Induction of Immunity to Neuroblastoma Early after Syngeneic Hematopoietic Stem Cell Transplantation Using a Novel Mouse Tumor Vaccine

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ABSTRACT
Autologous HSCT has resulted in improved event-free survival in patients with advanced neuroblastoma, but most of these patients still relapse. We previously reported that transient transfection of mouse neuroblastoma cells with plasmid DNA vectors encoding immune costimulatory molecules generates cell-based vaccines capable of inducing potent antitumor T cell immunity. In this study, we explored the effectiveness of tumor vaccine administration soon after HSCT. Soon after transplantation, only vaccinated mice that had received an adoptive transfer of syngeneic T cells survived tumor challenge. Tumor protective immunity in the transplant recipients was dependent on CD4+ and CD8+ T cells, and tumor-reactive T cells in the spleens of vaccinated mice could be detected in IFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays. Our data indicate that the adoptive transfer of T cells was absolutely required for induction of protective immunity by the tumor vaccine. Adoptive transfer of T cells accelerated T cell reconstitution, but it also resulted in increased percentages of CD4+CD25+Foxp3+ cells soon after HSCT. Treatment of HSC transplant recipients with an anti-CD25 mAb before tumor vaccination inhibited antitumor immunity and significantly decreased the number of IFN-γ-secreting tumor-specific CD4 T cells. However, physical depletion of CD25+ cells from the adoptively transferred splenocytes appeared to increase the efficacy of tumor vaccination. Collectively, these results demonstrate that anti-neuroblastoma immunity can be induced soon after HSCT using a novel cell-based cancer vaccine. However, sufficient numbers of T cells must be added to the graft to achieve protective antitumor immunity, and depletion of CD25+ T cells from adoptively transferred T cells might provide some additional benefit. These translational studies will aid in our development of post-HSCT vaccines for neuroblastoma.

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KEY WORDS
Hematopoietic stem cell transplantation • Tumor vaccine • Plasmid vectors • Immune stimulatory molecules • Adoptive immunotherapy • Regulatory T cells

INTRODUCTION
Neuroblastoma is the most common extracranial solid tumor in children, accounting for 8% of all childhood cancers [1]. It still has one of the highest death rates of all pediatric cancers and is responsible for approximately 15% of childhood cancer deaths. Despite aggressive treatment, the outcome remains poor for many patients with neuroblastoma. Autologous HSCT has resulted in improved event-free survival for patients with severe disease. However, >50% of patients develop recurrent neuroblastoma from residual disease or from contaminating tumor cells in autologous grafts. Because of the high incidence of relapse, more effective treatments targeting minimal residual disease are required.

It has been demonstrated that neuroblastoma cells express tumor antigens that might be recognized by cytotoxic T cells [2-4]. However, in vivo the tumor may be poorly immunogenic due to an absence of critical immune costimulatory molecules. Neuroblastoma cells that have been genetically modified to ex-
press various immune stimulatory molecules have been employed to induce antitumor immunity in experimental animal models. Because individual immune stimulatory molecules might trigger diverse patterns of cellular activation that contribute differentially to the induction and effecter phases of the immune response, combinations of immune stimulatory molecules could provide synergistic or additive effects to tumor immune responses. Our experiments have demonstrated synergistic antitumor effects by combining the co-stimulatory molecules CD80 and CD86 or CD80 and CD137L (4-1BB ligand) [5,6]. We also showed that high-level transfection of neuroblastoma cells to express a panel of 4 immune costimulatory molecules (CD54, CD80, CD86, and CD137L) transformed the tumor cells into a tumor vaccine capable of stimulating a potent T cell response [7]. This vaccine increased the numbers of detectable tumor-specific splenic CTLs in treated animals, and it induced a more effective antitumor response than did tumor cells expressing only CD80 and CD86.

In this study we explored the effectiveness of tumor vaccine administration soon after high-dose therapy and HSCT because this may be an ideal setting to induce effective tumor immunity due to decreased disease burden [8], altered immune regulation [9], and altered T cell homeostasis [10]. Accelerated lymphoid reconstitution of donor or host origin may overcome inherent defects in T cell signaling [11], defects in APC function including antigen processing and/or presentation, or defective T cell costimulation by APCs [12,13]. Manipulation of the T cell repertoire by immunization during post-HSCT immune reconstitution might skew the T cell repertoire toward particular antigen specificities [14]. Results of animal studies have also suggested that vaccination during homeostatic proliferation could facilitate an immune response to weak self-antigens and enhance T cell-mediated antitumor immunity [15-18].

The immediate post-HSCT period is accompanied by immune deficiency as a result of smaller immune effector cell numbers and impaired lymphocyte function [10]. We hypothesized that efficient antitumor immune responses could be induced soon after HSCT only if syngeneic T cells were adoptively transferred at the time of transplantation. In this study, we show that the combination of high-dose TBI, HSCT, T cell add-back, and early post-transplantation tumor vaccination generates potent anti-neuroblastoma immunity in a manner that could be exploited in future clinical trials.

METHODS

Mice

The following strains of mice (6–8 wk of age) were purchased from Jackson Laboratories (Bar Harbor, Me): A/J, C57BL/6 (B6) (CD45.2+; Thy1.2+), congenic B6.PL-Thy1a (CD45.2+; Thy1.1+), and B6-45.1 (CD45.1+; Thy1.2+). The animals were housed in the Medical College of Wisconsin Biomedical Resource Center (Milwaukee, Wis), which has been accredited by the American Association for Accreditation of Laboratory Animal Care.

Tumor Cells

Neuro-2a, a mouse neuroblastoma of strain A origin, was obtained from the American Type Culture Collection (ATCC; Manassas, Va). The tumor cells express MHC class I antigens, but are MHC class II negative. An aggressive subclone, designated AGN2a, was derived through sequential in vivo and in vitro passaging [5]. An MHC class II+ AGN2a cell line (designated AGN2a-CIITA) was derived by stably transfecting AGN2a with a plasmid expression vector (pcDNA3.1[−]; Invitrogen, Carlsbad, Calif) encoding the MHC class II transactivator (CIITA) gene (provided by Dr Suzanne Ostrand-Rosenberg at The University of Maryland, Baltimore County). SaI, a fibrosarcoma cell line derived from an A/J mouse, was obtained from the ATCC.

Antibodies

The following mAbs, with or without a fluorescent label, were obtained from BD Biosciences (BD Biosciences Pharmingen, San Diego, Calif): anti-CD4 (clones GK1.5 and RM4-5), anti-CD8 (clone 53-6.7), anti-CD16/CD32 (clone 2.4G2), anti-CD25 (clones 7D4 and PC61), anti-CD45.1 (clone A20), anti-CD45.2 (clone 104), anti-CD54 (clone 3E2), anti-4-1BB (clone TKS-1), anti-CD80 (clone 16-10A1), anti-CD86 (clone 37.51), anti-CD90.2 (Thy1.2; clone 53-2.1), and anti-rat IgG2a (clone RG7/1.30). Control antibodies included purified mouse IgG2b and rat IgG2b. Anti-CD90.1 (Thy1.1, clone HIS51) and anti-Foxp3 (clone FJK-16s) mAbs were obtained from eBioscience (eBioscience, San Diego, Calif).

Hybridomas producing anti-CD25 mAb (clone PC61), anti-CD4 mAb (clone GK1.5), and anti-CD8 mAb (clone 2.43) were obtained from the ATCC. These mAbs were produced in our laboratory using Integra CL 1000 bioreactors (Chur, Switzerland). Anti-Thy1.2-, anti-CD44, anti-CD8+, and anti-PE-conjugated microbeads used for immunomagnetic cell separation were purchased from Miltenyi Biotec (Miltenyi Biotec, Auburn, Calif).

Flow cytometry was used to analyze gene-modified AGN2a cells for cell surface expression of immune stimulatory molecules as described previously [7]. Antibody-stained cells were analyzed with a Becton Dickinson FACScan flow cytometer, and the resulting data were analyzed using FlowJo software (Tree Star, San Carlos, Calif).
**T Cell Enrichment**

AJ spleens were collected and processed into single-cell suspensions. The splenocytes were incubated with anti-Thy1.2-, anti-CD8-, or anti-CD4-conjugated microbeads (Miltenyi Biotec), and the T cells were positively selected using a Miltenyi automated immunomagnetic sorter (autoMACS). The level of T cell enrichment was determined by flow cytometric analysis. The Thy1.2-enriched cells typically consisted of >95% T cells, and the purity of CD4- and CD8-enriched T cells was >98%. Alternatively, CD25+ cells were physically depleted from splenocytes by sequential incubation with PE-conjugated anti-CD25 mAb (clone PC61) and anti-PE-conjugated microbeads (Miltenyi Biotec), followed by negative selection using an autoMACS cell sorter.

**Syngeneic HSCT and Tumor Vaccination**

Femurs and tibiae were obtained from donor AJ mice, and BM was harvested by flushing the bones with DMEM. For HSCT, AJ recipient mice were lethally irradiated (1100 cGy) and 24 h later given a single i.v. injection of 10^7 BM cells with or without 2 × 10^7 added splenocytes or 5 × 10^6 Thy1.2-enriched T cells. In some experiments, the Thy1.2-enriched T cells were administered 3 d after HSCT. In other experiments, lethally irradiated (1000 cGy) C37BL/6 mice received transplants by i.v. injection with 10^7 BM cells from B6.PL-CD45.1 mice plus 2 × 10^7 splenocytes from B6-CD45.1 mice.

For vaccination, on days 7 and 14 after HSCT, recipient AJ mice were given subcutaneous injections of 2 × 10^6 irradiated (5000 cGy) AGN2a cells. Experimental groups of mice were vaccinated with AGN2a cells that had been transfected 24 h previously with separate plasmids containing gene inserts for CD54 (pcDNA3.1/Hygro vector; Invitrogen), CD80, CD86, and CD137L (each in pCI-neo vectors; Promega, Madison, Wis) using nucleofection (Amaxa Biosystems, Koeln, Germany) as described previously [7]. Control mice were vaccinated with AGN2a cells nucleofected with unmodified pcDNA3.1/Hygro and pcCI-neo plasmids. One week to 3 wk after the last vaccination, the mice were challenged subcutaneously with 10^7 to 5 × 10^8 viable AGN2a cells and followed for tumor development. Mice were considered moribund and killed when a tumor exceeded 250 mm^2.

In some experiments, mice were treated with 250 µg of anti-CD25 mAb 4 d after HSCT. In other experiments, mice were depleted of T cell subsets in vivo by treating them with subset-specific mAbs during the induction or effector phases of the vaccine-induced immune response. Mice were injected i.p. with 250 µg anti-CD4 (GK1.5) or anti-CD8 (2.43) mAbs on days 4, 7, 10, and 14 after HSCT (immune induction phase experiments) or on days 32 and 35 after HSCT (immune effector phase experiments). To assess the involvement of T cell subsets in the induction phase of the vaccine-induced immune response, the mice were challenged with viable tumor cells 1 wk after the second tumor vaccination. For the effector phase experiments, the mice were challenged with viable tumor cells 3 wk after the second tumor vaccination.

**IFN-γ ELISPOT Assays**

To assess numbers of tumor-specific IFN-γ-secreting CD8+ or CD4+ T cells, enzyme-linked immunosorbent spot (ELISPOT) assays were done using the Mouse IFN-γ ELISPOT Kit from BD Biosciences Pharmingen. The numbers of spots were quantitated with an ImmunoSpot Analyzer using included acquisition and analysis software (CTL Analyzers, LLC, Cleveland, Ohio).

**Treg Cell Suppression Assays**

To verify the suppressive activity of CD4+CD25+ T cells, 5 × 10^7 CD4+CD25− responder T cells were plated in microtiter wells alone or with different numbers of CD4+CD25+ cells. CD4+CD25+ cells and CD4+CD25− responder T cells were isolated from splenocytes using the CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. The levels of CD4+CD25+ T cell enrichment were determined by flow cytometric analysis. The enriched CD4+CD25+ T cells were typically >96% pure (see Figure 7). Responder cells were stimulated with anti-CD3/anti-CD28 mAb-coated Dynabeads (DYNAL Biotech ASA, Oslo, Norway) at a bead-to-responder T cell ratio of 1:4. After 72 h of culture, 3H-thymidine was added to each well for an additional 18 h. 3H-thymidine incorporation was measured on a β-scintillation counter, and the results were expressed as mean counts per minute of triplicate wells.

**Statistics**

Survival curves were compared by log-rank analysis. Student t test was used to analyze the number of T cells. P < .05 was considered statistically significant.

**RESULTS**

**Generation of Effective Antitumor Immunity Soon after HSCT Requires Adoptive Transfusion of T Cells**

We previously reported that transient transfection of mouse neuroblastoma cells with plasmid DNA vectors encoding a panel of immune stimulatory molecules (CD54, CD80, CD86, and CD137L) generates a cell-based vaccine capable of inducing potent antitu-
mor T cell immunity [7]. In this study, we evaluated whether this tumor vaccine could generate a protective antitumor immune response in syngeneic HSC transplant recipients if administered immediately after transplantation. Studies of T cell reconstitution in mice that had been treated with lethal TBI and a syngeneic HSCT showed that the immediate post-transplantation phase was accompanied by a significant T cell deficiency (data not shown). We hypothesized that adoptive transfer of T cells would be required for the success of tumor vaccination in the early post-transplantation period. To test this hypothesis, A/J mice were lethally irradiated 1 d before HSCT (day –1), infused with 10^7 unmanipulated syngeneic BM cells on day 0, and infused with or without 5 × 10^6 purified T cells (Thy1.2-enriched) at day 3. Mice were vaccinated subcutaneously with 2 × 10^6 irradiated AGN2a tumor cells nucleofected to express CD54, CD80, CD86, and CD137L (designated as 4P-AGN2a cells) on days 7 and 14 after HSCT. Control mice were vaccinated with AGN2a cells that were nucleofected with equal amounts of “empty” plasmids (ie, containing no transgene inserts). Other controls consisted of nonvaccinated BM recipients. One week after the last vaccination, mice were challenged subcutaneously with 10^4, 10^5, or 10^6 viable AGN2a cells and followed for tumor development. Nonvaccinated mice were unable to resist even the lowest challenge dose of 10^4 AGN2a cells (Figure 1), and T cell transfer failed to protect these mice from tumor challenge. Tumor vaccination without T cell adoptive transfer also failed to protect mice from tumor challenge. In contrast, mice given adoptive T cell transfer and vaccination with nucleofected 4P-AGN2a cells were significantly protected from tumor challenge at all 3 challenge doses (100%, 78.5% and 78.5% survival
of mice challenged with $10^4$, $10^5$ and $10^6$ AGN2a cells, respectively; $P < .001$ versus nonvaccinated mice). AGN2a cells nucleofected with empty vectors also significantly enhanced the survival of adoptive T cell-transferred mice challenged with the lower doses of $10^4$ or $10^5$ tumor cells (88.9% and 33.3% of mice, respectively; $P < .01$; Figure 1A,B). Similar results had been previously obtained when empty vector/nucleofected AGN2a cells were tested as a vaccine in normal mice without transplants [7]. These results confirmed our hypothesis that T cell adoptive transfer is required for tumor vaccination to be effective in mice soon after HSCT.

Optimal Tumor Vaccine-Induced Immunity was Obtained using $2 \times 10^7$ Nonseparated Splenocytes as the Source of T Cell Adoptive Transfer

Next we sought to determine whether “untouched” T cells, in the form of nonseparated splenocytes, would be as effective at facilitating tumor immunity as purified T cells that had been positively selected by immunomagnetic bead sorting. Mice were subjected to HSCT as before, but purified T cells ($5 \times 10^6$) or splenocytes ($2 \times 10^7$ or $4 \times 10^6$) were coinfused with the BM, instead of given 3 d after transplantation. A dose of $5 \times 10^6$ T cells approximates the T cell content in $2 \times 10^7$ splenocytes (data not shown). On days 7 and 14 after HSCT, the mice were vaccinated subcutaneously with $2 \times 10^6$ irradiated 4P-AGN2a cells. A control group of mice given HSCT, but no T cell transfer or vaccine, was included in the experiments. All mice were challenged subcutaneously on day 21 after HSCT with $5 \times 10^6$ viable AGN2a cells. Adoptive transfer of $2 \times 10^7$ splenocytes appeared to provide a better vaccine-induced antitumor response than did $5 \times 10^6$ purified T cells, as indicated by increased, although not statistically different, survival (56% versus 20% survival; $P = .058$; Figure 2). These results suggested that the immunomagnetic positive selection had an effect on survival and/or function of the adoptively transferred T cells or that the splenocytes contained non-T cells capable of increasing the vaccine-induced antitumor effect. Increasing the dose of splenocytes given at the time of HSCT from $2 \times 10^7$ to $4 \times 10^7$ cells did not further improve survival after tumor challenge (Figure 2). Based on these results, a simplified protocol using $2 \times 10^7$ nonseparated splenocytes as the source of T cell add-back was chosen for the remaining experiments in this study.

Adoptive Transfer of Splenocytes and BM from Donors with Established Tumors Generates Vaccine-Induced Antitumor Immunity

To more closely mimic the clinical setting and to test if the presence of established AGN2a tumors results in tolerized tumor-reactive immune cells, mice with established neuroblastoma were used as donors for BMT and adoptive spleen cell transfer. A/J donor mice were inoculated subcutaneously with $1 \times 10^6$ AGN2a cells 12 days before BM and splenocyte harvest. Lethally irradiated A/J recipients underwent transplantation with BM and splenocytes harvested from the tumor-bearing or non-tumor-bearing A/J donors. At the time of tissue harvest, the tumor cell-inoculated mice had palpable tumors that were $>100$ mm$^3$; percentages of CD25$^+$ Foxp3$^+$ CD4$^+$ Treg cells in the spleens of tumor-bearing mice were not significantly increased compared with those in tumor-free mice (data not shown). Seven days after HSCT, recipients were vaccinated with $5 \times 10^6$ viable AGN2a cells. Data represent combined results of 2 or 3 separate experiments, and groups consisted of 10-16 total mice per group.

Figure 2. Adoptive transfer of nonseparated splenocytes provided a better vaccine-induced antitumor effect than did T cells purified by positive selection using immunomagnetic sorting. Lethally irradiated A/J mice underwent transplantation with $10^7$ syngeneic BM cells with/without $5 \times 10^6$ positively selected Thy1.2$^+$ T cells, $2 \times 10^6$ nonseparated splenocytes, or $4 \times 10^6$ nonseparated splenocytes. One week after HSCT, mice given adoptive T cell/spleen cell transfer were vaccinated twice weekly with $2 \times 10^6$ irradiated 4P-AGN2a cells. One week after the second vaccination (day 21 after HSCT) all mice were challenged with $5 \times 10^6$ viable AGN2a cells. Data represent combined results of 2 or 3 separate experiments, and groups consisted of 10-16 total mice per group.
tumor-bearing mice as the source of adoptive cell transfer to simplify the experimental design.

**Anti-neuroblastoma Response Induced by Vaccination with 4P-AGN2a Cells Soon after HSCT Depends on CD4 and CD8 T Cells**

To determine which T cells are responsible for the protective antitumor immune responses, mice were depleted of CD4 or CD8 cells in vivo using mAbs. For HSCT, lethally irradiated A/J mice underwent transplantation with BM cells admixed with \(2 \times 10^7\) splenocytes from normal A/J donors. The transplanted mice were vaccinated with nucleofected 4P-AGN2a cells on days 7 and 14 after HSCT. Anti-CD4 or anti-CD8 mAb was then administered during the induction phase (days 4, 7, 10, and 14 after HSCT) or during the effector phase (18 and 21 d after last vaccination and 32 and 35 d after HSCT). More than 99% of CD8\(^+\) and 95% of CD4\(^+\) T cells were depleted by the mAb treatment (data not shown). Mice were challenged with \(10^6\) live AGN2a cells 21 d (induction phase) after HSCT or \(5 \times 10^5\) live AGN2a 35 d (effector phase) after HSCT.

Approximately 80% of non-antibody-treated controls survived the tumor challenge (Figure 4A,B). Depletion of CD4 or CD8 T cells during the induction phase of the immune response significantly reduced survival of the vaccinated mice (\(P < .001;\) Figure 4A), indicating that both T cell subsets are important for generating the antitumor response.

As expected, CD8\(^+\) T cells were found to be important effectors of the antitumor immune response (Figure 4B; \(P = .0001\) versus non-antibody-treated mice). However, CD4\(^+\) T cells also appear to play a role in the effector phase of the immune response because the ability to resist tumor challenge was almost completely abrogated by treatment with anti-CD4 mAb (Figure 4B; \(P = .002\) versus non-antibody-treated mice).

**Immunization with 4P-AGN2a Cells Elicits Tumor-Reactive IFN-\(\gamma\)-Producing CD8\(^+\) and CD8\(^+\) T Cells in HSC Transplant Recipients**

T cell tumor reactivity elicited soon after HSCT by 4P-AGN2a vaccination was assessed in vitro using IFN-\(\gamma\) enzyme-linked immunosorbent spot (ELISPOT) assays. Lethally irradiated mice underwent transplantation with BM with/without splenocytes with/without tumor vaccination (days 7 and 14 after HSCT). An additional control group consisted of nontransplanted mice given 2 weekly vaccinations with 4P-AGN2a cells. Five days after the last vaccination, spleens were harvested and CD8\(^+\) and CD8\(^+\) T cells purified by immunomagnetic sorting. The purified cells were then analyzed for the presence of tumor-reactive, IFN-\(\gamma\)-producing cells in ELISPOT assays. Results of these assays, plotted as the number of IFN-\(\gamma\) spots per \(10^5\) T cells, are shown in Figure 5. Mice given the combination of BM, spleen cells, and vaccination had significantly increased frequencies of IFN-\(\gamma\)-producing, tumor-specific CD8\(^+\) T cells compared with mice given BM and vaccination (no spleen cells), mice given BM and splenocytes without vaccination, or nontransplanted/vaccinated mice (\(P < .001\) or \(P < .01;\) Figure 5A). Post-HSCT vaccination without spleen cell adoptive transfer also had significantly increased CD8\(^+\) IFN-\(\gamma\) spot frequencies compared with cells from nonvaccinated mice (\(P < .01;\) Figure 5A). Relatively low CD8\(^+\) IFN-\(\gamma\) spot frequencies were observed in response to the control MHC-matched SaI tumor cells. The creation of MHC class II\(^+\) AGN2a cells by stable transfection of the human MHC class II transactivator (CIITA) gene allowed us to also assess tumor-reactive, IFN-\(\gamma\)-producing CD4\(^+\) T cells by ELISPOT. We found that CD4\(^+\) IFN-\(\gamma\) spot frequencies in response to AGN2a-CIITA cells were significantly higher in cells from mice given BM, spleen cells, and vaccination than in cells from all other groups of mice, including the nontransplanted/vaccinated group (\(P < .001;\) Figure 5B). Together, results of the ELISPOT assays confirmed the involvement of tumor-reactive CD4\(^+\) and CD8\(^+\) T cells in the post-transplantation induction of antitumor immunity by the 4P-AGN2a tumor vaccine.
Total spleen cell numbers and percentages of splenic T cell subsets were also analyzed to compare levels of T cell reconstitution between the experimental groups. Adoptive transfer of splenocytes with HSCT accelerated T cell reconstitution as reflected by significantly increased splenic CD4 and CD8 T cell numbers compared with mice given HSCT only (Table 1). Further, HSC transplant recipients given adoptive T cell transfer and vaccination had 10-20 times more tumor-reactive CD4 and CD8 T cells in their spleens than did vaccinated mice receiving only BM cells (data not shown).

Effects of Treg Inhibition in the HSC Transplant Recipients or Physical Depletion of Treg Cells from the Transferred Donor-Derived Lymphocytes

CD4+CD25+Foxp3+ regulatory T (Treg) cells have been found to be important suppressors of immune reactivity in different disease settings including cancer [19,20]. Experiments in our laboratory have found that a single treatment with anti-CD25 mAb before tumor vaccination augments the antitumor response to AGN2a (manuscript in press). Because the persistence/presence of CD4+CD25+Foxp3+ Treg cells could have a negative effect on our ability to effectively vaccinate mice soon after HSCT, we wanted to (a) examine whether these cells were able to survive lethal TBI or expand soon after HSCT from donor-derived cells and (b) test whether anti-CD25 mAb treatment before early post-HSCT tumor vaccination could enhance the vaccine-induced immune response.

First, we assessed the effect of lethal TBI and HSCT on host-derived, donor BM-derived, and donor spleen cell-derived CD4+ and CD8+ T cell numbers compared with mice given HSCT only (Table 1). Further, HSC transplant recipients given adoptive T cell transfer and vaccination had 10-20 times more tumor-reactive CD4 and CD8 T cells in their spleens than did vaccinated mice receiving only BM cells (data not shown).
the mice had been infused with $2 \times 10^7$ splenocytes, they remained lymphopenic until 3 wk after transplantation (Figure 6A). The lymphopenia was reflected by low splenic CD4$^+$ T cell numbers (Figure 6B). Only small numbers of CD4$^+$CD25$^+$ and CD4$^+$Foxp3$^+$ cells could be detected in the first 2 wk after HSCT, and these cells were primarily host-derived on day 3 after HSCT, but they switched to being mostly derived from the adoptively transferred T cells by day 7 (Figure 6C,D). Interestingly, residual host-derived CD4$^+$CD25$^+$ and CD4$^+$Foxp3$^+$ cells appeared to expand and/or accumulate in the spleen 3-5 wk after HSCT. BM-derived CD4$^+$CD25$^+$ and CD4$^+$Foxp3$^+$ cells could not be detected until 3 wk after HSCT, and they expanded in number, but still constituted only a minority of the total CD4$^+$CD25$^+$ and CD4$^+$Foxp3$^+$ cells at 5 wk. This implies that no single time point in the HSCT procedure can be assumed to be free of Treg influences. In normal nontransplanted mice, it has been shown that approximately 10% of splenic CD4$^+$ cells coexpress CD25 or Foxp3 [21,22]. We observed similar percentages in the spleens of BM donors or recipients (Figure 6E,F; day 0). During the 35-d period after HSCT, percentages of residual host CD4$^+$ cells that coexpressed CD25 or Foxp3 showed a biphasic increase. The adoptively transferred CD4$^+$ cells showed a gradual increase in CD25 expression over time, but they had a much more pronounced increase in Foxp3 expression that peaked at approximately 35% on day 21 after HSCT. These results indicated that, despite the state of profound lymphopenia during the first 2 wk after HSCT, increased percentages of splenic CD4$^+$ cells coexpressed CD25 and Foxp3, representing an apparent skewing toward increased percentages of Treg cells in the residual host and adoptively transferred T cells. To test if the CD4$^+$CD25$^+$ cells present in mice soon after HSCT were capable of exhibiting regulatory (or suppressive) function, CD25$^+$ cells were isolated by immunomagnetic sorting from normal mice (Figure 7; no HSCT) or from mice given HSCT 14 d earlier with syngeneic BM cells plus 2 x 10$^7$ added splenocytes (Figure 7; BM + S). When the purified CD25$^+$ cells were analyzed by flow cytometry, cells were >99% CD25$^+$, >96% of cells expressed CD4, and >92% of cells were Foxp3$^+$ (Figure 7A). In functional assays, CD25$^+$ from the mice given syngeneic HSCT (BM + S) suppressed the polyclonal proliferation of CD4$^+$CD25$^+$ responder cells as effectively as CD25$^+$ cells from normal mice (no HSCT; Figure 7B), indicating that the CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in mice given HSCT were functional.

Based on our observations, we hypothesized that treatment of HSC transplant recipients with anti-CD25 mAb before tumor vaccination would result in

![Figure 5.](image)

**Figure 5.** Vaccination with 4P-AGN2a cells soon after HSCT elicits tumor-reactive IFN-γ-producing CD4$^+$ and CD8$^+$ T cells. A/J mice underwent transplantation with 10$^7$ syngeneic BM cells plus $2 \times 10^7$ splenocytes (BM + S) or BM alone (BM – S). Mice were then vaccinated on days 7 and 14 after HSCT with CD54/80/86/137L-AGN2a cells (+ Vax) or not vaccinated (– Vax). A group of normal nontransplanted mice given 2 weekly tumor vaccinations (No HSCT + Vax) was also included in the experiments for comparison. Five days after the second vaccination, all mice were killed, splenic T cell subsets were isolated by immunomagnetic sorting, and CD8$^+$ (A) and CD4$^+$ (B) cells were assayed for tumor-reactive IFN-γ-secreting cell frequencies by ELISPOT. Data are from 1 of 2 replicate experiments. *P < .001 and †P < .01 versus BM + S + Vax; #P < .01.

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*Mice were transplanted with 10$^7$ syngeneic BM cells plus 2 x 10$^7$ splenocytes (BM + S) or BM alone (BM – S). Mice were then vaccinated on days 7 and 14 after HSCT with CD54/80/86/137L-AGN2a cells (+ Vax). A group of normal nontransplanted mice given 2 weekly tumor vaccinations (No HSCT + Vax) was included in the experiments for comparison. Five days after the second vaccination, spleens were collected and the absolute T cell numbers were assessed. Data for each group represents the mean ± SEM from 6-8 total mice (combined from 2 independent experiments). †P < .01, BM + S + Vax versus BM – S + Vax.
increased antitumor immunity. To test this hypothesis, lethally irradiated A/J mice were given HSCT with BM cells plus splenocytes. Some of the transplanted mice were then treated with 250 μg of anti-CD25 mAb 3 d before the first of 2 weekly vaccinations with CD54/80/86/137L-AGN2a cells. The goal of this strategy was to inhibit endogenous and infused CD25+ Treg cells. Seven days after the second tumor vaccination, the HSC transplant recipients were challenged with 10^6 (Figure 8A) or 5 × 10^6 (Figure 8B) live AGN2a cells. Tumor vaccination significantly protected mice from tumor challenge (all vaccinated groups versus nonvaccinated group; P < .001). In contrast to previous results in normal mice, where treatment with anti-CD25 mAb improved antitumor immunity (manuscript in press), anti-CD25 mAb treatment soon after HSCT diminished the tumor vaccine-induce immune response as indicated by de-
Figure 7. CD4^{+}CD25^{+} cells isolated from mice soon after HSCT exhibited regulatory function in vitro. CD4^{+}CD25^{+} T cells were isolated from spleens of normal mice (No HSCT) or from transplanted mice 14 d after HSCT with syngeneic BM cells plus 2 × 10^7 added splenocytes (BM + S). A, Purity of isolated cells was assessed by flow cytometry, where CD4, CD25, and Foxp3 expressions are shown in 2-color histograms. B, In vitro suppressor assays were used to assess regulatory cell function. Briefly, 5 × 10^4 CD4^{+}CD25^{+} responder T cells were cocultured with 1.25 × 10^4 anti-CD3/anti-CD28 mAb-coated Dynal beads plus the indicated ratios of purified CD4^{+}CD25^{+} cells. ^{3}H-thymidine incorporation is shown as mean counts per minute ± SD of triplicate wells.
creased survival (Figure 8A,B). Although the decreases in survival did not reach statistical significance, the results suggested that our hypothesis was incorrect and further suggested that the anti-CD25 mAb treatment might have inhibited CD25⁺ effector T cells generated in the vaccinated hosts. To address this possibility, phenotypic and functional studies were done on tissues of anti-CD25 mAb-treated and vaccinated mice.

Flow cytometric analysis of tissues (blood, lymph nodes, and spleen) from anti-CD25 mAb-treated mice 10 d after antibody treatment indicated that CD25 expression was significantly decreased on CD4⁺ cells (Table 2). Percentages of Foxp3⁺CD4⁺ T cells were also decreased in anti-CD25 mAb-treated mice compared with non-anti-CD25 treated mice, but to a lesser degree (Table 2). Results of IFN-γ ELISPOT assays indicated that early post-HSCT anti-CD25 mAb treatment significantly decreased the frequency of tumor-reactive CD4⁺ T cells (Figure 8C).

As an alternative strategy to address the possible influence of CD25⁺ Treg cells on early post-HSCT tumor vaccination, we physically depleted CD25⁺ cells from the adoptively transferred splenocytes using immunomagnetic sorting. This approach was based on results displayed in Figure 6C and 6D showing that most CD4⁺CD25⁺Foxp3⁺ cells soon after HSCT were derived from the adoptively transferred T cells. Immunomagnetic sorting eliminated >95% of the CD25⁺ T cells in the transferred population and approximately 70% of Foxp3⁺CD4⁺ T cells (data not shown). Lethally irradiated A/J mice were given HSCT with BM cells admixed with nondepleted splenocytes or splenocytes depleted of CD25⁺ cells. Fourteen days or 21 d after 2 weekly vaccinations with 4P-AGN2a cells, the HSC

![Figure 8. Treatment of HSC transplant recipients with anti-CD25 mAb before early post-transplantation tumor vaccination negatively influenced the antitumor response. A/J mice underwent transplantation with 10⁷ syngeneic BM cells plus 2 × 10⁷ splenocytes. Transplanted mice were vaccinated on days 7 and 14 after HSCT with irradiated 4P-AGN2a cells. Some recipient mice were treated with anti-CD25 mAb 3 d before the first vaccination. A group of nonvaccinated control mice was included. Mice were then challenged on day 21 after HSCT with (A) 10⁶ or (B) 5 × 10⁶ viable AGN2a cells. Survival data represent combined results of 2-3 separate experiments, including a total of 9-16 mice in each group. C, CD4⁺ T cells were isolated from vaccinated mice 7 d after the initial vaccination (day 14 after HSCT) and tested in ELISPOT assays to determine frequencies of IFN-γ-producing cells in response to MHC class II⁺ (AGN2a-CIITA) tumor cells. ELISPOT data are from 1 of 2 replicate experiments. *P < .05.](image-url)
transplant recipients were challenged with $5 \times 10^6$ live AGN2a cells. Recipients of CD25-depleted splenocytes had better survival than did recipients of nondepleted splenocytes (77% versus 50%; Figure 9), but the increased survival did not reach statistical significance ($P = .14$).

To determine the influence of CD25 depletion on CD25$^+$Foxp3$^+$CD4$^+$ cell reconstitution, percentages of CD25$^+$CD4$^+$ and Foxp3$^+$CD4$^+$ in blood, lymph nodes, and spleen were examined (Table 2). Two weeks after HSCT and adoptive transfer of splenocytes, both groups of mice (BM + S versus BM + S [CD25 depl]) contained similar percentages of Foxp3$^+$CD4$^+$ and CD25$^+$CD4$^+$ T cells in the blood and lymph nodes, but decreased percentages of Foxp3$^+$CD4$^+$ and CD25$^+$CD4$^+$ T cells were detected in the spleens of mice given CD25-depleted splenocytes. These results suggested that CD25$^+$Foxp3$^+$CD4$^+$ cells in the transferred splenocytes expanded and upregulated expression of CD25. This also indicates that optimization of Treg cell depletion from a transferred T cell population awaits the development of cell surface markers others than CD25.

### DISCUSSION

Adoptive cellular therapy in the context of HSCT has the potential to boost the effectiveness of cell-based cancer vaccines. In this study, we tested whether vaccination with genetically modified neuroblastoma cells could generate anti-neuroblastoma immunity when administered early (ie, during the first 2 wk) after lethal irradiation and syngeneic HSCT. We discovered that potent antitumor immunity could be generated early after HSCT; however, the transplant recipients also required an adoptive transfer of T cells for the vaccine to be effective.

Tumor vaccination 3–6 wk after HSCT in mice had been shown by others to induce a superior antitumor immune response compared with vaccination of nontransplanted animals [15]. In contrast, immediate post-transplantation vaccination (1-2 wk after HSCT) in those experiments showed no significant tumor rejection due to a state of profound immune deficiency. Another report showed that antitumor immune responses could be elicited by a tumor lysate-pulsed dendritic cell vaccine initiated on day 7 after BMT without adoptive transfusion of T cells [17]. However, success of the vaccine depended on the presence of mature T cells in the transplanted BM and complete tumor elimination required 3 weekly vacci-

### Table 2. Treg Cell Reconstitution after HSCT

<table>
<thead>
<tr>
<th>Treg Subset</th>
<th>Tissue</th>
<th>BM + S</th>
<th>BM + S (CD25 depl)</th>
<th>BM + S + anti-CD25 mAb</th>
<th>Normal controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD25$^+$ CD4$^+$</td>
<td>Blood</td>
<td>42.9 ± 4.1</td>
<td>40.7 ± 4.7</td>
<td>1.4 ± 1.2</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>22.0 ± 1.4</td>
<td>22.0 ± 1.7</td>
<td>8.4 ± 3.4</td>
<td>12.5 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>44.0 ± 1.1</td>
<td>38.4 ± 1.3</td>
<td>1.2 ± 0.2</td>
<td>13.5 ± 1.4</td>
</tr>
<tr>
<td>Foxp3$^+$ CD4$^+$</td>
<td>Blood</td>
<td>43.0 ± 5.4</td>
<td>40.0 ± 6.5</td>
<td>36.7 ± 4.0</td>
<td>7.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>LN</td>
<td>21.6 ± 0.9</td>
<td>20.3 ± 0.6</td>
<td>17.6 ± 0.3</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Spleen</td>
<td>43.0 ± 1.0</td>
<td>35.2 ± 0.8</td>
<td>33.0 ± 0.1</td>
<td>13.4 ± 0.9</td>
</tr>
</tbody>
</table>

LN indicates lymph node.

*Mice were transplanted with $10^7$ syngeneic BM cells plus $2 \times 10^7$ splenocytes (BM + S) or BM plus CD25-depleted splenocytes (BM + S [CD25 depl]). Some mice transplanted with BM cells and splenocytes were treated with 250 μg anti-CD25 mAb 4 d after HSCT (BM + S + anti-CD25 mAb). Normal nontransplanted mice were included as a control group. Tissue samples were collected 14 d after HSCT. Data for each group represent mean percentages of CD4$^+$ cells that coexpressed CD25 or Foxp3 ± SEM; 6-8 mice were analyzed for each group (combined from 2 independent experiments).

$P < .05$; $4P < .01$, BM + S versus BM + S (CD25 depl).

$§P < .05$; $\|P < .01$, BM + S versus BM + S + anti-CD25 mAb.

![Figure 9](image-url)
nations. Our results show that adoptive transfer of syngeneic T cells plus tumor vaccination facilitates the generation of a protective antitumor response (Figure 1). The adoptively transferred T cells accelerated T cell reconstitution (Table 1), and the frequencies of tumor-reactive CD4+ and CD8+ T cells in the spleens of transplanted/vaccinated mice were significantly increased compared with nontransplanted mice (Figure 5), suggesting that the immune response was more effectively driven toward tumor reactivity by early post-transplantation vaccination. Similar to our results in nontransplanted mice [7], vaccination with tumor cells that had been nucleofected with “empty” plasmid vectors also protected mice from lower tumor challenge doses (Figure 1A,B). Nucleofection with empty vectors did not induce any change in MHC class I or II, CD54, CD80, CD86, or CD137L molecule expression on the tumor cell surface (data not shown). One explanation for the increased tumor immunity is that the bacterial plasmid backbones contain immunostimulatory CpG motifs. It has been shown that 1 plasmid vector used in our experiments (pCDNA3 vector) contains immunostimulatory CpG motifs [23], and CpG motifs have been previously shown by investigators to facilitate the activation of innate and acquired immune responses toward tumors [24]. As previously documented, the nucleofection process results in the immediate introduction of thousands of plasmid DNA copies into each cell [7], making this explanation plausible.

Our results are clinically relevant because autologous HSCT in patients is similarly accompanied by a period of pronounced immune deficiency. After autologous HSCT, patients typically have low lymphocyte counts and impaired T cell immune function during the first 3 mo after transplantation [10], and attempts to vaccinate soon after transplantation are likely hampered due to the immune deficient state. Early post-transplantation immune deficiency may also contribute to increased risk of relapse from minimal residual disease. Retrospective studies have suggested that superior clinical survival may be associated with more rapid lymphocyte recovery after transplantation. The rate of immune reconstitution has been shown to be an independent marker of time to progression in patients with advanced ovarian cancer after PBSCT [25]. A recently published randomized clinical study showed that a single early post-transplantation infusion of vaccine-primed and costimulated autologous T cells in conjunction with immunization improves post-transplantation immunodeficiency and can facilitate the generation of pneumococcal immunity [26]. The results of these 2 studies suggest that a rapid post-transplantation recovery of the immune system in a situation of minimal residual disease will be a prerequisite for effective antitumor immunotherapy. Further, they demonstrate the feasibility of T cell adoptive transfer at the time of or soon after HSCT.

Immune tolerance is a major barrier to effective immunization against tumor, and tumor establishment has been associated with tolerance to tumor antigens [27,28]. Our results demonstrated that immune cells from mice with established tumors were capable of facilitating vaccine-induced neuroblastoma immunity soon after HSCT (Figure 3). The splenocytes used for adoptive transfusion were collected from mice with 100 mm² 12-d AGN2a tumors. Although it has been shown in other mouse cancer models that tumor progression can be accompanied by increased frequencies of Treg cells in peripheral lymphoid tissues [29,30], we detected no significant increase in CD25+Foxp3+CD4+ Treg cells in the spleens of mice bearing 12-d AGN2a tumors. Further, we were unable to detect increased percentages of CD25+Foxp3+CD4+ Treg cells in mice with larger AGN2a tumors (~250 mm²) (unpublished data). Although our data suggest that tumor-reactive T cells in mice with established neuroblastoma are not eliminated or irreversibly tolerated in the peripheral lymphoid tissues, we cannot rule out the possibility that results would be different in mice bearing even larger tumor burdens. We currently hypothesize that immunization with our cell-based vaccine during the process of homeostatic expansion soon after HSCT facilitates activation and proliferation of low-affinity tumor-reactive T cells [31-35] and that this allows “tolerized” T cells to react against tumor-self antigens. These data have important implications as we tailor our future studies toward a model of established neuroblastoma, where we plan to test treatment of tumor-bearing mice with the combination of high-dose therapy, HSCT, adoptive immunotherapy (with cells from tumor-bearing donors), and tumor vaccination.

CD8 and CD4 T cells are involved in the effector phase of the vaccine-induced immune response soon after HSCT (Figure 4B). This was surprising because the AGN2a tumor cells do not express MHC class II molecules. We speculate that the involvement of CD4+ T cells in the effector response could be (a) by providing helper activity to aid in the prompt activation of CD8+ cytotoxic effector cells or tissue macrophages or (b) by directly serving as effector cells through tumoricidal cytokine production. Mechanisms that have been previously implicated in CD4 antitumor effector function include secretion of IFN-γ, which has been shown to mediate tumor rejection by its antitumor and antiangiogenic properties [36,37], or activation of macrophages capable of suppressing tumor growth [38,39].

Animal data from Antony et al [40] suggested that smaller numbers of Treg cells are in part responsible for the enhanced efficacy of tumor immunotherapy in
lymphodepleted hosts. It has been shown that depletion or inhibition of CD4+CD25+ cells by anti-CD25 mAb administration can enhance tumor rejection [41-43], and that tumor vaccination combined with Treg cell depletion/inhibition can increase vaccine efficacy [44,45]. Recently, we found that anti-CD25 mAb treatment can improve vaccine-induced antitumor responses to AGN2a (manuscript in press). In the present report, we investigated the effect of lethal TBI, HSCT, and adoptive T cell transfer (splenocytes) on CD4+CD25+ and CD4+Foxp3+ cells of host and donor origin. As far as we are aware, this is the first time this type of detailed study has been done in mice soon after HSCT. During the period of T cell lymphopenia soon after HSCT, increased percentages of splenic CD4+CD25+Foxp3+ cells capable of eliciting functional Treg activity (Figure 7) were observed in mice transplanted with BM and splenocytes, and most of these cells appeared to be derived from the transferred T cells (Figure 6). However, anti-neuroblastoma immunity could still be efficiently induced soon after HSCT. Due to the increased frequency of CD4+CD25+Foxp3+ cells, we wished to determine if inhibition of residual Treg cells could further influence vaccine efficacy. To do this, we tried 2 different strategies. First, we treated HSC recipients with anti-CD25 mAb before the initial tumor vaccination because this same strategy had increased vaccine-induced immunity in normal mice (unpublished observations). In the second approach, CD25-positive cells in the adoptively transferred splenocytes were physically depleted before infusion. Treatment of HSC recipients with anti-CD25 mAb negatively influenced vaccine-induced antitumor immunity (Figure 8A,B), suggesting that the antibody treatment inhibited effector cell function in this setting. To support this conclusion, IFN-γ ELISPOT analysis demonstrated that in vivo anti-CD25 mAb treatment resulted in decreased numbers of tumor-reactive CD4+ T cells in vaccinated HSC recipients (Figure 8C). It is unclear why prevaccination anti-CD25 treatment inhibits immune effector cells in the early post-HSCT setting but enhances tumor reactivity in the normal setting. Perhaps the difference is related to increased expression of IL-2 receptors on T cells in a lymphopenic environment [46].

Different results were seen in experiments testing the second strategy because infusion of CD25-depleted splenocytes increased survival of tumor-vaccinated mice (Figure 9). Although depletion of the CD25-positive cells from adoptively transferred cells did not influence the percentages of Foxp3+CD4+ cells in the peripheral blood or lymph nodes 14 d after HSCT, percentages of Foxp3+CD4+ cells were significantly decreased in the spleen (Table 2). Unfortunately, we did not evaluate the tissues at earlier time points, and we may have missed more profound differences early on that could influence tumor vaccination. As a cautionary note, the increased survival of mice receiving CD25-depleted splenocytes (27% increase over mice given nondepleted splenocytes) was not statistically significant. Because approximately 30% of Foxp3+CD4+ cells do not coexpress CD25, it is possible that elimination of these cells and CD25+Foxp3+CD4+ cells from the adoptively transferred T cells by some other means would further enhance tumor immunity. More specific Treg cell reagents are clearly needed to better target these cells for elimination/inhibition.

In summary, our results demonstrate that anti-neuroblastoma immunity can be induced soon after HSCT by tumor vaccination, but sufficient numbers of T cells must be included in the graft to achieve protective antitumor immunity. CD4+ and CD8+ T cells are involved in the induction and effector phases of the vaccine-induced immune response, and early post-HSCT vaccination resulted in increased frequencies of tumor-reactive T cells compared with non-transplanted mice that had been vaccinated. Although percentages of Foxp3+CD25+CD4+ T cells were increased soon after HSCT, protective tumor immunity could still be induced. However, elimination of CD25+ cells from splenocytes (as a source of T cells) added to the graft appears to enhance vaccine efficacy. These results provide experimental data in support of a multifaceted clinical approach for the treatment of neuroblastoma combining high-dose therapy, autologous HSCT, adoptive immunotherapy (T cell add-back), and post-transplantation tumor vaccination.

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