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Biology Contribution

Anti-PD-1 Blockade and Stereotactic Radiation Produce Long-Term Survival in Mice With Intracranial Gliomas

Jing Zeng, MD,* Alfred P. See, BS,† Jillian Phallen, BS,† Christopher M. Jackson, BA,† Zineb Belcaid, MS,† Jacob Ruzevick, BS,† Nicholas Durham, BS,‡ Christian Meyer, MD, PhD,§ Timothy J. Harris, MD, PhD,* Emilia Albesiano, PhD,† Gustavo Pradilla, MD,† Eric Ford, PhD,* John Wong, PhD,* Hans-Joerg Hammers, MD, PhD,‡ Dimitris Mathios, MD,† Betty Tyler, BS,† Henry Brem, MD,† Phuoc T. Tran, MD, PhD,* Drew Pardoll, MD, PhD,‡ Charles G. Drake, MD, PhD,‡ and Michael Lim, MD†

*Departments of *Radiation Oncology and Molecular Radiation Sciences, †Neurosurgery, ‡Immunology, and §Oncology, Johns Hopkins Medical Institutes, Baltimore, Maryland*

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Summary

Using an intracranial mouse glioma model, we tested using stereotactic radiosurgery (SRS) to augment an antitumor immune response against glioblastoma multiforme. Our data suggest that this SRS-based immunotherapy paradigm has the potential to induce and maintain antitumor immunity against primary intracranial neoplasms, overcoming the immunosuppressive milieu of the tumor while minimizing the immunosuppressive effects associated with radiation of normal

Purpose: Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults, and radiation is one of the main treatment modalities. However, cure rates remain low despite best available therapies. Immunotherapy is a promising modality that could work synergistically with radiation, which has been shown to increase antigen presentation and promote a proinflammatory tumor microenvironment. Programmed-death-1 (PD-1) is a surface receptor expressed on activated and exhausted T cells, which mediate T cell inhibition upon binding with its ligand PD-L1, expressed on many tumor types including human GBMs. We tested the combination of anti-PD-1 immunotherapy with stereotactic radiosurgery in a mouse orthotopic GBM model.

Methods and Materials: We performed intracranial implantation of mouse glioma cell line GL261 transfected with luciferase into C57BL/6 mice. Mice were stratified into 4 treatment groups: (*1*) control; (*2*) radiation only; (*3*) anti-PD-1 antibody only; and (*4*) radiation plus anti-PD-1 antibody. Overall survival was quantified. The mice were killed on day 21 after implantation to assess immunologic parameters in the brain/tumor, cervical lymph nodes, and spleen.

Results: Improved survival was demonstrated with combination anti-PD-1 therapy plus radiation compared with either modality alone: median survival was 25 days in the control arm, 27 days in the anti-PD-1 antibody arm, 28 days in the radiation arm, and 53 days in the radiation plus anti-PD-1 therapy arm ($P<.05$ by log-rank Mantle-Cox). Long-term survival was seen only in the combined treatment arm, with a fraction (15%-40%) of animals alive at day 180+ after treatment. Immunologic data on day 21 after implantation showed increased tumor infiltration by cytotoxic T cells (CD8+/interferon-γ+/tumor necrosis factor-α+) and decreased regulatory

Reprint requests to: Michael Lim, MD, Johns Hopkins Hospital, 600 N. Wolfe St, Neurosurgery - Phipps 123, Baltimore, MD 21287. Tel: (410) 614-1627; E-mail: mlim3@jhmi.edu

Conflict of interest: Dr Charles Drake has served as a consultant to Bristol-Myers Squibb in the past. Authors Michael Lim, Charles Drake, Drew Pardoll, and Jing Zeng have applied for a patent related to the work in this study. The other authors declare no conflict of interest.

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tissues. Mechanistically, the antitumor response is primarily mediated by expansion of the cytotoxic CD8+ T cell population.

T cells (CD4+/FOXP3) in the combined treatment group compared with the single modality arms.

Conclusions: The combination of PD-1 blockade and localized radiation therapy results in long-term survival in mice with orthotopic brain tumors. These studies provide strong preclinical evidence to support combination trials in patients with GBM. © 2013 Elsevier Inc.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults, with an associated median survival of 14.6 months despite aggressive, multimodality treatment (1). Although the central nervous system (CNS) has historically been regarded as immunologically privileged, a growing body of evidence suggests that immune cells can traverse the blood-brain barrier and effect vigorous immune responses in the brain (2). Given the propensity of GBM cells to migrate beyond the bulk tumor margins, the precision with which immune cells home to and eliminate tumor cells makes immunotherapy an attractive treatment modality.

Trials of immunotherapy for GBM have not yet achieved their full potential. One reason for the relative lack of efficacy thus far is that GBM has developed an array of strategies for evading and suppressing antitumor immune responses. Programmed-death-1 (PD-1, CD279), an immune checkpoint surface receptor expressed on lymphocytes, has been implicated as a mediator of immune suppression in a variety of tumors, including GBM (3). Binding of PD-1 to its ligands B7-H1 (PD-L1) or B7-DC (PD-L2) induces apoptosis or exhaustion of activated immune cells, and blocking this interaction has been shown to enhance antitumor activity (3).

Radiation therapy (RT) counteracts the immunosuppressive tumor microenvironment by increasing major histocompatibility complex (MHC) class I expression, enhancing the presentation of normally suppressed tumor-associated antigens, increasing the expression of proinflammatory cytokines, promoting dendritic cell maturation, and downregulating Fas ligand expression (4, 5). Fractionated RT is the standard of care for GBM but has been associated with several immunologic and neurologic side effects. Conversely, stereotactic radiosurgery (SRS) allows for delivery of a conformal, high dose of radiation in a single session, and thus it may counteract the immunosuppressive tumor microenvironment while avoiding the leucopenia induced by conventional RT (6). We hypothesized that single-session focal RT could work synergistically with PD-1 blockade to generate a robust antitumor immune response against intracranial gliomas.

Methods and Materials

Cells

GL261-Luc cells were grown in Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum + 1% penicillin-streptomycin at 37°C in a humidified incubator maintained at 5% CO_2 and 5% O_2 (Gibco).

Tumor model

Female C57BL/6J mice (Harlan), 6 to 8 weeks old, were used for orthotopic glioma experiments as previously described (7). To establish syngeneic gliomas, 130,000 GL261-Luc cells were stereotactically injected in a 1-μL volume into the left striatum over 1 minute into the following coordinates: 1 mm anterior, 1 mm lateral from bregma, and 3 mm deep from the cortical surface. Tumor burden was monitored by luciferase imaging on day 7 after implantation, and the mice were randomly allocated into treatment arms based on tumor radiance, so that the average tumor radiance in each group was roughly equivalent. The animals were killed when they showed predetermined signs of neurologic deficits (failure to ambulate, weight loss >20% body mass, lethargy, hunched posture). The tumor take rate was 100%. Each arm had 6 to 9 mice in survival experiments. All experiments were repeated at least in triplicate.

Anti-PD-1 antibodies

Hamster antimurine PD-1 monoclonal antibody-producing hybridoma G4 was used to produce antibodies as previously described (8). Hamster Immunoglobulin isotype (Rockland) antibody was administered to animals receiving RT monotherapy as a control. Dosing was 10 mg/kg.

Radiation therapy and computed tomographic imaging

For in vivo experiments, mice with established GL261-luc tumors were treated using the Small Animal Radiation Research Platform (SARRP) previously described (9). The anesthesized mice underwent computed tomographic (CT) imaging on the SARRP to identity burr holes from tumor implantation, which was used to aim 10-Gy radiation in a 3-mm beam centered on the tumor (Fig. 1). The dose rate was 1.9 Gy/min. The tumors were 1 to 1.5 mm in diameter on the day of irradiation (Fig. E1).

Analysis of tumor-infiltrating cells

To identify tumor-infiltrating immune cells in GL261-luc tumors, the mice were killed on day 21 after implantation. The brains and spinal cords were removed and homogenized in Roswell Park Memorial Institute (RPMI) medium, filtered through a 40-μm nylon cell strainer (BD Falcon), centrifuged at 1000 rpm, and resuspended in 4 mL 30% Percoll. The cells were overlaid onto a Percoll gradient (30%/37%/60%) and centrifuged at 1200 rpm for 20 minutes. Lymphocytes were collected from the 37%/60% interface and washed twice in phosphate-buffered saline (PBS), then stimulated for 6 hours with RPMI + phorbol myristate acetate (PMA) + ionomycin (Sigma-Aldrich) + Golgi-stop (BD Biosciences) at 37°C. After incubation, the cells were washed with PBS and stained with anti-CD4 (clone GK1.5) and anti-CD8 (clone 53-6.7). The cells were then fixed, permeabilized (eBioscience), and stained intracellularly with FOXP3 (clone FJK-16s), tumor necrosis factor (TNF)-α (clone MP6-XT22), and interferon (IFN-γ) (clone XMG1.2). Appropriate isotype controls were used. The cells were analyzed by fluorescence

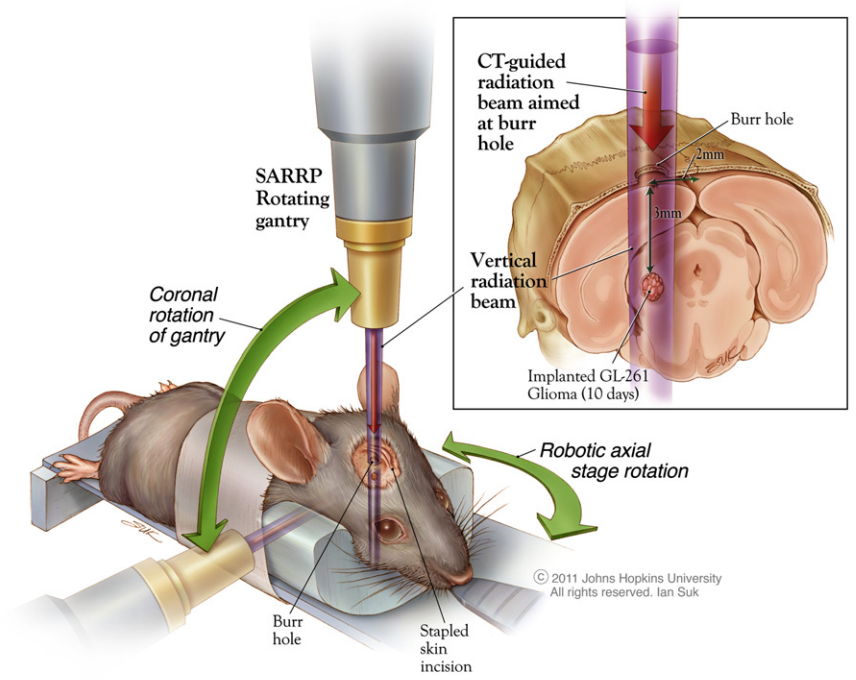

Fig. 1. Orthotopic mouse glioma model. Artistic depiction of stereotactic radiation beam (3-mm beam) aimed at intracranial GL261-luc tumor using burr hole as guidance for tumor location. CT = computed tomography; SARRP = Small Animal Radiation Research Platform. Printed with permission from Ian Suk-Johns Hopkins University.

activated cell sorting (FACS) analysis using a FACSCalibur flow cytometer (BD Biosciences). All antibodies were purchased from eBioscience. Data were analyzed with FlowJo software.

Immunoblot analysis

The GL261-luc cells were obtained and lysed. Fifty μg protein was loaded into 4% to 15% tris(hydroxymethyl) aminomethane-HCL Ready Gel (BioRad), separated, and transferred onto a polyvinylidene fluoride membrane (Millipore). These membranes were then probed with antibody for PD-L1/B7-H1 (clone MIH5) from eBiosciences. After incubation and washes, the membranes were detected with GE ECL Plus kit (GE Life Sciences) and BioRad Gel Doc. Raji cells were used as positive controls.

In vitro experiments

The GL261-luc cells were seeded at 100,000 cells per well into 6-well plates and then irradiated 24 hours later with 0-10 Gy using GammaCell with ^{137}Cs source, dose rate 50 cGy/min. Cells were obtained and analyzed for PD-L1, intercellular adhesion molecule (ICAM)-1, and MHC class I cell surface expression by flow cytometry after radiation on days 1, 2, and 3 (eBioscience). The cells were costained with propidium iodide to eliminate dead cells from analysis. For CXCL16 measurements, 2000 GL261-luc cells were seeded into 96-well plates, allowed to rest for 24 hours, and then irradiated as described above. On day 2 after radiation, fresh medium was placed into the wells for 4 hours and then obtained for CXCL16 measurement by enzyme-linked immunoassay (RayBiotech). The CXCL16 level was normalized to the number of viable cells present as measured by CellTiter-Blue (Promega).

CD4 and CD8 depletion

The mice underwent GL261-luc intracranial implantation and were injected with either GK1.5 α-CD4 mAb (BioXcell) or 53.6.72 α-CD8 mAb (BioXcell) at 200 μg per mouse on days 5-7 after implantation. On day 10, 2 mice from the CD4 depletion group and 2 from the CD8 depletion group were killed, and >99% depletion was confirmed by flow cytometry. An additional dose of depleting antibodies was administered on day 14 after implantation.

Flank rechallenge

One million GL261-luc cells were injected subcutaneously into the animals' bilateral flanks in 200 μL of mixed PBS and Matrigel in a 1:1 ratio (BD Biosciences). Four mice "cured" of their brain tumors with radiation plus anti-PD-1 therapy (90 days after implantation) were challenged, and 4 naïve mice were used as controls. The mice underwent luciferase imaging on day 10. Tumor measurements were taken every 2 to 3 days, and tumor volume was calculated (length × width × height × π/6). The mice were killed when the tumors reached 1000 mm^3.

Statistics

Survival was analyzed using Kaplan-Meier survival curves, and curves were compared with the log-rank Mantel-Cox test using GraphPad Prism. For comparison of cell numbers and percentages between treatment groups, an unpaired *t* test was used. *P* values <.05 were considered significant.

Results

Focal RT and PD-1 blockade produced long-term cures in mice with intracranial gliomas

We used the SARRP and anti-PD-1 antibodies to test the hypothesis that combined focal RT and immunotherapy could mediate a treatment effect in an orthotopic glioma model. After implantation with 130,000 GL261-luc cells (day 0), tumor engraftment was confirmed with luciferase imaging (day 7). Using the SARRP, 10 Gy radiation was administered with a 3-mm beam aimed at the burr hole (day 10). The animals were treated with: sham treatment, RT alone (plus isotype antibody), anti-PD-1 alone, or RT plus anti-PD-1 antibodies (Fig. 2A). Tumor growth was reassessed on day 21 with luciferase (Fig. 2B). Sample images are shown for 4 distinct mice per treatment arm on day 7 (before treatment) and day 21 (after treatment), illustrating the trend that mice in the control group tended to have the highest bioluminescent signal, and mice in the RT+PD-1 blockade group had the weakest signal. Survival data corroborated the growth patterns observed with luciferase imaging (Fig. 2C). The untreated mice had a median survival of 26 days, and RT improved the median survival to 27 days ($P = .03$ on comparison of Kaplan-Meier survival curves), and anti-PD-1 monotherapy prolonged survival to 30 days ($P = .04$). With RT+PD-1 blockade, the median survival increased to 52 days ($P < .001$), with 15% to 40% of mice becoming long-term survivors (>90 days after implantation). Our data show a synergistic improvement in survival with combination RT+PD-1 blockade.

Clinical trials with anti-PD-1 have suggested that tumor-specific B7-H1 expression may be an important biomarker of anti-PD-1 efficacy (10). We evaluated B7-H1 expression on GL261 by flow cytometry and Western blotting, demonstrating significant B7-H1 (Fig. 3A and B).

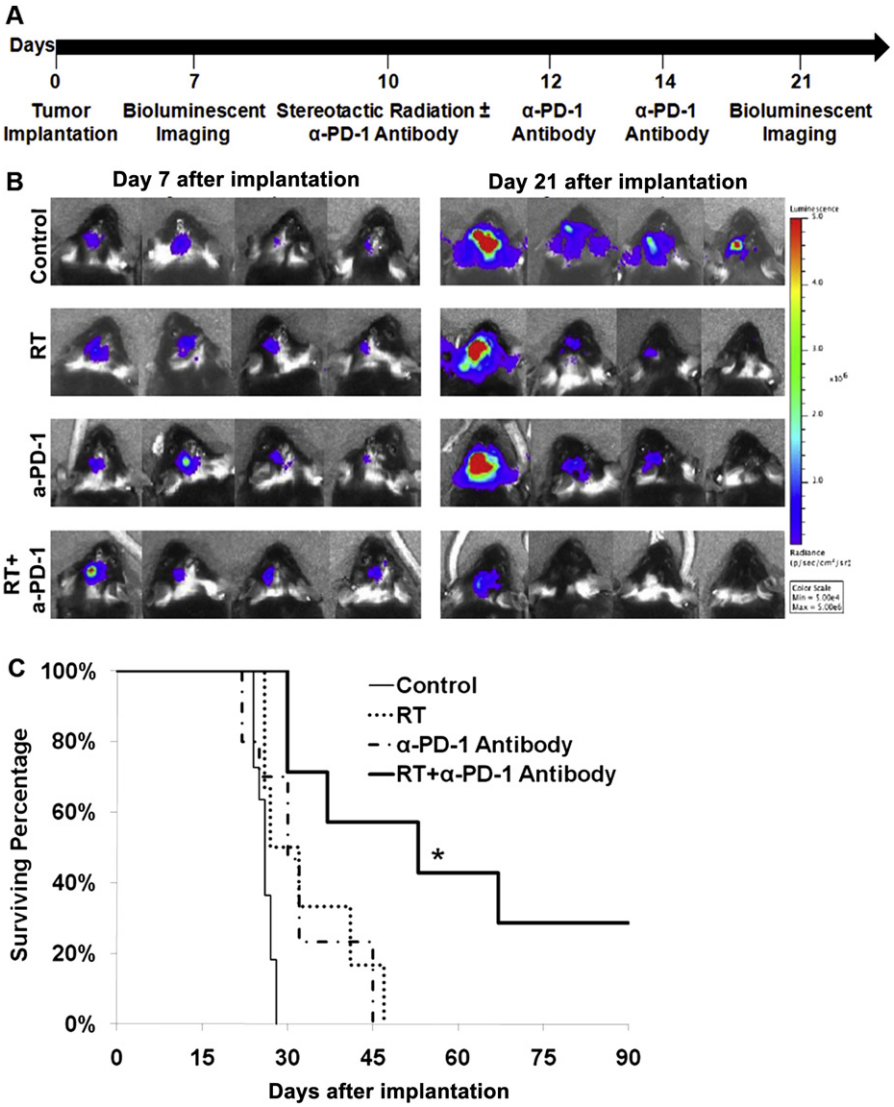

Fig. 2. Anti-PD-1 antibody plus radiation therapy (RT) cures mice with intracranial GL261-luc tumors. (A) Experimental timeline. (B) Luciferase imaging of 4 distinct mice per treatment arm before treatment (day 7) and after treatment (day 21), divided by treatment group. All images at same scale. All mice individually matched on days 7 and 21. (C) Kaplan-Meier survival curve. $P < .05$ between RT+anti-PD-1 antibody arm and all other arms. All experiments repeated in triplicate with >6 mice per arm.

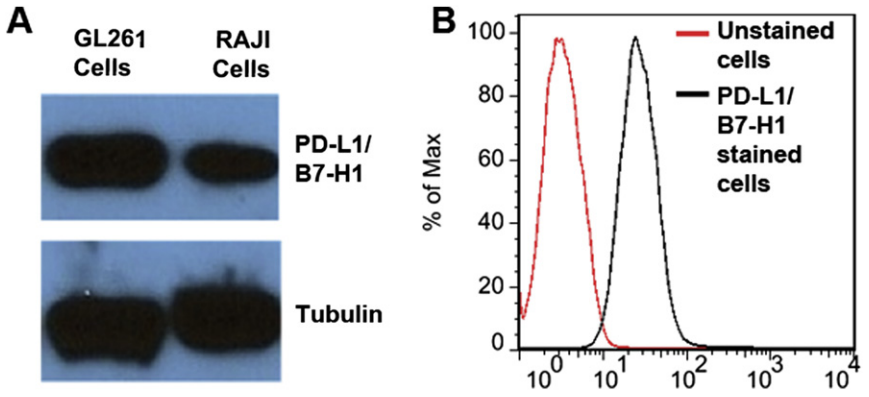

Fig. 3. (A) Immunoblot showing presence of PD-L1/B7-H1 in GL261-luc cells. (B) Flow cytometry showing cell surface presence of PD-L1/B7-H1 on GL261-luc cells.

Combination therapy increased the cytotoxic to regulatory T cell ratio

To identify the mechanisms underlying observed treatment effect of RT+PD-1 blockade, we removed the brains, spleens, and cervical lymph nodes of mice after treatment. No differences in the number or activation state of CD8 or CD4 cells were observed in the spleens or cervical lymph nodes (data not shown). The brains of mice receiving RT+anti-PD-1 antibody harbored increased CD8 effector T cells (CD8/IFN-γ/TNF-α) compared with other arms ($P<.01$) (Fig. 4A). The mice receiving RT harbored decreased numbers of regulatory T cells (Tregs, CD4/FOXP3) compared with unirradiated mice ($P=.03$) (Fig. 4B). There was no statistically significant difference between the RT group and the RT+anti-PD-1 group. The ratio of effector cells to Tregs has been correlated with therapeutic outcome in multiple tumor models (11). We saw a significant increase in the cytotoxic T cell to Treg ratio in mice receiving combination therapy (Fig. 4C).

These data suggest that an influx of CD8 effector T cells may be the major immunologic mechanism mediating the combined treatment effect. To test this hypothesis, we depleted mice of CD8 or CD4 cells before treating with RT+anti-PD-1 therapy. When the mice were depleted of CD8+ cells, the survival benefit of combination therapy was abrogated (Fig. 4D). Unlike the CD8-depleted mice, the CD4-depleted mice achieved a marginal survival benefit with combination therapy, although this response was less robust than that observed in mice not undergoing CD4 or CD8 depletion ($P=.048$).

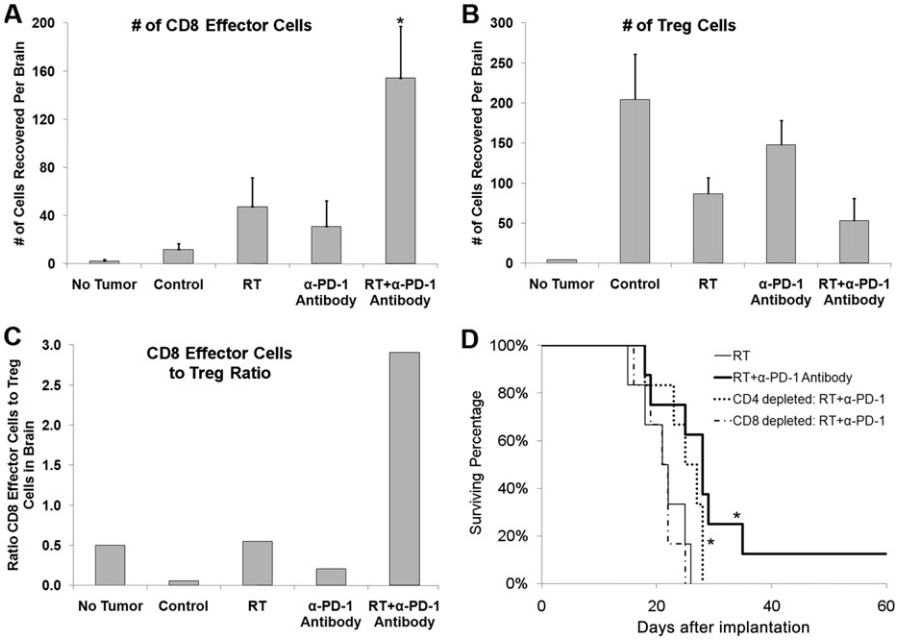

Fig. 4. Mice treated with RT+anti-PD-1 antibody show increased cytotoxic T cells (CD8 effector cells) and decreased regulatory T cells (Treg). (A) Mice treated with radiation therapy (RT)+anti-PD-1 antibodies have highest number of CD8 effector (CD8/IFN-γ/TNF-α) cells in the brain. (B) There are fewer regulatory T-cells (Treg, CD4/FOXP3) in mice brains that received radiation. (C) Ratio of cytotoxic to regulatory T cells is highest in RT+anti-PD-1 mice. (D) Without CD8 cells, adding anti-PD-1 antibodies to RT showed no survival benefit. Without CD4 cells, there was still a benefit to adding anti-PD-1 antibodies, less than seen in wild-type mice. Error bars represent standard error of the mean (SEM). Asterisks represent a statistically significant difference. All experiments repeated in triplicate with >6 mice per arm.

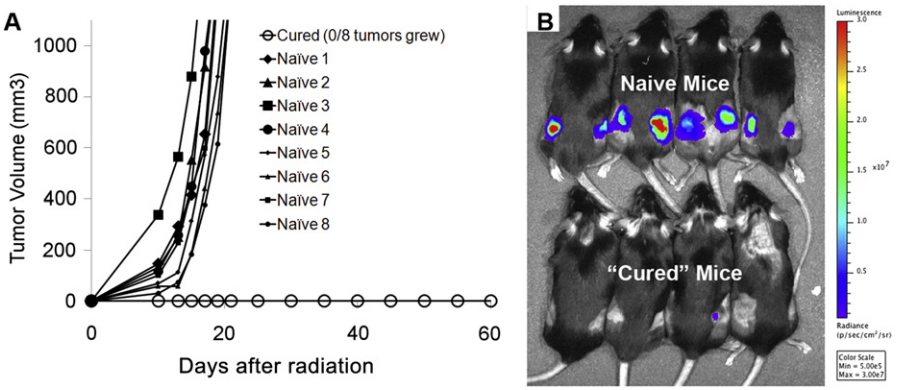

Fig. 5. (A) Mice "cured" of their brain tumors 90 days after implantation were rechallenged with 1 million GL261-luc cells per flank and compared with naïve mice. Flank tumors in naïve mice reached 1000 mm^3 by day 20, but none of the "cured" mice grew tumors by day 60. (B) Luciferase imaging of flank rechallenged mice on day 10 after implantation show strong signal in naïve mice (*top row*) and weaker signal in "cured" mice (*bottom row*).

Radiation altered the immune profile of GL261-luc cells in vitro

To elucidate the mechanisms underlying the synergistic RT+anti-PD-1 treatment effect, we evaluated how radiation alters the immune profile of GL261-luc cells. First, we quantified the expression of membrane-bound and soluble inflammatory molecules. Consistently with previous studies, we found that radiation induced the upregulation of surface MHC class I expression by day 3 after irradiation with 10 Gy, increasing from 4% to 16% ($P = .01$ by unpaired t test). ICAM-1 expression also increased after irradiation from 5% to 32% ($P < .001$) and seemed to peak by day 2. Soluble CXCL16 release was increased by 10 Gy irradiation. By day 2 after radiation, CXCL16 secretion increased approximately 12-fold from 0.3 pg/mL/10^4 cells to 3.5 pg/mL/10^4 cells ($P < .001$). These findings indicate that 10 Gy irradiation enhances the proinflammatory profile of GL261 gliomas.

Combination therapy with focal RT and PD-1 blockade resulted in immunologic memory

We tested mice for long-term immunity against glioma cells by rechallenging naïve and "cured" mice (animals surviving >90 days after intracranial tumor implantation) with flank injections of GL261-luc cells. In naïve mice, 100% of the flank tumors (8/8) reached >1000 mm^3 by day 21 after implantation (Fig. 5A). By contrast, tumors did not develop in any of the "cured" mice by day 60 after implantation. The luciferase imaging results on day 10 after implantation corresponded with tumor size measurements (Fig. 5B). These data suggest that "cured" mice retain long-term, systemic immunity against GL261-luc glioma cells.

Discussion

We showed a pronounced treatment effect against intracranial tumors using a novel paradigm of single-session focal RT combined with PD-1 blockade. Although radiosurgery alone has not demonstrated efficacy as a primary therapy for GBM (12), we hypothesized that focal radiation might be ideal in a combination immunotherapy regimen. The SARRP afforded a unique opportunity to test this hypothesis by delivering a single, high dose of focal RT in an animal model. Our results suggest that radiosurgery plus PD-1 blockade generates robust antitumor activity against primary intracranial gliomas.

In our experiments, compared with normal mice of similar age, mice that became long-term survivors after treatment showed no differences in body weight or posture, nor did they show any gross neurologic deficits in movement or feeding after age 18 months. Although more detailed toxicity analysis must be preformed for human clinical trials, when one considers the mortality and morbidity of GBMs, these findings represent a novel treatment paradigm that may constitute a significant addition to the GBM immunotherapy repertoire.

Previous studies have shown that fractionated RT synergizes with CTLA-4 blockade to produce tumor regression and long-term survival in a variety of extracranial cancer models (13, 14). Our results support and build on these data by demonstrating that blockade of other immune checkpoints, such as PD-1, may also synergize with RT. In contrast to prior studies using fractionated RT, our data indicate that single-session focal RT is effective in combination with PD-1 against intracranial tumors. Given the distinct immunologic environment of the CNS, these results could not have been predicted a priori. In fact, patients with brain metastases were excluded from phase 3 clinical trials of ipilimumab; although recent reports suggest that ipilimumab may have activity against intracranial melanoma lesions (15). Although these reports are anecdotal, they are encouraging and support the potential for efficacy of our treatment paradigm in a clinical trial setting.

Although neither PD-1 blockade nor single-session focal RT alone eradicated intracranial gliomas, the combination of these therapies generated robust, durable responses. Previous studies have shown that α-CTLA-4 therapy has activity against murine gliomas (16); however, α-CTLA-4 may not be ideal for combination therapy because of a high incidence of immune-related adverse events (17). Conversely, early trials of anti-PD-1 monoclonal antibodies have reported an elevated response rate and a comparably favorable side effect profile compared with α-CTLA-4 therapy (18). Of note, fractionated RT has been reported to be superior to single-session RT when used in combination with CTLA-4 (14). These results, however, were obtained in non-CNS tumors and did not have the benefit of direct targeting as afforded

by the SARRP. Future studies are needed to determine the optimal RT regimen in this treatment paradigm.

In our experiments, the survival benefit conferred by the addition of PD-1 blockade was abrogated by the depletion of cytotoxic T cells. Other studies examining immunotherapy in murine CNS tumor models have also found a similar dependence on the CD8 population (19). Smith et al (19) found that both CD4 and CD8 cells were essential for the combined immunologic effect of GM-CSF and IFN-γ against intracranial GL261 mouse gliomas. However, in our study, CD4-depleted mice still derived a survival benefit with the addition of PD-1 blockade to radiation, but the survival improvement was not as robust as in wild-type mice. This suggests that the immunologic pathways activated by PD-1 and GM-CSF/IFN-γ differ, and further delineation of the mechanism of action will help elucidate the distinction.

Anti-PD-1 in combination with focal RT leads to reliable rejection of GL261 tumors in long-term survivors. We hypothesize that the generation of immunologic memory in our treatment scheme resulted primarily from RT. Mechanistically, we envision that the release of multiple tumor-associated antigens in a proinflammatory environment acts as a vaccine, leading to the generation of immunologic memory. Future studies are needed to determine whether anti-PD-1 therapy is critical to the generation and/or maintenance of immunologic memory in this paradigm.

We demonstrated that radiation increases the expression of MHC class I, CXCL16, and ICAM in GL261 cells. Taken together, these results suggest that RT increases the proinflammatory profile of glioma cells. Our data demonstrate the importance of CD8 T-cell activity in the observed treatment effect. The ratio of CD8 to Treg cells, which has been suggested as a marker of responsiveness to immune therapies (11, 20), was significantly increased in the mice receiving combination therapy.

Conventional therapies have proved only marginally effective against GBM. Here we present the evidence for a novel approach with the potential to induce and maintain antitumor immunity against primary intracranial tumors. SRS is safe and is routinely used to deliver RT to intracranial lesions. Although the infiltrative nature of GBM precludes the use of SRS as a primary treatment modality, our results suggest that SRS should be reexamined in combination with PD-1 blockade in a clinical trial setting, for the appropriate patient population.

References

1. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;352:987-996.
2. Prins RM, Soto H, Konkankit V, et al. Gene expression profile correlates with T-cell infiltration and relative survival in glioblastoma patients vaccinated with dendritic cell immunotherapy. *Clin Cancer Res* 2011;17:1603-1615.
3. Parsa AT, Waldron JS, Panner A, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* 2007;13:84-88.
4. Klein B, Loven D, Lurie H, et al. The effect of irradiation on expression of HLA class I antigens in human brain tumors in culture. *J Neurosurg* 1994;80:1074-1077.
5. Newcomb EW, Demaria S, Lukyanov Y, et al. The combination of ionizing radiation and peripheral vaccination produces long-term survival of mice bearing established invasive GL261 gliomas. *Clin Cancer Res* 2006;12:4730-4737.
6. Grossman SA, Ye X, Lesser G, et al. Immunosuppression in patients with high-grade gliomas treated with radiation and temozolomide. *Clin Cancer Res* 2011;17:5473-5480.
7. Sonabend AM, Velicu S, Ulasov IV, et al. A safety and efficacy study of local delivery of interleukin-12 transgene by PPC polymer in a model of experimental glioma. *Anticancer Drugs* 2008;19:133-142.
8. Hirano F, Kaneko K, Tamura H, et al. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005;65:1089-1096.
9. Wong J, Armour E, Kazanzides P, et al. High-resolution, small animal radiation research platform with x-ray tomographic guidance capabilities. *Int J Radiat Oncol Biol Phys* 2008;71:1591-1599.
10. Brahmer JR, Drake CG, Wollner I, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 2010;28:3167-3175.
11. Waitz R, Solomon SB, Petre EN, et al. Potent induction of tumor immunity by combining tumor cryoablation with anti-CTLA-4 therapy. *Cancer Res* 2012;72:430-439.
12. Crowley RW, Pouratian N, Sheehan JP. Gamma knife surgery for glioblastoma multiforme. *Neurosurg Focus* 2006;20:E17.
13. Demaria S, Kawashima N, Yang AM, et al. Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. *Clin Cancer Res* 2005; 11:728-734.
14. Dewan MZ, Galloway AE, Kawashima N, et al. Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. *Clin Cancer Res* 2009; 15:5379-5388.
15. Hodi FS, Oble DA, Drappatz J, et al. CTLA-4 blockade with ipilimumab induces significant clinical benefit in a female with melanoma metastases to the CNS. *Nat Clin Pract Oncol* 2008;5:557-561.
16. Fecci PE, Ochiai H, Mitchell DA, et al. Systemic CTLA-4 blockade ameliorates glioma-induced changes to the CD4+ T cell compartment without affecting regulatory T-cell function. *Clin Cancer Res* 2007;13: 2158-2167.
17. Weber JS, O'Day S, Urba W, et al. Phase I/II study of ipilimumab for patients with metastatic melanoma. *J Clin Oncol* 2008;26:5950-5956.
18. Wolchok JD, Yang AS, Weber JS. Immune regulatory antibodies: are they the next advance? *Cancer J* 2010;16:311-317.
19. Smith KE, Fritzell S, Badn W, et al. Cure of established GL261 mouse gliomas after combined immunotherapy with GM-CSF and IFN-gamma is mediated by both CD8+ and CD4+ T-cells. *Int J Cancer* 2009 Feb 1;124:630-637.
20. Mostbock S, Lutsiak ME, Milenic DE, et al. IL-2/anti-IL-2 antibody complex enhances vaccine-mediated antigen-specific CD8+ T cell responses and increases the ratio of effector/memory CD8+ T cells to regulatory T cells. *J Immunol* 2008;180:5118-5129.