Directly Selected Cytomegalovirus-Reactive Donor T Cells Confer Rapid and Safe Systemic Reconstitution of Virus-Specific Immunity Following Stem Cell Transplantation

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(See the editorial commentary by Peralis, on pages 58–60.)

Background. Adoptive transfer of virus-specific T cells may accelerate reconstitution of antigen-specific immunity and limit the morbidity and mortality of viral infections following allogeneic hematopoietic stem cell transplantation. The logistics of producing virus-specific T cells has, however, limited the application of cellular therapies, particularly following the introduction of more-recent regulatory stipulations.

Methods. We investigated the ability of cytomegalovirus-specific T cells, directly isolated from donor leucapheresates on the basis of interferon \( \gamma \) secretion, to restore antiviral immunity in a group of 25 patients following related-donor transplantation in a single-arm phase I–II study. Selected cells were administered early following transplantation, either after the detection of cytomegalovirus DNA by polymerase chain reaction–based surveillance or prophylactically between day 40 and day 50.

Results. Cell selection was successful in all cases, yielding a product biased towards CD4\(^+\) over CD8\(^+\) T cells. The target cell dose of \( 1 \times 10^8 \) CD3\(^+\) T cells/kg of recipient weight contained a median of 2840 cytomegalovirus-specific CD4\(^+\) cells/kg and 630 cytomegalovirus-specific CD8\(^+\) cells/kg, with a median purity of 43.9% interferon \( \gamma \)–secreting cells. Expansions of both CD4\(^+\) and CD8\(^+\) cytomegalovirus-specific T cells were observed in vivo within days of adoptive transfer. These cells were predominantly terminally differentiated effector-memory cells and showed the same T cell receptor variable \( \beta \) chain (TCRBV) –restriction as the infused cells. They offered protection from reinfection in the majority of patients.

Conclusions. These data indicate that application of cytomegalovirus-specific T cells generated by direct selection using \( \gamma \)-capture is both feasible and effective in a clinical environment. These simple in vitro methodologies should allow more widespread application of virus-specific T cell immunotherapies.

Prophylactic or preemptive strategies employing anti-viral drugs can effectively reduce the incidence of cytomegalovirus (CMV) disease following allogeneic hematopoietic stem cell transplantation (HSCT) [1, 2]. However, currently available antiviral drugs are associated with significant toxicities, and their use results in an increase in late CMV reactivation and disease resultant upon delayed anti-CMV immunological recovery [3]. These considerations led to attempts to restore antiviral immunity by adoptive transfer of CMV-specific T cells [4]. Since the initial proof-of-concept studies, performed in Seattle using donor CMV-specific CD8\(^+\) T cell clones [5, 6], a number of groups have explored strategies allowing more rapid and efficient generation of CMV-reactive T cells [6–13]. Most studies have suggested that reconstitution of CMV-specific
immunity can be hastened with adoptive cellular therapy, but despite these successes, the translation of cellular therapy into the clinic has been limited by the technical difficulties associated with their generation and, more recently, by enhanced regulatory requirements.

We used a method to produce CMV-specific T cells that more readily complies with current regulatory guidance. The process involved a 16–20 h incubation of donor-derived peripheral blood mononuclear cells (PBMCs) with CMV-pp65 protein or 6-h incubation with a pool of peptides derived from pp65, followed by isolation of T cells secreting interferon γ (IFN-γ). We describe the characteristics of these CMV-reactive products and the outcomes following administration to patients after allogeneic HSCT.

PATIENTS AND METHODS

Eligibility

Patients who had undergone allogeneic HSCT from a CMV-seropositive related donor were eligible. The institutional Ethics Committee approved the study protocol, and the procedures followed were in accordance with the ethical standards of the Helsinki Declaration. Patients and donors gave written informed consent. Patients were excluded if they had active graft-versus-host disease (GVHD) of greater than grade I at the planned time of infusion or organ dysfunction as measured by serum creatinine level >200 μM/L, bilirubin level >50 μM/L, or liver transaminase levels >3 times the upper limit of the reference interval.

Study Design

The trial was a single-arm open-label phase I–II design. Primary end points were infusional toxicities and the incidence of GvHD. Acute GVHD was assessed according to the Glucksberg criteria, and chronic GvHD was graded as limited or extensive. Antiviral efficacy, assessed by avoidance of conventional antiviral drug usage during the primary reactivation episode and the incidence of subsequent viral reactivation episodes, was the major secondary end point.

Selection of IFN-γ–Secreting Cells

Donors underwent a nonmobilized leukapheresis lasting ~2 h for the isolation of PBMCs 15 days after collection of mobilized peripheral blood. PBMCs were washed and resuspended in Roswell Park Memorial Institute 1640 medium (Invitrogen) supplemented with 10% normal human A/B serum (NBS; Colindale) at a density of 10^6 cells/mL: 10^9 PBMCs were incubated overnight with recombinant pp65 (Miltenyi-Biotec) or for 6 h with 600 ng of a pool of overlapping peptides from CMVpp65 (CMV Peptivator; Miltenyi-Biotec). Cells were then labeled with IFN-γ capture reagent (Miltenyi-Biotec) and warmed to 37°C for 45 min to re-initiate IFN secretion, followed by incubation with anti-IFN-γ microbeads (Miltenyi-Biotec) to allow immunomagnetic selection of IFN-secreting cells (CliniMACS; Miltenyi-Biotec). Cells were cryopreserved in 2 dose aliquots: 1 × 10^4 and 3 × 10^4 CD3^+ T cells/kg of recipient weight in 50-mL cryogenic bags (Baxter Healthcare). The entire process time from receipt of apheresis to cryopreservation of product was completed in <24 h. Upon receipt of microbiological sterility tests (BacTec; Becton Dickinson UK), the aliquots were released as “fit for infusion” at ~21 days after HSCT.

Patient Monitoring

Routine screening for CMV infection was performed on whole blood by quantitative polymerase chain reaction (PCR) for CMV DNA once per week. Lymphocyte subsets (CD3^+CD4^+, CD3^+CD8^+ and CD3/CD56^−) and absolute numbers of CMV-reactive CD4^+ and CD8^+ T cells were analyzed once per week for the first month, then once per month until 6 months after infusion. CMV-reactive T cells were identified in vitro by flow cytometric detection of intracellular IFN-γ synthesis in CD4^+ and CD8^+ T cells after a 4-h incubation with recombinant pp65, as previously described [12]. Briefly, after co-incubation of PBMCs with CMV pp65, cells were surface-labelled with CD45RA FITC, CD4 Pe-Cy5, CD3 APC-H7 (all supplied by BD/Pharmingen), and CCR-7 Alexa750 (Invitrogen). Cells were then fixed and permeabilized (Intrastain; Dako-Cytomation) and labelled with anti-IFN-γ PE. After washing, the cells were re-suspended in PBS and analyzed immediately by flow cytometry (FACS Aria running Diva 6.0; BDIS). Patients expressing either one or both of HLA-A*0201 or HLA-B*0702 were additionally monitored using CMV pp65-specific class I human leukocyte antigen–pentamers (Proimmune).

In some cases, sufficient cells were available for the determination of the degree of oligoclonality within the CMV-reactive T cell population. PBMCs stimulated with CMV pp65, as described above, were labeled with T cell receptor variable beta chain (TCRBV)-specific antibodies (IOTest Beta Mark; Beckman/Coulter) and CD3 Pe-Cy5. Fixed/permeabilized cells were labeled with anti-IFN-γ APC, washed, and analyzed immediately by flow cytometry, as described above. The TCRBV usage within the CMV-reactive and CMV-nonreactive subsets was determined and compared with that of an aliquot of the infused donor product.

Antiviral Drugs

Management of CMV infection after cellular therapy was guided by quantitative PCR. Conventional antiviral drugs were administered according to institutional guidelines (ganciclovir at 5 mg/kg administered intravenously twice daily or foscarnet, if low blood counts precluded this, ie, neutrophils < 1 × 10^9/L or platelets < 50 × 10^9/L) if there was a doubling of the CMV load.
7 days after initial detection of viral DNA and the viral load was >3000 copies/mL or if the absolute viral load was >5000 copies/mL.

**RESULTS**

**Cellular Therapy Products**

Adequate numbers of CMV-specific T cells were isolated from all apheresates to allow production of both planned doses. All doses were free from aerobic and anaerobic contamination. The purity of IFN-γ secreting cells and the yields varied considerably between products. The median purity was 43.9% (range, 1.4%–81.8%), and the median yield was 41.7% (range, 0.6%–373.0%). There was no correlation between the frequency of CMV-reactive T cells in the starting population and the yield or purity of the final product (data not shown).

The majority of products were selected following overnight stimulation with recombinant CMV pp65 protein, but 3 products were produced using the pooled peptides, which became available to current good manufacturing practice (cGMP)–grade only later during the study. After stimulation with peptide, antigen presentation is not as dependent on endogenous uptake and processing as it is after stimulation with protein, and it likely occurs, at least in part, via direct loading onto externalized major histocompatability complex molecules. Optimal incubation times are therefore shorter. However, because of the logistics of cell harvesting and transport to the processing laboratory, it was not possible to stimulate and select on the same day as collection. Peptide stimulation was therefore performed after overnight storage of the apheresate, whereas protein-stimulated products were prepared immediately after collection and stimulated overnight. The time from collection to completion of separation was therefore comparable for both stimulants. Both protein and peptide stimulation led to the activation of both CD4+ and CD8+ CMV-specific T cells, with predominance of CD4+ T cells in 81% of products. Product purity was significantly improved in the peptide-stimulated products (Figure 1), and yield was improved through better viability. The improvement in purity was most marked for CD8+ T cells (P = .004) for which cross-presentation of antigens from pp65 protein is relatively inefficient, and failed to reach statistical significance for CD4+ T cells (P = .07), for which uptake, processing, and presentation of endogenous proteins via the class II pathway is more efficient. There was no correlation between the number of CD4+ and CD8+ T cells generated (data not shown).

**Patient Characteristics**

Twenty-five patient/donor pairs gave consent, and 18 patients received CMV-specific T cells. Three patients were ineligible to receive cells because of GvHD, 2 died before CMV infection, 1 experienced engraftment failure, and 1 had a donor who was unsuitable. Conditioning regimens included fludarabine: melphalan:alemtuzumab (n = 12), cyclophosphamide:anti-thymocyte globulin (n = 1), cyclophosphamide:total body irradiation (n = 4), and busulphan:cyclophosphamide (n = 1). Seventeen of the enrolled patients received the first dose of 1 × 10^6 CD3+ T cells/kg according to protocol. One patient did not receive cells prior to day 50 and had them returned preemptively on day 63. None of the patients required infusion of the second dose of CMV-specific T cells. The median infused dose of CMV-specific CD4+ cells was 2840 cells/kg (range, 280–6880 cells/kg), and the median dose of CMV-specific CD8+ cells was 630 cells/kg (range, 60–3990 cells/kg). No patients had clinical GvHD or CMV disease at time of infusion. One patient had previous grade I cutaneous GvHD, which was quiescent at the time of infusion. All patients were receiving cyclosporine at therapeutic levels at the time of infusion. In 11 cases, the cells were administered preemptively after detection of CMV DNA, and in 7 patients, they were administered prophylactically (Table 1). Preemptive infusions were given at a median of 28 days (range, 19–63 days) after transplantation, and prophylactic infusions were given at a median of 42 days (range, 41–48 days) after transplantation. Ten (91%) of the patients treated preemptively and 3 (43%) of those treated prophylactically were seropositive for CMV before receiving a transplant (P = .047).

**Toxicites**

No infusional toxicities occurred. Five patients developed grade I acute GvHD that responded to topical steroids. Two patients developed grade II and 1 patient developed grade III acute GvHD.
that required treatment with intravenous and oral steroids (Table 1). Two of these cases occurred in the group of 3 patients who received T cell–replete transplants, and only 1 case occurred in the group of 15 patients who received T cell–depleted grafts. Three patients experienced extensive chronic GvHD, although again 2 patients were in the group that received T cell–replete transplants. There was no correlation between GvHD and purity of the cellular therapy product (median purity in those with grade II–IV or extensive chronic GvHD, 56.5% [range, 2.7%–81.8%], compared with 35.3% [range, 1.4%–81.8%] in those without; \(P = .139\), by 2-tailed Mann-Whitney U test).

**Antiviral Responses**

None of the 7 patients who were treated prophylactically required antiviral drug therapy within the next 6 months, despite 3 episodes of CMV infection in 1 patient (Table 1). One patient who had remained free from infection up to 6 months subsequently required systemic steroids for extensive chronic GvHD and experienced 3 infection episodes that required therapy. Of the 11 patients who were treated preemptively, 2 required no antiviral drug treatment, 2 received foscarnet (for 14 and 15 days), 6 received ganciclovir (median duration of therapy, 14 days; range, 11–27 days), and 1 received ganciclovir followed by foscarnet (for a total of 33 days). Two of these patients required treatment for subsequent CMV infection, 1 after initiation of systemic steroids for grade II acute GvHD.

**Antiviral Immune Reconstitution**

In vivo expansion of CMV-reactive T cells was observed in all 11 patients who received cells preemptively (Figure 2). CMV-reactive CD4\(^+\) cells were undetectable in 6 of 11 patients prior to infusion and present at very low levels in 1 of the others (0.3 cells/\(\mu\)L). Two patients had substantial populations of CMV-reactive CD4\(^+\) T cells (>20 cells/\(\mu\)L) at the time of infusion. CMV-reactive CD4\(^+\) cells were present in 2 of 6 patients with previously undetectable levels within 1 week of infusion and in 5 of 6 patients within 2 weeks of infusion. CMV-reactive

**Figure 2.** Incidence of cytomegalovirus (CMV)–specific CD4\(^+\) and CD8\(^+\) T cells reconstitution. The proportion of patients with detectable peripheral blood CMV-specific CD4\(^+\) (open squares) and CD8\(^+\) (black triangles) is shown for each analysis time point.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Recipient</th>
<th>CMV serostatus</th>
<th>T cell depletion</th>
<th>Day of infusion (after transplantation)</th>
<th>Acute GvHD &gt;Gd I</th>
<th>Chronic GvHD</th>
<th>First treatment episode, days after transplantation:drug</th>
<th>Second treatment episode, days after transplantation:drug</th>
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<td>Day 97–126: GCV</td>
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<td>41</td>
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**NOTE.** CMV, cytomegalovirus; FOS, foscarnet; GCV, ganciclovir; Gd, grade; NEG, negative; POS, positive; ValGCV, valganciclovir.
CD8+ T cells were undetectable in 8 of 11 patients prior to infusion, but they were detected in 5 of these within 1 week and in 7 of these within 2 weeks. The other patient had received the lowest dose of CMV-specific T cells (700 CD4+ cells/kg and 60 CD8+ cells/kg) and was the slowest to reconstitute both CD4+ and CD8+ T cell immunity. In this patient, CMV-specific CD4+ T cells were detectable at 3 weeks after infusion and CD8+ T cells were detectable at 8 weeks, by which time, the patient had an additional CMV infection that required another 28 days of antiviral therapy.

In patients treated prophylactically, CD4+ CMV-specific T cells were undetectable in 3 of 7 patients and CD8+ CMV-specific T cells were undetectable in 5 of 7 patients prior to infusion. In those patients with detectable levels prior to infusion, levels were 1.4–3.9 cells/μL and 2.2–21.5 cells/μL for CD4+ and CD8+ cell populations, respectively. Virus-specific CD4+ and CD8+ T cells were detectable in all but 1 patient by 1 week and in all patients by 2 weeks after infusion.

Of interest, both CD4+ and CD8+ CMV-specific T cells were detectable before infusion (2.8–20.4 cells/L and 2.6–21.5 cells/L, respectively) in all 3 patients who underwent T cell–replete transplantation (2 of whom were treated preemptively, and 1 of whom was treated prophylactically), whereas CD4+ cells were undetectable in 9 of 15 patients and CD8+ cells were undetectable in 13 of 15 patients who underwent T cell depleted transplantation.

The peak levels of CMV-reactive T cells achieved during follow-up were higher in those patients who experienced CMV infection, particularly in the CD8+ compartment (Figure 3A and 3B). In these patients, the level of CD8+ CMV-reactive T cells increased above levels previously suggested to be protective against CMV infection (10 × 10^6 cells/L for NLV-specific T
cells) [14, 15] in all but 1 patient. There was no correlation between the dose of infused CMV-specific T cells and peak CMV-specific T cell number for either CD4⁺ or CD8⁺ T cells, even when analyses were performed according to whether CMV viremia did (Spearman’s rho, .4406 [P = .152] and .0280 [P = .931] for CD4⁺ and CD8⁺ cells, respectively) or did not (Spearman’s rho, .3714 [P = .497] and .6000 [P = .242]) occur. Two of the patients who experienced subsequent episodes of CMV infection had peak levels of both CD4⁺ and CD8⁺ CMV-reactive T cells that were not significantly lower than those in Figure 5. Cytomegalovirus (CMV)–specific T cells differentiate from central memory to effector memory phenotype in vivo. Flow cytometric dotplot A shows the CD45RA versus CCR7 phenotype of CMV-reactive T cells in a donor product after gating on CD3 expression and binding of a human leukocyte antigen–A2 restricted CMV pentamer reagent. Plots B, C, D, and E show the expansion and contraction in CMV-specific CD8⁺ T cells at 1, 3, 4, and 6 weeks after receipt of transplant, respectively, in line with the peripheral viremia in the vertical line graph on the left-hand side. Plots F, G, H, and I show the CD45RA/CCR7 phenotypes of the CMV-reactive T cells in the peripheral blood at the same time points.
patients who did not experience reinfection (Figure 3A and 3B), but both patients subsequently received lympholytic doses of steroids.

The virus-specific immune reconstitution of the 2 patients who were treated preemptively and required no antiviral drug treatment are summarized in Figure 4. Both had relatively low-level viremia that was rapidly cleared in association with expansion of CMV-specific populations.

The predominant CMV-reactive T cells in the infused product were effector-memory cells (CD45RA^2CCR7^2; T_{EM}) with some central-memory cells (CD45RA^2CCR7^1; T_{CM}) (Figure 5A). Rapid expansion of CMV-specific CD8^+ T cells in vivo (Figure 5B–5E) was associated with an increase in CD45RA^+CCR7^+ T_{CM} (Figure 5F), followed by expansion of both T_{CM} and T_{EM} (Figure 5G–5I) coincident with reduction in the level of viremia. Within 4 weeks of cell therapy (Figure 5D and 5H), the predominant anti-CMV T cells were T_{EM} and terminally differentiated CD45RA^+CCR7^- T_{EF} and the viremia had resolved. At this time, a population of CD45RA^+CCR7^- naive CMV-reactive T cells appeared that were likely to have derived from the hematopoietic progenitor cells in the initial donor graft. At week 6 after infusion, the

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**Figure 6.** TCRBV usage within the cytomegalovirus (CMV)–reactive T cell pool. Histogram A shows the proportion of T cells with each of the TCRBV restrictions detected by flow cytometry (IOTest Beta Mark; Beckman Coulter) in the non-CMV–reactive fraction of an individual donor apheresate. In contrast, the CMV-reactive T cell in the same donor product show considerable skewing of the BV repertoire (B), with only 13 of the 25 BV chains represented. CMV-specific T cells recovered from the peripheral blood of the patient after in vivo expansion 2 months after receiving the CMV-specific product shown in histogram B showed the same TCRBV restriction as the CMV-reactive T cells in the original product (C).
proportion of CMV-specific CD8⁺ T cells in the peripheral blood had reduced, and these cells showed a CD45RACCR7 profile typical of CMV-seropositive individuals (ie, largely T(EM)).

These data do not prove that the CMV-reactive T cells expanding in vivo derived from the therapeutic product. Stronger evidence in support of the role of the infused cells was provided by assessing the TCRBV restriction of the CMV-reactive cells in the infused product and those in vivo. Figure 6A shows the polyclonality of TCRBV usage in the CMV nonreactive cells from the product. In contrast, the CMV-reactive cells from the same product show restricted oligoclonal TCRBV usage (Figure 6B), which was shared by the CMV-reactive T cells recovered from the matched recipient after infusion (Figure 6C).

**DISCUSSION**

Widespread application of adoptive cellular therapy for CMV infection following allogeneic HSCT has been limited by the logistics and costs of the original culture techniques. We demonstrate that a simple, fast, and relatively inexpensive technique can be used to generate clinically relevant numbers of antiviral T cells for adoptive immunotherapy in a setting compatible with cGMP. Infused products had an excellent toxicity profile, with no apparent increase in incidence or severity of acute or of chronic GvHD above that seen in comparable untreated patients [16].

The immunodominance of the peptides derived from pp65 with respect to T cell immunity is well established, although many other CMV proteins, such as the immediate-early (IE) antigen, are immunogenic, and some individuals have modest or undetectable pp65-directed responses [17–20]. Our isolation method was restricted to pp65-responding T cells, but we were, nevertheless, able to generate an adequate CMV-reactive T cell dose from all donors. Furthermore, all but 1 patient demonstrated expansion of CD4⁺ or CD8⁺ CMV-specific T cells within 2 weeks of infusion. It remains possible that stimulation of the donor apheresate with peptides from the IE protein would have generated a stronger response in this case, although it remains uncertain whether the use of IE peptides for isolation would generate a quantitatively better product for adoptive immunotherapy [17, 19, 21].

Although the majority of patients treated preemptively required 1 treatment episode with antiviral drugs, this likely reflects the tempo of expansion of transferred cells in vivo, as reflected by the peak expansion lagging behind clearance of viremia (Figure 4). The presence of viral antigens appears important in driving T cell expansion and determining the magnitude of such responses (Figure 3). These findings are entirely consistent with our previous experience using T cell lines, where the lack of impact on the primary infection episode in terms of requirement for antiviral drugs did not appear to reflect a lack of development of durable functional antiviral immunity thereafter [11, 12]. The safety of these infusions was comparable to that of CMV-specific T cell lines and of directly isolated CMV-specific CD8⁺ T cells [7]. Furthermore, CMV-reactive T cell expansions were achieved safely in patients treated prophylactically, only 1 of whom required subsequent antiviral drug treatment, albeit with the recognition that the majority of these patients were CMV seronegative before transplantation, had not shown evidence of infection by 40 days after transplantation, and were therefore at lower risk of CMV infection.

In summary, the γ-capture technique is a convenient and technically simple procedure for the isolation of CMV-specific CD4⁺ and CD8⁺ T cells to cGMP standards from a short second apheresis of the patient’s original donor. This is relatively simple for recipients of sibling donor grafts but is more difficult in the case of volunteer nonrelated donors, where selection from an aliquot of the progenitor cell harvest may provide a viable alternative strategy. Our study suggests that products can be generated from the majority of CMV-seropositive donors and safely applied in the related donor setting, resulting in rapid CMV-directed immune reconstitution, particularly in the presence of active viral replication. Questions remain as to whether those receiving T cell replete grafts are likely to benefit and whether CMV-seronegative patients require prophylactic intervention. A phase III randomized study has been initiated in the United Kingdom to more formally address the issue of efficacy and to expand upon the toxicity data (NCT01077908), focusing on CMV-seropositive patients who receive T cell–depleted grafts.

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**References**