-enriched CTL culture from donor ST were tested at different E:T ratios in chromium release assay. CD8-enriched CTL (B) retained the cytotoxicity displayed by the bulk CTL (A), while the CD4-enriched CTL (C) did not.

PBMC. These findings are of importance to centers applying adoptive immunotherapy for viral infections in SCT patients.

Over the past decade, there has been a decrease in morbidity and mortality caused by CMV disease in posttransplant SCT, and this is largely attributable to the availability of pharmacological agents effective against CMV infections.^{43,44} Ganciclovir has been used to prevent CMV reactivation, either at the onset of laboratory evidence of CMV infection (by culture, polymerase chain reaction [PCR], or antigenemia assay) or as prophylaxis.^{7,17,18} However, ganciclovir-related granulocytopenia

has been reported in 30% to 60% of bone marrow transplant patients, complicating the routine use of this drug for prophylaxis in all CMV seropositive patients.^{7,17-19} This side effect is of particular concern in patients for whom slow engraftment is a potential problem and the myelosuppressive effects of this medication could lead to graft failure. As a result of drug-induced cytopenia, patients receiving ganciclovir can have increased transfusion requirements. The use of this drug has also been associated with delayed reconstitution of CMV-specific CTL posttransplant and an increased incidence of late CMV disease.⁴⁵ The limitations of anti-CMV pharmacotherapy have promoted interest in adoptive immunotherapy as an alternative means for the prevention and treatment of CMV disease in SCT patients.

The application of adoptive immunotherapy has evolved as a result of an increased understanding of the cellular immune response to viral pathogens. Infected cells process endogenously synthesized viral polypeptides and present viral epitopes on the cell surface.^{46,47} CD8⁺ CTL recognize these epitopes in an HLA class I-restricted context and are activated to proliferate and lyse the infected cells. Thus, the prerequisite for adoptive immunotherapy is to have suitable APC that can present relevant viral epitopes. Regarded as professional APC, BLCL express high levels of class I and II HLA molecules and costimulatory molecules such as intercellular adhesion molecule (ICAM)-1, B7/BB1, and lymphocyte function-associated antigen (LFA)-3,⁴⁸⁻⁵⁰ in addition to key EBV antigens. Probably due to this reason, BLCL have been used to generate EBV-specific CTL cultures reliably by different laboratories, and as a result, adoptive immunotherapy is now performed routinely at some centers for EBV-LPD in TCD-SCT patients. The relative ease with which EBV CTL are cultivated using donor BLCL led us to investigate the possibility of using the same APC to generate CMV-specific CTL.

CMV pp65 was chosen as CMV-specific antigen based on its immunological and virological properties. The majority of CMV-specific CTL isolated from CMV seropositive patients recognize pp65.^{29,32,33} As a component of the virion tegument, pp65 is introduced into the host cells during virus entry. The exogenous pp65 has been shown to be presented by HLA class I molecules and targeted by CD8⁺ CTL, possibly before the viruses multiply.⁵¹ Therefore, targeting this antigen may limit the spread of CMV infection at an early time point of CMV reactivation. Recently, attempts have been made to elucidate CMV HLA allele-specific epitopes for developing peptide vaccines. One nonamer pp65 epitope was shown to be immunogenic when presented in the context of HLA 0201.⁴² Although peptide-pulsed APC would be a promising tool to generate CTL culture for adoptive immunotherapy, it is limited, at least at this stage of development, to certain HLA alleles. In contrast, BLCLpp65 have the potential to present various pp65 epitopes, and it would be universally applicable to donors with diverse HLA alleles.

We chose a well-characterized recombinant retrovirus (MSCV) to achieve pp65 expression in BLCL. Retroviral vectors transduce rapidly dividing cells, such as BLCL, with high efficiency,³⁵ making it possible to establish pp65-expressing BLCL in a timely fashion. All of the BLCL that we tested were readily transduced by this virus, and all of the pp65-transduced BLCL stimulated specific cytotoxicity in the

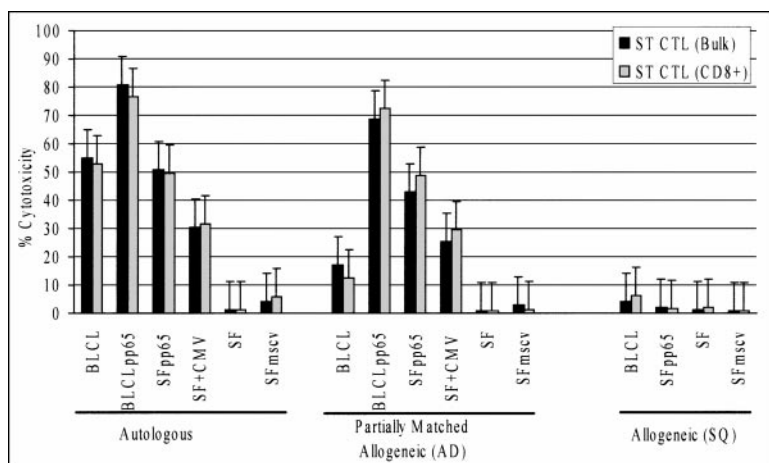


Fig 6. CMV pp65-specific cytotoxicity was HLA class I-restricted. Bulk and CD8-enriched CTL from donor ST showed cytotoxicity against HLA class I partially matched allogeneic targets (AD), which shared HLA A2 and B44 with donor ST. There was no specific lysis of the complete allogeneic targets (SQ). (E:T ratio, 12.5:1)

EBV/CMV seropositive CTL cultures regardless of the heterogeneity of pp65 expression. Furthermore, MSCV is replication-defective, and careful selection of producer cells should eliminate the problem of contamination with replication-competent viruses resulting from fortuitous recombination. MSCVpp65 encoded only 2 products, the target antigen pp65 and a selection marker that appeared not to elicit a strong CTL reaction. A minimal number of transgenes encoded by a vector translates into fewer antigens presented and hence fewer nonrelevant CTL expanded.

Our method for generating CMV/EBV-specific CTL cultures has unique advantages and is feasible in terms of both resources and time. This approach would technically simplify adoptive T-cell transfer, as EBV- and CMV-specific CTL are generated simultaneously in the same culture. The use of BLCL as APC circumvents the problem of procuring skin biopsies from the donors and the time to grow fibroblasts. In addition, CMV/EBV CTL primed by BLCLpp65 can be cultivated in FBS, which is readily available, does not carry the risk of exposure to human infectious agents, and is presently in use by other investigators in adoptive immunotherapy. The CMV/EBV CTL generated in this study were not reactive against noninfected, allogeneic SF, suggesting that the lengthy procedure of CTL cloning may not be necessary. Time required to generate bispecific CTL cultures could be further shortened by transducing BLCL with high-titer retrovirus produced by the new generation of vector production systems.^{52,53} High titer retrovirus should greatly improve the transduction efficiency and as a result dispense with the need for G418 selection of the transduced cells.^{52,53} Alternatively, retroviral transduction could be performed at the same time as EBV transformation, eliminating the time required to generate pp65-expressing BLCL.

It should be noted that with BLCLpp65 as stimulators, CMVpp65-specific CTL cultures were generated only from CMV-seropositive, but not seronegative, individuals. Similar results have also been observed by others when CMV-infected fibroblasts were used as APC.²⁰ These results appear to suggest that B cells, similar to fibroblasts, are able to reactivate the memory CTL, but not to prime naïve T cells. Thus, BLCL may not be suitable as APC to present CMV antigens for CMV seronegative individuals. Work is ongoing in our laboratory to

determine which APC can prime in vitro expansion of CMV-specific CTL from seronegative donors.

REFERENCES

1. Tosato G: The Epstein-Barr virus and the immune system. *Adv Cancer Res* 49:75, 1987
2. Britt WJ, Alford CA: Cytomegalovirus, in Fields B, Knipe DM, Howley PM (eds): *Fields Virology*. Philadelphia, PA, Lippincott-Raven, 1996, p 2493
3. Miller W, Flynn P, McCullough J, Balfour HJJ, Goldman, Haake R, McGlave P, Ramsay N, Kersey J: Cytomegalovirus infection after bone marrow transplantation: An association with acute graft-versus-host disease. *Blood* 67:1162, 1986
4. Papadopoulos EB, Ladanyi M, Emanuel D, Mackinnon S, Boulad F, Carabasi MH, Castro-Malaspina H, Childs BH, Gillio AP, Small TN, Young JW, Kernan NA, O'Reilly RJ: Infusions of donor leukocytes to treat Epstein-Barr virus-associated lymphoproliferative disorders after allogeneic bone marrow transplantation. *N Engl J Med* 330:1185, 1994
5. Shapiro RS, McClain K, Frizzera G, Gajl-Peczalska KJ, Kersey JH, Blazar BR, Arthur DC, Patton DF, Greenberg JS, Burke B, Ramsay NKC, McGlave P, Filipovich AH: Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation. *Blood* 71:1234, 1988
6. Wingard JR, Piantadosi S, Burns WH, Zahurak ML, Santos GW, Saral R: Cytomegalovirus infections in bone marrow transplant recipients given intensive cytoreductive therapy. *Rev Infect Dis* 12:S793, 1990 (suppl 7)
7. Winston DJ, Ho WG, Barton K, Du Mond C, Ebeling DF, Buhles WC, Champlin RE: Ganciclovir prophylaxis of cytomegalovirus infection and disease in allogeneic bone marrow transplant recipients. Results of a placebo-controlled, double-blind trial. *Ann Intern Med* 118:179, 1993
8. Zutter MM, Martin PJ, Sale GE, Shulman HM, Fisher L, Thomas ED, Durnam DM: Epstein-Barr virus lymphoproliferation after bone marrow transplantation. *Blood* 72:520, 1988
9. Meyers JD, Flournoy N, Thomas ED: Cytomegalovirus infection and specific cell-mediated immunity after marrow transplant. *J Infect Dis* 142:816, 1980
10. Lucas KG, Small TN, Heller G, Dupont B, O'Reilly RJ: The development of cellular immunity to Epstein-Barr virus after allogeneic bone marrow transplantation. *Blood* 87:2594, 1996
11. Lucas KG, Burton RL, Zimmerman SE, Wang J, Cornetta KG, Robertson KA, Lee CH, Emanuel DJ: Semiquantitative Epstein-Barr virus (EBV) polymerase chain reaction for the determination of patients

at risk for EBV-induced lymphoproliferative disease after stem cell transplantation. *Blood* 91:3654, 1998

12. Keever CA, Welte K, Small T, Levick J, Sullivan M, Hauch M, Evans RL, O'Reilly RJ: Interleukin 2-activated killer cells in patients following transplants of soybean lectin-separated and E rosette-depleted bone marrow. *Blood* 70:1893, 1987
13. Reusser P, Riddell SR, Meyers JD, Greenberg PD: Cytotoxic T-lymphocyte response to cytomegalovirus after human allogeneic bone marrow transplantation: Pattern of recovery and correlation with cytomegalovirus infection and disease. *Blood* 78:1373, 1991
14. Riddell SR, Greenberg PD: Principles for adoptive T cell therapy of human viral diseases. *Annu Rev Immunol* 13:545, 1995
15. Heslop HE, Ng CY, Li C, Smith CA, Loftin SK, Krance RA, Brenner MK, Rooney CM: Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes. *Nat Med* 2:551, 1996
16. Rooney CM, Smith CA, Ng CY, Loftin S, Li C, Krance RA, Brenner MK, Heslop HE: Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation. *Lancet* 345:9, 1995
17. Goodrich JM, Bowden RA, Fisher L, Keller C, Schoch G, Meyers JD: Ganciclovir prophylaxis to prevent cytomegalovirus disease after allogeneic marrow transplant. *Ann Intern Med* 118:173, 1993
18. Boeckh M, Gooley TA, Myerson D, Cunningham T, Schoch G, Bowden RA: Cytomegalovirus pp65 antigenemia-guided early treatment with ganciclovir versus ganciclovir at engraftment after allogeneic marrow transplantation: A randomized double-blind study. *Blood* 88:4063, 1996
19. Salzberger B, Bowden RA, Hackman RC, Davis C, Boeckh M: Neutropenia in allogeneic marrow transplant recipients receiving ganciclovir for prevention of cytomegalovirus disease: Risk factors and outcome. *Blood* 90:2502, 1997
20. Borysiewicz LK, Morris S, Page JD, Sissons JG: Human cytomegalovirus-specific cytotoxic T lymphocytes: Requirements for in vitro generation and specificity. *Eur J Immunol* 13:804, 1983
21. Schrier RD, Oldstone MB: Recent clinical isolates of cytomegalovirus suppress human cytomegalovirus-specific human leukocyte antigen-restricted cytotoxic T-lymphocyte activity. *J Virol* 59:127, 1986
22. Riddell SR, Greenberg PD: The use of anti-CD3 and anti-CD28 monoclonal antibodies to clone and expand human antigen-specific T cells. *J Immunol Methods* 128:189, 1990
23. Walter EA, Greenberg PD, Gilbert MJ, Finch RJ, Watanabe, KS, Thomas ED, Riddell SR: Reconstitution of cellular immunity against cytomegalovirus in recipients of allogeneic bone marrow by transfer of T-cell clones from the donor. *N Engl J Med* 333:1038, 1995
24. Riddell SR, Watanabe KS, Goodrich JM, Li CR, Agha ME, Greenberg PD: Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science* 257:238, 1992
25. Rowe M, Young LS, Crocker J, Stokes H, Henderson S, Rickinson AB: Epstein-Barr virus (EBV)-associated lymphoproliferative disease in the SCID mouse model: Implications for the pathogenesis of EBV-positive lymphomas in man. *J Exp Med* 173:147, 1991
26. Griffiths PD, Grundy JE: Molecular biology and immunology of cytomegalovirus. *Biochem J* 241:313, 1987
27. Borysiewicz LK, Hickling JK, Graham S, Sinclair J, Cranage MP, Smith GL, Sissons JG: Human cytomegalovirus-specific cytotoxic T cells. Relative frequency of stage-specific CTL recognizing the 72-kD immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. *J Exp Med* 168:919, 1988
28. Gilbert MJ, Riddell SR, Li CR, Greenberg PD: Selective interference with class I major histocompatibility complex presentation of the major immediate-early protein following infection with human cytomegalovirus. *J Virol* 67:3461, 1993
29. Wills MR, Carmichael AJ, Mynard K, Jin X, Weekes MP, Plachter B, Sissons JG: The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: Frequency, specificity, and T-cell receptor usage of pp65-specific CTL. *J Virol* 70:7569, 1996
30. Gilbert MJ, Riddell SR, Plachter B, Greenberg PD: Cytomegalovirus selectively blocks antigen processing and presentation of its immediate-early gene product. *Nature* 383:720, 1996
31. Li C, Yang X, Tu W, Riddell SR: Human cytomegalovirus matrix protein PP150 is efficiently presented as one of target antigens for cytotoxic T lymphocyte recognition. *Chin Med J* 110:397, 1997
32. McLaughlin-Taylor E, Pande H, Forman SJ, Tanamachi B, Li CR, Zaia JA, Greenberg PD, Riddell SR: Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. *J Med Virol* 43:103, 1994
33. Boppa SB, Britt WJ: Recognition of human cytomegalovirus gene products by HCMV-specific cytotoxic T cells. *Virology* 222:293, 1996
34. Pande H, Campo K, Tanamachi B, Zaia JA: Human cytomegalovirus strain Towne pp65 gene: Nucleotide sequence and expression in *Escherichia coli*. *Virology* 182:220, 1991
35. Hawley RG, Lieu FH, Fong AZ, Hawley TS: Versatile retroviral vectors for potential use in gene therapy. *Gene Ther* 1:136, 1994
36. Danos O, Mulligan RC: Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 85:6460, 1988
37. Markowitz D, Goff S, Bank A: Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* 167:400, 1988
38. Emanuel D, Peppard J, Stover D, Gold J, Armstrong D, Hammerling U: Rapid immunodiagnosis of cytomegalovirus pneumonia by bronchoalveolar lavage using human and murine monoclonal antibodies. *Ann Intern Med* 104:476, 1986
39. Sanchez V, Angeletti PC, Engler JA, Britt WJ: Localization of human cytomegalovirus structural proteins to the nuclear matrix of infected human fibroblasts. *J Virol* 72:3321, 1998
40. Evans TJ, Jacquemin MG, Farrell PJ: Efficient EBV superinfection of group I Burkitt's lymphoma cells distinguishes requirements for expression of the Cp viral promoter and can activate the EBV productive cycle. *Virology* 206:866, 1995
41. Lu M, Zhang N, Maruyama M, Hawley RG, Ho AD: Retrovirus-mediated gene expression in hematopoietic cells correlates inversely with growth factor stimulation. *Hum Gene Ther* 7:2263, 1996
42. Diamond DJ, York J, Sun JY, Wright CL, Forman SJ: Development of a candidate HLA A*0201 restricted peptide-based vaccine against human cytomegalovirus infection. *Blood* 90:1751, 1997
43. Schmidt GM, Horak DA, Niland JC, Duncan SR, Forman SJ, Zaia JA: A randomized, controlled trial of prophylactic ganciclovir for cytomegalovirus pulmonary infection in recipients of allogeneic bone marrow transplants: The City of Hope-Stanford-Syntex CMV Study Group. *N Engl J Med* 324:1005, 1991
44. Stocchi R, Ward KN, Fanin R, Baccarani M, Apperley JF: Management of human cytomegalovirus infection and disease after allogeneic bone marrow transplantation. *Haematologica* 84:71, 1999
45. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR: 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, 1994
46. Morrison LA, Lukacher AE, Braciale VL, Fan DP, Braciale TJ: Differences in antigen presentation to MHC class I- and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J Exp Med* 163:903, 1986
47. Braciale TJ, Morrison LA, Sweetser MT, Sambrook J, Gething MJ, Braciale VL: Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol Rev* 98:95, 1987
48. Cassell DJ, Schwartz RH: A quantitative analysis of antigen-

presenting cell function: Activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation. *J Exp Med* 180:1829, 1994

49. Dennig D, Lacerda J, Yan Y, Gasparetto C, O'Reilly RJ: ICAM-1 (CD54) expression on B lymphocytes is associated with their costimulatory function and can be increased by coactivation with IL-1 and IL-7. *Cell Immunol* 156:414, 1994

50. Gimmi CD, Freeman GJ, Gribben JG, Sugita K, Freedman AS, Morimoto C, Nadler LM: B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci USA* 88:6575, 1991

51. Riddell SR, Rabin M, Geballe AP, Britt WJ, Greenberg PD: Class I MHC-restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. *J Immunol* 146:2795, 1991

52. Pear WS, Nolan GP, Scott ML, Baltimore D: Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 90:8392, 1993

53. Naviaux RK, Costanzi E, Haas M, Verma IM: The pCL vector system: Rapid production of helper-free, high-titer, recombinant retroviruses. *J Virol* 70:5701, 1996