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

Takanori Teshima, Nicolas Mach, Geoffrey R. Hill, Luying Pan, Silke Gillessen, Glenn Dranoff, and James L. M. Ferrara

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-2b, CD45.2+), SJL (H-2d, CD45.1+), B6SJLF1 (H-2b, CD45.1+/2+), LP/J (H-2e, 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 x 10^7) and 1-2 x 10^6 nylon wool purified splenic T cells were resuspended in 0.25 ml of Leibovitz’s L-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 myeloepoiesis 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-2b), syngeneic to B6 mice, were cultured in DMEM containing 10% FCS, 50 units/ml penicillin, and 50 mg/ml streptomycin. GM-CSF-secreting B16 cells (300 mg/mL 10^6 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 his mobilization assay (33). Mice were immunized s.c. on the abdomen with 5 x 10^7 irradiated (33 Gy), GM-CSF-secreting or wild-type B16 cells in HBSS (Life Technologies, Inc.) and challenged 1 week later with 1 x 10^6 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, NK1.1, DX5 were purchased from PharMingen (San Diego, CA). Cells were first incubated with MoAbs 2.4G2 (rat antimouse FcγR MoAbs) for 15 min at 4°C to block nonspecific FcγR 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 FACScan (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+; B6SJLF1: 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 x 10^6 splenic T cells were plated in 96 flat-bottomed plates and cultured for 5 days with 2 x 10^6 B16 stimulators in 200 μl of supplemented 10% FCS in DMEM. Wild-type B16 cells were treated with IFN-γ 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 [3H]thymidine (1 μCi 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 μg/ml; PharMingen) for 3 days or with 10^3 irradiated (20 Gy) peritoneal cells for 5 days.

ELISA. 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 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-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.

51Cr Release Assays. Responder splenocytes (1 x 10^6 T cells/ml) were cultured with B16 stimulators (10^6/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 x 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-γ-treated B16 targets (2 x 10^5) or 2 x 10^6 ConA blasts prepared from murine splenocytes were labeled with 100 μCi of 35Cl for 2 h and plated at 10^4 or 10^5 cells per well in U-bottomed 96-well plates (Costar). Effector cells were added in quadruplicate at varying E:T ratios. 51Cr activity in supernatants taken 4 h later was measured in an 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 percentage of specific 51Cr release (% was calculated as 100 x (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 x 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 39%; P < 0.05) and was as potent at 6 weeks as in naive animals (TFS, 79%). Immunophenotyping of splenocytes revealed that numbers of CD4+, CD8+, and B220+ cells at 6 weeks after BMT were significantly greater than at 4 weeks (P < 0.01), but were comparable with numbers at 8 weeks after BMT (Fig. 1B). CD4+ T-cell number returned 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.
**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-2k), LP (H-2d), and SJL mice (H-2d) were challenged with 1 × 10^6 live B16 cells. None of the B6 or B6SJLF1 animals rejected B16 tumors, whereas the majority of LP and SJL mice rejected them, which demonstrated that B16 cells expressed sufficient amounts of MHC and/or MiHAs to induce an allogeneic immune response (Table 1). As expected, vaccination further enhanced the ability of nontransplanted SJL and LP mice to reject B16 cells (100% rejection after vaccination).

We then tested whether donor T cells from SJL could reject B16 cells after allogeneic BMT. B6SJLF1 and B6 recipients were transplanted after 11 Gy TBI with 5 × 10^6 BM and 1 × 10^6 splenic T cells from SJL and LP donors, respectively. After allogeneic BMT using these strain combinations, significant GVHD was developed.

<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>
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</table>

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 nontransplanted animals (TFS, 61.5% versus 0%; P < 0.0001) and in recipients of syngeneic BMT (TFS, 33.3% versus 0%; P < 0.0001; Fig. 1B). 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 MiHAs. B6 recipients 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 nontransplanted 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^+ lymphocytes were not significantly different compared with recipients of syngeneic BMT.
Vaccination after Allogeneic TCD BMT Generates Potent Antitumor Immunity.  

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 naïve 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 vitro and that vaccine efficacy is abolished by the immunodeficiency associated with GVHD.

Vaccination with a GM-CSF Whole Tumor Cell Vaccine Does Not Break Tolerance to Host Antigens after Allogeneic TCD BMT.  

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-\(\gamma\), 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 naïve 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\(\rightarrow\)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.
Table 2  Immunophenotypic and functional T-cell responses to vaccination

Data represent mean ± SD of three animals from one of two similar experiments.

<table>
<thead>
<tr>
<th>BMT No</th>
<th>No Syn</th>
<th>Syn Allo</th>
<th>Allo Allo</th>
<th>Allo TCD</th>
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<tr>
<td>GVHD vaccination</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>No</td>
<td>+</td>
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Immunophenotype (× 10^6/spleen) |

<table>
<thead>
<tr>
<th></th>
<th>CD4+</th>
<th>CD8+</th>
<th>DX5+</th>
<th>B20+</th>
<th>Gr-1+</th>
<th>CD11b+</th>
<th>T-cell proliferation (× 10^3 cpm)</th>
</tr>
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<tbody>
<tr>
<td>+B16</td>
<td>21.9 ± 1.6</td>
<td>16.0 ± 0.8</td>
<td>3.4 ± 0.4</td>
<td>54.5 ± 2.7</td>
<td>2.8 ± 0.7</td>
<td>2.2 ± 0.3</td>
<td>2.1 ± 0.5</td>
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<tr>
<td>+B6S1LF1</td>
<td>6.9 ± 1.4</td>
<td>5.9 ± 1.8</td>
<td>99.2 ± 12.6</td>
<td>54.7 ± 12.3</td>
<td>2.8 ± 1.2</td>
<td>2.5 ± 0.6</td>
<td>14.0 ± 0.0</td>
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<tr>
<td>+aCD3</td>
<td>392.2 ± 31.9</td>
<td>471.0 ± 50.1</td>
<td>53.4 ± 8.6</td>
<td>54.7 ± 12.3</td>
<td>2.2 ± 1.2</td>
<td>2.3 ± 0.8</td>
<td>70.6 ± 1.1</td>
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T-cell cytokine response |

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<thead>
<tr>
<th></th>
<th>GM-CSF (pg/ml)</th>
<th>IFN-γ (units/ml)</th>
<th>IL-2 (units/ml)</th>
<th>IL-4 (pg/ml)</th>
<th>IL-5 (pg/ml)</th>
<th>IL-10 (pg/ml)</th>
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</thead>
<tbody>
<tr>
<td>+B16</td>
<td>79.1 ± 15.1</td>
<td>UD</td>
<td>33.8 ± 3.3</td>
<td>UD</td>
<td>6.1 ± 0.8</td>
<td>UD</td>
</tr>
<tr>
<td>+B6S1LF1</td>
<td>1283 ± 42</td>
<td>UD</td>
<td>36.7 ± 1.1</td>
<td>UD</td>
<td>1.0 ± 0.1</td>
<td>UD</td>
</tr>
<tr>
<td>+aCD3</td>
<td>105.8 ± 26</td>
<td>UD</td>
<td>362 ± 18</td>
<td>UD</td>
<td>1801 ± 192</td>
<td>UD</td>
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<td></td>
<td>2059 ± 296</td>
<td>UD</td>
<td>1027 ± 198</td>
<td>UD</td>
<td>927 ± 296</td>
<td>UD</td>
</tr>
</tbody>
</table>

T-cell proliferation was assessed 6 weeks post-BMT and splenocytes were harvested 1 week later. Control mice were not immunized (three mice/group).

* Recipients were vaccinated 6 weeks post-BMT and splenocytes were harvested 1 week later. Control mice were not immunized (three mice/group).

* P < 0.01 compared with naive.

* 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 10^5 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
injections (group D). Thus, the presence of tumor antigens during immune reconstitution did not prevent the development of antitumor immunity stimulated by this vaccine protocol.

**Antitumor Activity Induced by Vaccination Post-BMT Is Long-Lasting.** To determine whether vaccination stimulated the development of long-lasting antitumor immunity, we challenged mice that had rejected an initial tumor inoculum of $10^6$ wild-type B16 cells at 5 months after immunization. We found that 67–75% of syngeneic BMT and 100% of allogeneic TCD BMT recipients eliminated the second tumor challenge, which demonstrated the induction of immunological memory by this vaccination strategy (Table 4).

### 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$^+$ properties of irradiated, GM-CSF-secreting B16 melanoma cells after syngeneic BMT (B6) 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 immunosuppression 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 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.

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

<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>
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<td>-</td>
<td>0/3 (0%)</td>
</tr>
<tr>
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<td>-</td>
<td>+</td>
<td>2/5 (40%)</td>
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<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**

<table>
<thead>
<tr>
<th>Table 4 Antitumor immunity induced by vaccination is long-lasting in transplant recipients</th>
<th>BMT</th>
<th>Tumor-free survivors at day 100 of rechallenge</th>
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<tr>
<td>Recipients</td>
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<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
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</tr>
<tr>
<td>B6SJLF1</td>
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<tr>
<td>B6SJLF1</td>
<td>Syngeneic</td>
<td>7/7 (100%)</td>
</tr>
<tr>
<td>B6SJLF1</td>
<td>Allogeneic TCD</td>
<td>7/7 (100%)</td>
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Fig. 4. Induction of B16-specific T-cell responses and antihist 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 ($2 \times 10^3$) were cultured for 5 days with $2 \times 10^3$ irradiated, IFN-$\gamma$-treated B16 cells or with $10^3$ irradiated SJL or B6SJLF1 peritoneal cells (Stim). Proliferation was determined by incubation of cells with $[^{3}H]$thymidine (1 $\mu$Ci) 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. (A) SJL, SJL + vax; (B) TCD BMT; (C) TCDBMT + vax. (A), TCD BMT; (B) B6SJLF1; (C) 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.
cine efficacy after allogeneic BMT; this experimental result is consistent with clinical studies evaluating posttransplant immunization against tetanus and poliovirus, in which impaired responses to vaccination were associated with chronic GVHD (43, 44). Thus, although GVHD has a known beneficial antitumor effects against hematological malignancies and certain solid tumors (45), its associated immunodeficiency 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 efficacy of vaccination evident after both allogeneic TCD BMT and syngeneic BMT. Reconstitution 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 stimulate immune reconstitution will be required in older patients with poor thymic function.

The tumor-specific T-cell production of GM-CSF, IFN-γ, 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 mechanisms 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). There is 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 suggest a central role of CD4+ T cells in the induction of antitumor immunity by GM-CSF-secreting whole tumor cell vaccine. Our studies 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 memory, 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 vaccination 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→B6SJL-F1 model presents a highly stringent test for GVHD exacerbation, because the donor and recipient differ at MiHAs 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 immunologically equivalent to viable tumor cells because irradiated B16 cells are known to have low MHC expression and are poor immunogens.

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 antitumor activity and GVHD in recipients of TCD BMT involve the establishment of tolerance to host antigens. Tumor challenge demonstrated that most naïve 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 detectable MiH and MHC II molecules (35). Studies of T-cell proliferative and cytotoxic responses to allogeneic targets and B16 tumors demonstrated that: (a) vaccination induced SJL T-cell responses directed 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 vaccination 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 immunized 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 activity 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 exposure 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 substantive immune reconstitution can be achieved in patients after BMT, this approach may be able to overcome the multiple immunological defects 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-
cacy of this strategy, perhaps in combination with other approaches such as donor lymphocyte infusions.

ACKNOWLEDGMENTS

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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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