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*J Immunol* 2006; 177:4159-4167; ; doi: 10.4049/jimmunol.177.6.4159

http://www.jimmunol.org/content/177/6/4159
DNA Immunization against Tissue-Restricted Antigens Enhances Tumor Immunity after Allogeneic Hemopoietic Stem Cell Transplantation

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Malignant relapse remains a major problem for recipients of allogeneic hemopoietic stem cell transplantation (HSCT). We hypothesized that immunization of allogeneic HSCT recipients against tissue-restricted Ags using DNA vaccines would decrease the risk of relapse without enhancing graft-vs-host disease (GVHD). Using the mouse B16 melanoma model, we found that post-HSCT DNA immunization against a single tumor Ag induces tumor rejection that is significantly greater than HSCT alone in a T cell-depleted MHC-matched minor Ag-mismatched allogeneic HSCT model (L.P. → B6). In treatment models, post-HSCT DNA immunization provides significantly greater overall survival than the vaccine alone. Donor leukocyte infusion further enhances tumor-free survival, including in treatment models. There was no GVHD in HSCT recipients treated with DNA vaccination and donor leukocyte infusion. Further analysis demonstrated that these effects are dependent on CD8+ T cells of donor origin that recognize multiple epitopes. These results demonstrate that DNA immunization against tissue-restricted Ags after allogeneic T cell-depleted HSCT can induce potent antitumor effects without causing GVHD. The Journal of Immunology, 2006, 177: 4159 – 4167.

Allogeneic hemopoietic stem cell transplantation (HSCT)5 is an important therapy with curative potential for a variety of malignant diseases, including leukemias, lymphomas, and some solid tumors (1). Despite continuing progress in reducing treatment-related mortality, malignant relapse remains a major problem and continues to reduce the overall outcome in recipients of allogeneic HSCT. Studies involving nonmyeloablative HSCT and donor leukocyte infusion (DLI) 6 have irrefutably demonstrated the important contribution of the donor immune system in a graft-vs-tumor effect that can prevent or treat malignant relapse (2–4).

The development of cancer vaccines is one of the most active areas under investigation in cancer immunotherapy and a number of vaccines are now in late-stage clinical testing. The immune system frequently recognizes self-molecules on cancer cells or in selected cases mutated self-proteins (5, 6). Differentiation Ags are self-Ags that distinguish a cell lineage from other cell types and are expressed at specific stages of differentiation and on cancer cells (7–11). Immune recognition has been intensively studied in melanoma, a cancer arising from melanocytes in the skin. There are several known melanoma differentiation Ags, including the melanosome membrane glycoproteins such as tyrosinase, tyrosinase-related protein-1 (TRP-1/gp75/TYRP1), TRP-2 (a DOPAchrome tautomerase), gp100/pmel17, and MelanA/MART-1 (8–10, 12). These Ags are recognized spontaneously by Abs and T cells and some solid tumors (1). Despite continuing progress in reducing treatment-related mortality, malignant relapse remains a major problem and continues to reduce the overall outcome in recipients of allogeneic HSCT. Studies involving nonmyeloablative HSCT and donor leukocyte infusion (DLI) have irrefutably demonstrated the important contribution of the donor immune system in a graft-vs-tumor effect that can prevent or treat malignant relapse (2–4).

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5 Abbreviations used in this paper: HSCT, hemopoietic stem cell transplantation; DLI, donor leukocyte infusion; GVHD, graft-vs-host disease; TRP, tyrosinase-related protein.

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1 This work was supported by Grants CA33049 (to M.-A.P., M.v.B., and A.N.H.), K08-CA10260 (to M.-A.P.), HL69929, HL72412, and CA107096 (to M.v.B.), CA59350, CA58621, CA33049, and CA47179 (to A.N.H.), T32-CA09512 (to A.D.C.), P20-CA103694 (to O.A.), by the National Institutes of Health, by Swim Across America (to M.-A.P., A.D., A.D.C., D.W.H., J.A.G.-P., M.E.E., J.D.W., and A.N.H.), the Byrne Fund from the Memorial Sloan-Kettering Cancer Center (to M.-A.P.), an American Society of Clinical Oncology Young Investigator Award (to A.D.C.), the Clinical Scholars Biomedical Research Training Program and the Charles A. Dana Foundation (to A.D.C.), the Mr. William H. Goodwin and Mrs. Alice Goodwin and the Commonwealth Cancer Foundation for Research and the Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center (to A.N.H., J.A.G.-P., M.E.E., and M.v.B.), by awards from the Emerald Foundation, the Leukemia and Lymphoma Society, and the Golfers against Cancer (to M.v.B.), a Damon Runyon-Lilly Clinical Investigator Award (to J.D.W.), a Fellowship award from the Cancer Research Institute (to M.E.E.), an Amy Streicher Manasevit Scholar Award from The National Marrow Donor Program and The Marrow Foundation (to O.A.), and the Deutscher Akademischer Austausch Dienst and Boehringer Ingelheim Fonds (to T.H.T.). A.N.H. has Damon Runyon/Eli Lilly mentorship support.

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Studies in mouse models have shown that graft-vs-tumor effects can be enhanced through posttransplant immunization against tumors (21–26). This early work in post-HSCT immunization has used cellular tumor vaccines and the presentation of multiple non-tumor Ags to the immune system could decrease the efficacy of the antitumor response and lead to recognition of non-tumor alloantigens and increased graft-vs-host disease (GVHD). In the present study, we describe the use of posttransplant DNA vaccines against tissue-restricted Ags to enhance antitumor immunity in the absence of GVHD.

Materials and Methods

Mice and stem cell transplantation

Female C57BL/6J (B6, H-2^d) and LP/J (H-2^k) mice were obtained from The Jackson Laboratory. Mice used in HSCT experiments were between 8 and 10 wk of age. Bone marrow cells were removed aseptically from femurs and tibias. Donor bone marrow was T cell-depleted by incubation with anti-Thy-1.2 for 30 min at 4°C followed by incubation with Low-TOX-M rabbit complement (Cedarlane Laboratories) for 40 min at 37°C. Cells were transplanted by tail vein injection into lethally irradiated recipients (1100 cGy total body irradiation from a 137Cs source as a split dose with a 3-h interval between doses). Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated drinking water (pH 3.0). HSCT protocols were approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee.

Abs and flow cytometry

Anti-murine CD16/CD32 Fc receptor block (2.4G2) and all of the following fluorochrome-labeled Abs against murine Ags were obtained from BD Pharmingen: Ly-9.1 (30C7), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD62L (MEL-14), CD122 (TM-B1), CD44 (IM7), CD45R/B220 (RA3-6B2), NK.1.1 (PK136), CD11b (M1/70), CD25 (PC61), CD69 (H1.2F3), IFN-γ (clone XMG1.2). Isotype controls include: rat IgG2a-κ (R35-95), rat IgG2b-λ (B39-4), rat IgG2b-κ (A95-1), rat IgG1-κ (R3-34), hamster IgG-group1-κ (A19-3), hamster IgG-group 1-λ (Ha4/8), and streptavidin-FITC, -PE, and -PerCP. FACS staining was performed as previously described (27). Cells were acquired on a FACSCalibur or LSR cytometer (BD Biosciences) with CellQuest software. Data were analyzed with FlowJo software (Tree Star).

Plasmid constructs

Human TRP-2 (supplied by Dr. S. A. Rosenberg and Dr. J. C. Yang (National Cancer Institute, Bethesda, MD) (28) and the mouse GM-CSF gene (provided by PowderJect Vaccines, Middleton, WI) were cloned into the WREG/BEN vector as previously described (29). VP22-Opt-TYRP1 DNA is a vaccine that has been optimized to generate CD8+ T cell responses, based on the concept of heteroclitic peptides (30). The coding sequence of mouse Tyrp1 was modified to optimize MHC class I binding to both Kb and Db and fused to VP22, an HSV-1 protein that has been shown to enhance vaccine potency (31, 32) (M. E. Engelhorn, manuscript in preparation). We have previously shown that immunization with the plasmid vaccine alone did not induce tumor rejection or Ag-specific responses (18–20).

DNA administration

Mice were immunized with helium-driven particle bombardment, as previously reported (29). Briefly, plasmid DNA was purified and coated onto 1-μm diameter gold particles (Alfa Aesar) and precipitated on bullets of viral DNA. Gold particles containing 1 μg of DNA were delivered to each abdominal quadrant using a helium-driven gene gun (Accell; PowderMed), for a total of 4 μg of DNA per mouse. Mice were immunized weekly for a total of three or four immunizations.

Mouse tumor studies

Tumor challenge experiments were conducted with melanoma B16 cells, as described previously (30). Briefly, 0.5 × 10^6 B16 F10 melanoma cells (gift from I. Fidler, M.D. Anderson Cancer Center, Houston, TX) were injected into the shaved right flank of the mice. Tumor diameters were measured by calipers every 2–3 days, and mice were sacrificed when the diameter exceeded 1 cm, tumors became ulcerated, or mice showed discomfort. Tumor-free survival was assessed from the day of tumor challenge. Kaplan-Meier survival curves were generated and compared using the log-rank test.

Generation of tumor lines for in vivo bioluminescent imaging

B16 cells were retrovirally transduced to express a triple fusion protein consisting of HSV thymidine kinase, enhanced GFP, and firefly luciferase (TGL) (provided by V. Ponomarev, Memorial Sloan-Kettering Cancer Center, New York, NY) (33). Transduced cells were expanded and indi
cidual clones with high enhanced GFP expression were sorted into 96-well plates using a MoFlo Cell Sorter (DakoCytomation). Bioluminescent signal intensity of single clones was determined in vitro and the brightest clone (termed B16-TGL) was used for subsequent experiments. For tumor challenge experiments, 1 × 10^5 B16-TGL cells were injected i.v. by tail vein injection. For imaging, mice were injected i.p. (150 mg/kg) with D-Luciferin (Xenogen), and 10 min after injection, anesthetized with isoflu
rane, placed supine in the IVIS bioluminescence imaging system (Xeno
gen), and recorded for 5 min. Pseudocolor images showing whole body distribution of bioluminescent signal were superimposed on conventional grayscale photographs.

FIGURE 1. T cell reconstitution in recipients of a MHC-matched allogeneic T cell-depleted HSCT. Le
thally irradiated (1100 cGy split dose) B6 mice (four to five per group) received 5 × 10^6 LP/J T cell-depleted bone marrow cells via tail vein injection. Spleens were harvested on days 14, 28, 42, and 56 for flow cytometric analysis. Total numbers (CD3, CD4, and CD8) and percentages (day 28) of CD4 and CD8 of donor and host origin (distinguished by the presence of a congenic marker, Ly9.1, present on LP/J but not on B6 mice) are shown.
stimulated with 1g/ml H9262 stained for CD8 and intracellular IFN-γ (Aldrich) was added 1 h after the peptide. Following stimulation, cells were coated with 100g/ml peptide in the presence of unbound Ab, and blocked with RPMI 1640/FCS for 2 h at 37°C. Purified CD8 T cells were plated at a concentration of 1 × 10^6 cells/well and stimulated with 1μg/ml peptide in the presence of 5 × 10^4 irradiated EL-4 cells. After incubation for 20 h at 37°C, plates were washed with PBS/0.05% Tween 20 and incubated with 100 μl/well biotinylated Ab against mouse IFN-γ (2μg/ml, clone 7-B6-1; MabTech). Plates were incubated for an additional 2 h at 37°C and spot development was performed as described (34). Spots were counted with an Automated ELISPOT Reader System with KS 4.3 software (Zeiss). A two-sample test was used to test the equality of means between groups for T cell responses measured in the ELISPOT assay or by intracellular cytokine flow cytometry.

Intracellular cytokine flow cytometry

Cells were stimulated for 16 h with 1 μg/ml peptide in the presence of irradiated EL-4 cells, at a ratio of 5:1. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added 1 h after the peptide. Following stimulation, cells were stained for CD8 and intracellular IFN-γ using the Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer’s instructions and analyzed on a FACS Calibur (BD Biosciences).

Assessment of GVHD

Survival was monitored daily. The severity of GVHD was assessed with a clinical GVHD scoring system as previously described (35, 36) and mice scoring 5 or greater were sacrificed.

Results

Peripheral and donor T cell reconstitution is detectable by day 28 after a T cell-depleted allogeneic HSCT

Previous studies have shown that T cell reconstitution is required for the induction of antitumor immunity to a vaccine given post-HSCT (21, 23). GVHD, as well as the therapies used for GVHD prophylaxis and/or treatment, are associated with immune suppression rendering immunization strategies ineffective (21). We therefore performed T cell depletion of the allograft in all experiments, which is the most effective strategy to prevent GVHD and avoid posttransplant immune suppression.

To determine the optimal time for posttransplant immunization, we performed longitudinal studies of T cell reconstitution following a T cell-depleted HSCT in a clinically relevant MHC-matched (but minor histoincompatibility Ag mismatched) allogeneic HSCT model (LPJ → B6) and found that, at day 28 after transplant, considerable numbers of T cells were detected in the spleen and ≥50% of all splenic T cells were of donor origin (Fig. 1). The majority of these cells had a naive phenotype (CD4^+CD25^-CD62L^+), CD8^+CD44^-CD62L^+), suggesting that these cells represented de novo generated donor T cells. Radioresistant T cells of host origin consisted primarily of populations of homeostatically expanding (CD4^+CD25^-CD62L^+), CD8^+CD44^-CD62L^+), and activated (CD4^+CD25^-CD62L^-, CD8^+CD44^-CD62L^-) T cells. The detection of substantial numbers of T cells, including de novo generated naive donor T
only immunized after allogeneic HSCT. DLI and vaccine had increased protection compared with mice that were vaccinated from that time point on is feasible. Post-HSCT DNA immunization induces tumor immunity

We have previously shown that the combination of human TRP-2 DNA and GM-CSF DNA is effective in established tumor models and models of minimal residual disease (19, 37). Based on the detection of donor-derived T cells as early as day +28 after allogeneic HSCT, we started immunizing mice 4 wk after T cell-depleted HSCT. Five days after the third weekly immunization, mice were challenged intradermally with B16, a syngeneic mouse melanoma. Increased tumor-free survival was seen in mice that were immunized post-HSCT with human TRP-2 DNA and GM-CSF DNA compared with untreated mice or mice that underwent HSCT alone (group 4 vs groups 1 or 2, \( p < 0.001 \)) (Fig. 2A). Tumor-free survival following post-HSCT DNA immunization was further enhanced with the use of VP22-Opt-TYRP1 DNA, a vaccine optimized for the activation of CD8\(^+\) T cells (Fig. 2B). These results demonstrate that post-HSCT DNA immunization against a single tissue-restricted Ag can provide significant protection from a tumor challenge.

In most experiments, there was no tumor rejection in mice that underwent HSCT alone (Fig. 2). When rejection was noted, it was <20% and could be due to the recognition of minor transplantation Ags on the melanoma cells. Consistent with this, B16 grew in <50% of LP/J mice, and typically regressed after initial growth up to 6 mm (data not shown).

**FIGURE 3.** Increasing the DLI dose enhances tumor-free survival without causing GVHD. B6 mice (five per group) were immunized post-allogeneic HSCT from LP/J donor mice with VP22-Opt-TYRP1 DNA as described in Fig. 2. DLI from immunized donors was performed on week 4, using increasing doses of splenocytes as indicated. Weight (A) and GVHD scores (B) were monitored weekly. No weight loss or clinical signs of GVHD were noted in any of three groups. In another experiment, DLI from immunized donors was performed on week 4, using 3 \( \times 10^6 \) splenocytes, followed by 3 weekly DNA immunizations with VP22-Opt-TYRP1 DNA (C) or human TRP-2 DNA (D). Mice (10 per group) were challenged intradermally with B16 melanoma 5 days after the last immunization. Mice that underwent DLI and vaccine had increased protection compared with mice that were only immunized after allogeneic HSCT.

**FIGURE 4.** CD8\(^+\), but not CD4\(^+\) T cells are required for tumor rejection. B6 mice (10 per group) were immunized post-allogeneic HSCT from LP/J donor mice weekly four times with VP22-Opt-TYRP1 DNA starting on week 4 post-HSCT. Following the last immunization, mice were injected with 250 \( \mu \)g of Abs against CD4 (GK1.5) or CD8 (2.43) 2 days before intradermal challenge with B16 melanoma. Depleting Abs were given weekly for wk.

DLI and post-HSCT DNA immunization enhance tumor rejection without increasing GVHD

We then analyzed whether posttransplant tumor vaccination could be improved with the addition of DLI (Fig. 2). A total of 3 \( \times 10^6 \) splenocytes from an immunized donor was administered before recipient immunization because this schedule has been shown to enhance the maintenance of antitumor responses (23). Using either human TRP-2 plus GM-CSF DNA (Fig. 2A) or VP22-Opt-TYRP1 DNA (Fig. 2B), there was a modest but statistically not significant increase in tumor rejection in mice that underwent DLI in addition to post-HSCT immunization (group 5 vs group 4, \( p = 0.5 \)). DLI alone did not increase posttransplant tumor-free survival in the absence of immunization. In addition, when splenocytes from nonimmunized donors were administered as DLI, tumor-free survival after posttransplant DNA immunization was decreased compared with mice that received DLI from immunized donors, although this did not reach statistical significance (\( p = 0.07 \); data not shown). In all subsequent DLI experiments, only immunized donors were used.

No GVHD was observed in mice that underwent post-HSCT DNA immunization with or without low-dose DLI after a T cell-depleted HSCT (data not shown). This result is in contrast to the induction of lethal GVHD observed in 50% of B6 mice that underwent a T cell-replete transplant (with 1 \( \times 10^8 \) T cells) from LP/J donors (data not shown). To further investigate whether DLI and DNA vaccines induced or increased GVHD in HSCT recipients, we performed a dose titration of DLI doses. Mice underwent DLI at week 4 after HSCT and received 3, 10, or 30 \( \times 10^6 \) splenocytes from donors immunized with VP22-Opt-TYRP1 DNA. Recipient mice then underwent DNA immunization with the same vector. All recipients survived and no weight loss or clinical signs of GVHD were noted in any of three groups (Fig. 3, A and B).

Because no GVHD was observed at the highest dose of splenocytes (Fig. 3A and data not shown), we assessed the effect of this
dose of DLI (3 x 10^6 splenocytes) on tumor-free survival in mice immunized after allogeneic T cell-depleted HSCT (Fig. 3, C and D). DLI increased tumor-free survival from 50% to 90% (p < 0.05) with VP22-Opt-TYRP1 DNA and from 10% to 70% (p < 0.007) with human TRP-2 DNA. Furthermore, with the addition of DLI, tumor-free survival was as good as (VP22-Opt-TYRP1) or superior (human TRP-2) to that typically observed in nontransplanted immunized mice. These results indicate that high-dose DLI can significantly enhance tumor-free survival in allogeneic HSCT recipients of post-HSCT DNA immunization without causing GVHD.

FIGURE 5. Post-HSCT DNA immunization induces tumor-specific CD8^+ T cells. B6 mice (five per group) were immunized post-HSCT from LP/J donor mice with human TRP-2 plus GM-CSF (hTRP2+GM-CSF) DNA (A and B, and E and F) or VP22-Opt-TYRP1 DNA (C and D, and G and H) using the schedule described in Fig. 2. Following the third immunization of the recipient, spleen and lymph node T cells were assayed for TRP2_{intr}specific (A and B) or Tyrp1_{intr}specific (C and D) responses by intracellular flow cytometry. The precursor frequencies in draining lymph nodes and in the spleen of mice that were immunized post-HSCT and also received 3 x 10^6 splenocytes from an immunized donor as DLI (HSCT + vaccine + DLI) was similar to or less than that in mice only receiving DNA immunization after HSCT (HSCT + vaccine). An ELISPOT assay showing spot-forming cells (SFC) demonstrated similar results following immunization with human TRP-2 plus GM-CSF (hTRP2+GM-CSF) DNA (E and F) or VP22-Opt-TYRP1 DNA (G and H), as well as recognition of B16 melanoma. The results presented are the average ± SEM. *, p < 0.05; **, p < 0.001, when compared with vaccine.

CD8^+ T cells are required for tumor rejection following post-HSCT DNA immunization with a vaccine optimized for MHC class I binding

To determine the contribution of CD4^+ and CD8^+ T cells to posttransplant tumor rejection by DNA vaccination, we depleted CD4^+ or CD8^+ T cells using mAbs before tumor challenge (effector phase). Tumor rejection was observed in 100% of vaccinated mice that underwent CD4 depletion and in nondepleted mice (Fig. 4). In contrast, mice in which CD8^+ T cells were depleted did not reject...
We then examined Ag-specific CD8\(^+\) T cell responses in draining lymph nodes and the spleen, to determine regional and systemic responses, respectively (Fig. 5). In draining lymph nodes of nontransplanted B6 mice immunized with human TRP-2 DNA, we detected 0.6% CD8\(^+\) T cells that recognized the TRP-2\(_{181-188}\) peptide using an intracellular cytokine assay. In nontransplanted B6 mice immunized with VP22-Opt-TYRP1 DNA, we detected responses to three CD8\(^+\) epitopes in the native Tyrp1 sequence (32). The precursor frequency in transplanted mice that were immunized (HSCT plus vaccine) was >2-fold higher in the spleen and almost 3-fold higher in draining lymph nodes compared with nontransplanted B6 mice immunized with vaccine alone (Fig. 5, E–H). Similar results were noted when T cell precursor frequencies were determined by ELISPOT assays (Fig. 5, E–H).

Using congenic markers to distinguish host from donor-derived T cells, we demonstrated that the peptide-specific T cells induced by post-HSCT DNA immunization were all of donor origin (Fig. 6A). In addition, similar to nontransplanted immunized B6 mice, we detected immune responses to three CD8\(^+\) epitopes in the native mouse Tyrp1 protein in mice that underwent post-HSCT immunization with VP22-Opt-TYRP1 DNA (Fig. 6B). These experiments provide evidence that DNA immunization post-HSCT can induce donor-derived tumor-specific CD8\(^+\) T cells against tumor Ags recognizing multiple epitopes, thus decreasing the risk of immune escape mutants.

**Post-HSCT immunization induces tumor rejection in treatment models**

In the tumor challenge experiments described, the tumor was injected after immunization. Although these prophylactic models establish the effects of post-HSCT DNA immunization, they do not mimic the clinical situation in which patients will be treated with minimal residual disease. We therefore performed experiments in which the tumor challenge was done after HSCT, but before DLI and DNA immunization (Fig. 7). Mice were challenged i.v. with B16 transduced with a vector containing a fusion reporter gene coding for HSV1 tyrosine kinase, enhanced GFP, and luciferase (B16-TGL). We monitored the in vivo tumor growth of B16-TGL by bioluminescent imaging and noted decreased tumor development in mice that underwent post-HSCT DNA immunization, with or without DLI (Fig. 7C). The tumor burden (measured as the median number of photons) also remained low in mice that had undergone post-HSCT DNA immunization (Fig. 7D). In addition to imaging the mice, we followed overall survival. Mice were monitored daily and sacrificed if they were in distress. All mice underwent necropsy and the presence of tumor was confirmed. There was a significant increase in overall survival in mice that underwent HSCT plus DNA vaccine with or without DLI compared with untreated mice, mice that underwent HSCT alone or nontransplanted mice that were immunized (\(p < 0.01\)) (Fig. 7B). Similar results were seen in mice challenged i.v. with nontransduced B16 (data not shown). The vaccine alone in nontransplanted mice did not provide significant protection, although this vaccine typically protects over 80% of mice in a standard prophylactic experiment. In conclusion, post-HSCT DNA immunization can significantly enhance overall survival in mice in treatment models.

**Discussion**

Recent experiments have suggested that cancer vaccines may enhance the efficacy of HSCT despite posttransplant immune deficiency (21–26, 38–40). In a mouse model for lymphoma, Borrello et al. (23) showed that immunizing mice with A20 lymphoma engineered to secrete GM-CSF induced superior tumor-free survival when given after a syngeneic HSCT compared with nontransplanted mice. Furthermore, this model was associated with Ag-specific T cell expansion in the posttransplant setting. Antitumor immunization was also effective after allogeneic HSCT in a mouse model for melanoma in which mice were immunized with B16 modified to secrete GM-CSF (21, 22). Survival was further improved when recipient mice were not only immunized, but also received DLI from immunized donors (22). These post-HSCT immunization studies made use of cellular tumor vaccines. In the setting of an allogeneic HSCT, the presentation of multiple non–tumor Ags to the immune system could decrease the efficacy of the antitumor response and lead to the recognition of non-tumor alloantigens resulting in increased GVHD. We hypothesized that
immunization against specific tumor Ags in the posttransplant setting may decrease the risk of relapse without enhancing the risk of GVHD. Indeed, we were able to demonstrate tumor rejection following immunization against a single tissue-restricted Ag (either TRP-2 or Tyrp1) that was not different from that observed after immunization with a whole cell B16-GM-CSF vaccine (data not shown), which would be expected to express multiple tumor Ags. Interestingly, tumor-free survival increased when four weekly DNA immunizations were given compared with three immunizations, consistent with ongoing immune reconstitution, because the benefit of additional immunization was only seen post-HSCT and not in nontransplanted mice.

One of the advantages of using vaccines against defined tumor Ags is the ability to monitor antitumor responses. As predicted, we demonstrated that tumor rejection was mediated by CD8\(^+\) T cells induced by a vaccine optimized for class I binding. Furthermore, we were able to detect donor-derived CD8\(^+\) T cells in draining lymph nodes and spleen that recognize native epitopes in TRP-2 and Tyrp1, as well as B16 melanoma. The DNA vaccines were able to induce recognition of more than one epitope, indicating

**FIGURE 7.** Post-HSCT DNA immunization enhances tumor-free survival and overall survival against an i.v. challenge with B16 melanoma given before immunization. B16 cells were transduced with a SFG vector containing a fusion reporter gene coding for HSV1-TK, enhanced GFP, and luciferase. B6 mice (14–15 per group) were challenged i.v. with the transduced B16 melanoma 25 days (25 T.C.) after allogeneic HSCT from LP/J donor mice. Mice then underwent five DNA immunizations with VP22-Opt-TYRP1 DNA, given every 5 days, starting at day 28, followed by a further three immunizations given weekly (A). A total of eight immunizations was given. One group of mice also received DLI (30 \(\times\) 10\(^6\) splenocytes) from immunized LP/J donor mice on day 27. B, Overall survival is increased in mice that underwent post-HSCT DNA immunization and is further enhanced by the addition of DLI. C, In vivo bioluminescent imaging of labeled B16 cells in anesthetized mice was determined after i.p. injection with luciferin. D, Median number of photons/sec/cm\(^2\)/steradian was measured at a given time point to demonstrate decreased tumor burden in mice immunized posttransplant.
that the response could overcome escape mutants that affect one or more epitopes within a tumor Ag.

We observed increased CD8\(^+\) T cell responses in immunized transplanted recipients compared with nontransplanted immunized mice. These results demonstrate that antitumor immunity can be induced through priming of de novo generated CD8\(^+\) T cells of donor origin, and are in agreement with recent studies that demonstrate the increased expansion of tumor-reactive T cell clones due to a combination of tumor-Ag-specific and homeostatic proliferation in lymphopenic mice (23) and patients (41).

Immune reconstitution is delayed in adult recipients of a T cell-depleted allogeneic HSCT (42), and this could represent an obstacle for effective tumor vaccination. Adoptive cell therapy (especially DLI) in the posttransplant setting can lead to increased tumor rejection in both autologous and allogeneic transplants (22, 23, 39). The importance of DLI in the treatment of relapse or posttransplant EBV lymphoma has been demonstrated in allogeneic HSCT patients (43–46). We therefore hypothesized that posttransplant adoptive cell therapy with donor-derived immunocompetent T cells that are specific for tissue-restricted Ags would lead to preferential expansion of tumor-reactive T cells and increased tumor rejection in HSCT recipients without causing GVHD. We chose to immunize donors to perform these proof-of-principle studies, realizing that for clinical applications alternative approaches such as in vitro priming would be more readily applicable. As expected, when DLI was administered to recipients before DNA immunization, a significant increase in tumor-free and overall survival was observed without GVHD, even in experiments in which the tumor was administered before DLI and immunization.

In our studies, we immunized against tissue-restricted Ags that are expressed by the tumor and normal host tissue. We have previously demonstrated that T cell tolerance/ignorance could be broken in nontransplanted mice following xenogeneic DNA immunization (18, 19), and have now extended these findings to post-HSCT DNA immunization to self-Ags. Because the tumor Ags in our models are self-Ags and, by definition, present before immunization, we do not expect that our DNA vaccination strategies will be affected by the presence of tumor Ags during posttransplant immune reconstitution before vaccination. Moreover, our studies in nontransplanted mice indicated that tumor rejection after DNA immunization correlated with tumor-free survival in a tumor-bearing model, in which the DNA immunization starts after the tumor challenge (19).

Finally, our results in the treatment model, in which the tumor was given before DNA immunization or DLI, were remarkable because tumor rejection was significant only in the setting of post-HSCT immunization and not in nontransplanted mice that were immunized. The fact that DNA immunization in a treatment model was more effective in HSCT-recipients than in nontransplanted mice may be explained by a combination of alloreognition and the expansion of tumor-specific T cells through homeostatic T cell proliferation in the lymphopenic host, with the latter probably being the most significant factor (47).

The studies by Teshima et al. (21, 22) and our studies did not demonstrate GVHD in recipients of an allogeneic T cell-depleted HSCT after DLI and vaccination with whole cell B16 GM-CSF or DNA vaccines. However, the induction or exacerbation of GVHD by immunizing against alloantigens remains a real concern for the clinical translation of whole cell tumor vaccines, and DNA vaccines that only target tissue-restricted Ags should be considered safer in this respect. This consideration could be particularly relevant to DLI strategies. We expect that performing DLI with donor cells from hosts that have undergone DNA immunization against differentiation Ags rather than with whole cell vaccines will carry a low risk of enhancing GVHD. Vaccination of the donor with whole tumor cells will most likely sensitize the donor not only to tumor Ags, but also potentially against alloantigens and increase the risk of GVHD upon transfer of donor cells to the HSCT recipient. Indeed, Anderson et al. (40) found increased GVHD when donors were immunized with an IL-2-secretory whole tumor vaccine before an allogeneic transplant. This finding was in contrast to experiments in which donors were immunized against a model tumor Ag, influenza nucleoprotein, with no increase in GVHD noted, even after the recipients were immunized with a whole cell vaccine expressing nucleoprotein (39). When the whole cell vaccine was given to donors before DLI, severe GVHD developed in the recipients. Interestingly, Ferrara and colleagues (22) did not find evidence for increased GVHD when they used DLI from donors vaccinated with the B16-GM-CSF vaccine. We did not observe any GVHD, even when we increased the DLI dose to 30 × 10\(^6\), a dose at which Ferrara’s group did observe GVHD (22). We predict that DNA vaccines compared with whole cell vaccines are even less likely to lead to GVHD, which would allow the safe administration of higher doses of DLI than after donor immunization with whole tumor cell vaccines.

In summary, our studies have demonstrated the ability of post-HSCT DNA immunization to generate effective T cell responses that contribute to increased tumor-free and overall survival, including in treatment models. These results and similar investigations provide a strong rationale for the development of novel therapeutic strategies that combine allogeneic HSCT, posttransplant tumor vaccination, and adoptive cell therapy in human clinical trials.

**Acknowledgment**

We thank the staff of the Research Animal Resource Center (Memorial Sloan-Kettering Cancer Center) for excellent animal care.

**Disclosures**

The authors have no financial conflict of interest.

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