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*Published online 4 November 2009
http://www.jimmunol.org/content/early/2009/11/04/jimmunol1.0900159*
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Vaccination with tumor Ags has not been an effective treatment for solid tumors. The goal of the current study was to determine whether a combination of vaccination and hematopoietic cell transplantation (HCT) can effectively treat primary, disseminated, or metastatic CT26 and MC38 murine colon tumors. Vaccination of tumor-bearing mice with irradiated tumor cells and CpG adjuvant failed to alter progressive tumor growth. However, mice bearing primary, disseminated lung, or metastatic liver tumors were uniformly cured after administration of total body irradiation, followed by the transplantation of hematopoietic progenitor cells and T cells from syngeneic, but not allogeneic vaccinated donors. Requirements for effective treatment of tumors included irradiation of hosts, vaccination of donors with both tumor cells and CpG, transfer of both CD4+ and CD8+ T cells along with progenitor cells, and ability of donor cells to produce IFN-γ. Irradiation markedly increased the infiltration of donor T cells into the tumors, and the combined irradiation and HCT altered the balance of tumor-infiltrating cells to favor CD8+ effector memory T cells as compared with CD4+CD25FoxP3+ T regulatory cells. The combination of vaccination and autologous hematopoietic cell transplantation was also effective in treating tumors. In conclusion, these findings show that otherwise ineffective vaccination to solid nonhematologic tumors can be dramatically enhanced by HCT. The Journal of Immunology, 2009, 183: 0000 – 0000.

Despite the potency and specificity of the immune system, vaccination with tumor Ags generally fails to eradicate cancer in mice and humans (1, 2). Currently, the most successful form of immunotherapy is adoptive cell therapy, which includes ex vivo activation of tumor-infiltrating lymphocytes (TILs)3 and reinfusion of these cells along with high doses of cytokines. This approach is limited by cytokine toxicity and by the limited range of tumors from which sufficient TILs can be obtained (melanoma) (3). We hypothesized that the adoptive transfer of T cells from mice vaccinated against tumor Ags into tumor-bearing mice given total body irradiation (TBI) would result in marked expansion of the T cells, and their subsequent infiltration and eradication of tumors.

The results of the current study show that mice that have developed disseminated tumors or bulky primary tumors established for 2 wk following inoculation with the CT26 colon carcinoma cells can be cured, when treated with a combination of tumor vaccination and hematopoietic cell transplantation (HCT), without ex vivo T cell activation or use of TILs. Prior attempts at effective treatment by vaccination of mice with unmodified CT26 cells have failed due presumably to the low immunogenicity of this tumor (4). Several strategies have been used to overcome this problem, including vaccination with GM-CSF-transfected CT26 cells, as well as with altered ligands with heteroclitic activity (4–7). Surprisingly, by combining vaccination with HCT, we reproducibly induced eradication of unmodified CT26 tumor cells that was dependent on the transfer of CD4+ and CD8+ T cells, and the ability of the transferred cells to produce IFN-γ.

Materials and Methods

Animals

Wild-type male BALB/c (H-2b) mice, male BALB/c RAG2−/− mice, wild-type male DBA2/J (H-2b) mice, and wild-type female C57BL/6 mice were purchased from The Jackson Laboratory. Mice were 5–8 wk old. The Stanford University Committee on Animal Welfare (Administration Panel of Laboratory Animal Care) approved all mouse protocols used in this study.

Cell lines

The CT26 cell line (an N-nitro-N-methylurethane-induced BALB/c murine colon carcinoma) was purchased from ATCC. The MC38 cell line (diethyl-hydrazine-induced C57BL/6 colon adenocarcinoma) was provided by D. Bartlett (University of Pittsburgh, Pittsburgh, PA) (8). Cell lines were maintained in RPMI 1640 complete medium supplemented with 10% FCS, l-glutamine, 2-ME, streptomycin, and penicillin. For intrasplenic injection, animals were anesthetized with ketamine/xylazine. Laparotomy was performed, and 5 × 10⁶ CT26 cells were injected in the spleen. Abdominal wall was closed with surgical sutures.
Vaccination

Five-week-old male BALB/c mice were immunized by s.c. injection of 1 × 10⁶ irradiated (10,000 cGy) CT26 cells and 30 μg of CpG. Five-week-old female C57BL/6 mice were immunized by s.c. injection of 1 × 10⁹ irradiated (10,000 cGy) MC38 cells and 30 μg of CpG. AH-1 peptide (30 μg per vaccination) used in this study was obtained from Sigma-Genosys. The peptide was >95% pure, as indicated by analytical HPLC. Lyophilized peptide was diluted in DMSO and stored at −20°C until use. Oligonucleotide containing unmethylated CG motifs (CpG; TCCATGACGTTCCTGACGT) was synthesized and phosphorothioate stabilized by Oligos Etc. The oligonucleotide was reconstituted in sterile pyrogen-free water and then diluted in PBS for in vivo injections. A total of 30 μg of ultrapure LPS (InvivoGen) was used in some experiments instead of CpG.

Irradiation

The irradiation was performed with a Philips x-ray unit (200 kV, 10 mA; Philips Electronic Instruments) at a rate of 84 cGy/min with a 0.5-mm Cu filter.

Donor cell preparation

Single-cell suspensions of bone marrow and spleen were prepared according to previously described procedures (9). Some samples were enriched either for CD4⁺ cells, CD8⁺ T cells or Thy1.2⁻ cells with anti-CD4, anti-CD8 magnetic microbeads (Miltenyi Biotec) or anti-Thy1.2-biotin mAbs (5a-8; Caltag Laboratories), and streptavidin-magnetic beads (Miltenyi Biotec), respectively, using the MidiMACS system (Miltenyi Biotec). Enriched cells were stained with anti-TCR allophycocyanin and anti-CD4 or anti-CD8 FITC mAbs to check for purity, and preparations were uniformly at least 95% pure.

Purified hematopoietic stem cells (HSCs) were obtained by modification of the methods described by Spangrude et al. (10). Thy-1⁻/low Lin⁻ c-Kit⁻/low Sca-1⁻ cells were sorted on a dual laser FACS (BD Biosciences) made available through the FACS shared-user group at Stanford University using FlowJo software (Tree Star) for data analysis. After sorting, cells were checked by FACS reanalysis and determined to be >99% pure.

Histopathology

Animals were killed when moribund as per Stanford Animal Welfare protocol guidelines, or at 100 days after transplantation if they survived without morbidity. Histopathological specimens were obtained from lungs and livers of hosts. Tissues were fixed in 10% formalin and stained with H&E, and images were obtained using an Eclipse E1000M microscope (Nikon), as described before (11).

Analysis of donor cell accumulation in host spleens and tumor nodules

Single-cell suspensions were prepared from spleens and tumor nodules of BALB/c mice. The recipients. The following reagents were used for flow cytometric analysis: unconjugated anti-CD16/32 (2.4G2; BD Biosciences), anti-CD4 FITC (RM4-5; BD Biosciences), anti-TCR allophycocyanin (H57-597; BD Biosciences), anti-CD8 allophycocyanin-Cy7 (53-6.7; BD Biosciences), anti-Thy1.1 PE-Cy7 (HIS51; eBioscience), anti-Thy1.2 biotin (5a-8; Caltag Laboratories) mAbs, and streptavidin-PE (BD Biosciences). All stainings were performed in PBS/1% calf serum in the presence of purified anti-CD16/32 mAbs.

Statistical analysis

Kaplan-Meier survival curves were generated using Prism software (SAS Institute), and statistical differences were analyzed using the log rank (Mantel-Cox) test. Statistical significance in differences between mean percentage of donor cells in host spleens and tumors was analyzed using two-tailed Student’s t test of means. For all tests, p < 0.05 was considered significant.

Results

Syngeneic HCT from vaccinated BALB/c donor mice can cure established CT26 colon tumors

Fig. 1A shows our experimental scheme, which uses HCT from tumor-vaccinated donors to treat CT26 colon tumors in syngeneic mice. In all instances, normal BALB/c donor mice were vaccinated s.c. with 10⁶ irradiated CT26 tumor cells mixed with 30 μg of CpG, an adjuvant that stimulates APCs via TLR-9 (12, 13). After 90 days, spleen and bone marrow cells were harvested, and transplanted i.v. into tumor-bearing BALB/c host mice following a single dose of TBI. Seven days before TBI, hosts had been given live tumor cells via s.c. (2.5 × 10⁶), i.v. (2 × 10⁵), or intraperitoneal (5 × 10⁵) routes. Fig. 1B shows the progressive growth of s.c. tumors in all untreated mice. Similarly, tumor-bearing recipients of 50 × 10⁶ bone marrow cells and 60 × 10⁶ spleen cells from unvaccinated donors had uniformly progressive tumors (Fig. 1C). In contrast, after HCT from vaccinated donors, tumor-bearing mice displayed a steady regression of tumor volume over a 100-day observation period (Fig. 1D), which remained stable until the end of the study (day 180; data not shown). Shortening the time interval between immunization of the donor and harvesting the graft from 90 to 14 days, but not to 50 days, resulted in lower antitumor effect (p = 0.005 and p = 0.3, respectively; log rank test; Fig. 1E). Omis- sion of CpG from the donor vaccine resulted in a further loss of efficacy (p = 0.01), and only 20% of hosts survived 100 days.

The same HCT strategy was also successful in recipients given tumor cells by i.v. administration. By day 7, tumor cells had disseminated into the lungs and formed multiple tumor clusters (Fig. 1H). By day 20, all untreated control mice succumbed to progressive disease with large, nearly confluent tumor nodules (Fig. 1, F and H). In contrast, recipients of HCT from vaccinated donors all survived at least 100 days, with no histologic evidence of residual tumor (Fig. 1H). Accordingly, improvement of survival was significant as compared with untreated mice (p < 0.01; Fig. 1F). When tumor cells were injected into the spleen, by day 7 tumor nodules became established in the parenchyma of the liver (Fig. 1I), and by day 14 there was evidence of blood vessel invasion (arrows, Fig. 1I). All untreated animals died by day 30 (Fig. 1G) with multiple visible, as well as microscopic, tumors. Treated mice survived beyond day 100 (Fig. 1G), easily exceeding the survival of untreated mice (p = 0.001). The liver of treated mice displayed no abnormalities and also no histologic evidence of residual tumor at day 60 (Fig. 1I). HCT from vaccinated donors also cured peritoneal carcinomatosis, which had been created by i.p. injection of 5 × 10⁶ tumor cells and which displayed multiple peritoneal nodules and ascites by the time of transplant (data not shown). All untreated mice died by day 20, and all transplanted mice survived at least 100 days without any peritoneal tumor growth.

Vaccination and HCT induce long-term antitumor immunity

To assess the duration of effect of vaccination combined with HCT, we challenged cured animals from the experiment in Fig. 1D with 2.5 × 10⁴ live tumor cells at day 120, as shown in the experimental scheme in Fig. 2A. The results show that these animals were completely protected and survived for at least 100 days (Fig. 2A). Moreover, harvesting of spleen and bone marrow cells from cured recipients at day 180 after HCT, and secondary transfer resulted in 80% of the new recipients surviving for more than 100 days (Fig. 2B). At least 100 days later, those secondary recipients were used as donors for another HCT into irradiated tumor-bearing tertiary hosts, which was also effective because all tertiary hosts survived more than 100 days (Fig. 2B). Thus, antitumor immunity generated by a single vaccination could eradicate tumors 370 days later (Fig. 2B).
Tumor eradication requires lethal irradiation of hosts, as well as transfer of CD4+ and CD8+ T cells from vaccinated donors. Fig. 3A illustrates the significant role of the host-conditioning regimen in our vaccine strategy. All tumor-bearing recipients of bone marrow and splenocytes from vaccinated donors were cured when conditioned with myeloablative TBI (800 cGy). In contrast, only 60% of hosts survived 100 days with a nonmyeloablative radiation dose (450 cGy; p < 0.05), whereas none survived more than 40 days without irradiation (p < 0.0001). Radiation causes lymphodepletion that might deplete host Treg cells that suppress antitumor immune responses. To test whether radiation mediates tumor eradication through such a mechanism, we studied unirradiated tumor-bearing RAG2−/− recipients that lack T cells. RAG2−/− BALB/c hosts were given myeloablative radiation or no radiation immediately before transplantation of cells from vaccinated donors. Fig. 3B shows that although there was a significant delay in mortality in nonirradiated RAG2−/− mice as compared with nonirradiated wild-type mice (p = 0.001), all mice died by day 65. Conditioning of the RAG2−/− mice with 800 cGy resulted in significant improvement in survival as compared with the nonirradiated mice (p = 0.001), and all hosts survived at least 100 days (Fig. 3B).

When IFN-γ−/− BALB/c mice were vaccinated and used as bone marrow and splenocyte donors, the survival of hosts was decreased as compared with wild-type syngeneic donors (p = 0.004; Fig. 3C). Likewise, survival was reduced in all five mice given grafts from vaccinated MHC-matched, minor Ag-mismatched DBA/2J donors (H-2d; p < 0.001; Fig. 3C). Although four of five animals had progressive tumor growth, one mouse displayed tumor regression, but succumbed due to graft-vs-host disease (GVHD). Substitution of whole tumor cells with the tumor-associated immunodominant AH-1 peptide (4) for vaccination of donors resulted in decreased survival (p < 0.0001; Fig. 3C). Use of LPS (30 μg) as an adjuvant for vaccination was just as effective as CpG, based on survival of hosts post-HCT (p = 1.0; Fig. 3C). Grafts from vaccinated donors consisting of bone marrow or FACS-purified c-kit+ Sca1high HSCs failed to prevent tumor progression (Fig. 3D). Addition of 5 × 10⁶ splenic T cells to the HSCs increased survival (p = 0.008) such that the majority of hosts were alive at 100 days without detectable tumors (Fig. 3D). When HSC grafts were supplemented with both CD4+ (3.5 × 10⁶) plus CD8+ T cells (1.8 × 10⁶), survival was improved (p < 0.008) as compared with supplementation with CD4+ or CD8+ T cells only (Fig. 3E).

The utility of HCT from vaccinated donors was further validated in studies of another colon cancer, MC38, which grows only in C57BL/6 (H-2b) mice (8). For these experiments, donor mice were vaccinated with 1 × 10⁶ MC38 tumor cells (Fig. 3F). Again, whereas syngeneic recipients of grafts from vaccinated donors were cured, recipients of transplants from unvaccinated donors did...
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not survive beyond 35 days ($p < 0.0001$). HCT from tumor-vaccinated donors could also significantly improve survival of animals with large (>10-mm) tumors established for 15 days ($p = 0.0005$; Fig. 3G). After 100 days, 60% of treated animals were completely tumor free.

Analysis of donor T cells in host tumors and spleens after transplantation: irradiation promotes T cell accumulation in tumors

To delineate the donor-derived cell populations involved in the antitumor response, we transplanted bone marrow and spleen cells from BALB/c Thy1.1 donors into tumor-bearing BALB/c Thy1.2 hosts, as depicted in Fig. 4A. To assure that there would be sufficient donor cells for analysis at day 28, HCT was performed in animals bearing tumors that had been established for 14 instead of 7 days. In irradiated recipients of vaccinated donor grafts, ~70% of T cells infiltrating the tumors were of donor origin (Fig. 4B and C), whereas donor T cells accounted for <2% of tumoral T cells when hosts were not irradiated ($p < 0.0001$). A similar facilitation of donor cell accumulation in the spleen was observed in irradiated vs nonirradiated hosts ($p < 0.01$; Fig. 4B and C). Differences in accumulation of total T cells from vaccinated and unvaccinated donors were not significant ($p > 0.05$). However, the majority of tumor T cells from vaccinated donors were CD8+ (p = 0.01; Fig. 4D and E). CD4+ T cells were in the majority in the spleens with both vaccinated and unvaccinated donors ($p < 0.0001$).

HCT alters the balance between regulatory and effector cells at the tumor site

Previous studies have shown that CD4+CD25+FoxP3+ Treg cells can suppress tumor immunity (14). Moreover, this suppression was mediated at the tumor site and was lost after intratumoral depletion of Treg cells (14). We showed above (Fig. 3B) that conditioning and HCT were required to cure tumors in RAG2−/− mice lacking Treg cells. Thus, the requirement for irradiation is not based on host Treg depletion.

However, Treg cells of donor or host origin may be capable of infiltrating tumors when wild-type hosts are used. We examined
Tumor vaccination becomes effective when combined with HCT, and vaccine-induced antitumor immunity is not prevented by the presence of growing tumors

FIGURE 4. Donor T cells accumulate in host tumors after transplantation. A, Experimental scheme. B, Thy 1.1 and Thy 2.2 analysis of single-cell suspensions of the tumors and spleens obtained on day 28 from tumor-bearing hosts given unvaccinated or vaccinated donor transplants with or without TBI. C, Mean percentages and SE of tumor-infiltrating Thy1.1 cells at day 28 in controls without TBI, as shown in the experimental scheme in Fig. 5A. Experimental mice were lethally irradiated and given HCT and vaccine-induced antitumor immunity is not prevented by the presence of growing tumors

Fig. 6A shows the experimental scheme used to determine the effect of vaccination alone on survival of tumor-bearing mice. Fig. 6B shows that the survival of vaccinated, but not HCT-treated, animals with 7-day tumors did not improve as compared with unvaccinated tumor-bearing animals, and all animals died by day 40. Moreover, when vaccinated nontumor-bearing animals were challenged with as few as 2.5 \times 10^6 CT26 cells 16 and 50 days after vaccination (Fig. 6C), only 20% of mice survived 100 days (Fig. 6D). Some degree of protection developed in animals vaccinated given the tumor challenge 90 days after vaccination, as indicated by the observation that 50% of animals remained tumor free (\( p = 0.03; \) Fig. 6D). Next, to assess the potential effect of larger tumors on the response to vaccination, we vaccinated mice with tumors growing for 7 days and then waited 14 additional days before resecting the growing tumors at day 21 (Fig. 6E). Bone marrow and splenocytes were harvested from donors on day 110 and transferred into lethally irradiated tumor-bearing hosts. All hosts survived with complete tumor regression for at least 100 days (Fig. 6F). Only 20% of hosts given transplants from unvaccinated donors survived 100 days, and the difference using vaccinated vs unvaccinated donors was significant (\( p < 0.05; \) Fig. 6F). Thus, HCT from vaccinated animals into syngeneic tumor-bearing hosts resulted in cure of tumors, indicating that growing tumors in donors do not prevent the development of potent antitumor effector cells in adoptive hosts.
Autologous HCT enhances tumor immunity after vaccination

A model of autologous HCT was studied, as shown in Fig. 6G. In this scheme, a group of donors was vaccinated 7 days after live tumor cell injection, splenectomized 7 days later, and conditioned with TBI immediately after recovery from surgery, and spleen cells were injected i.v. within 6 h after TBI. Bone marrow cells were not required for rescue of these myeloablated hosts, because mouse spleen cells contain both immune cells and HSCs. Donors that received the autologous transplants had significantly improved survival as compared with those without transplants, and ~40% survived at least 100 days with complete tumor regression (p < 0.001; Fig. 6F). Thus, large 14 day tumors were either cured or their growth significantly delayed after autologous HCT.

Discussion

These data show for the first time that it is possible to eradicate primary, metastatic, or disseminated solid tumors by treating tumor-bearing hosts with HCT containing sensitized T cells from vaccinated donors. Although there is growing evidence that hematologic cancers can be effectively treated with a combination of tumor vaccination and HCT (15, 16), the effect of such treatment on solid tumors has not been tested. Our outcome measure for tumor immunity in the current study was eradication of the CT26 or MC38 colon tumors. Investigating the specific tumor Ag epitopes recognized by sensitized T cells after whole tumor cell vaccination is the subject of our continuing studies.

Allogeneic HCT from tumor Ag-vaccinated donors was not effective in our model, although this approach has been reported to induce complete remissions of primary melanoma tumors or metastatic breast tumors in mice (17, 18). An important limitation of allogeneic HCT is the development of GVHD, which occurs in a severe form in ~30–50% of humans who receive this therapy (19–21). GVHD is likely to be aggravated by vaccinating donors with tumors expressing host alloantigens. By combining tumor vaccination of the donor with syngeneic HCT for the treatment of primary and metastatic colon cancer in mice, we not only avoided GVHD, but also achieved a potent and durable antitumor response. The results demonstrate a powerful synergy between tumor vaccination and HCT in two genetically distinct mouse strains with different colon tumors. A similar synergy has been reported in mice with primary melanoma treated with ex vivo expanded, T cell Ag receptor transgenic CD8+ T cells specific to a tumor Ag in combination with syngeneic HCT (22, 23).

The latter approach required infusion of IL-2 to facilitate the expansion and persistence of the T cells, and was not used for metastatic tumors (22, 23). In our model, a robust antitumor immune response could be transferred to tumor-bearing mice without ex vivo T cell expansion or treatment of the mice with cytokines. The finding that CD4+ and CD8+ T cells needed to be included in the transplant to achieve cures indicates that effective vaccination requires epitopes recognized by both types of T cells. Such epitopes were lacking in a vaccine consisting of the immunodominant AH-1 peptide and CpG, which may explain why this vaccine was ineffective, in contrast to vaccines containing whole tumor cells, which are a source of multiple CD4 and CD8 epitopes. CD4+ T cells provide help to memory CD8+ T cells by enhancing their immune potency, expansion, and persistence after exposure to Ag (24). The efficacy of the transferred donor cells in the current study required that they produce IFN-γ, because cells obtained from IFN-γ−/− donors lost potency.

Irradiation of tumor-bearing hosts was also required for tumor cures, and markedly augmented the expansion of transplanted T
cells in the spleen and their infiltration into tumors. Because lethal irradiation was considerably more effective than sublethal irradiation, HSCs had to be included in the transplants to rescue hosts and their infiltration into tumors. Because lethal irradiation combined with HCT is highly effective, even in the presence of T cell subsets infiltrating the tumors rather than killing, the balance of the subsets was determined in tumor-bearing mice with or without HCT. Mice given irradiation and HCT had a 10-fold higher ratio of CD8+ effector memory T cells:Treg cells in the tumors as compared with control mice without HCT. Thus, the HCT procedure not only increases the absolute number of T cells that infiltrated the tumors, but also favors the T cell subsets that kill tumor cells vs the subset that suppresses tumor immunity.

Tumor vaccination without HCT was not effective against established tumors. However, vaccination of tumor-bearing animals provided long-term, transferrable immunity, which could be enhanced by HCT. These data suggest that patients whose primary tumors are resected, but remain at high risk for relapse, might benefit from early vaccination combined with HCT in the event of relapse.

Acknowledgments
We thank G. Fisher for advice and G. Letsinger for help with manuscript preparation. We acknowledge Dr. J. Slansky form the University of Colorado for providing Ld-AH-1 tetramers.

Disclosures
The authors have no financial conflict of interest.

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