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Antiviral Responses following L-Leucyl-L-Leucine Methyl Esther (LLME)-Treated Lymphocyte Infusions: Graft-versus-Infection without Graft-versus-Host Disease

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Although allogeneic hematopoietic progenitor cell transplant (HPCT) is curative therapy for many disorders, it is associated with significant morbidity and mortality, which can be related to graft-versus-host disease (GVHD) and the immunosuppressive measures required for its prevention and/or treatment. Whether the immunosuppression is pharmacologic or secondary to graft manipulation, the graft recipient is left at increased risk of the threatening opportunistic infection. Refractory viral diseases in the immunocompromised host have been treated by infusion of virus-specific lymphotyces and by unmanipulated donor lymphocyte infusion (DLI) therapy. L-leucyl-L-leucine methyl ester (LLME) is a compound that induces programmed cell death of natural killer (NK) cells, monocytes, granulocytes, most $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells. We have undertaken a study of the use of LLME-treated DLI following T cell-depleted allogeneic HPCT, specifically to aid with immune reconstitution. In this ongoing clinical trial, we have demonstrated the rapid emergence of virus-specific responses following LLME DLI with minimal associated GVHD. This paper examines the pace of immune recovery and the rapid development of antiviral responses in 6 patients who developed viral infections during the time period immediately preceding or coincident with the administration of the LLME DLI.

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KEY WORDS: Hematopoietic progenitor cell transplant, Donor lymphocyte infusion, Immune reconstitution opportunistic infection

INTRODUCTION

Although allogeneic hematopoietic progenitor cell transplant (HPCT) is curative therapy for many disorders of lymphohematopoiesis, it is associated with significant morbidity and mortality [1-5]. Much of this risk can be traced to graft-versus-host disease (GVHD) and the immunosuppressive measures required for its prevention and/or treatment [6,7]. T cell depletion (TCD) is an effective means of reducing or eliminating GVHD, but if too rigorous, can also result in delayed immune reconstitution. Whether the immune suppression is pharmacologic or secondary to graft manipulation, the graft recipient is left quantitatively or functionally lymphopenic, and at increased risk of life-threatening/fatal opportunistic infection. T lymphocytes, and in particular, $CD4^+$ T lymphocytes are the most affected, and their absence leaves the patient at particular risk for viral infection.

Refractory viral diseases in the immunocompromised host have been treated by infusion of virus-specific lymphocytes. Infusion of ex vivo activated and/or expanded antivirus T cells has been used for the treatment of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) [8,9]. Donor lymphocyte infusion (DLI) therapy has been used after transplant for a variety of infections including persistent adenovirus [10], CMV [11], EBV-lymphoproliferative disease (LPD) [12,13], and hepatitis [14,15]. Both virus-specific and unmanipulated donor lymphocytes have been documented to help restore T cell numbers [16,17], and

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to successfully treat infection or relapse [17-19]. It has been shown that recipients of allogeneic HPCT who are deficient in $CD8^+$ cytotoxic T lymphocytes specific for CMV are specifically at risk for active infection with CMV. In 1 study, patients who had received CMV-specific clones of $CD8^+$ T cells derived from donor bone marrow (BM) had improved reconstitution of their cellular immunity against CMV [20]. Although the infusions of the donor-derived CMV-specific clones were well tolerated and all patients had reconstituted CMV-specific cytotoxic T lymphocytes by day 49 following HPCT, the virus-specific $CD8^+$ CTL response persisted only in those patients in whom $CD4^+$ helper responses recovered. Virus-specific CTLs have been found to be effective therapy for prevention of other posttransplant complications including EBV-LPD [21,22]. Unfortunately, the infrastructure and resources required to make this type of approach broadly applicable in the clinical setting are not widely available, and the use of unmanipulated DLI therapy is often complicated by the development of GVHD. Alternative approaches are needed.

L-leucyl-L-leucine methyl ester (LLME) is a compound taken up by cells through saturable facilitated transport [23,24], and once intracellular, accumulates in lysosomes and endosomes where dipeptidyl peptidase I (DPP_I) converts LLME to pro-apoptotic metabolites. Because DPP_I is expressed primarily by cytotoxic granule-containing leukocytes, LLME induces programmed cell death of natural killer (NK) cells, monocytes, granulocytes, the majority of $CD8^+$ T cells, and a small fraction of $CD4^+$ T cells [24]. LLME has been effective in preventing GVHD in mice and humans, but its human application had been limited by toxicity to hematopoietic stem cells when unseparated BM was treated prior to infusion [25-27]. In a phase I trial, HLA-matched or mismatched BM grafts were incubated ex vivo with LLME prior to infusion [26]. Although LLME was effective in preventing GVHD, toxicity to BM colony forming units (CFUs) was noted, and 1 patient died from secondary graft failure. The study was therefore terminated. Aside from the adverse effects of LLME on hematopoietic stem cells (HSCs) directly exposed to the agent, other infusional or delayed toxicities were not observed. Use of DLI following T cell-depleted transplantation circumvents the problem of HSC exposure to LLME. Additionally, this facilitates administration of precisely defined doses of LLME-treated T cells, which may allow for further reduction in GVHD risk, as well as accelerated $CD4^+$ T cell reconstitution.

We have undertaken a study of the use of LLME-treated DLI following T cell-depleted allogeneic HPCT specifically to aid with immune reconstitution. In this clinical trial, we have demonstrated the rapid emergence of virus-specific responses following LLME DLI with minimal associated GVHD. This paper presents data detailing the pace of immune recovery and the rapid development of antiviral responses in the 6 patients from the larger cohort who developed viral infections during the time period immediately preceding or coincident with the administration of the LLME DLI.

MATERIALS AND METHODS

Eligibility

From February 2002 through July 2007, all patients at Thomas Jefferson University Hospital (TJUH) who had received a T cell-depleted (CD34-enriched) HPCT were offered participation in a phase I study of LLME-treated DLI to improve immune reconstitution. The study was subsequently opened to patients at the Medical College of Wisconsin. Patients were eligible if they had an HLA-matched sibling donor, HLA-matched unrelated donor, or an HLA partially matched (single-antigen mismatch, 2-antigen mismatch, or haplodisparate) related donor. Patients were required to have undergone HPCT at least 28 days earlier and have a nondetectable antithymocyte globulin (ATG) level (<2 μg/mL of serum for rabbit ATG) within the prior 21 days. To be enrolled, patients must have demonstrated neutrophil engraftment as defined by an absolute neutrophil count (ANC) greater than 500 cells/μL for 3 consecutive days, but have persistent CD4 lymphopenia (absolute $CD4^+$ T cell count (ALC) <100 cells/μL). If a $CD4^+$ T cell count was not obtainable, an absolute lymphocyte count <100 cells/μL was considered prime facie evidence of $CD4^+$ T cells <100 cells/μL. Patients were excluded if there was evidence of overt relapse of the underlying malignancy or persistent disease or active GVHD. The presence of opportunistic infection was not a contraindication to enrollment in this study. The study was approved by the institutional review boards at Thomas Jefferson University and the Medical College of Wisconsin and informed consent was obtained for all participating patients.

Conditioning Regimen and Supportive Care

All 6 patients reviewed in this paper received a CD34-enriched HPCT following a preparative regimen of fludarabine (Flu; 30 mg/m^2, days −6 through −2), cytarabine (2 g/m^2, days −6 though −2), and melphalan (Mel; 200 mg/m^2, day −1). All patients received rabbit ATG (Thymoglobulin®, Sang-Stat) during the peritransplant period. Prophylactic antimicrobials consisted of an amphotericin product, valacyclovir, and trimethoprim-sulfamethoxazole (TMP-SMX). All patients received transfusion support with leukodepleted and irradiated cellular blood

products. Patients who were CMV-seronegative received CMV-seronegative blood products.

ATG Levels

Patients had blood drawn for ATG levels prior to ATG administration, on the day of transplant, on day 7 following HPCT and generally weekly thereafter as described previously [28]. At the beginning off this trial, patients became eligible to receive LLME DLI when the ATG level was <1.0 μg/mL. Subsequently, based on further information about the pharmacokinetics and mechanisms of action of ATG, the protocol was revised to allow for inclusion when the ATG level became <2.0 μg/mL.

LLME Treatment

LLME was synthesized under GMP conditions by Bachem (Switzerland). Immediately prior to use the dry LLME powder was prepared in Normosol R at a 500 μM concentration. Washed cells (blood or apheresis product) were incubated with LLME at a concentration of 10×10^6 cells/mL for 60 minutes. Treated cells were washed once at 4°C in Normosol, and then twice at room temperature. Only products with ≥80% depletion of NK cells (CD $16/56^+$, $CD3^-$) determined by flow cytometry were released for infusion. The treatment dose was based on the number of viable $CD3^+$ cells.

Immunophenotyping

Intracellular staining for perforin and surface staining for lymphocyte subsets were performed, using standard staining techniques as previously described [29,30]. Flow cytometric analysis was performed using the FACScan (Becton Dickinson, Fullerton, CA).

Antiviral Testing

Ex vivo ELISPOT assay

Peripheral blood monouclear cells (PBMC) were separated from heparinized whole blood or buffy coats using Ficoll-Hypaque density gradient centrifugation, and cells were used either fresh or thawed from cryopreserved aliquots. The ex vivo ELISPOT assay for detection of interferon (IFN)-γ was based on the protocol previously described [31]. Ninety-six-well polyvinylidene difluoride backed plates (Millipore, Bedford, MA) were coated overnight at 4°C with 15 μg/mL of anti-IFN-γ monoclonal antibody (MAb) 1-D1 K (Mabtech, Stockholm, Sweden). Wells were washed and blocked with RPMI supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, glutamine, and 10 mM HEPES (R10) for 1 hour at 37°C. Next, each antigen was incubated with 250,000 PBMCs in 100 μL R10 in triplicate wells for 6 to 48 hours at 37°C in 5% CO_2. Wells were washed extensively with phosphate-buffered saline (PBS), 0.05% Tween 20, 1 μg/mL of biotinylated anti-IFN-γ MAb 7-B6-1 (Mabtech) was added, and plates incubated at room temperature for 3 hours. Next, wells were washed and incubated with a 1:1000 dilution of alkaline phosphatase conjugate (Mabtech) for 2 hours. Wells were washed again and incubated with alkaline phosphatase substrate (Vector Labs, Burlingame, CA) for 30 minutes; the reaction was stopped with tap water. After wells were air dried, large spots with fuzzy borders were counted under a dissecting microscope. The number of spot-forming cells (SFCs) per 10^6 PBMC was calculated as the mean number of SFCs in triplicate microwells ×4. Antigen-specific precursor frequencies were calculated as the mean number of SFCs in the antigen-stimulated microwells minus the mean number of SFCs in the control microwells.

Viral Titers

Whole blood samples were taken on all patients weekly to screen for the reemergence of CMV using quantitative real-time DNA PCR as previously described [32-35]. Assays were performed by the clinical laboratory at TJUH or at Viracor (Lee's Summit, MO). When clinically indicated, whole blood samples were also analyzed for adenovirus, EBV, and BK virus also using quantitative real-time DNA PCR [36-39]. In patients who developed reactivation of any virus as demonstrated by a rising PCR titer, samples were followed weekly until resolution, as evidenced by at least 2 consecutive undetectable titers.

CDR3-Size Spectratype Analysis/Spectratype Complexity Index

As previously described [40], peripheral blood lymphocytes (PBL) were enriched from donor and patient PB samples by centrifugation over Ficoll-Histopaque. $CD4^+$ and $CD8^+$ subsets were separated by standard antibody-panning techniques and the enriched subset populations were solubilized in Ultraspec (Biotex Laboratories, Houston, TX). Vβ spectratype analysis of separated PBL from untreated donor samples served as the reference point for full repertoire complexity, and was used as the standard of comparison for the LLME-treated, as well as posttransplant samples. At the time of the posttransplant analysis, all patients exhibited >90% donor chimerism, as determined by molecular analysis of short tandem repeats [41].

Total RNA was isolated from Ultraspec samples and cDNA was prepared, as previously described [40]. Seminested polymerase chain reaction (PCR) was performed using a panel of human Vβ sense oligoprimers and 2 Cβ antisense oligoprimers, the second Cβ being fluorescently labeled. The PCR products were run on a sequencing gel and analyzed by the

Genotyper Genescan software program (Applied Biosystems, Foster City, CA). The complexity index within a Vβ family was determined as a percentage of the number of peaks found in its spectratype histogram in relation to the number of peaks in the corresponding donor Vβ family histogram. Any Vβ family with a complexity index of ≥85% was considered to be fully complex. Histogram peaks were identified by the Genotyper Genescan program. Any donor or patient sample exhibiting fewer than 12 evaluable Vβ family spectratypes was excluded.

Statistical Methods

Data from the ELISPOT assays were analyzed using a paired *t*-test to compare the mean number of spots in the quadruplicate control and experimental (antigen-stimulated) microwells. The results were compared between responders using the Mann-Whitney *U*-test. Data comparisons for Vβ repertoire analysis were analyzed by the nonparametric Wilcoxon rank-sum analysis. Value of $P \leq .05$ were considered statistically significant.

RESULTS

Patients

Twenty-three patients with hematologic malignancies were enrolled in a study of LLME-treated DLI to accelerate immune reconstitution after receiving CD34-enriched HPCT. Six of these 23 patients represented a unique subset in that they each developed 1 or more viral infections during the time period immediately before or concurrent with the administration of the LLME DLI. These patients demonstrated a rapid and consistent pattern of response to their viral infections, which is the subject of this report.

Five of these 6 patients received HPCT from HLA identical sibling donors, whereas 1 received HPCT from an unrelated donor. One patient (patient #1) was treated with steroids for adrenal insufficiency and idiopathic pneumonia syndrome (IPS) and expired 35 days following LLME DLI (134 days s/p HPCT). A second patient (patient #3) also developed IPS and was treated with steroids. This patient expired 100 days following LLME DLI (148 days s/p HPCT). Although both of these patients have been included in this analysis of antiviral responses, neither is considered evaluable for immune reconstitution because of the administration of corticosteroids for the treatment of IPS.

LLME Treatment and Lymphocyte Subset Depletion

Following LLME treatment of the DLI product as described earlier, patients received a targeted number

Table 1. Patient Characteristics

	Age/ Sex	Disease	Graft Type	LLME DLI		Infection				Time to CD4 > 100 (days)	GVHD
				CD3+ T Cell Dose/kg	Days s/p HPCT	Virus	Days s/p HPCT	Resolved?	Other Therapy for OI		
1	65/M	ALL—1st CR	Sib	1×10^6	99	EBV-PTLD	113	Partial*	Rituximab	NA	No
2	23/M	CML—2nd CP	Sib	1×10^6	102	Adeno, CMV	91, 105	Yes	Ganciclovir, Cidofovir, IVIg	21	No
3	53/M	CML—2nd CP	Sib	1×10^7	64	CMV	28	Yes	Foscarnet + IVIg	57	Grade 3†
4	61/M	ALL—Ph+-1st CR	Sib	1×10^7	48	CMV	26	Yes	Ganciclovir/ valganciclovir, IVIg	34	No
5	28/M	AML—1st relapse	Sib	1×10^7	72	EBV-PTLD	75	Yes	Rituximab	10	No
6	45/M	AML—2nd relapse	URD	1×10^6	69	CMV	24	Yes	Valganciclovir	NA	No

HPCT indicates hematopoietic progenitor cell transplant; GVHD, graft-versus-host disease; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; AML, acute myelogenous leukemia; CR, complete remission; CP, chronic phase; Ph+, Philadelphia chromosone; Sib, HLA identical sibling donor; IRD, unrelated donor; EBV-PTLD, Epstein Barr virus related posttransplant lymphoproliferative disease; Adeno, adenovirus; CMV, cytomegalovirus; OI, opportunistic infection; IVIg, intravenous immuno globulin.

Six patients developed viral infections concurrent with or immediately preceding LLME DLI. All had resolution or partial resolution of the viral disease. Four of six developed rapid recovery of $CD3^+/CD4-$ cells. Two patients, who expired at days 35 and 46 s/p LLME DLI of other causes did not.

*Patient expired from pulmonary and renal complications prior to complete resolution of EBV-PTLD.

†This patient had no GVHD following his first dose of LLME DLI (1×10^7/kg), but did not achieve a CD4 count >200/μL. He was, therefore, offered a second dose of DLI (actual dose 4.1×10^7/kg) and received it 43 days after the first dose. He subsequently developed grade III GVHD within a few days of the second DLI administration. The trial was subsequently changed to allow for further DLI only if the CD4 count was < 100/μL.

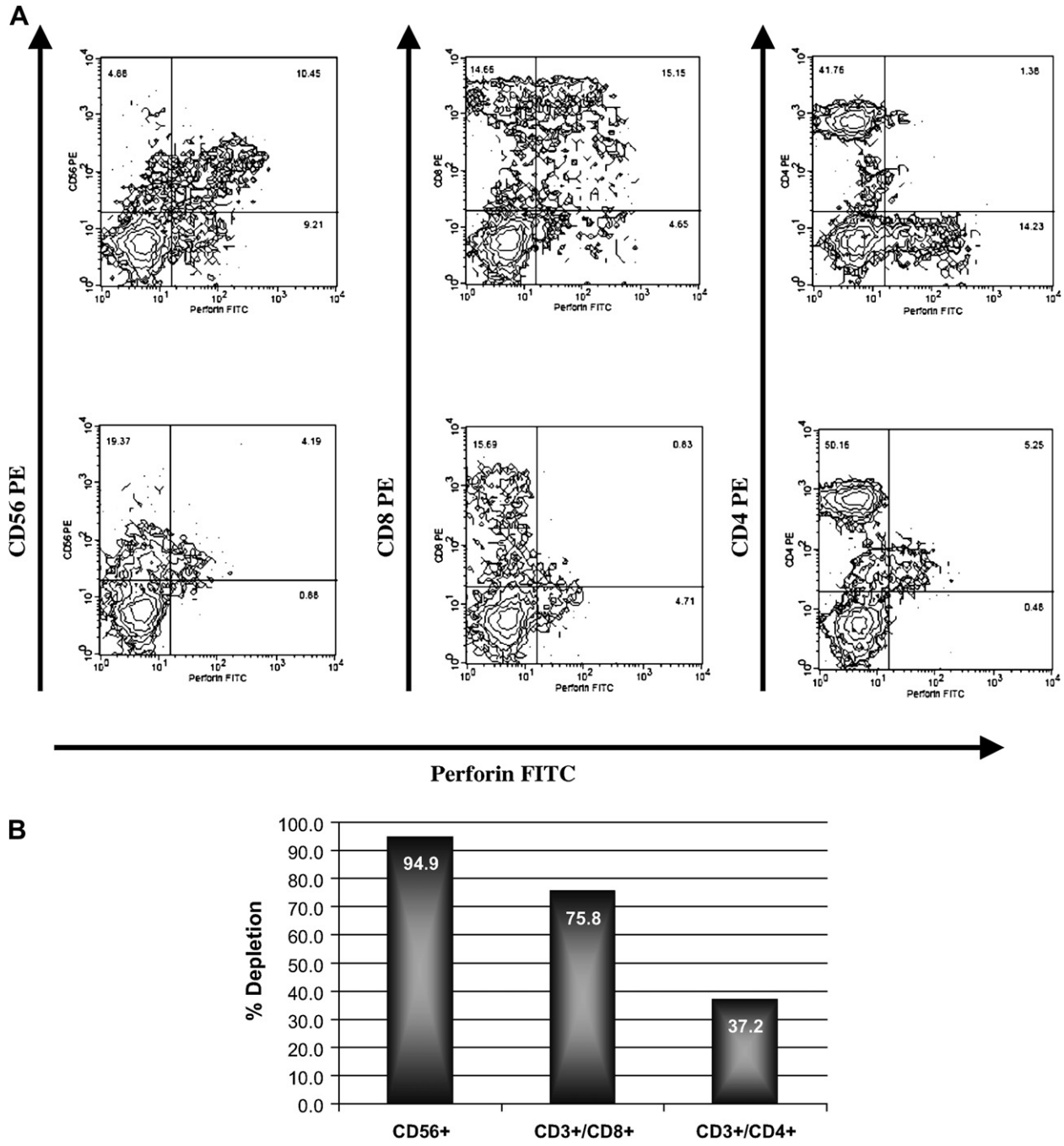
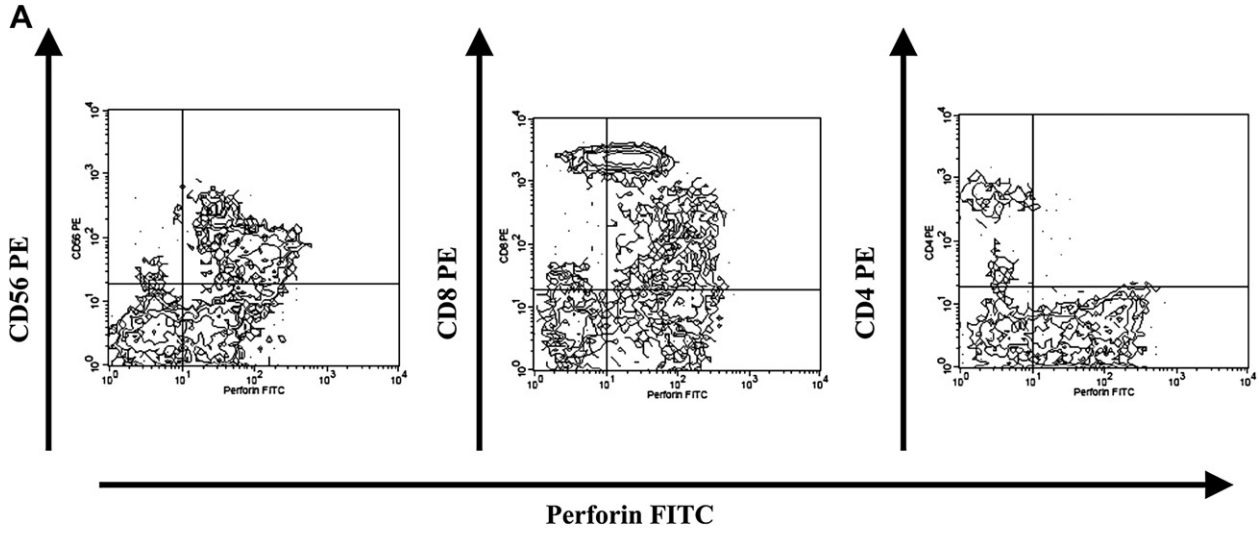


Figure 1. LLME depletion. Flow cytometry of product prior to LLME treatment shows the presence of NK (CD56 or $16^+/CD3^-$) cells, and $CD3^+/CD8^+$ cells, and $CD3^+/CD4^+$ cells. However, following LLME treatment, cells that contain perforin, namely, the NK cells and a portion of $CD8^+$ cells, are markedly depleted. The CD4 cell population is only minimally depleted in contrast (A). Following treatment with LLME, products showed adequate depletions of NK and $CD8^+$ cells with a median 95.2% (range: 79.9-100) depletion of NK cells, 76.1% (range: 35.1-92.8) of $CD3^+/CD8^+$ cells, but only 31.0% (0-88.8) of $CD3^+/CD4^+$ cells (B).

of $CD3^+$ cells/kg (Table 1). Initial cohorts of recipients of unrelated donor grafts received a dose of 10^5 $CD3^+$ cells/ kg, whereas the first cohort of patients receiving HLA identical sibling stem cell grafts received a dose of 10^6 $CD3^+$ cells/ kg. Dose escalation was allowed for each individual patient if that patient did not develop GVHD and had also not recovered a $CD4^+$ T cell count of >200/μL. If at least 3 patients in 1 cohort received LLME-treated DLI at a dose without toxicity/GVHD, the next cohort received a dose 1 log higher. The patients described here received either 10^6 CD3+ cells/kg (HLA identical unrelated donor– n = 1; HLA identical sibling donors, n = 2) or 10^7 $CD3^+$ cells/kg (HLA identical sibling donors, n = 3).

Following treatment with LLME, flow cytometric analysis showed as expected, a selective depletion of cells containing perforin (Figure 1A). The products showed adequate depletions of NK and $CD8^+$ cells

A



B

Time (Day pre-or post-LLME DLI)	CD3-/CD16+ or 56+ (cells/ul)	CD3+/CD8+ (cells/ul)	CD3+/CD4+ (cells/ul)
-8	190.29	0.00	0.30
+7	30.69	0.00	0.00
+13	117.12	1.22	0.00
+27	276.72	15.90	69.97
+34	**861.03**	**553.77**	**115.31**
+43	619.19	398.66	156.55

Figure 2. Reconstitution of perforin-positive cells. Following LLME DLI, a product containing perforin-negative cells, patients were able to reconstitute both perforin-negative and then perforin-positive cells. For the sample patient seen here, recovery of $CD3^+/CD4^+$ cells was apparent by day 37 after LLME DLI (A). Earlier time points were measured (B) showing recovery of the same populations of cells but at lower numbers. The recovery of NK cells heralded (third dot plot and third column) preceded the recovery of CD8 cells. Reconstitution of CD8 cells included both perforin-positive and perforin-negative cells and preceded the recovery of CD4-positive cells.

with a median 95.2% (range: 79.9-100) depletion of NK cells, 76.1% (range: 35.1-92.8) depletion of $CD3^+/CD8^+$ cells, but only 31.0% (0-88.8) depletion of $CD3^+/CD4^+$ cells (Figure 1B).

Immune Reconstitution

At the time of the first LLME-treated DLI treatment, all patients demonstrated neutrophil engraftment, but not lymphocyte engraftment. Two patients (#1, #3) were not evaluable based on corticosteroid use as described above. Posttreatment flow cytometric analysis showed recovery of NK cells first, as would be expected following T cell-depleted HPCT, including both perforin positive and perforin negative cells, followed by recovery of $CD8^+$ and then $CD4^+$ cells (Figure 2). Following the first dose of LLME-treated lymphocytes, 4 of 6 patients who were actively fighting viral infections, achieved a $CD3^+/CD4^+$ count >100 cells/μL by 27.5 days postinfusion (range: 10-57 days) (Table 1). This recovery was not only rapid but sustained. Data from a sample patient (#2) who developed adenovirus infection including hepatitis with transaminases 5-10 times the upper limit of normal is shown (Figure 3A and B). Following LLME DLI, reconstitution of lymphocyte subsets was prompt and persistent. By 21 days after receiving DLI, he had achieved a CD4 count of 333 cells/μL, and has since maintained this count above 100 cells/μL (Figure 3A). His recovery included reemergence of both memory and naïve T cells (Figure 3B), which have also been sustained. In another patient (#5) who had not achieved 100% donor chimerism at the time he developed EBV-posttransplantation lymphoproliferative disorders (PTLD), the pace of $CD4^+$ T cell recovery was also rapid and sustained, but the CD4 response consisted primarily of memory T cells ($CD45\ RO^+/CD4^+$). He subsequently converted to 100% donor cells, and maintained a stable number of both memory and naïve $CD4^+$ T cells (Figure 3C).

Antiviral Responses

We assayed for antiviral responses in the 6 patients who developed infections following CD34-enriched HPCT. These included reactivation of EBV (n = 2), CMV (n = 4), and adenovirus (n = 1). The infections developed at a median of 51.5 days (range: 28-113

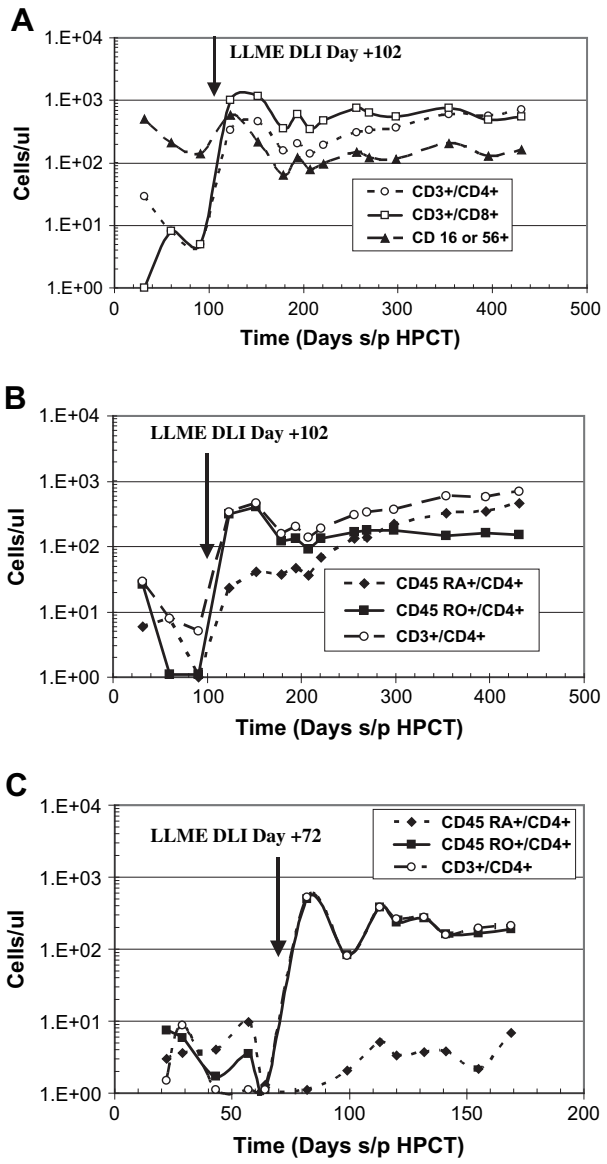


Figure 3. Immune reconstitution following LLME DLI. Patients developed rapid and sustained reconstitution of lymphocyte subsets following LLME DLI. As shown in this sample patient, this included $CD3^+/CD4^+$, $CD3^+/CD8^+$, and $CD3^-/CD56$ or 16^+ cells (A) as well as both memory and naïve CD4 cells (B). In 1 patient who had not converted to 100% donor cells at the time he received LLME DLI, the recovery of memory CD4 cells was more prominent (C).

days) following the CD34-enriched HPCT. Clinically, 5 of 6 patients had a complete response as demonstrated by resolution of the viral PCR titer. One patient (#1) who developed idiopathic pneumonia syndrome and required high dose steroids, had a partial resolution of his EBV titers, but had not completely resolved them prior to his death from multisystem organ failure, 35 days after receiving his first dose of LLME DLI. Sample data are shown for 2 patients, 1 (#2) of whom developed adenovirus hepatitis (Figure 4A and B), and another (#4) who developed CMV infection (Figure 4C and D). In both cases, one can see rapid resolution of the viral PCR titers (Figure 4b and d). Similar resolution of viral PCR titers were seen in 3 other patients (#3, #5, and #6) at a range of 11-32 days (data not shown). The final patient (patient #1) had partial resolution of EBV pcr titer (94% reduction) prior to his death from idiopathic pneumonia syndrome.

In addition, ELISPOT data demonstrated the development of viral-specific response following LLME DLI therapy. The patient with adenovirus infection (patient #2) developed an adenovirus-specific IFN-γ response by his $CD4^+$ cells (Figure 4B). The patient (patient #4) who developed the CMV infection was found to have both a CMV-specific $CD8^+$ T cell response (anti-A2 peptide) and a $CD4^+$ T cell response (CMV ag) (Figure 4D), but no response to adenovirus (not shown). Similarly, ELISPOT data from patient #5 with EBV-PTLD showed an EBV specific response following the LLME DLI (data not shown).

These responses were detected at a median of 11.5 days (range: 4-22) following LLME DLI therapy, and were associated with regression of the viral infection and resolution of circulating viral DNA by a median of 20 days (range: 11-42) in the 5 patients who did resolve their infections.

LLME DLI was given in conjunction with appropriate pharmacologic therapy in all cases. Patients with EBV-PTLD (n = 2) received rituximab, patients with CMV (n = 4) or adenovirus (n = 1) received foscarnet, ganciclovir, or valganciclovir, as clinically indicated based on renal insufficiency and count recovery. Three of 4 patients with CMV also received i.v. immuno globulin as adjunctive therapy.

Development of GVHD or Opportunistic Infection

Severe GVHD (grade III) developed in only 1 of these 6 patients (#3), a recipient of an HLA identical sibling graft. This patient had received an initial dose of 10^7 LLME DLI, but had not reached the target of 200 $CD4^+$ cells/μL, and thus received a second LLME-treated DLI 43 days later. One week following the second dose of LLME DLI, at 4.1×10^7 $CD3^+$ cells/kg (10^8) he developed GVHD of the skin and gastrointestinal (GI) tract.

Spectratyping

CDR3-size spectratype analysis was used to analyze PB samples to determine if there were any significant differences in the complexity of the reconstituting $CD4^+$ and $CD8^+$ T cell subsets, between donor and recipient (Figure 5). Reconstitution of a complex repertoire was seen in all tested patients following LLME DLI. There were no significant differences in the level of VB family complexity between the donor and "reconstituted" host.

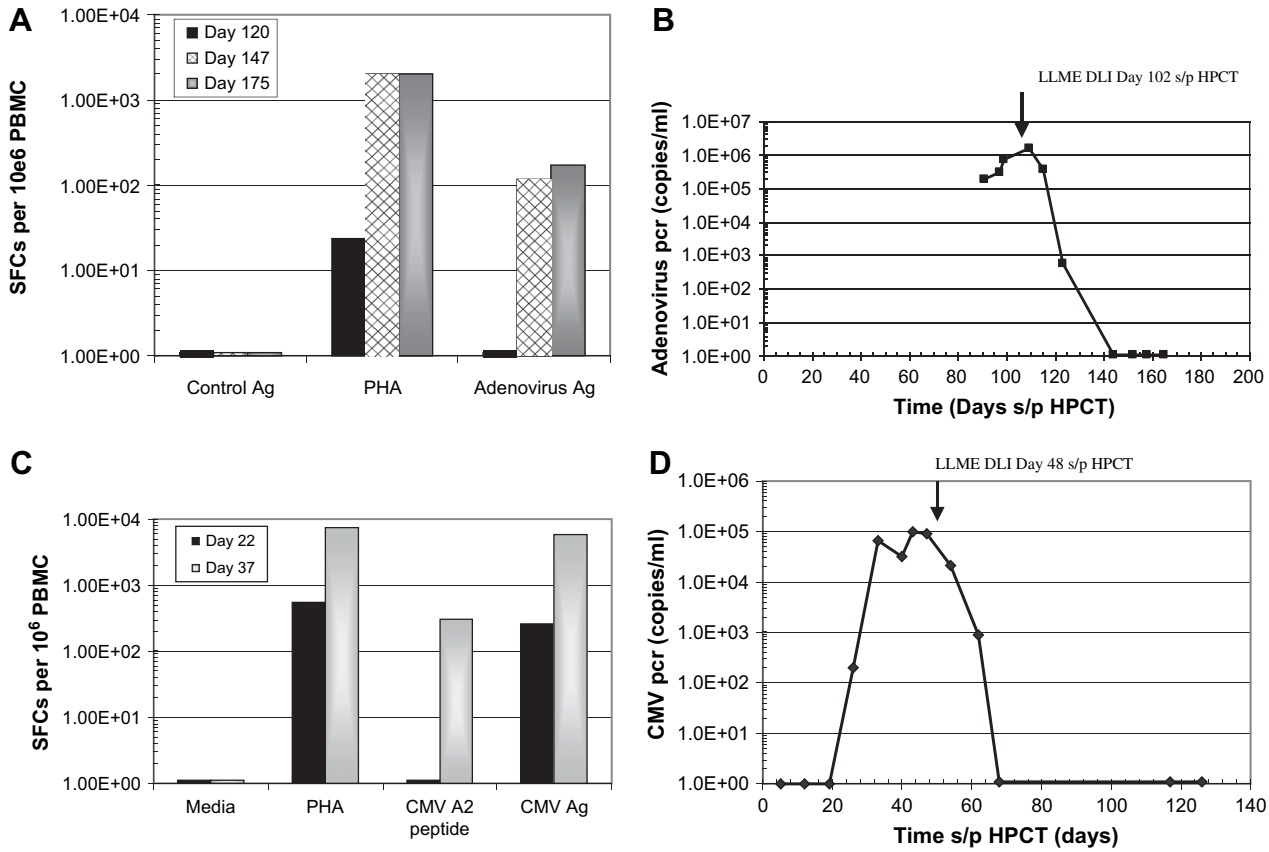


Figure 4. Antiviral responses. Sample data are shown for 2 patients. In panels A and B, one sees the data from a patient who developed adenovirus hepatitis. Shortly after receiving the LLME DLI, he began to clear the adenovirus with a 10-fold drop in PCR titers by 11 days s/p DLI (113 days s/p HPCT), and complete resolution by 42 days s/p DLI (144 days s/p HPCT). This was associated with an adenovirus specific CD4 IFN-γ response that was evident at 147 and 175 days post-DLI. Sample from a second patient, who had developed CMV infection, is shown in panels C and D. Once again, the patient had rapid resolution of CMV PCR titers with a significant drop within 6 days and complete resolution by 20 days s/p DLI (68 days s/p HPCT). He was also found to have developed both a CMV-specific CD8 response (anti-A2 peptide) and CD4 response (CMV ag), but no response to adenovirus.

DISCUSSION

Immune recovery following HPCT is slow, particularly following CD34-enriched or T cell-depleted HPCT. Prior to immune recovery, patients are susceptible to opportunistic infection including CMV, adenovirus, EBV-PTLD, and BK virus. In patients who receive unmanipulated grafts, GVHD pharmacoprophylaxis and treatment may also lead to profound immune suppression and similar types of infections. These infections lead to significant morbidity and mortality following HPCT. In a phase I study using LLME DLI, 6 patients developed significant viral infections during the immediate pre-DLI time period. Following the LLME DLI, we have seen rapid reconstitution of lymphocyte subsets including $CD3^+/CD8^+$, $CD3^+/CD4^+$, and both $CD45RA^+$ and $CD45RO^+$. This recovery, unlike that seen with infusion of cultured virus- specific CTLs, is sustained. It is associated with the emergence of virus-specific clones, but also with the development of a normal repertoire of T cells. Furthermore, it is associated with an acceptable risk of GVHD compared to unmanipulated DLI.

LLME treatment is simple and not labor-intensive. Within a few hours of phlebotomy or leukapheresis, the product is ready for infusion. Thus, a patient in need of DLI would have to wait only a few hours longer for this type of product than for an unmanipulated product. This could be a significant advantage over the use of virus-specific CTLs, which may take days or weeks to prepare and require facilities not available at many institutions. Infusions of virus-specific CTLs can lead to rapid resolution of CMV disease. However, the anti-CMV response is difficult to sustain in patients who are otherwise still immune compromised and lymphopenic. We have shown here that following LLME DLI, the anti-CMV response is rapid and also sustained.

In addition to the inability to maintain the antiviral response, virus-specific CTLs do not provide immune competence against other pathogens. In contrast, LLME DLI, together with pharmacologic antiviral therapy (foscarnet, cidofovir, ganciclovir), fosters rapid virus-specific responses that are sustained. In addition, these patients have quantitative evidence of immune recovery across a panel of lymphocyte subsets including cytotoxic cells, memory, and naïve cells.

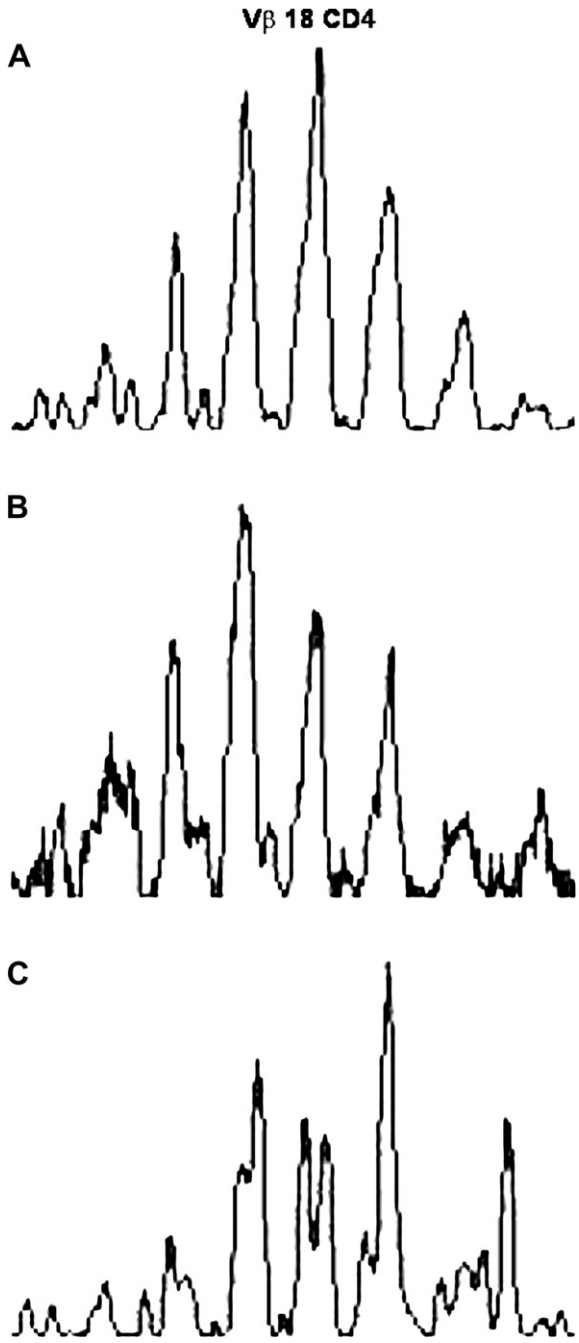


Figure 5. Vβ spectratyping. Representative histograms of Vβ spectratype analysis of the $CD4^+$ T cells enriched from the peripheral blood of 1 of the patients/pairs. The repertoire complexity of the donor sample before LLME treatment (A), after LLME treatment (B), and reconstituting in the patient post-LLME DLI (C) exhibit similar levels of complexity.

The LLME specifically depletes the stem cell graft of perforin containing cells capable of mediating cytotoxocity, providing a product that is enriched for $CD4^+$ cells, but depleted of NK cells, and perforin containing $CD8^+$ cells. Thus, the infused product is relatively depleted of cytotoxic cells that might cause GVHD or be targeted against specific pathogens while it maintains T cells with helper activity. Although $CD8^+$ T cells mediate direct cytotoxicity against viral-infected targets, their generation in vivo is dependent upon viral-specific $CD4^+$ helper T cells. Thus, in the presence of replicating viral pathogens, our $CD4^+$ T cell-deficient patients receiving LLME DLI were able to mount an appropriate immune response within several days after the infusion.

In the setting of active viral infection, immune recovery following LLME DLI is relatively rapid. The median time to recovery of a CD4 count >100/μL was 27.5 days in these patients. This contrasts with the slower recovery of patients treated with LLME DLI who were not infected with viruses (data not shown). The more rapid recovery kinetics of virally infected patients likely reflects an initial reactive lymphocytosis directed toward the specific viral pathogen, while these patients as well as the uninfected patients subsequently undergo a broader, infection-independent expansion of the infused T lymphocytes. In addition, the response was durable. Patients did not relapse in terms dropping lymphocyte counts. More importantly, no patient had relapse of viral disease at any time following LLME DLI. This contrasts with patients who have received only pharmacologic therapy or virus-specific CTLs who unfortunately often suffer relapse of the viral disease [9].

Although GVHD has long been associated with DLI, in this small group of patients this has not been an issue, except for 1 patient who did receive a higher dose of cells and likely was developing GVHD from the initial DLI just as a second larger DLI was administered. These patients were treated as part of a study that had as its primary endpoint the development of a CD4 count >100 and as a secondary endpoint the development of GVHD. It would be premature to comment on the risk of GVHD with LLME DLI until a larger cohort of patients have received this therapy. However, in the small number of patients described here, we have seen encouraging results with very little GVHD.

In the absence of severe GVHD, LLME-treated DLI appears to be a viable treatment option that has simplicity along with a rapid and durable immune recovery as major advantages. In addition, the rapid reconstitution of more broad-based T cell immunity including a complex T cell repertoire is also an advantage. Further trials are required to assess the utility of this therapy in the treatment of established viral infections.

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