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Leucyl-Leucine Methyl Ester–Treated Haploidentical Donor Lymphocyte Infusions Can Mediate Graft-versus-Leukemia Activity with Minimal Graft-versus-Host Disease Risk

Michael H. Hsieh, Gabor Varadi, Neal Flomenberg, Robert Korngold

Kimmel Cancer Institute, Jefferson Medical College, Philadelphia, Pennsylvania

Correspondence and reprint requests: Robert Korngold, PhD, Kimmel Cancer Institute, Jefferson Medical College, 233 South 10th St, Philadelphia, PA 19107 (e-mail: R.Korngold@mail.jci.tju.edu).

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ABSTRACT

L-leucyl-L-leucine methyl ester (LLME) prevents GVHD in several animal models by depleting dipeptidyl peptidase I (DPPI)–expressing cytotoxic cellular subsets. However, clinical application has been hampered by difficulties in stem cell engraftment following treatment of donor bone marrow inocula with LLME at the concentrations necessary to purge DPPI-expressing T-cells. Noting that T-cells can mediate graft-versus-leukemia (GVL) responses via both perforin (usually co-expressed in cytotoxic granules with DPPI) and Fas ligand pathways in a murine model, we hypothesized that LLME might be useful for treatment of delayed DLIs for potential GVL activity with a decreased risk of GVHD induction. In regard to the clinical setting, the ex vivo use of LLME for this purpose would circumvent any toxicity issues for donor stem cells, because by that time patients would have already achieved successful engraftment. For our preclinical studies, we used the haploidentical C57BL/6 (B6) ($H2^b$) → ((B6 × DBA/2)F_1 ($H2^{b/d}$) murine model with lethally irradiated hosts that had received transplants of T-cell–depleted bone marrow cells and were challenged with the MMD2-8 myeloid leukemia line ($H2^d$) of DBA/2 origin. A DLI of LLME-treated donor splenocytes, from B6 mice presensitized to recipient alloantigens, was administered in varying doses 14 days post–marrow transplantation, and the potential for both GVHD and GVL activity was assessed. All mice that received any dose of LLME-treated DLI survived indefinitely, without evidence of cachexia nor B-cell hypoplasia, in contrast to the severe and lethal GVHD induced by mock-treated DLI. Histological analysis largely correlated with the symptomatic findings and revealed no GVHD-like lesions in the spleens of LLME-treated DLI recipients, although some mice displayed various degrees of hepatic mononuclear infiltration. Most notably, mice given LLME-treated DLI also experienced DLI dose-dependent increases in survival against the challenge with the MMD2-8 leukemia. LLME-treated splenocytes remained immunocompetent, as these cells could proliferate in response to mitogens and to restimulation with ovalbumin when used as a recall antigen. In conclusion, LLME-treated DLI possesses immune potential and, in particular, GVL activity without inducing clinically evident GVHD.

KEY WORDS

Allogeneic hematopoietic cell transplantation • Graft-versus-host disease • Graft-versus-leukemia • Donor lymphocyte infusions

INTRODUCTION

It is now well recognized that donor T-cells can mediate graft-versus-leukemia (GVL) responses in the setting of allogeneic hematopoietic stem cell transplantation (HSCT) [1]. However, many of those same alloreactive T-cells may also induce graft-versus-host disease (GVHD), with its associated high level of morbidity and mortality. Consequently, in recent years much work has been directed toward development of approaches that can separate the donor T-cell–derived elements of GVL activity from the deleterious development of GVHD.

One approach toward achieving this separation is to try to take advantage of the differential dependency of cytotoxic T-cells and their involvement in particular GVHD and GVL responses. In regard to GVHD, immunohistochemical studies of epidermal tissue from allogeneic bone marrow

transplant recipients have correlated acute GVHD lesions with the presence of perforin-containing cytolytic T-cells [2,3]. Furthermore, in murine models of GVHD using lethally irradiated hosts across major histocompatibility complex (MHC) barriers, it has been demonstrated through the use of perforin and Fas ligand (FasL)–deficient donor mice that perforin expression is an important factor for lethal disease development mediated by either one or both $CD4^+$ and $CD8^+$ T-cells [4-6]. On the other hand, in other models FasL pathways tend to dominate the involvement in GVHD, although perforin expression still contributed to the pathological development at some level [7,8]. Finally, in MHC-matched minor histocompatibility antigen allogeneic recipient mice, perforin appeared to be more important to the development of severe GVHD-associated cachexia, but less involved in hepatic or cutaneous disease [9].

In relation to GVL responses, the importance of perforin, FasL, and tumor necrosis factor-α (TNF-α) to the overall mechanism of resistance may largely depend on the T-cell subset mediating the effect and the nature of the tumor cells under study. Of particular interest is the capacity of the tumor cells to express MHC class II, likely required for FasL and perforin-expressing $CD4^+$ T-cells, and Fas or TNF receptors with active downstream elements for apoptosis induction by either $CD4^+$ or $CD8^+$ T-cells. It is not surprising that murine GVL responses to tumors such as the MHC class II–negative P815 mastocytoma line, which are mediated by $CD8^+$ cytotoxic T-lymphocytes (CTL), are heavily dependent on perforin pathways, as this is a dominant cytolytic mechanism for those effector cells [6,8]. In contrast, many human acute and chronic myelogenous leukemia cells express MHC class II and Fas molecules, which may make them susceptible to $CD4^+$ $FasL^+$ effector T-cells. Recently, in murine models for GVL responses to myeloid leukemia lines, we demonstrated that $CD4^+$ T-cells could effectively use either perforin or FasL cytolytic pathways, with the latter being dominant in at least 1 setting [10,11]. Considering these GVL results with the collective data concerning involvement of perforin pathways at significant levels in GVHD development, we hypothesized that elimination of perforin-positive donor T-cells would allow effective GVL responses to a myeloid leukemia challenge with minimal GVHD risk.

A previously attempted method to reduce GVHD caused by T-cells in stem cell grafts has been the application of L-leucyl-L-leucine methyl ester (LLME). LLME is a lysosomotropic agent that is taken up by cells and converted to pro-apoptotic metabolites by the action of intracellular dipeptidyl peptidase I (DPPI), and it thus depletes cells containing cytotoxic granules and perforin, most notably relevant T-cell subsets [12]. LLME has been shown to reduce or prevent GVHD in both mouse [13,14] and canine models [15]. However, LLME was abandoned as GVHD prophylaxis after a clinical trial indicated that the concentrations necessary to most effectively purge GVHD-inducing T-cells in bone marrow grafts ex vivo resulted in failure to engraft the human stem cells [16].

Noting the effectiveness of LLME in eliminating perforin-positive T-cells (mostly $CD8^+$ and a small portion of $CD4^+$ cells [12,17]), yet the potential toxic effects on stem cell engraftment, we considered whether we could avoid this problem by using LLME to prevent delayed donor lymphocyte infusion (DLI)–associated GVHD. DLI was designed to provide the benefits of a donor T-cell–mediated GVL response with less risk of severe GVHD, theoretically because the stimulatory environment and inflammatory cytokine milieu following preconditioning regimens have had time to recede several months after HSCT. The efficacy of DLI has been well established in preventing or treating relapse of chronic myelogenous leukemia and shows promise with acute myelogenous leukemia and acute lymphocytic leukemia [18,19]. However, the approach is still plagued by relatively high frequencies of human GVHD. In regard to the application of LLME, the advantage of ex vivo treatment of DLI cells rather than of donor inoculum at time of HSCT would be to decrease GVHD potential without any possibility of stem cell toxicity, because the patient would already have successful donor engraftment.

To test the effects of LLME treatment on DLI, we used the haploidentical B6 ($H2^b$) → ((B6 × DBA/2)F_1 ($H2^{b/d}$) irradiation model, reconstituted at day 0 with B6 anti-Thy-1 treated (T-cell–depleted) bone marrow (ATBM) and challenged on day 13 with the MMD2-8 myeloid leukemia line of DBA/2 ($H2^d$) origin. Administration on day 14 of a DLI composed of splenocytes from B6 mice that had been presensitized to host alloantigens, resulted in dose-dependent GVHD mortality. In contrast, LLME-treated DLI did not cause GVHD, as assessed by multiple criteria, including lethality, cachexia, lymphoid hypoplasia, and B-cell hyporesponsiveness to lipopolysaccharide (LPS) stimulation, even at a maximum dose of 1.5×10^8 splenocytes. Pathological analysis indicated only mild infiltration of mononuclear cells in the livers of some mice given intermediate or high doses of LLME-treated DLI, but there was no evidence of GVHD in the spleens. Of most importance, LLME-treated DLI splenocytes were still immunocompetent and could still respond to stimulation in vitro to concanavalin A (ConA), to alloantigens of host type and third party, and to a recall antigen. Furthermore, LLME-treated DLI mediated significant and DLI dose-dependent in vivo GVL activity against the MMD2-8 leukemia and significantly prolonged survival.

MATERIALS AND METHODS

Mice

Male C57BL/6 (B6; $H2^b$) and (B6 × DBA/2)F_1 (B6D2; $H2^{b/d}$) mice were purchased from the National Cancer Institute Research and Development Center (Frederick, MD). B6 mice were used as donors between the ages of 7 and 12 weeks, and B6D2 mice were used as recipients between the ages of 8 and 16 weeks. Mice were kept in a sterile environment in microisolators at all times and were provided with acidified water and autoclaved food ad libitum.

Cell Lines

WEHI 164 (CRL 1751), a methylcholanthrene-induced fibrosarcoma, was obtained from the American Type Culture Collection (Manassas, VA). MMD2-8, a *c-myc*–transformed myeloid leukemia line, was cloned from the ascites of a DBA/2 mouse that had been injected with a *c-myc*–encoding Moloney murine leukemia virus (MMLV) construct, as previously described [20].

Media

Phosphate-buffered saline (PBS) supplemented with 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO) was used for all in vitro manipulations of the donor bone marrow and splenocytes, unless otherwise noted. Immediately prior to injection, cells were washed and resuspended in PBS alone. Culturing of cell lines and in vitro assays were performed in RPMI-1640, supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 5.5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin (complete media).

GVL Survival Assay

All B6 donor mice used for DLI survival assays were presensitized to recipient alloantigens by intraperitoneal (i.p.) administration of 1.5 to 2.0×10^7 B6D2 splenocytes 12 days prior to use. LLME treatment of DLI was based on previously outlined protocols [13]. Briefly, single cell suspensions of spleens were lysed with Gey's balanced salt lysing solution containing 0.7% NH_4Cl for removal of red blood cells (RBC). Splenocytes were then washed and suspended at 2.5 to 10×10^6 cells/mL in PBS containing 250 μM LLME (Sigma). Cells were incubated for 15 minutes at room temperature and then washed and resuspended in ice-cold PBS. Splenocytes were promptly placed in culture or infused in vivo. For mock-treated DLI, splenocytes were subjected to identical conditions except that no LLME was added to the incubation media.

Bone marrow cells were obtained from the femurs and tibias of B6 mice by flushing with PBS. To prepare ATBM, cells were incubated with J1j monoclonal antibody (MoAb) (1:100 dilution of ascites; TIB 184, American Type Culture Collection) and guinea pig complement (1:25; Rockland, Boyertown, PA) for 45 minutes at 37°C and were washed twice in PBS containing 0.1% BSA before final resuspension in PBS.

B6D2 mice designated as recipients were given a total of 13 Gy of ionizing radiation, given as a split dose of 6.5 Gy each separated by 4 hours, using a Shepherd Mark-I-68A ^{137}Cs source (1.43 Gy/min). One hour after the final exposure, these mice were injected intravenously (i.v.) with 2×10^6 donor ATBM cells. On day 13 posttransplantation of ATBM, half of the recipient mice were challenged with 10^5 MMD2-8 leukemia cells i.p. The following day, LLME- or mock-treated DLI was prepared, as described above, and administered i.v. to appropriate recipients. Mice were checked daily for morbidity and mortality, median survival times (MST) were determined, and statistical comparisons were performed between experimental groups on data pooled from 2 separate experiments using the nonparametric Tukey multiple analysis of variance.

Monoclonal Antibodies

Ascitic fluid containing MoAb specific for Thy-1.2 (J1j) was used for preparation of ATBM cells. For phenotyping of MMD2-8 cells and splenocytes, fluorescein isothiocyanate (FITC)–conjugated or phycoerythrin (PE)–conjugated versions of the following MoAb were purchased from Pharmingen, San Diego, CA, and specific for: CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD11b/Mac-1 (M1/70), CD13 (R3-242), CD45R/B220 (RA3-6B2), CD80/B7-1 (1G10), CD86/B7-2 (GL1), 2B4, Ly-6G/GR-1 (RB6-8C5), $H2K^d$ (SF1-1.1), $H2K^b$ (AF6-88.5), and a rat immunoglobulin (Ig) G2a isotype control (R35-95). Biotinylated anti-Fas MoAb (Jo2) and a hamster IgG isotype standard (Ai9-3), both from Pharmingen, as well as streptavidin (SA)-PE (Caltag, South San Francisco, CA) were also used.

Flow Cytometry

Appropriate volumes of 25 μL of MoAB were each incubated with 2 to 5×10^5 cells in a 96-well U-bottom microtiter plate for 25 minutes at 4°C, centrifuged at 1500 rpm for 3 minutes, and washed with PBS containing 0.1% BSA and 0.01% sodium azide (wash buffer). When applicable, SA-PE or a secondary antibody was added in a volume of 25 μL for 25 minutes at 4°C, followed by 2 washes with wash buffer. The fluorescence analysis was performed on an EPICS Profile II analyzer (Coulter, Hialeah, FL) in the Kimmel Cancer Center Flow Cytometry Facility. The percentage of positive cells and the arithmetic mean fluorescence intensity were calculated for each sample.

For analysis of the effects of LLME treatment on splenocyte subpopulations, cells were incubated with LLME or PBS alone, as described above, and then placed in complete media at 37°C for 3 hours to allow apoptosis to occur. Splenocytes were then stained with fluorochrome-labeled MoAb and subjected to flow cytometric analysis.

For assessment of FasL expression after mitogenic stimulation, LLME-treated B6D2 splenocytes were cultured at 4×10^6 cells/mL in 4 mL in 6-well plates with complete media containing 10 mg/mL ConA. The cells were harvested after 48 hours and stained with either Fas-Fc or an isotype control (Alexis, San Diego, CA), followed by anti-Fc MoAb and then SA-PE, and analyzed by flow cytometry.

TNF Receptor Reverse Transcriptase–Polymerase Chain Reaction

Total cellular RNA was prepared from MMD2-8 leukemia cells. TNF receptor and GADPH primer sequences have been previously described [21] and were provided by Dr. Keith Kelley, University of Illinois, Urbana, IL. Reactions were performed using a Perkin-Elmer Applied Biosystems GeneAmp PCR System 9700. Cycling conditions were 30 minutes at 42°C; 2 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 45 seconds at 55°C, and 45 seconds at 72°C; and final extension for 7 minutes at 72°C. Product size was determined by electrophoresing the samples on an agarose gel and staining with ethidium bromide.

TNF-α Sensitivity Assays

Assay conditions to determine the sensitivity of MMD2-8 to exogenous TNF-α–mediated cytotoxicity were based on previous studies [21,22]. MMD2-8 and WEHI164 cells were cultured overnight at 2×10^4 cells/well in a volume of 100 μL in 96-well flat bottom plates. Medium (100 μL) containing either actinomycin D alone (0.5 μg/mL final) or actinomycin D and titrated concentrations of recombinant murine TNF-α (Peprotech, Rocky Hill, NJ) was added to appropriate quadruplicate wells. After 22 hours, 70 μL was aspirated from each well prior to addition of 50 μL of tetrazolium salt (MTT) (final concentration 1 mg/mL). After a 4-hour incubation at 37°C, 50 μL of medium was aspirated

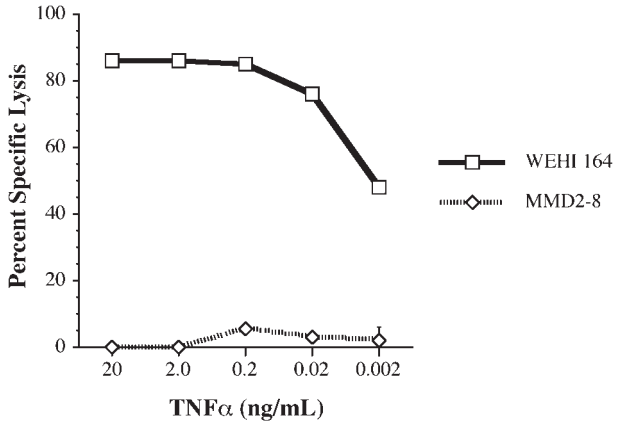

Figure 1. Insensitivity of MMD2-8 cells to TNF-α–mediated cytotoxicity. MMD2-8 myeloid leukemia and WEHI 164 cells were incubated with various concentrations of TNF-α and a fixed amount of actinomycin D (0.5 μg/mL final). The percent specific lysis was analyzed by an MTT assay, as described in Materials and Methods. Means and standard deviations (all but one <5% of the mean) were calculated from quadruplicate wells for all culture conditions.

and 150 μL of acidified isopropanol (0.04N HCl) was added to each well. The medium in each well was then extensively pipetted to dissolve crystals prior to measurement of each well's absorbance at 540 nm.

Cytotoxic Assays

B6 splenocytes were depleted of RBC with Gey's balanced salt lysing solution and then treated with LLME or mock-treated, as described above. Cells (5×10^6) were cultured in vitro for 5 days in upright 25 cm^2 tissue culture flasks with 5×10^6 irradiated (15 Gy) B6D2 splenocytes and 1:20 of T-STIM culture supplement (Becton Dickinson Labware, Franklin Lakes, NJ) in a total of 10 mL of complete medium. MMD2-8 cells were labeled with ^{51}Cr-sodium chromate (ICN Radiochemicals, Irvine, CA) and used as targets in a 6-hour ^{51}Cr-release assay. To inhibit Fas-mediated cytotoxicity, 5 μg/mL of a Fas fusion protein composed of human recombinant fas combined with the Fc region of human IgG1 (Fas-Fc) and 1 μg/mL of an antihuman IgG1-Fc antibody (enhancer protein) used for cross-linking (Alexis) were added to relevant wells. As a control, 5 μg/mL of isotype-matched Fc fragment (Accurate Chemical, Westbury, NY) was substituted for the Fas-Fc. Percent specific lysis was calculated as 100× experimental CPM minus spontaneous release CPM (target cells in media alone), divided by maximum release CPM (target cells in cetrimide) minus spontaneous release CPM.

Proliferation Assays

B6 mice were immunized with ovalbumin (OVA) (150 μg; Sigma) in Complete Freund's Adjuvant containing H37RA (Difco, Detroit, MI) subcutaneously 2 to 3 weeks before priming them to recipient B6D2 splenocytes (1.5×10^7 i.p.). Two weeks later, splenocytes from these mice were mock- or LLME-treated, and the cells (2.5×10^5) were cultured in 96-well round-bottom plates with either ConA (2 μg/mL), *Escherichia coli* LPS (20 μg/mL), B6D2 or C3H irradiated (15 Gy) splenocytes (5×10^5), or OVA (75 μg/mL). Proliferative responses were determined after 3 to 5 days of culture by measuring [^{3}H]-TdR incorporation following an overnight pulse/label period. Quadruplicate samples were used to calculate the mean incorporation and standard deviations. A similar approach was used for examining the responsiveness to LPS polyclonal B-cell activation of B6D2 recipient splenocytes harvested 1 and 10 weeks post-DLI. These cells were stimulated in vitro (10^5 per well) with 100 μg/mL LPS.

Histology

Mice were killed 10 weeks post-DLI, and tissues were fixed in 10% formalin, embedded and sectioned (7 μm thick) in paraffin wax, stained with hematoxylin and eosin, and evaluated by light microscopy. Liver infiltrates were considered "mild" if up to 25% of portal tracts featured mononuclear infiltrates, and "severe" if >25% of portal tracts were affected.

RESULTS

MMD2-8 Phenotype and Sensitivity to TNF-α

The *c-myc*–induced MMD2-8 leukemia cell line was assessed phenotypically via flow cytometric analysis and found to express high levels of surface H2K^d (98.4%), CD11b/Mac-1 (99.4%), CD86/B7-2 (96.7%), CD13 (99.2%), CD11a (72%), and CD95/Fas (96.8%). The cells expressed intermediate levels of surface CD80/B7-1 (59.8%), Ly6G/GR-1 (47.9%), and Ie^d (35.9%); low levels of CD45R/B220 (14.6%); and no FasL. This phenotype is consistent with the monocytic-myeloid cell lineages known to be produced through transduction with the *c-myc*–encoding retrovirus [20,23]. The moderate to high expression of costimulatory and both MHC classes I and II molecules suggested that MMD2-8 could be capable of antigen presentation, and the high level of surface Fas protein expression was consistent with sensitivity to FasL-mediated apoptosis.

To determine whether MMD2-8 cells expressed TNF type I or II receptors, reverse transcriptase–polymerase chain reaction analysis was performed, because functional expression levels of these surface molecules can be below the threshold of detection by flow cytometry [24]. Production of mRNA for both TNF receptors was evident (data not shown). Despite the presence of these receptors, MMD2-8 cells were resistant to exogenous TNF-α–mediated cytotoxicity over a concentration range of 0.002 to 20 ng/mL, in comparison to the WEHI164 cell line, which was highly susceptible at all tested concentrations (Figure 1). These findings suggested that MMD2-8 cells might be incapable of transmitting apoptotic signals via the TNF receptor pathway.

MMD2-8 Cells Can Be Lysed by LLME-Treated Allogeneic CTL in a Fas-Dependent Manner

For determination of whether LLME-treated splenocytes were capable of lysing MMD2-8 cells in vitro, MMD2-8 cells were labeled with ^{51}Cr-sodium chromate and used as targets in a standard 6-hour ^{51}Cr-release assay with either in vitro B6D2-stimulated LLME- or mock-treated B6 splenocytes as the CTL effectors (Figure 2). B6D2

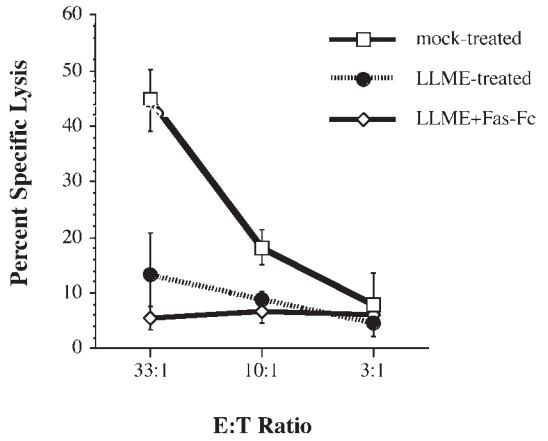

Figure 2. Lysis of MMD2-8 cells by mock- and LLME-treated allogeneic splenocytes. LLME or mock-treated B6 splenocytes were stimulated with irradiated B6D2 splenocytes in a mixed lymphocyte reaction and used as effectors against ^{51}Cr-labeled MMD2-8 target cells in a standard 6-hour ^{51}Cr-release assay. To inhibit Fas-mediated cytotoxicity, 5 μg/mL of Fas-Fc and 1 μg/mL of enhancer were added to one of the LLME-treated groups, whereas an isotype matched Fc fragment control was added to the other group. The mean percent specific cell lysis was determined from triplicate wells, and the data are representative of 2 similar experiments.

LPS-stimulated lymphoblasts were tested in parallel as targets using the same effector cells. Mock-treated splenocytes could effectively lyse B6D2 LPS-stimulated lymphoblasts, whereas LLME-treated effectors could not (data not shown). Mock-treated CTL could also efficiently lyse MMD2-8 target cells (peak killing of 44.7% at an effector:target [E:T] ratio of 33:1), whereas LLME-treated effectors exhibited weaker activity (peak killing of 13.2% at an E:T of 33:1, $P < .01$). However, this weaker LLME-treated CTL activity appeared to be Fas-dependent, because addition of Fas-Fc, but not the irrelevant control Fc fusion protein, suppressed lysis to background levels (5.4% killing at an E:T of 33:1), although this failed to reach statistical significance ($P > .05$). These results suggested that LLME-treated splenocytes could potentially mediate GVL activity against MMD2-8 target cells, and possibly through direct Fas-dependent cytotoxicity.

Phenotype of LLME-Treated Splenocytes

LLME- and mock-treated splenocytes from B6D2-presensitized B6 mice were stained with fluorochrome-labeled MoAb and analyzed by flow cytometry. Most CD8$^+$ T-cells (95.1%) were depleted, as well as most CD11b/Mac1$^+$ (94.6%) and all 2B4$^+$ (100%) cells, which are myeloid and natural killer (NK) cell markers, respectively (Table 1). CD4$^+$ T-cells appeared essentially unaffected, as well as B220$^+$ cells (B-cell marker), but both were proportionally increased because of the loss of the other cellular subsets. These findings were in agreement with the well-established effects of LLME [13].

For determination of whether LLME-treated B6 splenocytes had the capacity to express FasL, cells were cultured with ConA (10 μg/mL) for 48 hours and then stained with Fas-Fc or an isotype control, followed by anti-Fc MoAb and then SA-PE. Both mock- and LLME-treated splenocytes could express FasL after mitogenic stimulation, but the latter exhibited a 61% increase in the percentage of positive-staining cells (16.8% and 27.0%, above isotype control staining of 3.9% and 3.6%, respectively; Figure 3). In addition, the intensity of FasL expression nearly doubled for the LLME-treated population (from mean fluorescence intensity values of 0.63 to 1.11). Without mitogenic stimulation, neither mock- nor LLME-treated splenocytes expressed detectable FasL.

The immunocompetency of LLME-treated B6 splenocytes was assessed by in vitro proliferative responses to mitogenic stimulation with either ConA or LPS, alloantigen stimulation with recipient B6D2 or third-party strain (C3H) splenocytes, and stimulation to a recall antigen, OVA (mice had been immunized with OVA 2 to 3 weeks before also priming them to recipient B6D2 alloantigens). The results indicated that LLME-treated splenocytes were capable of responding to all types of stimulation, although they did so at a higher level and with somewhat delayed kinetics compared to the mock-treated control group (Figure 4). This observation may be due to the actually higher proportion of CD4$^+$ T-cells, and perhaps even B-cells serving as antigen-presenting cells, in the splenocyte population following LLME treatment. In summary, LLME depleted the majority of CD8$^+$ cells, in addition to myeloid and NK cells, and possibly a small number of CD4$^+$ cells, but preserved a population of cells that was capable of expressing FasL and of responding to mitogenic stimulation, recipient and third-party alloantigens, and a recall antigen.

LLME-Treated DLI Does Not Cause Lethal GVHD or Associated Cachexia

B6D2 mice were lethally irradiated (13 Gy, split dose) and reconstituted with B6 ATBM cells and 2 weeks later were given graded doses (2, 4, and 15 × 10^7) of DLI in the form of B6D2-presensitized B6 splenocytes that were either LLME- or mock-treated ex vivo. Mice given mock-treated DLI exhibited significant dose-dependent GVHD with low

Table 1. *Flow Cytometric Analysis of Cell Lineages Comprising LLME-Treated Splenocytes**

Marker	Percent Positive Cells	
	LLME-Treated	Mock-Treated
CD4	30.1	29.1
CD8	0.6	12.2
B220	56.8	42.0
Mac-1	0.3	5.5
2B4	0	4.0

*For analysis of the effect of LLME treatment on B6 splenocyte subpopulations, pooled cells were incubated with LLME or PBS alone (mock) and then held in complete media at 37°C for 3 hours to allow LLME-induced apoptosis to occur. Preparations were stained with FITC-labeled MoAb and analyzed by flow cytometry. The values represent the percent positive cells after subtraction of backgrounds obtained by incubation with a FITC-labeled rat IgG2a control MoAb (2.0% for LLME- and 1.8% for mock-treated groups).

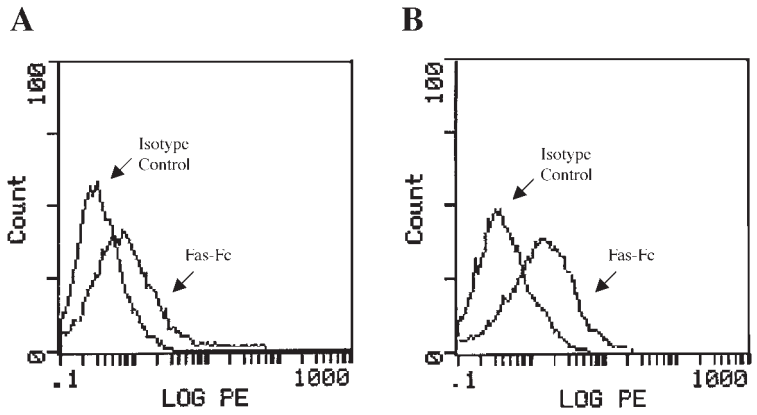

Figure 3. Expression of FasL on mock-treated (A) and LLME-treated (B) splenocytes after culture with ConA (10 μg/mL) for 48 hours and staining with Fas-Fc protein or an isotype control, followed by anti-Fc MoAb and then SA-PE.

percentages of long-term survival, from 0% to 45%, and MST of day 53 (2×10^7), day 16 (4×10^7), and day 14 (15×10^7; Figure 5A). In contrast, all mice receiving any dose of LLME-treated DLI survived indefinitely ($P < .05$ compared to all doses of mock-treated DLI). Other typical parameters of GVHD, such as cachexia and weight loss, were evident in all mock-treated groups, but not in those recipients of LLME-treated DLI. For example, mice receiving the highest dose of 15×10^7 LLME-treated DLI maintained and gradually increased their mean body weight throughout the initial few weeks of observation and paralleled, if not exceeded, the weights of the ATBM reconstituted recipients ($P > .05$ at all time points; Figure 5B). It should also be noted that LLME-treated splenocytes were not "dose adjusted" for cellular subset equivalency to their mock-treated control counterpart groups. Nevertheless, the same question is approached by the dose titration, whereby 1.5×10^8 LLME-treated splenocytes failed to mediate lethal GVHD with almost twice the number of CD8 cells as found in the mock-treated 4×10^7 dose level (9.0 versus 4.8×10^6 cells, respectively), which mediated severe GVHD.

Analysis of GVHD-Related Spleen and Liver Pathology

Spleen and liver samples were harvested from B6D2 mice 10 weeks after DLI administration. The tissues were embedded and sectioned in paraffin wax and stained with hematoxylin and eosin. All of the mice that received a low dose (2×10^7 splenocytes) of mock-treated B6 DLI exhibited

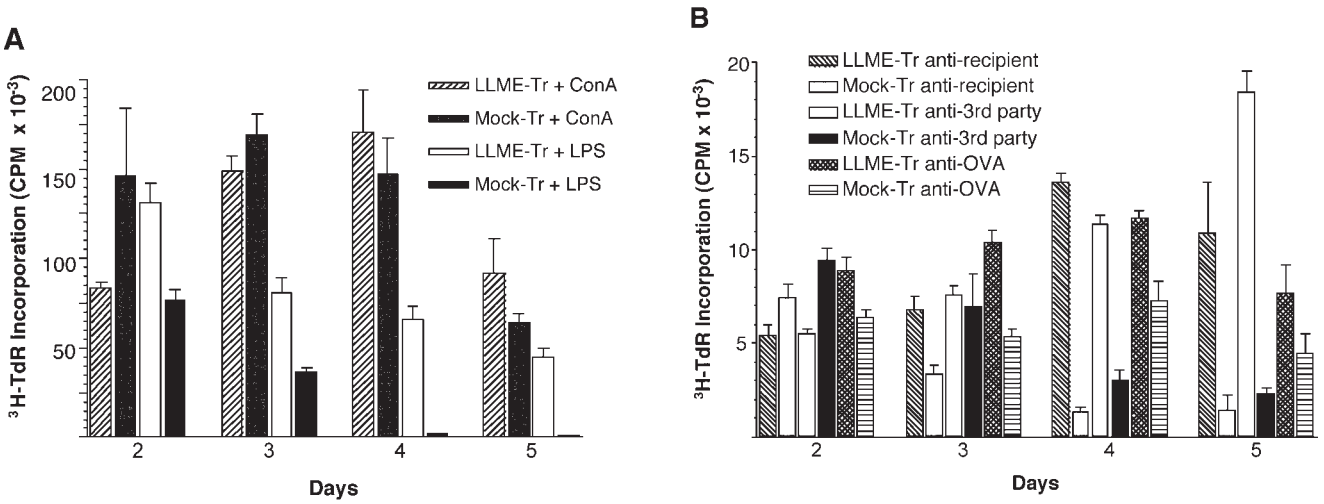

Figure 4. Proliferative responses of LLME-treated donor splenocytes. The immunocompetency of LLME-treated B6 splenocytes was assessed by in vitro proliferative responses to mitogenic stimulation with either ConA or LPS (A), alloantigen stimulation with recipient B6D2 or third-party strain (C3H) splenocytes (B), and stimulation to OVA, when used as a recall antigen. B6 mice were immunized with OVA 2 to 3 weeks before further presensitization to recipient B6D2 splenocytes (1.5×10^7 i.p.). Two weeks later, the mock- or LLME-treated splenocytes were cultured with either ConA (2 μg/mL), LPS (20 μg/mL), B6D2 or C3H irradiated (15 Gy) splenocytes, or OVA (75 μg/mL). Proliferative responses were determined after 2 to 5 days of culture by determining the mean [^{3}H]-TdR incorporation from quadruplicate samples. The data are representative of 2 similar experiments. There was no significant difference between the LLME and mock-treated responses to ConA stimulation on any of the days ($P > .05$). Significantly higher responses were obtained with the LLME-treated groups to LPS on all of the days ($P < .05$); to recipient stimulation on days 3 to 5 ($P < .02$); to third-party stimulation on days 4 and 5 ($P < .001$); and to OVA on days 2 to 4 ($P < .03$).

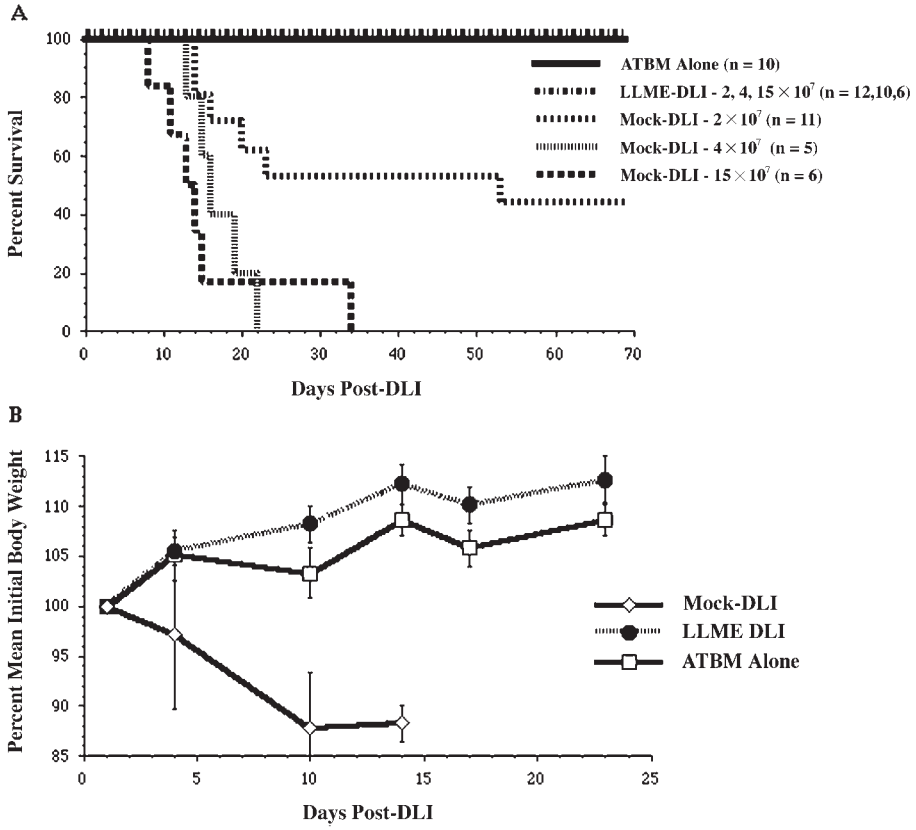

Figure 5. Effect of LLME-treated allogenic DLI on GVHD development. B6D2 mice were lethally irradiated, reconstituted with ATBM cells, and 2 weeks later given graded doses (2, 4, or 15 × 10^7) of host-presensitized mock- or LLME-treated B6 splenocyte DLI. A, Survival of mice given DLI. The data were pooled from 2 similar experiments with combined n values indicated in the legend. B, Percent mean initial body weights of mice given the 15 × 10^7 dose of DLI. The data are from 1 representative experiment (n = 6 per group).

massive disruption of splenic architecture, including loss of follicular organization (Figure 6A) and marked periportal infiltrates in the liver (Figure 6B), consistent with GVHD (80% of the mice had extensive infiltration; 20% had mild infiltration). In contrast, the spleens of a majority (66%) of the mice that were given 2 × 10^7 LLME-treated B6 DLI displayed normal splenic architecture with intact follicles (Figure 6C), whereas the remainder displayed only mild distortions. Livers were free of periportal infiltrates (Figure 6D), similar to those of control mice given only ATBM cells without DLI (not shown). At higher LLME-treated DLI doses of either 4 × 10^7 or 1.5 × 10^8 splenocytes, the majority of mice (66%) still exhibited no indications of GVHD pathology in the spleen (Figure 6E for the intermediate dose), with the remainder again expressing only minor involvement. Results in the liver were variable in both dose groups, with some mice (15%) still free of periportal infiltrates (Figure 6F, at the 4 × 10^7 dose), whereas others had livers with scant (70%) to extensive (15%) periportal infiltrates (not shown).

LLME-Treated DLI Maintains Donor Chimerism without Lymphocyte Hypoplasia

B6D2 mice, 10 weeks after receiving doses of either 2, 4, or 15 × 10^7 host-presensitized, LLME-treated B6 DLI, had no obvious lymphoid hypoplasia in either the T-cell ($CD4^+$ or $CD8^+$) or B-cell ($B220^+$) compartments, compared to animals given ATBM cells alone without DLI. The mean number of total cells per spleen ranged between 2.16 and 3.33 × 10^7 cells with donor chimerism between 88.4% and 98.4% (Table 2). In contrast, recipients of even a low dose of mock-treated DLI had marked lymphoid hypoplasia with an average of only 6.8 × 10^6 total cells/spleen, of which only 21.8% were B-cells. The remaining 42% of the cells were $Ly6G/Gr\text{-}1^+$ (granulocytes) and macrophages, a phenotype most likely related to the immunopathological development of GVHD. Overall, these results suggested that LLME-treated DLI could maintain, if not enhance, donor chimerism without inducing GVHD-associated hypoplasia in the late post-DLI period.

LLME-Treated DLI Does Not Cause GVHD-Associated B-cell Hyporesponsiveness

To determine whether there was GVHD-associated B-cell impairment in the splenic population of mice given LLME-treated DLI, at 1 week posttransfer, splenocytes were tested in vitro for mitogenic proliferative responses to LPS. At the peak response after 4 days of culture, splenocytes from B6D2 mice given 4 × 10^7 mock-treated B6 DLI proliferated poorly in response to LPS with a mean of 7127 cpm, compared to cells from mice given 4 × 10^7 LLME-treated DLI or no DLI (22,931 and 33,205 cpm, respectively; $P < .05$) (Figure 7A). This pattern was also maintained when

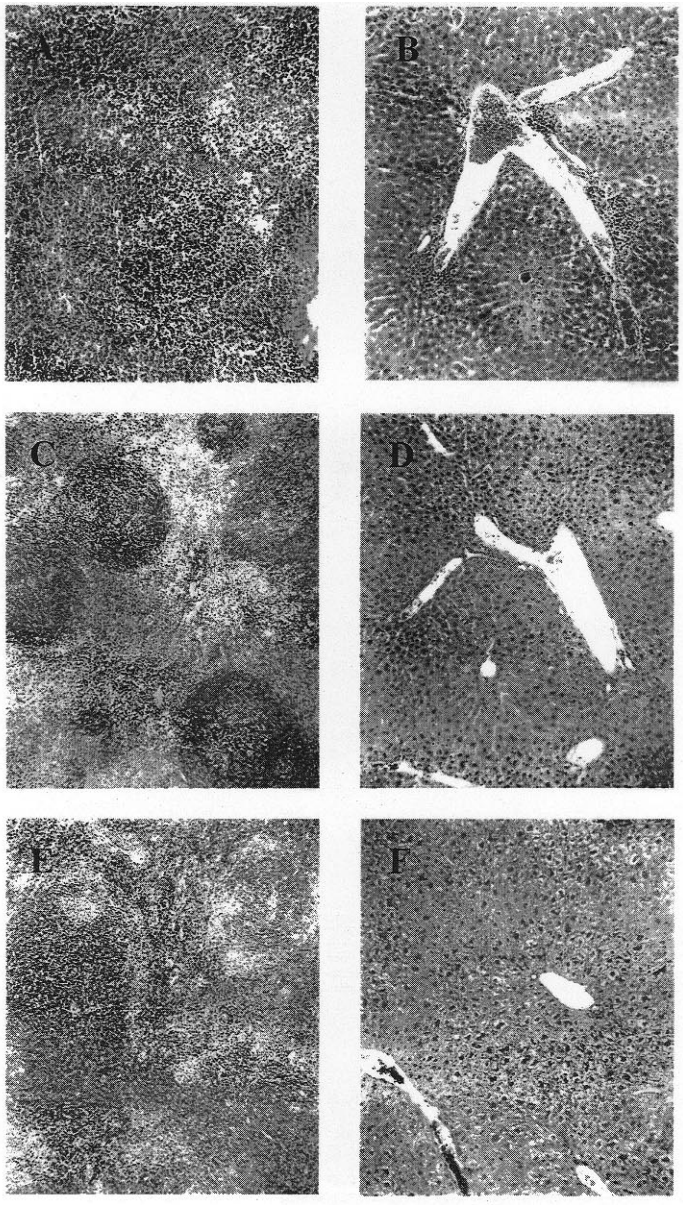

Figure 6. Histological analysis of spleens and livers from mice that received DLI. Whole spleens and liver samples were harvested from mice surviving 10 weeks after DLI, fixed in formalin, embedded and sectioned in paraffin, and stained with hematoxylin and eosin. Spleen (A) and liver (B) from a mouse that received a low dose (2×10^7 cells) of mock-treated splenocytes as DLI. Spleen (C) and liver (D) from a mouse given a low dose (2×10^7 cells) of LLME-treated splenocytes as DLI. Spleen (E) and liver (F) from a mouse given a medium dose (4×10^7 cells) of LLME-treated splenocytes as DLI (original magnification ×10 for all panels).

splenocytes were assayed on days 3 and 5 of culture. The LLME-treated DLI was 31% lower ($P < .05$) than the no-DLI group, but only on day 4 of assay, and, by comparison, splenocytes from both groups responded less than splenocytes from normal B6D2 mice.

LPS responses were also studied 10 weeks post-DLI, using a maximum dose of host-presensitized B6 DLI (1.5×10^8 splenocytes/mouse). At 3 to 5 days of culture, splenocytes from mice given LLME-treated DLI responded to LPS similarly to those cells from mice without DLI, with a slight 25% decrease on day 4 (58,668 and 78,360 cpm, respectively; $P < .05$; Figure 7B). These responses were well within the range observed for normal splenocytes. Cells from mock-treated mice were not tested because by this time the mice had succumbed to GVHD.

LLME-Treated DLI Mediates GVL Activity

DLI has been found to be an effective approach for the prevention or control of leukemic relapse by providing a donor T-cell–mediated GVL response. It was, therefore, imperative to test for the retention of this GVL capability following the administration of LLME-treated DLI in our experimental model. Lethally irradiated B6D2 mice reconstituted with B6 ATBM cells alone (no DLI) and challenged

Table 2. *Enumeration and Phenotypic Analysis of Splenocytes of Mice Given DLI**

Group	No. of Cells, $\times 10^{-6}$	% Donor	%CD4	%CD8	%B220
Normal B6	51.6	n/a	22.0	13.7	45.7
Normal B6D2	60.0	n/a	21.5	12.8	55.7
ATBM/No DLI	27.9	82.9	31.0	14.9	48.2
Mock-treated DLI (2×10^7)	6.8	90.4	26.6	9.6	21.8
LLME-treated DLI (2×10^7)	32.8	88.4	27.1	12.3	58.2
LLME-treated DLI (4×10^7)	21.6	98.4	20.1	11.0	49.7
LLME-treated DLI (1.5×10^8)	33.3	89.4	27.9	20.2	41.6

*Splenocytes were harvested and pooled from B6D2 recipients 10 weeks post-DLI with mock- or LLME-treated B6 donor lymphocytes. Viability was determined by trypan blue exclusion, and cells were counted and phenotyped by flow cytometry with appropriate FITC-labeled MoAb. Analysis of $H2K^b$ and $H2K^d$ expression was used to measure donor chimerism. All values are expressed as the mean from pooled group samples (n = 3-5), and the data are from a representative experiment.

with 1×10^5 MMD2-8 myeloid leukemia cells on day 13 had an MST of 26 days postchallenge with no long-term survivors (Figure 8). Mice similarly MMD2-8 challenged but given a medium dose of mock-treated DLI (4×10^7 B6 host-presensitized splenocytes) on day 14 had an MST of 13 days postchallenge, with no survivors. These mice exhibited clinical symptoms of GVHD before their demise. Mice given leukemia challenge and 4×10^7 LLME-treated DLI exhibited a significantly prolonged MST of 57 days in comparison to either the ATBM control ($P < .05$) or the mock-treated ($P < .05$) groups, with 27% surviving at the termination of the experiment on day 70. Sample mice from these survivors had no macroscopic evidence of leukemia upon autopsy (data not shown).

Leukemia-challenged mice given a maximum mock-treated DLI dose of 1.5×10^8 B6 host-presensitized splenocytes had a MST of 15 days with no survivors (Figure 7). In contrast, mice injected with LLME-treated DLI at that dose had a MST of 60 days with 50% survival at 70 days. At time of sacrifice, there was no evidence of widespread leukemia. Instead, 2 of 3 sample mice from the survivors had evidence of encapsulated leukemia in the peritoneum at the original injection site. Collectively, these findings strongly suggest that LLME-treated DLI is capable of mediating GVL effects without overt development of GVHD, as reflected in significantly prolonged survival of transplant recipient mice.

DISCUSSION

GVHD remains a constant threat in the setting of HSCT, whether mediated by T-cells contaminating the stem cell graft or purposefully given in the form of DLI to

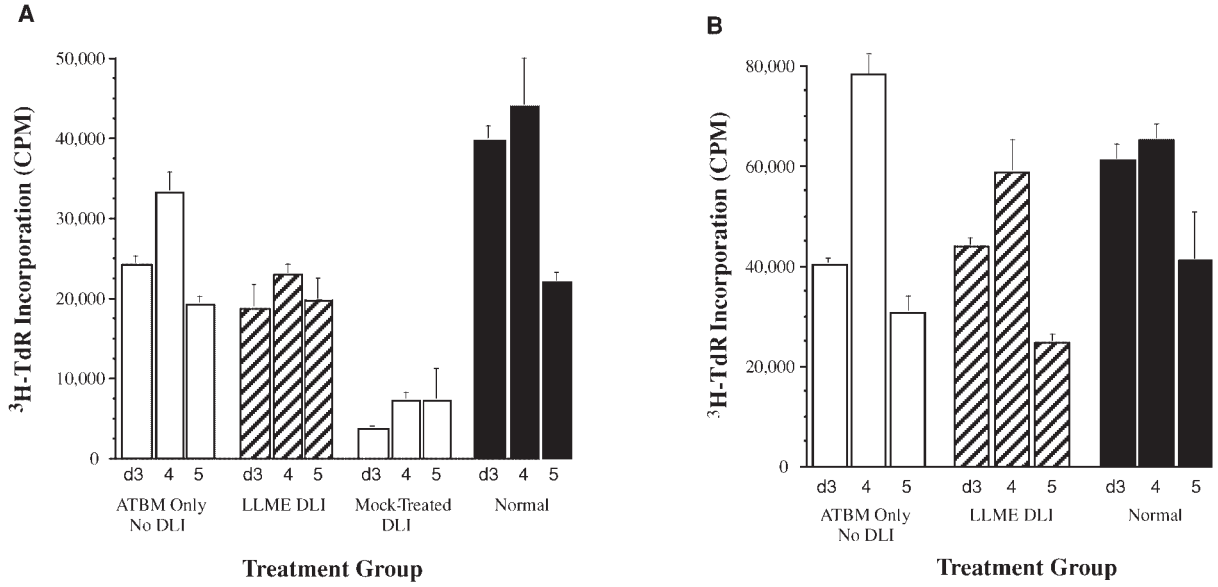

Figure 7. LPS-induced proliferation of splenocytes from mice given DLI. A, LPS-induced proliferation of splenocytes from B6D2 mice 1 week post-DLI administration. Splenocytes from mice given ATBM cells alone (no DLI) or 4×10^7 host-presensitized LLME- or mock-treated B6 splenocytes as DLI were harvested and incubated alone or with LPS. Quadruplicate wells were pulsed overnight with [^{3}H]-TdR, samples were collected on days 3 to 5 of culture, and mean cpm and standard deviations were determined. Background samples of cells incubated without LPS ranged between 159 and 516 cpm. B, LPS-induced proliferation of splenocytes from B6D2 mice 10 weeks post-DLI administration. Assays were performed as described above, except that the original DLI dose was 1.5×10^8 host-presensitized LLME-treated B6 splenocytes. Background samples of cells incubated without LPS ranged between 151 and 1643 cpm.

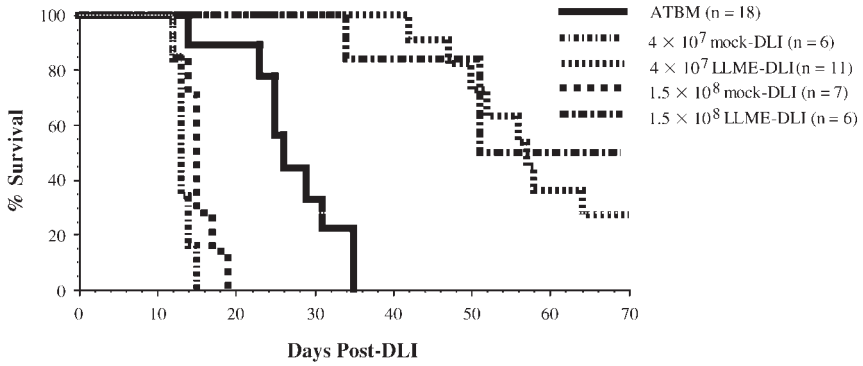

Figure 8. GVL capacity of LLME-treated DLI. B6D2 mice were lethally irradiated and reconstituted with B6 ATBM cells, and 13 days later all mice were challenged with MMD2-8 myeloid leukemia cells (i.p.). The following day, mice were given mock- or LLME-treated B6 DLI (4 or 15 × 10^7 host-presensitized splenocytes). Mice were monitored daily for survival, and the results represent pooled data from 2 similar experiments. The sample size for each group is as indicated in the legend.

counteract leukemic relapse. Ex vivo LLME treatment of donor bone marrow has been extensively investigated as a means of GVHD prophylaxis. The agent has been found to completely prevent the development of GVHD in multiple models, including several allogeneic MHC- and minor histocompatibility antigen–mismatched strain combinations [13] and fully allogeneic MHC-mismatched systems [14]. In these models, mice did not experience cachexia or other clinical measures of GVHD, and the only microscopic signs of disease were mild to moderate cholangitis and transient skin infiltrates with residual dermal sclerosis [13,25]. Because GVHD-associated cachexia is classically associated with TNF-α release [26,27] and LLME depletes DPPI-expressing T-cells and myeloid cells, perhaps a significant proportion of TNF-α–producing leukocytes also expresses DPPI. This possibility may warrant further investigation.

It should be noted, however, that LLME only partially prevented lethal GVHD in certain murine settings, including an MHC class II–disparate model [B6 → ((B6 × bm12) F_1] [28]; a host-presensitized, semiallogeneic MHC-mismatched combination (B6 → (B6D2; the same system used in this study) [29]; and a fully allogeneic MHC-mismatched model (C3H → (B6) [30]. All 3 studies were performed by administering LLME-treated T-cells at the time of bone marrow transplantation, whereas in the results reported here, cells were given as DLI 2 weeks posttransplantation, weakening the GVHD potential of the donor cells. Recognizing the fact that in the semiallogeneic study, LLME treatment only extended MST but did not increase the proportion of long-term survivors, this model was nevertheless adapted for assessing LLME-treated DLI capability because of its relevance to clinical haploidentical HSCT.

LLME has likewise yielded somewhat mixed results in canine studies. An initial study suggested that in vitro allogeneic proliferative responses as well as progenitor cell colony counts were reduced by LLME treatment of canine peripheral blood and marrow, respectively [31]. In another study, administration of stem cell factor to dogs receiving MHC-matched, LLME-treated bone marrow transplants failed to enhance engraftment [32]. Yet an investigation of the effects of LLME on canine GVHD demonstrated that the agent was partially capable of preventing disease [15].

These findings in animal models complemented in vitro studies with human cells, in which LLME could deplete human NK cells, lymphokine-activated killer cells, monocytes, certain T-cells, and granulocytes, but also reduced human colony-forming unit (CFU) potential [17,33]. An attempt to prevent the apparent human stem cell toxicity of LLME by incubating CFU with interleukin-1 prior to exposure resulted in increased CFU–granulocyte, erythrocyte, megakaryocyte, macrophage (GEMM), but not CFU–granulocyte-macrophage (GM) or burst-forming unit, erythroid (BFU-E) viability [34]. In this regard, it is likely that the effect of LLME on stem cells is indirectly mediated by its effect on accessory cells necessary for their survival, rather than via direct toxicity on the stem cells themselves, because they appear to lack DPPI activity (I. Weissman, MD, Stanford University, personal communication, November 2000). Alternatively, it is possible that the observed failures of engraftment actually represented immunologic rejection of the marrow, as can occur after marrow manipulations such as T-cell depletion.

In a phase I clinical trial conducted to test the ability of LLME to prevent GVHD, bone marrow grafts were incubated ex vivo with LLME prior to infusion into patients undergoing HLA-matched or -mismatched transplantation [16]. The highest dose of LLME tested (500 μM) yielded a significant effect on acute GVHD development, with only 1 of 3 patients exhibiting grade I symptoms. Unfortunately, this dose of LLME also resulted in suppression of CFU-GM, and 1 patient died from secondary graft failure, halting the study.

Based on the sizable body of work previously performed on LLME and the potential problems encountered in stem cell engraftment, we hypothesized that the more recent development of DLI therapy might be more amenable to the use of the agent as a means of GVHD prophylaxis. Any effects of LLME on donor stem cells are irrelevant in the setting of a DLI graft given to a patient who has already had successful engraftment following HSCT. However, GVHD is still a major concern with the DLI approach, considering that the overall incidence of disease development is approximately 55% of all patients, half of whom experience grade II and a quarter who experience grades III to IV levels of severity with a high proportion of fatalities [18,19]. In

smaller but more recent studies with HLA-identical DLI patients, the incidence of severe acute GVHD ranged between 8% and 40% [35,36]. Clearly, DLI-associated GVHD remains a clinical problem, and, thus, LLME treatment in this modality may be beneficial.

In the murine model studied here, we demonstrate that LLME treatment of DLI can prevent lethal GVHD and associated cachexia, even at a physiologic maximum dose of 1.5×10^8 host-presensitized cells (Figure 4). This result is in stark contrast to the rapid GVHD-induced deaths of mice receiving an equivalent dose of mock-treated DLI (MST, 14 days). This observation is interesting considering that non–perforin/granzyme-dependent cytolytic mechanisms (ie, FasL/Fas) may still cause some level of GVHD in other systems [7-9,37]. In the first of 2 possibilities to account for this difference in the DLI setting, there is likely to be a decreased level of inflammatory cytokines available to upregulate Fas expression on relevant cells in target tissues. Second, all of the other studies reporting FasL-mediated GVHD effects involved either perforin-, granzyme-, or DPPI-deficient mice that merely lacked the single gene product. In contrast, LLME treatment of donor splenocytes would likely eliminate all T-cells that contain cytotoxic granules and DPPI, including those that might also coexpress FasL or possess other inflammatory capabilities, such as the production of TNF-α. Thus, either or both possibilities could result in a vastly reduced GVHD potential of the LLME-treated DLI population.

Histological analysis indicated that LLME treatment of DLI could prevent GVHD-related pathology in the spleens of recipients, but the treatment did variably result in some hepatic periportal infiltrates at the highest donor cell doses (see Results). The latter effect could be due to the activity of FasL-bearing T-cells, because they have been postulated to play a critical role in hepatic GVHD by targeting constitutively Fas-expressing hepatocytes [27,30]. The gastrointestinal tract is another organ in which Fas can be constitutively expressed. However, gut samples did not exhibit histological evidence of GVHD (data not shown), and the lack of any weight loss in LLME-treated DLI recipients compared to the control ATBM group (Figure 5) argues against any pathological involvement in that GVHD target organ.

The data clearly suggest that mice receiving LLME-treated DLI can still mediate a GVL response without concomitant clinical GVHD (Figure 8). Although the mechanism of this effect is still unclear, it does not appear to involve TNF-α, because the MMD2-8 leukemia cells are insensitive to TNF-α–mediated lysis (Figure 1) and also produce their own TNF-α cytokine. The FasL/Fas pathway is still a likely candidate for GVL activity because of the high levels of constitutive Fas expression on the MMD2-8 cells (Table 1). Alternatively, other lytic pathways may be operative, such as the TNF-related apoptosis-inducing ligand (TRAIL) [38]. Both activated human and mouse T-cells express TRAIL [39,40], and this ligand can induce apoptosis by binding to a number of receptors on target cells, including the Apo2/designated death receptor-4 (DR4)/TRAIL receptor-1 [41] and DR5/TRAIL receptor-2 [42]. In this regard, it has been demonstrated that mouse $CD4^+$ T-lymphocytes expressing TRAIL can lyse myeloid cells in a Fas-independent manner [43]. The precise mechanism of the LLME-treated DLI GVL effect observed in our model system is under further investigation, including the phenotype of the effector cell in anti-MMD2-8 CTL assays. We will also be examining whether multiple infusions of LLME-treated DLI at lower doses can further enhance the GVL effect.

In regard to other potential mechanisms by which LLME-treated cells could mediate GVL activity, it is formally possible that transferred $CD4^+$ T-cells could still provide cytokine help for the true effector cells, eg, NK cells. However, LLME also effectively eliminates NK cells, and it is unlikely that there are any residual host lymphocytes, including NK cells, after the high-dose radiation exposure (13 Gy, split dose), although NK cells are the most rapidly reconstituting lymphoid population after human marrow transplantation [44]. Another remote possibility is that the DLI transfer contains residual LLME at concentrations sufficient to directly kill myeloid leukemia cells in vivo. However, NK cells, the most LLME-sensitive leukocyte population, are depleted in vivo only at a concentration of ≥500 μg/g body wt of LLME, whereas in vitro depletion occurs efficiently in 9 μg/mL solutions (D. L. Thiele, MD, University of Texas Southwestern Medical Center, personal communication, 2000). Considering that cells designated for use as DLI were incubated in 250 μM (approximately 9 μg/mL) LLME and washed twice with 50 mL PBS, it is highly unlikely that mice received sufficient LLME to deplete any cells.

The most interesting aspect of the LLME-treated DLI approach is that the cells retain a significant level of immunoresponsiveness, as demonstrated in earlier studies [13,29,33] and in the current investigation. LLME treatment of donor splenocytes left a large proportion of $CD4^+$ T-cells and B-cells intact (Table 2), and these cells were still responsive in vitro to ConA or LPS mitogenic stimulation, as well as to specific recall antigen (OVA) stimulation (Figure 4). Furthermore, in addition to the above-mentioned retention of GVL potential, splenocytes from recipient mice still exhibited adequate proliferative responsiveness to LPS stimulation at both 1 and 10 weeks post-DLI transfer, arguing against any GVHD-induced B-cell dysfunction (Figure 7). These findings were also consistent with previous reports using the same murine model but with administration of LLME-treated B6 splenocytes at the time of marrow transplantation [29]. However, our data are in contrast to another study suggesting that GVHD-induced B-cell dysfunction is FasL-dependent and not reliant on perforin-mediated mechanisms [45]. We apparently do retain some FasL-mediated cytolytic potential (Figure 2), albeit at a level that might be below the threshold to cause immunosuppressive B-cell dysfunction.

Because the ex vivo LLME treatment of donor lymphocytes can retain a significant level of $CD4^+$ T-cell and B-cell immune functionality, the approach may be highly useful in resolving the problem of poor immune reconstitution following haploidentical HSCT [44,46-48]. $CD4^+$ T-cell counts often remain at suboptimal levels (<150 counts/mL) for up to 6 months or longer in these patients, and, as a result, they are at high risk for opportunistic infections. Because LLME spares the majority of $CD4^+$ T-cells, the transfer of these cells could potentially restore adequate immunoresponsiveness, without high GVHD risk, until de novo immune capability can be generated. DLI is already administered to prevent or treat opportunistic infections, as

well as leukemic relapse [47,49]. Ex vivo LLME treatment of a DLI population would theoretically enable earlier post-HSCT transfers and at higher doses to more effectively restore immunocompetence to the patient. In this context, it is noteworthy that recovery of $CD8^+$ T-cells may occur quite rapidly after conventional or T-cell–depleted HSCT, whereas $CD4^+$ T-cell recovery is far more delayed in both patient groups. The preservation of $CD4^+$ T-cells in the DLI thus might complement the endogenous lymphocyte redevelopment occurring in vivo. In light of these potential advantages and the preclinical results, a phase I clinical trial has been initiated to test the ability of LLME-treated DLI to accelerate functional immune recovery after HSCT while minimizing the risks of developing severe GVHD.

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