Tumor Cell Vaccine Elicits Potent Antitumor Immunity after Allogeneic T-Cell-depleted Bone Marrow Transplantation

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ABSTRACT

Allogeneic bone marrow transplantation (BMT) is currently restricted to hematological malignancies because of a lack of antitumor activity against solid cancers. We have tested a novel treatment strategy to stimulate specific antitumor activity against a solid tumor after BMT by vaccination with irradiated tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF). Using the B16 melanoma model, we found that vaccination elicited potent antitumor activity in recipients of syngeneic BMT in a time-dependent fashion, and that immune reconstitution was critical for the development of antitumor activity. Vaccination did not stimulate antitumor immunity after allogeneic BMT because of the post-BMT immunodeficiency associated with graft-versus-host disease (GVHD). Remarkably, vaccination was effective in stimulating potent and long-lasting antitumor activity in recipients of T-cell-depleted (TCD) allogeneic bone marrow. Recipients of TCD bone marrow who showed significant immune reconstitution by 6 weeks after BMT developed B16-specific T-cell-cytotoxic, proliferative, and cytokine responses as a function of vaccination. T cells derived from donor stem cells were, therefore, able to recognize tumor antigens, although they remained tolerant to host histocompatibility antigens. These results demonstrate that GM-CSF-based tumor cell vaccines after allogeneic TCD BMT can stimulate potent antitumor effects without the induction of GVHD, and this strategy has important implications for the treatment of patients with solid malignancies.

INTRODUCTION

Intensive chemo-radiotherapy alone mediates the antitumor effects of autologous BMT, but the conditioning regimen together with additional graft-versus-tumor effects help to eliminate malignancy after allogeneic BMT (1, 2). However, relapse after BMT remains a major clinical problem, and because residual disease after BMT is frequently resistant to cytotoxic therapies, improved patient outcomes will likely require novel treatment approaches (3, 4).

Recently, a number of promising cancer vaccination strategies have been developed that significantly augment antitumor immunity in multiple rodent tumor systems (5, 6). Vaccination with modified whole tumor cells as the antigen source has been explored as a means to prime systemic antitumor immunity. Among the various schemes tested, we have shown that vaccination with irradiated tumor cells engineered to secrete murine GM-CSF elicits potent, specific, and long-lasting antitumor immunity in murine models of melanoma, sarcoma, colon carcinoma, renal cell carcinoma, and lung carcinoma (7). The efficacy of GM-CSF-secreting vaccines has also been observed in rodent models of prostate carcinoma, bladder carcinoma, metastatic and primary brain cancer, myeloma, lymphoma, and acute leukemia (4, 7–15). GM-CSF-based vaccines require the participation of both CD4- and CD8-positive T lymphocytes and likely involve improved tumor antigen presentation by host macrophages and dendritic cells (7). The principles delineated in these preclinical studies have proven relevant to patients with advanced renal cell carcinoma or malignant melanoma (16, 17). In a recent Phase I study of 21 metastatic melanoma patients, vaccination with irradiated, autologous tumor cells that were engineered to secrete GM-CSF consistently stimulated the development of tumor-specific CD4+ and CD8+ T lymphocytes and plasma cells that induced extensive tumor necrosis, fibrosis, and edema (17).

The efficacy of any cancer immunotherapy is likely related to the overall tumor burden (18). A previous investigation of vaccination with irradiated leukemia cells engineered to express CD86 demonstrated that therapeutic outcomes could be improved by first reducing the tumor burden with chemotherapy (19). These observations suggest that definitive clinical testing of cancer vaccines should be attempted in the setting of minimal residual disease, which could be achieved by autologous or allogeneic BMT. Although the ability of BMT to induce minimal residual disease has been well documented, relatively little attention has been directed to studying tumor vaccination in this context. This situation likely reflects the finding that BMT results in a significant immunodeficiency that may compromise the efficacy of vaccination. Immune reconstitution after BMT is characterized by a recapitulation of lymphoid ontogeny and a lack of sustained transfer of clinically significant donor T- and B-cell immunity (18, 20). Multiple quantitative and qualitative T- and B-cell defects have been described after both autologous and allogeneic BMT (18, 21), although, with the passage of sufficient time, most abnormalities resolve, except in the presence of chronic GVHD which is associated with immunosuppression in both humans and mice (21–23).

Despite the delay in immune reconstitution after BMT, some evidence suggests that vaccination may still be possible in this setting. Effective immunization with a live attenuated vaccine against measles, mumps, and rubella has been reported 2 years after BMT (24). Vaccination of both the donor and recipient against hepatitis B and tetanus has resulted in enhanced immunity in BMT recipients (25, 26).

Immunization of a donor with a myeloma-associated paraprotein resulted in a tumor-specific immunity to the allogeneic BMT recipients (27). Collectively, these findings suggest that the development of antitumor immunity post-BMT may be feasible.

To investigate whether whole tumor cell vaccination strategies can be efficaciously used in combination with BMT to stimulate an antitumor effect, we have examined the ability of immunization with irradiated, GM-CSF-secreting B16 murine melanoma cells to generate specific antitumor immunity after BMT. Our findings establish that this vaccination scheme elicits potent antitumor effects after T-cell-depleted allogeneic BMT without the induction of GVHD.
MATERIALS AND METHODS

Mice. Female C57BL/6 (B6, H-2^b, CD45.2^+), SJL (H-2^b, CD45.1^+), B6.SJL-F1 (H-2^b, CD45.1^+/2^+), LPJ (H-2^b, CD45.2^+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The age of mice used as BMT recipients ranged between 11 and 16 weeks. Mice were housed in sterilized microisolator cages and received filtered water and normal chow and autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT.

BMT. Mice were transplanted according to a standard protocol as described previously (28). Briefly, on day 0, mice received 11 Gy total body irradiation (TBI; 137Cs source), split into two doses separated by 3 h to minimize gastrointestinal toxicity. BM cells (5 \times 10^6) and 1–2 \times 10^6 nylon wool purified splenic T cells were resuspended in 0.25 ml of Leibovitz's 15 media (Life Technologies, Inc., Gaithersburg, MD) and injected i.v. into recipients. SJL and LP were used as donors in allogeneic BMT models. In some experiments, allogeneic BM was depleted of T cells (TCD) by incubating cells with anti-Thy-1.2 MoAbs 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 less than 0.01% T cell in the BM. This protocol provides complete donor myelopoiesis after TCD BMT when donor and recipient differ at multiple minor histocompatibility loci (29, 30). No evidence of GVHD after TCD BMT is seen by histological examination, as published previously (31).

Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly by a scoring system that sums changes in five parameters: weight loss, posture, activity, fur texture, and skin integrity (maximum index, 10) as described previously (32). Scores of less than 1.0 are not specific and do not indicate clinically significant GVHD.

Tumor Vaccination and Challenge. B16-F10 melanoma cells (H-2^b), syngeneic to B6 mice, were maintained in DMEM containing 10% FCS, 50 units/ml penicillin, and 50 mg/ml streptomycin. GM-CSF-secreting B16 cells (300 ng/ml cells/24 h) were generated using the retrovirus vector MFG as described previously (7). No replication component retrovirus is generated with this system, as determined by the hils mobilization assay (33). Mice were immunized s.c. on the abdomen with 5 \times 10^5 irradiated (33 Gy), GM-CSF-secreting or wild-type B16 cells in HBSS (Life Technologies, Inc.) and challenged 1 week later with 1 \times 10^5 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 (7). Tumor growth was monitored every other day, and mice were killed when challenge tumors reached 1 cm in longest diameter. In some experiments, 10^5 irradiated (50Gy) B16 cells were s.c. injected into recipients on days 0, 7, 14, and 21 after BMT.

FACS Analysis. FITC-conjugated MoAbs to mouse CD45.2, CD4, CD11b, Gr-1, and PE-conjugated CD45.1, CD8, B220, NK1.1. DX5 were purchased from PharMingen (San Diego, CA). Cells were first incubated with MoAbs 2.4G2 (rat antimouse FcR MoAbs) for 15 min at 4°C to block nonspecific FcR binding of labeled antibodies, then with the relevant MoAbs 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 FACSscan (Becton Dickinson Immunocytometry Systems, San Jose, CA). Irrelevant IgG2a/b MoAbs were used as a negative control. Ten thousand live events were acquired for analysis. Donor T-cell engraftment was determined by the percentages of CD45.1/CD45.2 cells among CD3^+ cells in 3 mice per group (SIL: CD45.1/CD45.2^-/+; B6SJL/F1: CD45.1^-/+CD45.2^-/+).

Cell Culture and Analysis of T-Cell Proliferative Response. 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 (34). After lysis of erythrocytes with ammonium chloride, cells were washed twice and resuspended in supplemented 10% FCS in DMEM. The percentage of CD4^- and CD8^- T cells in this fraction were estimated by FACS analysis and were normalized for CD4^+ plus CD8^- T-cell numbers. The percentages of CD4^- and CD8^- T cells in the spleens of vaccinated and control group did not differ significantly. For the measurements of T-cell proliferation to B16 cells, 2 \times 10^5 splenic T cells were plated in 96 flat-bottomed plates and cultured for 5 days with 2 \times 10^6 B16 stimulators in 200 \mu l of supplemented 10% FCS in DMEM. Wild-type B16 cells were treated with IFN-\gamma for 24 h to increase expression of MHC class I and II molecules on their surface (35), washed twice, and irradiated (100 Gy). After 4 days of culture, supernatants were harvested from the culture for cytokine measurements, and cells were then pulsed with \textsuperscript{3}H thymidine (1 \muCi per well) for an additional 16 h. Proliferation was determined on a 1205 Betaplate reader (Wallac, Turku, Finland). For the measurements of T-cell proliferative responses to alloantigens or anti-CD3 MoAbs, splenocytes were cultured with plate-bound anti-CD3 MoAbs (5 \mug/ml; PharMingen) for 3 days or with 10^3 irradiated (20 Gy) peritoneal cells for 5 days.

ELISA. ELISA for GM-CSF, IFN-\gamma, 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 the specific primary MoAbs and detected by biotin-labeled secondary MoAbs. 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 sensitivity of the assays was 5 pg/ml for GM-CSF, 0.1 units/ml for IFN-\gamma and IL-2, 10 pg/ml for IL-4, 4–8 pg/ml for IL-5, and 62.5 pg/ml for IL-10.

\textsuperscript{3}Cr Release Assays. Responder splenocytes (1 \times 10^6 T cells/ml) were cultured with B16 stimulators (10^3/ml) in 24-well culture plate (Costar, Cambridge, MA) in the presence of 10 units/ml human IL-2 (Pharmacia Diagnostics Inc., Silver Spring, MD) for 5 days. Cells were then layered over Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged at 800 \times g for 15 min. Cells were collected from the interface and washed twice before suspension in supplemented 10% FCS in RPMI medium. The percentage of CD8^- T cells was estimated by FACS analysis, and the counts were normalized for CD8^- T-cell numbers. IFN-\gamma-treated B16 targets (2 \times 10^5) or 2 \times 10^6 ConA blasts prepared from murine splenocytes were labeled with 100 \muCi of \textsuperscript{51}Cr for 2 h and plated at 10^5 or 10^4 cells per well in U-bottomed 96-well plates (Costar). Effector cells were added in quadruplicate at varying E:T ratios. \textsuperscript{51}Cr activity in supernatants taken 4 h later was measured in a auto-gamma counter (Packard Instrument Company, Meriden, CT). Maximal and background release were determined by the addition of 2% Triton X-100 or media to the targets. The percent of specific \textsuperscript{51}Cr release (%) was calculated as 100 \times (sample count – background count)/maximal count – background count.

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, and the Mantel-Cox log-rank test was used to analyze survival data. P < 0.05 was considered statistically significant.

RESULTS

Immune Reconstitution Is Critical for the Induction of Antitumor Immunity Elicited by GM-CSF Tumor Cell Vaccine. To determine the relationship between immunological reconstitution and responsiveness to vaccination, we performed a time course analysis of vaccination after syngeneic BMT. B6 recipients were transplanted with 5 \times 10^5 BM from syngeneic B6 donor mice after 11 Gy TBI. BMT recipients were then immunized with irradiated, GM-CSF-secreting or wild-type B16 cells at either 4 or 6 weeks after BMT. Mice were challenged with live B16 cells 1 week after immunization.

As expected, tumor challenge was uniformly lethal in control animals vaccinated with irradiated, wild-type B16 cells; the kinetics of tumor development was similar between transplant recipients and naive mice (Fig. 1A). By contrast, vaccination with GM-CSF-secreting B16 cells resulted in substantial antitumor immunity at both 4 and 6 weeks after BMT (P < 0.001). Antitumor immunity was greater at 6 than at 4 weeks (TFS, 77% versus 79%, P < 0.05) and was as potent at 6 weeks as in naive animals (TFS, 79%). Immunophenotyping of splenocytes revealed that numbers of CD4^- and CD8^+ cells were increased 3-fold at 6 weeks as compared with 4 weeks after BMT (Fig. 1B).

CD4^- T-cell numbers increased to normal level by 6 weeks post-BMT, whereas CD8^+ cell counts remained below normal at all time points. B-cell numbers recovered to normal by 4 weeks and reached supranormal levels at 6 weeks. These results suggested that immune reconstitution of T cells was critical for the generation of antitumor immunity post-BMT.

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GM-CSF-based Tumor Cell Vaccines Did Not Elicit Antitumor Immunity 6 Weeks after Allogeneic BMT. Allogeneic BMT is an effective form of immunotherapy for a number of hematological malignancies. To determine whether our tumor vaccination strategy could be used in the context of allogeneic BMT, we first tested whether B16 cells, which express low levels of MHC class I and II molecules in vitro (35), could induce tumor-specific immune responses. Naive B6 (H-2b), B6SJLF1 (H-2b/s), LP (H-2b), and SJL (H-2d) mice rejected tumor challenge at day 100 is shown.

Fig. 1. Immune reconstitution is critical for the induction of antitumor immunity elicited by GM-CSF tumor cell vaccine. B6 mice were transplanted with 5 × 10^6 BM from syngeneic B6 donor mice after 11 Gy of TBI. A, B6 recipients were immunized with 5 × 10^5 irradiated, wild-type (○, SynBMT, wild-type vaccine; n = 15) or GM-CSF-secreting B16 cells 4 weeks (●, SynBMT (4w), GM-CSF vaccine; n = 18) or 6 weeks (□, SynBMT (6w), GM-CSF vaccine; n = 17) after syngeneic BMT and challenged 1 week later with 1 × 10^6 live B16 cells. Naive B6 mice were also immunized with 5 × 10^5 irradiated, wild-type B16 cells (△, No BMT, wild-type vaccine; n = 6) or GM-CSF-secreting B16 cells (▲, No BMT, GM-CSF vaccine; n = 14). Tumor growth was monitored up to day 100, and mice were killed when tumors reached 1 cm in longest diameter. Data represent results from two similar experiments. SynBMT, syngeneic BMT. *P < .05 versus 4 weeks. B, immune reconstitution of the spleen after BMT (n = 5/group). ○, naive; □, 4w post-BMT; ●, 6w post-BMT; △, 8w post-BMT. Data represent mean ± SD. *, P < .01 compared with 6 weeks.

Table 1 Tumorigenicity of B16 cells in naive mice and recipients of allogeneic BMT

<table>
<thead>
<tr>
<th>Strain</th>
<th>No vaccine</th>
<th>Vaccine</th>
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<tbody>
<tr>
<td>Naive</td>
<td>0/16 (0%)</td>
<td>13/19 (69%)</td>
</tr>
<tr>
<td>B6</td>
<td>0/12 (0%)</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td>B6SJLF1</td>
<td>6/8 (75%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>LP</td>
<td>7/11 (64%)</td>
<td>8/8 (100%)</td>
</tr>
<tr>
<td>SJL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMT recipient</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LP→B6 (7w post-BMT)*</td>
<td>0/8 (0%)</td>
<td></td>
</tr>
<tr>
<td>SJL→B6SJLF1 (1w post-BMT)*</td>
<td>0/6 (0%)</td>
<td></td>
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<tr>
<td>(7w post-BMT)*</td>
<td>0/5 (0%)</td>
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</table>

* BMT recipients of bone marrow and T cells from allogeneic SJL donors were challenged at either 1 or 7 weeks post-BMT.

Nonetheless, none was able to reject the B16 melanoma when injected 1 or 7 weeks after BMT (Table 1).

Vaccine efficacy was then assessed after allogeneic BMT. Recipients were immunized 6 weeks after BMT with GM-CSF-secreting B16 cells. Controls were not vaccinated. Mice were challenged with live, parental B16 cells 1–4 weeks later, which were lethal in control animals. Vaccination resulted in substantial antitumor immunity in both non-transplanted animals (TFS, 61.5% versus 0%; P < 0.0001) and in recipients of syngeneic BMT (TFS, 33.3% versus 0%; P < 0.0001; Fig. 2A). By contrast, 0% of the vaccinated recipients of allogeneic BMT survived challenge. In addition, vaccination failed to alter the kinetics of tumor development in recipients of allogeneic BMT, which demonstrated a lack of primary antitumor activity. These results demonstrate that immunization with irradiated, GM-CSF-secreting B16 cells fail to stimulate antitumor immunity after allogeneic BMT.

Similar results were obtained in a second BMT strain combination, LP→B6, in which the strains differ only in MHC. LP donors were transplanted after 11 Gy TBI with 5 × 10^6 BM and 1 × 10^6 splenic T cells from syngeneic B6 or allogeneic LP donors. After wild-type tumor challenge, none of the control animals survived without tumor beyond day 30, and no allogeneic graft-versus-tumor activity was evident after allogeneic BMT (Fig. 2B). Although vaccination stimulated protective antitumor immunity in 50% of non-transplanted animals (P < 0.05) and 25% of syngeneic BMT recipients (P < 0.05), 0% of allogeneic BMT recipients rejected the tumor challenge (P < 0.05 versus syngeneic BMT).

GVHD-associated Immunodeficiency Limits Vaccine Efficacy after Allogeneic BMT. GVHD is known to cause significant delays in immunological reconstitution after BMT (21–23), and we hypothesized that poor immunological reconstitution in the context of GVHD impaired antitumor activity. The effect of vaccination on tumor-specific T-cell responses was analyzed in vitro 1 week after vaccination (Table 2). The phenotype of lymphocytes in the spleen was not affected by the vaccination. Immunophenotyping of splenocytes 7 weeks post-BMT revealed severely reduced T- and B-lymphocyte numbers in recipients of allogeneic BMT with significant GVHD as described previously (36, 37), whereas numbers of CD4^+ populations were not significantly reduced.
T cells, natural killer cells, B cells, and myeloid cells, but not CD8+ cells, were normal 7 weeks after syngeneic BMT. Culture of splenocytes harvested 1 week after immunization showed marked T-cell proliferative responses to B16 cells in vaccinated but not in control animals. In addition, vaccination did not prime T cells to respond to B6SJLF1 peritoneal cells or anti-CD3 cross-linking, which indicated vaccination-specific induction of antitumor reactivity. Whereas T cells from vaccinated recipients of syngeneic BMT proliferated as potently as cells from naive animals, recipients of allogeneic BMT showed little detectable B16-specific T-cell proliferation, even when T-cell numbers were normalized prior to culture. These results demonstrate that functional immune reconstitution of T-cell responses to B16 is associated with tumor eradication in vivo and that vaccine efficacy is abolished by the immunodeficiency associated with GVHD.

Vaccination after Allogeneic TCD BMT Generates Potent Antitumor Immunity. TCD of the donor inoculum is able to prevent the immunosuppression associated with GVHD after allogeneic BMT, but it also impairs immune reconstitution in clinical BMT (38). We, therefore, asked whether TCD of semiallogeneic BM could also provide for sufficient immune reconstitution to provide antitumor immunity in this allogeneic BMT model. B6SJLF1 recipients were transplanted after 11 Gy TBI with 5 × 10^6 TCD BM from SJL donors. Vaccination stimulated the development of striking antitumor activity (Fig. 2A; TFS, 70.6 versus 0%; P < 0.001), equivalent to that observed in nontransplanted vaccinated animals (TFS, 61.5%). Similar effects were found after BMT across MiHA differences (LP→B6), in which vaccination after TCD BMT also resulted in substantial levels of antitumor activity (TFS, 28.6%; Fig. 2B).

The effect of vaccination on tumor-specific T-cell responses was analyzed in vitro 1 week after vaccination (Table 2). Allogeneic TCD BMT recipients showed normal numbers of all cell phenotypes except CD8+ cells by 6 weeks after BMT. T-cell proliferation to B16 stimulators in these animals was restored to normal levels. A recent study demonstrated that GM-CSF-based B16 cell vaccine require both Th1 and Th2 cytokine responses for the induction of maximal antitumor immunity (39). We, therefore, examined T-cell cytokine responses to vaccination after BMT. Analysis of the conditioned media obtained from cocultures of splenocytes from vaccinated animals and B16 stimulators revealed substantial levels of GM-CSF, IL-4, IL-5, IL-10, IFN-γ, and IL-2, similar to the profile observed in tumor-infiltrating lymphocytes stimulated by GM-CSF-based tumor vaccines in human melanoma patients (17). Cytokine responses in vaccinated TCD BMT recipients were never less than responses after syngeneic BMT and often equivalent to that seen in vaccinated naive animals. The development of proliferation and cytokine production to B16 in vitro correlated closely with the efficacy of the vaccine and tumor destruction in vivo. Comparable results were obtained in the LP→B6 system (data not shown). These results demonstrate that dual Th1 and Th2 cytokine responses that are closely associated with the development of antitumor immunity against B16 tumor can be induced by vaccination after BMT, including allogeneic TCD BMT.

Vaccination with a GM-CSF Whole Tumor Cell Vaccine Does Not Break Tolerance to Host Antigens after Allogeneic TCD BMT. Theoretically, whole tumor cell vaccines could present a significant risk of exacerbating GVHD by focusing increased reactivity to histocompatibility antigens shared by the tumor and host. To determine the effect of vaccination on GVHD severity, we monitored the survival and clinical GVHD score (range, 0–10) of immunized allogeneic BMT recipients, as described previously (32). GVHD was severe in the SJL→B6SJLF1 BMT model, with 36% mortality from GVHD by the time of vaccination (Fig. 3A). Clinical scores of GVHD severity in surviving allogeneic animals ranged from 5 to 7 by 4 weeks after allogeneic BMT, but it was mild or absent in recipients of syngeneic or TCD BMT (Fig. 3B). Importantly, vaccination did not exacerbate GVHD in any group, and, in particular, it did not cause increased skin disease or, depigmentation, as has been reported in other strategies to eliminate B16 tumors (40). Similar results were observed in the LP→B6 BMT model across MiHA differences, in which GVHD was relatively mild, and only 15% of the animals died by the time of vaccination (Fig. 3C). As expected, clinical GVHD scores were low, but even mild GVHD was not intensified by vaccination (Fig. 3D). Together, these findings demonstrate that GM-CSF-
Table 2. Immunophenotypic and functional T-cell responses to vaccination

<table>
<thead>
<tr>
<th>BMT</th>
<th>No</th>
<th>No</th>
<th>Syn</th>
<th>Syn</th>
<th>Allo</th>
<th>Allo</th>
<th>Allo</th>
<th>TCD</th>
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<tr>
<td>GVHD</td>
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Data represent mean ± SD of three animals from one of two similar experiments. 

<table>
<thead>
<tr>
<th>Immunophenotype (× 10^6/spleen)</th>
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<tbody>
<tr>
<td>CD4+</td>
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<td>CD8+</td>
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<tr>
<td>DX5+</td>
</tr>
<tr>
<td>A20+</td>
</tr>
<tr>
<td>Gr-1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD11b&lt;sup&gt;c&lt;/sup&gt;</td>
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T-cell proliferation (× 10^5 cpm)<sup>d</sup>

| +B16 | 2.1 ± 0.5 | 99.2 ± 12.6<sup>c</sup> | 3.5 ± 0.8 | 1007 ± 15.2<sup>c</sup> | 0.7 ± 0.5 | 1.6 ± 0.7 | 3.1 ± 0.6 | 97.3 ± 13.0<sup>c</sup> |
| +B6SJLF1 | 6.9 ± 1.4 | 5.9 ± 1.8<sup>c</sup> | 6.4 ± 1.6 | 50 ± 0.1<sup>c</sup> | ND | ND | ND | ND |
| +αCD3 | 392.2 ± 31.9 | 471.0 ± 50.1<sup>c</sup> | 382.0 ± 20.6<sup>c</sup> | 4052 ± 14.4<sup>c</sup> | ND | ND | 142.5 ± 21.1<sup>c</sup> | 380.0 ± 41.9<sup>c</sup> |

T-cell cytokine response<sup>e</sup>

| GM-CSF (pg/ml) | 79.1 ± 15.1 | 1283 ± 42<sup>c</sup> | 105.8 ± 26<sup>c</sup> | 771 ± 36<sup>c</sup> | ND | ND | 57.0 ± 6.6<sup>c</sup> | 1574 ± 123<sup>c</sup> |
| IFN-γ (units/ml) | UD | 33.8 ± 3.3<sup>c</sup> | UD | 16.1 ± 0.8<sup>c</sup> | ND | ND | UD | 17.2 ± 0.5<sup>c</sup> |
| IL-2 (units/ml) | UD | 0.5 ± 0.1<sup>c</sup> | UD | 1.0 ± 0.1<sup>c</sup> | ND | ND | UD | 3.9 ± 0.5<sup>c</sup> |
| IL-4 (pg/ml) | UD | 421 ± 30<sup>c</sup> | UD | 362 ± 18<sup>c</sup> | ND | ND | UD | 383 ± 31<sup>c</sup> |
| IL-5 (pg/ml) | UD | 2678 ± 12<sup>c</sup> | UD | 1801 ± 192<sup>c</sup> | ND | ND | UD | 1545 ± 201<sup>c</sup> |
| IL-10 (pg/ml) | UD | 2883 ± 296<sup>c</sup> | 1027 ± 159<sup>c</sup> | ND | ND | UD | 1823 ± 116<sup>c</sup> | |

<sup>a</sup> = no; +, yes; Syn, syngeneic; Allo, allogeneic; UD, undetectable; ND, not determined.
<sup>b</sup> Recipients were vaccinated 6 weeks post-BMT and splenocytes were harvested 1 week later. Control mice were not immunized (three mice/group).
<sup>c</sup> P < 0.01 compared with naive.
<sup>d</sup> Splenocytes from three to four animals per group were combined. (2 × 10^6) T cells were cultured for 5 days with 2 × 10^4 irradiated, IFN-γ-treated B16 cells, 10^5 irradiated B6SJLF1 peritoneal cells, or with anti-CD3 MAb's for 3 days in quadruplicate. Proliferation was determined by incubation of cells with [3H]thymidine (1 μCi) for the last 16 hours of culture.
<sup>e</sup> Cytokine levels in supernatants harvested from 4-day cultures with B16 stimulators. Splenocytes from control animals did not produce detectable levels of cytokines.
based tumor cell vaccines do not exacerbate GVHD when administered after allogeneic BMT.

In light of this absence of GVHD after vaccination, we evaluated T-cell responses to host antigens in vitro in these recipients of allogeneic TCD BMT. Analysis of donor engraftment at 4 and 6 weeks after BMT in peripheral blood disclosed mixed donor/host chimerism of CD3⁺ T cells in TCD BMT recipients (29.1 ± 4.1% and 67.5 ± 8.1% donor type at 4 and 6 weeks, respectively), although myeloid cells were completely of donor origin by 4 weeks post-BMT. Overall donor engraftment was 74 ± 5% donor at 4 weeks and 84 ± 4% donor at 6 weeks after BMT. Splenocytes were harvested 1 week after vaccination, and T-cell proliferative and cytotoxic responses were analyzed (Fig. 4). T cells from unvaccinated naive SJL proliferated in response to B6SJLF1 peritoneal cells, but T cells from TCD BMT recipients did not (Fig. 4A), which confirmed the acquisition of tolerance to host antigens after allogeneic TCD BMT. After vaccination, TCD BMT recipients proliferated to B16 stimulators in vitro without proliferating to B6SJLF1 antigens, which demonstrated that vaccination induced B16-antigen specific T-cell responses (Fig. 4A). Similar results were obtained in CTL assays (Fig. 4, B and C). Vaccination produced equivalent cytotoxic responses to B16 tumors after allogeneic TCD BMT and syngeneic BMT (data not shown). As expected, T cells from vaccinated SJL mice lysed B6 ConA blasts but did not lyse SJL ConA blasts. Although unvaccinated SJL mice possessed little detectable cytotoxicity against B16, vaccination significantly enhanced this cytotoxicity, similar to observations in immunized melanoma patients (17). Vaccination did not augment cytolytic activity against B6 ConA blasts, confirming tolerance to host antigens in vitro. Thus, despite mixed donor/host chimerism after allogeneic TCD BMT, GM-CSF-based tumor cell vaccines were able to stimulate effective antitumor immunity and did not elicit immune responses to host alloantigens either in vitro or in vivo.

In these experiments, immune reconstitution occurred in the absence of tumor, perhaps preventing the acquisition of tolerance to tumor antigens. To examine this possibility, we repeated this experiment with tumor present during immune reconstitution. After allogeneic TCD BMT (SJL→B6SJLF1), recipients were given s.c. injections with 10⁵ irradiated B16 cells on days 0, 7, 14, and 21 of BMT. Mice were subsequently vaccinated with irradiated, GM-CSF-secreting B16 cells at 6 weeks post-BMT and were challenged at 7 weeks post-BMT and monitored for survival, clinical scores, and tumor development (Table 3). TFS of BMT recipients receiving both repeated injections of irradiated B16 cells (Group E) and vaccination was equivalent to that of recipients receiving vaccination without such
GM-CSF TUMOR CELL VACCINE AFTER BMT

Fig. 4. Induction of B16-specific T-cell responses and antihost tolerance after TCD BMT. A, naive SJL and recipients of TCD BMT were vaccinated. Splenocytes were harvested one week after vaccination and spleens from three animals per group were combined. T cells (2 × 10^6) were cultured for 5 days with 2 × 10^7 irradiated, IFN-γ-treated B16 cells or with 10^9 irradiated SJL or B6SJLF1 peritoneal cells (Stim). Proliferation was determined by incubation of cells with [3H]thymidine (1 μCi) for the last 16 h of culture. Data are shown as mean ± SD of stimulation index (cpm in culture with B16 or B6SJLF1/cpm in culture with SJL) from quadruplicate culture. ○ SJL; □ SJL + vax; □ TCD-BMT; □ TCD-BMT + vax. B and C, cytotoxicity of splenic T cells from naive SJL (B) and from TCD BMT recipients (C) cultured for 5 days with B16 stimulators as determined in a standard 4-h 51Cr release assay against B16 targets, B6 ConA blasts (B6-Spl), or SJL ConA blasts (SJL-Spl). Data represent results from two similar experiments. ○, − Vax, Target B16; ●, + Vax, Target B16; △, − Vax, Target B6-Spl; ◻, + Vax, Target B6-Spl; ■, + Vax, Target SJL-Spl.

Table 3 Presence of tumor antigens during immune reconstitution did not induce tolerance to tumor

Naive mice or BMT recipients of allogeneic TCD BM received injections of 10^6 irradiated B16 cells at days 0, 7, 14, and 21 post-BMT. Subsequently mice were vaccinated at 6 weeks and challenged at 7 weeks after BMT.

<table>
<thead>
<tr>
<th>Group</th>
<th>BMT</th>
<th>Irradiated tumor injection</th>
<th>Vaccination</th>
<th>Tumor-free survivors at day 30 of challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>B</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>0/4 (0%)</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>4/8 (50%)</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4/10 (40%)</td>
</tr>
</tbody>
</table>

* −, no; +, yes.

Table 4 Antitumor immunity induced by vaccination is long-lasting in transplant recipients

Vaccinated mice that rejected initial tumor challenges were rechallenged with 1 × 10^6 live B16 cells 5 months after vaccination, and the numbers of surviving animals were reported.

<table>
<thead>
<tr>
<th>Recipients</th>
<th>BMT</th>
<th>Tumor-free survivors at day 100 of rechallenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>No</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>B6 SJL F1</td>
<td>Yes</td>
<td>8/12 (67%)</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6 SJL F1</td>
<td>No</td>
<td>3/4 (75 %)</td>
</tr>
<tr>
<td>B6 SJL F1</td>
<td>Yes</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>B6 SJL F1</td>
<td>Allogeneic TCD</td>
<td>7/7 (100%)</td>
</tr>
</tbody>
</table>

Discussion

The ability of cancer vaccines to enhance antitumor immunity after BMT are influenced by the toxicities of the conditioning regimen, the requirement for immunological reconstitution, and the immunosuppression associated with allogeneic BMT. To study the complex interactions of these variables, we have examined the vaccination properties of irradiated, GM-CSF-secreting B16 melanoma cells after BMT. GM-CSF-based vaccines require both CD4+ and CD8+ T cells for successful immunization (7), and thus, present a stringent test of immunological function post-BMT.

In preliminary studies, we examined the relationship between immunological reconstitution and responsiveness to vaccination by performing a time course analysis of immunization with irradiated, GM-CSF-secreting B16 cells after syngeneic BMT (B6→B6). Vaccination generated substantial levels of antitumor immunity by 4 weeks and full levels by 6 weeks post-BMT, demonstrating a rapid recovery from the toxicities of the conditioning regimens. Splenic CD4+ T cells recovered in significant numbers by 4 weeks and reached normal levels by 6 weeks, whereas CD8+ T cells achieved only 50% of normal levels by 8 weeks post-BMT. These findings confirm recent observations in a different BMT model (41), demonstrate that immune reconstitution is critical for effective vaccination, and underscore the correlation between T-cell recovery and vaccination efficacy. Although elimination of B16 tumor has been reported to occur independently of CD4+ cells (40), our results confirm that vaccination with GM-CSF-secreting B16 cells results in both CD4+ and CD8+ T-cell sensitization to tumor.

We then examined the ability of vaccination to generate antitumor immunity after allogeneic BMT. Immunization 6 weeks after syngeneic BMT with GM-CSF-secreting B16 cells generated potent antitumor immunity, as measured by both tumor protection and by B16-specific T-cell responses in vitro. However, when allogeneic BMT recipients were vaccinated, no antitumor activity was induced in two different BMT models. The absence of antitumor activity correlated with the immune suppression associated with GVHD; spleens obtained from allogeneic BMT recipients showed marked lymphoid hypoplasia and functional T-cell defects that are typical of GVHD-associated immune deficiency (21, 22, 36, 37, 42). It, therefore, seemed likely that GVHD-associated immunodeficiency limits vac-
cine efficacy after allogeneic BMT; this experimental result is con-
istent with clinical studies evaluating posttransplant immunization
against tetanus and poliovirus, in which impaired responses to vacci-
nation were associated with chronic GVHD (43, 44). Thus, although
GVHD has a known beneficial antitumor effects against hematolo-
gical malignancies and certain solid tumors (45), its associated immu-
nodedeficiency may inhibit efforts to enhance tumor eradication through
this type of vaccination strategy after allogeneic BMT.

Remarkably, this vaccination strategy was extremely effective after
allogeneic BMT when the donor inoculum was depleted of T cells to
prevent GVHD and resulted in mixed chimerism. This efficacy was
manifest in terms of both tumor protection and the development of
T-cell responses specific for B16 melanoma antigens. The induction
of tumor-specific cytokine production, proliferation, and cytotoxicity
after vaccination was closely associated with efficiency of vaccination
evident after both allogeneic TCD BMT and syngeneic BMT. Reconsti-
tution to normal levels of CD4+ T cells (but not CD8+ T cells) was
observed by 6 weeks after TCD BMT as well as after syngeneic BMT.
These findings demonstrate that TCD that prevents the development
of GVHD, allows sufficient reconstitution of T cells from donor stem
cells and can thereby restore the efficacy of vaccination. In this case,
a functional thymus is critical for repopulation of the periphery with
competent T cells because expansion of donor T cells is not an option
after TCD BMT. Unfortunately, such rapid reconstitution is unlikely
to occur in adult humans, in which the age-related reductions in
thymic regenerative capacity often result in incomplete restoration of
T-cell homeostasis after TCD BMT (46). Novel approaches to stimu-
late immune reconstitution will be required in older patients with
poor thymic function.

The tumor-specific T-cell production of GM-CSF, IFN-\(\gamma\), IL-2,
IL-4, IL-5, and IL-10 does not fit a classic Th1 or Th2 cytokine
pattern and suggests that multiple immunological effector mecha-

nisms are induced by GM-CSF-based vaccines. Pathological studies
of the skin at vaccination sites and challenge sites in mice and humans
receiving GM-CSF-secreting tumor cell vaccine have revealed an
extensive local influx of T cells, B cells, macrophages, dendritic cells,
and eosinophils (7, 17, 39). It has recently been demonstrated that
vaccination with GM-CSF-secreting B16 cells required both Th1 and
Th2 cytokines from CD4+ T cells for the induction of maximal
antitumor immunity (39). This cytokine profile has also been observed
in human Phase I clinical trials of vaccination with irradiated, GM-
CSF-secreting melanoma cells (17). These observations strongly sug-
gest a central role of CD4+ T cells in the induction of antitumor
immunity by GM-CSF-secreting whole tumor cell vaccine. Our stud-
es demonstrate that transplanted mice can generate both Th1 and Th2
cytokine responses after BMT as well as nontransplanted mice. The
efficacy of vaccination after syngeneic or allogeneic TCD BMT was
also comparable with that seen in nontransplanted mice, which may be
explained by the nearly normal quantitative and qualitative immune
reconstitution in these animals.

Interestingly, the protective antitumor immunity induced by GM-
CSF vaccination was long-lasting and displayed immunological mem-
ory, evidenced by the ability of vaccinated mice to reject a tumor
challenge 5 months later. Clinical studies of BMT patients show a loss
of donor-derived immunity (20, 27, 44), which suggests the need for
antigenic stimulation to an immune system that is newly generated
from donor BM cells, hence the recommendation of post-BMT vac-
cination against infectious agents (47).

To determine whether vaccination with GM-CSF-secreting B16
cells broke tolerance to host antigens, we evaluated a group of
immunized mice for progression of GVHD. The SJL→B6SJLJF1
model presents a highly stringent test for GVHD exacerbation, be-
cause the donor and recipient differ at MHC I and II loci in addition
to MiHAs. Although immunization was performed in mice that had
already developed significant GVHD, this cellular-based vaccine
caused no exacerbation of GVHD. Although GVHD in the LP→B6
BMT model (disparate MiHAs only) was less intense than in the other
model system, again vaccination had no significant influence on the
course of GVHD. Vaccination also did not induce GVHD after TCD
BMT in either strain combination. Lastly, our experiments determined
that the presence of tumor cells during immune reconstitution, as
might occur during clinical BMT when some malignant cells survive
high-dose conditioning, does not induce tolerance to tumor antigens
and does not prevent the efficacy of vaccine. However it should be
noted that administration of irradiated B16 cells may not be immu-
nologically equivalent to viable tumor cells because irradiated B16
cells are known to have low MHC expression and are poor immuno-
genics.

Our studies confirm and extend recent observations in a different
allogeneic BMT model when the use of a cellular-based vaccine
provided tumor-specific immunity in vivo without exacerbation of
GVHD (48). The mechanisms underlying the dissociation of antitu-
mor activity and GVHD in recipients of TCD BMT involve the
establishment of tolerance to host antigens. Tumor challenge demon-
strated that most naive SJL (MHC- and MiHA-discordant) and LP
(MiHA-discordant) mice, but not B6 (syngeneic) donor mice rejected
a lethal inoculum of B16 melanoma. This observation shows that B16
cells express a sufficient amount of MiHAs or MHC to stimulate the
immune system, although B16, a well-known tumor, has little detect-
able MHC I and MHC II molecules (35). Studies of T-cell prolifer-
ative and cytotoxic responses to allogeneic targets and B16 tumors
demonstrated that: (a) vaccination induced SJL T-cell responses di-
rected against B16-associated antigens; (b) donor T cells derived from
SJL TCD BMT were tolerant of host B6 antigens; and (c) vaccination
with B16 GM-CSF cells did not break tolerance of host antigens by
donor T cells. Tolerance of host antigens was associated with the
presence of mixed chimerism in TCD BMT recipients, and induction
of mixed chimerism has now become a major strategy to induce
tolerance after allogeneic BMT (49). These results show that vacci-
nation is capable of stimulating donor T cells to generate antitumor
immunity despite their acquisition of tolerance to host antigens in the
recipient thymus, which prevents GVHD after vaccination. However,
our data regarding B16 may not be representative of all tumors
because of its low MHC expression and the profound role of natural
killer cells in its rejection (50).

In other systems, antitumor effects are closely associated with
GVHD. A recent study in which allogeneic BMT donors were immu-
nized with IL-2-secreting tumor cells demonstrated a concomitant
increase in both antitumor activity and GVHD (51). By contrast, our
experiments clearly show that vaccination of recipients with GM-
CSF-secreting tumor cells after TCD BMT generates antitumor ac-
tivity that is separable from GVHD. Immunization of recipients rather
than donors may have several advantages; vaccinations can: (a)
be administered after the acquisition of tolerance to host antigens by
donor cells; (b) stimulate the newly developing immune system,
resulting in long-lasting immunity; and (c) avoid unnecessary ex-
posure of healthy donors to tumor cells and foreign proteins such as
alloantigens. Because TCD is associated with a marked reduction in
the frequency and intensity of GVHD and antitumor activity (52), the
ability of tumor vaccination to increase antitumor immunity without
GVHD in this setting has important clinical implications. If substan-
tive immune reconstitution can be achieved in patients after BMT, this
approach may be able to overcome the multiple immunological de-
fects associated with progressive cancer and, in so doing, enhance the
overall potency of tumor vaccines. The work presented here provides
a framework for crafting clinical trials aimed at evaluating the effi-

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cacy of this strategy, perhaps in combination with other approaches such as donor lymphocyte infusions.

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REFERENCES

Tumor Cell Vaccine Elicits Potent Antitumor Immunity after Allogeneic T-Cell-depleted Bone Marrow Transplantation

Takanori Teshima, Nicolas Mach, Geoffrey R. Hill, et al.


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