The Allogeneic Effect Revisited: Exogenous Help for Endogenous, Tumor-Specific T Cells

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ABSTRACT

The “allogeneic effect” refers to the induction of host B cell antibody synthesis or host T cell cytotoxicity, including tumoricidal activity, by an infusion of allogeneic lymphocytes. We show that treatment of mice with cyclophosphamide (Cy) followed by CD8+ T cell-depleted allogeneic donor lymphocyte infusion (Cy1 CD82 DLI) induces regression of established tumors with minimal toxicity in models of both hematologic and solid cancers, even though the donor cells are eventually rejected by the host immune system. The optimal antitumor effect of Cy1 CD82 DLI required the presence of donor CD4+ T cells, host CD8+ T cells, and allo-antigen expression by normal host but not tumor tissue. The results support a model in which a donor CD4+ T cell-mediated graft-versus-host (GVH) reaction effectively awakens antitumor immunity among Cy-resistant host CD8+ T cells. These events provide the cellular mechanism of the “allogeneic effect” in antitumor immunity. Cy1 CD82 DLI may be an effective and minimally toxic strategy for awakening the host immune response to advanced cancers.

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KEY WORDS

Allogeneic • Graft-versus-host disease • Adoptive immunotherapy • Cyclophosphamide • Lymphocyte subsets • graft-versus-tumor effects

INTRODUCTION

Allogeneic blood or marrow transplantation (allo-BMT) is a well-established therapy for hematologic malignancies. The graft-versus-leukemia (GVL) effect of alloBMT is perhaps the best evidence that T cells can mediate the destruction of advanced, metastatic human malignancies via direct killing of tumor cells by donor T cells reactive to host histocompatibility antigens [1-8]. Despite the curative potential of allogeneic T cells, alloBMT as a procedure is severely hampered by 3 factors: (1) toxicity; (2) lack of efficacy, especially in solid tumors; and (3) limited availability of human leukocyte antigen (HLA)-identical donors. The conditioning regimen, infection, and GVHD all contribute to toxicity, and lack of efficacy manifests itself as progression of the underlying malignancy after transplantation [9,10].

Recently, there have been reports of disease responses in patients with hematologic [11-13] or solid [14] malignancies despite rejection of the donor leukocytes. Earlier studies had shown that antitumor effects can be mediated by donor lymphocyte infusions (DLI) in patients receiving no or minimal conditioning. Interestingly, although alloBMT has not been conclusively shown to be effective in the treatment of solid tumors, allogeneic or even xenogeneic lymphocyte infusions have induced objective responses in as many as 20%-30% of patients with advanced solid malignancies [15]. These observations raise the question of whether transiently engrafting lymphocytes mediate clinical responses via direct killing of tumor cells. Alexander and colleagues [16] were the first to speculate that cells of the host mediate the antitumor effect of allogeneic lymphocytes. Katz et al. [17,18] subsequently found that allogeneic DLI prolonged the survival of guinea pigs subsequently challenged with a host strain leukemia, even when the challenge occurred after the
allogeneic lymphocytes had been rejected by the host immune system. This was dubbed the “allogeneic effect” of antitumor immunity, yet the underlying cellular mechanisms of this effect have been poorly defined.

Here, we have developed a mouse model to investigate the cellular interactions involved in the antitumor effect of transiently engrafting lymphocytes. These studies reveal a potential collaboration in which donor CD4+ T cells cooperate with, and effectively awaken, host CD8+ T cells to induce tumor regression in both hematologic and solid malignancies. Therefore, infusion of allogeneic lymphocytes after minimal conditioning can mediate an antitumor effect against a wide spectrum of malignancies with reduced toxicity compared with standard allogeneic bone marrow transplantation.

MATERIALS AND METHODS

Animals

C57BL/6 (B6; H-2d), BALB/c (H-2d, Thy1.2+/-), BALB/c X B6 (CB6) F1 (H-2d/b), and B6 X C3H (B6C3) F1 (H-2d/b) mice were all obtained from the National Cancer Institute (Frederick, MD). Mice were maintained in microisolator cages and were fed ad libitum with autoclaved laboratory chow and acidified water. All mice were approximately 6 to 12 weeks of age at the time of treatment. All manipulations were performed in a laminar flow hood. The Animal Care and Use Committee of the Johns Hopkins University approved all procedures on animals in accordance with guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). In specified GVHD experiments, mice were weighed semiweekly beginning at the time of adoptive transfer of lymphocytes.

Tumor Cell Lines

A20 is a spontaneous B cell leukemia/lymphoma of BALB/c origin [19]. Cells were obtained originally from American Type Culture Collection (ATCC; Manassas, VA), and were maintained in culture and administered intravenously (i.v.) at the lethal dose of 1 x 106 as previously described [20]. Cells were cultured in vitro in Eagle Hanks Amino Acid (EHAA) medium (Biofluids, Rockville, MD), 10% fetal calf serum (FCS; GIBCO BRL, Gaithersburg, MD), 5 M 2-mercaptoethanol (2-ME), glutamine, and antibiotics (complete medium; CM). RENCA, a murine carcinogen-induced renal cell carcinoma of BALB/c origin and B16, a murine melanoma cell line of C57Bl/6 origin, were used in specified experiments and maintained in vitro in RPMI 1640 (Life Technologies, Grand Island, NY), supplemented with 10% heat-inactivated FCS (HyClone, Logan, UT), 5 x 10-5 M 2-ME, 20 mmol/L HEPES, 30 Ag/mL gentamicin (Schering Corporation, Kenilworth, NJ), and 0.2% sodium bicarbonate.

Cell Preparations

Donor spleens were removed aseptically and pressed through a nylon mesh to obtain single cell suspensions. In some experiments, T cells were depleted from donor splenocytes by incubation with antibodies to CD4 (RL172.4; gift of Dr Albert Bendelac, U. Chicago, Chicago, IL) and/or CD8 (3.155; ATCC), and guinea pig complement (GIBCO BRL), as previously described [21]. Cell suspensions were vigorously pipetted, counted, and washed in sterile phosphate-buffered saline (PBS) prior to injection. The purity of depleted cells was greater than 99.4% in all experiments.

Adoptive Transfer of T Cells

Cyclophosphamide (Cy; Bristol- Myers, Evansville, IN) was dissolved in PBS to a concentration of 20 mg/mL and administered at a dose of 200 mg/kg intraperitoneally (i.p.) before or after tumor inoculation as specified in each experiment. Twenty-five million splenocytes, prepared as above, were injected i.v. into the lateral tail vein unless otherwise specified.

In Vivo Depletion of T Cells

Mice were depleted of CD8+ T cells in vivo by giving 1.4 mg 2.43 (rat antimouse CD8 monoclonal antibody) [22,23] i.p. on days 9 and 30 after tumor inoculation. The 2.43 antibody was produced and purified from the supernatant of a bioreactor cell culture at the National Cell Culture Center (Division of Biostat International, Worcester, MA) and was diluted in sterile PBS prior to injection. Control mice were injected with sterile PBS only.

Antibodies and Flow Cytometry

Antibodies for flow cytometry were anti-CD4 (allophycocyanin, peridinin chlorophyll protein [PerCP], and phycoerythrin-Cy5 [Cy5]), Thy1.1 (FITC, PerCP and phycoerythrin [PE]), Thy1.2-allophycocyanin and -fluorescein isothiocyanate (FITC), H-2Kb FITC, CD-45 PerCP, CD8-PE, and -FITC, and B220-FITC (eBioscience, San Diego, CA). All antibodies were purchased from BD Biosciences (Mountain View, CA) unless otherwise specified. Gated events were collected on a FACSCalibur (Becton Dickinson, San Jose, CA) and analyzed using CellQuest software (Becton Dickinson).

Analysis of Leukocyte Chimerism

At designated times after DLI, blood was obtained from the lateral tail vein, or animals were killed and suspensions of spleen and/or bone marrow were prepared. Erythrocytes from the peripheral blood were lysed by using ammonium chloride buffer before initiation of staining. For determination of lineage-specific chimerism in BALB/c chimeras, 1 million cells were stained with fluorescein (FITC)-conjugated antibody.
to H-2K\textsuperscript{b} or H-2K\textsuperscript{d}-FITC with or without phycoerythrin (PE)-conjugated antibodies to CD4 or CD8 (all from Becton Dickinson). In each experiment, samples of peripheral blood from at least 3 host strain mice not receiving transplants were stained for donor H-2 or Thy antigens. The mean + (3 × SEM) of the percentage of FITC cells in hosts not receiving transplants was calculated (and was <0.5% for every experiment), and any transplant recipient containing a greater percentage of FITC cells than this value was considered to have donor cell engraftment.

### Lymphocyte Quantification

Trucount\textsuperscript{TM} Tubes (BD Biosciences) were used for determining absolute counts of leukocytes in blood. The tubes contain a known number of lyophilized 4.2-mm fluorescent beads. In specified experiments, peripheral blood was obtained from the lateral tail vein at specified times after adoptive lymphocyte transfer. Trucount\textsuperscript{TM} was performed according to the manufacturer’s recommendations. Briefly, 20 μL containing CD45-PerCP antibody was distributed to each Trucount\textsuperscript{TM} tube. Then 50 μL of peripheral blood from each mouse was added, gently mixed, and incubated for 15 minutes at room temperature in the dark. The lysis and fixation were obtained by adding 450 μL of FACS Lysing solution\textsuperscript{TM} (BD Biosciences). Samples were analyzed within 1 hour on a FACS Calibur (Becton Dickinson). Samples were mixed gently immediately prior to analysis. Absolute cell count was calculated using the following formula:

\[
\frac{\text{# of events in region containing cell}}{\text{# of events in absolute count bead region}} \times \frac{\text{# of beads per test}}{\text{test volume}} = \text{absolute count of cell}
\]

![Graphs A, B, C, D](image)

**Figure 1.** Cy + Non-engrafting CD8-depleted DLI abrogates risk of GVHD, induces anti-tumor immunity, and prolongs survival of animals with established, metastatic hematologic and solid tumor malignancies. (A, B) CD8 depletion of DLI abrogates risk of GVHD without compromising anti-tumor immunity. BALB/c mice (H-2\textsuperscript{d}; n=10/group) either received 10\textsuperscript{6} A20 lymphoma cells IV on Day 0 alone (△) or were conditioned with Cy 200 mg/kg IP on day –1 and received 10\textsuperscript{6} A20 lymphoma cells IV on Day 0 (○). Additional mice were then treated with 5 × 10\textsuperscript{7} whole spleen cells from fully MHC-mismatched C57BL/6 (H-2\textsuperscript{b}) donors, either undepleted (■) or depleted of CD4\textsuperscript{+} T cells (●), CD8\textsuperscript{+} T cells (▲), or both (□). Results for 1A: (▲) versus (□) \(P = .04\), (●) versus (□) \(P = .3\). Results for 1B: (▲) versus (○) versus (□) \(P < .0001\), (●) versus (□) versus \(P = .0002\), (○) versus (□) \(P = .17\). (C) Cy + CD8-depleted allogeneic DLI prolongs survival of animals with established, metastatic lymphoma. BALB/c mice (H-2\textsuperscript{d}; n=10/group) received 10\textsuperscript{6} A20 cells IV on Day 0 either alone (■), or followed by treatment with Cy 200 mg/kg IP on day 14 (●), or Cy 200 mg/kg IP on day 14 and 5 × 10\textsuperscript{7} spleen cells from fully MHC-mismatched C57BL/6 (H-2\textsuperscript{b}) donors, depleted of CD8\textsuperscript{+} T cells on Day 15 (▲). Results show: (●) versus (■) \(P < .0001\), (▲) versus (■) \(P = .02\). (D) Cy + CD8-depleted allogeneic DLI prolongs survival of animals with established, metastatic renal cell carcinoma. BALB/c mice (H-2\textsuperscript{d}; n=10/group) received 10\textsuperscript{6} RENCA cells IV alone on Day 0 either alone (■), or followed by treatment with Cy 200 mg/kg IP on day 14 (●), or Cy 200 mg/kg IP on day 14 and 5 × 10\textsuperscript{7} spleen cells from fully MHC-mismatched C57BL/6 (H-2\textsuperscript{b}) donors, depleted of CD8\textsuperscript{+} T cells on Day 15 (▲). Results show: (●) versus (■) \(P = .01\), (▲) versus (●) \(P = .001\). All experiments were repeated at least once.
where * can be found on the TruCOUNT Absolute Count Tube foil pouch label.

**Statistical Analysis**

All survival data were analyzed by the nonparametric rank sum test of Wilcoxon. $P < .05$ was considered statistically significant. In mice receiving both tumor and donor lymphocytes, the mechanism of death (tumor versus GVHD) was verified by necropsy of representative animals (data not shown).

Medians and SEMs were calculated for lymphocyte and chimerism data and analyzed using Sigma Plot, v9.0 (Systat Software, San Jose, CA). All experiments were repeated at least once.

**RESULTS**

**Cyclophosphamide plus CD8+ T Cell-Depleted DLI is Effective against Early and Established, Metastatic Hematologic, or Solid Cancers**

To model the antitumor effects of transiently engrafting allogeneic lymphocytes, mice were conditioned with cyclophosphamide (Cy) only, a drug that is minimally toxic to host hematopoietic stem cells. In the first set of experiments, Cy was administered the day before tumor challenge, with or without concomitant lymphocyte transfusion, so that any antitumor effects observed could be unequivocally attributed to the lymphocytes and not the drug. In a tumor survival experiment employing BALB/c recipients and fully MHC-incompatible B6 donors, administration of Cy followed by 50 million whole splenocytes led to severe acute GVHD (aGVHD), marked by hunched posture, ruffled fur, weight loss, diarrhea, and ultimately death (Figure 1A). In contrast, animals receiving the same number of splenocytes depleted of CD8+ cells (CD8− DLI) had no clinical evidence of GVHD and survived longer than animals receiving Cy plus splenocytes depleted of both CD4+ and CD8+ cells (median survival, 60 versus 30 days; $P = .04$). Recipients of CD4+ cell-depleted splenocytes did not survive significantly longer than recipients of T cell-depleted splenocytes (median survival, 56 versus 30 days; $P = .30$). Therefore, donor CD4+ T cells are required for the optimal antitumor effect of Cy + CD8-depleted DLI.

In a second tumor survival experiment employing BALB/c recipients and fully MHC-incompatible B6 donors (Figure 1B), animals that received CD8− DLI survived significantly longer than mice receiving Cy alone (median survival, 73 versus 19.5 days; $P < .0001$). Additionally, recipients of CD8− DLI survived significantly longer than mice receiving CD4−CD8− DLI (median survival, 73 versus 22 days; $P = .0002$). Furthermore, there was no difference between mice receiving no DLI (Cy only) and CD4−CD8− DLI (median survival, 19.5 versus 22 days; $P = .17$). This experiment demonstrates that CD4−CD8− DLI does not provide any antitumor benefit over no DLI. Deaths in mice in Figures 1A and 1B that received A20 alone, or A20 with Cy conditioning +/−CD8− DLI or CD4−CD8− DLI were secondary to tumor, as confirmed by necropsy or visualization of protuberant abdomens and/or hindleg paralysis, whereas deaths in mice receiving whole spleen DLI were solely from GVHD. Recipients of CD4− DLI died either early from GVHD or later from tumor.

To investigate the therapeutic potential of Cy + CD8− DLI, disseminated tumor was established by intravenous (i.v.) injection 14 days prior to any treatment. Compared to animals receiving no treatment, A20- or RENCA (renal cell carcinoma)-bearing BALB/c mice treated with Cy alone survived a median of 34 (Figure 1C) or 6 days (Figure 1D) longer ($P < .0001$ and $P = .01$) respectively. Addition of CD8− DLI from B6 donors further prolonged survival by a median of 10 (Figure 1C; $P = .02$) and 7 (Figure 1D; $P = .001$) days, respectively. These data demonstrate that transiently engrafting allogeneic lymphocytes achieve modest but significant prolongations of survival in mice with disseminated hematologic or solid malignancies.

**CD8− DLI Engrafts Transiently and Does Not Cause GVHD or Leukopenia**

The 2 most common complications of transfusing viable allogeneic lymphocytes into unconditioned human recipients are severe aGVHD and bone marrow aplasia, both of which require the sustained engraftment of the donor cells [6,24]. To characterize the toxicities of Cy + DLI, tumor-free BALB/c mice were conditioned with Cy, transfused with 50 million B6 splenocytes, and were monitored serially for survival, and leukocyte count in the peripheral blood. Additionally, A20-bearing BALB/c mice were conditioned with Cy, transfused with 50 million B6 splenocytes, and were monitored for donor CD4+, CD8+, and B cell chimerism. Recipients of either whole or CD4+ T cell-depleted splenocytes in nontumor bearing mice had GVHD-associated mortality (Figure 2C), and leukopenia prior to death (Figure 2D). In contrast, splenocytes depleted of CD8+ cells, alone or together with CD4+ cells, did not induce either GVHD-associated mortality or sustained leukopenia in nontumor bearing mice. In A20-bearing mice, recipients of either whole or CD4+ T cell-depleted splenocytes had sustained engraftment of donor CD4+ (Figure 2A), CD8+ (data not shown), and B cells (Figure 2B), whereas recipients of splenocytes depleted of CD8+ cells, alone or together with CD4+ cells engrafted only transiently. The effect of donor T cell subset depletion on donor chimerism was the same in tumor-free versus tumor-bearing recipients of donor lymphocyte infusions (data not shown). Therefore, in immunocompetent recipients, the combination
of Cy + CD8− allogeneic DLI induces antitumor effects without DLI-associated toxicities.

**A Direct Graft-versus-Tumor (GVT) Reaction Is Not Required for Prolongation of Survival by Cy + CD8− DLI**

A variety of mechanisms have been proposed to account for the antitumor effect of transiently engrafting lymphocytes, including a direct GVT effect [25,26], or the stimulation of endogenous antitumor immunity [16-18]. In some instances, it has been shown that a host-versus-graft reaction [27-29] can augment antitumor immunity. We wished to characterize the antitumor effect of a graft-versus-host (GVH) reaction in the absence of any T cell-mediated host-versus-graft or GVT effects. To do so, CB6 F1 mice bearing the B16 melanoma, of B6 origin, were treated with Cy followed by nothing or DLI from syngeneic CB6 F1 or parental strain B6, or BALB/c donors. Whereas BALB/c donors would induce both GVH reactions and GVT effects, and CB6 F1 donors would provide neither, the B6 donors are syngeneic to the tumor and would induce only a GVH reaction. Recipients of DLI from either parental strain prolonged survival compared to recipients of either no DLI (Figure 3; \( P = .04 \) and \( P = .06 \) for B6 and BALB/c donors, respectively) or DLI that was syngeneic to the recipient (\( P = .04 \) and \( P = .03 \) for B6 and BALB/c donors, respectively). The results with B6 DLI demonstrate that a GVH reaction is sufficient to prolong survival in the absence of a direct GVT effect. The failure of CB6 F1 DLI to prolong survival demonstrates that host expression of alloantigens is required for the induction of antitumor immunity.
Experiments were repeated at least once. CD4+15 from syngeneic (CB6F1) (■), or Cy 200 mg/kg i.p. on day 14 and 5 days after tumor inoculation. Results show: (●), 1 haplotype matched (C57Bl/6) (○), or fully MHC mismatched (BALB/c) (□) donors, depleted of CD8+ T cells. The table included describes the GVT and GVH relationships between the donor and recipient strains given that B16 melanoma is of B6 background. Results show: (●) v (■) P = .04, (○) v (●) P = .06, (■) v (■) P = .04, (○) v (□) P = .03. All experiments were repeated at least once.

**Figure 3.** Tumor expression of alloantigens is not required for the beneficial effect of Cy plus CD8-depleted DLI. CB6 F1 mice (H-2d), n = 10/group) received 3 x 10^6 B16 melanoma cells i.v. on day 0 followed by treatment with Cy 200 mg/kg i.p. on day 14 (■), or Cy 200 mg/kg i.p. on day 14 and 5 x 10^7 spleen cells on day 15 from syngeneic (CB6F1) (■), 1 haplotype matched (C57Bl/6) (○), or fully MHC mismatched (BALB/c) (□) donors, depleted of CD8+ T cells. The table included describes the GVT and GVH relationships between the donor and recipient strains given that B16 melanoma is of B6 background. Results show: (●) v (■) P = .04, (○) v (●) P = .06, (■) v (■) P = .04, (○) v (□) P = .03. All experiments were repeated at least once.

**Host CD8+ T Cells Participate in the Antitumor Effect of Cy + CD8− DLI**

CD8+ T cells have been shown to be capable of mediating antitumor immunity in both mouse models [30,31] and in human clinical trials [32]. Because CD4+ T cells deliver help to CD8+ T cells by “licensing” antigen-presenting cells [33–35], we postulated that a donor CD4+ T cell-mediated GVH reaction effectively licenses host APCs to provide help to host-derived, antitumor CD8+ T cells. To characterize the role of host CD8+ T cells in the antitumor effect of Cy + CD8− DLI, the A20 therapy experiment of Figure 1C was repeated but with the addition of separate groups of mice treated with the in vivo CD8-depleting monoclonal antibody 2.43 on days 9 and 30 after tumor inoculation. Table 1 shows the effect of antibody treatment on peripheral blood lymphocyte populations in recipients of Cy + CD8− DLI, and Figure 4 shows the effect of antibody treatment on the antitumor effect of Cy + CD8− DLI. Several conclusions emerge from these data. First, depletion of host CD8+ T cells was associated with a higher peak and longer duration of donor CD4+ T cell chimerism (Table 1), implicating a role for host CD8+ T cells in determining the kinetics of donor graft rejection. Second, the survival of animals treated with Cy alone was longer in antibody-untreated than in CD8-depleted mice (Figure 4A; median survival, 99 versus 51 days, P = .005). This result suggests that host CD8+ T cells contribute to the antitumor effect of Cy. Third, CD8-depleted recipients of Cy + CD8− DLI survived longer than CD8-depleted recipients of Cy alone (Figure 4A; median survival >130 versus 51 days, P = .005), suggesting that allogeneic donor CD4+ T cells mediate an antitumor effect that does not require host CD8+ T cells. Fourth, Cy + CD8− DLI was marginally more effective in antibody-untreated than in antibody-treated recipients (Figure 4A; median survival >130 days for both groups; P = .08). This result raises the possibility that host CD8+ T cells participate in the antitumor effect of Cy + CD8− DLI, and demonstrates that prolonged survival of donor cells does not guarantee a more potent antitumor effect.

In the A20 model described above, a clear role of host CD8+ T cells in the antitumor effects of CD8− DLI was difficult to discern because donor CD4+ T cells could kill tumor cells directly and because the 2.43 antibody prolonged donor cell survival in addition to depleting host CD8+ T cells. To eliminate the possibility of a direct GVT effect of DLI, we examined the effect of host CD8+ cell depletion in B16-bearing CB6 F1 recipient of Cy ± B6 CD8− DLI, which is syngeneic to the tumor (Figure 4B). When host CD8+ T cells were not depleted, recipients of Cy + CD8− DLI survived significantly longer than recipients of Cy alone (Figure 4B; median survival 56.5 versus 70 days, P = .02). In contrast, when host CD8+ T cells were depleted, the recipients of Cy + CD8− DLI did not survive significantly longer than recipients of Cy alone (Figure 4B; median survival 52.5 versus 53.5 days, P = .41). This result demonstrates that host CD8+ T cells are required for the antitumor effect of CD8− DLI in the treatment of metastatic B16 melanoma.

Additionally, because the antitumor effect of B6 CD8− DLI is abrogated completely by depleting host CD8+ T cells (Figure 4C), it rules out any direct GVT effects from the donor B6 cells; otherwise, this effect would have been seen even in the absence of host CD8+ T cells.

**DISCUSSION**

The results presented here demonstrate that transiently engrafting allogeneic lymphocytes can mediate the regression of established solid or hematologic malignancies with minimal associated toxicity. Treatment of tumor-bearing animals with Cy was required...
to unmask the antitumor activity of CD8-depleted DLI. This antitumor activity involves at least 2 distinct mechanisms: (1) a direct GVT effect that requires CD4$^+$ T cells in the DLI and alloantigen expression by the tumor itself, and (2) an indirect antitumor effect mediated by host CD8$^+$ T cells and requiring a GVH reaction against nonmalignant host tissue.

Although it is possible that the relative contribution of CD4 and CD8 cells in GVHD and GVT is model specific, we have found that the combination of Cy followed by CD8$^+$ DLI is effective at inducing tumor regression without producing GVHD in 2 fully MHC-mismatched and 1 MHC-haploidentical strain combination (data not shown). We do find, however,

Table 1. Lymphocyte Subsets after Cy + CD8$^+$ DLI: Effect of In Vivo CD8 Depletion

<table>
<thead>
<tr>
<th>Day after CD8 DLI</th>
<th>Median % CD8$^+$ Cells in Blood (SEM)</th>
<th>Median % donor CD4$^+$ Chimerism (SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Depletion</td>
<td>CD8$^+$ Depleted</td>
<td>$P$</td>
</tr>
<tr>
<td>7</td>
<td>2.9 (0.76)</td>
<td>0.12 (0.03)</td>
</tr>
<tr>
<td>14</td>
<td>5.3 (3.2)</td>
<td>0.30 (0.43)</td>
</tr>
<tr>
<td>21</td>
<td>ND*</td>
<td>0.24 (0.13)</td>
</tr>
<tr>
<td>28</td>
<td>ND</td>
<td>2.0 (0.69)</td>
</tr>
</tbody>
</table>

*ND, not determined

BALB/c mice (H-2$^d$; n = 10/group) either depleted in vivo of CD8$^+$ T cells with 2.43 antibody on day 9 and every 3 weeks, or undepleted, received Cy 200 mg/kg i.p. on day 14, and 5 x 10$^7$ spleen cells from fully MHC-mismatched C57BL/6 (H-2$^b$) donors, depleted of CD8$^+$ T cells on day 15. Donor CD4$^+$ cell chimerism (% of total CD4$^+$ cells) was measured weekly, from days 7-28, by staining tail blood with fluorochrome-conjugated antibodies against CD4 and H-2K$^b$ (donor). Percentage of host CD8$^+$ T cells in tail blood was also measured.

Figure 4. The antitumor effect of Cy plus CD8-depleted allogeneic DLI requires host CD8$^+$ T cells and is mediated by both a direct GVT and an indirect GVHD effect. (A) CD8$^+$ DLI mediates antitumor immunity through a GVT effect that requires direct tumor expression of alloantigens. BALB/c mice (H-2$^d$; n = 10/group) received 10$^6$ A20 cells i.v. on day 0, either alone (■) or followed by treatment with 2.43 antibody on day 9 and every 3 weeks (∆), or Cy 200 mg/kg i.p. on day 14 (▲), or 2.43 antibody on day 9 and every 3 weeks, and Cy 200 mg/kg i.p. on day 14 (∆), or Cy 200 mg/kg i.p. on day 14 and 5 x 10$^7$ spleen cells from fully MHC-mismatched C57BL/6 (H-2$^b$) donors, depleted of CD8$^+$ T cells on day 15 (■), or 2.43 antibody on day 9 and every 3 weeks, Cy 200 mg/kg i.p. on day 14, and 5 x 10$^7$ spleen cells from fully MHC-mismatched C57BL/6 (H-2$^b$) donors, depleted of CD8$^+$ T cells on day 15 (■). Results show: (▲) v (∆) $P = .005$, (■) v (∆) $P = .005$, (■) v (■) $P = .02$, (B, C) Cy + CD8-depleted DLI syngeneic to the tumor is sufficient to provide antitumor immunity. CB6F1 mice (H-2$^b$; n = 10/group) received 1.5 x 10$^4$ B16 melanoma cells i.v. on day 0 either alone (■) or followed by treatment with 2.43 antibody on day 9 and every 3 weeks (∆), or Cy 200 mg/kg i.p. on day 14 (▲), or 2.43 antibody on day 9 and every 3 weeks, Cy 200 mg/kg i.p. on day 14, and 5 x 10$^7$ spleen cells from 1 haplotype matched C57BL/6 (H-2$^b$) donors, depleted of CD8$^+$ T cells on day 15 (■), or 2.43 antibody on day 9 and every 3 weeks, Cy 200 mg/kg i.p. on day 14, and 5 x 10$^7$ spleen cells from 1 haplotype matched C57BL/6 (H-2$^b$) donors, depleted of CD8$^+$ T cells on day 15 (■). Results show: (■) v (▲) $P = .02$, (■) v (∆) $P = .41$. All experiments were repeated at least once.
that when the recipients’ tumor is MHC Class II⁺, there are both direct and indirect antitumor effects of donor CD4⁺ T cells, whereas the antitumor effect against MHC Class II⁻ tumors is indirect only, that is, mediated by host CD8⁺ T cells. We specifically chose to study a variety of tumor models including both hematologic and solid tumors of BALB/c background (A20 and RENCA, respectively), as well as a solid tumor of C57BL/6 background (ie, B16) to make our conclusions more generalizable and applicable to a variety of cancer types.

Survival is not always a proper measure of a GVT effect. However, we performed parallel transfers of allogeneic cells into tumor-free mice, and found that the only groups with mortality were those that received DLI containing CD8⁺ T cells. These deaths were GVHD related, as mice exhibited ruffled fur, hunched posture, diarrhea, and weight loss (data not shown). Tumor-free mice that received Cy with or without CD8⁻ DLI appeared healthy and survived long term. From this, we conclude that CD8⁺ T cells are required for GVHD-associated mortality, and that deaths among tumor-bearing mice not receiving CD8⁺ T cells were attributable to complications of progressive tumor.

Previous studies have demonstrated an antitumor effect of allogeneic or even xenogeneic lymphocyte infusions given to unconditioned or minimally conditioned humans [13,15,36-44] or experimental animals [18,26,45,46]. A variety of mechanisms have been invoked to explain tumor regression induced by transiently engrafting DLI, including transient GVT responses mediated by donor T cells or NK cells [12,26,46-48], conditioning effects on host immunity [49,50], or even abrogation of host tolerance by cytokines liberated during the rejection reaction [27,29]. Moreover, immunosuppressive conditioning has been shown to augment the capacity of adoptively transferred allogeneic cells to induce GVHD [51] or GVT effects [46], and recent studies have shown that lymphopenia-induced proliferation per se is sufficient to augment antitumor immunity by adoptively transferred T cells [52,53]. We are currently investigating why Cy pretreatment is required to unmask the antitumor activity of CD8⁻ DLI in animals with established tumors. Preliminary experiments demonstrate that Cy mitigates the inhibitory influence of tumor-induced, Foxp3⁺ regulatory T cells (H.J.S. and E.J.F., unpublished data), but other explanations remain possible, such as the enhancement of tumor- and allo-antigen presentation resulting from the Cy-induced apoptosis of tumor and host cells. Any or all of these mechanisms may be contributing to the antitumor effect of transiently engrafting DLI in our models. Indeed, results in the A20 model demonstrate that at least some of the antitumor effect of CD8-depleted DLI is independent of host CD8⁺ T cells (Figure 4), and thus may be produced directly by donor lymphocytes. However, when the donor lymphocytes were syngeneic to the tumor, an antitumor effect of allogeneic lymphocyte infusion that is dependent upon the presence of host CD8⁺ T cells became evident, suggesting that donor CD4⁺ T cells and host CD8⁺ T cells cooperate to induce tumor regression. This implies that the reason that CD8⁻-depleted recipients of Cy + CD8⁻ DLI survived longer than CD8⁻-depleted recipients of Cy alone in the A20 model (Figure 4A), but not in the B16 melanoma model (Figures 4B-C), may be explained by direct tumor recognition of MHC class II on A20 tumor cells, an event that would not occur with B16 because it does not express MHC Class II molecules. Taken together, the results of Figure 4 suggest that CD8⁻ DLI mediates antitumor effects through 2 distinct mechanisms—a direct GVT effect that requires direct tumor expression of alloantigens, and an indirect antitumor effect that is mediated by host CD8⁺ T cells and does not require tumor expression of alloantigens. Therefore, CD8⁺ T cells of host origin may play a critical role in producing tumor regression in the context of a GVH reaction, as described here, or in a host-versus-graft reaction, as described previously by Sykes and colleagues [29].

The idea that a GVH reaction could awaken a dormant antitumor response from the host was first proposed by Alexander and colleagues [16]. The term “allogeneic effect” was originally coined to describe how a GVH reaction could substitute for cognate T cell help in the secondary antibody response to a hapten [54], but was extended to describe how an allogeneic lymphocyte infusion could augment host resistance to a subsequent tumor challenge, even at a time when the donor cells had been rejected. Because of limitations in existing technology, these early studies could not define the precise cellular interactions involved in the antitumor effect of transiently engrafting lymphocytes [17,18,45]. The current study describes a novel cooperation between donor CD4⁺ and host CD8⁺ T cells in mediating an antitumor effect against a subsequent tumor challenge as well as against an established burden of either a solid or hematologic malignancy. Because CD4⁺ T cells provide “help” to CD8⁺ cytotoxic T cells by activating APCs [33-35], it seems reasonable to propose that alloreactive donor CD4⁺ T cells recognize and activate host APCs, which in turn, can activate tumor-specific host CD8⁺ T cells even after the donor CD4⁺ T cells have been rejected. This model is consistent with the finding that alloantigen expression by nonmalignant host tissue is sufficient for an antitumor effect of parental DLI given to an F₁ host (Figure 3A).

Allogeneic BMT has an established role in the treatment of a variety of hematologic malignancies through the induction of a GVT effect. Although there has been no dearth of attempts to apply alloBMT to the treatment of advanced solid tumors, results have
been mixed at best. A putative GVT effect of allogeneic transplant has been demonstrated against a variety of solid tumors [55], including renal cell carcinoma [56], breast cancer [57,58], colon cancer [59], and ovarian carcinoma [60], but the antitumor effect usually occurs in the context of GVHD, and no conclusive benefits in overall or event-free survival have been reported [61]. In contrast, Cy + CD8^+ DLI induced antitumor effects against systemically disseminated solid tumors with minimal toxicity. Although encouraging, these results need to be confirmed in more clinically relevant models, such as the treatment of endogenous rather than transplanted tumors. We postulate that distinct populations of T cells mediate antitumor effects after allogeneic transplant versus Cy + CD8^+ DLI. The antitumor effectors after myeloablative transplant are thought to be donor T cells reactive to host minor histocompatibility antigens. Although these T cells can cure hematologic malignancies through a lymphohematopoietic GVH reaction that destroys both normal and malignant blood cells, there is no a priori reason for them to be able to distinguish between normal and malignant solid tissue. In contrast, at least some of the antitumor effectors of Cy + CD8^+ DLI are host CD8^+ T cells, which are presumably tolerant to normal peripheral self tissues. It is possible that the systemic activation of host APCs by alloreactive donor CD4^+ T cells could also unmask the activity of autoreactive T and/or B cell clones. Although we have not rigorously evaluated the mice for autoimmune phenomena, none was clinically evident (data not shown).

The results described herein demonstrate that chemotherapy, with or without adoptive cellular immunotherapy, works best in the presence of an intact host CD8^+ T cell compartment. Indeed, the participation of host CD8^+ T cells in the antitumor response to chemotherapy (Figure 4A) may explain why the pretreatment absolute lymphocyte count correlates with the response to chemotherapy of lymphoma [62] and solid tumors [63]. By the same token, it is also possible that chemotherapy-induced lymphopenia underlies the inexorable decline in responsiveness of metastatic solid tumors to cytotoxic drugs. If, as our results suggest, the optimal response to immunotherapy requires an intact host immune system, then the sequence of trying immunotherapy after the failure of standard chemotherapy would appear to be counterproductive. Likewise, the strategy of intensive, nonselective lymphodepletion prior to adoptive immunotherapy [32,64,65] makes sense only when donor cells are the sole mediators of antitumor immunity.

Finally, our results support a strategy of awakening dormant antitumor immunity against advanced cancer by providing exogenous CD4^+ T cell help for endogenous tumor-specific CD8^+ T cells [66]. The capacity of a GVH reaction to provide this help suggests that allogeneic lymphocytes are a readily available tool for breaking functional tolerance to advanced cancers in the clinic.

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