Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes

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The naive phenotype of cord blood (CB) T cells may reduce graft-versus-host disease after umbilical cord blood transplantation, but this naivety and their low absolute numbers also delays immune reconstitution, producing higher infection-related mortality that is predominantly related to CMV, adenovirus (Adv), and EBV. Adoptive immunotherapy with peripheral blood–derived virus-specific cytotoxic T lymphocytes (CTLs) can effectively prevent viral disease after conventional stem cell transplantation, and we now describe the generation of single cultures of CTLs from CB that are specific for multiple viruses. Using EBV-infected B cells transduced with a clinical-grade Ad5f35CMVpp65 adenoviral vector as sources of EBV, Adv, and CMV antigens, we expanded virus-specific T cells even from CB T cells with a naive phenotype. After expansion, each CTL culture contained both CD8+ and CD4+ T-cell subsets, predominantly of effector memory phenotype. Each CTL culture also had HLA-restricted virus-specific cytotoxic effector function against EBV, CMV, and Adv targets. The CB CTLs recognized multiple viral epitopes, including CD4-restricted Adv-hexon epitopes and immunosubdominant CD4- and CD8-restricted CMVpp65 epitopes. Notwithstanding their naive phenotype, it is therefore possible to generate trivirus-specific CTLs in a single culture of CB, which may be of value to prevent or treat viral disease in CB transplant recipients. This study is registered at www.clinicaltrials.gov as NCT00078533. (Blood. 2009;114:1958-1967)

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for selected patients with high-risk hematologic malignancies. However, a significant proportion of patients—especially nonwhites—lack a marrow or peripheral blood stem cell donor. Umbilical cord blood (CB) has therefore emerged as an important alternative source of stem cells for patients who receive an allotransplant.1,2

The major advantages of CB transplants compared with unrelated adult donor stem cells include more rapid procurement of the graft, the requirement for less-stringent HLA matching, the higher likelihood of finding a match for ethnic minorities, and a decreased incidence of graft-versus-host disease. Although a major disadvantage of CB is the low stem cell dose, which results in delayed engraftment, there is also delayed immune recovery, particularly in mismatched unrelated cord blood transplantations with the use of antithymocyte globulin.3 This is attributed to the small numbers of T cells transferred, the absence of memory T cells within the CB grafts, and the apparent hypersensitiveness of CB antigen-presenting cells. Consequently, CB recipients are susceptible to an array of viral and other infections that are the leading cause of death in these patients.3,7

Cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (Adv) are particularly problematic in the immunocompromised recipient and are associated with significant rates of morbidity and mortality.5,10 Although antiviral drugs can be beneficial for CMV and EBV, effective agents are unavailable for Adv; all have substantial toxicities and drug resistance may occur. These deficiencies in conventional therapeutics have increased interest in an immunotherapeutic approach to viral disorders, and adoptive transfer of monoculture-derived multivirus-specific cytotoxic T-lymphocytes (CTLs) from CMV-seropositive donors can prevent and treat CMV, EBV, and adenoviral disease after HSCT without significant toxicities.11

There are significant conceptual obstacles to extending this approach to the recipients of CB transplants. Only limited numbers of CB T cells are available for manipulation, and these cells have a naive phenotype, making them incapable of conferring protection against CMV, EBV, and Adv during engraftment.3,12 Hence, the development of virus-protective T-cell therapy for CB transplant recipients requires the priming and extensive expansion of naive T cells rather than the more limited and simple direct expansion of a preexisting virus-specific memory T-cell population. This task is made even more challenging because CB T cells have lower cytotoxic activity and higher activation-induced cell death than do peripheral blood–derived T cells.13,14 These limitations have prevented the successful generation of virus-specific CTLs in sufficient numbers for clinical use as prophylactic therapy of CMV, EBV, and adenoviral infections after CB transplantation (CBT).
It has become evident that, in addition to antigen presentation and intercellular costimulation, the ex vivo priming of antigen-specific T cells requires the presence of appropriate soluble cytokines. Hence, IL-7, IL-15, and IL-12, respectively, decrease the antigen concentration threshold, direct T cells toward a central memory phenotype, and influence the polarization of T cytokotoxic type 1 and T helper type 1 cells. In combination these cytokines augment the generation of antigen-specific CTLs from naive T cells.  

We now describe how Ad5f35pp65-transduced CB-derived antigen-presenting cells can be used to generate large numbers of T cells that are specific for both CMV and Adv even from the naive T-cell population in human CB. Incorporation of EBV-transformed B-lymphoblastoid cell lines in the antigen-presenting cells further allows the Adv/CMV specificity of the CB T cells to be extended to EBV. The cells we describe have broad epitope specificity and are cytotolytic to CMV, Adv, and EBV target cells. Hence, they may be protective in vivo.

### Methods

#### CB units

Virus-specific CTLs were generated from CB units obtained frozen from the M. D. Anderson Cancer Center Cord Blood Bank or fresh from mothers who had consented to the protocol approved by the Institutional Review Board (IRB) of Baylor College of Medicine. To ensure the future clinical feasibility of this approach, we also froze the fresh CB units in DMSO containing 50% human serum before use for the generation of CB-derived CTLs, thereby mimicking the likely clinical setting. All CB samples were typed by the HLA laboratory of The Methodist Hospital, and we used CB from donors with multiple HLA types (Table 1). To further ensure that the approach would be feasible clinically, a total of only 40 million CB mononuclear cells (which can be obtained from the 20% fraction of frozen CB units) were thawed and used in the manufacturing process. According to our IRB approval, clinical protocol patients must have a single CB unit matched with the patient at 4, 5, or 6 of 6 HLA class I (serologic) and II (molecular) antigens. The unit must be cryopreserved in 2 fractions, with a minimum of 2.5 × 10^7 total nucleated cells/kg preduring the fraction which will be used for the primary transplant. The remaining fraction will be used to generate the CTLs to give at day 30 or beyond as described in section entitled “Generation of multivirus-specific cultures derived from umbilical CB.” This cell dose has been found to support acceptable engraftment in both pediatric and adult patients and is a commonly used minimal cell dose target in the CB transplantation community.  

### Generation of dendritic cells from cord blood

Umbilical CB was thawed and then purified by Ficoll (Lymphoprep; Nycomed) gradient separation. CB mononuclear cells (CBMCs; 3 × 10^6) were washed twice and resuspended in CellGenix media (CellGenix USA) and plated at 5 × 10^6 cells/well in dendritic cell (DC) medium (CellGenix media plus 2 mM l-glutamine; Glutamax; Invitrogen) in a 6-well plate (Costar) for 2 hours at 37°C in a humidified CO2 incubator. Nonadherent cells were removed by rinsing with 1× PBS (GibcoBRL) and refrozen. Loosely adherent cells were cultured in DC media with 800 U/mL GM-CSF (Sargramostim Leukine; Immunex) and 500 U/mL IL-4 (R&D Systems) for 7 days. IL-4 and GM-CSF were again added on day 3. On day 5, cells were harvested for transduction.

#### Transduction of DCs

As our source of CMV and Adv antigens, we used a clinical-grade recombinant Adv type 5 vector pseudotyped with an Adv type 35 fiber, that encoded CMVpp65.  

On day 5, the CB-derived DCs were transduced with the clinical-grade Ad5f35CMVpp65 vector at a multiplicity of infection of 10 IU/cell for 2 hours and matured in a cytokine cocktail of GM-CSF, IL-4, IL-1β, TNF-α, IL-6, (R&D Systems) and PGE2 (Sigma-Aldrich) for 2 days. On day 7, the DCs were harvested, irradiated (30 Gy) and then used to stimulate virus-specific CTLs.

#### Generation and transduction of EBV-transformed B-cell lines from umbilical CB

Frozen CBMCs were thawed, washed, and resuspended in 45% RPMI (Hyclone) and 45% CLICKS (Irvine Scientific) with 10% human serum before use for the generation of CB-derived EBV-specific CTLs. As our source of CMV and Adv antigens, we used a clinical-grade Ad5f35CMVpp65 vector at a multiplicity of infection of 100 IU/cell for 90 minutes at 37°C. The cells were then resuspended at 5 × 10^6 cells/mL of complete media (RPMI [Hyclone] plus human serum and Glutamax) and transferred to a 24-well plate at 2 mL/well and cultured for 2 days before use as stimulator cells.

#### Generation of multivirus-specific cultures derived from umbilical CB

Fetal CBMCs were thawed, washed, and resuspended in 45% RPMI (Hyclone) and 45% CLICKS (Irvine Scientific) with 10% human serum plus Glutamax-I (CTL medium). Cells were resuspended at 2 × 10^6/mL and cocultured with autologous, irradiated transduced DCs at a ratio of 20 CBMCs to 1 DC in the presence of the cytokines IL-7, IL-12 (R&D Systems), and IL-15 (CellGenix), all at 10 ng/mL. Cultures were restimulated on day 10 with irradiated Ad5f35CMVpp65-transduced LCL at a responder-to-stimulator ratio of 4:1 plus IL-15 (10 ng/mL) and 1 week later with irradiated autologous Ad5f35CMVpp65-transduced LCL at a responder-to-stimulator ratio of 4:1. IL-2 (50-100 U/mL; Proleukin; Chiron) was added 3 days after the second stimulation and then twice weekly.

To define the origin of the virus-specific T-cell population we sorted CD45RA/CCR7 double-positive (DP) and double-negative (DN) CD3+ T-cell populations with the use of flow cytometry and stimulated the DP and DN populations with transduced DCs followed by transduced LCLs.

#### Peripheral blood–derived CTL lines

CTL lines from peripheral blood were prepared from stem cell donors who gave informed consent, in accordance with the Declaration of Helsinki, on enrollment in our clinical trial of virus-specific T cells for the treatment of...
viral infection after transplantation. All protocols were approved by the Baylor College of Medicine IRBs and the National Marrow Donor Program. For the purposes of this analysis, we have characterized 11 of these CTL lines.

**Immunophenotyping**

CTL lines were analyzed with monoclonal antibodies to CD19, CD4, CD8, CD56/16, TCRβ, TCRγ, CD45RA, CD3/28, CD62L, CD4/25, and CCR7 (Becton Dickinson). Samples were acquired on a FACScan flow cytometer (Becton Dickinson), and the data were analyzed with the use of CellQuest software (Becton Dickinson).

**Enzyme-linked immunospot assay**

Enzyme-linked immunospot (ELISPOT) assay analysis was used to determine the frequency and function of T cells secreting IFN-γ in response to PepMixes (Jeni) for CMVpp65, AdvHexon, AdvPenton, and CMVIE1, which contain all 15mer peptides of each viral antigen in one pool. ELISPOT assays were performed on the CTL lines. In addition, to define the CD4- and CD8-restricted CMVpp65-specific activity we pulsed CD8+ or CD4+ T cells (sorted from CTL lines with flow cytometry) with CMV peptides. CBMCs stimulated with CMVIE1 PepMix and staphylococcal enterotoxin B (1 μg/mL; Sigma-Aldrich) served as controls. Spot-forming cells were enumerated by Zellnet Consulting and compared with input cell numbers to obtain the frequency of virus-reactive T cells.

**Multimers and peptides**

To detect CMVpp65-specific T cells in the CTL lines, we used the soluble HLA-peptide HLA-A*0201-MLN tetramer prepared by the Baylor College of Medicine Tetramer Core Facility. Peptides were synthesized by the Baylor College of Medicine Protein Core Facility or by Proimmune Inc. Tetramer staining of CTLs (5 × 10^6) was previously described. For each sample, 100,000 cells were analyzed using CellQuest software.

Panels of 20mer peptides (overlapping by 15 amino acids) covering the entire amino acid sequence of CMVpp65 from the AD169 strain and Adv-hexon from serotype 5 were synthesized. For CMVpp65, 22 peptide pools comprising 2 to 12 15mer peptides were prepared, so that each 20mer peptide was represented in 2 pools. For Adv-hexon, 11 peptide pools contained 17 or 18 peptides, so that each 20mer peptide was represented in one pool, and we then screened the positive pools as previously described. These CMVpp65 and Adv-hexon peptide libraries were designed to identify all possible HLA class I–restricted epitopes, which have a length of 9 to 11 amino acids, and to also identify HLA class II–restricted epitopes, which have lengths of 13 to 17 amino acids.

**Cytotoxicity assay**

CTLs were tested for specific cytoxicity against autologous LCLs, PHA blasts pulsed with CMVpp65 PepMix (Jeni), or Adv-hexon PepMix. As control target cells we used unpulsed PHA blasts, PHA blasts pulsed with irrelevant peptides, and HLA class I–mismatched LCLs. 51Cr-labeled target cells were mixed with effector cells at doubling dilutions to produce the effector-to-target (E/T) ratios specified. Target cells incubated in complete medium or 5% Triton X-100 (Sigma-Aldrich) were used to determine spontaneous and maximal 51Cr release, respectively. After 4 hours (LCLs and PHA blasts) or 6 hours (fibroblasts), supernatants were collected, and radioactivity was measured on a gamma counter. The mean percentage of specific lysis of triplicate wells was calculated as 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release).

**Statistical analysis**

The Student t test was used to test for significance in each set of values, assuming equal variance. Mean values plus or minus SDs are given unless otherwise stated.

**Results**

Expanded CB-derived CTLs are virus specific and polyclonal

We prepared T-cell lines from 9 different CB units. After growth on Ad5f35CMVpp65-transduced DCs and LCLs in the presence of IL-2, IL-7, and IL-15, we harvested a median of 85.5 × 10^6 T cells (range, 60 × 10^6 to 2.65 × 10^7 T cells) by day 21 of culture. All of these cells numbers would be sufficient for current T-cell adoptive transfer protocols, in which 1 × 10^7/m² effectively reconstitute immunity to CMV,
EBV, and Adv-hexon and for product safety, function, and identity testing (Figure 1A). These cells were 78.7% CD8+/H11001 (range, 46%-94%) and 31% CD4+/H11001 (range, 12%-54%; Figure 1B). Flow cytometric analysis of memory markers showed a predominance of CD45RO+/H11001 CD62L+/H11002 T cells (mean, 87.9% ± 5%) with a smaller population of CD45RA+/H11002 CD62L+/H11001 T cells (mean, 14.6% ± 13%; Figure 1B).

We next used cytotoxicity and IFN-γ ELISPOT assays to discover whether the T-cell lines were indeed trivirus-specific CTLs. Cytotoxic activity was tested against a panel of 51Cr-labeled autologous and allogeneic target cells and showed that the CTL lines killed PHA blasts only if they were pulsed with CMVpp65 PepMix or Adv-hexon PepMix (40% and 13%, respectively, at an E/T of 20:1; Figure 1C). Furthermore, autologous EBV-LCLs were also killed in this assay, showing an EBV-specific T-cell component. There was no cytotoxic activity against unpulsed PHA blasts, PHA blasts pulsed with CMVIE1 PepMix (Figure 1C), or HLA-mismatched EBV-LCLs (data not shown).

Similarly, Figure 1D shows that after 3 stimulations, virus-specific T-cell lines (n = 9) can secrete IFN-γ in response to EBV, CMVpp65-, and Adv-hexon–expressing target cells with a mean (± SD) of 157 (± 134), 209 (± 210), and 74 (± 65) spot-forming cells per 10^5 T cells, after incubation with EBV-LCL, CMV-pp65, and Adv-hexon/penton peptides, respectively. In contrast, T cells did not respond to CMVIE1, the irrelevant peptide control.

**Virus-specific CTLs are expanded from the naive CCR7+/CD45RA+ T-cell population**

We derived the virus-specific CTLs from CB, which should lack an EBV-, CMV-, or Adv-specific memory T-cell compartment. To determine whether the virus-specific T cells were indeed expanded from T cells with a naive phenotype (ie, CCR7+/CD45RA+ T-cell populations), the mononuclear cells from 3 cord units (2 from CMV-seronegative mothers and 1 from a CMV-seropositive mother) were incubated with antibodies for CD45RA and CCR7. We used flow cytometry to sort T cells that were either DP for both CD45RA and CCR7 or DN for CD45RA and CCR7 (mean, 14.6% ± 13%; Figure 1B).

![Figure 2. Virus-specific cytotoxic T lymphocytes from CB are derived from the CD45RA+/CCR7+ naive T-cell population.](image-url)

Each of these populations was then stimulated with transduced autologous antigen-presenting cells. Indicated are percentage of CD45RA+/CCR7+ T cells versus percentage of CD45RA+/CCR7+ T cells that were selected. (B) After 3 stimulations, the phenotype of the CTL lines derived from cells that were either double positive for both CD45RA and CCR7 (CD45RA+/CCR7+) or double negative for CD45RA and CCR7 (CD45RA-/CCR7-) was determined by flow cytometry. (C) Virus-specific activity of the CTL lines was determined after 3 stimulations by the IFN-γ ELISPOT assay in response to direct stimulation with CMVpp65 PepMix (CMVpp65), CMVIE1 PepMix (CMVIE1), Adenovirus hexon PepMix (Adv hexon), irradiated autologous EBV-LCL at an E/T ratio of 4:1 (EBV) or PMA-ionomycin (PMA-I). ELISPOT analysis of a T-cell line derived from the CD45RA and CCR7 double-positive fraction versus a T-cell line derived from the double-negative fraction from a representative CB unit from a CMV-seronegative mother is shown. Mean values (± SDs) of triplicate experiments are reported.
Table 2. Adenovirus specificity of CB virus-specific T-cell lines

<table>
<thead>
<tr>
<th>Cord ID</th>
<th>HLA typing cord</th>
<th>Adv hexon epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB2</td>
<td>A2;24/B7</td>
<td>Penton specificity only</td>
</tr>
<tr>
<td>CB3</td>
<td>A2;29/B35;44</td>
<td>556-580: VYDPHVQPKFAIKLLNLPSGYT</td>
</tr>
<tr>
<td>CB6</td>
<td>A2;33/B40(60);60</td>
<td>76-95: TQSYSSKAFTLAVGDNRNLVD</td>
</tr>
<tr>
<td>CB7</td>
<td>A11;30/B18;5</td>
<td>Hexon pools 1, 2, 3 and penton specificity</td>
</tr>
<tr>
<td>CB8</td>
<td>A2;3/B6;18</td>
<td>Hexon pools 6, 10 and penton specificity</td>
</tr>
<tr>
<td>CB9</td>
<td>A29;31/B7;51</td>
<td>796-815: RNFPQPMRSQVDDTKYKDYQ</td>
</tr>
</tbody>
</table>

both CD45RA+CCR7+ and CD45RA-CCR7- populations secreted IFN-γ in response to PMA-ionomycin. However, only T cells derived from the CD45RA and CCR7 DP population secreted IFN-γ in response to EBV, CMV, and Adv antigen-expressing target cells.

**Virus-specific CTLs derived from CB recognize immunodominant CD4-restricted Adv-hexon epitopes**

To map the Adv-hexon epitope specificity of the CTL lines, we used an overlapping peptide library representing the entire sequence of the Adv serotype 5 hexon protein.18,20 For the initial screening, CTL lines were stimulated by overnight incubation with the 188 overlapping hexon peptides, divided into 11 pools, containing 17 or 18 peptides per pool.

The results from Adv-hexon peptide pool screening of 6 evaluable CTL lines are summarized in Table 2. Of the 6 CTL lines, 3 had reactivity against one Adv-hexon epitope, and 2 lines had reactivity against multiple hexon epitopes. Three lines had reactivity against Adv penton. The hexon epitopes identified in these CB CTL lines were the same CD4-restricted epitopes as those identified in the multivirus CTL lines generated from adult peripheral blood.18

Figure 3 shows the details of such analyses from 2 of these CB-derived CTL lines. One line reacts predominantly against pool 1 (Figure 3A) and the other against pool 10 (Figure 3B). Subsequent analysis with individual 20mer peptides contained in these pools shows that the CTL reactivity in pool 1 could be mapped to overlapping peptides 15 (aa 71-90 VDREDYTAISKAFRTLVGD) and 16 (aa 76-95 TAISKAFRTLVGDNRVL; Figure 3C), whereas CTL reactivity directed against pool 10 could be mapped to overlapping peptide 6 (aa 791-814 MYSSFRNFPQPMRSQVVD-DTK) and peptide 7 (aa 796-815 RNFPQPMRSQVDDTKYKDYQ: Figure 3D). Thus, CB-derived CTLs have a similar pattern of epitope recognition to adult blood-derived CTLs. Furthermore, these epitopes are CD4-restricted with apparent immunodominance because they have been previously detected in multiple CTL lines derived from peripheral blood.18

**Virus-specific CTLs derived from CB recognize unconventional CD4- and CD8-restricted CMVpp65 epitopes**

We characterized the CMVpp65 epitope specificity of the CTLs by incubating them with CMVpp65 peptide pools20 and measuring IFNγ release by ELISPOT assays. Table 3 shows that evaluable virus-specific lines derived from umbilical CB can secrete IFNγ in response to several different CMVpp65 peptides, representing discrete epitopes on the CMVpp65 antigen.

A representative example is detailed in Figure 4. This CTL line (HLA type: A2.29B35.44) had a detectable T-cell response to 20mer peptides from pools 7, 8, 9, 10, 11, 13, 14, and 18 (Figure 4A). The 20mer peptides common to these 3 pools were peptides 7, 8, 22, 23, 69, and 70 (Figure 4B). As shown in Figure 4C, screening of these 20mer peptides showed T cells specific for at least 2 epitopes. They were the known HLA A0201-restricted epitope MNPSINV (confirmed by tetramer as shown in Figure 4D) and 2 other epitopes (ALFFFDIDLLLQGRGQYSE and DTPVLPHET-RLLQTIHVRV) spanning the regions 347 to 358 and 31 to 51, respectively. By contrast, analysis of this CTL line for the known HLA A2-restricted immunodominant CMVpp65 peptide (NLVPVMATV) was negative. Furthermore, 7 of the 7 CTL lines that were HLA A2 and/or A24 and/or B7 positive lacked T cells specific for the immunodominant A2 NLVPVMATV epitope and/or the B7 TPRVTTGGGAM and RPHERNGFTVL epitopes and/or the A24 QYDPVAAFL epitope (Table 3). These results are in contrast to CTL lines generated from peripheral blood donors21 with these HLA types in which 11 of 11 CTL lines exhibited specific activity against these immunodominant epitopes (Table 4).

To characterize the EBV-specific T-cell response, we screened the CTL lines for known immunodominant epitopes in EBNA 3, BZLF1, and LMP.23 We found, however, no evidence for such dominant epitope-specific T cells (data not shown).

**Discussion**

This study provides the first direct evidence that antigen-specific T cells targeting multiple viral epitopes from Adv, CMV, and EBV can be primed simultaneously from CB T cells with a naive phenotype. In addition, the multivirus-specific T cells recognize an array of epitopes after only 2 weeks of expansion in vivo. Notably, the pattern of CMV epitopes recognized appears to be different to “adult”-derived virus-specific T cells, whereas the recognition of Adv epitopes is the same.

Park et al26 have previously shown that CMV-specific T cells can be generated in vitro from umbilical CB, but the antigen and epitope specificity of the CMV-specific CTL was largely uncharacterized. Moreover, it was unclear whether the CMV-specific CTLs they detected were derived from T cells with a naive phenotype, or acquired CMV epitopes during in vitro expansion. Notably, the pattern of CMV epitopes recognized appears to be different to “adult”-derived virus-specific T cells, whereas the recognition of Adv epitopes is the same. CB-derived T cells can respond to endogenous antigens, such as minor histocompatibility antigens that are disparate between mother and fetus, and to exogenous antigens, such as parasites and environmental allergens.28,29 They may also respond to viral antigens if the mother is seropositive or has been vaccinated. Hence, antigens in the maternal environment may prime fetal T cells transplacentally and elicit antigen-specific T-cell responses.30,31 In our study, however, it was evident that the responses we obtained were derived in vitro priming of the
Figure 3. Specificity of CB CTL lines for adenovirus hexon. CB-derived CTL lines were screened by ELISPOT assay against 11 pools of hexon peptides (20mers overlapping by 15 aa) to identify which peptide pools were being recognized by each CTL line. (A) The CTL line CB6 showed specific IFN-γ release specifically against peptide pool 1. (B) The CTL line CB9 showed specific IFN-γ release specifically against peptide pool 10. Results are expressed as spot-forming cells (SFCs)/10^5 CTLs. (C) To identify the stimulating 20mer peptides, CTL line CB6 was rescreened against the individual peptides contained in pool 1. The CTL reactivity against pool 1 were mapped to the overlapping peptides 15 (VDREDTAYSYSKرافTLAVGD) and 16 (TAYSYSKرافTLAVGD)NRLVLD. (D) In contrast, the reactivity in CTL line CB9 was mapped to peptides 6 (MYSFPRNFOPMSRQVVDDTK) and 7 (NRFOPMSRQVVDDTKYKDYQ) in pool 10. Results are expressed as SFCs per 10^5 CTLs.

Table 3. CMVpp65 epitope specificity of cord blood virus-specific T-cell lines

<table>
<thead>
<tr>
<th>Cord ID</th>
<th>HLA typing cord</th>
<th>CMVpp65 epitope(s)</th>
<th>Predicted immunodominant CMVpp65 epitope(s) not identified</th>
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<tbody>
<tr>
<td>CB1</td>
<td>A2;3/B44;51</td>
<td>31-51: DTPVLPHETRLLQTG1HHRV</td>
<td>495-503: NLVPMATV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>126-146: INVHHYPSAAERKLRHNLPA</td>
<td></td>
</tr>
<tr>
<td>CB2</td>
<td>A2;24/B7</td>
<td>120-129: MLNIPINSV</td>
<td>495-503: NLVPMATV</td>
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<tr>
<td></td>
<td></td>
<td>351-371: LLDQGPOYSEHPTFT</td>
<td>341-349: QYDPAVAALF</td>
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<td></td>
<td></td>
<td>495-503: NLVPMATV</td>
<td>417-426: TPRVTGGGAM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>265-275: RHERNGGFTVL</td>
<td>265-275: RHERNGGFTVL</td>
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<tr>
<td>CB3</td>
<td>A2;29/B35;44</td>
<td>31-51: DTPVLPHETRLLQTG1HHRV</td>
<td>495-503: NLVPMATV</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>347-358: ALFFFDIDILL</td>
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<td>CB5</td>
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<td>151-171: ASKQGMRQARLTVSGLAWR</td>
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<td>CB9</td>
<td>A29;31/B7;51</td>
<td>116-131: LPLKMLNIPINSW1HYPASA</td>
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<td></td>
<td></td>
<td>265-275: RHERNGGFTVL</td>
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N/A indicates not applicable.
Table 4. CMVpp65 epitope specificity of peripheral blood virus-specific T-cell lines

<table>
<thead>
<tr>
<th>Peripheral blood CTL ID</th>
<th>HLA typing</th>
<th>CMVpp65 epitope(s)</th>
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<tbody>
<tr>
<td>PB1</td>
<td>A1;24/B51;57</td>
<td>QYDPVAALF 369-379; FTSQYRIQGKL 113-121: VYALPLKML</td>
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<td>A1;80/B7;53</td>
<td>TPRVTGGGAM</td>
</tr>
<tr>
<td>PB3</td>
<td>A2/B8;49</td>
<td>NLVPVMATV</td>
</tr>
<tr>
<td>PB4</td>
<td>A2402,3201/B1302,3501</td>
<td>QYDPVAALF</td>
</tr>
<tr>
<td>PB5</td>
<td>A2;68/B35;40</td>
<td>NLVPVMATV</td>
</tr>
<tr>
<td>PB6</td>
<td>A1;2/B8;15</td>
<td>NLVPVMATV</td>
</tr>
<tr>
<td>PB7</td>
<td>A2;66/B27(66);52(52)</td>
<td>NLVPVMATV</td>
</tr>
<tr>
<td>PB8</td>
<td>A2;3/B7;44</td>
<td>NLVPVMATV</td>
</tr>
<tr>
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<td>A2;B40(61);46</td>
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</tr>
<tr>
<td>PB10</td>
<td>A2;B35;39</td>
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</tr>
<tr>
<td>PB11</td>
<td>A2;3/B1501;5301</td>
<td>NLVPVMATV</td>
</tr>
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Figure 4. Identification of CMVpp65 epitopes with the use of a CMVpp65-peptide library. (A) CTLs (10^5/well) from CB3 (HLA-A2, A29, B35, B44) were stimulated with a CMVpp65-peptide library pooled into 22 pools. Responses were measured in an 18-hour IFN-γ ELISPOT assay. Shown is mean and SD of duplicate wells. (B) All peptides were divided into 22 pools in such a way that each peptide is present in 2 pools. This method allows determining those single peptides that probably induced responses to the peptide pools. Thus, responses to pools 7, 8, and 13 or 9, 10, and 11, or 14 and 15 and 16 may be induced by single peptides 7 and 8 or 9 and 10 or 11, respectively (C) Testing of these individual 20mers identifies the sequence of peptides 7 and 8 or 9 and 10 as most probably the overlapping 15 amino acids, as the CTL epitope. In addition, the T-cell line mapped to a known HLA-A2-restricted epitope (MLNIPSVIN) contained in peptides 23 and 24. (D) The polyclonal CB-derived virus-specific CTL line in which this epitope had been identified was stained with an HLA-A*0201 MLNIPSINV tetramer. Indicated is the percentage of tetramer-positive cells within the CD8 T-cell population.
CMV-infected fetuses are also deficient in CD8

studies appear to reflect physiologic events in humans, because

spreads were analyzed for sex chromosome signal pattern.

ing). A total of 200 interphase nuclei and 5 metaphase

cells were analyzed using Quips Pathvysion (Applied Imag-

ition of 100

/H11003

images were captured using 10

/H11003

stained with 4,6-diamidino-2-phenylindole (DAPI) and the

to the manufacturer’s protocols. The slides were counter-

Abbott Laboratories. Hybridization was performed according

some Y labeled with spectrum green were obtained from

spectrum orange and the satellite III (Yp12) region of chromo-

satellite centromeric region of chromosome X labeled with

origin we used fluorescence in situ hybridization (FISH) with

CB3 were of neonatal and not maternal origin; moreover, placental tissue from our donors was

Adv and CMV negative by polymerase chain reaction) analysis and

by immunohistochemistry, further excluding the possibility of

priming in utero (data not shown).12

Although our ex vivo–expanded CB-derived multivirus-specific

T cells developed a memory phenotype profile that was similar to

peripheral blood–derived multivirus-specific T cells,11 their epitope

specificity could be strikingly different. Thus, none of the CB-

derived CTL lines recognized the strongly immunodominant

epitopes that are universally identified in the peripheral blood

CMVpp65 CTL lines generated from CMV-seropositive donors

(Table 4), with a preference instead for unconventional and even

novel CMVpp65 epitopes. CB T cells may be a transitional

population between thymocytes and adult T cells,28 so that the

specificities we describe may thus represent a “default response” by

recent thymic emigrants, which have not undergone postnatal

selection for more specific and, perhaps, higher affinity antiviral

reactivity.22,32 This hypothesis is supported by the broad Vβ

repertoire we observed in CB-derived CTLs with the use of

spectratyping (data not shown). Murine studies showing that

promiscuous low-affinity TCR/MHC-peptide interactions on the

surface of functionally immature neonatal T cells leads to an

intensive “burst” of short-lived cellular immunity before the

establishment of conventional T-cell memory mediated by high-

affinity T cells.33 Our own human in vitro and the murine in vivo

studies appear to reflect physiologic events in humans, because

CMV-infected fetuses are also deficient in CD8+ T cells respond-

ing to the canonical, immunodominant, B7-restricted RPH and

A2-restricted NLV epitopes.31

Notwithstanding the recognition of noncanonical epitopes,

CB-derived CMV-specific CTLs are capable of specifically lysing

CMVpp65-pulsed targets. Moreover, these usually “subdominant”

CMV epitopes still successfully compete against Adv-hexon

epitopes, because CTLs responding to CMV-pp65 continue to

exceed in number the CTLs responding to Adv-hexon, a character-

istic they share with memory cell–derived CTLs directed to

canonical pp65 epitopes.

Although the specificity of CB-naive T-cell responses to CMV

were markedly different from adult seropositive peripheral blood

T cells, the responses of the 2 T-cell populations to Adv were,

perhaps surprisingly, indistinguishable. With the use of pools of

overlapping 20mer peptides for hexon and pp65, we identified

T cells specific for the same immunodominant CD4-restricted

hexon epitopes found in adult seropositive donors, such as the

hexon peptides that span amino acids 791 to 815 (MYSFFRN-

FQPMRSQVVDKTKYDYO).18 We do not know why CB and

peripheral blood T cells should share epitope recognition for Adv

but not CMV, but the difference probably reflects the profoundly
different biology of the 2 viruses. Adv is an acute infection that

elicits strong innate immunity, followed by an adaptive CD4-

restricted hexon-specific T-cell response that in turn maximizes the

range of epitopes recognized by CD4+ and CD8+ effector cells.18

As a latent virus, however, CMV recruits a dominant and oligo-
estral CD8+ response.34 CB T cells are biased toward a T helper

type 2 phenotype,27 which may support development of a conven-
tional adenoviral CD4-specific T-cell response, but which, in

combination with the immaturity of the responding CB T cells,

can direct the CMV response away from the canonical epitopes.

Our explanation leads us to predict that our CB T-cell responses
to EBV, a latent virus like CMV, would also be to nondominant

epitopes. An evaluation for noncanonical responses is more

difficult for EBV than for CMV, because T cells from most

responders recognize epitopes from multiple latent and early lytic

cycle EBV antigens. Nevertheless, our screen of CTL lines for

known immunodominant epitopes in EBNA 3, BZLF1, and LMP25

failed to show any of the anticipated epitope-specific T cells (data

not shown) despite a functional cytokine and cytotoxic response to

HLA-matched EBV-LCLs (Figure 1C-D). These observations are

at least consistent with the differences we propose between CB

naive T-cell responses to acute and latent viral antigens.

Although we were able to generate multivirus-specific CTLs

from CB, success was only achieved when we supplemented the

cultures with IL-7, IL-12, and IL-15. Such supplementation is

unnecessary for the generation of virus-specific CTLs from adult

memory T cells11 and may simply indicate a lack of memory T-cell

precursors a deficiency of CB APC secretion of these cytokines, or

both. Neonatal DCs certainly appear to have a deficiency in IL-12

production, and we have shown how cytokine supplementation can

overcome these limitations.35 In the current studies, it is possible

that the altered epitope recognition we observed in the CMV

responses is a consequence of the addition of these supplementary

cytokines. This seems unlikely because the sole explanation for the

phenomenon not only in view of the supporting data from murine

and human studies described earlier but also because the responses to

Adv-hexon epitopes were unchanged. Hence, we believe these

differences more likely reflect intrinsic differences in the responsi-

iveness of naive CB versus memory T cells derived from CMV-

seropositive donors.

Although antiviral pharmacotherapy may help prevent or treat

CMV36,37 and CD20-specific antibodies may control EBV-

associated lymphoproliferation,38 these drugs are expensive, toxic,
and often ineffective because of primary or secondary resistance. Moreover, infections with adenoviruses are increasingly reported after both allogeneic HSCT and CBT, and effective treatments are not currently available. Strategies to reduce the incidence of viral infections after CBT include using grafts with the best HLA match and higher TNC, changes in conditioning, and graft-versus-host disease prophylaxis. However, adoptive transfer of peripheral blood–derived T-cell lines enriched in cells recognizing CMV, EBV, and Adv can reproducibly control infections resulting from all 3 viruses after allogeneic HSCT in a cost-effective manner. We suggest that our ability to generate virus-specific CTLs from CB against a plethora of epitopes recognized by both CD4+ and CD8+ T cells should minimize the risk of viral escape and maximize therapeutic benefit on administration of these cells to CB recipients at risk of severe viral disease.

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Authorship

Contribution: P.J.H. and C.R.Y.C. conducted the in vitro studies and contributed to the writing of the paper; B.S., A.M.L., M.K., M.S., and W.D. helped develop the protocol and conduct the in vitro studies; J.J.M. performed V8 spectrotyping analysis; H.L. provided statistical support; A.P.G. helped with scale up for Good Manufacturing Practice (GMP); C.M.R., H.E.H., and G.D. helped with data analysis and provided advice on experimental design; M.K.B. helped with data analysis and contributed to the writing of the paper; E.J.S. provided advice on ex vivo use of cord blood and contributed to the writing of the paper; and C.M.B. developed the study, supervised the experiments, and contributed to the writing of the paper.

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References


Functionally active virus-specific T cells that target CMV, adenovirus, and EBV can be expanded from naive T-cell populations in cord blood and will target a range of viral epitopes

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