Pretreatment of Murine Donor Grafts With L-Leucyl-L-Leucine Methyl Ester: Elimination of Graft-Versus-Host Disease Without Detrimental Effects on Engraftment

By Bruce R. Blazar, Dwain L. Thiele, and Daniel A. Vallera

Incubation of murine bone marrow and splenocytes with the dipeptide methyl ester, L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), which results in the selective depletion of cytotoxic T cells and their precursors, natural killer cells, and monocytes, completely protected 30 recipients of fully allogeneic donor grafts from lethal graft-versus-host disease (GVHD). These results were comparable with those obtained in 30 recipients of anti-Thy 1.2 plus complement (C')-treated donor marrow. However, in contrast to antibody- and C'-dependent T-cell depletion, which reduces the level of donor cell engraftment in our model system, we did not observe such effects using Leu-Leu-OMe marrow pretreatment. As compared with the 24 H-2 typed recipients of anti-Thy 1.2 + C'-treated donor grafts, the 29 H-2 typed recipients of Leu-Leu-OMe–treated donor grafts had significantly ($P < .001$) higher percentages of donor cells (mean = 93% *v* 74%) and significantly ($P < .001$) lower percentages of host cells (mean = 6% *v* 15%) posttransplantation. In vitro limiting dilution assay (LDA) was performed to assess the comparative efficacy of cytolytic T-lymphocyte (CTL) precursor depletion by Leu-Leu-OMe or anti-Thy 1.2 + C' pretreatment. We observed greater levels of CTL precursor depletion in Leu-Leu-OMe treated as compared with anti-Thy 1.2 + C'-treated bone marrow plus spleen cells (BMS) obtained from nontransplanted mice. This suggests that the in vivo results cannot simply be attributed to a less efficacious functional inactivation of cytolytic T-cell precursors by Leu-Leu-OMe treatment as compared with anti-Thy 1.2 + C' treatment. Immunoreconstitution was similar in recipients of Leu-Leu-OMe–treated grafts and anti-Thy 1.2 + C'–treated grafts 100 days posttransplant. In our opinion, Leu-Leu-OMe marrow pretreatment deserves further investigation as a methodology to achieve GVHD prevention without significantly reducing the propensity toward host cell repopulation.

DESPITE THE in vivo use of immunosuppressive drugs such as methotrexate and cyclosporin A, graft-versus-host disease (GVHD) remains a significant complication in bone marrow transplantation (BMT), even when the procedure is restricted to histocompatible siblings.[1] Removal of the GVHD-causing T-lymphocyte population from donor marrow in preclinical studies in rodents,[2-6] dogs,[7,8] and primates,[9] and in human clinical trials,[1,10-12] has successfully reduced the incidence and overall severity of GVHD. However, T-cell depletion (TCD), regardless of the technique, has significantly increased the incidence of graft rejection 10% to 30% in recipients of histocompatible sibling donor grafts, whereas without TCD the incidence is 0.5% to 3%.[1,10-12] Although these findings may argue against TCD, more selective or less effective TCD might eliminate GVHD-causing cells without removing cells essential to the engraftment process.

The dipeptide methyl ester, L-leucyl-L-leucine methyl ester (Leu-Leu-OMe), is a metabolite generated by the lysosomal processing of L-leucine methyl ester by myeloid cells such as polymorphonuclear leukocytes or mononuclear phagocytes.[13] Incorporation of Leu-Leu-OMe into cytolytic T lymphocytes (CTL) and their precursors as well as into natural killer cells (NK) and monocytes, results in cell death.[13,14] The ability of Leu-Leu-OMe to spare cells with helper cell function[15,16] renders it effective for dissecting the role of T-cell subpopulations in GVHD and engraftment.

We have previously described a murine BMT model in which histoincompatible donor marrow is pan T-cell depleted and recipients are conditioned for BMT with 8.0 Gy of total body irradiation (TBI).[17] In this model, GVHD is lethal with recipient death occurring 2 to 6 weeks post-BMT. The same model can be modified to produce a varying degree of host cell repopulation by the recipient. Using this system, we found that Leu-Leu-OMe treatment of class I + class II major histocompatibility complex (MHC) disparate donor marrow eliminates a population of cells that mediates GVHD, similar to pan-T depletion with anti-Thy 1.2 + complement (C'). Under these identical marrow pretreatment conditions, the level of donor cell chimerism was not reduced, in contrast to pan-T depletion. These results suggest that GVHD prevention may be accomplished without interfering with donor cell engraftment.

MATERIALS AND METHODS

Mice. B10.BR (H-2^k) and C57BL/6 (H-2^b) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and the National Institutes of Health (Bethesda, MD). They were maintained in the University of Minnesota mouse colony. Animals were housed in conventional cages with filter lids, fed a fat-supplement diet, and given antibiotic-supplemented water for 2 days before

From the Departments of Pediatrics, Divisions of Bone Marrow Transplantation and Pediatric Oncology; Therapeutic Radiology, Section on Experimental Cancer Immunology; and the Institute of Human Genetics, University of Minnesota Hospitals and Clinics, Minneapolis; and the Department of Internal Medicine, Liver Unit, University of Texas Southwestern Medical Center at Dallas.

Submitted February 24, 1989; accepted October 4, 1989.

Supported in part by US Public Health Service Grant Nos. PO1-CA-21737, T32-CA-09445, RO1-CA-31618, RO1-CA-36725, R01-AI-24639, and R23-A1-19254 awarded by the National Institutes of Health; the American Cancer Society Research Grant No. IM-502; the Minnesota Medical Foundation; and the Children's Cancer Research Fund. B.R.B. is a recipient of Clinical Investigator Award No. K08 AM1509. This paper is no. 14 in a series on murine bone marrow transplantation across the MHC barrier and no. 7 in a series on murine engraftment. This is Center for Experimental Transplantation and Cancer Research Manuscript no. 55.

Address reprint requests to Bruce R. Blazar, MD, Box 109 UMHC, Harvard St at East River Rd, University of Minnesota, Minneapolis, MN 55455.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" *in accordance with 18 U.S.C. section 1734 solely to indicate this fact.*

0006-4971/90/7503-0016$3.00/0

pretransplant immunosuppression and for 1 month posttransplant. Recipients were at least 8 weeks old; donors were at least 4 weeks old.

Recipient pretransplant conditioning. Our conditioning protocols for these strain combinations have been previously described.[17] For GVHD experiments, recipients were irradiated to a total dose of 8.0 Gy using a 220 KeV x-ray source at a dose rate of 0.45 Gy/min. For engraftment experiments, recipients received 7.0 to 7.25 Gy TBI.

Bone marrow pretreatment. Our procedure has been previously described in detail.[17-19] C57BL/6 marrow with or without splenocytes (1:1 ratio) were suspended at a concentration of 20×10^6 mL. The donor inoculum was depleted of T cells by incubation with a pan-T cell monoclonal antibody (MoAb) anti-Thy 1.2 (H0-13-4-9, American Tissue Culture Collection, [ATCC], Rockville, MD; diluted 1:25,000) for 30 minutes, at 4°C, followed by an additional 45 minutes at 37°C with low background baby rabbit C′ (Pel-Freez, Rogers, AK; diluted 1:6). Marrow treated with Leu-Leu-OMe and control marrow were handled identically to anti-Thy 1.2 + C′–treated marrow except for the omission of anti-Thy 1.2. After treatment, all aliquots were washed, resuspended at 10^7 cells/mL, and incubated an additional 15 minutes at room temperature. For the Leu-Leu-OMe–treated marrow only, Leu-Leu-OMe was added to a final concentration of 250 μmol/L.[20] At this concentration, all cytolytic and monocytic functions were eliminated[14-16] (see Results). All marrow groups were then washed once in cold media and adjusted to 100×10^6 cells/mL for GVHD experiments or 40×10^6 cells/mL for engraftment experiments. One-half milliliter was injected into each recipient via the caudal vein. Recipients were monitored daily for survival and weight loss during the 100-day observation period.

Limiting-dilution assay (LDA) of splenocyte CTL precursor frequencies. For LDA analysis of CTL precursors, single cell suspensions of bone marrow plus spleen cells (BMS), obtained from C57BL/6 (donor strain) mice, were sham-treated, treated with 250 μmol/L Leu-Leu-OMe, or treated with anti-Thy 1.2 + C′ as described above, and resuspended in supplemental Dulbecco's Modified Eagle's Media for LDA analysis as previously described.[21] Various numbers of responder splenocytes were plated with 10^6 irradiated (30 Gy) B10.BR (host strain) stimulator splenocytes and 100 U recombinant murine interleukin-2 (IL-2, a generous gift of Hoffman-LaRoche, Nutley, NJ). After 8 days, each well was assayed for cytolytic activity against ^{51}Cr-labeled B10.BR splenic blasts (Con A-stimulated). Wells were scored as positive if they released more ^{51}Cr (mean + 3 SD) than microwells containing stimulator spleen cells and IL-2, but no responder cells.

Minimal estimates of the CTL precursor frequencies were obtained from the Poisson distribution relationship between the responding cell number per culture and the logarithm of the percentage of nonresponding (negative) cultures. When plotted according to the zero-order Poisson equation, the data define a straight line with a slope equal to the frequency of CTL precursors.

Chimerism analysis. In the first engraftment experiment, chimerism was assessed with complement-dependent microcytotoxicity analysis[6,17] using noncrossreactive anti-H-2 specific MoAbs (anti-H-2^b: clone 28-8-6, reference 22; anti-H-2^k: clone 12-2-S, reference 23) between days 38 through 41. Mice were retyped on days 53 through 56 post-BMT to further analyze engraftment stability.[24,25] For the second engraftment experiment, chimerism of peripheral blood mononuclear cells was analyzed at a later time period than experiment 1, between days 53 through 70 post-BMT. Engraftment was quantified by analyzing the cell surface binding of anti-H-2 MoAbs linked to fluorochromes on a FACScan (Becton-Dickinson, Mountain View, CA). Anti-H-2^k (clone 11-4.1, mouse immunoglobulin G2a [IgG2a], ATCC, Rockville, MD) or anti-H-2^b (clone EH144, mouse IgG2b, provided by Dr T.V. Rajan, Albert Einstein University, New York, NY) were directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE), respectively, as previously described.[26] An irrelevant mouse myeloma protein (UPC_{10}), mouse IgG2a; Litton Bionetics, Charleston, SC) was conjugated to FITC or PE to determine the degree of background binding. To prevent nonspecific binding by mouse macrophage and lymphocyte Fc receptors, all samples were first incubated with a saturating concentration (40 μg/mL) of an anti-Fc receptor specific MoAb (clone 2.4G2, generously provided by Dr Richard Hodes and Susan Sharrow, National Institutes of Health) for 15 to 30 minutes at 4°C.[27] The peripheral blood mononuclear cells were then incubated with an optimal concentration of fluorochrome-labeled antibody for 45 minutes at 4°C. Cells were washed three times and resuspended for analysis. Background binding using UPC_{10}-FITC and UPC_{10}-PE was subtracted from the results obtained with the anti–H-2 specific fluorochrome-labeled antibodies, and percent donor or host cells were calculated as described.[28] Zero to 1% of the control peripheral blood mononuclear cells stained positive with the irrelevant anti–H-2 antibody linked to fluorochrome, while 99% to 100% of cells were positive for the relevant anti–H-2 specific antibody linked to fluorochrome.

Hematologic evaluation of recipients post-BMT. Peripheral blood, 150 to 250 μL, was obtained by retro-orbital venipuncture on days 7, 14, and 28 post-BMT. Leukocyte number and morphology were determined by examination of Wright-Giemsa stained slides.[28-31] Hematocrit values were determined by capillary tube red cell to plasma volume ratios after centrifugation.

Immunoreconstitution studies. Immunoreconstitution was tested in mice surviving 100 days post-BMT. (1) Rejection of donor and third-party (BALB/c: H-2^d) skin was tested (n = 3 mice/group). Our skin grafting procedure has been previously described.[32] (2) NK function was tested against NK sensitive YAC-1 tumor targets.[17] Percent cytotoxicity was determined at effector to target ratios of 100:1, 50:1, 25:1, and 12.5:1. (3) CTL function was assessed as described,[17] except that 10^4 P815 (H-2^d) myeloma tumor target cells (NK-resistant) were substituted for YAC-1 target cells. Effector: target ratios were 50:1, 25:1, 12.5:1, and 6.25:1.

Statistical analyses. Group comparisons of continuous data were made by Student's *t*-test. Survival data were analyzed by lifetable methods using the Mantel-Peto-Cox summary of chi square.[33]

RESULTS

Efficacy of Leu-Leu-OMe treatment and anti-Thy 1.2 + C′ treatment in depleting CTL precursor cells. To determine the comparative efficacy of Leu-Leu-OMe treatment and anti-Thy 1.2 + C′ treatment to deplete CTL precursors, we performed an LDA on treated bone marrow plus splenocytes (BMS). A representative experiment showing the CTL precursor frequency is depicted in Fig 1. A frequency of 1/4,601 was measured for the sham-treated control splenocytes. Anti-Thy 1.2 + C′ treatment reduced the frequency to 1/61,489. Leu-Leu-OMe pretreatment of the same population reduced the frequency to 1/1,214,759. In a second experiment, both the anti-Thy 1.2 + C′-treated and Leu-Leu-OMe–treated BMS populations had comparable CTL precursor frequencies of less than 1/1,000,000, greater than two logs of depletion as compared with the sham-treated controls (frequency = 1/6,596). In neither instance was the CTL precursor frequency of the anti-Thy 1.2 + C′–treated BMS population less than the frequency of the Leu-Leu-OMe–treated BMS population. An analysis of 11 experiments in which anti-Thy 1.2 + C′ or Leu-Leu-OMe pretreatment of

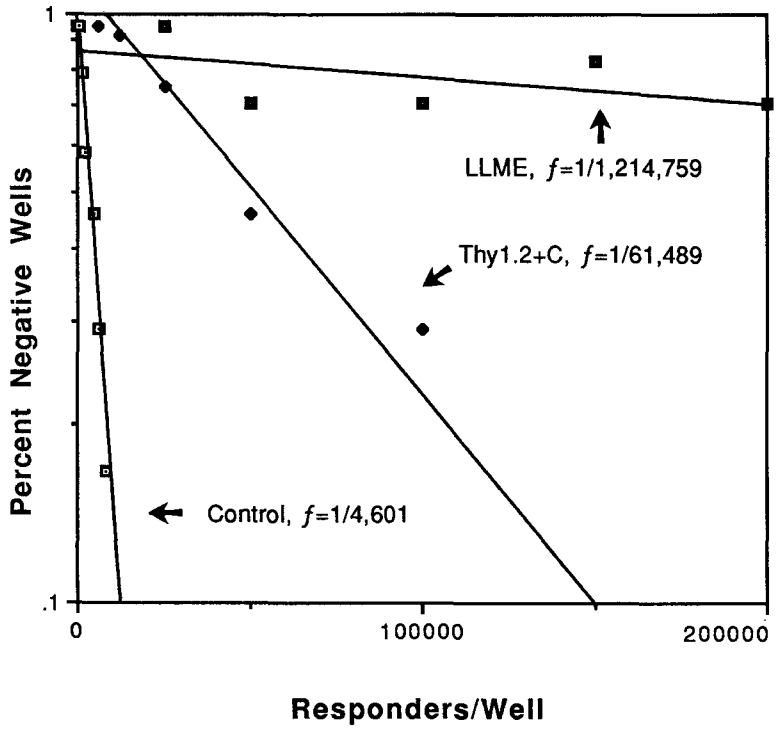

Fig 1. Effect of Leu-Leu-OMe treatment and anti-Thy 1.2 + C′ treatment on CTL precursor elimination as assessed by LDA analysis. A representative experiment is depicted. BMS from C57BL/6 mice were sham-treated (controls), treated with anti-Thy 1.2 + C′, or treated with Leu-Leu-OMe (LLME). CTL precursor frequencies (f) were calculated. The number of responder cells/well is plotted against the percent of negative wells. The straight lines are linear regression lines.

bone marrow (with or without supplemental splenocytes) was used for T-cell depletion before BMT showed comparable cell yields for these groups. Taken together, the data do not show that a higher number of CTL precursors were infused in the Leu-Leu-OMe pretreated donor preparation as compared with the anti-Thy 1.2 + C′–treated donor preparation.

Efficacy of Leu-Leu-OMe treatment of bone marrow/splenocytes in preventing GVHD induction across class I + class II MHC barriers. To study the ability of Leu-Leu-OMe to prevent GVHD, donor C57BL/6 grafts were pretreated and injected into irradiated B10.BR recipients. The survival curves are depicted in Figs 2A (experiment 1) and 2C (experiment 2). None of the recipients of Leu-Leu-OMe–treated donor cells in either experiment (n = 30) developed GVHD. In the control group receiving nontreated grafts, signs of GVHD (ruffled fur, weight loss, diarrhea, runting, alopecia) were noted between days 12 and 20 post-BMT. No control mice survived 37 days post-BMT (overall median survival = 27 days), which was significantly ($P < .0001$) shorter than survival in the Leu-Leu-OMe–treated group. Treatment of donor grafts with anti-Thy 1.2 + C′ protected all but one mouse from GVHD-induced mortality. An additional four deaths occurred in this group on day 4 post-BMT, before the onset of GVHD in the control group. These early deaths were presumed to be non-GVHD related. During the entire 100-day observation period, no signs of acute or chronic GVHD (alopecia, ruffled fur, diarrhea, weight loss, leukopenia, anemia, inability to reject skin graft or mount a CTL or NK response, or increased mortality as a result of immunodeficiency disorders) appeared in either the Leu-Leu-OMe–treated or anti-Thy 1.2 + C′-treated groups.

Mean weight curves also indicate that Leu-Leu-OMe or anti-Thy 1.2 + C′ treatment of donor grafts protected mice from GVHD. As shown in Figs 2B (experiment 1) and 2D (experiment 2), mice in the control group experienced a progressive weight loss beginning 7 to 11 days post-BMT, consistent with the onset of acute GVHD. In contrast, recipients of grafts treated with Leu-Leu-OMe or anti-Thy 1.2 + C′ steadily gained weight.

We performed extensive histopathologic analysis of tissue samples including skin, liver, gastrointestinal tract, and lung and have been unable to detect subclinical GVHD in recipients of pan-T depleted marrow/splenocyte grafts in contrast to recipients of nonmanipulated marrow grafts (unpublished data). Moreover, studies varying the number of supplemental splenocytes (approximately 20% T cells) used to induce GVHD from 1×10^5 to 25×10^6 per mouse (n = 8/group) show a dose response in GVHD-induced mortality. As few as 10^6 splenocytes (approximately 2×10^5 T cells) caused 100% lethality by day 70 post-BMT (data not shown). Supplemental splenocytes, 10^5 (approximately 2×10^4 T cells), resulted in a 37.5% GVHD-induced lethality by day 70 post-BMT. Also evident in these groups of mice was a progressive weight loss beginning between days 14 through 18 in all groups.

Serotyping was performed to determine whether repopulating cells were of donor or host origin. In the first experiment, the 11 surviving recipients of anti-Thy 1.2 + C′–treated grafts and the 17 surviving recipients of Leu-Leu-OMe–treated grafts had comparable donor cell numbers (mean ± 1 standard error of the mean = 91 ± 3 *v* 92 ± 3, respectively) and host cell numbers (16 ± 3 *v* 20 ± 5, respectively). In experiment 2, the donor cell numbers (93 ± 1 *v* 96 ± 1) and

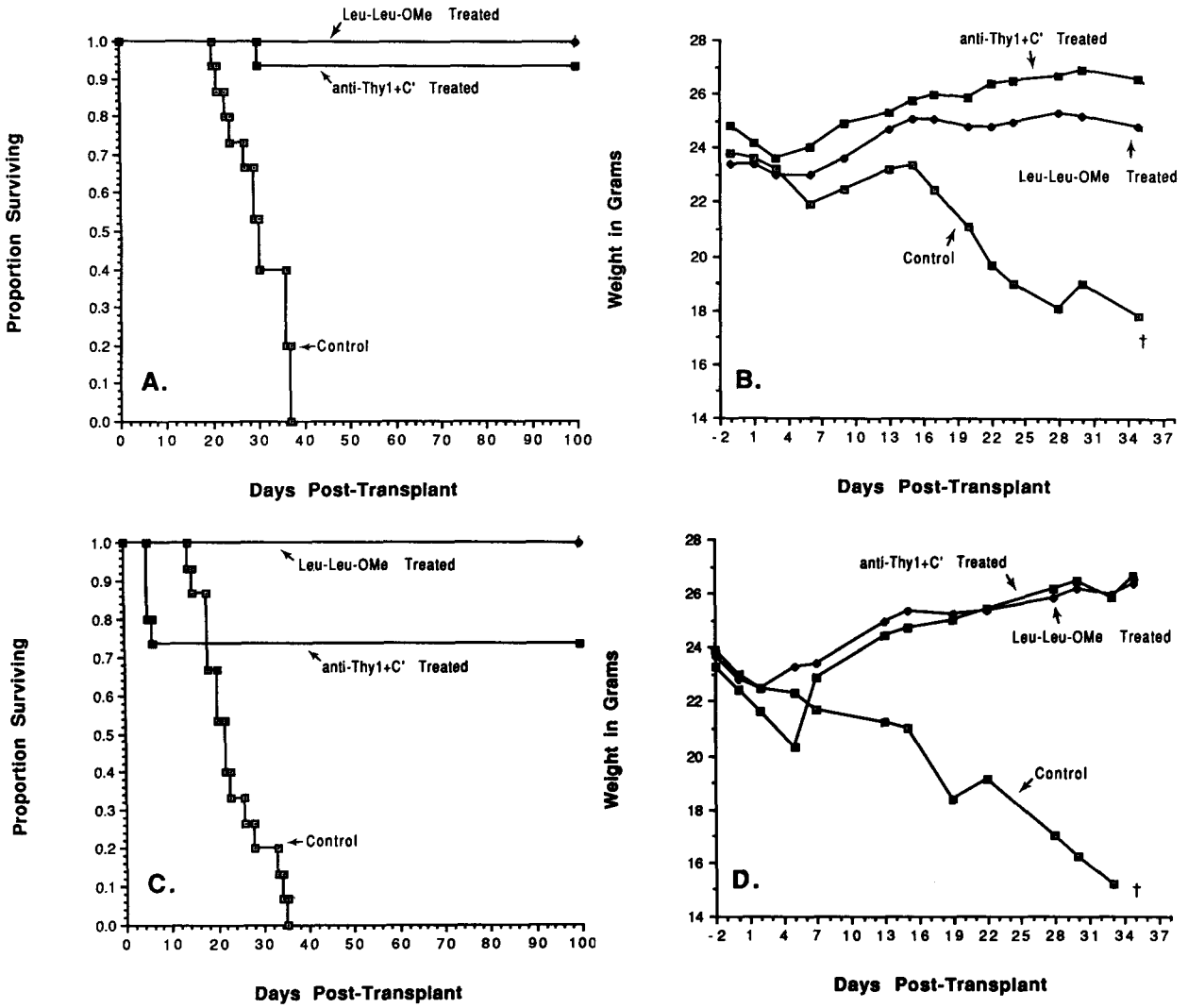

Fig 2. Effect of Leu-Leu-OMe treatment and anti-Thy 1.2 + C' treatment on GVHD induction. Donor C57BL/6 marrow and splenocytes (1:1 ratio) were treated with Leu-Leu-OMe or anti-Thy 1.2 + C' and then washed. Cells, 50 × 10^6, were injected into B10.BR recipients conditioned with 8.0 Gy TBI. Two experiments are shown containing 15 animals/group. Groups included recipients of Leu-Leu-OMe–treated grafts or anti-Thy 1.2 + C'–treated grafts, or controls. (A) One experiment in which actuarial survival is plotted. (B) The mean weights of animals in (A) at various time points post-BMT. The dagger indicates the time by which all animals had died. (C and D) A second experiment identical in procedure to the first.

host cell numbers (0 ± 0 *v* 0 ± 0) were similar for the 14 surviving recipients of anti-Thy 1.2 + C'–treated grafts and the 15 surviving recipients of Leu-Leu-OMe–treated grafts. None of the control mice in either experiment survived long enough to obtain serotyping data.

Leu-Leu-OMe treatment but not anti-Thy 1.2 + C' treatment permits donor cell engraftment to occur in recipients of H-2 disparate donor grafts. For engraftment studies, we used our previously described modifications of the C57BL/6 into B10.BR system.[17]

Cumulative donor cell data indicate a significantly higher level of chimerism among recipients of Leu-Leu-OMe–treated grafts or control grafts as compared to recipients of anti-Thy 1.2 + C'–treated grafts (Table 1). Percent donor cells was significantly ($P < .001$) lower in 24 recipients of anti-Thy 1.2 + C'–treated marrow (74 ± 4) as compared with 29 recipients of Leu-Leu-OMe–treated marrow (93 ± 1) or 27 controls (99 ± 2). Although cumulative donor cell data suggest that recipients of Leu-Leu-OMe–treated grafts may have experienced slightly impaired engraftment when compared with controls, cumulative host cell data are not consistent with such an impairment. Twenty-four recipients of Leu-Leu-OMe–treated grafts had comparable host cell values (6 ± 1) to those for 27 recipients of control grafts (4 ± 1), and values for both groups were significantly ($P < .001$) lower when compared with those for recipients of anti-Thy 1.2 + C'–treated grafts (15 ± 2). Because these data were obtained in mice at intervals up to 2.5 months post-BMT and at two time points, we conclude that the observed differential effect of pan-T depletion and Leu-Leu-

Table 1. Comparison of the Effects of Murine Engraftment When Donor Marrow Is Treated With Leu-Leu-OMe or Pan-T Cell Depleted

Experiment	Group*	No. BMT	No. H-2 Typed†	% Donor‡	% Host‡
1	Anti-Thy + C′	18	11	77 ± 9	8 ± 1§
	Leu-Leu-OMe	18	17	99 ± 1‖	3 ± 1
	Control	18	14	99 ± 1‖	9 ± 2
2	Anti-Thy + C′	14	13	72 ± 4	19 ± 2
	Leu-Leu-OMe	14	12	85 ± 2¶	9 ± 2‖
	Control	14	13	99 ± 0‖	0 ± 0‖
1-2 (pool)	Anti-Thy + C′	32	24	74 ± 4	15 ± 2
	Leu-Leu-OMe	32	29	93 ± 1‖	6 ± 1‖
	Control	32	27	99 ± 1‖	4 ± 1‖

B10.BR ($H\text{-}2^k$) recipients were conditioned with 7.0 to 7.25 Gy TBI. The next day, recipients received 20×10^6 bone marrow cells from C57BL/6 ($H\text{-}2^b$) donors.

*Mice received bone marrow that was treated in vitro with C′ (controls), anti-Thy 1.2 + C′, or 250 μmol/L Leu-Leu-OMe as described in Materials and Methods.

†All mice surviving the first 5 weeks post-BMT were H-2 phenotyped for engraftment by microcytotoxicity or by FACS.

‡Data are expressed as mean % ± 1 SEM.

§H-2 phenotyping using the anti-host specific MoAb was not possible. Retyping of eight remaining recipients 2 weeks later is shown. The mean percent donor cells at that time was 87%.

‖$P < .001$ as compared with recipients of anti-Thy 1 + C′-depleted marrow grafts.

¶$P < .01$ as compared with recipients of anti-Thy 1 + C′-depleted marrow grafts.

OMe marrow pretreatment were persistent. Analysis of individual experiments was consistent with our findings with the cumulative data.

Hematologic reconstitution in recipients of Leu-Leu-OMe–treated, anti-Thy 1.2 + C′–treated or control marrow grafts. Detailed hematologic data are presented in Table 2. During the entire study period, erythroid and total leukocyte recovery in the control group and Leu-Leu-OMe–treated group and erythroid recovery (hematocrit) in all groups were not significantly different. Total leukocyte recovery in the anti-Thy 1.2 + C′–treated group was consistently lower than in the other two groups. Recipients of anti-Thy 1.2 + C′–treated grafts had impaired leukocyte reconstitution, which was most evident 28 days post-BMT. These mice (n = 14) had significantly ($P \leq .002$) lower leukycyte numbers than recipients (n = 12) of Leu-Leu-OMe–treated grafts or recipients (n = 13) of control grafts. Differences in leukocyte numbers were primarily due to lymphocyte regeneration. Recipients of anti-Thy 1.2 + C′–treated grafts had significantly lower ($P < .001$) absolute lymphocyte counts (ALC) than recipients of control grafts on day 14 and day 28 post-BMT. The ALC for recipients of Leu-Leu-OMe–treated grafts was not significantly different than for controls on day 28 post-BMT, although recipients of Leu-Leu-OMe–treated grafts did have a lower ALC on day 14 post-BMT. Absolute neutrophil counts (ANC) were not significantly different among the three groups at any time point post-BMT.

Immunoreconstitution in recipients of Leu-Leu-OMe–treated, anti-Thy 1.2 + C′–treated, or control marrow grafts. We measured the time to skin graft rejection of third-party grafts and the capacity of day 100 post-BMT recipients to tolerate donor skin grafts. C57BL/6 skin grafts were tolerated without evidence of scarring, retraction, or necrosis in nine recipients (n = 3 mice/group) during a 5-week observation period. In contrast, third-party grafts were rejected in all nine recipients within 14 days. No differences were noted in the speed of skin graft rejection between recipients of Leu-Leu-OMe–treated, anti-Thy 1.2 + C′–treated, or control marrow grafts.

Splenic NK and CTL function was measured in mice surviving 100 days after transplantation in two experiments.

Table 2. Hematologic Recovery of Mice That Received Pan-T Cell Depleted, Leu-Leu-OMe-Treated, or Control Marrow Grafts

	Day 7 Post-BMT			Day 14 Post-BMT			Day 28 Post-BMT		
	Thy + C	LLME	Control	Thy + C	LLME	Control	Thy + C	LLME	Control
Leukocytes ($\times 10^3/\mu L$)	0.5 ± 0.1*	0.7 ± 0.2	0.7 ± 0.3	2.0 ± 0.2	2.3 ± 0.4	3.0 ± 0.3	4.5 ± 0.5	8.3 ± 0.9†	9.0 ± 0.6†
Neutrophils (%)	38 ± 5	46 ± 6	41 ± 3	38 ± 2	41 ± 5	31 ± 4	28 ± 4	27 ± 4	20 ± 3
Lymphocytes (%)	62 ± 5	53 ± 7	59 ± 4	60 ± 2	57 ± 5	67 ± 4	71 ± 4	72 ± 4	79 ± 3
ANC ($\times 10^3/\mu L$)	0.2 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	1.1 ± 0.4	0.9 ± 0.1	1.2 ± 0.2	2.6 ± 0.7	1.7 ± 0.2
ALC ($\times 10^3/\mu L$)	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	2.2 ± 0.2†	3.2 ± 0.4	5.7 ± 0.5†	7.2 ± 0.7†
Hct (g/dL)	44 ± 0.5	44 ± 0.4	43 ± 0.4	44 ± 0.4	45 ± 1.4	43 ± 0.3	45 ± 0.8	44 ± 0.5	45 ± 0.5

Hematologic recovery was assessed in mice that were conditioned with 7.0 to 7.25 Gy TBI and transplanted with 20×10^6 marrow cells. Marrow cells were treated with anti-Thy 1 + C′ or Leu-Leu-OMe (LLME) at a final concentration of 250 μmol/L as described in Materials and Methods.

Abbreviations: Pan-T, anti-Thy 1.2 + C′ depleted; LLME, Leu-Leu-OMe; ANC, absolute neutrophil count; ALC, absolute lymphocyte count; Hct, hematocrit.

*Data are expressed as mean ± 1 SEM.

†$P < .01$ as compared with pan-T cell depleted recipients.

Recipients of Leu-Leu-OMe–treated marrow, sham-treated marrow, or normal nontransplanted murine splenocytes had comparable NK function. (For example, at an effector: target ratio of 100:1, cytolysis averaged 73.0%, 82.7%, or 80.1%, respectively.) They also had comparable CTL function. At an effector target ratio of 50:1, specific cytolysis averaged 87.0%, 95.3%, or 77.9%, respectively. Treatment of normal donor murine splenocytes with 250 μmol/L Leu-Leu-OMe was effective in eliminating NK and CTL function.

DISCUSSION

We first determined whether chemical pretreatment of donor marrows/splenocytes with Leu-Leu-OMe prevented lethal GVHD observed in B10.BR recipients of fully allogeneic C57BL/6 donor marrow grafts. Studies to date have suggested that lethal acute GVHD induced across a class I + class II MHC barrier is mediated mostly, but not entirely, by $CD5^+(Lyt1^+)$, $CD4^+(L3T4^+)$, $CD8^-(Lyt\ 2^-)$ T lymphocytes.[19,34,35] Although the $CD4^+$ population is dominated by cells capable of proliferation and cytokine production, some $CD4^+$ cytolytic cells are theoretically capable of mediating a donor anti-host reaction.[15]. Since pretreatment of donor grafts with Leu-Leu-OMe eliminates multiple types of cells that mediate cytolysis regardless of cell surface phenotype, while preserving cells that produce cytokines (predominantly $CD4^+$ with a minority of $CD8^+$ cells), Leu-Leu-OMe treatment provides a useful method for testing the relative importance of these cell functions in both GVHD and engraftment.

Previous studies demonstrated the efficacy of 250 μmol/L Leu-Leu-OMe for preventing lethal GVHD in naive F1 ($H\text{-}2^{d,b}$ or $H\text{-}2^{b,k}$) recipients of parental ($H\text{-}2^b$: C57BL/6) marrow/splenocyte donor grafts.[15] Furthermore, the in vitro suppressive effect of splenocytes isolated from mice undergoing GVHD across minor antigen barriers was inhibited by leucine methyl ester.[36] Leucine methyl ester is converted to Leu-Leu-OMe in lysosomotropic vesicles in myeloid cells[13] and is the toxic product that kills monocytes, NK, and CTL.[37,38]

In this study, a single brief pretreatment of fully allogeneic donor marrow/splenocyte grafts with Leu-Leu-OMe completely prevented lethal GVHD in all 30 recipients. There was no evidence of clinical or subclinical GVHD during the entire observation period of 100 days, in contrast to chronic skin GVHD previously seen in F1 recipients of parental donor grafts.[20] We compared Leu-Leu-OMe pretreatment to standard C′ dependent T-cell depletion using the MoAb anti-Thy 1.2. All control mice died by day 37 post-BMT in our study. The clinical appearance of murine recipients of Leu-Leu-OMe–treated grafts and murine recipients of anti-Thy 1.2 + C′–treated grafts was similar. However, survival was slightly lower in mice that received anti-Thy 1.2 + C′–treated grafts as compared with mice that received Leu-Leu-OMe–treated grafts.

We previously reported that donor NK alone are not important to the development of GVHD in this donor-recipient strain combination;[17] therefore, observed efficacy of Leu-Leu-OMe treatment in GVHD prevention cannot be attributed solely to NK removal. Moreover, since removal of donor $CD4^+$ and $CD8^+$ cells has protected recipients of fully allogeneic bone marrow/splenocytes from lethal GVHD similarly to anti-Thy 1.2 + C′,[19] the efficacy of Leu-Leu-OMe may result largely from the elimination of cytolytic $CD4^+$ and $CD8^+$ effector cells and their precursors. In vitro measurement of CTL precursors by LDA in these studies showed that Leu-Leu-OMe treatment of donor strain BMS eliminated greater than two logs of CTL precursors. The removal of $CD4^+$ CTL or CTL precursors may explain the partial protective effect seen by our group in previous studies.[19] However, the selective removal of $CD8^+$ cells was of minimal benefit in GVHD prevention.[19] Taken together, these data suggest that GVHD can be prevented either by removing the presumptive effector CTL, NK, and their precursors, (eg, by Leu-Leu-OMe treatment) or by removing donor alloantigen-stimulated cytokine-producing cells necessary for the in vivo expansion of GHVD effector cells and their precursors (eg, by anti-CD4 + C′ and anti-CD8 + C′, or anti-Thy 1.2 + C′ treatment). The cooperation between $CD4^+$ cells and $CD8^+$ T cells has also been observed in recipients of minor antigen disparate donor grafts[39] or anti-Thy 1.2 + C′–treated class I disparate donor grafts supplemented with $CD8^+$ cells and IL-2 (normally produced mainly by $CD4^+$ cells).[40]

Since Leu-Leu-OMe pretreatment of donor marrow grafts was an effective means of GVHD prophylaxis, we sought to determine if this strategy of removing cells based on their functional (as opposed to phenotypic) characteristics would result in a similar reduction in the level of chimerism as observed when using anti-Thy 1.2 + C′ for T-cell depletion. The occurrence of mixed chimerism with persistence and expansion of host cell populations in B10.BR recipients of C57BL/6 is dependent on the administration of a fixed dose of both TBI and TCD marrow.[17] Recipient host cell repopulation was favored by reducing the TBI dose and lowering the donor marrow cell dose according to previously established protocols.[6,17,24,25,28-31,35,41] These maneuvers significantly increased the proportion of repopulating recipient host cells when donor grafts were depleted of T cells with anti-Thy 1.2 + C′, but not when grafts were untreated.

The cumulative engraftment data from our studies are most consistent with a substantial reduction in chimerism resulting from anti-Thy 1.2 + C′ depletion and not Leu-Leu-OMe treatment or treatment with C′ alone. The slight reduction in the overall percent donor cells in the Leu-Leu-OMe–treated group as compared with controls was not observed in the first experiment, and may be ascribed to minor differences in the number of marrow cells infused, marrow composition, or slight variations in the in vivo myeloablation and/or immunosuppression delivered by TBI. In contrast, the reduced engraftment caused by anti-Thy 1.2 + C′ was reproducible and highly significant in overall analysis.

The observed differences in engraftment between anti-Thy 1.2 + C′ and Leu-Leu-OMe pretreatment do not appear to be merely a reflection of a less efficient elimination of the total number of CTL precursors by the latter technique. Specifically, the number of CTL precursors infused per mouse in the recipients of Leu-Leu-OMe pretreated inocula was likely to be equal to or less than the number infused in

recipients of anti-Thy 1.2 + C′–treated inocula based on both analysis of LDA data and quantiation of cell yields pre- and posttreatment in 11 separate BMT experiments. We favor the explanation that a true functional discrimination of the GVHD-causing cells from the engraftment promoting cells is possible by using this chemical compound, which spares helper T cells (in contrast to anti-Thy 1.2 + C′ treatment) while eliminating CTL precursor cells, since (1) the allogeneic engraftment observed after Leu-Leu-OMe marrow treatment is consistent with the uninhibited in vivo bone marrow proliferation seen in a 5-day in vivo ^{125}I-5-iodo-2′-deoxyuridine incorporation assay using recipients of syngeneic donor marrow;[20] and (2) published evidence also supports the feasibility of complete alloengraftment without GVHD, indicating that these processes can be dissociated in other experimental systems.[42-45] In these studies, selective TCD of syngeneic marrow administered with non–T-cell depleted fully allogeneic marrow protected mice from clinically apparent GVHD without decreasing the number of donor cells engrafted.

Peripheral blood hematologic recovery among recipients of Leu-Leu-OMe–treated grafts was not different from controls except for a moderate reduction in ALC on day 14 post-BMT, which was not evident on day 28 post-BMT. In contrast, recipients of anti-Thy 1.2 + C′–treated grafts had a significant reduction in ALC on days 14 and 28 post-BMT that was largely independent of the effects of pan TCD on engraftment. Others have reported that canine recipients of autologous marrow grafts incubated with up to 4,000 μmol/L Leu-Leu-OMe had similar leukocyte recovery as compared with recipients of nonmanipulated marrow grafts.[46] Taken together, these findings suggest that hematopoiesis is not adversely affected by Leu-Leu-OMe treatment at doses that effectively prevent GVHD in mice, while anti-Thy 1.2 + C′ depletion is detrimental to lymphocyte recovery.

On day 100 post-BMT, NK and CTL function, initially eliminated in the donor inoculum by Leu-Leu-OMe treatment, was restored to the level of recipients of sham-treated or normal nontransplanted controls. This supports the findings of others.[47] In addition, recipients of Leu-Leu-OMe–treated marrow rejected third party skin grafts and tolerated donor skin grafts. These results suggest that Leu-Leu-OMe treatment of donor marrow does not impair long-term immunoreconstitution. However, subtle defects may exist that are not detectable by these assays.

In summary, Leu-Leu-OMe treatment of donor grafts eliminates cells with a specific function independent of cell surface antigen expression. A method of GVHD prevention using Leu-Leu-OMe would have certain advantages over techniques that depend on cell surface antigen expression and lysis by MoAbs directed against specific epitopes. We recognize that there is a complex relationship between donor marrow cells and the host milieu that dictates the level of chimerism and that strategies designed to test the donor graft component alone examine only one component of the engraftment process. However, the possible differential sensitivity of GVHD-causing splenocytes and putative engraftment promoting donor cells to Leu-Leu-OMe deserves further investigation in additional animal models and strain combinations.

ACKNOWLEDGMENT

We gratefully acknowledge the scientific contributions of Christie Soderling, Sue Brief, and Dr Patricia Taylor for excellent technical assistance. The editorial skills of M. J. Hildreth are appreciated.

REFERENCES

1. Martin PJ, Hansen JA, Storb R, Thomas ED: Human marrow transplantation: An immunological perspective. Adv Immunol 40: 379, 1987

2. Cantor H: The effects of anti-theta antiserum upon graft-versus-host activity of spleen and lymph node cells. Cell Immunol 3:461, 1972

3. Rodt HV, Thierfelder S, Eulitz M: Suppression of acute secondary disease by heterologous anti-brain serum. Blut 25:385, 1972

4. Tyan ML: Modification of severe graft-versus-host disease with antiserum to the theta antigen or to whole serum. Transplantation 15:601, 1973

5. Korngold R, Sprent J: Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J Exp Med 148:1687, 1978

6. Vallera DA, Soderling C, Carlson G, Kersey JH: Bone marrow transplantation across the major histocompatibility barriers in mice. Effect of elimination of T cells from donor grafts by treatment with monoclonal Thy 1.2 plus complement or antibody alone. Transplantation 31:218, 1981

7. Kolb HJ, Rieder I, Rodt H, Netzel B, Grosse-Wilde H, Scholz S, Schaffer E, Kolb H, Thierfelder S: Anti-lymphocyte antibodies and marrow transplantation: VI. Graft-versus-host tolerance in DLA-incompatible dogs after in vitro treatment of bone marrow with absorbed antithymocyte globulin. Transplantation 27:242, 1979

8. Storb R: The role of T cells in engraftment: Experimental models, clinical trials. Prog Bone Marrow Transplant. New York, NY, Liss, 53:23, 1987

9. Wagemaker G, Vriesendorp HM, van Bekkum DW: Successful bone marrow transplantation across major histocompatibility barriers in rhesus monkeys. Transplant Proc 13:218, 1981

10. Butturini A, Gale RP: T-cell depletion in bone marrow transplantation for leukemia: Current results and future directions. Bone Marrow Transplant 3:185, 1988

11. Poynton CH: T cell depletion in bone marrow transplantation. Bone Marrow Transplant 3:265, 1988

12. Martin PJ, Kernan NA: T cell depletion for GVHD prevention in humans, in Burakoff SJ, Deeg HJ, Ferrara JLM, Atkinson MK (eds): Graft-versus-Host Disease: Research and Clinical Management. New York, NY, Dekker, 1989 (in press)

13. Thiele DL, Lipsky PE: Regulation of cellular function by products of lysosomal enzyme activity: Elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes. Proc Natl Acad Sci (USA) 82:2468, 1985

14. Thiele DL, Lipsky PE: The immunosuppressive activity of L-leucyl-L-leucine methyl ester: Selective ablation of cytotoxic lymphocytes and monocytes. J Immunol 136:1038, 1986

15. Thiele DL, Charley MR, Calomeni JA, Lipsky PE: Lethal graft-vs-host disease across major histocompatibility barriers: Requirement for leucyl-leucine methyl ester sensitive cytotoxic T cells. J Immunol 138:51, 1987

16. Thiele DL, Bryde SE, Lipsky PE: Lethal graft-vs-host disease induced by a class II MHC antigen only disparity is not mediated by cytotoxic T cells. J Immunol 141:3377, 1988

17. Blazar BR, Soderling CCB, Koo GC, Vallera DA: Absence of a facilitory role for NK 1.1-positive donor cells in engraftment across a major histocompatibility barrier in mice. Transplantation 45:876, 1988

18. Vallera DA, Soderling CCB, Kersey JH: Bone marrow transplantation across major histocompatibility barriers in mice. II. T-cell requirement for engraftment in total lymphoid irradiation-conditioned recipients. Transplantation 33:243, 1982

19. Pietryga DW, Blazar BR, Soderling CCB, Vallera DA: The effect of T-subset depletion on the incidence of lethal graft-versus-host disease in a murine major histocompatibility complex mismatched transplantation system. Transplantation 43:442, 1987

20. Charley M, Thiele DL, Bennet M, Lipsky PE: Prevention of lethal murine graft versus host disease by treatment of donor cells with L-leucyl-L-leucine methyl ester. J Clin Invest 78:1415, 1986

21. Vallera DA, Soderling CCB, Orosz CG: Assessment of immunocompetence by limiting dilution analysis in long-term T cell depletion chimeras transplanted across the MHC barrier. Transplantation 40:311, 1985

22. Ozato K, Sachs DH: Monoclonal antibodies of mouse MHC antigens: III. Hybridoma antibodies reacting to antigens of the $H\text{-}2^b$ haplotype reveal genetic control of isotype expression. Transplantation 126:317, 1981

23. Ozato K, Mayer N, Sachs DH: Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. J Immunol 124:533, 1980

24. Soderling CCB, Song CW, Blazar BR, Vallera DA: A correlation between conditioning and engraftment in recipients of MHC mismatched T-cell depleted murine bone marrow transplants. J Immunol 35:941, 1985

25. Blazar BR, Soderling CCB, Vallera DA: Assessment of multi-organ system engraftment by genotypic typing using restriction fragment length polymorphisms. J Immunol 137:3338, 1986

26. Anderson PM, Blazar BR, Bach FH, Ochoa AC: Anti-CD3 + interleukin-2 stimulated murine killer cells: *In vitro* generation and *in vivo* anti-tumor activity. J Immunol 142:1383, 1989

27. Unkeless JC: Characterization of a monoclonal antibody directed against mouse macrophage and lymphocyte Fc receptors. J Exp Med 150:580, 1979

28. Blazar BR, Widmer MB, Cosman D, Sassenfeld HM, Vallera DA: Improved survival and leukocyte reconstitution without detrimental effects on engraftment in murine recipients of human recombinant granulocyte colony-stimulating factor following transplantation of T-cell depleted histoincompatible bone marrow. Blood 74:2264, 1989

29. Blazar BR, Widmer MB, Soderling CCB, Urdal DL, Gillis S, Robison LL, Vallera DA: Augmentation of donor bone marrow engraftment in histoincompatible murine recipients by granulocyte/macrophage colony-stimulating factor. Blood 71:320, 1988

30. Blazar BR, Widmer MB, Soderling CCB, Gillis S, Vallera DA: Enhanced survival but reduced engraftment in murine recipients of recombinant granulocyte/macrophage colony-stimulating factor following transplantation of T-cell depleted histoincompatible bone marrow. Blood 72:1148, 1988

31. Vallera DA, Blazar BR: Depressed leukocyte reconstitution in histoincompatible recipients of T-cell depleted marrow pretreated with interleukin-3. Transplantation 46:616, 1988

32. Vallera DA, Youle RJ, Neville DM, Soderling CCB, Kersey JH: Monoclonal antibody-toxin conjugates for experimental GVHD prophylaxis. Reagents selectively reactive with T-cells and not murine stem cells. Transplantation 36:73, 1982

33. Mantel N: Evaluation of survival data in two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163, 1966

34. Vallera DA, Soderling CCB, Kersey JH: Bone marrow transplantation across major H-2 barriers. III. Treatment of donor graft with monoclonal antibodies directed against Lyt determinants. J Immunol 128:871, 1982

35. Vallera DA, Blazar BR: T cell depletion for graft-versus-host disease (GVHD) prophylaxis: A perspective on engraftment in mice and humans. Transplantation 47:751, 1989

36. Wall DA, Hamberg SD, Reynolds DS, Burakoff SJ, Abbas AK, Ferrara JLM: Immunodeficiency in graft-versus-host disease; I. Mechanisms of immune suppression. J Immunol 140: 2970, 1988

37. Thiele DL, Kurosaka M, Lipsky PE: Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: Delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. J Immunol 131: 2282, 1983

38. Thiele DL, Lipsky PE: Modulation of human natural killer cell function by L-leucine methyl ester: Monocyte-dependent depletion from human peripheral blood mononuclear cells. J Immunol 134:786, 1985

39. Korngold R, Sprent J: Variable capacity of $L3T4^+$ cells to cause lethal graft-versus-host disease across minor histocompatibility barriers in mice. J Exp Med 165:1552, 1987

40. Sprent J, Schaefer M, Gao E-K, Korngold R: Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences; I. $L3T4^+$ cells can either augment or retard GVHD elicited by $Lyt\text{-}2^+$ cells in class I-different hosts. J Exp Med 167:556, 1988

41. Blazar BR, Soderling CCB, Robison LL, Vallera DA: Short course total lymphoid irradiation to facilitate engraftment of T-cell depleted marrow across a major histocompatibility barrier in mice. Transplantation 46:324, 1988

42. Sykes M, Sharabi Y, Sachs DH: Achieving alloengraftment without graft-versus-host disease: Approaches using mixed allogeneic bone marrow transplantation. Bone Marrow Transplant 3:379, 1988

43. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Stephany D, Sachs DH: Effect of selective T cell depletion of host and/or donor bone marrow on lymphopoietic repopulation, tolerance, and graft-versus-host disease in mixed allogeneic chimeras (B10+ B10.D2 into B10). J Immunol 136:28, 1986

44. Sykes M, Sheard MA, Sachs DH: Effect of T cell depletion in radiation bone marrow chimeras. II. Requirement for allogeneic T cells in the reconstituting bone marrow inoculum for subsequent resistance to breaking of tolerance. J Exp Med 168:661, 1988

45. Sykes M, Chester CH, Sachs DH: Protection from graft-versus-host disease in fully allogeneic chimeras by prior administration of T cell-depleted syngeneic bone marrow. Transplantation 46:327, 1988

46. Raff RF, Severns E, Storb R, Martin P, Graham T, Sandmaier B, Schuening F, Sale G, Appelbaum FR: L-Leucyl-L-Leucine methyl ester treatment of canine marrow and peripheral blood cells. Transplantation 46:655, 1988

47. Thiele DL, Calomeni JA, Lipsky PE: Leucyl-leucine methyl ester treatment of donor cells permits establishment of immunocompetent parent $\rightarrow F_1$ chimeras that are selectively tolerant of host alloantigens. J Immunol 139:2137, 1987