Graft-versus-Leukemia and Graft-versus-Host Reactions after Donor Lymphocyte Infusion Are Initiated by Host-Type Antigen-Presenting Cells and Regulated by Regulatory T Cells in Early and Long-Term Chimeras

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ABSTRACT

Regulatory T (T_{reg}) cells and host antigen-presenting cells (APCs) have been implicated in graft-versus-host disease (GVHD) and the graft-versus-leukemia (GVL) effect after donor lymphocyte infusion (DLI), but their relative contributions remain unclear in early versus long-term complete donor or mixed murine allogeneic hematopoietic stem cell (HSC) chimeras. We have previously demonstrated that donor HSC-derived Thy1^{+} T_{reg} cells, consisting primarily of CD4^{+}CD25^{+} cells, play an important role in the suppression of graft-versus-host (GVH) reactivity when DLI is given to complete donor chimeras 28 days after HSC transplantation. Data presented here demonstrate that protection against GVHD exerted by Thy1^{+} T_{reg} cells is less evident with time and eventually is not required in long-term complete donor chimeras because of an absence of host-type APCs to activate alloreactive T cells. Lethal GVHD was observed when Thy1^{+} T_{reg} cells were depleted from complete donor chimeras given by DLI at day 28, 35, or 42; however, T_{reg} cell depletion and DLI at day 70 no longer induced GVHD-associated mortality. Moreover, the failure of DLI to induce GVHD with T_{reg} depletion correlated with a loss of the DLI-induced GVL effect in long-term (day 100) complete donor chimeras. In contrast to the results from complete donor chimeras, GVL reactivity in day 100 mixed chimeras was robust after DLI. Loss of a DLI-induced GVL effect in long-term complete donor chimeras was attributed to the absence of host APCs because the infusion of exogenous host-type dendritic cells partially restored both DLI-induced GVL and GVH reactions in day 100 complete donor chimeras. The GVL and GVH reactions restored by infusion of host dendritic cells in day 100 complete donor chimeras were at least partially regulated by T_{reg} cells because transient depletion of CD25^{+} cells increased both the GVL effect and the severity of GVHD after DLI. Taken together, these data suggest that T_{reg} cells can regulate DLI-induced GVL and GVH reactions in both early and long-term complete donor chimeras, and a state of mixed chimerism is superior to complete donor chimerism because host-type APCs facilitate a DLI-induced GVL effect without severe GVHD.

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KEY WORDS

Donor lymphocyte infusion • Graft-versus-leukemia effect • Graft-versus-host disease • Bone marrow transplantation • Regulatory T cells • CD4^{+}CD25^{+} • Antigen-presenting cells • Dendritic cells

INTRODUCTION

Donor lymphocyte infusion (DLI) has become a standard form of immunotherapy for treating leukemia relapse after hematopoietic stem cell transplantation (HSCT) in humans. It is particularly effective for inducing remission in chronic myeloid leukemia patients but is less beneficial for other types of leukemia [1-5]. The ability of immune cells in the DLI inoculum to react against leukemia cells is referred to as the graft-versus-leukemia (GVL) effect [2]. DLI has also been used to facilitate immune reconstitution, treat chronic viral infections such as Epstein-Barr virus and
cytomegalovirus, correct immunodeficiencies, and increase donor chimerism in mixed donor/host chimeric animals or humans [3].

Graft-versus-host disease (GVHD), a major complication of DLI therapy, typically correlates with desirable GVL reactivity and response rates [1-5]. Hence, common target antigens (Ags) and/or effector cells have been suspected in the processes of GVHD and GVL after DLI, although the GVL effect has been separable from GVHD in experimental animal models [6-9]. In a large retrospective analysis, acute or chronic GVHD occurred in up to 60% of DLI-treated patients and accounted for 10% of deaths in these patients [4]. More than 90% of complete responders developed acute GVHD, and 88% of responders developed chronic GVHD [4]. In contrast, remissions have been rarely observed in patients without GVHD after DLI. Fortunately, GVHD in this setting has frequently been mild to moderate and responsive to immunosuppressive therapy. Moreover, adoption of a dose-escalation DLI strategy has been shown to decrease the risk for developing life-threatening GVHD [4].

DLI has allowed for the infusion of donor T cells at numbers that would typically induce lethal GVHD if given at the time of HSCT. Elucidation of the immunologic mechanisms involved in regulating graft-versus-host (GVH) reactivity is needed to optimize clinical usage for DLI-based immunotherapy. As possible mechanisms, findings from clinical and experimental studies have implicated avoidance of the cytokine storm initiated by pretransplantation conditioning, decreased numbers of host Ag-presenting cells (APCs) at the time of DLI, and development of regulatory T (T_{reg}) cells [10-13], but APC and T_{reg} dynamics (eg, short-, intermediate-, or long-term after transplantation) and their relative contributions at each stage have not been fully addressed. Recently, diminished DLI-induced GVL reactivity was seen in long-term major histocompatibility complex (MHC)–matched or mismatched complete hematopoietic stem cell (HSC) chimeras [14,15]. Similar findings have also been made in long-term MHC-mismatched chimeras that had converted from mixed to complete donor chimerism after an earlier DLI [16]. In this study, MHC-mismatched C57BL/6 (B6)–into-AKR complete donor chimeras and B6-into-AKR mixed donor/host chimeras were used to address T_{reg} cell dynamics and DLI-induced GVL/GVH effects in early versus long-term HSC chimeras. First, we examined DLI-induced GVHD at several time points after HSCT (eg, days 28, 35, 42, 49, and 70) and the influence of Thy1^{+} T_{reg} cell depletion on GVHD at each time point. Second, we compared DLI-induced GVL in early (day 28) versus late (day 100) complete donor chimeras. Third, we examined GVL reactivity in long-term complete donor and mixed donor/host chimeras. Finally, we investigated the effect of in vivo CD25^{+} T_{reg} cell depletion on DLI-induced GVL/GVHD and whether host-type APCs, in the form of dendritic cells (DCs), given to long-term complete donor chimeras could influence GVL and GVH reactions.

**MATERIALS AND METHODS**

**Mice**

AKR/J (H-2^{k}, Thy1.1^{+}), AKR/Cum (H-2^{k}, Thy1.2^{+}), C57BL/6 (B6; H-2^{b}, Thy1.2^{+}), and B6.PL-Thy1/Cy (B6.PL; H-2^{b}, Thy1.1^{+}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the Biomedical Resource Center at the Medical College of Wisconsin (Milwaukee, WI).

**Reagents and Monoclonal Antibodies**

The following monoclonal antibodies (mAbs) were purchased from BD Pharmingen (San Diego, CA): fluorescein isothiocyanate (FITC)–conjugated anti-H-2^{k} (clone AF6-88.5), FITC–anti-CD11c (clone HL3), phycoerythrin (PE)–conjugated anti-CD5e (clone 145-2C11), PE–anti-CD25 (clone PC61), PE–anti-CD45R/B220 (clone RA3-6B2), PE–anti-CD11b (clone M1/70), PE–anti-Ly6G/6C (clone RB6-8C5), allophycocyanin-conjugated anti-CD4 (clone RM4-5), and PE–Cy5–anti-CD8a (clone 53-6-7). In vivo–depleting anti-CD25 (clone PC61) and anti-Thy1.2 (clone 53-2.1) mAbs were produced from hybridoma cells obtained from the American Type Culture Collection (Bethesda, MD). These mAbs were produced in our laboratory by using a Vivascreen bioreactor system (Hanover, Germany). Rabbit complement was purchased from Cedarlane Laboratories Ltd. (Hornby, Ontario, Canada). Anti-CD11c (N418)–conjugated immunomagnetic beads were purchased from Miltenyi Biotec Inc. (Auburn, CA). Collagenase D was obtained from Roche Applied Sciences (Indianapolis, IN). Bovine serum albumin and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO). Fetal bovine serum, phosphate-buffered saline, high-glucose Dulbecco’s modified eagle medium, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, sodium pyruvate, l-glutamine, l-arginine, l-asparagine, folic acid, non-essential amino acids, and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA).

**In Vitro Isolation of DCs**

Spleens harvested from AKR mice were digested with collagenase D (1 mg/mL) for 45 minutes at 37°C in a 5% carbon dioxide incubator. The splenocytes were then washed, incubated with anti-CD11c–conjugated immunomagnetic beads for 15 minutes at 4°C, and purified by positive selection with an automated Miltenyi Biotec immunomagnetic cell sorter. The purity of CD11c^{+} DCs was >98%.

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Establishment of Complete Donor and Mixed Donor/Host HSC Chimeras

To establish complete donor HSC chimeras, AKR/J hosts were lethally irradiated with 1100 cGy 1 day before HSCT. Fresh bone marrow (BM) cells harvested from B6 donors were depleted of mature T cells in vitro by using anti-Thy1.2 mAb and rabbit complement. A total of $10^7$ T cell–depleted nucleated BM cells were given intravenously (IV) to each recipient via the tail veins. To establish mixed donor/host HSCT chimeras, AKR/Cum mice were lethally irradiated with 1100 cGy 1 day before HSCT, and a single intraperitoneal (IP) injection of anti-Thy1.2 mAb (500 µg) was given on the day of HSCT to prevent graft rejection. Fresh BM cells harvested from B6 and AKR/Cum donor mice were mixed at a donor-host ratio of 3:1, and a total of $10^7$ non–T cell–depleted BM cells (ie, $7.5 \times 10^6$ from B6 and $2.5 \times 10^6$ from AKR/Cum) were injected IV. Levels of donor HSC engraftment were confirmed 3 weeks after transplantation by flow cytometric analysis of peripheral blood leukocytes by using the following cell-surface markers: FITC–anti–H-2Kb, FITC–anti–H-2Kk, PE–anti-CD3, PE–anti-B220, PE–anti-CD25, PE–Cy5–anti-CD8, and allophycocyanin–anti-CD4.

DLI and Leukemia Challenge

In all DLI studies, $3 \times 10^7$ splenocytes isolated from B6.PL mice (Thy1.1+) were administered IV at various time points after HSCT (days 28, 35, 42, 49, 70, and 100) to complete donor chimeras or to long-term mixed chimeras (day 100). Some DLI recipients were injected IP with anti-Thy1.2 mAb (500 µg) on the day of DLI to deplete donor BM-derived Thy1.2+ T cells in vivo. Treatment with anti-Thy1.2 mAb results in $>98\%$ depletion of Thy1.2+ cells (Figure 1). Other recipients were treated IP with anti-CD25 mAb 7 days (500 µg at day 93) and 4 days (250 µg at day 96) before DLI to deplete CD25+ cells in vivo. The optimal dose of anti-CD25 mAb needed to achieve maximal depletion of CD25+ T cells was determined in preliminary experiments (data not shown). In some day 28 or day 100 complete donor host HSC chimeras, $2.5 \times 10^6$ or $5 \times 10^6$ purified AKR DCs were infused alone or together with a DLI. To assess GVL reactivity, mice were challenged IV with $10^5$ AKR-derived M2 leukemia/lymphoma cells 1 week after DLI (ie, day 35 or day 107). The origin and characteristics of the M2 tumor, a T-cell leukemia/lymphoma that spontaneously arose in a male AKR/J mouse, have been previously described [17]. Mice that died from GVHD or tumor progression were differentiated from one another on the basis of clinical symptoms and systemic autopsy findings.

T-Cell and DC Phenotyping

Peripheral blood was collected from early (day 28) and long-term (day 100) complete donor HSC chimeras to assess the presence of donor BM-derived CD4+ and CD8+ T cells, as well as CD4+CD25+ T_reg cells, by flow cytometry. Peripheral blood leukocytes were stained with the following panel of cell-surface markers: FITC–anti–H-2Kb, PE–anti-CD3 or PE–anti-CD25, PE–Cy5–anti-CD8, and allophycocyanin–anti-CD4. To assess the purity of isolated DCs, the cells were stained with a combination of FITC–anti-CD11c and PE–anti-CD11b. The samples were analyzed on a Becton Dickinson FACSCalibur flow cytometer (San Jose, CA).

Statistics

Survival curves were compared by log-rank analysis. Relative risk comparisons were performed by using Fisher exact tests. Body weight loss values were compared by using the t test. Significance was defined as $P < .05$.

RESULTS

Induction of DLI-Induced GVHD after T_reg Depletion Waned with Time

Previous studies from our group showed that significant lethal acute GVHD occurs after DLI in MHC-mismatched recipients until the fourth week after HSCT and that new thymus-derived donor T_reg cells are in large part responsible for suppressing the GVH reactivity at 4 weeks after HSCT [11]. However, it was unclear whether T_reg cells continue to play an important role at later time points. Therefore, experiments were performed to elucidate the kinetics of Thy1+ T_reg cells in suppressing GVHD development in longer-term chimeras. DLI given to MHC-mismatched recipients at days 28, 35, 42, 49, or 70 resulted in little or no GVH-related mortality (Figure 1; DLI groups). In vivo depletion of Thy1+ T_reg cells by treatment of BM chimeras with anti-Thy1.2 mAb immediately before DLI resulted in severe and lethal GVHD when DLI was given at days 28, 35, and 42 (Figure 1; anti-Thy1.2 + DLI groups). In contrast, after T_reg depletion, DLI induced relatively mild GVHD at day 49 and no GVHD at day 70. Flow cytometric analysis of splenocytes from BM chimeras at each DLI time point indicated that anti-Thy1.2 mAb treatment resulted in $>98\%$ depletion of peripheral Thy1.2-expressing T cells (Figure 1).

The DLI-Induced GVL Effect Observed Early (Day 28) after HSCT Was Absent in Long-Term (Day 100) Complete Donor Chimeras

To study whether the DLI-induced GVL effect correlated with development of GVHD, early (day 28)
and long-term (day 100) complete donor chimeras were treated with DLI and challenged with M2 leukemia cells 1 week later, ie, at days 35 and 107, respectively. As shown in Figure 2, the GVL effect was robust in day 28 chimeras (Figure 2A), but it was completely absent in day 100 chimeras (Figure 2B). In
DLI-treated day 28 chimeras, 11 of 14 mice were tumor free for more than 70 days after challenge, with some long-term body weight loss (Figure 2C). Among the 3 deaths that occurred in this group, 2 (days 14 and 39 after tumor challenge) resulted from tumor progression, whereas the third (day 56) was attributed to GVHD. Akin to non–DLI-treated controls (BM transplantation only), all DLI-treated day 100 chimeras (12/12) died rapidly from tumor progression after challenge (Figure 2B), and corresponding acute body weight loss was observed in these animals (Figure 2D).

Depletion of CD25<sup>+</sup> T<sub>reg</sub> before DLI Failed to Restore the GVL Effect in Long-Term Complete Donor HSC Chimeras

Previous studies from our group revealed that CD4<sup>+</sup>CD25<sup>+</sup> cells comprise the most potent Thy1<sup>+</sup> T<sub>reg</sub> population in experimental DLI models [17,18]. When donor-derived T cells in the peripheral blood of day 28 and day 100 complete donor BM chimeras were phenotyped for CD3, CD4, CD8, and CD25 expression, the percentage of CD25<sup>+</sup> cells within the CD4 population (ie, putative T<sub>reg</sub> cells) was relatively higher in day 100 chimeras (9.7%-12.3%; n = 3) than in day 28 chimeras (5.7%-7.1%; n = 3) or naive B6 mice (7.7%-9.5%; n = 5), even though similar percentages of CD3<sup>+</sup>CD4<sup>+</sup> (15.5%-27.7% versus 17.4%-28.8%) and CD3<sup>+</sup>CD8<sup>+</sup> (5.7%-7.1% versus 5.7%-6.7%) were detected in day 28 and day 100 chimeras, respectively. To determine whether the presence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> contributed to the disappearance of DLI-induced GVL in long-term chimeras, day 100 chimeras were depleted of CD25<sup>+</sup> cells in vivo before DLI and subsequently challenged with M2 leukemia cells 1 week after DLI (ie, day 107). CD25<sup>+</sup> cell depletion failed to restore GVL reactivity (Figure 3). All anti-CD25/DLI–treated mice (n = 11) died from tumor growth after challenge (Table 1), and the kinetics of mortality in this group were similar to those of non–CD25-depleted mice given DLI (Figure 3A). Tumor progression was associated with cachexia (Figure 3B).

DLI-Induced GVL Activity Occurred in Long-term Mixed HSC Chimeras without GVHD

To investigate whether the disappearance of a DLI-induced GVL effect in long-term complete donor chimeras is related to a deficiency of stimulatory host alloantigens, B6/AKR long-term mixed HSC chimeras were generated. By transplanting recipients with a mixture of donor and host BM at a 3:1 ratio, 75% donor hematopoietic chimerism could be achieved (data not shown). The mixed chimeras were given (DLI + M2 group) or not given (M2 group) a DLI at day 100 after BM transplantation and then challenged with AKR M2 leukemia cells 1 week later (day 107). In contrast to complete chimeras, a robust GVL effect was observed in mixed chimeras given DLI, with 13 of 16 mice surviving tumor free for more than 70 days.
after tumor inoculation (Figure 4A). An absence of body weight loss in these mice correlated with an absence of GVH-associated mortality (Figure 4B), and it was confirmed that the 3 deaths were due to tumor progression. Long-term mixed chimeras not given a DLI were unable to resist the leukemia challenge, and all mice in this group died between 15 and 30 days after tumor challenge (Figure 4).

Host-Type DCs Coinfused with DLI Partially Restored GVL and GVH Effects in Long-Term Complete Donor Chimeras

Results in previous experiments suggested that the persistence of host-type APCs in day 100 mixed HSC chimeras facilitated a GVL effect in DLI-treated animals and that a lack of host APCs in day 100 complete donor chimeras was responsible for the absence of a GVL effect in DLI-treated animals. In this study, the presence of host-type DCs was shown to partially restore GVL and GVH effects in long-term complete donor chimeras.

Table 1. Outcomes for DLI-Treated and Tumor-Challenged Day 100 Complete Donor Chimeras

<table>
<thead>
<tr>
<th>Group</th>
<th>Anti-CD25</th>
<th>Host DCs</th>
<th>Mortality*</th>
<th>MST (d)†</th>
<th>Causes of Death (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No</td>
<td>No</td>
<td>100% (12/12)</td>
<td>15</td>
<td>Tumor (12), GVHD (9)</td>
</tr>
<tr>
<td>2</td>
<td>No</td>
<td>2.5 × 10^4</td>
<td>81.8% (9/11)</td>
<td>27</td>
<td>Tumor (9), GVHD (0)‡</td>
</tr>
<tr>
<td>3</td>
<td>No</td>
<td>5.0 × 10^4</td>
<td>80% (8/10)</td>
<td>38</td>
<td>Tumor (1), GVHD (7)</td>
</tr>
<tr>
<td>4</td>
<td>Yes</td>
<td>No</td>
<td>100% (11/11)</td>
<td>15</td>
<td>Tumor (11), GVHD (0)</td>
</tr>
<tr>
<td>5</td>
<td>Yes</td>
<td>2.5 × 10^4</td>
<td>66.7% (8/12)</td>
<td>25</td>
<td>Tumor (2), GVHD (6)‡</td>
</tr>
<tr>
<td>6</td>
<td>Yes</td>
<td>5.0 × 10^4</td>
<td>100% (10/10)</td>
<td>41</td>
<td>Tumor (0), GVHD (10)</td>
</tr>
</tbody>
</table>

*These data were obtained from the experiments in Figures 3, 5, and 6.
†MST, median survival time.
‡As analyzed by using Fisher exact tests, the relative risk of dying from tumor was significantly greater in group 2 than in group 5 (P = .003), and the relative risk of dying from GVHD was significantly greater in group 5 than in group 2 (P = .014). Log-rank comparisons of the survival curves for groups: 1 vs. 2, P = .019; 1 vs. 3, P = .0014; 1 vs. 4, P = NS (not significant); 4 vs. 5, P = .00488; 4 vs. 6, P = .0022; 2 vs. 5, P = NS; 3 vs. 6, P = NS.
GVL effect. To address this further, host-type DCs (2.5 or 5 \times 10^6 per mouse) were coinfused with a DLI into day 100 chimeras to determine whether a DLI-induced GVL effect could be restored by infusion of host APCs. Although coadministration of 2.5 \times 10^6 DCs with the DLI did not result in significantly better survival after tumor challenge as compared with non–DLI-treated chimeras (Figure 5A), the median survival time in DLI-treated mice was increased from 15 to 27 days (Table 1, groups 1 and 2). When the DC dose was increased to 5 \times 10^6 cells, DLI coadministration resulted in a significant survival benefit as compared with non–DLI-treated chimeras (Figure 5B), and the median survival time in DLI-treated mice was further increased to 38 days (Table 1, group 3). It is interesting to note that although all deaths in the chimeras given 2.5 \times 10^6 DCs and DLI (n = 9) were attributed to tumor progression, most deaths in chimeras given 5 \times 10^6 DCs and DLI (7 of 8 deaths) were attributed to GVHD (Table 1). Significantly increased body weight loss in the long-term chimeras given 5 \times 10^6 DCs and DLI (DLI + DC + M2 group; Figure 5D) as compared with chimeras not given DLI (DLI + M2 group; Figure 5D) correlated with the increased GVHD reactivity. However, no enhancement of GVHD was seen in DLI-treated day 28 complete donor chimeras coinfused with 5 \times 10^6 fresh host-type DCs (Figure 6).

**Long-Term Chimeras Depleted of CD25^+ T regulatory (T_reg) Cells before Coinfusion of Host DCs and DLI Had Increased GVL Reactivity at the Expense of GVHD**

We next examined whether DLI-mediated GVH/GVL reactions initiated by host DC administration are influenced by donor CD25^+ T_reg cells. To address this, day 100 complete donor chimeras were treated with 2 injections of anti-CD25 mAb at days 93 and 96 after HSCT to deplete CD25^+ cells in vivo. CD25-depleted or -nondepleted mice were treated either with DCs alone (2.5 or 5 \times 10^6 cells) or DCs plus DLI at day 100, and all mice were subsequently challenged with 10^3 AKR M2 leukemia cells at day 107. In vivo depletion of CD25^+ cells before DLI (no host DCs) had no effect on GVL reactivity (Table 1, group 1 versus 4). As shown in Figure 7A and 7B, DLI/DC coadministration modestly increased 70-day survival as compared with chimeras given DCs without DLI, but the increases in survival were not significant, and depletion of CD25^+ cells had no effect on survival. However, depletion of CD25^+ cells did significantly increase GVL reactivity in DLI/DC-treated chimeras.
count for the ability to give patients relatively large numbers of donor T cells with less severe GVHD than would be anticipated if similar numbers of T cells were given at the time of HSCT.

In the experiments shown in Figure 1, we extended our previous findings in day 28 complete donor HSC chimeras to examine the importance of Thy1\(^+\) T\(_{reg}\) cells at later time points. We confirmed our previous observations in day 28 chimeras demonstrating suppression of DLI-induced GVH reactivity by Thy1\(^+\) regulatory cells, and depletion of donor Thy1\(^+\) cells in day 35 and day 42 chimeras similarly resulted in lethal acute GVHD, thus indicating that Thy1\(^+\) T\(_{reg}\) cells still played an important role in suppressing DLI-induced GVH reactivity at these later time points. However, by day 49 after HSCT, the role of Thy1\(^+\) T\(_{reg}\) cells was no longer evident. This could be due to turnover of APCs from host to donor type during this time period, thus resulting in decreased activation of alloreactive T cells given as DLI. The presence of host-type APCs has been shown by others to be an absolute requirement for the induction of GVHD in murine HSCT models [12,15,25,26], and DLI in humans and dogs with mixed chimerism has resulted in GVHD [27,28]. Alternatively, it has been suggested that the persistence of T\(_{reg}\) cells requires the ongoing presence of cognate Ags [29]. Thus, it is possible that T\(_{reg}\) cells specific for host allo-Ags decrease in frequency as APCs turn over. However, the GVH reactivity induced in our long-term complete donor chimeras by coadministration of host-type DCs and DLI seemed to be regulated in part by CD25\(^+\) T\(_{reg}\) cells (Table 1), thus indicating that these cells persisted in the long-term chimeras. CD4\(^+\)CD25\(^+\) T\(_{reg}\) cells have been used to effectively prevent or treat GVHD after HSCT or DLI [18,19,30-34]. A potential problem with this approach is that accumulating evidence has suggested that CD4\(^+\)CD25\(^+\) T\(_{reg}\) cells can promote tumor growth/progression [35-38]. Therefore, depletion of CD4\(^+\)CD25\(^+\) cells may have a synergistic benefit with DLI therapy to clear tumor cells, particularly when the DLI-induced anti-tumor response alone is too weak to completely eradicate the tumor cells.

An absence of GVHD in DLI-treated long-term complete chimeras after Thy1\(^+\) T\(_{reg}\) cell depletion (Figure 1E) correlated with an absence of GVL reactivity in these animals (Table 1, group 2 versus 5; P = .003), but any survival benefit was negated by significantly increased GVH reactivity in these animals (P = .014).

### DISCUSSION

In early animal studies, immune-suppressive/regulatory cells were implicated as playing a role in the suppression of GVH reactivity after DLI [10]. Subsequent studies from our group and others demonstrated that various types of donor-derived cells and even residual host cells, including non-T cells, may be involved in regulating development of DLI-induced GVHD [11,18-24]. However, donor Thy1\(^+\)CD4\(^+\)CD25\(^+\) cells seem to be the most potent T\(_{reg}\) population according to studies in our laboratory examining the relationship of DLI-induced GVHD and deficiency of particular donor-derived T-cell subsets [11,18]. DLI is typically administered weeks or months after HSCT to treat tumor relapse in humans [4]. Therefore, it is conceivable that donor reconstitution of the host thymus has occurred in some DLI-treated patients and that donor-derived T\(_{reg}\) cells play a role in blunting DLI-induced GVH reactivity. This may in part ac-
in day 28 complete donor chimeras (Figure 2A) and in day 100 mixed chimeras (Figure 4A). Mapara et al. [15,16] similarly reported DLI-induced GVL reactivity without GVHD in day 56 mixed chimeras and a superior GVL effect in mixed chimeras as compared with complete donor chimeras. Other data have indicated that GVH reactions in mixed chimeric mice after DLI may be confined to the lymphohematopoietic tissues, which may account for the absence of DLI-induced GVHD in these animals [39]. Clinically, acute myeloid leukemia patients have responded better to DLI therapy than patients with acute lymphocytic leukemia, possibly as a result of direct tumor Ag presentation by myeloid leukemia cells that express immune costimulatory molecules [1]. It is interesting to note that we found that deficient GVL reactivity in long-term complete HSC chimeras could be partially restored by coadministration of host-type DCs and DLI (Figure 5A and B), but the increased GVL effect occurred at the expense of more GVHD (Table 1). This suggests the involvement of common target Ags. Our experimental results seem to favor the hypothesis that turnover of APCs from host type to donor type results in diminished GVH and GVL reactivities after DLI therapy, which is consistent with findings from other research groups [25,26]. Moreover, the absolute requirement for host-type APCs to elicit a GVL effect was elegantly demonstrated by the work of Mapara et al. [15] showing that GVL was robust when host-type DCs expressed MHC class I Ags but absent when host-type DCs in mixed chimeric mice were deficient in MHC class I. In one clinical study involving the use of prophylactic DLI to treat tumors after T cell–depleted BM transplantation, myeloablated patients were shown to tolerate higher dose of T cells and develop less severe GVHD when DLI was performed at 45 days instead of 30 days after transplantation [40]. However, DLI has mostly been used to treat residual or relapsed malignancies, and there are no conclusive data regarding the timing of administration and DLI-induced toxicity and/or GVL efficacy. Furthermore, benefits from DLI treatment are complicated by patient heterogeneity, tumor burden, the T-cell dose(s), and other therapies [1-5]. In practice, restoration of GVL/GVH activities by coinfusion of fresh host-type DCs would be difficult in humans because the DCs would need to be generated and cryopreserved before transplantation.

**Figure 7.** Depletion of CD25+ cells increased GVH/GVL reactivity in long-term BM chimeras coinfused with host DCs and DLI. Survival (A and B) and body weight changes (C and D) are shown for CD25-depleted or -nondepleted day 100 complete donor chimeras given 2.5 × 10⁶ (A and C) or 5 × 10⁶ (B and D) host-type DCs with or without DLI followed by challenge with AKR M2 leukemia cells at day 107. The data for the anti-CD25-treated groups represent the combined results of 3 independent experiments. Each group was included in at least 2 of the 3 experiments that were all performed within a 6-month time period. The total numbers of mice in each group are shown in (C) and (D). Survival curves for the DC + M2 and DLI + DC + M2 groups are the same as those shown in Figure 5, and they are shown to provide comparative survival data for the other experimental groups.
Data in Figure 4, as well as results from other investigators, have suggested that a state of mixed chimerism is important to facilitate a GVL effect without significant GVHD after DLI therapy in long-term murine chimeras [15,16]. Nonmyeloablative conditioning regimens can facilitate the establishment of mixed chimerism with less toxicity than myeloablative regimens, but mixed chimerism is associated with a higher risk for tumor relapse because of the development of donor/host T-cell tolerance [41]. Fortunately, antitumor responses have been effectively generated after DLI therapy in both myeloablative and nonmyeloablative settings [41]. Unlike mice, mixed chimeric patients frequently develop GVHD after DLI, and the incidence and severity of GVHD in these patients has been lower than or equivalent to that of complete chimeric patients [27,42]. In contrast to the results from complete chimeric patients, no significant differences in GVHD development were observed in mixed chimeric patients whether DLI was performed within the first 100 days after transplantation or beyond 180 days after transplantation [27,40]. These observations could be explained by the continuous presence of host APCs in mixed chimeric patients. Studies are needed to address the discrepancy between the findings in mice and humans, and a thorough clinical analysis is needed to examine the relationship between DLI-induced GVL/GVHD and the timing of DLI, as well as the relationship of DLI-induced GVL and GVHD in patients with mixed multilineage chimerism.

In summary, a state of mixed chimerism was superior to complete donor chimerism, because preservation of host-type APCs seemed to be essential for initiating DLI-induced GVL/GVH reactions in our studies. These results are consistent with previous findings by other investigators [15]. Infusion of host-type DCs partially restored a DLI-induced GVL effect in long-term complete chimeras, but restoration of GVL reactivity was accompanied with an increased risk for GVHD. Our data indicate that donor-derived T_{reg} cells can regulate DLI-induced GVL and GVH reactions in both early and long-term complete donor chimeras. The role of donor BM–derived T_{reg} cells in our mixed chimeric DLI model is under investigation, and we are interested in testing whether modulating the functional activity of T_{reg} cells can optimize GVL reactions in mixed chimeras.

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