Donor CD4-Enriched Cells of Th2 Cytokine Phenotype Regulate Graft-Versus-Host Disease Without Impairing Allogeneic Engraftment in Sublethally Irradiated Mice

By Daniel H. Fowler, Kazuhiro Kurasawa, Rhett Smith, Michael A. Eckhaus, and Ronald E. Gress

We have recently shown that donor CD4-enriched cells of Th2 cytokine phenotype, generated by treating mice in vivo with a combination of interleukin-2 (IL-2) and IL-4, prevent lipopolysaccharide-induced, tumor necrosis factor-α-mediated lethality during graft-versus-host reaction. To assess the potential regulatory role of such Th2-type cells in lethal graft-versus-host disease (GVHD) and graft rejection, we used a fully allogeneic murine transplant model using sublethally irradiated hosts (B6 → C3H, 500 cGy). Such recipients generated a strong host-versus-graft response, as reflected by their ability to reject T-cell–depleted inocula. The administration of T-cell–containing donor whole spleen inocula resulted in allograft rejection, but such recipients developed lethal GVHD. However, mice receiving sequential donor whole spleen (day 0) and CD4-enriched, Th2-type (day 1) populations engrafted, and had prolonged survival with protection from histologically defined tissue injury associated with GVHD. The findings in this fully allogeneic model thus extend our previous observations and indicate that the transfer of donor Th2-type cells may be an important strategy for regulating GVHD. Furthermore, the sequential "Th1→Th2-type" donor cell transfer described in this report represents a novel approach for abrogating graft rejection with concomitant control of GVHD and illustrates the importance of kinetics in the interaction of functionally distinct donor T-cell populations.

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DONOR CELLS OF THE Th2 TYPE REGULATE GVHD

Table 1. Donor Inocula Sufficient to Overcome Graft Rejection Results in Lethal GVHD: The Delayed Administration of Donor CD4-Enriched Cells of Th2 Cytokine Phenotype Reduces Lethal GVHD Without an Increase in Graft Rejection

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>TBI (cGy)</th>
<th>B6 Donor Inocula</th>
<th>Delayed B6 Inocula</th>
<th>Graft Rejection*</th>
<th>Long-Term Survivors (&gt;30 d/t)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 &quot;Th1&quot; (GVHD Control)</td>
<td>500</td>
<td>Th1†</td>
<td>—</td>
<td>7/46 (15.2%)</td>
<td>2/39 (5.1%)</td>
</tr>
<tr>
<td>2 &quot;TCD&quot;</td>
<td>500</td>
<td>TCD§</td>
<td>—</td>
<td>8/6 (100%)</td>
<td>NA</td>
</tr>
<tr>
<td>3 &quot;Th2&quot;</td>
<td>500</td>
<td>Th2</td>
<td></td>
<td></td>
<td>9/9 (100%)</td>
</tr>
<tr>
<td>4 &quot;Th1 + Th2&quot;</td>
<td>500</td>
<td>Th1 + Th2</td>
<td></td>
<td></td>
<td>0/11 (0%)</td>
</tr>
<tr>
<td>5 &quot;Th1 → Th2&quot;</td>
<td>500</td>
<td>Th1</td>
<td>Low-dose Th2 (day 1)</td>
<td>0/8 (0%)</td>
<td>1/9 (11.1%)</td>
</tr>
<tr>
<td>6 &quot;Th1 → Th2&quot;</td>
<td>500</td>
<td>Th1</td>
<td>Th2 (day 1)#</td>
<td>4/32 (12.5%)</td>
<td>12/29 (41.3%) (P &lt; .0001)**</td>
</tr>
<tr>
<td>7 &quot;Th1 + Th2&quot;</td>
<td>500</td>
<td>Th1</td>
<td>Th2 (day 3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 &quot;Th1 → Th2&quot;</td>
<td>500</td>
<td>Th1</td>
<td>CD4-Th2 (day 1 or day 3)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
</tr>
<tr>
<td>9 &quot;Th1 → CD4-depleted Th2&quot;</td>
<td>500</td>
<td>Th1</td>
<td>CD4-depleted Th2 (day 1 or day 3)</td>
<td>0/8 (0%)</td>
<td>0/8 (0%)</td>
</tr>
</tbody>
</table>

Abbreviations: TBI, total body irradiation; NA, not applicable.
† Rejecting mice had greater than 95% host-type chimerism.
‡ TCD inoculum consisted of 90 × 10^6 whole spleen cells from B6 mice, administered IV 4 to 6 hours after host irradiation.
§ TCD inoculum consisted of 50 × 10^6 CD4-enriched spleen cells from IL-2/IL-4-treated B6 mice.
|| This inoculum consisted of 50 × 10^6 CD4-enriched spleen cells from IL-2/IL-4-treated B6 mice.
¶ This group received 5 to 25 × 10^6 CD4-enriched spleen cells from IL-2/IL-4-treated B6 mice.
# This inoculum consisted of 50 to 150 × 10^6 CD4-enriched spleen cells from IL-2/IL-4-treated B6 mice.
** P value calculated by Student's two-sided t-test assuming unequal variances, in comparison to group 1.
†† This inoculum consisted of 50 × 10^6 CD4-enriched spleen cells from untreated B6 mice.
‡‡ The CD4-enriched population from IL-2/IL-4 treated B6 mice was additionally depleted of Thy1.2+ cells, and 50-150 × 10^6 cells were injected IV.

MICE.
Cs7IL6 (B6, H-2b) and C3H/HeJ (C3H, H-2b) mice were bred at the Frederick Cancer Research Center (Frederick, MD). All mice used were 8 to 20 weeks of age and were maintained in a specific pathogen-free facility at the National Institutes of Health (NIH; Bethesda, MD).

In vivo generation of donor CD4-enriched cells of Th2 cytokine phenotype. Donor-type B6 mice were injected for 5 days (intraperitoneally [IP] twice daily [TID]) with recombinant human IL-2 (rIL-2; 25,000 Cetus Units; provided by Chiron Corp, Emeryville, CA) and recombinant murine (rmIL-4; 500 ng; Peprotech, Inc [Rocky Hill, NJ]; provided by Dr Scott Duram, National Cancer Institute, Bethesda, MD). CD4-enriched spleen cells from cytokine-treated control mice were obtained by harvesting of spleens into a single-cell suspension, removal of B cells (goat antiserum against bioparticles; Advanced Magnetics, Cambridge, MA), and depletion of CD8+ cells by treatment with complement-sensitized from clone 83-12-5 (anti-CD8), and guinea pig complement (GIBCO, Grand Island, NY). The TCD population from cytokine-treated mice was generated by complement treatment of the spleen single-cell suspension after incubation with supernatant from clone HO-13-4 (anti-Thy 1.2).20

Allogeneic cell transfers. Recipient C3H mice were sublethally irradiated (500 cGy; 137Cs γ radiation source; Gamma Cell 40; Atomic Energy of Canada, Ltd, Ottawa, Canada) at a dose rate of 115 cGy/min. Four to six hours later, recipients were injected intravenously [IV] with a single-cell suspension of 90 × 10^6 unfractionated spleen cells from untreated B6 donor mice; alternatively, some mice received TCD B6 spleen cells (Table 1, group 2). GVHD control mice received no additional treatment (group 1; "Th1"); other mice received additional donor CD4-enriched cells of Th2-type either immediately (day 0; group 4), 24 hours (day 1; groups 5 and 6), or 72 hours (day 3; group 7) after the initial allogeneic transfer; control mice received additional donor CD4-enriched cells from untreated mice (group 8).

Pathologic examination of tissues. Cohorts of mice receiving only the allogeneic whole spleen cell inoculum ("Th1-type") or additional donor CD4-enriched cells of Th2 cytokine phenotype ("Th1 → Th2-type") were killed on day 14 posttransplant, and tissues were taken for histopathologic analysis (Table 1, groups 1 and 6, respectively; such subjects were also evaluated for chimerism, as described below). Liver, small intestine, colon, and skin were placed in 10% neutral buffered formalin and imbedded in paraffin, and 6-μm thick sections were stained with hematoxylin and eosin (H & E). The samples were examined in a blinded fashion by light microscopy, and each organ was graded semiquantitatively as 0 to 4+ (no change to severe GVHD). Grading of hepatic GVHD was grade 0, no lesions; grade 1+, minimal periductal and perivascular lymphoid cell infiltrates; grade 2+, minimal periductal and perivascular lymphoid cell infiltrates; grade 3+, moderately cellular periductal and perivascular lymphoid infiltrates, associated with individual hepatocyte degeneration; and grade 4+, intensely cellular, bridging periductal and perivascular lymphoid infiltrates, degeneration of individual biliary epithelial cells and infiltration of biliary epithelium with lymphoid cells, and prominent individual hepatocyte degeneration. Grading of intestinal (small and large) GVHD was grade 0, no lesions; grade 1+, rare crypt epithelial cell degeneration; grade 2+, small numbers of crypt epithelial cell degeneration, associated with an increase in...
intraepithelial lymphocytes; grade 3+, most crypts with small to moderate numbers of degenerative epithelial cells, increased numbers of intraepithelial lymphocytes, and presence of crypt abscesses; and grade 4+, most crypts with moderate numbers of degenerative epithelial cells, extensive intraepithelial lymphocytes and crypt abscesses, and evidence of epithelial erosion or ulceration. Skin was rarely positive for any lesions of GVHD, and was thus not included in the analysis.

Chimerism analysis by flow cytometry (FCM). Mice receiving only the TCD or CD4-enriched, Th2-type inocula (Table 1, groups 2 and 3) were killed on day 14 for chimerism analysis. All other treatment groups were observed for lethality, and subjects surviving more than 60 days posttransplant were then killed and evaluated. Splenic single-cell suspensions were prepared, stained, and analyzed by two-color FCM analysis as previously described. Reagents used included biotinylated and fluorescein isothiocyanate (FITC)-labeled anti-CD4 and anti-CD8 (GenTrak, Inc, Plymouth Meeting, PA) and FITC-conjugated anti-\( \text{H-2}^b \) (clone 20-8-6; generously provided by Julie Titus, NIH). Levels of donor chimerism were calculated as the percentage of donor chimerism = (donor cells/host + donor cells)) \times 100\%. Graft rejection was defined as greater than 95\% host-type chimerism; engrafted recipients typically had greater than 90\% donor-type chimerism.

Statistical analysis. Mice were examined daily for morbidity and mortality and were humanely killed if preterminal symptoms of GVHD developed. Survival data were plotted (see Fig 2) and analyzed by the nonparametric rank sum analysis of Wilcoxin \( P \) values of \(<0.05 \) were considered significant; long-term survivors were included in the analysis. Mean time to lethality was calculated by analysis of the time of death in those recipients undergoing lethality in the first 30 days posttransplant (long-term survivors were excluded from this analysis). Recipients that rejected the allograft were not included in the lethality analyses.

RESULTS

The administration of B6 donor whole spleen cells ("Th1-type") is necessary for the alloengraftment of sublethally irradiated C3H recipients. Sublethally irradiated C3H recipients (500 cGy) had preservation of an HVG rejection response, as shown by their ability to reject TCD B6 allogeneic inocula (Table 1, group 2; 0/6 mice engrafted at day 14 posttransplant). We first determined whether B6 donor naive whole spleen cells would modulate graft rejection in this model. Given that alloreactive lymphocytes produce Th1-type cytokines\(^2\) and typically result in a cell-mediated immune response in vivo (involving both CD4\(^+\) and CD8\(^+\) T lymphocytes),\(^3\) allotransfers containing unfractionated, naive splenic T cells are here referred to as "Th1-type" inocula. As shown in Fig 1A, recipients of B6 whole spleen inocula (90 \( \times \) 10\(^6\) cells; "Th1") were alloengrafted (>90\% donor-type lymphoid chimerism). This result was consistently observed, as only 7 of 46 (15.2\%) of such recipients rejected the allograft (Table 1, group 1); the administration of lower doses of B6 whole spleen cells resulted in a higher rate of graft rejection (data not shown). As a result of these observations, all experiments used an allogeneic inoculum of 90 \( \times \) 10\(^6\) unfractionated whole spleen cells to consistently overcome graft rejection and establish alloengraftment in the sublethally irradiated C3H recipients.

We have recently shown\(^6\) that the in vivo treatment of donor B6 mice for 5 days with a combination of IL-2 and IL-4 generates a CD4\(^+\)-enriched population of Th2 cytokine phenotype, as defined by increased production of IL-4 and IL-10 and decreased production of IL-2 and IFN-\( \gamma \). In an LPS-induced model of lethal GVHR, this Th2-type donor population protected mice from otherwise lethal allogeneic inocula. To evaluate whether such Th2-type cells might also regulate the HVG rejection reaction, sublethally irradiated C3H mice received donor CD4\(^+\)-enriched cells from IL-2/IL-4-treated B6 donor mice. As shown in Fig 1B ("Th2"), such recipients lacked any appreciable level of allochime-

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Fig 1. Sublethally irradiated C3H recipients reject B6 donor CD4-enriched cells of Th2 cytokine phenotype, but are engrafted by donor Th1-type or Th1 - Th2-type inocula. C3H mice were irradiated (500 cGy) and various donor B6 inocula were administered IV 4 to 6 hours later. Recipients were killed on day 14 posttransfer, spleen cells were harvested, FCM analysis was performed, and the level of allochimerism was calculated as described in Materials and Methods. In (A), the "Th1" inoculum consisted of 90 \( \times \) 10\(^6\) whole spleen cells from untreated B6 mice (a representative example of Table 1, group 1). (B) shows a representative example from Table 1, group 3; the "Th2" inoculum consisted of 50 \( \times \) 10\(^6\) CD4-enriched cells from B6 mice injected in vivo for 5 days (IP TID) with a combination of rhlL-2 125,000 Cetus Units/dose and rmIL-4 (500 ng/dose). (C) shows a representative example from Table 1, group 7; these "Th1 - Th2" recipients were injected on day 0 with the whole spleen B6 inocula (90 \( \times \) 10\(^6\) cells) and additional Th2-type inocula (50 \( \times \) 10\(^6\) CD4-enriched cells from IL-2/IL-4-treated B6 mice) on day 3 posttransplant.
The delayed administration of donor cells of Th2 cytokine phenotype reduces lethal GVHD. C3H mice were sublethally irradiated (500 cGy), and $9 	imes 10^6$ B6 donor whole spleen cells were injected on day 0 (curve C; GVHD control, "Th1"); Table 1, group 1; $n = 39$). Some mice received additional donor CD4-enriched cells of Th2-type on day 1 (curve A; "Th1 → Th2, day 1"); Table 1, group 6; $n = 29$; curve A v C: $P < .0001$) or on day 3 (curve B; "Th1 → Th2, day 3"); Table 1, group 7; $n = 18$; curve B v C: $P = .99002$). Recipients were observed for lethality; survival results were pooled from five independent experiments. $P$ values were determined by the nonparametric rank sum analysis of Wilcoxin.

Donor whole spleen inocula sufficient to overcome rejection generate lethal GVHD. As discussed above, allograftment was consistently observed in recipients of $90 \times 10^6$ naive unfractionated whole spleen cells. However, successful engraftment of this T-cell-containing donor inoculum resulted in acute lethality in the vast majority of recipients (Table 1, group 1; "Th1"); 37/39 (94.9%) of such allografted recipients died in the first 30 days posttransplant). As shown in the survival curve in Fig 2, mice engrafted with this inoculum underwent an acutely lethal process (median time to lethality of 14.5 days). Day 14 histopathologic analysis of hepatic, small intestinal, and colonic tissue from cohorts of this treatment group established the diagnosis of GVHD (see below).

**Donor whole spleen inoculum** influenced survival, some C3H recipients received the additional administration of donor CD4-enriched cells of Th2 cytokine phenotype might regulate GVHD. The administration of donor Th2-type cells, in fact, did improve survival for recipients of the day 0 whole spleen allogeneic inoculum. Protection from lethality was greatest when the Th2-type inocula was administered 24 hours after the whole spleen inocula (Fig 2, "Th1" v "Th1 → Th2, day 1"); $P < .0001$; recipients of the day 1 Th2-type inocula were also more likely to become long-term survivors (Table 1; 2/39 ["Th1"] v 12/29 ["Th1 → Th2, day 1"]; $P < .0001$). The dose of the day 1 Th2-type inoculum was important for the demonstration of protection from acute lethality; only 1 of 9 mice receiving less than $50 \times 10^6$ Th2 type donor cells survived more than 30 days posttransplant (Table 1, group 5). However, increasing the day-1 Th2-type dose to greater than $50 \times 10^6$ cells did not increase the rate of survival (4/11 recipients of $150 \times 10^6$ Th2-type cells were protected, whereas 8/18 recipients of $50 \times 10^6$ Th2-type cells were protected).

To assess whether timing of the Th2-type donor inoculum influenced survival, some C3H recipients received the additional Th2-type cells on day 3. Although lethal GVHD was attenuated in recipients of the day 3 Th2-type inocula (Fig 2; "Th1" v "Th1 → Th2, day 3"); $P = .0002$), long-term survival was not statistically different from that of GVHD controls (Table 1; 2/39 ["Th1"] v 4/18 ["Th1 → Th2, day 1"]; mice survived >30 days posttransplant; $P = .125$). Additionally, the time to lethality in recipients of the day 3 Th2 inocula was minimally, but not significantly, delayed (median time to lethality of 16.9 v 14.5 days for mice receiving only the whole spleen inocula; $P = .100$). Thus, the Th2-
Table 2. Compared With Th1 Recipients, Th1 → Th2 Recipients Are Protected From Multisystem GVHD

<table>
<thead>
<tr>
<th>Donor Cells Administered</th>
<th>Splenic Engraftment (day 14)</th>
<th>Histopathologic Score (day 14)*</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Whole Spleen (90 x 10^6) (day 0)</td>
<td>CD4+ Spleen Cells (x 10^6)</td>
</tr>
<tr>
<td>Th1 no. 1§</td>
<td>+</td>
<td>11.9</td>
</tr>
<tr>
<td>Th1 no. 2</td>
<td>+</td>
<td>8.4</td>
</tr>
<tr>
<td>Th1 no. 3</td>
<td>+</td>
<td>9.6</td>
</tr>
<tr>
<td>Th1 no. 4</td>
<td>+</td>
<td>13.4</td>
</tr>
<tr>
<td>Th1 → Th2 no. 15</td>
<td>+</td>
<td>47.6</td>
</tr>
<tr>
<td>Th1 → Th2 no. 2</td>
<td>+</td>
<td>31.5</td>
</tr>
<tr>
<td>Th1 → Th2 no. 3</td>
<td>+</td>
<td>35.2</td>
</tr>
<tr>
<td>Th1 → Th2 no. 4</td>
<td>+</td>
<td>30.4</td>
</tr>
</tbody>
</table>

* Tissues were processed, evaluated in a blinded fashion, and scored on a 0 through 4 scale (no change to severe GVHD).
† Donor CD4+ and CD8+ lymphoid splenic engraftment was determined by FCM analysis, and calculated using the formula: Donor CD4 + Donor CD8/(Donor + Host) (CD4) + (Donor + Host) (CD8).
‡ "Th1" mice (Table 1, group 1) received whole spleen cells from B6 mice (day 0; 90 x 10^6, injected IV).
§ "Th1 → Th2" mice (Table 1, group 8) received 90 x 10^6 donor B6 whole spleen cells (IV; day 0) and 150 x 10^6 CD4-enriched cells from B6 mice treated in vivo with a combination of rhIL-2 and rmIL-4 (doses as outlined in Materials and Methods).

type cell regulation of lethal GVHD was most effective when administered shortly (day 1) after the whole spleen inocula. Interestingly, the coadministration (day 0) of both naïve whole spleen and Th2-type cells conferred no protection from lethal GVHD (Table 1, group 4; lethality in 11/11 recipients).

To determine whether the CD4+ T-cell population from cytokine-treated mice was necessary for the observed protection from lethal GVHD, the CD4-enriched spleen population from IL-2/IL-4-treated donor mice was depleted of CD4+ cells. Recipients of such inocula (Table 1, group 9) were not protected from acute lethality (0/8 survivors), thus establishing that the CD4+ cells from IL-2/IL-4-treated mice were necessary for protection from GVHD. Furthermore, to evaluate whether the cytokine phenotype of donor CD4+ cells was related to the observed reduction in GVHD, a separate control group received the additional administration of naïve donor CD4+-enriched cells. Such recipients were not protected from lethality (Table 1, group 8; 0/8 survivors), thereby indicating that the Th2 cytokine phenotype of additional donor CD4+ cells was necessary for demonstrating protection from lethality.

Donor CD4-enriched cells of Th2 cytokine phenotype protect recipients from the histopathologic liver and intestinal lesions of GVHD. After establishing that the delayed administration of additional donor CD4-enriched cells of Th2 cytokine phenotype prolonged survival, we asked whether this protection was associated with a reduction in GVHD as defined by histologic criteria. Cohorts of mice were killed on day 14 posttransplant, and tissues were processed and semiquantitatively scored in a blinded fashion for lesions of GVHD. Moderate to severe GVHD-induced liver and intestinal changes were observed in 3 of 4 recipients of the whole spleen cell inocula (Table 2; "Th1†††"). Figure 3A illustrates the liver lesions found in these mice; the extensive periportal infiltration of lymphocytes and lymphoblasts, with associated multifocal, single-cell hepatocellular necrosis are consistent with severe hepatic GVHD. In contrast, Fig 3B shows that recipients of additional donor cells of Th2-type (Table 2, "Th1 → Th2†††") had a reduction in hepatic GVHD lesions; the liver is relatively normal in appearance, with only minimal periportal mononuclear cell infiltration and no evidence of hepatocellular necrosis. As shown in Table 2, the protection from hepatic GVHD conferred by the donor Th2-type cells was consistently observed; in fact, 2 of 4 mice in this cohort had completely normal appearing livers, without histologic evidence for GVHD (multiple sections from 2 separate liver lobes were evaluated).

In addition to protection from hepatic GVHD, Th1 → Th2 recipients were relatively spared of small intestinal and colonic lesions of GVHD. Figure 4 shows photomicrographs of H & E-stained intestinal tissue sections from representative Th1 and Th1 → Th2 recipients. Figure 4A (Th1 recipient) illustrates the increased numbers of intraepithelial lymphocytes and clusters of necrotic crypt epithelial cells characteristic of severe intestinal GVHD. Similar lesions were seen in both the small intestine and colon; these recipients also had multifocal mucosal erosion and foci of ulceration in areas of the Peyer’s Patches (illustrated in Fig 4B). In contrast, the Th1 → Th2 recipients were relatively protected from both small intestinal and colonic lesions of GVHD; the histologically normal small intestine of one such recipient is illustrated in Fig 4C. This protection from intestinal GVHD was marked, as 3 of 4 mice had no evidence of small intestinal or colonic GVHD. Because the intestine was harvested as an “intestinal roll,” the entire length of the intestine was available for analysis, and it is thus unlikely that sampling error could account for the lack of lesions observed in the Th1 → Th2 recipients. Importantly, these differences in histologic GVHD could not be explained by differential alloengraftment; ie, all mice in the day-14 histologic analysis had predominant allochimerism by FCM analysis, and the Th1 → Th2 recipients actually had increased absolute numbers of donor cells engrafted (as shown in Table 2, splenic engraftment analysis).
DONOR CELLS OF THE TH2 TYPE REGULATE GVHD

Because GVHD is known to alter thymocyte subpopulations,20 most notably causing a diminution in the number and percentage of CD4⁺CD8⁺ thymocyte precursors, we performed FCM on thymocytes from day 14 Th1 and Th1 → Th2 recipients. Figure 5A shows data from a representative Th1 recipient and illustrates that these mice displayed the characteristic decrease in the CD4⁺CD8⁺ thymocyte population seen with GVHD. In contrast, 4 of 4 of the Th1 → Th2 recipients had preservation of this CD4⁺CD8⁺ thymocyte precursor subpopulation (Fig 5B). Because allotyping of the thymocyte populations was unsuccessful due to low levels of class I surface expression, it is unknown whether the double-positive precursor subpopulation seen in Th1 → Th2 recipients was of donor or host origin.

Analysis of long-term survivors (>60 days postransplant). To assess the engraftment and clinical status of survivors of the Th1 → Th2 donor cell regimen, subjects surviving more than 60 days were killed for analysis. Such long-term survivors typically showed persistence of allogeneic lymphoid chimerism (average of 92.3% donor-type splenic lymphoid chimerism, n = 8); however, two mice had predominant host-type chimerism (donor type chimerism of 2% and 16.2%). Multilineage engraftment was likely, because spleen cells in the nonlymphoid gate were also of donor type (data not shown; direct staining for nonlymphoid markers was not performed). The immune status of such long-term survivors was markedly abnormal, as evidenced by very low thymocyte (<2 × 10⁶ cells per thymus) and splenocyte numbers (<10 × 10⁶ cells per spleen). Additionally, such long-term survivors had marked reductions in the percentage and absolute numbers of splenic CD4⁺ lymphocytes and low proliferative and cytokine responses (data not shown).

DISCUSSION

We have recently shown18 that donor cells of Th2 cytokine phenotype regulate Th1-driven, LPS-induced lethal GVHR (unirradiated parent-into-F1 model); the protection conferred by the Th2-type cells was characterized by regulation of both cytokine (IFN-γ and TNF-α) and cellular (CD8⁺ allografting) events associated with lethal GVHR. In this study, we extend our previous observations and conclude that donor cells of Th2 cytokine phenotype regulate GVHD in fully allogeneic, irradiated recipients. Th2-type cell regulation of GVHD was observed by prolonged survival and, importantly, through a reduction in the histopathologic tissue damage characteristic of GVHD. Additionally, our observation that sublethally irradiated recipients of fully major histocompatibility complex (MHC)-mismatched donor whole spleen and Th2-type cells engrafted with reduced GVHD shows that Th1/Th2 interactions in vivo are important considerations for the administration of functional donor T-cell populations in transplant settings in which bidirectional alloreactivity exists (graft rejection and GVHD). As a result, we propose that a strategy incorporating a “Th1- → Th2-type” donor cell administration might represent a novel strategy for the abrogation of graft rejection with regulation of GVHD.

The T cells mediating graft rejection and GVHD, the primary barriers for the broadened clinical application of AlloBMT, appear to exist in a competitive, dynamic balance.
Fig 4. Day-14 histologic analysis. Characteristic intestinal lesions of GVHD are reduced in recipients of Th1-→Th2-type donor cell inocula. (A) is a representative example of H & E-stained small intestinal sections from sublethally irradiated C3H recipients of the B6 whole spleen cell inocula (Table 2, "Th1") and illustrates the mononuclear cell infiltration and crypt epithelial cell necrosis (see arrowhead) seen in mice undergoing lethal GVHD. Additionally, as shown in the representative example in (B), these recipients had multifocal mucosal ulceration superficial to Peyer’s Patches (marked by P). In contrast, (C) illustrates that recipients of additional B6 donor CDC-enriched cells of Th2-type (Table 2, "Th1 + Th2") had preservation of crypt architecture without evidence for mucosal lesions. Small and large intestinal sections were semiquantitatively scored for GVHD and the results are shown in Table 2. (Original magnification for [A] and [C] ×200; for [B] ×50.)

For example, the critical role that donor T lymphocytes may play in abrogating host rejection responses has been identified²⁷ and recently characterized in a murine fully allogeneic bone marrow transplantation model.²⁸ In that study, it was shown that donor CD8⁺ cells were approximately five times more efficient than CD4⁺ cells in abrogating graft rejection. Given that CD8⁺ responses in vivo are driven by CD4⁺ cells of Th1-type²⁵ and that CD8⁺ alloreactive lymphocytes may secrete primarily Th1-type cytokines (although CD8⁺ cells secreting Th2-type cytokines have been reported³⁵), a donor Th1-type immune response may be most capable of overcoming rejection responses. In this study, we evaluated whether donor Th2-type cells might regulate HVGR; no engraftment was observed in these recipients (Table 1, group 3), thus supporting the concept that Th1-type donor cells are important in the abrogation of rejection. The precise mechanism whereby donor T cells counteract rejection responses is not clear; some studies have shown that donor...
We thus conclude that the parameters of timing and dose are important considerations for strategies attempting to regulate ongoing Th1-type alloresponses with cells of Th2 cytokine phenotype. CD8+ cells generate class I-restricted antihost cytotoxic responses, whereas others have shown that "veto inactivation" of host cells by donor cells expressing cytotoxic granules can occur (data from our laboratory show that the donor Th2-type cells used in this study do not generate significant in vitro cytotoxic responses in allogeneic MLR/CML reactions or possess veto cell function; K. Kurasawa, unpublished observations).

In this study, because of the relatively low host irradiation administered, a strong HVG rejection response against allogeneic inocula was generated. Donor T-cell-containing inocula resulting in engraftment in less than 100% of recipients consistently resulted in lethal GVHD (Table 1, group 1; rejection was observed in 7/46 [15.2%] of recipients; lethal GVHD was observed in 37/39 [94.9%] of engrafted recipients). This model thus illustrates a critical problem in AlloBMT, ie, the "therapeutic index" for allogeneic T-cell containing inocula (ie, a T-cell dose that will consistently abrogate HVG without GVHD) may in some settings be marginal.

Given this limitation, our observation that the delayed administration of donor CD4-enriched cells of Th2 cytokine phenotype improved survival in recipients of allogeneic inocula has important implications. The sequential Th1->Th2-type donor treatment was most protective when the Th2-type cells were administered shortly (day 1) after the initial whole spleen inoculum and when doses of greater than 50 × 10^6 Th2-type cells were administered. Our finding that delaying the administration of Th2-type cells to day 3 resulted in a reduction in the protection from GVHD suggests that Th1/Th2 interactions in the transplant setting are of primary importance during the generation of alloresponses. Interestingly, coadministration of the Th2-type cells with the whole spleen inoculum (day 0) did not protect recipients from lethal GVHD (Table 1, group 4); this observation illustrates the complex nature of interacting T-cell populations in vivo and further stresses the importance of timing in the interaction of functionally distinct donor T-cell populations. We thus conclude that the parameters of timing and dose are important considerations for strategies attempting to regulate ongoing Th1-type alloresponses with cells of Th2 cytokine phenotype.

The model presented here generates a very aggressive lethal GVHD (median survival time of 14.5 days) with strong histopathologic features of GVHD, and thus represents a stringent model to evaluate methods to regulate allogeneic responses in vivo. As a result, the observed protection from hepatic, small intestinal, and colonic infiltration and necrosis conferred by donor Th2-type cells was revealing (Table 1 and Figs 3B and 4C). GVHD-induced multiorgan tissue damage contributes greatly to the morbidity and mortality associated with AlloBMT. Intestinal lesions are especially important in severe GVHD; some investigators have reasoned that bacterially derived LPS endotoxin, introduced systemically via GVHD-induced intestinal lesions, results in cytokine-mediated tissue damage (in particular, TNF-α-mediated pathology) and subsequent mortality from GVHD. The absence of intestinal pathology in Th1->Th2 recipients thus may have been an important determinant in the observed survival advantage for this cohort. Interestingly, a recent report in a murine parasite model provides additional evidence that cells of Th2 cytokine phenotype regulate intestinal lesions caused by pathogenic Th1-type responses.

The regulation of GVHD conferred by the Th2-type inoculum did not appear to negatively effect the extent or stability of alloengraftment. In fact, as illustrated in the day-14 splenic analysis shown in Table 2, the reduced hepatic and intestinal lesions observed in recipients of the Th2-type inoculum occurred concomitant with an increase in the absolute number of donor lymphoid cells engrafted. Additionally, the majority of long-term surviving recipients of the Th2-type inoculum showed persistence of donor-type lymphoid engraftment. These findings thus suggest that the allogeneic reactions accounting for the abrogation of graft rejection and the mediation of GVHD may be distinct. Given that the mechanism of GVHD involves a cytokine cascade, whereas the abrogation of rejection is largely mediated by cytolytic or veto mechanisms, the administration of donor Th2-type cells may therefore regulate cytokine events without compromising the cellular components of the allograft that promote engraftment.

Given that the thymus is an important and very sensitive target tissue of GVHD, the partial preservation of thymic histology, numbers, and subpopulations observed in recipi-
ents of the Th1→Th2-type donor inoculum provides further evidence that the Th2-type cells regulated GVHD. In light of a recent study\(^7\) that illustrates the deleterious effects of GVHR-induced thymic damage on positive and negative selection, this observation has potential relevance for attempts to preserve immunity posttransplant. Although day-14 thymic parameters in the Th1→Th2 recipients were preserved relative to GVHD control mice, long-term survivors (>60 days posttransplant) of this Th1→Th2 donor inoculum showed evidence for severe thymic-related immunosuppression. Because immunosuppression is probably the most sensitive indicator of GVHD,\(^1\) the observed thymic dysfunction and generalized immunosuppression in long-term survivors suggests that, although the Th2-type cells prolonged survival and reduced the histologic changes of GVHD, such survivors most likely had sublethal or chronic GVHD. The regulation of GVHD by donor Th2-type cells was therefore incomplete; we are currently evaluating whether multiple doses or alternative sources of Th2-type cells, or the systemic administration of Th2-type cytokines, might enhance the Th2-effect and result in more complete regulation of GVHD.

The mechanism whereby the Th2-type cells regulate GVHD is currently under investigation and is not completely understood at this time. However, we have previously shown that donor Th2-type cells regulate both cytokine (IFN-γ and TNF-α) and cellular (CD8\(^+\) alloengraftment) processes associated with GVHR.\(^1\)\(^4\) Recently, we have shown that the Th2-type cells used in these experiments reduce the generation of CD8\(^+\) cells and cytolytic activity in an in vitro allogenic MLR/CML culture sytem (K. Kurasawa, unpublished observations). Because donor CD8\(^+\) cells are a key effector cell of GVHD, Th2-type cell regulation of CD8\(^+\) proliferation and/or function is one potential mechanism for the protection observed in this model. The diminished cellular infiltration of host tissues observed on histopathologic evaluation of Th1→Th2 recipients further suggests that the Th2-type cells may regulate GVHD by altering cellular processes in vivo. Additionally, because of evidence that the elaboration of cytokines (in particular, TNF-α\(^2\)) is an important contributor to the tissue damage observed in GVHD, regulation of cytokines in vivo may have contributed to the preserved tissue histology observed in recipients of the Th2-type inocula. Given our previous observation that Th2-type donor cells reduce LPS endotoxin-induced TNF-α production in vivo during GVHR, regulation of TNF-α and/or other macrophage-derived inflammatory cytokines may have contributed to the protection from GVHD. This possibility is currently under investigation.

This report, by showing that the delayed administration of donor CD4±enriched cells of Th2 cytokine phenotype prolongs survival and reduces histopathologic GVHD-induced tissue lesions, introduces a novel strategy for the regulation of alloreactivity in vivo. Our results illustrate the importance of timing in the administration of functionally distinct donor lymphoid populations and suggest that a strategy incorporating the sequential administration of Th1- and Th2-type donor cells can achieve the abrogation of graft rejection with subsequent regulation of GVHD. Such a "Th1→Th2 strategy" might have application for attempts to decrease graft rejection in the setting of unrelated donor transplants or in transplants using less-intensive preparative regimens. Whether a Th1→Th2-type donor strategy, by increasing the therapeutic index of allogeneic T-cell transfers, can be used to mediate a graft-versus-leukemia effect is of interest and is under investigation.

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