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ORIGINAL RESEARCH

Combination PD-1 blockade and irradiation of brain metastasis induces an effective abscopal effect in melanoma

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ABSTRACT

Nearly half of melanoma patients develop brain metastases during the course of their disease. Despite advances in both localized radiation and systemic immunotherapy, brain metastases remain difficult to treat, with most patients surviving less than 5 months from the time of diagnosis. While both treatment regimens have individually shown considerable promise in treating metastatic melanoma, there is interest in combining these strategies to take advantage of potential synergy. In order to study the ability of local radiation and anti-PD-1 immunotherapy to induce beneficial anti-tumor immune responses against distant, unirradiated tumors, we used two mouse models of metastatic melanoma in the brain, representing BRAF mutant and non-mutant tumors. Combination treatments produced a stronger systemic anti-tumor immune response than either treatment alone. This resulted in reduced tumor growth and larger numbers of activated, cytotoxic $CD8^+$ T cells, even in the unirradiated tumor, indicative of an abscopal effect. The immune-mediated effects were present regardless of BRAF status. These data suggest that irradiation of brain metastases and anti-PD-1 immunotherapy together can induce abscopal anti-tumor responses that control both local and distant disease.

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Introduction

Metastatic melanoma is a highly aggressive disease that is associated with poor survival.[1,2] Brain metastases (BM) are common, with 20% of patients presenting and 50% developing them during the course of disease.[3-5] Radiotherapy is a major treatment modality of melanoma, with successful control of local lesions often occurring in up to 80% of patients.[4,6,7] Despite successful control of localized disease, however, patients frequently develop metastases, including in the brain, that result in overall treatment failure.

Immunotherapies that boost the patients' own anti-tumor immune responses have revolutionized the management of melanoma and show promise against both intra and extracranial melanoma.[8-10] These findings are part of an evolving understanding of the brain as an immune-accessible tissue.[11,12] Immune-checkpoint inhibitors, including antibody blockade of programmed death-1 (PD-1) signaling are now widely used against melanoma due to their efficacy in recent clinical trials.[13-15] Anti-PD-1 treatment can result in dramatic successes in melanoma patients, with response rates from 25 to 45%.[16-22] Anti-PD-1 treatment has also demonstrated efficacy in other forms of cancer including bladder, colorectal, and certain hematopoietic malignancies.[23-26] PD-1 expression is induced on T cells in response to signals in the tumor microenvironment including chronic antigen exposure. Signaling through PD-1 delivers negative signals to T cells, which reduces cytotoxic function and contributes to immune tolerance.[27] Blockade of PD-1, therefore, prevents T cell inhibition and supports anti-tumor immune responses.[28,29]

In the current study, we used two models of mouse melanoma representing BRAF wildtype (wt) and mutant tumors to model the clinical management of cancer patients with brain metastases of these two major types of melanoma. One of the tumor models utilized B16-F10 cells, which develop aggressive tumors *in vivo* that are well-studied in pre-clinical models of immunotherapy, and which express wild-type BRAF.[30,31] The other model made use of D4M cells, which were derived from a spontaneous melanoma induced in a transgenic mouse expressing mutant BRAF (V600E) combined with genetic knock-out of the tumor suppressor gene Pten.[32,33] This actively transforming $BRAF^{V600E}$ mutation is a common feature of nearly 50% of human melanomas, and drives disease progression, highlighting the clinical relevance of this model.[34-36]

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We used these cell lines in two models of metastatic melanoma. In one model, two contralateral flank tumors were established subcutaneously in mice. In subsequent experiments, tumor cells were injected into the brain and flanks of mice to model brain metastasis and extracranial disease. Mice were then treated with anti-PD-1 combined with localized radiation to the head (to target the brain tumor) or to one of the flank tumors (for the bilateral flank tumor model).[37-40] In the bilateral flank tumor model, we found that the combination of irradiation and anti-PD-1 treatment resulted in the greatest survival of mice, and that even growth of the unirradiated flank tumor was significantly delayed in the group receiving combined irradiation and anti-PD-1. Strikingly, similar results were observed in models of brain metastasis using both B16-F10 and D4M tumor lines, in which the brain was irradiated during PD-1 treatment. To our knowledge, this is the first time that such response has been demonstrated in this kind of model.

We hypothesized that this delay was due to the enhancement of a systemic anti-tumor immune response which induced an abscopal effect on the flank tumor. Indeed, we found that the tumor tissue in the unirradiated flanks of anti-PD-1 treated mice, in both the head irradiation- and flank irradiation models, had significantly greater T cell infiltrates and expression of markers associated with cytotoxic immunity. These data demonstrate that anti-PD-1 therapy combined with irradiation of only a single metastatic lesion can result in a beneficial immune response that can affect unirradiated distal tumors. Collectively, our novel findings indicate that combined radiation and anti-PD-1 checkpoint blockade therapy can have beneficial effects on stimulating systemic anti-tumor immune responses to induce regression of distant tumors.

Results

Combination radiation and anti-PD-1 therapy induces a systemic anti-tumor response

The abscopal effect is a phenomenon in which the combination of systemic immunotherapy and irradiation of localized lesions causes a systemic anti-tumor immune response that affects the growth of distal, non-irradiated tumors. Though a rare but real occurrence in the clinic, this effect has been observed in a number of pre-clinical mouse models of melanoma.[41,42] To determine the immunologic changes underlying this process, we began by performing similar studies with contralateral flank tumors to observe whether similar responses could be induced with both our D4M and B16 models of melanoma. In Figure 1A, B16 tumors were established in each flank of mice. Mice were then treated with anti-PD-1, and one of the tumors was irradiated (8 Gy/4 fractions). We chose this dosing schedule because previously published studies have indicated that fractionated radiation dosages induce greater immunity than single doses, and maximize abscopal effects.[43] Anti-PD-1 treatment continued once every 5 days for the duration of the experiment. As expected, the irradiated tumor exhibited decreased growth. Likewise, while anti-PD-1 and irradiation each resulted in a significant delay in growth, the combination of the two treatments resulted in the greatest effect in both the directly irradiated and distant, unirradiated tumors. In order to determine whether significantly delayed growth of non-irradiated, contralateral tumors was due to anti-tumor immune responses, tumor tissue was harvested from mice at the conclusion of the experiment, dissociated by digestion with collagenase and DNase, and stained for T cells and quantified by flow cytometry (Figure 1B). These studies indicated that delayed tumor growth was associated with an increased $CD8^+$ T cell infiltrate, and correspondingly enhanced anti-tumor immune responses.[44,45]

Combination anti-PD-1 and radiation induced an abscopal effect in a brain metastasis model of melanoma

Radiotherapy is frequently used for treatment of brain metastases. Given the ability of radiation to induce and enhance anti-tumor immune responses, we sought to combine radiation with immunotherapy to enhance systemic anti-tumor immune responses. D4M melanoma cells were injected both intracranially and in the flank of immune competent syngeneic mice. Fourteen days post-injection, mice were irradiated to the head (8 Gy in 4 fractions) after verifying by bioluminescence imaging that they had equally sized luminescent tumors. For mice receiving anti-PD-1, antibody treatment began five days prior to irradiation (summarized in Figure 2A), as recent studies indicated that the optimal timing of checkpoint blockade is just prior to irradiation, presumably so that the antibody is present in the tissues at the time of therapy.[46-48] Survival was followed over the next 25 days and is illustrated in Figure 2B. Animals were removed from the study when tumor growth or neurological complications exceeded veterinary guidelines. These data indicate that while radiation and anti-PD-1 were slightly beneficial alone, the combination had the greatest impact on animal survival with about 20% of the mice still being alive at day 25. To observe the effect of radiation and anti-PD-1 treatment on individual, we assessed their growth for 10 days following radiation using calipers (for flank tumors) or by bioluminescence imaging (for flanks and brain). Interestingly, the flank tumors on mice treated with anti-PD-1 and irradiation to the head were most growth inhibited, and progressed much less than the tumors on mice given either treatment alone, suggesting that the beneficial effects of combined therapy can be seen soon after radiation treatment ends (Figure 2C). We then followed growth of the flank tumor in all four treatment groups out over a longer period of time (20 days post radiation), and found that the group treated with both radiation and anti-PD-1 antibody continued to have a significant delay over either treatment alone. Of note, we found that head irradiation alone was not sufficient to have a long-term effect on growth of the flank tumor, which suggests that head irradiation served to enhance the effect of the anti-PD-1 treatment on the non-irradiated flank tumor (Figure 2C and D). Similar results were observed using bioluminescence imaging of the flank tumors on Day 20. At this time point, we also observed that both radiation and anti-PD-1 treatment (and the combination) were sufficient to reduce the BM

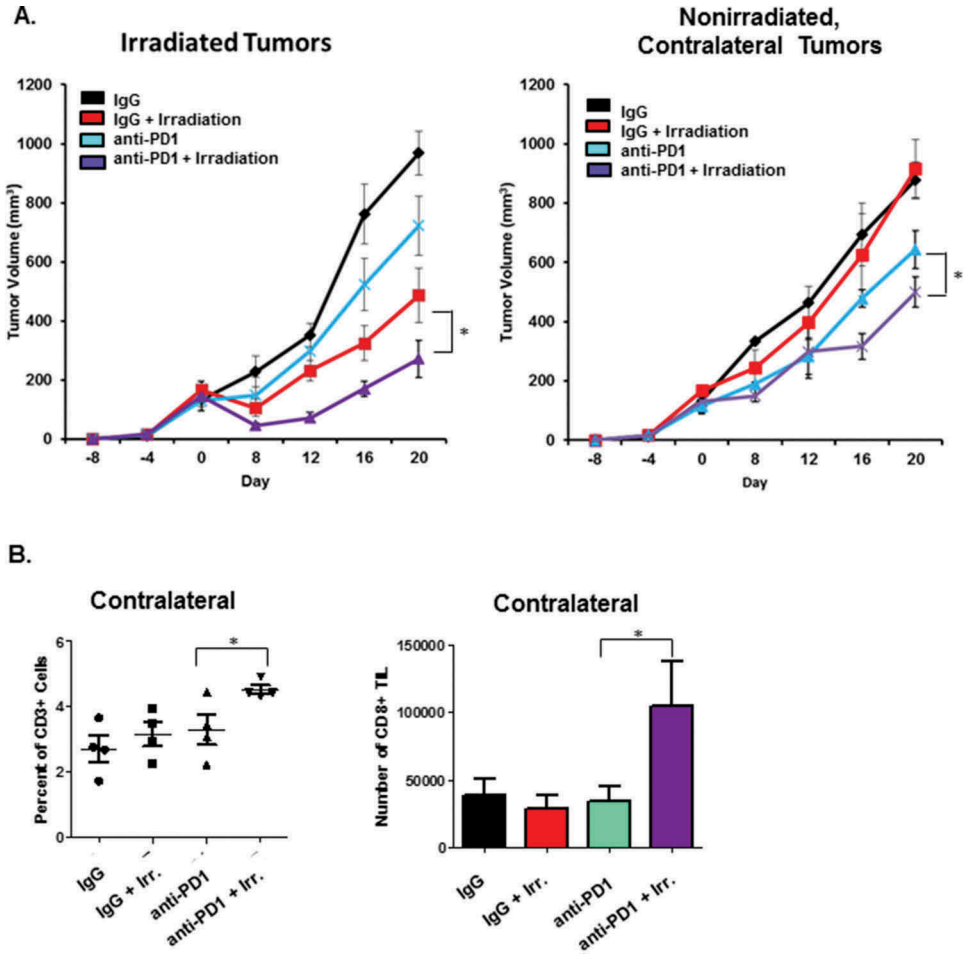

Figure 1. Combination radiation and anti-PD-1 therapy induces an abscopal effect in contralateral flank tumors. A. 1.5×10^5 B16-F10 tumor cells were injected in to each flank of wildtype C57BL/6 mice. Nine days later, mice received 150 μg anti-PD-1 antibody or IgG via intraperitoneal injection. After five days, indicated mice received 8 Gy in 4 fractions to one flank tumor. Growth of the irradiated (left) and non-irradiated contralateral tumor (right) was followed for 20 days. B. At the conclusion of the experiment, tumor tissues were harvested and digested to a single cell suspension with collagenase/hyaluronidase. T cell phenotype and frequency was determined using staining for readout by flow cytometry. Indicated study is representative of the independent experiments. *P < 0.05 for the indicated comparisons at day 20 in A. and between anti-PD-1 and anti-PD-1 + head irradiation groups in B.

tumor to a near-undetectable levels when measured by bioluminescence imaging. (Figure 2E and F).

BM irradiation and anti-PD-1 therapy enhance anti-tumor immunity

Having observed that the combination of radiation and anti-PD-1 antibody could combine to significantly delay tumor growth in distant, non-irradiated D4M tumors we next sought to determine whether the tumor delay was associated with increased immune infiltrate into the tumor tissues. Immunofluorescence assays of brain tumors harvested 15 days after irradiation revealed significantly greater numbers of both $CD8^+$ and $CD4^+$ T cells in PD-1 treated groups and a significant increase in $CD8^+$ T cells in anti-PD-1 plus irradiation treated mice over anti-PD-1 alone (Figure 3A and B). At the conclusion of the experiment, flank tumors were harvested, dissociated by collagenase digestion and assayed for immune cell infiltrate by flow cytometry. These studies revealed that the number and percentage of $CD4^+$ T cells was largely unchanged by either radiation or anti-PD-1 treatment or the combination (Figure 3D–F). In contrast, we found significantly more $CD8^+$ T cells in both frequency and number in the combination PD-1 antibody and irradiation treated group, and a modest increase in the anti-PD-1 alone group (Figure 3G–I). We also assessed Ki-67 expression on tumor-resident T cells and found significantly higher expression on both $CD4^+$ and $CD8^+$ T cells in the combination treatment group (Figure 3C). When $CD8^+$ TIL were re-stimulated *ex vivo* using anti-CD3/CD28, we found that T cells from anti-PD-1 alone treated tumors produced more interferon-γ (IFNγ) than either control IgG or radiation alone, but $CD8^+$ T cells from combination-treated tumors produced dramatically more. Together these data indicate that the optimal delay in flank tumor growth observed in animals treated with both head irradiation and anti-PD-1 antibody we associated with an enhanced anti-tumor immune response that was mediated by $CD8^+$ cytotoxic T cells (Figure 3J). In addition to increased IFNγ, we also found significantly greater t-bet expression in anti-PD-1 combination groups as compared to IgG alone, and a trend toward greater granzyme B expression in these T cells (Figure 3K). Lastly, we assessed the effects

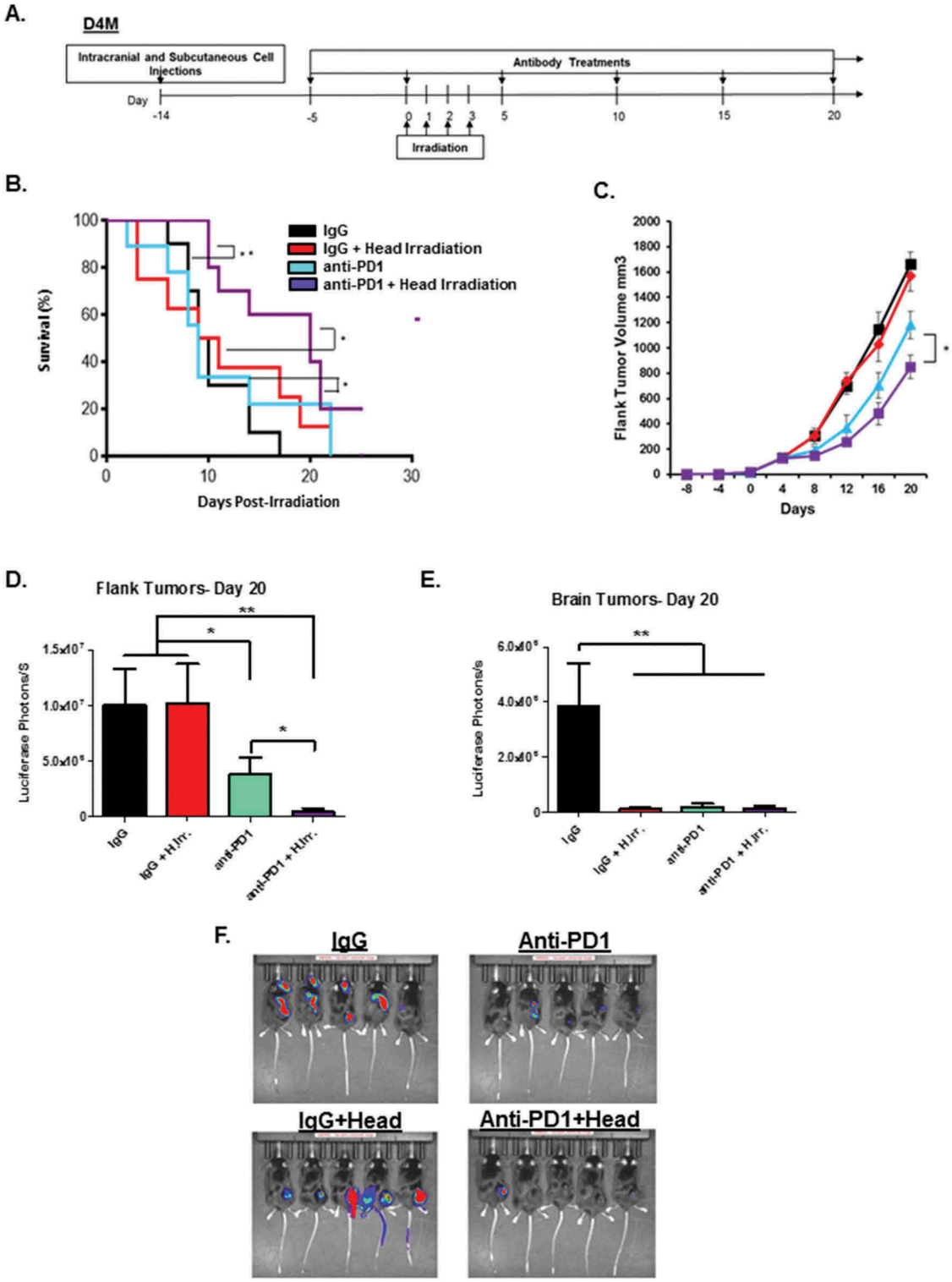

Figure 2. Irradiation and PD-1 blockade therapy induce an additive effect in a model of metastatic melanoma of the brain. A. Graphical representation of the experimental setup and treatment schedule for head irradiation experiments. B. Mice receiving concomitant D4M tumors implanted intracranial and in the flank were treated with IgG or anti-PD-1 antibody followed by four doses of 2 Gy irradiation to the head according to the experimental plan in A. Mice were then followed for 25 days post irradiation, and survival is indicated by the Kaplan-Meier plot. C. Growth in flank tumor size as measured by volume for the indicated groups is represented. On day 20, IVIS bioluminescence imaging of the animals was performed and graphs represent the intensity of luminescence of flank tumors (D.) and brain tumors (E.). Representative bioluminescence images at this time point. (F.) *p < 0.05 and **p < 0.01 for comparisons between the indicated groups.

of radiation, anti-PD-1 antibody treatments or the combination on other common regulatory immune cells in the tumor microenvironment. The tumor microenvironment has been demonstrated to contain significantly high numbers of cells like $CD4^+$ regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC), and their presence is associated with immunosuppression and poor anti-tumor immune responses.[49,50] In our model of

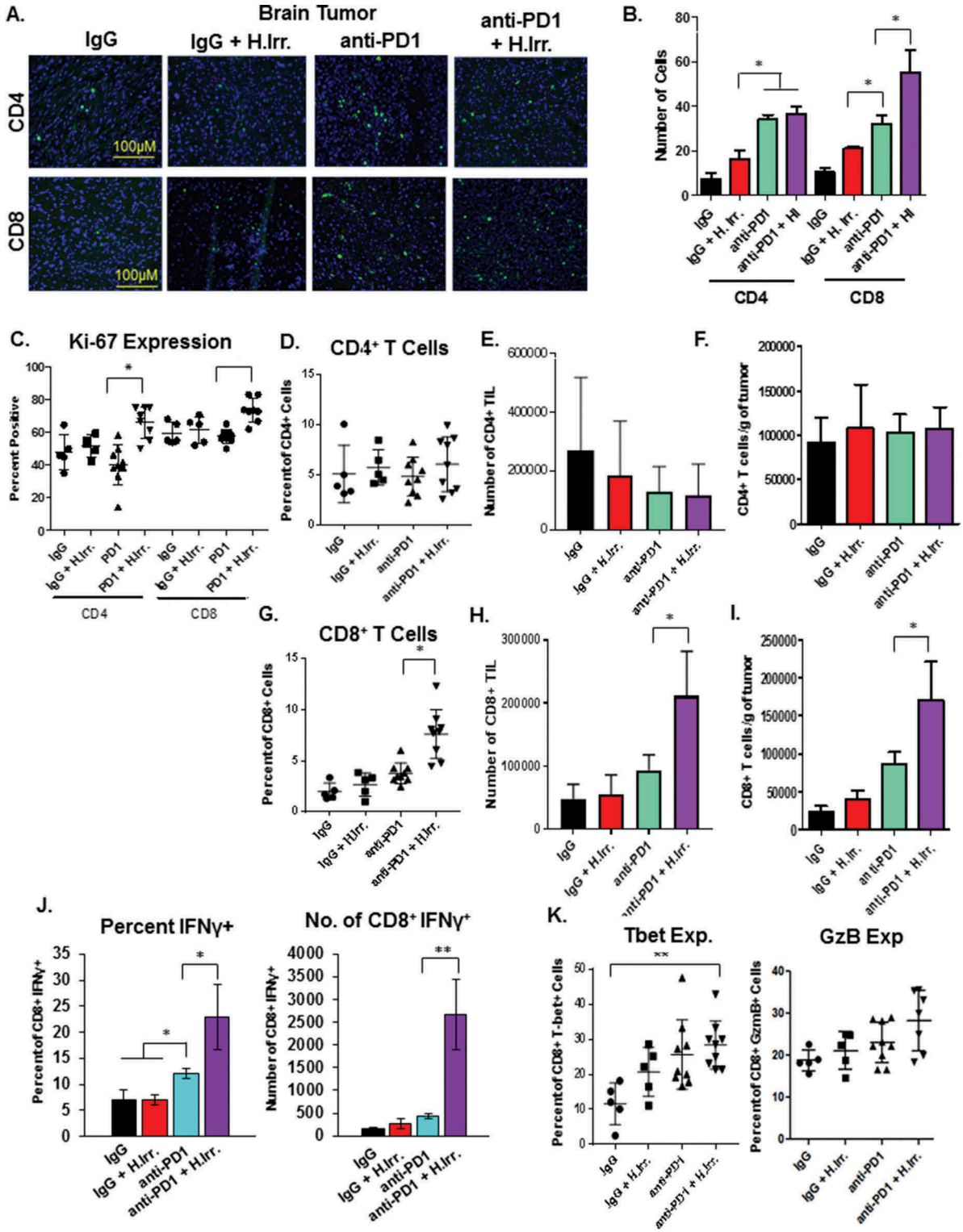

Figure 3. Abscopal effects induced by radiation and anti-PD1 combination therapy are associated with increased anti-tumor immunity. A. At the conclusion of the previous experiment, brain tissue was sectioned and T cell markers stained for immunofluorescence microscopy. B. Quantification of the number of cells in at least four independent fields for each treatment group. C. Flank tumor tissue was harvested and dissociated by collagenase/hyaluronidase digestion and immune cell markers were stained for readout and quantification by flow cytometry. Ki-67 expression was assessed on the indicated T cell subsets by permeabilization and staining followed by readout by flow cytometry. D-I. T cell subset percentages in non-irradiated flank tumor tissue were compared to total cell recovery to determine total number of T cells and ratio of CD8T cells to regulatory CD4T cells (Treg). $CD4^+/CD8^+$ percentages in D. and G. are of total $CD45^+$ cells. J. $CD8^+$ T cells from non-irradiated flank tumors were restimluated *ex vivo* with plate-bound anti-CD3 and anti-CD28 antibodies followed by intracellular cytokine staining for interferon-gamma (IFNγ) staining. K. Expression of the indicated markers of cytotoxic T cell function on $CD8^+$ T cells from non-irradiated flank tumor tissue were stained following permeabilization and read-out by flow cytometry. *p ≤ 0.5 and **p ≤ 0.01 for indicated comparisons between anti-PD-1 and anti-PD-1 plus head irradiation treatment groups according to a student's t test.

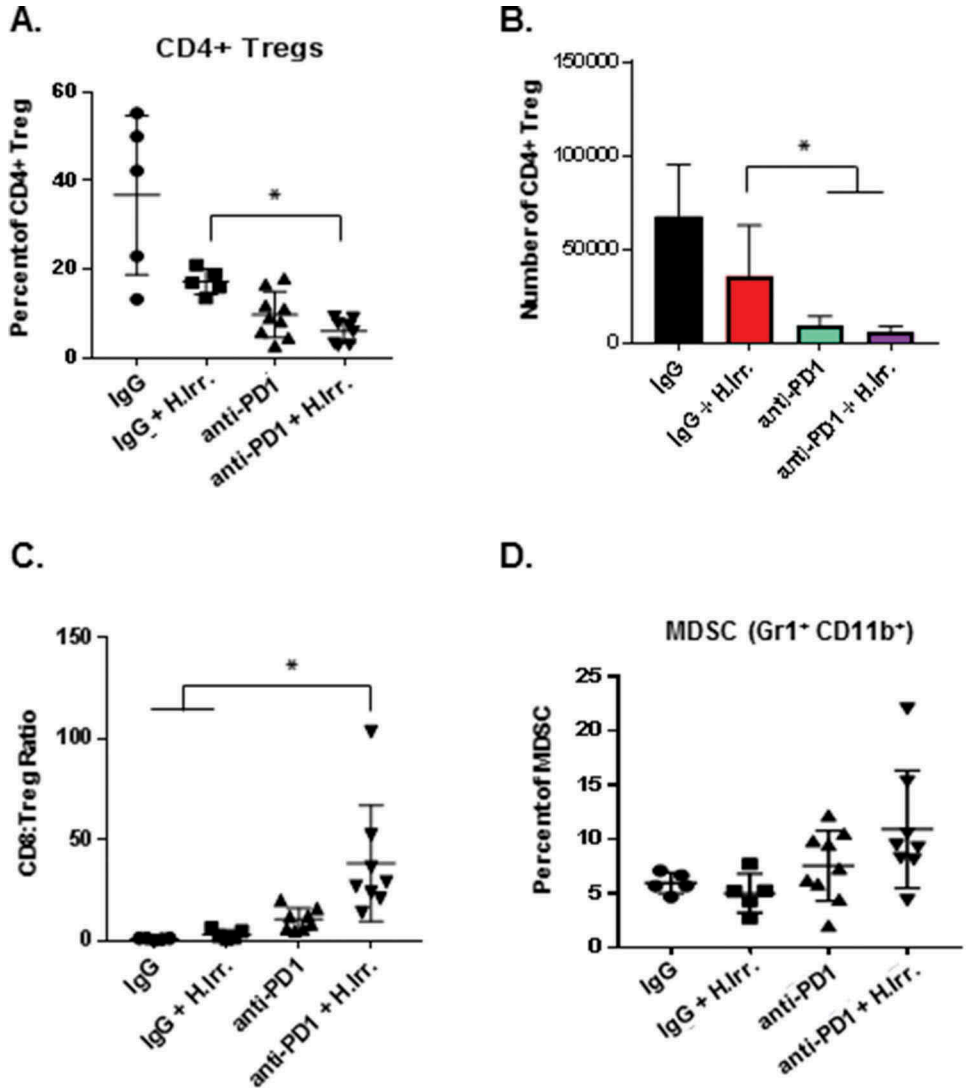

Figure 4. Effect of combination BM irradiation and PD-1 treatment on regulatory immune cell populations in distant flank tumors. A. Tissue from flank D4M tumors were harvested at the conclusion of the experiment described in Figure 3, dissociated by collagenase/hyaluronidase digestion, and stained for regulatory T cell markers before being quantified by flow cytometry. Regulatory T cells were defined as $CD25^{hi}$ $Foxp3^{+}$ cells among $CD3^{+}$ $CD4^{+}$ T cells. B. Based on the percentages of the flow stain, the numbers of Treg per tumor and the ratio of these cells to the number of $CD8^{+}$ T cells was determined (B, C). D. The percentage of myeloid derived suppressor cells (MDSCs) was determined in the tumors by anti-body staining and flow cytometry. Cells were gated on $CD45^{+}$ $Gr1^{+}$ $CD11b^{+}$ populations. *$p \leq 0.5$ for the indicated comparisons to the anti-PD-1 plus irradiation group.

irradiation plus anti-PD-1 therapy of BM, we found that while anti-PD-1 treatment alone was able to slightly decrease then percentage of $CD4^{+}$ Treg in the flank tumor, the combination of PD-1 plus head irradiation resulted in the greatest, most significant decrease. (Figure 4A) This decrease was also observed in the number of these cells (Figure 4B). We then assessed the ratio of the number of $CD4^{+}$ Treg and $CD8^{+}$ T cells in each treatment group and found that the combination therapy group had significantly larger ratio of $CD8^{+}$ T cells to Treg (Figure 4C). Lastly, we assessed the percentage of $GR1^{+}$ $CD11b^{+}$ MDSC among $CD45^{+}$ cells in the tumor and found that while both anti-PD-1 alone and combination treatment resulted in a slight increase in MDSC, these differences were not statistically significant from IgG treated groups (Figure 4D). This result suggests that anti-PD-1 treatment alone may not affect MDSC cell frequency, since these cells express the ligand of PD-1, PD-1L. Overall, these data suggest that the reduced tumor growth effects of combination anti-PD-1 therapy and irradiation reduce tumor growth by inducing a stronger $CD8^{+}$ cytotoxic T cell response directed against the non-irradiated tumor.

Combination therapy induces an abscopal effect in multiple melanoma models

We next extended our model of immunotherapy of metastatic melanoma of the brain and flank with using the B16 model of melanoma which expresses wildtype BRAF. Tumors were established intracranially and subcutaneously in the flank similar to those using D4M cells. Likewise, anti-PD-1 antibody or IgG control treatment began five days before radiotherapy, which followed the same dosing schedule used previously (4 fractions of 2 Gy each). Following brain tumor growth by bioluminescence imaging, we found that, over the

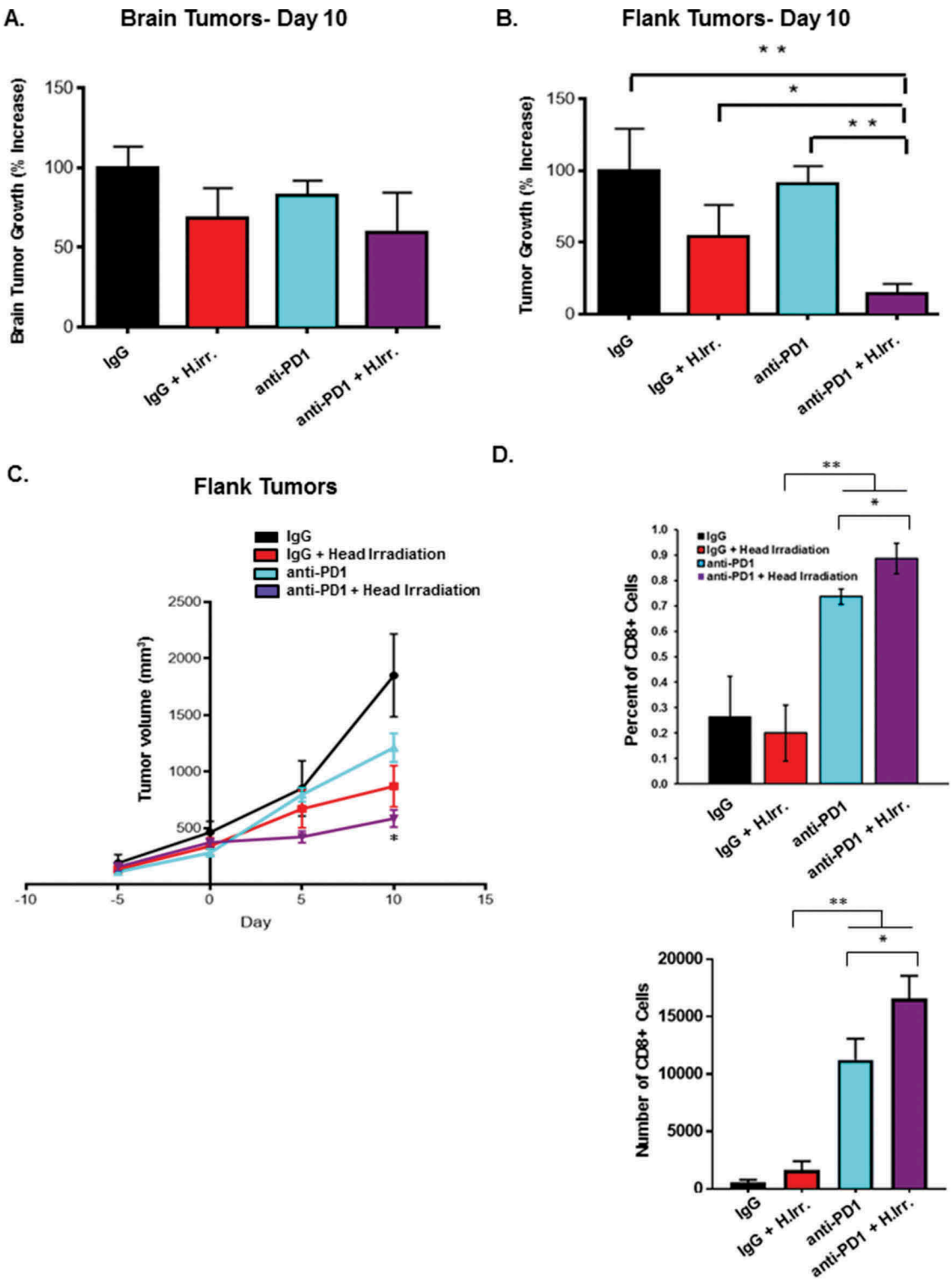

Figure 5. Anti-PD-1 treatment and irradiation induces an abscopal effect in B16 tumors. B16 tumors were established intracranially and subcutaneously in the flank similar to those using D4M cells. Brain (A.) and flank (B.) tumor growth was followed by bioluminescence imaging and graphs represent percent in growth. C. D. At the conclusion of the experiment, tumors were harvested and dissociated by collagenase/hyaluronidase digestion followed by staining for $CD8^+$ T cell markers. Cells were then read-out and quantified by flow cytometry. *$p < 0.5$ and **$p < 0.01$ for the indicated comparisons between treatment groups. For the tumor growth graph. *$p < 0.05$ for the combination group vs. others at the final time point using ANOVA analysis.

course of the first 10 days following treatment, that radiation or anti-PD-1 or the combination was able to modestly slow tumor growth as compared to IgG alone control, with only slight differences between anti-PD-1 and combination groups (Figure 5A). When we assessed growth of the flank tumors by bioluminescence imaging over the course of the first 10 days post-radiation, we found that the combination treatment had the greatest effect on tumor growth, which was significantly less than both the anti-PD-1 alone and radiation plus IgG control groups; a similar finding to what we observed using

the D4M model. Consistent with the D4M model, the effects of combination treatment on flank tumor growth were visible over the duration of the experiment (Figure 5B and C). These data from 2 different mouse models suggest that the benefit of combination therapy is independent of BRAF mutational status and may be generalizable. Lastly, to verify that the delay in tumor growth observed in treatment groups was due to enhanced anti-tumor immune responses, we assessed the frequency and number of $CD8^+$ T cells present in the flank tumors at the conclusion of the experiment by dissociation in collagenase followed by flow cytometry. Similar to our previous D4M studies, anti-PD-1 treated groups had dramatically greater frequency and numbers of $CD8^+$ T cells present in the tumor tissue, while the combination head irradiation and anti-PD-1 treatment group had significantly greater T cell infiltrate than anti-PD-1 alone (Figure 5D). Together these data confirm our observation that the radiation of a distant melanoma tumor in the brain in combination with systemic anti-PD-1 therapy can enhance a systemic anti-tumor T cell response that can have beneficial effects on other metastatic tumors in the same animal.

Discussion

In the present study we report that anti-PD-1 antibody therapy in combination with radiation of mouse intracranial brain melanoma tumors can induce an abscopal effect slowing the growth of a non-irradiated flank tumor in the same animal that is greater than the use of the antibody alone. Consistent with clinical observations, anti-PD-1 therapy alone can significantly inhibit tumor growth in our model, but when combined with radiotherapy of the intracranial tumor, anti-PD-1 dramatically enhances the anti-tumor immune response against the unirradiated flank tumor as well. Likewise, we found that radiation alone was unable to induce any measurable change in the size or immune composition of non-irradiated flank tumors; however the combined radiation and anti-PD-1 therapies induced systemic immune responses in nearly every animal. These results are similar to those reported for other, recent preclinical studies that used anti-PD-1 (as well as other checkpoint blockade antibodies), though ours is the first to include irradiation of melanoma brain tumors specifically.[43,51-53]

The phenomenon of the radiation-induced abscopal effect was first described by Mole in 1953, but this rarely occurs in the clinic when using only routine radiotherapy regimens.[54] Increasing appreciation that these occasional tumor regressions were likely immune-mediated, together with recent insights into how irradiation actually enhances the immunogenicity of tumor tissue, has renewed interest in using immunotherapies to amplify these responses. In fact, immune involvement is increasingly appreciated as a major mechanism of radiotherapy efficacy.[55] For instance, radiation-induced cell death has been shown to release proteins such as HMGB1 that can act as immunological danger signals that can enhance anti-tumor T cell responses.[56] Indeed, clinical studies have found a positive correlation between serum levels of these danger signal proteins and overall survival.[57]

The brain and central nervous system have traditionally been considered immune-privileges sites based on the ability of the blood brain barrier to largely inhibit the influx of both immune effectors and antigen presenting cells[58]. More recent studies, however, indicate that both antigen-presenting cells and activated T cells are more adept at infiltrating central nervous tissues than previously thought.[11,12,59-61] Further, BM were shown to contain large numbers of T cells and the extent of T cell infiltration correlates with survival prognosis.[62,63] Pre-clinical animal data have also demonstrated that radiation can increase the permeability of the blood-brain barrier.[64] Further, immunoglobulins such as antibodies administered as part of immunotherapy were found to penetrate the blood brain barrier into nerve and brain tissue, where they exert their beneficial effects on tissue-resident T cells, particularly after radiotherapy.[65] These observations influenced our decision to initiate anti-PD-1 administration prior to radiation treatment, and suggest that optimal clinical use of these treatments should follow similar timing schedules.

The use of radiation for the treatment of brain metastases in melanoma is an increasingly common procedure with stereotactic techniques that have high rates of local control up to 80% and have fewer complications compared to whole-brain irradiation.[66,67] Despite these local successes, disease progression often occurs in distant metastases, which underscores the need for combinatorial strategies that have systemic effects, including immunotherapy. Recent, promising retrospective studies found that the combination of radiosurgery with ipilimumab (anti-CTLA-4, a related immune checkpoint signaling protein) raised the median survival of metastatic melanoma patients with brain metastases from 4.9 to 21 months, though these studies could only speculate about immune involvement in this phenomenon.[68-70] Whether the positive benefits of combining radiation of brain metastases with immunotherapy were true synergistic effects or worked separately was not addressed by these studies. The animal model data in our current study indicate that these two treatment strategies work together.

Lastly, the applicability of this treatment strategy to both BRAF mutant and wildtype forms of melanoma is also notable in that non-mutated BRAF tumors have fewer options for targeted therapy (due to the absence of mutant BRAF), making anti-PD-1 and radiation combination therapy an important strategy to treat both kinds of melanoma together. In conclusion, our findings highlight the benefit of combining immune checkpoint blockade of the PD-1/PDL-1 signaling axis with radiotherapy of BM for treating not just localized tumors in the brain, but also of distant metastases via induction of an abscopal effect. Our data further suggest that these two increasingly common treatment modalities for metastatic melanoma would have synergistic effects in the clinic, and provides the basis for the use of other combinations of radiation and immunotherapy in future treatment strategies.

Materials and methods

Cell lines

The B16-F10 tumor cell line has been previously described and was obtained from the American Type Culture Collection. D4M melanoma cells have been previously described and were a generous gift of Dr. Molly Jenkins (Dartmouth University).[32] Both cell lines were transduced to express firefly luciferase as previously described.[71] All cells were maintained in RPMI with 10% Fetal Bovine Serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 2 mmol/L nonessential amino acids and 100 u/ml penicillin/streptomycin in an incubator containing 10% CO_2. 1 μg/ml puromycin was used to select for transduced cells.

Mouse tumor implantation, irradiation, and TIL isolation

C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). For tumor growth experiments, 150,000 B16 or D4M tumor cells were implanted subcutaneously in 100 μl sterile saline and tumor growth was monitored by measuring tumor diameter with calipers every third day for the duration of the experiment. For intracranial tumors 2×10^4 tumor cells were injected as previously described.[40] Briefly, cells were implanted into the right frontal lobes of wildtype C57BL/6 mice. For bioluminescence imaging, mice were given d-luciferase (Promega) via i.p. injection before imaging using an IVIS imaging system. Animals were immobilized with isoflurane during this procedure. For antibody-treatment, anti-PD-1 antibody or an IgG control were injected at 150 μg/dose i.p. starting five days before irradiation and continuing every fifth day for the duration of the experiment. For irradiation, animals were immobilized via a single injection of ketamine and xylazine and irradiation was delivered to the mouse head or flank tumor using a Pantak X-ray irradiator. Lead shielding was used to limit radiation exposure to other areas of the body. Four 2 Gy doses were administered daily for a total radiation dose of 8 Gy. Tumor-infiltrating lymphocytes were isolated by disruption of tumor tissue first by mincing with crossed scalpels under sterile conditions followed by enzymatic digestion as described above. Live cells were isolated from debris by centrifugation over a Ficoll gradient prior to staining for flow cytometry or cryopreservation for further analysis. All animals were housed in the Biological Resources Unit of the Cleveland Clinic Lerner Research Institute according to Office of Laboratory Animal Welfare guidelines and experiments were conducted under an approved Institutional Animal Care and Use Committee protocol.

Antibodies, immunofluorescence, and flow cytometry

Fluorescently conjugated, anti-mouse antibodies were purchased from Biolegend (San Diego, CA) (CD3-PerCP, CD8 FITC, CD8 APC, PD-1 FITC, CD45 AlexaFluor700, Ki-67 APC or PerCP Cy5.5, CD25 FITC, Foxp3 PE, Gr1 APC, CD11b AlexaFlour 647, Interferon-γ PE, Granzyme B APC, Tbet APC). Prior to staining, all cells were treated with anti-FcγIII/CD16 antibody from Biolegend according to the manufacturer's recommended protocol (human/mouse TrueStain FCX). Antibody staining was performed in phosphate-buffered saline with 0.1% fetal bovine serum or bovine serum albumin. Data were collected on FACS Calibur or LSR II instruments and analyzed with the FlowJo data analysis software (FlowJo Inc, Salem OR). Anti-PD-1 antibody (mDX400) used *in vivo* was and the IgG control were provided by Merck & Company via a Materials Transfer Agreement and has been described previously.[72] Immunofluorescent staining of cells and tissues sections was performed as described.[73] Quantification of immunofluorescence images was performed using ImageJ software (https://imagej.nih.gov/ij/).

Statistics

Means of all groups were compared for statistical differences by Student's t test or a One-Way Analysis of Variance (ANOVA). A Bonferroni t test was used, following the ANOVA, to understand the statistical difference between two groups, when more than two groups were compared. Data was presented as means ±SD. Significance levels were set to $p < 0.05$.

Disclosure statement

Merck & Co., Inc., Kenilworth, NJ USA provided anti-PD-1 antibody and financial support for the study.

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