

Donor Leukocyte Infusion from Immunized Donors Increases Tumor Vaccine Efficacy after Allogeneic Bone Marrow Transplantation¹

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ABSTRACT

Donor T cells play a critical role in mediating both harmful graft-versus-host disease (GVHD) and beneficial graft-versus-tumor effect after allogeneic bone marrow transplantation (BMT). We have recently demonstrated a novel treatment strategy to stimulate specific antitumor activity with preservation of tolerance to host antigens after T cell-depleted allogeneic BMT by vaccination of recipients with irradiated B16 melanoma cells engineered to secrete granulocyte-macrophage colony-stimulating factor. In this murine system, donor leukocyte infusion from a donor immunized with the recipient-derived B16 vaccines enhanced clinical activity of tumor vaccines without exacerbating GVHD. CD4⁺ T cells are essential for this enhancement. *In vitro* analysis of splenocytes from donor leukocyte infusion donor mice demonstrated that immunization of donors with the recipient-derived B16 vaccines elicited potent T-cell proliferation and cytokine responses specific to B16 antigens. These results demonstrate that immunization of donors with recipient-derived tumor vaccines preferentially induces tumor-specific T-cell responses and that vaccination of both donors and recipients can generate potent antitumor immunity without exacerbating GVHD. This strategy has important implications to prevent recurrence of malignancies after BMT.

INTRODUCTION

A number of promising tumor vaccination strategies to augment antitumor immunity have recently been tested in multiple rodent tumor systems (1). Tumor vaccination strategies include: (a) altered whole tumor cell vaccines, which are genetically engineered to express immunoregulatory molecules, such as cytokines and costimulatory molecules; (b) peptide/protein/ganglioside vaccines; (c) DNA/RNA vaccines; and (d) DC-based vaccines. Because the most relevant tumor antigens to stimulate effective immune responses are still unknown, whole tumor cell vaccines continue to be most commonly used in clinical studies (2, 3). Whole tumor cell vaccines have great potential to stimulate broad immune responses against multiple antigens. A broad response is more likely to result in prolonged effects, because different clones with selective antigen loss can reside within tumor tissues (4, 5).

GM-CSF³ is one of the most efficacious molecules used to create whole tumor cell vaccines that have been tested in mice (5, 6). Modification of the tumor microenvironment with GM-CSF-based vaccines improves tumor-antigen presentation in the development of specific cellular and humoral immunity and compromises tumor vasculature with soluble factors and leukocytes (5, 7). The principles

derived from these preclinical studies have proved relevant to patients with advanced renal cell carcinoma, malignant melanoma, prostate cancer, or pancreatic cancer (8–11).

Allogeneic BMT is the treatment of choice for a number of hematological and some solid tumors. Relapse of malignancy after BMT, however, remains a major clinical problem. Improved patient outcomes require novel treatment approaches, because residual disease after BMT is often resistant to cytotoxic therapies (12, 13). Recently, two groups have established a novel treatment strategy in murine models to stimulate tumor-specific responses in recipients of syngeneic BMT through irradiated tumor cells that secrete GM-CSF (14, 15). This strategy has also proved successful in stimulating tumor-specific responses after allogeneic BMT while preserving tolerance to host antigens, but the immunosuppression associated with active GVHD could abrogate the effect of the vaccine (15, 16). Remarkably, this vaccination strategy was extremely effective after allogeneic BMT when the donor inoculum was TCD because the prevention of GVHD by TCD permitted reconstitution of T cells from donor stem cells and restored the efficacy of vaccination (15). In humans, however, immune reconstitution after TCD BMT is often incomplete because of the age-related reductions in thymic regenerative capacity (17). Therefore, novel approaches to enhance clinical activity of the vaccine are required.

Cellular immunotherapy with DLIs has been used recently to induce a GVT effect in patients with post-BMT relapse (18). In this setting, GVHD appears to be less severe than that observed when similar numbers of donor T cells are infused at the time of BMT (19, 20). Mechanisms responsible for decreased GVHD after DLI may include: (a) avoidance of the “cytokine storm” induced by pretransplant conditioning (21, 22); (b) disappearance of recipient-derived, antigen-presenting cells (23); and (c) generation of regulatory T cells in the newly developing immune system at the time of DLI (24, 25). In an attempt to improve the clinical activity of tumor vaccines given to BMT recipients, we evaluated whether DLI from donors immunized with irradiated recipient-derived tumor cell vaccines would show improved therapeutic efficacy without exacerbating GVHD in a well-defined mouse model.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6, H-2^b, and CD45.2⁺), SJL (H-2^s and CD45.1⁺), B6SJLF1 (H-2^{bbs} and CD45.1^{+/2+}), and LP (B6, H-2^b, and CD45.2⁺) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The age range of mice used as BMT recipients was between 10 and 20 weeks.

BMT. Mice were transplanted according to a standard protocol as described previously (26). On day 0 of BMT, mice received 11 Gy of TBI (¹³⁷Cs source) split into two doses, separated by 3 h to minimize gastrointestinal toxicity. TCD BM cells (5 × 10⁶) from SJL donor mice were injected i.v. into B6SJLF1 recipients. TCD of BM was performed by incubating cells with anti-Thy-1.2 mAbs at 4°C for 30 min followed by low toxicity rabbit complement treatment for 40 min at 37°C. This two-round TCD procedure resulted in <0.01% T cell in the BM. Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyper-chlorinated drinking water for the first 3 weeks post-BMT, replaced by filtered water thereafter.

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³The abbreviations used are: GM-CSF, granulocyte-macrophage colony-stimulating factor; BMT, bone marrow transplantation; GVHD, graft-versus-host disease; TCD, T cell depleted; DLI, donor lymphocyte infusion; GVT, graft-versus-tumor; TBI, total body irradiation; BM, bone marrow; mAb, monoclonal antibody; IL, interleukin; TFS, tumor-free survival; DC, dendritic cell.

DLI. Spleen cells harvested from SJL donor mice were used as the source of DLI. In some experiments, CD4⁺ T cells, CD8⁺ T cells, or B220⁺ B cells isolated from spleens using immunomagnetic beads and AutoMACS separation (Miltenyi Biotec, Bergisch Gladbach, Germany) were given as DLI. Cell purity was analyzed by flow cytometry to verify >90% purity. DLI was given i.v. to BMT recipients 4 weeks after BMT. Some DLI donors were immunized s.c. with irradiated GM-CSF-secreting B16 cells once, 1 week before DLI.

Tumor Vaccination and Challenge. B16-F10 melanoma cells (H-2^b), syngeneic to B6 mice, were maintained in medium as described (15). GM-CSF-secreting B16 cells (300 ng/10⁶ cells/24 h) were generated using the retrovirus vector MFG as described previously (6). No replication component retrovirus is generated with this system, as determined by the *his* mobilization assay (27). Mice were immunized s.c. on the abdomen with 5 × 10⁵ irradiated (33 Gy) GM-CSF-secreting B16 cells (vaccine) in HBSS and challenged 1 week later with 1 × 10⁶ live wild-type B16 cells s.c. on the back. Irradiation of GM-CSF-secreting B16 cells did not abrogate production of GM-CSF *in vitro* over the course of 7 days (6). Tumor growth was monitored every other day, and mice were sacrificed when challenge tumors reached 1 cm maximum diameter.

Systemic and Histopathological Analysis of GVHD. Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by a scoring system, which sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index = 10) as described previously (28). This index is a more sensitive index of GVHD severity than weight loss alone, a parameter which has been found to be a reliable indicator of systemic GVHD in multiple murine models (28). Acute GVHD was also assessed by detailed histopathological analysis of liver, a primary GVHD target organ. Sections of liver (right lobe) were fixed in 10% buffered formalin. Specimens were then embedded in paraffin, cut into 5-μm-thick sections, and stained with H&E for histological examination. Slides were coded without reference to prior treatment and examined systematically by a single pathologist (C. L.) using a semiquantitative scoring system as described previously (29).

Flow Cytometric Analysis. FITC- or phycoerythrin-conjugated mAbs to mouse CD3, CD4, CD8, CD45.1, and B220 were purchased from PharMingen (San Diego, CA). Cells were preincubated with mAbs 2.4G2 (rat antimouse FcγR mAbs) for 15 min at 4°C to block nonspecific FcγR binding of labeled antibodies, then incubated with the relevant mAbs for 30 min at 4°C. Finally, cells were washed twice with 0.2% BSA in PBS, fixed with 1% paraformaldehyde in PBS, and analyzed by EPICS Elite ESP cell sorter (Beckman-Coulter, Miami, FL). Irrelevant IgG_{2a/b} mAbs were used as a negative control. Live events (10,000) were acquired for analysis. Donor T-cell engraftment was determined by the relative percentages of CD45.1⁺/CD45.2⁻ cells among CD3⁺ cells in 3 mice per group (SJL: CD45.1⁺/CD45.2⁻, B6SJLF1: CD45.1⁺/CD45.2⁺).

Cell Culture and ELISA. Splenocytes were harvested from animals 7 days after vaccination, and three spleens combined from each group. All of the media and culture conditions were as described previously (15). After lysis of erythrocytes with ammonium chloride, cells were washed twice and resuspended in supplemented 10% FCS in DMEM (Life Technologies, Inc., Gaithersburg, MD). The percentage of CD4⁺ and CD8⁺ T cells in this fraction was estimated by flow cytometric analysis and normalized for CD4⁺ plus CD8⁺ T-cell numbers. The percentages of CD4⁺ and CD8⁺ T cells in the spleens of vaccinated and control groups did not differ significantly. Splenic T cells (2 × 10⁵/well) were cultured in 96-well plates with either 2 × 10⁴/well irradiated (100 Gy) B16 stimulators or 1 × 10⁵/well irradiated (20 Gy) peritoneal cells leaved from SJL or B6SJLF1 mice in 200 μl of supplemented 10% FCS in DMEM. Wild-type B16 cells were treated with IFN-γ for 24 h to increase surface expression of MHC class I and II molecules (30), washed twice, and used as B16 stimulators. After 4 days of culture, supernatants were harvested from the culture for cytokine measurements, and cells were then pulsed with [³H]thymidine (1 μCi/well) for an additional 16 h. Proliferation was determined on a 1205 Betaplate reader (Wallac, Turku, Finland).

ELISA for GM-CSF, IFN-γ, IL-2, IL-4, IL-5, and IL-10 were performed according to the manufacturer's protocol (PharMingen). Briefly, samples were diluted 1:1 to 1:4, and each cytokine was captured by their specific primary mAbs and detected by their specific biotin-labeled secondary mAbs. Assays were developed with streptavidin and substrate (KPL, Gaithersburg, MD).

Plates were read at 450 nm using a microplate reader (Bio-Rad Labs, Hercules, CA). Samples and standards were run in duplicate, and the lower limit of detection was 5 pg/ml for GM-CSF, 0.1 units/ml for IFN-γ and IL-2, 10 pg/ml for IL-4, 4–8 pg/ml for IL-5, and 62.5 pg/ml for IL-10.

Statistical Analysis. Survival curves were plotted using Kaplan-Meier estimates. The Mann Whitney *U* test was used for the statistical analysis of *in vitro* data and clinical scores, whereas the Mantel-Cox Log-rank test was used to analyze survival data. *P* < 0.05 was considered statistically significant.

RESULTS

Vaccination with Recipient-derived B16 Tumor Vaccines to Allogeneic DLI Donors Stimulates B16-specific T-Cell Responses.

Theoretically, whole tumor cell vaccines given to allogeneic donors could exacerbate GVHD by stimulating reactivity to histocompatibility antigens shared by the tumor and host. Therefore, we first examined whether immunization to DLI donors with recipient-derived tumor vaccines could induce a tumor-specific response *in vitro*. One week after vaccination of SJL mice with irradiated GM-CSF-secreting B16 cells, T-cell proliferative and cytokine responses were measured *in vitro*. T cells from unimmunized SJL (H-2^s) mice responded to B6SJLF1 (H-2^{bxs}) stimulators but not to B16 (H-2^b) stimulators, confirming that B16 tumors were poorly immunogenic (Fig. 1). After vaccination, T cells vigorously proliferated and produced large amounts of both Th1 and Th2 cytokines (IFN-γ, IL-2, GM-CSF, IL-4, IL-5, and IL-10) to B16 stimulators. Vaccination did not stimulate proliferation to F1 stimulators but did stimulate weak Th2 responses. These results demonstrate that vaccination with recipient-derived tumor vaccines preferentially stimulate tumor-specific T-cell responses.

DLI from Immunized Donors Increases the Efficacy of Vaccination Given to Recipients without Exacerbating GVHD.

We next examined the effects of DLI on GVHD and GVT activity after allogeneic TCD BMT in a MHC-haplotype-mismatched model of BMT [SJL (H-2^s) donors into B6SJLF1 (H-2^{bxs}) recipients]. In this model, GVHD was severe when donor T cells were added to BM inoculum, and ~50% of recipients died of GVHD by day 60 after BMT (15). B6SJLF1 recipients were transplanted with 5 × 10⁶ TCD BM from allogeneic SJL donors after 11 Gy of TBI. Four weeks after BMT, donor engraftment in the peripheral blood at that time revealed complete donor myeloid engraftment and mixed donor/host chimerism of CD3⁺ T cells (29 ± 4% donor). The timing and dose of DLI were based on results published previously (20). We first tested both high-dose DLI (3 × 10⁷ splenocytes) and low-dose DLI (3 × 10⁶ splenocytes) given at 4 weeks after BMT. High-dose DLI, but not low-dose DLI, induced significant GVHD. Clinical GVHD scores after high-dose DLI were significantly higher than controls (2.8 ± 0.3 *versus* 0.4 ± 0.2, *P* < 0.05). It is well known clinically that DLI sometimes induces GVHD (31, 32). Thus, low-dose DLI was used in the following experiments.

After TCD BMT, recipients were vaccinated and also injected with low-dose DLI. One week later, all recipients were challenged with 1 × 10⁶ live wild-type B16 cells, which were uniformly lethal to unimmunized B6SJLF1 mice (15). Vaccination to recipients alone resulted in 22% TFS (Fig. 2). Nonimmune DLI given concurrently with vaccination did not impact the efficacy of the vaccine or exacerbate GVHD, but DLI from immunized donors significantly improved the efficacy of vaccination (TFS 60 *versus* 22%, *P* < 0.05). In contrast, DLI alone did not confer any significant antitumor immunity, regardless of immunization status of the donor (data not shown). Importantly, immunized DLI did not cause clinical GVHD, as assessed by clinical GVHD score (0.6 ± 0.1 *versus* 0.6 ± 0.2). These results demonstrate that immunized DLI increases the efficacy of the tumor vaccine given to recipients without braking tolerance to host antigens.

Fig. 1. Induction of B16-specific T-cell proliferative and cytokine responses in SJL mice. Naive SJL mice were vaccinated with irradiated, GM-CSF-secreting B16 cells. Splenocytes were harvested 1 week after vaccination. T cells (2×10^5) were cultured with 2×10^4 irradiated, IFN- γ -treated B16 cells or 10^5 irradiated B6SJL F1 peritoneal cells. After 4 days of culture, supernatants were harvested from the culture for cytokine measurements by ELISA, cells were then pulsed with [3 H]thymidine (1 μ Ci) for an additional 16 h, and proliferation was determined. Data represent results from two similar experiments and are shown as mean \pm SD from quadruplicate cultures. Cytokines were undetectable when T cells were cultured with SJL stimulators (data not shown). -vax, without vaccination; +vax, with vaccination; UD, undetectable.

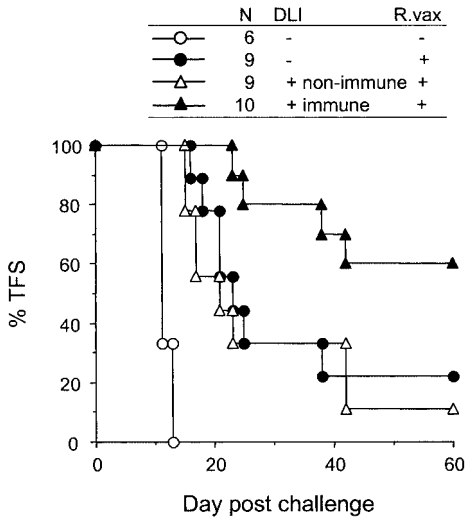
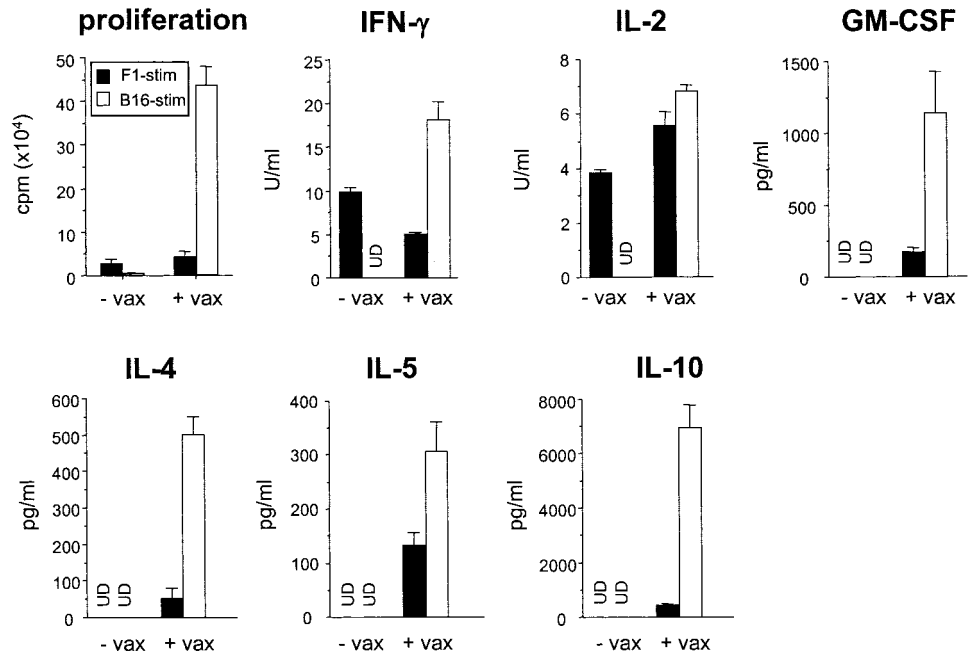


Fig. 2. Immunized DLI augments efficacy of recipient vaccination without worsening GVHD. B6SJL F1 mice were transplanted with 5×10^6 TCD BM from SJL donor mice after 11 Gy of TBI. Four weeks after BMT, recipient mice received DLI (3×10^6 splenocytes) from either unimmunized (*nonimmune*) or immunized (*immune*) SJL donor mice, were immunized with 5×10^5 irradiated, GM-CSF-secreting B16 cells, and challenged 1 week later with 1×10^6 live B16 cells. Immunization to donors was performed 1 week before DLI. % TFS after challenge is shown. R.vax, recipient vaccination.

CD4⁺ T Cells in the DLI Inoculum Are Essential in Eliciting Antitumor Activity in BMT Recipients. We then investigated which cell components in the DLI inoculum are responsible for enhanced vaccine efficacy. SJL donor mice were immunized with the recipient-derived B16 tumor vaccines 1 week before DLI as shown in Table 1. CD4⁺ T cells, CD8⁺ T cells, and B220⁺ B cells were positively selected by AutoMACS from spleens of immunized DLI donors. DLI of either 3×10^6 T cells (T-DLI: 2×10^6 CD4⁺ T cells plus 1×10^6 CD8⁺ T cells), 2×10^6 CD4⁺ T cells (CD4-DLI), 1×10^6 CD8⁺ T cells (CD8-DLI), or 2×10^6 B220⁺ B cells (B-DLI) was injected in BMT recipients concurrently with vaccination, followed by tumor challenge 1 week later. The CD4:8 ratio of 2:1 was chosen based on the normal ratio in naive SJL spleens. Vaccination

without DLI produced 11% TFS, and the addition of immunized T-DLI again significantly increased the efficacy of recipient vaccination (Fig. 3; TFS 50 versus 11%, $P < 0.01$). This effect of immunized T-DLI was attributable solely to the presence of CD4⁺ T cells in the DLI inoculum: the TFS of recipients who received immunized CD4-DLI concurrent with recipient vaccination was 57%. By contrast,

Table 1 Experimental schedule

Post-BMT	BMT Donor SJL	DLI Donor SJL	BMT/DLI recipient B6SJL F1
0w	BM harvest		BMT
3w		\pm Vaccination	
4w		Spleen harvest	\pm DLI, \pm vaccination
5w			Tumor challenge

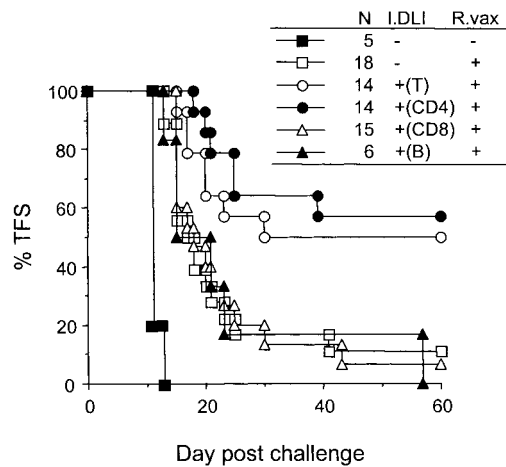


Fig. 3. CD4⁺ T cells from immunized donors are responsible for antitumor immunity. SJL donor mice were immunized, and 1 week later, CD4⁺ T cells, CD8⁺ T cells, and B220⁺ B cells were positively isolated from spleens. B6SJL F1 recipients of allogeneic TCD BMT received DLI of either 2×10^6 CD4⁺ T cells, 1×10^6 CD8⁺ T cells, T cells (2×10^6 CD4⁺ T cells plus 1×10^6 CD8⁺ T cells), or 2×10^6 B220⁺ B cells 4 weeks after BMT. Recipients were immunized at the same time and challenged 1 week later. % TFS was shown from two similar experiments. I.DLI, immunized DLI; R.vax, recipient vaccination.

Table 2 Immunized CD4-DLI does not exacerbate GVHD

Recipient vaccination	+	+
Immunized CD4-DLI	-	+
Clinical score (0–10)	0.2 ± 0.2	0.3 ± 0.2
Pathology scores of the liver (0–40)	3.0 ± 2.0	1.3 ± 0.6

immunized CD8-DLI and B-DLI did not increase the efficacy of recipient vaccination (TFS: 0 and 7%, respectively). Lastly, immunized CD4-DLI given at the time of recipient vaccination did not exacerbate GVHD as assessed by clinical scores and by histopathological scores of the liver 4 weeks after DLI (Table 2).

DISCUSSION

We have shown previously that immunization of BMT recipients with GM-CSF-based tumor vaccines can elicit long-lasting antitumor immunity by using a B16 murine melanoma model (15). In the current study, we tested whether administration of DLI at the time of recipient vaccination could generate additional antitumor activity in BMT recipients. DLI from immunized donors alone did induce neither GVHD nor GVT activity in this system, although it has been shown that tumor-specific T-cell immunity in donors could be transferred to BMT recipients (33–35). Our result is consistent with clinical studies showing a loss of donor-derived immunity in BMT recipients, which suggest the need for additional immunization to recipients (34, 36). In our study, however, DLI from immunized donors given at the time of vaccination greatly enhanced its efficacy. Immunization of both donors and recipients was essential for an optimal effect, suggesting that the improved antitumor immunity involves adoptive transfer of activated/effector cells, as well as restimulation of these effector cells by vaccination. We also found that CD4⁺, not CD8⁺, T cells in the DLI inoculum are primarily responsible for this enhanced effect. Although much attention has been given to the role of CD8⁺ cytotoxic T cells in antitumor immune responses, several lines of evidence suggest an important role for CD4⁺ T cells in mediating systemic antitumor effector functions (37–39). Our results are consistent with a previous study that demonstrated a critical role for CD4⁺ T cells in the effective induction of antitumor immunity with irradiated GM-CSF-secreting vaccine (40). However, the differences between CD4⁺ and CD8⁺ cells may be unique to this model, and it remains to be determined if this response can be generalized to other tumor/transplant systems. In humans, these results are consistent with clinical data showing that low numbers of CD4⁺ DLI can induce GVT activity against chronic myelogenous leukemia and multiple myeloma (41).

The precise mechanisms underlying the enhanced antitumor effects of the combination of vaccination and immunized DLI are yet to be determined. Recent studies have demonstrated that naive T cells differentiate into CCR7⁺ central memory T (T_{CM}) cells and CCR7⁻ effector memory T (T_{EM}) cells after antigenic stimulation *in vivo* (42). T_{CM} cells, which reside in secondary lymphoid organs, lack immediate effector function but efficiently stimulate DCs and differentiate into T_{EM} cells on secondary stimulation (42, 43). It is not known whether CD4⁺ cells function as direct antitumor effectors in our model, but CD4⁺ cells harvested from spleens of immunized mice may represent T_{CM} cells that can rapidly differentiate into T_{EM} cells after restimulation by vaccination. The CD4⁺ T cell fraction also includes CD4⁺ NKT cells, which also have a critical role in the generation of antitumor immunity (44). We have shown recently that GM-CSF-secreting tumor vaccines markedly enhance the expression of CD1d on DCs (45) and that the efficacy of the vaccine is ablated

in CD1d-deficient mice.⁴ Thus, CD1d-restricted CD4⁺ NKT cells from the donor might be responsible, at least in part, for the improved antitumor immunity.

Administration of a recipient-derived whole tumor cell vaccine to allogeneic donors has been shown to increase the risk of GVHD in a recent murine study (46). SJL donor differs from B6SJL.F1 recipients at MHC I and MHC II loci in addition to MiHA. T-cell depletion of the donor marrow prevents the development of GVHD. When 2 × 10⁶ donor T cells are added to BM inoculum, severe acute GVHD develops and ~50% of recipients die of GVHD by day 60 after BMT (15). Immunized, low-dose DLI to TCD BMT recipients did not induce GVHD in our model. Therefore, we examined whether recipient-derived B16 (H-2^b) vaccines could induce B16-specific immunity in SJL (H-2^s) donors. *In vitro*, splenic T cells from vaccinated SJL mice proliferated vigorously and secreted both Th1 and Th2 cytokine responses to B16 stimulators but not to host (H-2^{bxs}) stimulators. This specificity may be attributable to low expression of MHC on B16 tumors, which can be a mechanism for escape from immune surveillance of the host (30). Therefore, lack of increased GVHD after DLI may be explained by the induction of B16-specific T-cell immunity in DLI donors. We have shown previously that vaccination of the recipients with such vaccines after allogeneic TCD BMT does not by itself induce GVHD (15).

The efficacy of DLI from vaccinated donors suggests that primary immune responses to B16 tumors are not directed at host alloantigens. Therefore, the antitumor activity induced by this vaccine might be exploited after allogeneic BMT, although the generalizability of this approach to other tumors needs to be determined. Because TCD BMT is associated with a marked reduction in the frequency and intensity of GVHD, as well as reduced antitumor activity (47), the ability of the combined strategy to increase antitumor immunity without GVHD has important clinical implications.

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