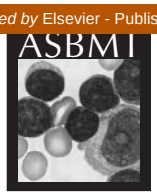
Biology of Blood and Marrow Transplantation 8:525-535 (2002)



CD25$^+$ Immunoregulatory T-Cells of Donor Origin Suppress Alloreactivity after BMT

Bryon D. Johnson, Marja C. Konkol, Robert L. Truitt

Department of Pediatrics, Medical College of Wisconsin, Milwaukee, Wisconsin

Correspondence and reprint requests: Bryon D. Johnson, PhD, Department of Pediatrics, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226 (e-mail: bjohnson@mcw.edu).

Received June 11, 2002; accepted July 29, 2002

ABSTRACT

We have previously identified donor-derived Thy1$^+$ αβ T-cell receptor (TCR)$^+$ CD4$^+$CD8$^-$ regulatory T-cells that suppress GVH reactivity induced by donor leukocyte infusion (DLI) after BMT. These cells develop in the recipient thymus and may play a role in the maintenance of donor-host tolerance after allogeneic BMT. In the present study, we sought to further characterize the T-cells responsible for the regulatory cell activity in our model. Lethally irradiated recipient AKR mice (H-2^k) received transplants of BM from CD25-deficient (–/–) C57BL/6 mice (H-2^b). Recipients of CD25-deficient BM developed more severe GVHD after DLI than did recipients of normal BM, a result that indirectly suggests that CD4$^+$CD25$^+$ regulatory T-cells are important to the suppression of GVH reactivity after allogeneic BMT. GVHD was accompanied by mortality, body weight loss, and elevated percentages of T-cells from the DLI in the peripheral blood in mice that received CD25-deficient BM compared to mice that received normal BM. Both CD40-CD40L and CD28-B7 costimulatory pathways have been implicated in the generation of CD25$^+$ regulatory T-cells. Therefore, we tested whether deficiency in either of these pathways affected the activity of donor BM–derived regulatory T-cells. The absence of CD40L did not affect the regulatory T-cells (ie, recipient mice were still protected from DLI-induced GVHD). In contrast, use of marrow from CD28-deficient mice resulted in complete loss of suppression of GVH reactivity. Thus, CD28 but not CD40L was critical for the generation and/or activation of immunoregulatory T-cells that suppressed GVHD induced by DLI. Together, the results of these experiments suggest that CD4$^+$CD25$^+$ regulatory T-cells suppress GVH reactivity after BMT and that CD28 expression is indispensable for the generation of these cells.

KEY WORDS

Donor leukocyte infusion • Bone marrow transplantation • Regulatory T-cells • Graft-versus-host disease

INTRODUCTION

There has been a renewed interest in suppressor, or regulatory, T-cells because new technologies and reagents allow for more specific identification of such cells. Regulatory T-cells include several phenotypes. One population that has received a great deal of attention consists of CD4$^+$ cells that constitutively express CD25, the α-chain of the interleukin (IL)-2 receptor. In mice, 5% to 10% of mature CD4$^+$ T-cells express CD25 [1,2]. Similar cells have been reported in humans [3-6]. These cells acquire their phenotypic characteristics and immunosuppressive function in the thymus [7,8], but little is known about how this acquisition occurs. Experimental data have suggested that CD28-B7 and CD40L-CD40 interactions are important in the generation of CD4$^+$CD25$^+$ cells because CD4$^+$CD25$^+$ cell numbers are significantly reduced in CD28-deficient and CD40-deficient mice [9,10]. Despite their small numbers, CD4$^+$CD25$^+$ cells have been shown by several investigators to exhibit potent immunosuppressive functions both in vitro and in vivo [11,12]. These cells play an important role in several settings, including autoimmune disease [1,13], allograft tolerance [14-16], syngeneic graft-versus-host disease (GVHD) [17], allergy [18], and tumor immunity [19-21]. Adoptively transferred CD4$^+$CD25$^+$ cells can also suppress GVH reactivity [15,22].

We previously described the presence of thymus-derived donor T-cells that suppress GVH reactions induced by post–bone marrow transplantation (BMT) infusion of donor T-cells [23], a form of adoptive immunotherapy typically referred to as donor leukocyte infusion (DLI) therapy. The regulatory T-cells were Thy1$^+$ and αβ T-cell receptor (TCR)$^+$ and consisted of 2 subpopulations: CD4$^+$CD8$^-$

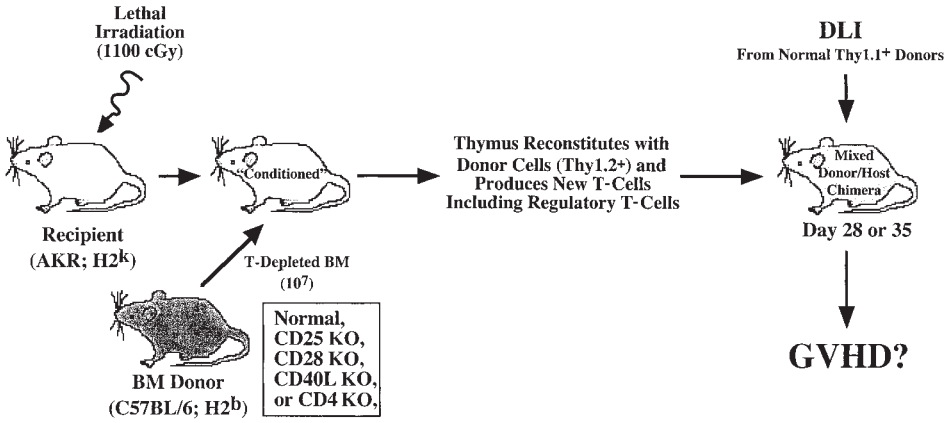


Figure 1. Experimental design. To test the roles of $CD25^+$, $CD4^+$, $CD28^+$, and $CD40L^+$ regulatory T-cells in the suppression of GVHD after DLI, the indicated genetically deficient (KO) mice were used as BM donors. $Thy1.2^+$ regulatory T-cells are produced de novo from donor BM–derived precursors in the reconstituted host thymus [23].

T-cells and $CD4^-CD8^-$ double-negative (DN) T-cells [23]. We hypothesized that the $CD4^+$ population consists of $CD4^+CD25^+$ regulatory T-cells. In the present study, we demonstrate that $CD25^+$ T-cells play an important role in suppression of GVH reactivity after DLI and provide indirect evidence that these cells are the $CD4^+$ regulatory T-cells previously identified by us. The results presented here also further implicate $CD4^-CD8^-$ DN cells as regulatory cells in our DLI model. Thymus-derived regulatory T-cells may help in the establishment and maintenance of donor-host tolerance after allogeneic BMT.

MATERIALS AND METHODS

Mice

Mice aged 4 to 6 weeks of the strains AKR/J ($H\text{-}2^k$, $Thy1.1^+$); AKR/Cum ($H\text{-}2^k$, $Thy1.2^+$); C57BL/6 ($H\text{-}2^b$, $Thy1.2^+$); B6.PL-*Thy1*^{a} ($H\text{-}2^b$; $Thy1.1^+$); C57BL/6-*Cd28*^{tm1Mak} (CD28 knock-out [KO])); C57BL/6-*Tnfsf5*^{tm1Imx} (CD40L KO); and C57BL/6-*Cd4*^{tm1MAK} (CD4 KO) were obtained from Jackson Laboratories (Bar Harbor, Maine). A C57BL/6-*IL2ra*^{tm1Dw} (CD25 KO) heterozygote breeding pair was also obtained from Jackson Laboratories, and the offspring were screened for homozygosity using polymerase chain reaction in a protocol provided by Jackson Laboratories. All animals were housed and cared for in the Medical College of Wisconsin's Animal Resource Center (Milwaukee, WI).

Experimental Design and Transplantation Protocols

The basic experimental design for these studies is shown in Figure 1. AKR mice (AKR/J or AKR/Cum) were conditioned for BMT with a lethal dose of 1100 cGy total body irradiation (TBI) using a small-animal cesium irradiator (Shepherd Mark I; JL Shepherd, San Fernando, CA). The irradiated mice underwent transplantation 18 hours later by intravenous (IV) injection with 10^7 T-depleted BM cells from C57BL/6 donor mice. The recipient mice were fully engrafted with donor hematopoietic cells, and by 3 weeks after BMT, all mononuclear cells in the lymphoid organs were of donor origin except for the T-cells, which were a mixture of donor and residual host T-cells (data not shown). The thymuses became fully donor engrafted and produced new mature donor T-cells, including the regulatory T-cells identified in our previous studies [23]. DLI (3×10^7 donor splenocytes) given to these recipients on day 28 after BMT induced only mild GVHD. In contrast, if donor regulatory T-cells were eliminated before the DLI, the recipients developed severe and lethal GVHD [23]. Selective elimination of donor regulatory T-cells was accomplished by treating mice before DLI with in vivo–depleting anti-Thy1.2 monoclonal antibody (MoAb) (clone 30-H12; American Type Culture Collection, Rockville, MD). This strategy eliminated regulatory T-cells, and $Thy1.1^+$ C57BL/6 (B6.PL-*Thy1*^{a}) splenocytes could be given as DLI without concern that they would be depleted by the anti-Thy1.2 MoAb.

The goal of the current study was to determine whether elimination of CD25, CD28, or CD40L molecules on the newly generated host-thymus–derived donor T-cells affected the regulatory T-cell activity. To eliminate these molecules, recipient AKR mice received transplants of BM from CD25-KO, CD28-KO, or CD40L-KO mice. We hypothesized that if any of these molecules were involved in generation and/or activation of the regulatory T-cells, then mice that received transplants of BM deficient for the particular molecule would develop severe GVHD after DLI (with 3×10^7 splenocytes from normal mice). In those experiments in which mice were given transplants of CD4-KO BM (to confirm previous results), a higher dose of donor cells (6×10^7 splenocytes) was given as DLI on day 35 after BMT. Control groups of mice received transplants of BM from normal C57BL/6 donors.

For experiments in which recipients received transplants of CD25 KO BM, the BMT protocol was modified because of engraftment problems. Recipient AKR/Cum mice ($Thy1.2^+$) were conditioned with lethal TBI and injected intraperitoneally (IP) with 1 mg of anti-Thy1.2 MoAb on the day of BMT to further suppress host-versus-graft reactivity. This approach resulted in consistent donor engraftment after BMT with CD25-KO BM.

To obtain BM, femurs and tibias were collected from C57BL/6 normal and KO donor mice. The bones were

flushed with Dulbecco modified Eagle culture medium. The BM cells were treated with anti-Thy1.2 MoAb (clone 30-H12) and complement (Low-Tox-M; Accurate Chemical and Scientific Corp, Westbury, CT) to remove T-cells. For DLI, splenocytes were collected from healthy Thy1.1⁺ C57BL/6 (B6.PL-*Thy1*^{a}) mice.

Phenotypic Analysis

On days 24 through 27 after BMT, blood samples were obtained from all mice that underwent transplantation in each experiment to assess engraftment status. Whole blood samples collected in EDTA were stained with a fluoroscein isothiocyanate (FITC)-conjugated donor major histocompatibility complex (MHC) class I–specific MoAb (FITC-anti-H2K^b) in conjunction with a phycoerythrin (PE)-conjugated B-cell–specific MoAb (PE–anti-B220). Red cells were eliminated with fluorescence-activated cell-sorting (FACS) lysing solution (BD Biosciences, San Jose, CA), and the stained mononuclear cells were fixed in 1% paraformaldehyde.

In some experiments, blood samples were collected 7 to 10 days after DLI to determine the percentage of DLI-derived donor T-cells. To detect these cells, the blood samples were stained with FITC–anti-H2K^b plus PE–anti-Thy1.1. This staining method allowed us to distinguish DLI-derived donor T-cells (H-2^b, Thy1.1) from BM-derived donor T-cells (H-2^b, Thy1.2).

Splenocytes from some BM donors were collected to assess CD4⁺CD25⁺ T-cell content. These cells were stained with FITC–anti-CD4 and PE–anti-CD25.

For immune reconstitution studies, splenocytes isolated from BM chimeras on day 28 after BMT were stained with FITC–anti-H2K^k (recipient MHC class I-specific) or FITC–anti-H2K^b (donor MHC class I-specific) in combination with PE–anti-B220, PE–anti-CD4, PE–anti-CD8, or PE–anti–Mac-1 to detect B-cells, T-cells, and monocytes of donor and host origin. In some experiments, the splenocytes were analyzed for CD4⁺CD25⁺ T-cell content by 3-color flow cytometry using FITC–anti-Thy1.1 (host T-cells) or

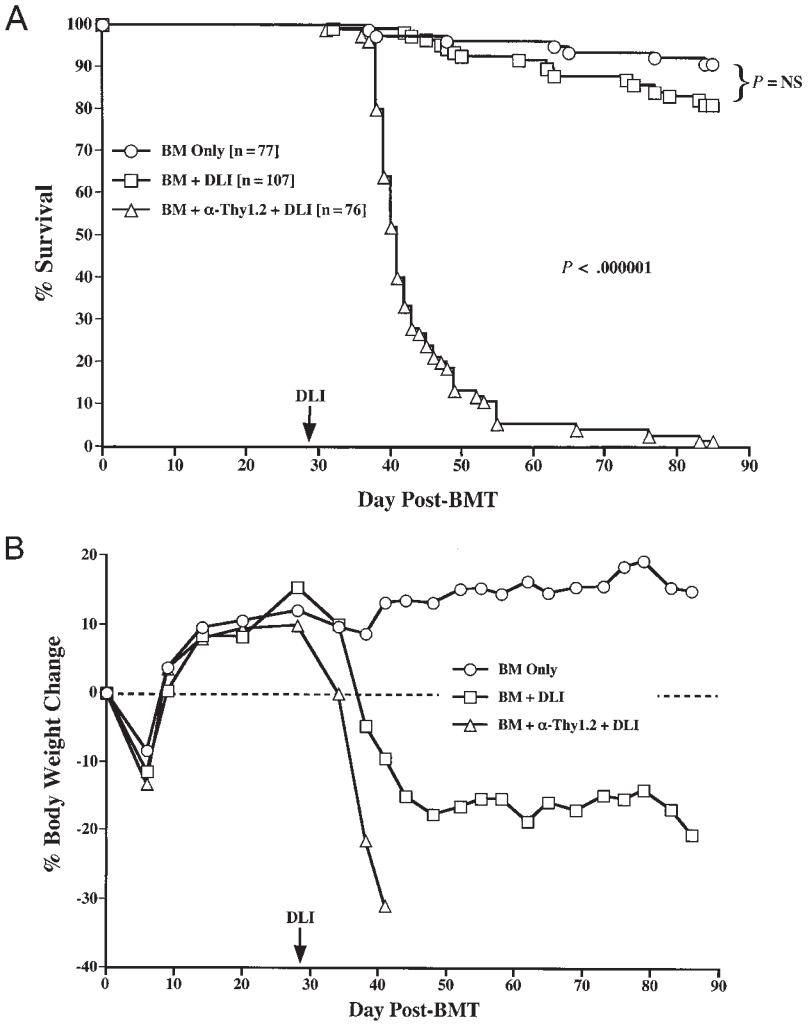


Figure 2. Thy1⁺ cells of donor origin suppress GVH reactivity after DLI. Lethally irradiated recipient AKR mice received transplants of 10^7 C57BL/6 BM cells and were then randomized to receive no further treatment (BM Only), DLI with 3×10^7 Thy1.1⁺ donor (B6.PL- *Thy1*^{a}) spleen cells on day 28 post-BMT (BM + DLI), or treatment with 0.5 to 1 mg of anti-Thy1.2 MoAb IP 4 and 2 days before DLI to deplete regulatory T-cells (BM + α-Thy1.2 + DLI). The mice were followed for survival (A) and body weight change (B). The survival curves are the combined data of 20 independent experiments. Body weight curves are the data from 1 representative experiment.

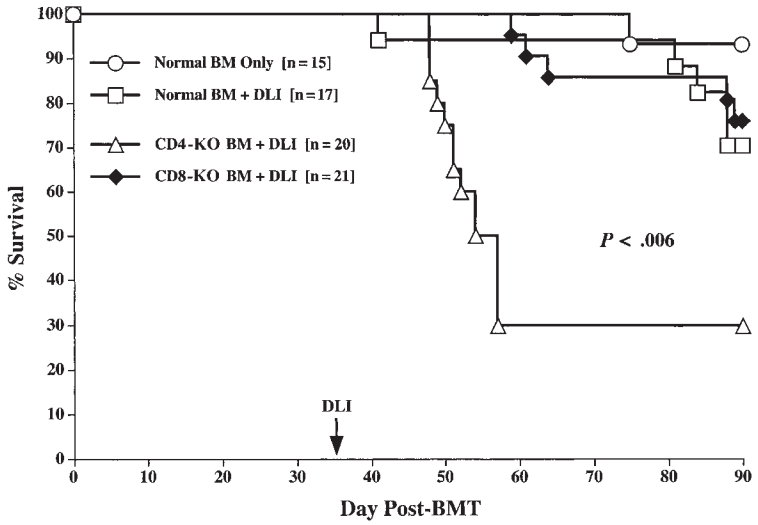


Figure 3. Confirmation that donor Thy1$^+$ regulatory T-cells include CD4$^+$ cells but not CD8$^+$ cells. Irradiated recipient AKR mice received transplants of 10^7 BM cells from normal, CD4-KO, or CD8-KO C57BL/6 donor mice. GVH-negative controls consisted of mice that received transplants of normal BM only (no DLI). The other groups were all given DLI with 6×10^7 normal Thy1.1$^+$ donor (B6.PL-*Thy1^a*) spleen cells on day 35 post-BMT. Survival curves are the combined data of 3 independent experiments.

FITC–anti-Thy1.2 (donor T-cells) in combination with APC–anti-CD4 and PE–anti-CD25.

All fluorochrome-conjugated MoAbs were purchased from BD Pharmingen (San Diego, CA). Flow cytometry was done using a Becton Dickinson FACScan or FACSCalibur flow cytometer (BD Biosciences). Five to 50×10^4 events were collected for 2- and 3-color analyses. Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).

Statistics

Survival curves were compared using the log-rank test. Spleen and thymus cell numbers; percentages of splenic B-cells, T-cells, Mac-1$^+$ cells, and CD4$^+$CD25$^+$ cells; and percentages of infused donor T-cells in blood samples were compared using the Student *t* test.

RESULTS

CD4$^+$ Regulatory T-cells Are Important to the Suppression of GVH Reactivity after DLI

Initial experiments were done to confirm previous findings that Thy1$^+$CD4$^+$ T-cells of donor origin suppressed GVH reactivity after DLI. The results from 20 independent experiments examining the effect of eliminating donor BM–derived Thy1$^+$ T-cells before DLI were combined (Figure 2A). Recipient AKR mice were given lethal TBI (1100 cGy), received transplants of 10^7 T-cell–depleted B6 BM cells, then randomized into 3 groups: (*a*) mice that received no additional treatment (BM only), (*b*) mice given DLI with 3×10^7 Thy1.1$^+$ donor (B6.PL-*Thy1^a*) spleen cells 28 days after BM transplantation, and (*c*) mice depleted of donor BM–derived T-cells before DLI by treatment with anti-Thy1.2 MoAb (–4 and –2 days before DLI). Use of lethal TBI resulted in rapid and complete donor engraftment in all cell lineages except for the T-cell compartment, which contained a mixture of donor and residual host T-cells (data not shown). Overall survival of mice receiving transplants of BM alone was 91% (Figure 2A). The mice maintained or gained body weight following early conditioning-related body weight loss (Figure 2B), indicating little or no GVHD. Mice given DLI on day 28 had worse overall survival outcomes than did mice not given DLI (81% versus 91%), but the difference was not statistically significant (Figure 2A). However, DLI-treated mice had some GVHD, as indicated by marked body weight loss after DLI (Figure 2B). Mice that were depleted of BM-derived donor T-cells before DLI developed severe GVHD, and only 1% (1/76) survived until day 85 after BMT (Figure 2A). Clinical onset of GVHD was accompanied by a sharp loss in body weight (Figure 2B), and the majority of recipients died within 10 to 20 days after DLI.

In the next series of experiments, to assess whether BM-donor–derived CD4$^+$ and/or CD8$^+$ cells were essential for preventing GVHD after DLI therapy, we transplanted BM from CD4-KO or CD8-KO B6 mice into host mice. The dose of donor cells from normal mice given as DLI was 6×10^7 splenocytes, and the time of DLI was day 35 after BMT. These parameters were changed to determine whether higher numbers of DLI cells would overwhelm the effect of CD4$^+$ regulatory T-cells on suppression of GVHD. Control mice received transplants of normal BM plus DLI. Mice receiving transplants of BM alone (no DLI) were included as GVH-negative controls. Transplantation with CD4-KO or CD8-KO BM did not adversely affect engraftment of donor hematopoietic cells (data not shown). The survival rate of mice receiving transplants of CD8-KO BM and given DLI was not significantly different from that of mice receiving transplants of normal BM and given DLI (Figure 3), a result indicating that donor CD8$^+$ cells were not involved in suppression of GVHD after DLI. In contrast, the survival rate of mice receiving transplants of BM from CD4-KO donors and given DLI was significantly lower

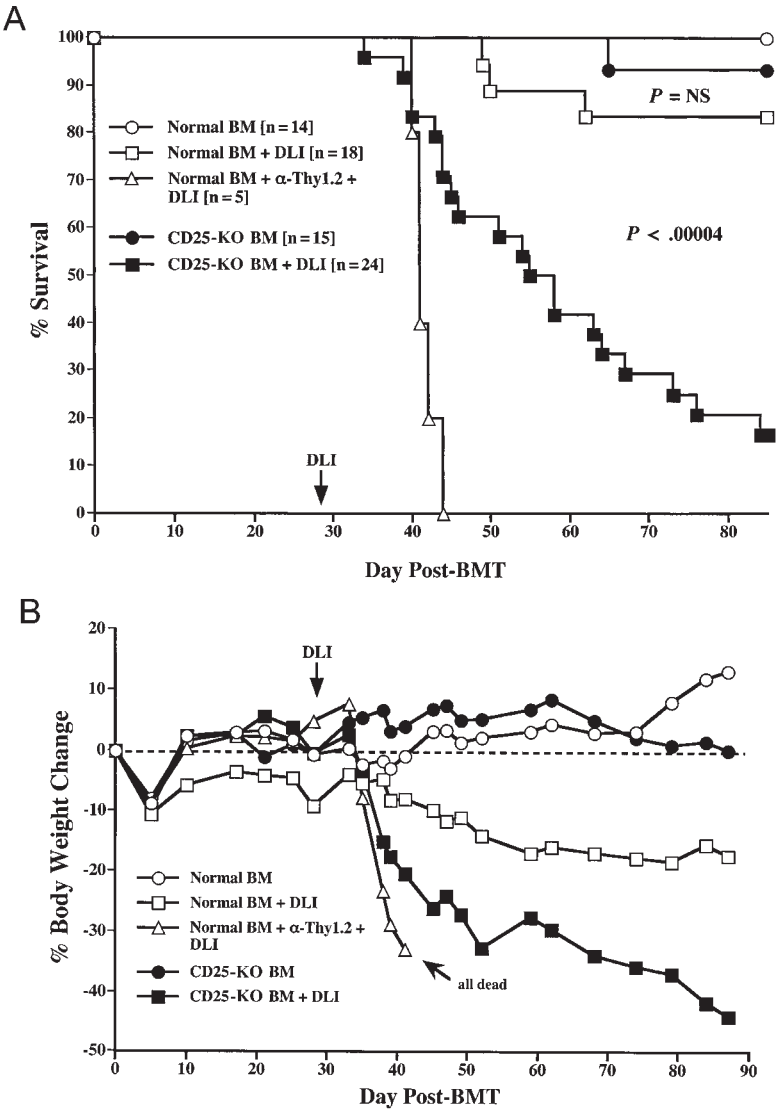


Figure 4. CD25⁺ cells are involved in suppression of alloreactivity after DLI. Irradiated AKR recipients received transplants of 10^7 BM cells from normal or CD25-KO C57BL/6 donor mice. GVH-negative controls consisted of mice that received transplants of normal BM or CD25-KO BM (no DLI). Mice treated with anti-Thy1.2 MoAb to eliminate donor regulatory cells were injected with 1 mg of antibody twice (days –4 and –2) before DLI. Groups of recipients given a DLI were injected with 3×10^7 normal Thy1.1⁺ donor (B6.PL-*Thy1*^{a}) spleen cells on day 28 post-BMT. The mice were followed for survival (A) and body weight change (B). Survival curves are the combined results of 3 independent experiments, and body weight curves are results from 1 representative experiment.

than that of mice receiving transplants of normal BM, a result suggesting that donor BM–derived CD4⁺ regulatory T-cells contributed to suppression of DLI-induced GVH reactivity. Results of these experiments confirm and extend our previous report that donor Thy1⁺CD4⁺ regulatory T-cells suppress GVH reactivity after DLI [23].

CD25⁺ Regulatory T-Cells Are Important to the Suppression of GVH Reactivity after DLI

To assess the role of CD25⁺ regulatory T-cells in suppression of GVH reactivity after DLI, we transplanted BM from CD25-KO mice into recipient AKR mice. If CD25⁺ regulatory T-cells were involved, then mice undergoing reconstitution with CD25-KO BM would develop more severe GVHD after DLI than mice receiving transplants of normal BM. Control groups included mice receiving transplants of normal BM or CD25-KO BM alone (GVH-negative controls), as well as a small group of mice receiving transplants of normal BM but depleted of donor regulatory T-cells by treatment with anti-Thy1.2 MoAb before DLI (GVH-positive controls). For DLI, mice were given a single IV injection of 3×10^7 normal Thy1.1⁺ donor splenocytes on day 28 posttransplantation. All mice in the experiments were tested for engraftment status on day 27 after BMT to ensure engraftment of donor cells. Combined survival data from 3 experiments are shown in Figure 4A, and body weight curves from 1 representative experiment are shown in Figure 4B.

Survival of GVH-negative controls that received transplants of normal or CD25-KO BM alone (no DLI) was

Table 1. *Percentages of CD4⁺CD25⁺ T-Cells Are Lower in Spleens of CD28-KO and CD40L-KO BM Donors than in Spleens of Normal BM Donors*

BM Donors*	n	% of CD25⁺ Splenic CD4⁺ Cells
Normal	4	9.7 ± 1.0
CD28 KO	4	2.6 ± 0.3†
CD40L KO	5	5.2 ± 1.3†

*Spleens of BM donors used in the experiments in Figures 5 and 6 were analyzed by flow cytometry for CD4⁺CD25⁺ T-cell content.

†$P < .05$ compared to normal BM donors.

>93% (Figure 4A), and body weights of these mice remained at or near pretransplantation levels (Figure 4B). In contrast, GVH-positive control mice, depleted of regulatory T-cells before DLI, all died with clinical evidence of severe GVHD (Figure 4, open triangles). Because mice that received transplants of CD25-KO BM and DLI had a significantly lower survival rate than did mice that received transplants of normal BM and DLI (17% versus 83%, Figure 4A), donor BM–derived CD25⁺ T-cells were implicated in suppression of DLI-induced GVH reactivity. The lower survival rate in DLI-treated mice that received transplants of CD25-KO BM was paralleled by increased body weight loss (Figure 4B). Blood samples were obtained from some mice 10 days after DLI to examine the percentages of circulating DLI-derived donor T-cells by flow cytometry. Donor T-cells derived from the DLI (Thy1.1⁺) could be distinguished from BM-derived donor T-cells (Thy1.2⁺) with donor-specific MHC class I–specific MoAb and anti-Thy1.1 MoAb. The peripheral blood of mice that received transplants of CD25-KO BM contained significantly higher percentages of DLI-derived donor T-cells than did peripheral blood of mice that received transplants of normal BM (20% versus 6%; $P < .05$), suggesting that the infused donor T-cells had expanded more in recipients of CD25-KO BM.

CD28, but Not CD40L, Is Necessary for the Generation of Donor-Derived Regulatory T-Cells that Suppress GVH Reactivity after DLI

To address the importance of CD28 and CD40L molecules for the generation of regulatory T-cells, we transplanted T-cell–depleted BM from CD28-KO, CD40L-KO, or normal B6 mice into lethally irradiated recipient AKR mice. Both CD28-KO and CD40L-KO mice, used as BM donors, had fewer CD4⁺CD25⁺ T-cells, as indicated by a significantly decreased percentage of CD4⁺CD25⁺ T-cells found in their spleens compared to the percentage found in normal B6 mice (Table 1). Notably, the decrease in CD4⁺CD25⁺ T-cells was greater in CD28-KO mice. On day 28 after BMT, DLI as a single injection of 3×10^7 normal Thy1.1⁺ B6 splenocytes was administered to some of the mice that had received transplants. Controls included mice that received BM transplants only (no DLI) and mice depleted of Thy1.2⁺ donor regulatory T-cells by administration of anti-Thy1.2 MoAb before DLI. Some BM chimeras were killed at the time of DLI to assess chimerism and immune reconstitution in the spleen and thymus (Table 2). For reconstitution, recipients of CD28-KO or CD40L-KO BM were administered donor hematopoietic cells at levels similar to those administered to recipients of normal BM (data not shown). Spleen and thymus cellularities of recipients of CD28-KO or CD40L-KO BM also were similar to those of recipients of normal B6 BM (Table 2). The percentages of splenic B-cells, T-cells, and Mac-1⁺ cells (macrophages/monocytes) in these BM chimeras were not significantly different from one another (Table 2). Collectively, these data indicated that BMT with CD28-KO or CD40L-KO BM did not alter donor engraftment or immune reconstitution.

The spleens of BM chimeras analyzed on day 28 after BMT were also examined for CD4⁺CD25⁺ cell content (Table 3). Although the non–T-cell compartments were completely donor reconstituted, the T-cells in these mice remained 32% to 49% of host origin. We examined the donor-derived and host T-cell compartments separately for CD25 expression. Recipients of CD28-KO BM had a significantly lower percentage of donor-derived splenic CD4⁺ cells that were CD25⁺ than did recipients of normal BM or CD40L-KO BM (Table 3). In contrast, CD25 expression on donor-derived CD4⁺ cells in recipients of CD40L-KO BM was not significantly different from that in recipients of normal BM. CD25 expression on host CD4⁺ T-cells was the same in all 3 groups of BM chimeras (Table 3).

Figure 5A shows the combined survival data from 3 independent experiments in which recipient AKR mice received transplants of CD28-KO BM with or without DLI. Greater than 90% of control mice that received transplants of BM only (normal or CD28 KO) survived long-term (Figure 5A, circles), and body weights of these mice remained above pretransplantation levels (Figure 5B). In contrast, mice depleted of regulatory T-cells before DLI (by treatment with

Table 2. *Immune Reconstitution in Recipients of CD28-KO or CD40L-KO BM Is Not Different from Immune Reconstitution in Recipients of Normal BM*

BM Chimeras†	n	Spleen Size, $\times 10^7$	Thymus Size, $\times 10^7$	Splenocytes, %*		
				B-Cells	T-Cells	Mac-1⁺ Cells
Normal	9	4.3 ± 1.4	10.1 ± 5.7	66.8 ± 14.0	22.9 ± 9.3	8.0 ± 3.4
CD28 KO	7	6.2 ± 1.5‡	7.0 ± 1.9	73.1 ± 2.2	20.1 ± 3.8	5.2 ± 1.2
CD40L KO	10	5.7 ± 1.0‡	8.9 ± 2.8	74.6 ± 3.0	20.0 ± 2.7	5.4 ± 1.8

*Spleens and thymuses were isolated on day 28 after BMT from AKR recipients that had received transplants of BM from normal, CD28-KO, or CD40L-KO C57BL/6 mice (see "Materials and Methods" for details).

†The splenic B-cells and Mac-1⁺ cells from all chimeras were completely of donor origin. In contrast, the T-cells from all chimeras consisted of both donor and residual host cells (51%-68% donor cells).

‡$P < .05$ compared to recipients of normal BM.

Table 3. *At the Time of DLI, Chimeras Receiving Transplants of CD28-KO BM Had a Significantly Lower Percentage of Splenic Donor CD4⁺ Cells that Were CD25⁺ than Did Chimeras Receiving Transplants of Normal BM or CD40L-KO BM*

BM Chimeras*	n	% of CD25⁺ Donor CD4⁺ Cells	% of CD25⁺ Host CD4⁺ Cells
Normal	**9**	**5.3 ± 1.9**	**12.4 ± 2.0**
CD28 KO	**7**	**2.6 ± 1.1†**	**11.6 ± 1.9**
CD40L KO	**10**	**4.6 ± 1.3**	**12.2 ± 2.3**

*Spleens were isolated on day 28 after BMT from AKR recipients that had received transplants of BM from normal, CD28-KO, or CD40L-KO C57BL/6 mice. T-cell chimerism in the spleens of all BM recipients on day 28 was mixed (51% to 68% donor T-cells).

†$P < .05$ compared to chimeras receiving transplants of normal BM or CD40L-KO BM.

anti-Thy1.2 MoAb) developed severe GVHD after DLI (Figure 5, triangles). Mice receiving transplants of normal BM and given DLI had a lower survival rate (76%) than did recipients of BM only (92%) (Figure 5A) and had increased body weight loss (Figure 5B). However, mice receiving transplants of CD28-KO BM and given DLI developed severe and lethal GVHD (Figure 5). The survival rate for this group was not significantly different from that of control mice depleted of regulatory T-cells with anti-Thy1.2 MoAb. The similarities in survival rates in these 2 groups were also reflected by similar patterns in body weight loss after DLI. Blood samples were collected from some DLI-treated mice 7 days after DLI to assess the percentage of circulating DLI-derived donor T-cells. The blood of mice that received transplants of CD28-KO BM contained a significantly higher percentage of infused donor T-cells than did the blood of mice that received transplants of normal BM (15% versus 4%; $P < .05$), demonstrating a correlation between increased percentages of infused donor T-cells and increased GVH-associated mortality. These results indicate that CD28 is critical for the generation of regulatory T-cells that suppress DLI-induced GVH reactivity.

Figure 6A shows survival data from an experiment in which recipient AKR mice were given transplants of CD40L-KO BM. Data from GVH-negative controls (mice given BM transplants only) and GVH-positive controls (mice depleted of donor regulatory T-cells with anti-Thy1.2 MoAb before DLI) are also shown. The key comparison is between mice receiving normal BM transplants and given DLI and mice receiving CD40L-KO BM transplant and given DLI. Long-term survival rates were not significantly different between these 2 experimental groups (Figure 6A), and body weight curves were similar (Figure 6B). Blood samples were collected from some DLI-treated mice 7 days after DLI to examine percentages of circulating infused donor T-cells. The percentages in the blood of mice that received CD40L-KO BM transplants were not different from percentages in mice that received normal BM (1.8% versus 2.1%, respectively). These results suggest that CD40-CD40L interactions are not involved in generation (or activation) of donor regulatory T-cells that suppress GVHD after DLI.

DISCUSSION

In the current study, we showed that CD25⁺ cells of donor origin are critical for the suppression of GVH reactivity after DLI (Figure 4). These cells may be involved in the establishment and maintenance of donor-host tolerance after allogeneic BMT. In the present study, we did not directly prove that the CD25⁺ cells were CD4⁺, but several lines of indirect evidence suggest that they were. First, the pattern of mortality and overall survival rates after DLI were similar in mice receiving transplants of either CD25-KO (Figure 4) or CD4-KO BM (Figure 3 and [23]). Second, CD4⁺CD25⁺ regulatory T-cells are generated in the thymus [7,8], and we previously reported that the regulatory T-cells in our DLI model are produced by the donor-repopulated host thymus [23]. Third, others have reported that the majority of T-cells that constitutively express CD25 (>90%) coexpress CD4 [1,2]. Likewise, most CD25⁺ cells present in our BM chimeras at the time of DLI were CD4⁺, suggesting that the Thy1.2⁺ regulatory T-cells were most likely CD4⁺CD25⁺.

Because CD28-B7 costimulatory interactions have been shown by others to be important in generation of CD4⁺CD25⁺ regulatory T-cells [9], we tested whether elimination of CD28 expression on donor T-cells interfered with the generation of regulatory T-cells in our DLI model. If CD28 was important for generation of the CD25⁺ regulatory T-cells, then the survival of DLI-treated mice that received transplants of CD28-KO BM should be similar to that of CD25-KO BM chimeras. However, none of the mice given transplants of BM from CD28-KO donors survived DLI, whereas 17% of those given transplants of BM from CD25-KO donors did ($P = .0086$). The survival and body weight curves of DLI-treated mice that received transplants of CD28-KO BM were nearly identical to those of mice depleted of "all" Thy1⁺ regulatory T-cells, suggesting that elimination of CD28 affected the generation of both CD4⁺CD25⁺ and the putative CD4⁻CD8⁻ DN regulatory T-cells. Salomon et al. [9] previously showed that both CD28-KO and B7-KO mice have a profound deficiency of CD4⁺CD25⁺ cells. We confirmed this finding in our CD28-KO BM donors (Table 1). Transplantation with BM from CD28-KO mice resulted in a deficiency of splenic donor-derived CD4⁺CD25⁺ cells after BMT (Table 3). We also found a deficiency of thymic CD4⁺CD8⁻CD25⁺ cells in these chimeras (data not shown), suggesting that there was a defect in thymic generation of these cells. Takahashi and coworkers found that the small numbers of CD4⁺CD25⁺ cells in CD28-KO mice are potently suppressive [24], indicating that CD28 expression on CD25⁺ regulatory T-cells is not required for suppressive activity. Collectively, these observations suggest that CD28 is important for the generation of CD25⁺ regulatory T-cells but not for functional activity. There may be a threshold number of CD4⁺CD25⁺ cells necessary to effectively suppress GVHD after DLI.

It has been shown that small numbers of T-cells injected into T-cell–deficient syngeneic mice expand to achieve homeostasis [25]. Because the mice in our DLI model are mixed T-cell chimeras, treatment with anti-Thy1.2 MoAb (before DLI) results in the depletion of 50% to 70% of the total T-cells. We interpret our data to indicate that the DLI-induced GVHD after depletion of the Thy1.2⁺ cells in vivo was due to the removal of regulatory T-cells. However,

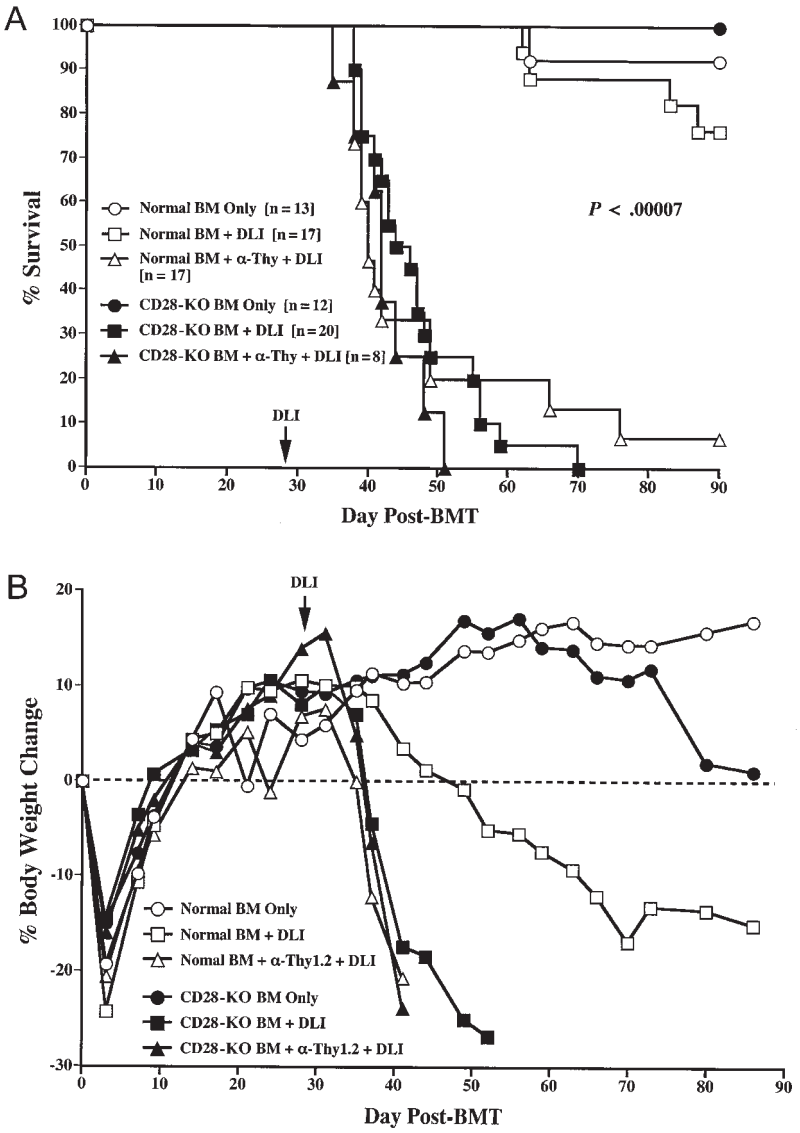


Figure 5. CD28 is necessary for the generation of donor-derived Thy1$^+$ regulatory T-cells. Irradiated recipient AKR mice received transplants of 10^7 BM cells from normal or CD28-KO C57BL/6 donor mice. GVH-negative controls consisted of mice that received transplants of normal BM or CD28-KO BM (no DLI). GVH-positive controls (regulatory T-cells depleted) consisted of mice treated with anti-Thy1.2 MoAb (0.5 mg IP) 4 and 2 days before DLI. DLI was administered as a single IV injection of 3×10^7 normal Thy1.1$^+$ donor (B6.PL-*Thy1^a*) spleen cells on day 28 post-BMT. The mice were followed for survival (A) and body weight change (B). Survival curves are the combined results of 3 independent experiments, and body weight curves are the result of 1 representative experiment.

an alternative hypothesis for the heightened DLI-induced GVHD after Thy1.2$^+$ T-cell depletion is that the induced T-cell deficiency creates a favorable environment for expansion of DLI T-cells and, consequently, heightened GVH reactivity. This hypothesis can be excluded for the following reason: mice given transplants of CD28-KO BM developed severe GVHD after DLI, but they did not have altered T-cell reconstitution at the time of DLI. The percentage of peripheral (splenic) T-cells was the same as that in mice given transplants of normal BM (Table 2). Although there were decreased percentages of donor CD4$^+$CD25$^+$ cells in mice given transplants of CD28-KO BM (Table 3), this decrease did not affect the overall T-cell number. These data argue against the T-cell–deficiency hypothesis and support our assertion that the increased rate of GVHD in mice given transplants of CD28-KO BM was due to a deficiency in regulatory T-cells.

Data from others has indicated that CD40-CD40L interactions also are important for generation of CD4$^+$CD25$^+$ regulatory cells [10]. Kumanogoh and colleagues showed that splenocytes from CD40-KO mice have an 80% reduction in CD4$^+$CD25$^+$ T-cell numbers [10]. We formulated a hypothesis based on these data, that if donor T-cells were incapable of expressing the ligand for CD40 in our DLI model, then we would see increased DLI-induced mortality. However, in recipients of CD40L-KO BM, survival rates after DLI were no different from those in recipients of normal BM that gives rise to regulatory T-cells (Figure 6A).

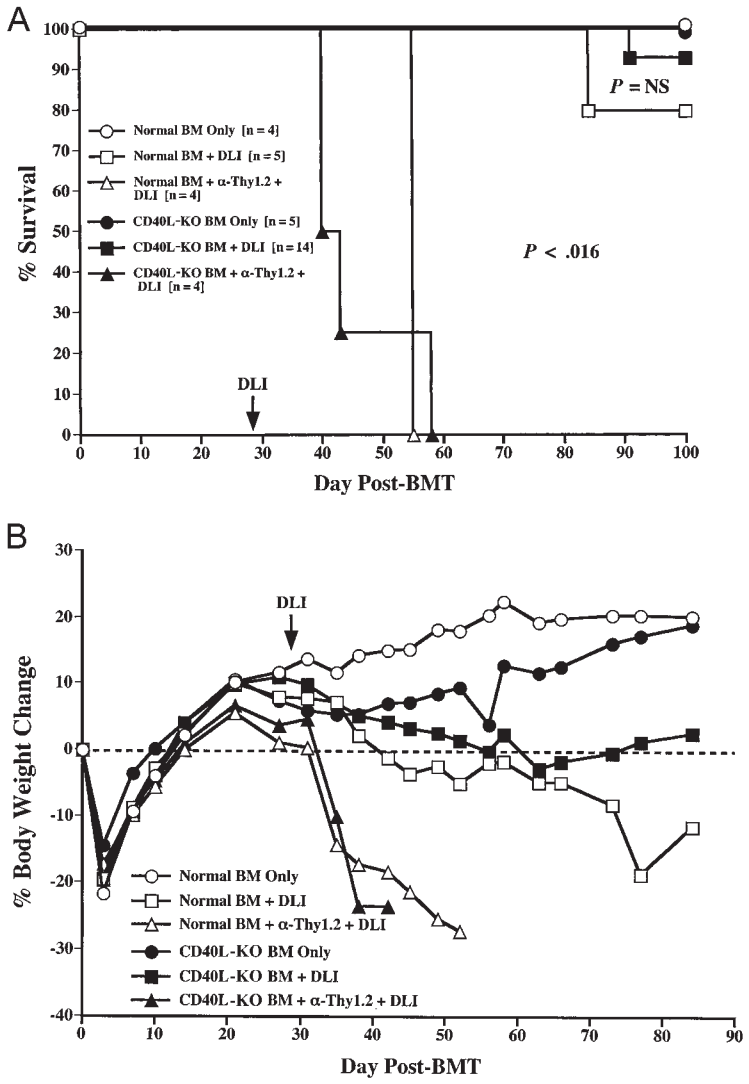


Figure 6. CD40L is not needed for the generation of donor regulatory T-cells. Irradiated recipient AKR mice received transplants of 10^7 BM cells from normal or CD40L-KO C57BL/6 donor mice. GVH-negative controls consisted of mice that received transplants of normal BM or CD40L-KO BM (no DLI). GVH-positive controls (regulatory T-cells depleted) consisted of mice treated with anti-Thy1.2 MoAb (0.5 mg IP) 4 and 2 days before DLI. DLI was administered as a single IV injection of 3×10^7 normal Thy1.1⁺ donor (B6.PL-*Thy1*^{a}) spleen cells on day 28 post-BMT. The mice were followed for survival (A) and body weight change (B). The survival curves and body weight curves are from a single experiment.

The CD40L-KO BM donors used in our experiments had decreased percentages of splenic $CD4^+CD25^+$ T-cells (approximately 50% of normal, Table 1), but spleens of BM chimeras given transplants of CD40L-KO BM did not have decreased percentages of donor-derived $CD25^+$ cells (Table 3). Thus, our data indicate that CD40L is not involved in generation of the regulatory T-cells that suppress DLI-induced GVHD. Reduction in $CD4^+CD25^+$ T-cell numbers is apparently more profound in CD40-deficient mice than in CD40L-deficient mice [10]. The reason for this difference is not known.

Our data are most consistent with the hypothesized presence of at least 2 populations of regulatory T-cells involved in suppression of GVHD after DLI. Both populations are $Thy1^+$ $\alpha\beta TCR^+$ [23] and natural killer (NK)1.1^- [26]. Based on the indirect evidence cited above, the first of these populations appears to comprise $CD4^+CD25^+$ T-cells. However, because the survival rates for DLI-treated mice given transplants of CD25-KO or CD4-KO BM were significantly higher than the survival rates of mice depleted of $Thy1.2^+$ cells before DLI (Figures 3 and 4), we speculate that a second population of $Thy1^+$ cells ($CD4^-$ and $CD25^-$) also may be involved in the suppression of GVH reactivity. Because donor $CD8^+$ T-cells do not appear to be involved (Figure 3 and [23]), the second regulatory population may consist of $CD4^-CD8^-$ DN T-cells. Such a finding would not be unexpected, because a variety of T-cell populations have been shown to be immunosuppressive, including $CD3^+NK1.1^-$ DN T-cells [27,28]. In normal mice, 1% to 5% of peripheral T-cells are DN [29]. It has been suggested that DN cells are derived from $CD8^+$ T-cells [30,31]. However, if DN cells are involved in suppression of GVHD after DLI in our models, our results indicate that they are not derived from $CD8^+$ cells, because mice given transplants of

CD8-KO BM did not develop severe GVHD after DLI (Figure 3). $CD4^+CD25^+$ cells isolated from mice given DLI were immunosuppressive in vitro (data not shown). Whether suppressive activity is present in $CD4^-CD8^-$ T-cells has not yet been determined.

In summary, we have shown that $CD25^+$ T-cells of donor origin suppress in vivo alloreactivity after DLI in mice. Collectively, the evidence suggests that these cells are similar, if not identical, to the thymus-derived $CD4^+CD25^+$ regulatory T-cells that have been shown by several investigators to be important in the suppression of autoimmune diseases. Our data also suggest that a second regulatory cell population comprised of $CD4^-CD8^-$ DN T-cells may be involved. Both regulatory cell populations are thymus derived, and CD28 is critical for their generation. Current efforts are focusing on isolation and ex vivo expansion of these regulatory cells for adoptive transfer to secondary recipients. Recent data from Taylor et al. [22] suggests that $CD4^+CD25^+$ regulatory cells can be expanded ex vivo and adoptively transferred in vivo to inhibit GVHD. Because the development of GVHD after DLI is still a significant problem [32], demonstration that adoptively transferred regulatory T-cells suppress GVH reactivity in our experimental DLI model could have significant implications for the clinical use of these cells in the treatment/prevention of GVHD after DLI.

ACKNOWLEDGMENTS

This work was supported by US Public Health Service Grants No. CA90286 and No. CA39854, as well as the Midwest Athletes Against Childhood Cancer (MACC) Fund (Milwaukee, WI).

REFERENCES

1. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor α-chains (CD25). *J Immunol.* 1995;155:1151-1164.
2. Asano M, Toda M, Sakaguchi N, Sakaguchi S. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med.* 1996;184:387-396.
3. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA. $CD4^+CD25^{high}$ regulatory cells in human peripheral blood. *J Immunol.* 2001;167: 1245-1253.
4. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. *J Exp Med.* 2001;193: 1303-1310.
5. Jonuleit H, Schmitt E, Stassen M, Tuttenberg A, Knop J, Enk AH. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J Exp Med.* 2001;193:1285-1294.
6. Taams LS, Smith J, Rustin MH, Salmon M, Poulter LW, Akbar AN. Human anergic/suppressive $CD4^+CD25^+$ T cells: a highly differentiated and apoptosis-prone population. *Eur J Immunol.* 2001;31:1122-1131.
7. Itoh M, Takahashi T, Sakaguchi N, et al. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol.* 1999;162:5317-5326.
8. Stephens LA, Mottet C, Mason D, Powrie F. Human $CD4^+CD25^+$ thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur J Immunol.* 2001;31:1247-1254.
9. Salomon B, Lenschow DJ, Rhee L, et al. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity.* 2000; 12:431-440.
10. Kumanogoh A, Wang X, Lee I, et al. Increased T cell autoreactivity in the absence of CD40-CD40 ligand interactions: a role of CD40 in regulatory T cell development. *J Immunol.* 2001;166: 353-360.
11. Sakaguchi S, Sakaguchi N, Shimizu J, et al. Immunologic tolerance maintained by $CD25^+$ $CD4^+$ regulatory T cells: their common role in controlling autoimmunity, tumor immunity, and transplantation tolerance. *Immunol Rev.* 2001;182:18-32.
12. Shevach EM, McHugh RS, Piccirillo CA, Thornton AM. Control of T-cell activation by $CD4^+$ $CD25^+$ suppressor T cells. *Immunol Rev.* 2001;182:58-67.
13. Takahashi T, Kuniyasu T, Toda M, et al. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Intl Immunol.* 1998;10:1969-1980.
14. Hara M, Kingsley CI, Niimi M, et al. IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J Immunol.* 2001;166:3789-3796.
15. Taylor PA, Noelle RJ, Blazar BR. $CD4^+CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J Exp Med.* 2001;193:1311-1317.
16. Sanchez-Fueyo A, Weber M, Domenig C, Strom TB, Zheng XX. Tracking the immunoregulatory mechanisms active during allograft tolerance. *J Immunol.* 2002;168:2274-2281.
17. Wu DY, Goldschneider I. Tolerance to cyclosporin A-induced autologous graft-versus-host disease is mediated by a $CD4^+CD2^{5+}$ subset of recent thymic emigrants. *J Immunol.* 2001;166:7158-7164.
18. Suto A, Nakajima H, Kagami S-I, Suzuki K, Saito Y, Iwamoto I. Role of $CD4^+$ $CD25^+$ regulatory T cells in T helper 2 cell-mediated allergic inflammation in the airways. *Am J Respir Crit Care Med.* 2001;164:680-687.
19. Onizuka S, Tawara I, Shimizu J, Sakaguchi S, Fujita T, Nakayama E. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor α) monoclonal antibody. *Cancer Res.* 1999;59:3128-3133.
20. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing $CD25^+CD4^+$ T cells: a common basis between tumor immunity and autoimmunity. *J Immunol.* 1999;163:5211-5218.
21. Steitz J, Bruck J, Lenz J, Knop J, Tuting T. Depletion of $CD25^+$ $CD4^+$ T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon α-induced, $CD8^+$ T-cell-dependent immune defense of B16 melanoma. *Cancer Res.* 2001;61:8643-8646.
22. Taylor PA, Lees CJ, Blazar BR. The infusion of ex vivo activated and expanded CD4(+)CD25(+) immune regulatory cells inhibits graft-versus-host disease lethality *Blood.* 2002;99:3493-3499.
23. Johnson BD, Becker EE, LaBelle JL, Truitt RL. Role of immunoregulatory donor T cells in suppression of graft-versus-host disease following donor leukocyte infusion therapy. *J Immunol.* 1999;163:6479-6487.
24. Takahashi T, Tagami T, Yamazaki S, et al. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med.* 2000;192:303-309.

25. Rocha B, Dautigny N, Pereira P. Peripheral T lymphocytes: expansion potential and homeostatic regulation of pool sizes and CD4/8 ratios in vivo. *Eur J Immunol.* 1989;19:905-911.
26. Johnson BD, Dagher N, Stankowski MC, Hanke CA, Truitt RL. Donor natural killer (NK1.1⁺) cells do not play a role in the suppression of GVHD or in the mediation of GVL reactions after DLI. *Biol Blood Marrow Transplant.* 2001;7:589-595.
27. Strober S, Cheng L, Zeng D, et al. Double negative ($CD4^-CD8^-\alpha\beta^+$) T cells which promote tolerance induction and regulate autoimmunity. *Immunol Rev.* 1996;149:217-230.
28. Zhang Z-X, Yang L, Young KJ, DuTemple B, Zhang L. Identification of a previously unknown antigen-specific regulatory T cell and its mechanism of suppression. *Nature Med.* 2000;6: 782-789.
29. Zhang ZX, Young K, Zhang L. $CD3^+CD4^-CD8^-$ αβ-TCR^+ T cell as immune regulatory cell. *J Mol Med.* 2001;79:419-427.
30. Erard F, Wild MT, Garcia-Sanz JA, Le Gros G. Switch of CD8 T cells to noncytolytic CD8-CD4- cells that make TH2 cytokines and help B cells *Science.* 1993;260:1802-1805.
31. Mehal WZ, Crispe IN. TCR ligation on CD8+ T cells creates double-negative cells in vivo *J Immunol.* 1998;161:1686-1693.
32. Collins RH, Shpilberg O, Drobyski WR, et al. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J Clin Oncol.* 1997;15:433-444.