Tumor-Specific Inhibition of In Situ Vaccination by Distant Untreated Tumor Sites

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Abstract

In situ vaccination is an emerging cancer treatment strategy that uses local therapies to stimulate a systemic antitumor immune response. We previously reported an in situ vaccination effect when combining radiation (RT) with intratumor (IT) injection of tumor-specific immunocytokine (IC), a fusion of tumor-specific antibody and IL2 cytokine. In mice bearing two tumors, we initially hypothesized that delivering RT plus IT-IC to the “primary” tumor would induce a systemic antitumor response causing regression of the “secondary” tumor. To test this, mice bearing one or two syngeneic murine tumors of B78 melanoma and/or Panc02 pancreatic cancer were treated with combined external beam RT and IT-IC to the designated “primary” tumor only. Primary and secondary tumor response as well as animal survival were monitored. Immunohistochemistry and quantitative real-time PCR were used to quantify tumor infiltration with regulatory T cells (Treg). Transgenic "DEREG" mice or IgG2a anti-CTLA-4 were used to transiently deplete tumor Tregs. Contrary to our initial hypothesis, we observed that the presence of an untreated secondary tumor antagonized the therapeutic effect of RT + IT-IC delivered to the primary tumor. We observed reciprocal tumor specificity for this effect, which was circumvented if all tumors received RT or by transient depletion of Tregs. Primary tumor treatment with RT + IT-IC together with systemic administration of Treg-depleting anti-CTLA-4 resulted in a renewed in situ vaccination effect. Our findings show that untreated tumors can exert a tumor-specific, Treg-dependent, suppressive effect on the efficacy of in situ vaccination and demonstrate clinically viable approaches to overcome this effect. Untreated tumor sites antagonize the systemic and local antitumor immune response to an in situ vaccination regimen. This effect is radiation sensitive and may be mediated by tumor-specific regulatory T cells harbored in the untreated tumor sites. Cancer Immunol Res; 6(7): 825–34. ©2018 AACR.

Introduction

In situ tumor vaccination utilizes local therapies to convert a patient’s own tumor into a nidus for enhanced presentation of tumor-specific antigens, so as to stimulate and diversify a systemic antitumor immune response (1, 2). Tumor-targeted radiotherapy (RT) may interact with the immune system through localized release of tumor-specific antigens, induction of tumor cell expression of immune susceptibility markers, and temporary local depletion of suppressive immune cell lineages (3–5). By modulating functional tumor immunogenicity at a targeted site and by rendering tumor antigens more accessible to immune recognition, RT may be incorporated into in situ tumor vaccination strategies. RT may augment the endogenous T-cell response to autochthonous tumors and their antigens (6, 7), improving local and systemic antitumor immunotherapeutic efficacy (8–10).

In mice bearing single tumors, we recently reported a cooperative in vivo interaction between local RT and the immune response to intratumor (IT) injection of a tumor-specific antibody + IL2 fusion protein (11). In that study, mice bearing well-established (5 weeks), poorly immunogenic, disialoganglioside D2-expressing (GD2+) syngeneic melanoma tumors were treated with a combination of 12 Gy RT and IT injection of the anti-GD2 hu14.18-IL2 immunocytokine (RT + IT-IC). With this treatment regimen, we observed an in situ vaccination effect resulting in complete tumor regression in 71% of mice. Mice that rejected their primary tumor after RT + IT-IC demonstrated a tumor-specific memory T-cell response enabling rejection of cross-reactive parental tumor lines that lacked the GD2 antigen targeted by IC, consistent with the generation of epitope spread (11).

We expected that treatment of a primary tumor with RT + IT-IC, in the context of a distant untreated secondary tumor, would result in regression of the primary tumor and we hypothesized that this would lead to a systemic antitumor immune response against the distant secondary tumor. To the contrary, in the...
models studied here, we report that the presence of an untreated distant tumor specifically suppresses the primary tumor response to local RT + IT-IC. We refer to this effect as "concomitant immune tolerance."

Materials and Methods

Tumor cell lines

B78-D14 (B78) melanoma was obtained from Ralph Reisfeld (Scripps Research Institute, La Jolla, CA) in 2002. B78 melanoma is a poorly immunogenic GD2+ cell line derived from B16 melanoma (12). Panc02 pancreatic tumor cells were obtained from the NCI in 2012. B78 and Panc02 cells, both syngeneic for C57BL/6 mice, were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Additionally, B78 cells also had 400 μg/mL of genetecin and 50 μg/mL hygromycin-B added to their media. Mycoplasma testing was done using PCR methodology previously described (13). All cells used were confirmed to be negative for Mycoplasma prior to injection into mice. Cell authentication was performed per ATCC guidelines using morphology, growth curves, and mycoplasma testing. Cells were typically cultured for no more than 5 passages prior to use (~2 weeks) and replicate freezer stocks were thawed and utilized for each experiment.

Panc02-GD2+ cells were Panc02 cells transduced to express GD2 using a retroviral vector that encodes the disialoganglioside (GD2) mini-operon (MP956:SGC.GD3synthase-2A-GD2synthase plasmid; a kind gift from Prof. Martin Pule, University College London, UK; ref. 14). After transduction, Panc02-GD2+ cells were labeled using PE-conjugated anti-GD2 (14G2a-PE, 357304, BioLegend) and sorted on a BD FACSAria flow cytometer to isolate Panc02-GD2+ cells with the highest GD2 expression. These cells were expanded in vitro and injected into mice for in vivo studies within five passages post-sorting. GD2 expression was confirmed by flow cytometry prior to tumor engraftment.

Murine tumor models

Mice were housed in accordance with the Guide for Care and Use of Laboratory Mice and experiments were performed under an animal protocol approved by the institutional animal care and use committee. C57BL/6 female mice at age 5 to 7 weeks were purchased from Taconic. C57BL/6-Tg (Foxp3 DTR/EGFPP) 23.2Spar/Mmjx “DEREG” mice were purchased from The Jackson Laboratory. Tumor depletion with diphtheria toxin was achieved following prior methodology (15) by daily intraperitoneal injection of 1 μg diphtheria toxin (Sigma) diluted in PBS for 2 days beginning the day of RT administration. Nondepleted control DEREG mice received intraperitoneal injection of PBS on the same schedule. DEREG mice were not followed for overall survival because of the potential confounding effect of Treg depletion on autoimmunity and consequent mortality (16).

B78 and Panc02 tumors were engrafted by subcutaneous flank injection of 2 × 10^5 tumor cells diluted in 100 μL phosphate-buffered saline (PBS). Tumor size was determined by precision caliper measurement. Tumor volume was approximated as (tumor volume in mm^3) = [(tumor width in mm)^2 × (tumor length in mm)/2]. Mice with visible primary and secondary tumors were randomized immediately prior to initiating treatment in all experiments. We defined the initial day of RT treatment as “day 1” of treatment in all experiments and in all figures. Unless otherwise stated, treatment of well-established tumors began ~5 weeks after primary tumor implantation and ~3 weeks after secondary tumor implantation. Unless otherwise stated, for experiments involving mice with two tumors, eligibility for randomization required having a primary (~5 week) tumor of ~200 to 320 mm^3 and a secondary (~3 week) tumor of ~40 to 60 mm^3. Approximately 5 to 10% of mice failed to develop a suitable primary or secondary tumor after injection, and we excluded these mice from randomization and treatment. In order to have sufficient numbers of eligible mice for randomization, approximately 10% more mice were implanted initially with two tumors for each experiment, to account for mice that would not be eligible for randomization.

We sacrificed mice when tumors exceeded 18 mm in any dimension, or whenever recommended by an independent animal health monitor for morbidity or moribund behavior. For in vivo mouse experiments, we normally conducted an initial pilot study followed by two repeats of each study with appropriately powered cohorts. For tumor response curves, we present data from the first of replicate fully powered experiments and we confirmed these results in replicate studies. For survival and complete response rate analyses, we pooled composite data from all relevant replicate experiments.

Radiotherapy

RT was delivered to tumors in vivo using a cabinet orthovoltage X-ray biological irradiator (X-RAD 320, Precision X-Ray, Inc.). RT was delivered, as indicated, beginning on experiment “day 1” in a single fraction to a calculated maximum dose of 12 Gy or in 3 daily fractions of 8 Gy. Mice were immobilized during RT administration using custom-designed lead jigs that exposed the dorsal flank tumor to irradiation and shielded non–tumor-bearing normal tissues and untreated distant tumor.

Antibodies and Immunocytoxine

Hu14.18-IL2 IC was provided by Apeiron Biologics via the NCI and has been previously described (17). Anti-mouse-CTLA-4 (IgG2a and IgG2b isotypes of the 9D9 clone) were provided by Bristol-Myers Squibb and previously described (18). The timing of anti-CTLA-4 IP injection relative to RT (days 3, 6, and 9) was selected based on our prior studies (11) and those of others showing a role for anti-CTLA-4 in enhancing the abscopal effect of RT (19) and reducing tumor-infiltrating Tregs in conjunction with RT (6). Gammmagard human IgG (Baxter) was utilized as a nonspecific IgG control for all tumor treatments and was tested against PBS treatment alone or following RT with no effect observed.

Immunohistochemistry

Immunohistochemistry was performed on tumors from 4 mice per treatment condition to quantify Foxp3+ tumor infiltrate. Tumors were harvested on day 6 after delivery of 12 Gy or sham RT to the primary tumor in mice bearing a primary or a primary and untreated secondary B78 melanoma tumor. Fresh tumor samples were dissected, cryo-embedded in OCT solution, and sectioned. Frozen sections were fixed in ~20°C acetone for 20 minutes, blocked in 5% normal rabbit serum, and labeled overnight at 4°C using a 1:1,000 dilution of anti-Foxp3 (clone FJK-16s, eBioscience), anti-CD8 (clone 53-6.7, eBioscience), or nonspecific isotype control antibody in 5% rabbit serum PBS with 0.01% Triton X-100. Foxp3 is used as a lineage marker for Tregs,
recognizing that this population is functionally heterogeneous and may include rare subsets of other cell types (20). Labeled cells were detected using the ImmPRESS peroxidase secondary and DAB substrate kits from Vector Laboratories. Slides were counterstained with hematoxylin. Three representative images were captured from the cortex of each tumor specimen at 200x magnification using an Olympus BX41 inverted microscope equipped with an Olympus XM10 digital camera. Images were viewed using CellSen Standard software and FoxP3- and CD8+ cells were quantified by an individual blinded to the treatment conditions.

Results

Concomitant immune tolerance in GD2+ melanoma and pancreatic tumor models

To test whether in situ vaccination with the combination of local RT + IT-IC might elicit a systemic antitumor response against an untreated macroscopic distant tumor, we engrafted C57BL/6 mice on the right flank with a “primary” GD2+, B78 melanoma tumor. Two weeks later, we engrafted the contralateral flank of these mice with a “secondary” B78 tumor. After three additional weeks, we treated these mice with 12 Gy RT to the primary tumor, while shielding the secondary tumor with lead. At the time of RT (day 1) the mean primary and secondary tumor volumes ± standard error (SE) for all treatment groups was 280.8 ± 24.5 mm3, and 40.5 ± 6.2 mm3, respectively. Animals without visible primary and secondary tumors were excluded. The primary tumor was then treated with daily 50 μg IT injections of the anti-GD2 hu14.18-F2 IC on days 6 to 10 after RT following a previously optimized dosing schedule (11). We treated control mice bearing a single B78 tumor with RT + IT-IC as above. Additional control mice bearing both primary and secondary B78 tumors were treated to the primary tumor with (a) 12 Gy and nonspecific IT- IgG, (b) sham RT and IT-IC, or (c) sham RT and IT-IgG.

We did not observe an immunotherapeutic effect on the distant tumor in mice that received combined RT + IT-IC to the primary tumor (Supplementary Fig. S1A). In fact, the presence of the untreated secondary tumor was associated with an inhibitory effect on the local response to RT + IT-IC at the primary tumor site (Fig. 1). In the presence of an untreated secondary tumor, primary tumor response to RT + IT-IC was not significantly different from that achieved with RT alone (Fig. 1A, P = 0.68). We also found no significant effect of RT + IT-IC compared with RT alone on overall survival (OS) in mice bearing a treated primary and a nontreated secondary tumor (Fig. 1B, P = 0.25). These findings are in contrast to our prior observations in mice bearing a primary B78 tumor alone, where this RT + IT-IC regimen improved response and OS compared with RT alone (11). Here, we also observed significantly improved primary tumor response and OS following RT + IT-IC in mice bearing a primary B78 tumor alone compared with those also bearing a nontreated secondary B78 tumor (Fig. 1A and B). This inhibitory effect of an untreated secondary tumor on the primary tumor response to RT + IT-IC was not unique to a 12 Gy × 1 fraction RT regimen. We observed a comparable effect when treating the primary tumor with IT-IC together with an 8 Gy × 3 fraction RT regimen (Supplementary Fig. S1B) that has been reported to be more immunogenic (21).

To test whether the size of a secondary tumor might affect concomitant immune tolerance, we compared the inhibitory effect of secondary tumors that were palpable but not grossly visible (≤4 mm3 on the day of RT) with that of moderate-sized secondary tumors that were comparable with those in Fig. 1A (~40 mm3, Supplementary Fig. S1C). For this study, we injected all primary tumors 5 weeks prior to RT and all secondary tumors three weeks prior to RT. The small secondary tumor group consisted of the “outlier” mice with very small, slow-growing secondary tumors. We observed a greater response of primary tumors to combined RT + IT-IC in mice bearing the smaller secondary tumors compared with those bearing the larger secondary tumors (Supplementary Fig. S1B, P < 0.001). In a distinct tumor model, we engrafted C57BL/6 mice on the right flank with syngeneic Panc02 pancreatic tumor cells transfected to express GD2 (Panc02-GD2+). We first confirmed a cooperative interaction between RT + IT-IC in mice bearing a single Panc02-GD2+ tumor (Supplementary Fig. S2). In this single tumor model, RT + IT-IC significantly improved tumor response compared with RT alone or IT-IC alone. RT + IT-IC yielded complete tumor regression in 69% (9/13) of mice compared with 0% in all other groups (0/13 in each group; P < 0.001).

Given the similar response to combined RT + IT-IC in mice bearing a single Panc02-GD2+ pancreatic tumor or a single B78 melanoma, we tested whether a secondary untreated Panc02-GD2- (wild-type Panc02) tumor might suppress the local
response to combined treatment of a primary Panc02-GD2⁺ tumor. Two weeks after primary Panc02-GD2⁺ tumor engraftment, we injected mice on the contralateral flank with Panc02-GD2⁺ cells to generate a secondary tumor. We compared response with primary tumor treatment with RT + IT-IC in these mice with that observed in mice bearing only a primary B78 melanoma tumor. A secondary Panc02-GD2⁺ tumor exerted a suppressive effect on the response of a primary Panc02-GD2⁺ tumor to locally administered RT + IT-IC (Fig. 1C). When data from replicate experiments are included, mice bearing a single Panc02-GD2⁺ tumor showed 69% complete tumor regression with RT + IT-IC, whereas none of 14 mice bearing an untreated secondary Panc02-GD2⁺ showed complete primary tumor regression (P < 0.001).

Concomitant immune tolerance is tumor specific and RT sensitive

To determine whether concomitant immune tolerance is a tumor-specific inhibitory effect, mice bearing a B78 primary tumor and a contralateral secondary Panc02-GD2⁺ tumor were treated with RT + IT-IC at the primary tumor. We observed no significant effect of this unrelated Panc02-GD2⁺ tumor on the response of the B78 primary tumor, compared with mice bearing a primary B78 tumor alone (Fig. 1D, P = 0.87). Similarly, a secondary B78 tumor did not significantly affect the response of a primary Panc02-GD2⁺ tumor to RT + IT-IC, compared with mice bearing a primary Panc02-GD2⁺ tumor alone (Fig. 1E, P = 0.75). These findings demonstrate reciprocal tumor specificity for concomitant immune tolerance, whereby a distant untreated secondary tumor may affect the response of a primary tumor to RT + IT-IC if its identical or related to that primary tumor, but not if the secondary tumor is unrelated to the primary tumor—even if the unrelated second tumor expresses the GD2 target of the IC.

In single tumor models, we previously demonstrated a role for RT in enabling an in situ vaccination effect when treating moderate or large sized murine tumors (200–500 mm³) with IT-IC (11). To test the influence of RT on the inhibitory effect of concomitant immune tolerance, we compared the effect of delivering 12 Gy RT to the primary tumor alone or to both the primary and secondary tumors in mice bearing two B78 tumors. When RT + IT-IC was given to the primary tumor, delivering RT to the secondary tumor eliminated concomitant immune tolerance, resulting in improved primary tumor response (Fig. 2A, P = 0.005; complete primary tumor response in 71%; 15/21) and improved overall survival (Fig. 2B, P < 0.001).

Regulatory T cells are necessary for concomitant immune tolerance

Concomitant immune tolerance is an RT-sensitive (Fig. 2), tumor-specific (Fig. 1) inhibitory effect of an untreated distant tumor on the response of a primary tumor to locally delivered immunotherapy. We next tested whether regulatory T cells (Treg), which are a tumor-specific and RT-sensitive inhibitory immune cell lineage (22, 23), might account, in part, for concomitant immune tolerance. Mice bearing a single B78 tumor were treated to this site with 12 Gy or sham RT and 6 days later this tumor was dissected. Using both immunohistochemistry (Fig. 3A) and...
To test the necessity of Tregs for concomitant immune tolerance, we used transgenic C57BL/6 "DEREG" mice in which the diphtheria toxin receptor is expressed downstream of the FoxP3 promoter, resulting in constitutive expression of this receptor on Tregs. This transgenic model enables depletion of Tregs upon treatment with diphtheria toxin (16). We implanted these mice with primary and secondary B78 tumors on contralateral flanks and delivered RT + IT-IC. These mice were randomly assigned to receive 2 daily intraperitoneal (IP) injections beginning the day of RT with either diphtheria toxin (to deplete Tregs) or PBS (15). Following Treg depletion, we observed improved primary (Fig. 3B, \( P < 0.001 \)) and secondary tumor (Fig. 3C, \( P = 0.003 \)) response to the combination of primary tumor RT + IT-IC, compared with control mice not depleted of Tregs (received IP-PBS). RT + IT-IC treatment in Treg-depleted mice resulted in a 60% (6/10) primary tumor complete response rate and 30% (3/10) secondary tumor complete response rate as compared with 10% (1/10; \( P = 0.01 \)) and 10% (1/10; \( P = 0.25 \)), respectively, in mice not depleted of Tregs. It is possible that Treg depletion alone can elicit a therapeutic effect; however, prior studies evaluating growth of B16 melanoma (parental to B78) in DEREG mice demonstrate that depletion of FoxP3+ cells does not impact the growth of tumors established for 2 or more weeks (29). These findings are consistent with the necessity of Tregs for concomitant immune tolerance.

CTLA-4 antibody overcomes concomitant immune tolerance and restores \textit{in situ} vaccination

To corroborate this role for Tregs, we compared the impact on concomitant immune tolerance of an IgG2a versus IgG2b isotype of anti-CTLA4. Although both of these antibodies bind to and antagonize CTLA-4, the IgG2a isotype depletes tumor Tregs to a greater extent (18). For this study, we again engrafted mice with primary and secondary B78 melanoma tumors and treated the primary tumor with RT + IT-IC. On days 3, 6, and 9 post-RT, we injected mice IP with 100 \( \mu \)g of either isotype of anti–CTLA-4 or nonspecific IgG control. We observed that the IgG2a anti–CTLA-4 could overcome concomitant immune tolerance and rescue the primary tumor response, whereas the IgG2b anti–CTLA-4 could not (Fig. 4A). Here, 90% of mice treated with RT + IT-IC to the primary B78 tumor had complete primary tumor response when also receiving anti–CTLA-4-IgG2a versus 20% of those receiving the IgG2b isotype of this antibody.

We confirmed that \textit{in situ} vaccination with RT + IT-IC results in not only a local response at the primary tumor when delivered
Concomitant immune tolerance is circumvented by specific depletion of Tregs. A, Immunohistochemistry for the Treg marker, FoxP3 (representative 400× images are shown) for tumors evaluated on day 6 after RT in mice with one (A1 and A2) or two (A3 and A4) tumors. Mice received no RT, or RT only to the primary tumor. The primary tumor is shown in A1-A3 and the secondary is shown in A4. Small arrows point out some of the FoxP3+ cells. B and C, DEREG mice express diphtheria toxin receptor under control of the Treg-specific FoxP3 promoter, enabling specific depletion of Tregs upon IP injection of diphtheria toxin (15). DEREG mice bearing primary and secondary B78 melanoma tumors were treated with RT+IT-IC to the primary tumor and IP injection of either diphtheria toxin or PBS (the first of replicate experiments are shown). Concomitant immune tolerance is eliminated following depletion of Tregs in these mice, resulting in improved B and C, secondary tumor response. n = number of mice per group. Tumor response experiments were performed in triplicate and representative single experiment data are shown. Histology was performed on tumor specimens from ≥5 mice per treatment condition and an independent replicate experiment was performed. **, P < 0.01; ***, P < 0.001.

together with IgG2a anti–CTLA-4 (Fig. 4B), but also triggers a systemic antitumor response at the untreated secondary tumor (Fig. 4C). In fact, the combination of systemic IgG2a anti–CTLA-4 and primary tumor RT + IT-IC resulted in improved animal survival compared with treatment with any doublet combination of these three treatments (Fig. 4D). In these experiments, the combination of systemic anti–CTLA-4 and primary tumor RT + IT-IC also rendered 41% of mice entirely disease-free (no residual primary or secondary tumor). In contrast, only 20% of mice were disease-free after the combination of RT + anti–CTLA-4, and no mice were rendered disease-free with IT-IC + anti–CTLA-4, RT + IT-IC, IT-IC, anti–CTLA-4, or RT. Immunohistologic assessment of tumor-infiltrating CD8+ and FoxP3+ cells at day 12 after RT in mice with a single B78 melanoma following 12 Gy + IT-IC or 12 Gy + IT-IC + IgG2a anti–CTLA-4 demonstrates an increase in the ratio of tumor-infiltrating CD8+:FoxP3+ cells with either treatment combination compared with untreated controls (Fig. 5A–C). This ratio is reduced in the treated primary tumor with either treatment regimen when an untreated secondary tumor is present, compared with mice receiving the same treatment in the absence of a second tumor. With the addition of anti–CTLA-4 (IgG2a) we observe a significant reduction in the FoxP3+ cells infiltrating a secondary tumor that is not directly treated (Fig. 5A), consistent with prior studies (18). In the primary tumor from these mice with two tumors, at day 12 after 12 Gy + IT-IC, we observe no difference in the extent of FoxP3+ cell infiltration with or without anti–CTLA-4 (IgG2a); however, at this tumor site, we observe an increase in the extent of CD8+ cell infiltration with the addition of anti–CTLA-4 (Fig. 5B). In these mice, with two tumors, this increase in CD8+ cells, but not in FoxP3+ cells, in the primary tumor in mice receiving anti–CTLA-4, is consistent with a relative in vivo-depleting effect of the anti–CTLA-4. Our results suggest that the ratio of tumor-infiltrating CD8+:FoxP3+ cells after treatment (Fig. 5C) is correlated with antitumor response and demonstrate a role of distant tumor sites in modulating this interaction between effector and suppressor T cells at a site of in situ vaccination.

Figure 3.
Figure 4.
Concomitant immune tolerance is overcome by treatment with IgG2a anti–CTLA-4, revealing a capacity of in situ vaccination to augment checkpoint blockade. A, C57BL/6 mice bearing B78 primary and secondary tumors were treated with RT + IT-IC and IP injection (days 3, 6, and 9 after RT) with control IgG or an IgG2a or IgG2b anti–CTLA-4. B and C, C57BL/6 mice bearing B78 primary and secondary tumors were treated with all possible combinations of RT + IT-IC and IgG2a anti–CTLA-4; results for (B) primary and (C) secondary tumors from the first of replicate experiments are shown. D, Aggregate animal survival from replicate experiments is shown. n = number of mice per group. Experiments were performed in triplicate. Aggregate results are reported for survival, and representative single experiment data are shown for tumor response. NS, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Discussion

Using syngeneic murine tumor models, we have identified a suppressive effect of distant untreated tumor sites on the local and systemic response to in situ tumor vaccination with primary tumor RT and IT injection of hu14.18-IL2. This concomitant immune tolerance exhibits reciprocal tumor specificity and requires an untreated secondary tumor that may serve as a nidus to maintain a reservoir of tumor-specific Tregs that can repopulate the primary tumor site after local RT. This tumor specificity is consistent with an adaptive Treg response; further characterization of these Tregs will be pursued in future studies. This concomitant immune tolerance is consistent with a report demonstrating that T-cell egress from tumors is a regulated process capable of affecting the immune microenvironment at distant tumor sites (30). In a B78 murine melanoma model, concomitant immune tolerance can be overcome by delivering RT to all sites of disease, selective Treg depleton, or combined treatment with an anti–CTLA-4 that partially depletes tumor Tregs. This should not be taken to indicate the absence of a potential role for other Treg-independent mechanisms or other suppressive immune cell lineages (such as myeloid-derived suppressor cells) in concomitant immune tolerance.

Our findings have implications for in situ tumor vaccination as a therapeutic strategy for any tumor that can be recognized by a tumor-reactive mAb. A separate combination of tumor-reactive mAb, IL2, checkpoint blockade and a tumor-antigen vaccine was shown to enable immune-mediated destruction of macroscopic tumors in mice (31). The preclinical results presented here, and in our prior report (11), demonstrate that large murine tumors, at a single site or at multiple sites, can be eradicated using a combined approach that includes an immunomodulatory dose of RT (that does not cause direct tumor shrinkage), together with IT-IC (providing tumor-reactive mAb and IL2) and Treg-depleting checkpoint blockade, acting together as an in situ vaccine. Here, we provide evidence that this combined modality approach can transform a targeted immunologically "cold" tumor with minimal tumor-infiltrating CD8+ cells into an immunologically "hot" tumor with a 10-fold increase in CD8+ tumor infiltrate following local RT + IT-IC and a 14-fold increase with this combination plus anti–CTLA-4.

In the setting of metastatic cancer, the rationale for in situ tumor vaccination is derived from the ability of the human immune system to respond systemically to local immunogenic stimuli. Unlike traditional vaccines against infectious pathogens, which are generally delivered prior to pathogen exposure, any in situ tumor vaccination for cancer therapy will be delivered in the face of preexisting disease and may be delivered in the setting of distant, known or subclinical, untreated tumor. Our results suggest that tumor-specific Tregs harbored in untreated tumors may pose a challenge to the efficacy of in situ vaccination. Furthermore, they suggest that therapeutic approaches to circumventing concomitant immune tolerance may lead to more effective systemic antitumor efficacy via in situ vaccination. This may include combination with an anti–CTLA-4, although we recognize that the ability of currently approved anti–CTLA-4 to deplete tumor-infiltrating Tregs in patients remains controversial. In preclinical studies, we are now investigating alternative strategies to achieve local Treg depletion in the tumor microenvironment, including systemic administration of tumor-selective, low dose, molecular targeted RT.

Human melanoma, neuroblastoma, and sarcoma tumors commonly express the GD2 antigen (32). Early clinical testing of hu14.18-IL2, given intravenously, has demonstrated safety, immune activation, and clinical antitumor activity for non-bulky tumor (33–35). However, the majority of patients with measurable or evaluable non-bulky disease do not respond. The preclinical findings presented in this report have translational

Figure 5.
The ratio of tumor-infiltrating CD8+FoxP3+ cells correlates with response to in situ vaccination. Immunohistochemistry was performed on tumor specimens collected at day 12 after either 12 Gy + IT-hu14.18-IL2 IC (red) or 12 Gy + IT-hu14.18-IL2 IC + IP IgG2a anti–CTLA-4 (blue) from mice bearing either a single B78 melanoma (open box) or two B78 melanoma tumors, including a primary (treated with RT and IT-IC; dotted box) and a secondary (not directly treated; filled box). Untreated mice bearing a B78 tumor implanted at the same time were analyzed in parallel (black). Box and whisker plots (in A and B) show aggregate individual data from replicate experiments including individual data points (circles), middle two quartiles (box), local maximum and minimum (whisker bars), and median (line) values for tumor-infiltrating (A) FoxP3+ and (B) CD8+ cells. Points outside the whisker bar are those extending beyond the middle quartile limits by >1.5 × the interquartile range. C, The ratio of mean values of tumor-infiltrating CD8+FoxP3+ cells from these studies is plotted. These mice were engrafted and treated at the same time as those in Supplementary Fig. S3B, and these experiments share the same untreated controls, which were analyzed on the day corresponding to day 2 for treated mice. Pertinent statistical comparisons are shown. These data are consistent with a correlation between the ratio of tumor-infiltrating CD8+FoxP3+ cells at a tumor site and the response to in situ vaccination. Histology was performed on tumor specimens from ≥ 3 mice per treatment condition and an independent replicate experiment was performed. NS, nonsignificant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.
implications for treatment of GD2+ cancers. We are now advancing a phase I clinical study in patients with metastatic melanoma, investigating the safety, immunologic, and antitumor effects of this RT + IT-IC in situ vaccination strategy together with systemic administration of anti–CTLA-4.

Clinical studies of T-cell checkpoint inhibitors such as anti–CTLA-4 indicate that, even in the context of metastatic disease, a subgroup of patients may experience complete and durable regression of disease (36, 37). This suggests that augmenting rates of response to checkpoint blockade may result in a beneficial impact on patient survival. Our data suggest that in situ vaccination with RT + IT-IC may be a viable approach to achieving such an objective in patients with metastatic disease, if concomitant immune tolerance can be overcome.

Disclosure of Potential Conflicts of Interest

Z.S. Morris has ownership interest in a patent related to molecular targeted radiation therapy for immune modulation and is a consultant/advisory board member for Archeus Technologies Inc. H. Loibner is CEO of Aepioron Biologies AG. A.J. Korman has ownership interest (including patents) in Bristol-Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

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Other (provided critical antibody reagents and information on their use): A.J. Korman

Acknowledgments

This work was supported by Sari Zirbel Memorial Fund, NIH grants CA032685, CA87025, CA166105, CA197078, GM067386, IUL1TR000427, and 1DP5OD024576, The University of Wisconsin ICTR Grants ITLI3R0029013 and KL1TR00428, The University of Wisconsin Carbone Cancer Center Core Grant, P30CA014520, The University of Wisconsin UW20/20 grant, The University of Wisconsin “The Ride” Scholar program, The Midwest Athletes for Childhood Cancer Fund, and The Stand Up To Cancer – St. Baldrick’s Pediatric Dream Team Translational Research Grant [SIU22-AACR-DT1113].

Stand Up To Cancer is a division of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the Scientific Partner of SU2C.

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Received July 7, 2017; revised February 21, 2018; accepted May 3, 2018; published first May 10, 2018.

References


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Zachary S. Morris, Emily I. Guy, Lauryn R. Werner, et al.