Tumor-specific inhibition of in situ vaccination by distant untreated tumor sites

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**Running title:** Distant tumor sites antagonize in situ vaccination

Synopsis (limit 38 words): 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.

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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 intra-tumor (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 (Tregs). 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.
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 radiation therapy (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 intra-tumor (IT) injection of a tumor-specific
antibody + IL2 fusion protein (11). In that study, mice bearing well-established (5 week), 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”.

Methods

Tumor cell lines

B78-D14 (B78) melanoma was obtained from Ralph Reisfeld (Scripps Research Institute)
in 2002. B78 melanoma is a poorly immunogenic GD2+ cell line derived from B16 melanoma
(32). 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), 2mM L-glutamine, 100U/mL penicillin and 100µg/mL
streptomycin. Additionally, B78 cells also had 400mcg/ml of genetecin and 50mcg/ml
hygromycin-B added to their media. Mycoplasma testing was done using PCR methodology
previously described (33). 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 (MP9956:SFG.GD3synthase-2A-
GD2synthase plasmid; a kind gift from Prof. Martin Pule, University College London) (34).
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 \textit{in vitro} and injected into mice for \textit{in vivo} studies within five passages post-sorting. GD2 expression was confirmed by flow cytometry prior to tumor engraftment.

\textit{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 5-7 weeks were purchased from Taconic. C57BL/6-Tg (Foxp3 DTR/EGFP) 23.2Spar/Mmjax “DEREG” mice were purchased from Jackson. Treg depletion with diphtheria toxin was achieved following prior methodology (21) by daily intraperitoneal injection of 1\(\mu\)g diphtheria toxin (Sigma) diluted in PBS for 2 days beginning the day of RT administration. Non-depleted 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 (20).

B78 and Panc02 tumors were engrafted by subcutaneous flank injection of \(2\times 10^6\) tumor cells diluted in 100\(\mu\)L 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)\(^2\) x (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 \(\sim\) 5 weeks after primary tumor implantation and \(\sim\) 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 ~200-320 mm$^3$ and a secondary (~3 week) tumor of ~40-60 mm$^3$. Approximately 5-10% 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.

**Radiation**

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 Immunocytokine**
Hu14.18-IL2 IC was provided by Apeiron Biologics via the NCI and has been previously described (35). Anti-mouse-CTLA-4 (IgG2a and IgG2b isotypes of the 9D9 clone) were provided by Bristol Myers Squibb and previously described (23). The timing of anti-CTLA-4 IP injection relative to RT (days 3, 6, 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 (36) and reducing tumor infiltrating Tregs in conjunction with RT (6). Gammagard human IgG (Baxter) was utilized as a non-specific 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 1:1000 dilution of anti-FoxP3 (clone FJK-16s, eBioscience), anti-CD8 (clone 53-6.7, eBioscience), or non-specific 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 (37). 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.

**Real-time quantitative PCR**

We extracted mRNA from flash frozen tumor samples and utilized quantitative real-time-PCR to compare levels of FoxP3 transcript relative to the tumor-specific GD2-synthase transcript in these samples. We have previously demonstrated that RT does not impact the expression of GD2 in B78 melanoma cells (11). Tumors were crushed using the CryoPrep™ System (Covaris). RNA was isolated using an RNEasy® Mini Kit (Qiagen). Reverse Transcription was conducted using the QuantiTect® Reverse Transcription Kit (Qiagen) and qPCR was conducted using Power Sybr® Green Master Mix (ThermoFisher) and quantified using the C1000™ Thermal Cycler by BioRad. Primer sequences were as follows: FoxP3-forward 5’-CAC CTA TGC CAC CCT TAT CC-3’ and reverse 5’-CGA ACA TGC GAG TAA ACC AA-3’; GD2 forward 5’-TAC CCA CCA TCA TCC CTA C-3’ and reverse 5’-GTG TCC GTA GTC GAT CAT AAC-3’; and PGK1forward 5’- TGT TCC CAT GCC TGA CAA GT-3’ and reverse: 5’-AGG CAT TCT CGA CTT CTG GG-3’ was used as a housekeeping gene control.

**Statistical methods**

Tumor response was monitored after RT and figures show the means and standard errors of the tumor volumes. The slopes of tumor response curves were estimated and compared between treatment groups using mixed effect models on log-transformed tumor volume. For all figures, mean tumor volume response curves are truncated whenever a mouse in that treatment group was euthanized. Mouse survival was monitored for up to 60 days after RT. Survival curves were generated using the Kaplan-Meier method and compared using log-rank tests. Rates of complete response (regression of the specified tumor of interest to a non-visible size at any time),
and disease-free status (absence of any detectable tumor at experimental completion) were compared between groups using chi-square tests or Fisher’s exact tests. FoxP3+ and CD8+ cell quantification per 200x field by immunohistochemistry were compared between groups using ANOVA and t tests. P values less than 0.05 were considered statistically significant. All analyses were performed using JMP and SAS statistical software (SAS Institute, Cary, NC).

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 280.8mm$^3$+/−24.5 mm$^3$ and 40.5mm$^3$+/−6.2 mm$^3$, 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-IL2 IC on days 6-10 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 non-specific 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 to RT alone on overall survival (OS) in mice bearing a treated primary and a non-treated 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 to 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 to those also bearing a non-treated 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 x 1 fraction RT regimen. We observed a comparable effect when treating the primary tumor with IT-IC together with an 8 Gy x 3 fraction RT regimen (Supplementary Fig. S1B) that has been reported to be more immunogenic (12).

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 (≤ 4mm³ on the day of RT) with that of moderate sized secondary tumors that were comparable to 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 to 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 to RT alone or IC alone; RT+IT-IC yielded complete tumor regression in 69% (9/13) of mice compared to 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 to primary tumor treatment with RT+IT-IC in these mice with that observed in mice bearing only a primary Panc02-GD2\(^+\) 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 to 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 to 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 it is 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 mm\(^3\)) 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 (Tregs), which are a tumor-specific and RT-sensitive inhibitory immune cell lineage (13, 14), 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 quantitative real-time PCR (Supplementary Fig. S3A) for the Treg marker, FoxP3, we observed depletion of FoxP3+ cells in B78 melanoma tumors 6 days after RT in single-tumor bearing mice. In contrast, in tumors collected 6 days after primary tumor RT from mice bearing an untreated secondary tumor, we did not observe depletion of FoxP3+ cells in the radiated primary B78 tumor. In fact, in mice bearing 2 tumors, we observed no significant change in intra-tumor FoxP3+ cells in the RT-treated primary tumors relative to either untreated secondary B78 tumors from the same mice or untreated primary B78 tumors from mice bearing a single tumor (Fig. 3A).

In a modified replicate of this study we evaluated the time course of changes in tumor-infiltrating FoxP3+ and CD8+ cells following 12 Gy RT (Supplementary Fig. S3B-G). We observed initial depletion of both FoxP3+ (Supplementary Fig. S3B) and CD8+ cells (Supplementary Fig. S3C) one day after RT in mice. This is consistent with the sensitivity of both effector and regulatory T cells to RT, compared to most tumor cells (15-18). This temporary local depletion is followed by an increase in tumor-infiltrating CD8+ cells between days 4-12 after RT, as has been observed in prior studies demonstrating a role for local RT in increasing T-cell priming and tumor infiltration (7, 19). This leads to an elevated ratio of CD8+:FoxP3+ cells when RT is delivered in the absence of an untreated distant B78 tumor (Supplementary Fig. S3D), peaking ~5 days after RT. This elevated CD8+:FoxP3+ cell ratio is not seen at this same time (~5 days after RT) in the irradiated tumor in mice also bearing an untreated secondary tumor (Supplementary Fig. S3G). This appears to reflect an accelerated repopulation of the radiated site with FoxP3+ cells when RT is delivered in the presence of a non-radiated distant
tumor, compared to the same treatment in mice without an additional tumor site

(Supplementary Fig. S3 B vs. E).

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 (20). 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 (21). 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 to 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 to 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 two or more weeks (22). These findings are consistent with the necessity of Tregs for concomitant immune tolerance.

**CTLA-4 antibody overcomes concomitant immune tolerance and restores in situ vaccination**

To corroborate this role for Tregs, we compared the impact on concomitant immune tolerance of an IgG2a vs 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 (23). For
this study, we again engrafted mice with primary and secondary B78 melanoma tumors and
injected the primary tumor with RT+IT-IC. On days 3, 6, and 9 post-RT, we injected mice IP with
100µg of either isotype of anti-CTLA-4 or non-specific 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 vs 20% of those receiving the IgG2b isotype of this antibody.

We confirmed that in situ vaccination with RT+IT-IC results in not only a local response
at the primary tumor when delivered 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 to 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. Immunohistological assessment of tumor-infiltrating CD8⁺ and FoxP3⁺
cells at day 12 after RT in mice with a single B78 melanoma tumor 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 to 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 to 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 (23). 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+ infiltrate with the addition of anti-CTLA-4 (Fig. 5B). In these mice with 2 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.

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 impacting the immune microenvironment at distant tumor sites (24). In a B78 murine melanoma model, concomitant immune tolerance can be overcome by delivering RT to all sites of disease, selective Treg depletion, 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 (25). 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 pre-existing disease and may be delivered in the setting of distant, known or sub-clinical, 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 \textit{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 radiation therapy.

Human melanoma, neuroblastoma, and sarcoma tumors commonly express the GD2 antigen (26). Early clinical testing of hu14.18-IL2, given intravenously, has demonstrated safety, immune activation and clinical antitumor activity for non-bulky tumor (27-29). However, the majority of patients with measurable or evaluable non-bulky disease do not respond. The preclinical findings presented in this report have translational implications for treatment of GD2$^+$ cancers. We are now advancing a phase I clinical study in patients with metastatic melanoma, investigating the safety, immunological and antitumor effects of this RT+IT-IC \textit{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 sub-group of patients may experience complete and durable regression of disease (30, 31). This suggests that augmenting rates of response to checkpoint blockade may result in a beneficial impact on patient survival. Our data suggest that \textit{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.

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References


**Figure 1. Tumor-specific inhibition of primary tumor response to the combination of local RT+IT-IC by a distant untreated tumor in murine melanoma and pancreatic tumor models.**

C57BL/6 mice bearing a syngeneic primary flank tumor +/- a secondary tumor on the contralateral flank were treated, as indicated, to the primary tumor only. Mean primary tumor volumes are displayed in panels A and C-E. **A**). In mice bearing a primary B78 melanoma tumor, the presence of an untreated secondary B78 tumor antagonized primary tumor response to RT+IT-IC. **B**) Kaplan-Meier survival curves are shown for mice in panel A plus replicate experiments. Nearly all mice were euthanized due to primary tumor progression. **C**) In mice bearing a primary Panc02-GD2\(^+\) pancreatic tumor, with or without a secondary Panc02-GD2\(^-\) tumor on the opposite flank, the presence of an untreated Panc02 secondary tumor suppressed the response of a primary Panc02-GD2\(^+\) tumor to RT+IT-IC. **D**) In mice bearing a primary B78 melanoma tumor, a secondary B78 tumor suppressed primary tumor response to RT+IT-IC but a secondary Panc02-GD2\(^+\) pancreatic tumor did not exert this effect. **E**) In mice bearing a primary Panc02-GD2\(^+\) tumor, a secondary Panc02-GD2\(^-\) tumor suppressed primary tumor response to combined RT and IT-hu14.18-IL2, whereas a B78 secondary tumor did not. \(n = \) number of mice per group. All experiments were performed in triplicate. Aggregate results are reported for survival and representative single experiment data are shown for tumor response. NS = non-significant, ***\(P < 0.001\).

**Figure 2. Concomitant immune tolerance is overcome by delivering RT to both tumor sites.** In mice bearing primary and secondary B78 tumors, the secondary tumor suppresses primary tumor response to primary tumor treatment with RT + IT-IC. This is overcome by delivering 12 Gy RT to both the primary and secondary tumors and IT-IC to the primary tumor, resulting in improved **A**) primary tumor response (the first of replicate experiments is shown) and **B**) aggregate animal
survival from replicate experiments. *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. **P < 0.01, ***P < 0.001.

**Figure 3. Concomitant immune tolerance is circumvented by specific depletion of Tregs. A.** Immunohistochemistry for the Treg marker, FoxP3 (representative 400x 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 (brown nuclei = FoxP3+, blue = hematoxylin counterstain). The graphs on the right display blinded quantification of FoxP3+ cells per 200x field, corresponding to the conditions shown in A1, A2, A3 and A4, respectively. 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 (21). 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) primary 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 ≥ 3 mice per treatment condition and an independent replicate experiment was performed. **P < 0.01, ***P < 0.001.

**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-CTLA4; 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 = non-significant, * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).

**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 > 1.5x 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-G 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 = non-significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 
Figure 2.

A

Primary Tumor Response

- 12 Gy to primary only (n = 5)
- 12 Gy to primary and secondary (n = 5)

B78 Melanoma primary and secondary tumors
All treated with IT-hu14.18-IL2 to the primary tumor

B

Survival rate (%)

- 12 Gy to primary only
- 12 Gy to primary and secondary

B78 Melanoma primary and secondary tumors
All treated with IT-hu14.18-IL2 to the primary tumor
Log-rank = 0.0001
n = 21
Figure 3.

(A) B78 primary only

No RT

12 Gy

Primary tumor

Secondary tumor

(A) Two B78 tumors

No RT

12 Gy

Primary tumor

Secondary tumor

(B) Primary Tumor Response

(Tumor volume (mm$^3$))

Non-depleted control (n = 5)

Treg depleted (n = 5)

(C) Secondary Tumor Response

(Tumor volume (mm$^3$))

All treated with 12 Gy + IT-hu14.18-IL2 to the primary tumor

B78 Melanoma primary and secondary tumor

"DEREG" transgenic mice

All treated with 12 Gy + IT-hu14.18-IL2 to the primary tumor

B78 Melanoma primary and secondary tumor

"DEREG" transgenic mice

ANOVA p < 0.001

Secondary Tumor Response

Non-depleted control (n = 5)

Treg depleted (n = 5)

**
Figure 4.

A. Primary Tumor Response

B. Primary Tumor Response

C. Secondary Tumor Response

D. Survival rate (%)
Tumor-specific inhibition of in situ vaccination by distant untreated tumor sites

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