Brief report

In vitro priming and expansion of cytomegalovirus-specific Th1 and Tc1 T cells from naive cord blood lymphocytes

Kyung-Duk Park, Luciana Marti, Joanne Kurtzberg, and Paul Szabolcs

Adoptive transfer of CMV-specific cytotoxic T cells (CTLs) expanded in vitro from memory donor T cells can reduce the incidence of CMV disease in allogeneic transplant recipients. However, this approach has been unavailable in the cord blood (CB) transplantation setting because CB T cells are antigen naive and biased toward Th2/Tc2 function. We developed a protocol to in vitro prime and expand CMV-specific CTLs from CB. T cells were primed with cytokines to trigger skewing toward Th1/Tc1 lineage before encountering monocyte and CD34+ progenitor-derived dendritic cells loaded with CMV antigen and its immune complex. CMV-pulsed cultures expanded significantly more over 4 to 6 weeks than CMV cultures despite identical cytokine milieu. T cells isolated from CMV+ cultures showed a preferential expansion of CD45RA−/RO+/CD27+ T cells compared to CMV− cultures. CMV-specific IFN-γ− and TNF-α−producing CD4+ (Th1) and CD8+ (Tc1) T cells were enriched after 3 to 4 weeks and CMV-specific cytotoxicity developed 1 to 2 weeks later.

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Introduction

Although cytomegalovirus (CMV) infection in immunocompetent individuals is usually minimally symptomatic, CMV may cause fatal disease in hematopoietic cell transplant (HCT) recipients. Adoptive transfer of CMV-specific cytotoxic T lymphocytes (CTLs), expanded from donor memory cells, can prevent CMV disease in HLA-identical HCT recipients. Monocyte-derived dendritic cells (Mo/DCs) and other antigen-presenting cells (APCs) have been used to elicit CMV-specific CTLs (for a review, see Bollard et al.). Although CMV-specific CTL generation is feasible in seropositive donors, it rarely succeeds in CMV-seronegative individuals.

Antiviral CTLs have been unavailable for umbilical cord blood transplant (UCBT) recipients because cord blood (CB), like seronegative donors, contains antigen-inexperienced T cells. Additionally, compared to CD45RA+CD28− ‘naive’ adult T cells, CB T cells are defective in the inducible expression of Th1 cytokines, primarily IFN-γ, as a result of Th2/Tc2 skewing imparted by placental factors (for a review, see Marchant and Goldman). Independently, diminished IL-12 expression by neonatal DCs impairs Th1 immunoreactivity. We now report that CMV-specific CTLs can be generated from CB along the Th1/Tc1 pathway.

Study design

DC generation and antigen loading

Fresh mononuclear cells (MNCs) from discarded CB units obtained with Institutional Review Board approval were cultured in RPMI 1640 plus 10% pooled human serum (PHS; NABI, Miami, FL). CD34+ progenitors were selected from nonadherent cells using CD34-PE (Becton Dickinson, San Jose, CA) and anti-PE microbeads (Miltenyi Biotech, Auburn, CA) over a MiniMACS column per the manufacturer’s instruction. CD34+ cells were frozen. Immature Mo/DCs developed from adherent monocytes in RPMI 1640 plus 1% PHS, GM-CSF, and IL-4 over 5 to 6 days. Unused MNCs were frozen and subsequently thawed weekly until exhausted to generate immature Mo/DCs as described except for the adherence media, LGM-3 plus 10% PHS (Clonetics, San Diego, CA). On “day −2” DCs were pulsed at 37°C with inactivated AD169 CMV lysate (5 μg/mL, Biodesign, Carmel, NY). One hour later 1 ng/mL CMV hyperimmune globulin (Medimmune, Gaithersburg, MD) was added for 6 to 8 hours, followed by maturation in TNF-α plus PGE2,11,12 for 24 to 36 hours. Once Mo/DCs were exhausted CD45RA−-derived DCs were generated in AIM V (Gibco, Grand Island, NY) plus 3% PHS, 20 ng/mL SCF, 100 ng/mL Flt-3L (both from Amgen, Thousand Oaks, CA), GM-CSF, and IL-4. After 12 to 14 days, adherent cells were subcultured in GM-CSF plus IL-4 for 4 to 5 days. Nonadherent cells remained in the original cytokines until subcultured.

Priming and expansion of CMV-specific CTL responses

CTL cultures were started on “day 0” when CMV-immune complex (IC)-loaded mature Mo/DCs and unpulsed/control Mo/DCs were mixed with autologous lymphocytes/CTL responders. Responders were generated from CD34+ cells thawed on “day −3,” depleted of human IgG adherent cells, and cytokine-primed in AIM V plus 3% PHS, 1 ng/mL IL-7, and 20 U/mL IL-12 (both from R&D Systems, Minneapolis, MN). CTL cultures were started at 1 × 106 cells/mL at 1:1 responder/DC ratio in AIM V plus 3% PHS plus IL-1β plus IL-2 plus IL-413 and 1 ng/mL IL-7 and 20 U/mL IL-12 with or without 1 μg/mL CMV lysate. Media/ cytokines, except for IL-1 and IL-4, were renewed on days 3 and 5. After day 7, 10 IU/mL IL-2 (R&D Systems) was supplemented besides IL-7 and IL-12, with or without CMV lysate. Media/cytokines were renewed at 2 to 3 per week and freshly prepared DCs with or without CMV lysate were added weekly.
Figure 1. CMV-IC–loaded CB DCs exhibit a potent T-cell stimulatory profile. Surface immunophenotype of day 7 MoDCs about 36 hours after loading with CMV lysate immune complex (CMV-IC) and subsequent maturation in PGE2 plus TNF-α. Representative of 6 independent experiments. Presented data were gated on forward side scatter (FSC/SSC) high cellular events conforming to DCs.

CMV-reactive CTL characterization

Cells (10⁵ cells/100 μL) from weekly restimulated CTL cultures was pulsed 3 days later with [3H]-thymidine.11 Stimulation index (SI) calculates the ratio of mean CMV-stimulated/unstimulated counts.

Immunophenotyping was performed weekly as described.14 For cytokine secretion DC plus responser cocultures were incubated with anti-CD28 and anti-CD49d antibodies (1 μg/mL) with or without 5 μg/mL CMV lysate (Advanced Biotechnologies, Columbia, MD) for 10 to 12 hours. Brefeldin A was present the last 8 to 10 hours.15 Reagents and antibodies were purchased from Becton Dickinson. CTL activity was quantitated by a 5-hour LDH-release assay per the manufacturer’s instructions (Cytotox96; Promega, Southampton, United Kingdom) against autologous immature CD34⁺–derived DC targets with or without CMV lysate.

Statistical analysis

The paired 2-tailed Student t test analyzed parallel culture conditions.

Results and discussion

CMV-IC–loaded CB DCs exhibit a potent T cell-stimulatory phenotype

DCs are uniquely capable to endocytose and process soluble antigens and present major histocompatibility class I–restricted peptides to CD8⁺ T cells in a process coined “cross-presentation.”15 Antigens opsonized with specific antibodies form ICs that can gain entry into DCs via Fcγ receptors16 resulting in superior antigen presentation.15 Immature Mo/DCs were generated from CB and pulsed with CMV lysate and CMV-ICs. Although CMV-IC generation was not verified experimentally, high concentrations of CMV antigens and CMV-hyperimmune globulin should inevitably lead to IC formation.17 Wild-type CMV infection may impair DC phenotype/function18; however, CMV-IC–loaded Mo/DCs expressed potent T cell-stimulatory phenotype (Figure 1).

Cytokine priming of CB T cells and phenotypic characteristics of CMV-specific CTLs

To overcome the type 2 bias of neonatal T cells imparted by reduced tryptophan levels and high progesterone, IL-10, and IL-4 at the maternal-fetal interface,8,19 CB T cells were cytokine-primed before encountering DCs. After about 5 weeks in CTL culture there was greater expansion (2.5 ×; range, 1.7–4.5 ×) in the CMV-pulsed cultures (P = .04, n = 5) than control (0.5 ×; range, 0.2–1.7 ×) despite identical cytokine milieu. CMV-pulsed T-cell proliferation exceeded controls (mean SI, 3.0 ± 3.7, P = .003, 24 time points/5 experiments). SI increased further within each experiment as cell death exceeded proliferation in controls while CMV-loaded cultures kept expanding (not shown).

Beyond 4 weeks CD45RO⁺/RA−/“memory” T cells dominated in reduced tryptophan levels and high progesterone, IL-10, and IL-4 (Table 1). By week 6, CD45RO replaced CD45RA⁺/CD62L⁺/“recent thymic emigrants” (< 10%, P = .003, n = 10 between weeks 4 and 6; not shown). “Memory CD8⁺” cells (CD45RA⁻/CD27⁺/CD8⁺) expanded significantly in CMV-IC–pulsed cultures compared to control (P = .03, n = 9 time points; Table 1). CD57 and CD28 expression remained unchanged, as all T cells regardless of culture conditions retained the CD28⁺/CD57⁺ phenotype. CD27 expression was retained in more than 95% of T cells (not shown).

Table 1. Phenotypic and functional changes in lymphocyte subsets after about 4 weeks of stimulation with or without CMV loading

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>Control culture, mean % ± SD</th>
<th>CMV-pulsed culture, mean % ± SD</th>
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<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
</tr>
<tr>
<td>CD3⁺</td>
<td>77 ± 11</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>CD4⁺/CD3⁺</td>
<td>78 ± 10</td>
<td>72 ± 4</td>
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<tr>
<td>CD8⁺/CD3⁺</td>
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<td>CD45RA⁻/CD8⁺</td>
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<td>71 ± 27</td>
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<td>65 ± 32</td>
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</tr>
<tr>
<td>CD45RA⁺/CD27⁺/CD8⁺</td>
<td>31 ± 30</td>
<td>28 ± 31</td>
</tr>
<tr>
<td>IL-2⁺/CD3⁺ T cells</td>
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</tr>
<tr>
<td>IFN-γ⁺/CD3⁺ T cells</td>
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<tr>
<td>TNF-α⁺/CD8⁺ T cells</td>
<td>ND</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

ND indicates not determined.
CMV-specific cytokine production and cytotoxicity develop in CMV-IC–pulsed cultures

CMV-specific, IFN-γ–producing cells appeared by the fourth week of stimulation and by week 5 they exceeded more than 10% of the total T-cell pool in 5 of 5 consecutive experiments (median, 26%). Similarly, TNF-α and IL-2 secretion was detected in response to CMV (Table 1; Figure 2A). Importantly, IFN-γ and TNF-α, which are critical for effective antiviral immunity, were produced by CD8+ and CD4+ cells. Nevertheless, under these conditions CD4+ responses dominated (TNF-α, P = .001; IFN-γ, P = .065, n = 7) corroborating other reports on viral lyse stimulation.20 CD4+ T cells may contribute to viral control independently.21,22 After at least 4 weeks of culture CMV-specific lysis could be detected (Figure 2B) provided CMV-specific IFN-γ– and TNF-α–secreting T cells were enriched to 10% or more. T cells from the CMV-IC–pulsed cultures killed CMV-pulsed autologous DCs significantly better than unmanipulated DCs (P = .03, n = 4).

Cytokine-priming and continued exposure of CB T cells and DCs to Th1/Tc1-promoting cytokines is probably essential for CTL generation from CB. IL-12 may be the most critical because it can induce 4-1BB expression on naive CB T cells to receive CD28-independent costimulation, vital for acquisition of CTL function besides correcting neonatal DC impairment. The overall phenotype in our CMV-IC–loaded cultures was similar to the IFN-γ–, TNF-α–secreting CD45RA−/CD28+/CD27+ cells described in healthy and HIV+ individuals during the acute phase of protective immunity to CMV.24 Starting with about 1 million T cells present in 2 to 3 mL CB,14 at most 100 to 1000 T cells may possess CMV reactivity.25 Following about 4 weeks in the described CTL culture if CMV-reactive T cells comprise about 25% of 2.5 × 106 cells, then about 600 000 CMV-specific lymphocytes could be generated. Clinical responses were reported after infusing 350 to 400 CMV-specific T cells/kg expanded from seropositive donors.2

In conclusion, this report documents the in vitro generation of virus-specific CTLs from naive human CB lymphocytes. Although currently time and labor intensive, our approach may lead to effective clinical strategies to prevent or treat opportunistic viral infections in the CB transplantation setting.

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