Cancer Research

In Situ Tumor Vaccination by Combining Local Radiation and Tumor-Specific Antibody or Immunocytokine Treatments

Zachary S. Morris[1], Emily I. Guy[1], David M. Francis[1], Monica M. Gressett[1], Lauryn R. Werner[1], Lakeesha L. Carmichael[2], Richard K. Yang[1], Eric A. Armstrong[1], Shyhmin Huang[1], Fariba Navid[3], Stephen D. Gillies[4], Alan Korman[5], Jacquelyn A. Hank[1], Alexander L. Rakhmilevich[1], Paul M. Harari[1], and Paul M. Sondel[1,6,7]

Abstract

Interest in combining radiotherapy and immune checkpoint therapy is growing rapidly. In this study, we explored a novel combination of this type to augment antitumor immune responses in preclinical murine models of melanoma, neuroblastoma, and head and neck squamous cell carcinoma. Cooperative effects were observed with local radiotherapy and intratumoral injection of tumor-specific antibodies, arising in part from enhanced antibody-dependent cell-mediated cytotoxicity (ADCC). We could improve this response by combining radiation with intratumoral injection of an IL2-linked tumor-specific antibody (termed here an immunocytokine), resulting in complete regression of established tumors in most animals associated with a tumor-specific memory T-cell response. Given the T-cell response elicited by combined local radiation and intratumoral immunocytokine, we tested the potential benefit of adding this treatment to immune checkpoint blockade. In mice bearing large primary tumors or disseminated metastases, the triple-combination of intratumoral immunocytokine, radiation, and systemic anti–CTLA-4 improved primary tumor response and animal survival compared with combinations of any two of these three interventions. Taken together, our results show how combining radiation and intratumoral immunocytokine in murine tumor models can eradicate large tumors and metastases, eliciting an *in situ* vaccination effect that can be leveraged further by T-cell checkpoint blockade, with immediate implications for clinical evaluation. *Cancer Res; 76(13); 3929–41. ©2016 AACR.*

Introduction

Radiation and tumor-specific antibodies (mAbs) are frequently used together in the treatment of human cancers. Nevertheless, the potential interaction of radiation with the antitumor immune effects induced by tumor-specific mAbs has not been well elucidated. Radiation elicits an antitumor effect through the induction of DNA damage, yet may also affect tumor immune tolerance (1). In rare instances, local radiation treatment can trigger a systemic or "abscopal" immune response at nonradiated tumor sites in patients with metastatic disease. Tumor-specific mAbs are commonly designed to antagonize a target molecule on tumor cells, but may also initiate a tumor-directed immune response by engaging Fcγ receptors (FcγR) on innate immune cells (2). Upon binding the Fc portion of mAb, these immune cells can destroy mAb-bound tumor cells through the process of antibody-dependent cell-mediated cytotoxicity (ADCC). Tumor-specific mAbs bound to dying tumor cells can also interact with FcγR on antigen-presenting cells, resulting in enhanced antigen presentation to the adaptive immune system, thereby augmenting activation of a T-cell response (3).

We have been exploring approaches to enhance the immune response induced by administration of mAb-based therapies that are able to selectively bind to specific antigens on the surface of tumor cells. Our focus has been on mAbs targeting disialoganglioside D2 (GD2), which is expressed in neuroblastoma and melanoma (4). Antibodies targeting GD2 are thought to elicit antitumor effects primarily through ADCC (5–7). Others and we have been exploring how increased activation of ADCC effector cells may augment this effect (8–11). We have investigated the effect of cytokines that activate natural killer (NK) cells and myeloid elements (12) and demonstrated that treatment with anti-GD2 mAb, combined with IL2 and GM-CSF, improves overall survival in children with neuroblastoma (13). These studies attest to the potential of combinatorial approaches to augment immune response to tumor-specific mAbs.

Multiple studies of clinically relevant murine tumor models indicate that the most immunogenic tumor antigens recognized by T cells are "private antigens" derived from mutated proteins in tumor cells (14, 15). *In situ* tumor vaccination is a therapeutic

[1]Department of Human Oncology, University of Wisconsin, Madison, Wisconsin. [2]Department of Biostatistics and Bioinformatics, University of Wisconsin, Madison, Wisconsin. [3]Department of Oncology, St. Jude Children's Hospital, Memphis, Tennessee. [4]Provenance Biopharmaceuticals, Carlisle, Massachusetts. [5]Bristol Myers Squibb, Redwood, California. [6]Department of Pediatrics, University of Wisconsin, Madison, Wisconsin. [7]Department of Genetics, University of Wisconsin, Madison, Wisconsin.

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Corresponding Author: Zachary Morris, University of Wisconsin, 1111 Highland Avenue, WIMR, 3rd floor, Madison, WI 53705. Phone: 608-263-8500; Fax: 608-263-9167; E-mail: zmorris@uwhealth.org

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strategy aimed at taking advantage of these antigens by converting a patient's tumor into a nidus for adaptive immunologic recognition (16). In this report, we test whether radiation might augment the antitumor immune response induced by tumor-specific mAbs in multiple tumor-bearing mouse models. We characterize a cooperative interaction between local radiation and intratumoral (IT) delivery of tumor-specific mAb therapeutics and demonstrate the capacity of this combined treatment to elicit an *in situ* vaccination effect that may be leveraged to improve the response to systemic T-cell checkpoint blockade.

Materials and Methods

Cells

B78-D14 (B78) melanoma is derived from B16 melanoma, as previously described (17) and was obtained from Ralph Reisfeld (Scripps Research Institute) in 2002. B16-F10 melanoma was obtained from the ATCC in 2005 and the Panc02 pancreatic tumor cells were obtained from the NCI in 2012. B78, B16, and Panc02 cells were grown in RPMI-1640 (Mediatech) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. NXS2 is a murine neuroblastoma hybrid cell line obtained from Ralph Reisfeld (Scripps Research Institute) in 1997 and grown as previously described (18). The acquired cetuximab-resistant clone, SCC1-C, was derived from UM-SCC1 cells (Thomas Carey, University of Michigan, Ann Arbor, MI) in 2009 and cultured as previously described (19). Cell authentication was performed per ATCC guidelines using morphology, growth curves, and *Mycoplasma* testing within 6 months of use.

Clonogenic and cytotoxicity assays

In vitro clonogenic (20) and ^{51}chromium-release cytotoxicity assays (21) were performed as previously described. For clonogenic assays, mAb, IC, or IgG were introduced at 1 μg/mL 30 minutes before radiation and maintained in media for the duration of experiments. For cytotoxicity assays, target cells were labeled with ^{51}chromium and incubated for 4 hours in the presence of 1 μg/mL cetuximab or control IgG with or without fresh peripheral blood mononuclear effector cells (21). ADCC was measured using a beta counter (Packard Matrix 9600) to quantify release of ^{51}chromium.

Murine tumor models

Animals were housed and treated under an animal protocol approved by the institutional animal care and use committee. Female mice were purchased at age 6 to 8 weeks from Taconic (C57BL/6 and Fcγ receptor-deficient C57BL/6.129P2-*Fcer1g*^{tm1Rav} N12), Harlan (NcR nude mice), and Jackson (A/J and FasL-deficient C57BL/6 Smn.C3-Tnfsf6gld/J).

B78, B16, NXS2, and Panc02 tumors were engrafted by subcutaneous flank injection of 2×10^6 tumor cells. For disseminated disease models 3.5×10^5 B16 cells were IV injected. Engraftment of SCC1-C was performed by subcutaneous flank injection of 3×10^6 cells = in 2:1 PBS:Matrigel (BD Biosciences).

Tumor size was determined using calipers and volume approximated as (width^2 × length)/2. Mice were randomized immediately before treatment. The day of radiation was defined as "day 1" of treatment. IT injections were made by a single percutaneous needle puncture followed by injection of a 100 μL volume with needle redirection to distribute injected material around the tumor. IT injections of 50 μg hu14.18K322A, cetuximab, hu14.18-IL2, or control IgG were delivered daily on days 6 to 10. Anti–CTLA-4 or control IgG was administered by 200 μg intraperitoneal (IP) injection days 3, 6, and 9. For NK cell depletion, IP injections of 500 μg NK1.1 mAb (clone PK136, ATCC) were delivered days 1, 5, 10, and 15. Depletion of T cells was performed as previously described (22).

Treatment began when tumors were well established (~200 mm^3), occurring approximately 5 weeks after tumor implantation for B78 melanoma. For "large" B78 tumors (~500 mm^3), treatment began approximately 7 weeks after implantation. Animals were sacrificed when tumors exceeded a pre-determined dimension. Mouse experiments were repeated in triplicate. Final replicates are presented for tumor response and aggregate data for survival; number of animals (n) per group is indicated.

Radiation

Radiation of cells *in vitro* was performed using a cesium source irradiator (JL Shepherd Model 109). Radiation was delivered to *in vivo* tumors by an X-RAD 320 (Precision X-Ray, Inc.). Mice were immobilized using custom lead jigs that exposed the dorsal right flank. For i*n vivo* experiments radiation was delivered in one fraction to a maximum dose of 12 Gy.

Antibodies and Immunocytokine

Hu14.18K322A was provided by Children's GMP, LLC (6). A monovalent Fab-only fragment of this was generated using a Pierce kit (Thermo Scientific, No. 44985). Cetuximab (Eli Lilly) and Gammagard non-specific human IgG (Baxter) were obtained through the University of Wisconsin pharmacy. Hu14.18-IL2 was provided by Apeiron Biologics (23). Anti–CTLA-4 (clone 9D9) was provided by Bristol Myers Squibb.

Immunohistochemistry

Immunohistochemistry was performed on at least nine distinct tumor sections from at least 3 mice for each treatment condition to characterize and quantify tumor immune cell infiltrate on day 12 following radiation as previously described (24).

Flow cytometry

Flow cytometry was performed as previously described (25) using a MacsQuant analyzer (Miltenyi Biotec). Cells were labeled as indicated using hu14.18 + PE-conjugated anti-human mAb (SAB1294; Open Biosystems), FITC-labeled anti-Fas/CD95 (15404D, BD Biosciences), respective isotype control (Human IgG, Baxter; FITC-labeled Hamster IgG, Pharmingen), and/or DAPI. FlowJo Software was used for analysis. Forward- and side-scatter gating identified single cells and DAPI exclusion identified live cells. For Fas-activation experiments, cells were radiated with 12 Gy and 6 days later were incubated for 18 hours with 1 μg/mL Fas-activating mAb (JO-2, BD Biosciences) or control IgG in normal media at 37°C. Cells were stained with propidium iodide without fixation and apoptotic fraction was defined by the ratio of propidium-positive to total cells.

Quantification of lung metastases

Animals injected IV with B16 melanoma were sacrificed 15 to 20 days after radiation. All animals within an experiment were sacrificed on a pre-determined day or earlier if they became moribund. Blinded quantification of metastatic foci per lung was performed (B16 tumors are black). The experiment was performed in triplicate and aggregate data are presented.

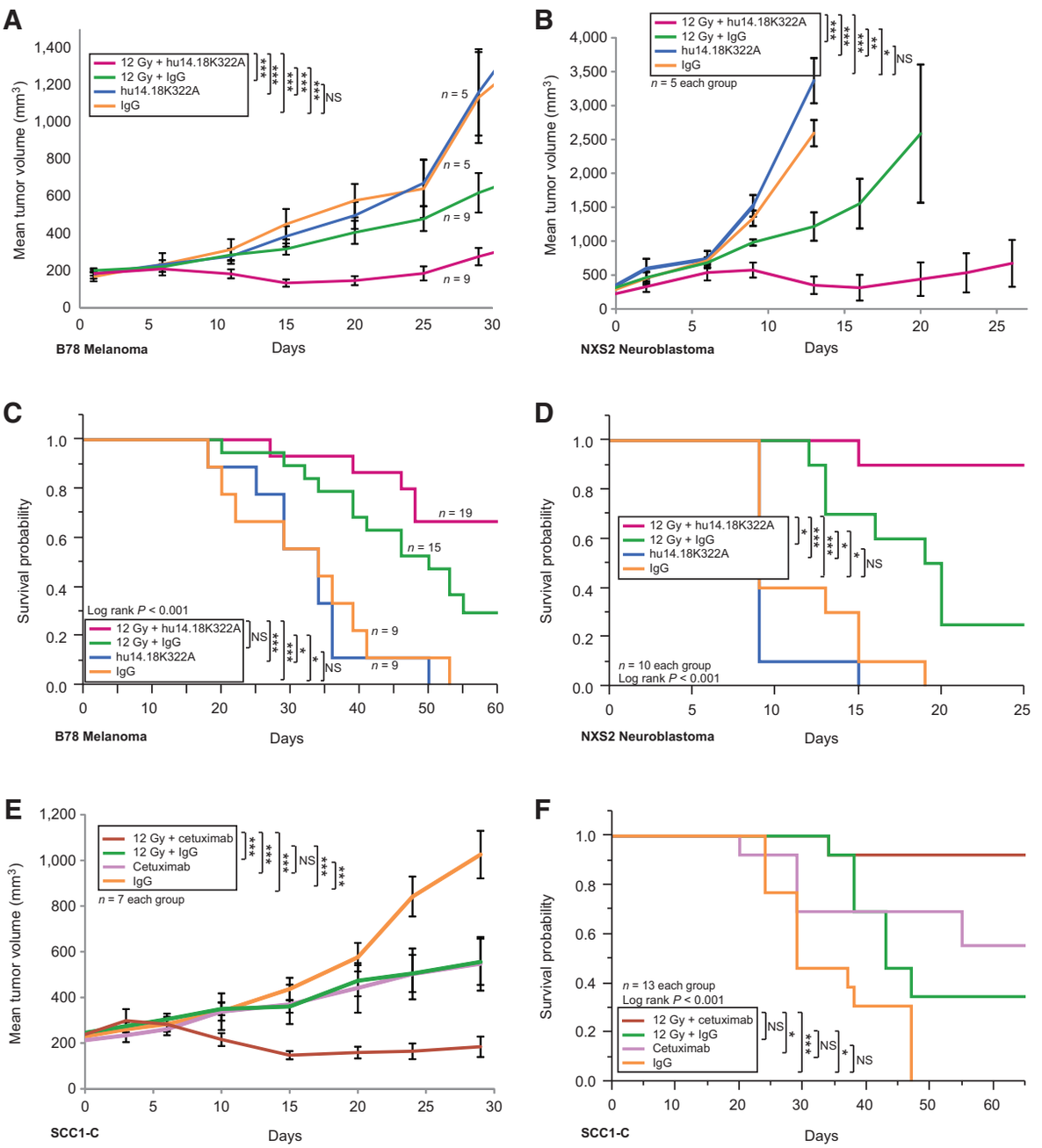


Figure 1.
Cooperative interaction of radiation and tumor-specific mAb in murine tumor models. Tumor growth curves are shown for C57BL/6 mice bearing B78 melanoma (A) and A/J mice bearing NXS2 neuroblastoma (B). In both cases, hu14.18K322A had no impact on tumor growth compared with IgG, but treatment with hu14.18K322A and radiation resulted in tumor shrinkage ($P < 0.0001$, day 15 vs. 6) compared with radiation plus IgG. Survival plots are shown for C57BL/6 mice bearing B78 melanoma (C) and A/J mice bearing NXS2 neuroblastoma (D). For B78, log-rank analysis demonstrates significant differences in survival and a nonsignificant trend toward improved survival with hu14.18K322A plus radiation compared with IgG plus radiation. For NXS2, improved survival is observed with hu14.18K322A plus radiation compared with IgG plus radiation. In nude mice bearing human SCC1-C tumor xenografts, the combination of radiation and cetuximab (E) significantly enhanced tumor response compared with cetuximab alone or radiation plus IgG, resulting in survival differences (F) on log-rank analysis with a trend ($P = 0.06$) toward improved survival with radiation plus cetuximab compared with radiation plus IgG. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, nonsignificant ($P \geq 0.05$).

Ex vivo interferon response assay

Ex vivo IFN response assays were conducted as previously described (26). Splenocytes from B78 tumor-bearing animals were harvested on day 12 of indicated treatment and cocultured with B16 cells for 5 days. Splenocytes were labeled with anti-CD4 and anti-CD8 mAbs, fixed, permeabilized, and stained for cytoplasmic IFN-γ (BD Pharmingen). Live T cells producing IFNγ were quantified relative to total live T cells by flow cytometry.

Statistical analyses

Tumor response and animal weight plots are displayed as means ± SE. Mixed effect models on log-transformed data were used to estimate and compare the slopes of tumor response and animal weight curves. Survival curves were generated using the Kaplan–Meier method and compared using log-rank tests. Results of immunohistochemistry, B16 metastasis, and IFN response experiments were evaluated using ANOVA with post-testing done using two-sample *t* tests. Clonogenic assays were evaluated by two-tailed two-sample *t* tests comparing treatment groups at each dose of radiation. Rates of complete response and tumor engraftment were compared between groups using χ^2 analysis. *P* values less than 0.05 were considered significant and are indicated in figures as ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, nonsignificant ($P \geq 0.05$). Analyses were performed using JMP and SAS software (SAS Institute).

Results

Cooperative interaction of radiation and tumor-specific mAbs

To examine whether radiation may enhance the antitumor immune response elicited by tumor-specific mAbs, we tested combinations of radiation and the anti-GD2 mAb hu14.18K322A in the treatment of macroscopic, syngeneic, GD-2–expressing tumors (B78 melanoma and NXS2 neuroblastoma) in two distinct murine strains (C57BL/6 and A/J, respectively). Animals were treated with either sham or single-fraction radiation (12 Gy) and 5 daily IT injections (50 μg/injection) of either anti-GD2 mAb or non-specific control IgG. Although hu14.18K322A alone had no perceptible impact on tumor growth in these macroscopic tumor models, treatment with this mAb following radiation resulted in increased tumor response and delay of tumor regrowth (Fig. 1A and B) and a trend toward, or significantly improved survival (Fig. 1C and D, respectively). Monitoring of animal weights (Supplementary Fig. S1A and S1B) demonstrated no added toxicity from combined IT-hu14.18K322A and radiation. In prior preclinical studies, we have demonstrated enhanced local and systemic anti-tumor immune response with IT as compared with intravenous (i.v.) delivery of anti-GD2 antibody therapeutics (24). Here, we confirmed that even when following radiation, IT injection of anti-GD2 mAb resulted in improved tumor response compared with i.v. delivery (Supplementary Fig. S2).

To explore the generalizability of an interaction between radiation and tumor-specific mAb, we used nude mice engrafted with the human head and neck cancer (HNC) tumor cell line, SCC1-C. We have previously demonstrated that these cells express EGFR at the plasma membrane, but are resistant to *in vitro* inhibition of proliferation by the anti-EGFR mAb cetuximab (19). Unlike most cetuximab-sensitive cells, we observed no intrinsic sensitization of these "cetuximab-resistant" cells to radiation following treatment with cetuximab *in vitro* (Supplementary Fig. S3A). On the other hand, these cells remain sensitive to cetuximab-mediated ADCC (Supplementary Fig. S3B). SCC1-C cells, therefore, represent a tool for enabling interrogation of the interaction between radiation and ADCC response to cetuximab in the absence of confounding effects from EGFR inhibition on cell proliferation and radiosensitivity. We treated mice bearing SCC1-C tumors with 12 Gy or sham radiation and 5 daily IT injections of cetuximab or control IgG (50 μg/injection) on days 6 to 10 after radiation. We observed modest tumor inhibition from cetuximab alone compared with control IgG and a strongly enhanced response to the combination of cetuximab and radiation compared with radiation and control IgG (Fig. 1E and F). This suggested generalizability for the cooperative interaction of radiation and ADCC.

NK cells are critical to the cooperative interaction of radiation and tumor-specific mAb

We have previously demonstrated that hu14.18 mAb elicits ADCC against both B78 and NXS2 cells (25, 27). The hu14.18K322A mAb has been further engineered to abrogate complement binding and is thought to primarily mediate a therapeutic effect through ADCC (6). Importantly, the GD2 antigen is not known to have a functional role in cell survival, proliferation, DNA damage response, or other cellular processes known to interact with radiation. In support of this, we have confirmed that hu14.18K322A does not affect the intrinsic sensitivity of B78 or NXS2 cells to radiation using *in vitro* clonogenic assays (Supplementary Fig. S4A and S4B). We have also confirmed that radiation does not increase the expression of GD2 in B78 or NXS2 cells (Supplementary Fig. S4C and S4D).

We evaluated whether the cooperative *in vivo* interaction of hu14.18K322A and radiation (Fig. 1A–D) resulted from ADCC. To confirm that this interaction required the GD2 antigen, we tested the combination of radiation and hu14.18K322A in a

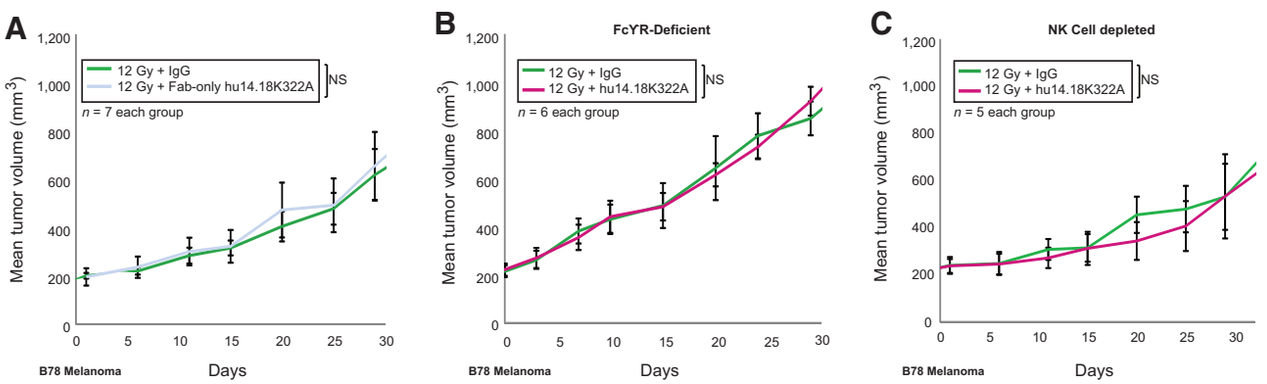


Figure 2.

The interaction of radiation and hu14.18K322A requires FcγR and NK cells. A, a Fab-only version of hu14.18K322A still capable of binding GD2 did not improve B78 tumor response ($P = 0.406$) compared with control IgG following radiation. In mice lacking the FcγR (B) or depleted of NK cells (C), full-length hu14.18K322A did not improve B78 tumor response compared with IgG following radiation ($P = 0.983$, $P = 0.301$, respectively).

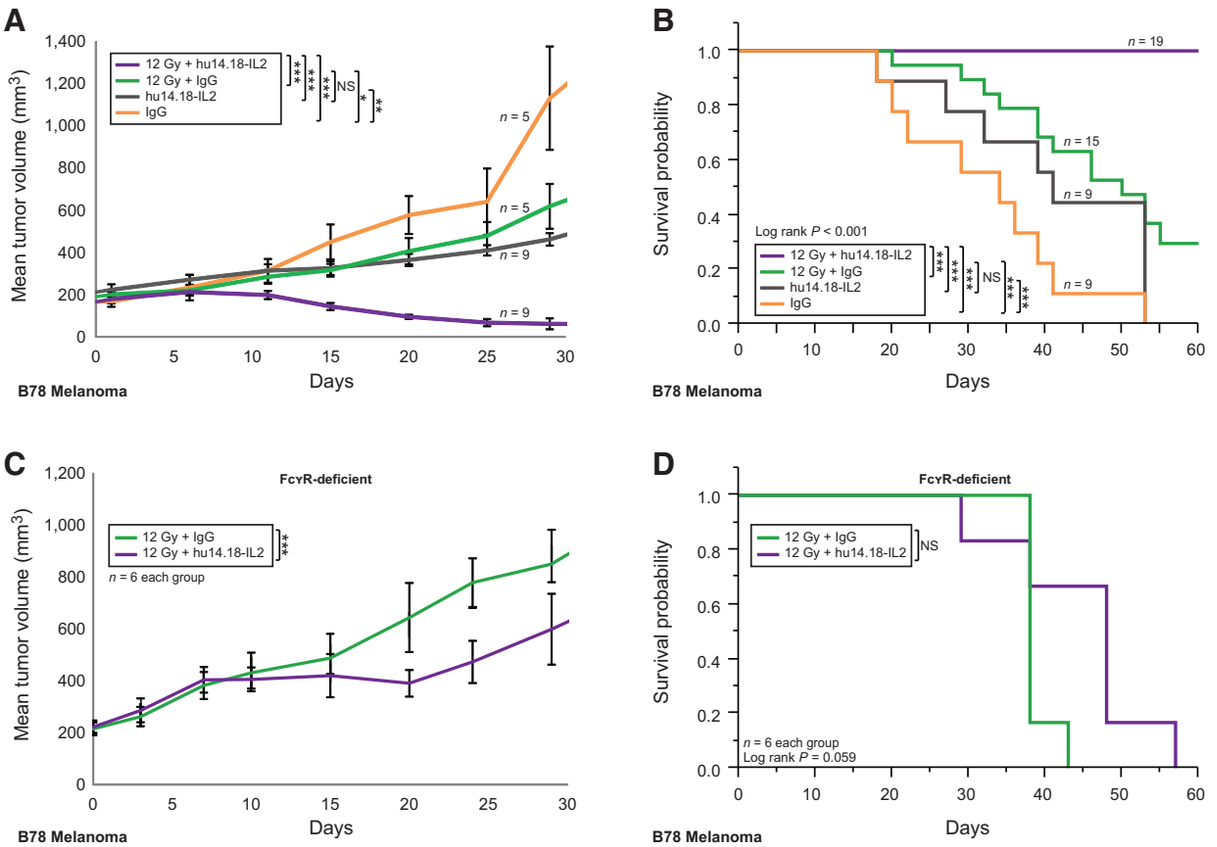


Figure 3.

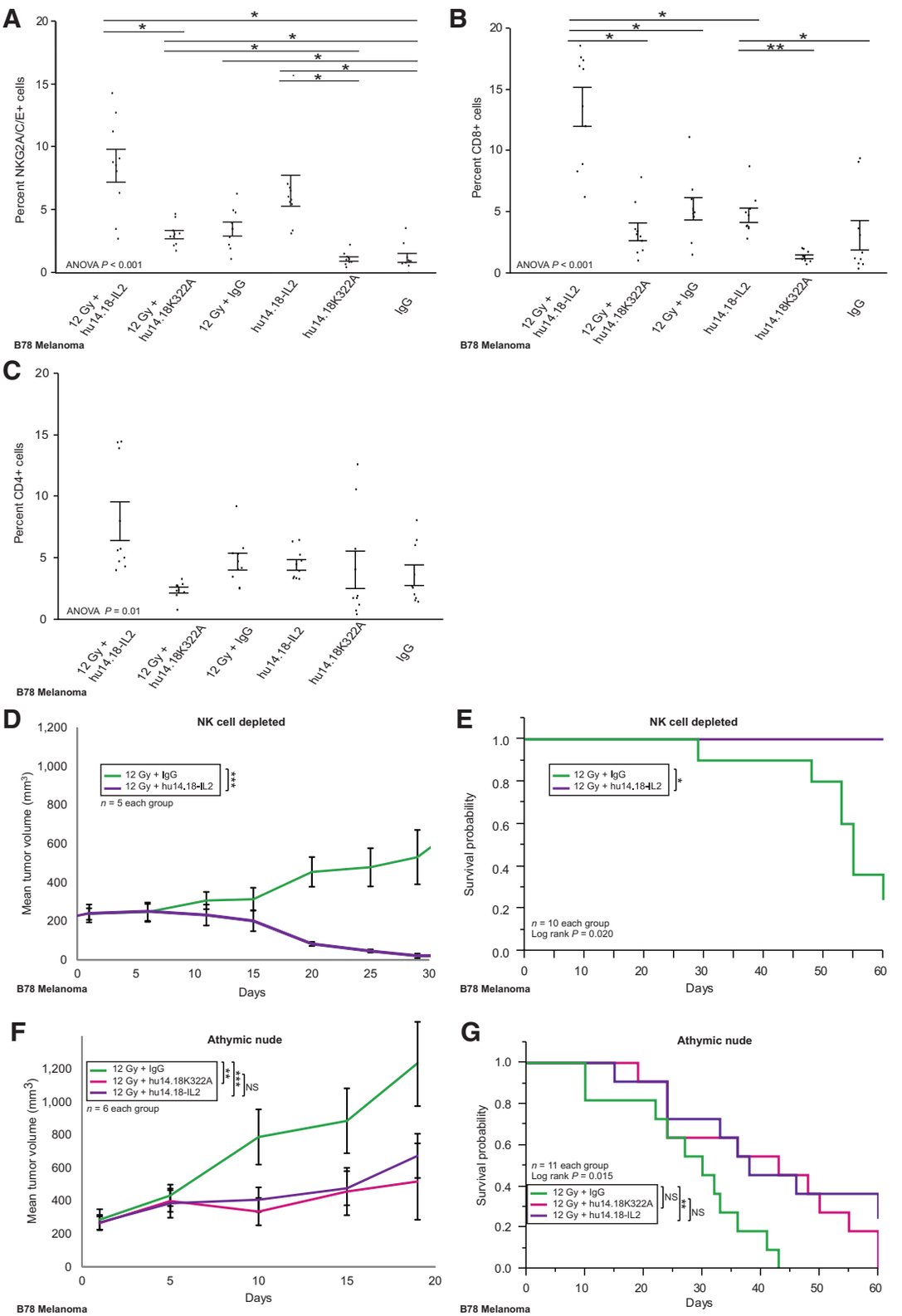
Improved tumor control and animal survival with radiation and IT-hu14.18-IL2. Treatment with radiation and hu14.18-IL2 resulted in tumor regression (A) and improved survival (B). In FcγR-deficient mice, radiation plus hu14.18-IL2 resulted in a significant but modest effect on tumor response (C; compared with radiation plus IgG), with no animals rendered disease-free and no significant impact on survival (D). Experiments in Figs. 1A and 3A as well as 2D and 3D were performed concurrently. Compared with radiation and hu14.18K322A, treatment with radiation and hu14.18-IL2 improved tumor response in wild-type ($P < 0.001$) and FcγR-deficient mice ($P < 0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, nonsignificant ($P \geq 0.05$).

syngeneic B16 melanoma tumor model. B16 is parental to the B78 cell line but lacks expression of the GD2 antigen (11). Not surprisingly, following radiation in this GD2-deficient model, we observed no effect of hu14.18K322A compared with control IgG, indicating a requirement for GD2 antigen in the interaction of radiation and hu14.18K322A (Supplementary Fig. S4E and S4F). A monovalent Fab-only version of hu14.18K322A, which retains the ability to bind GD2 but is unable to engage FcγR, did not affect tumor response when given following radiation (Fig. 2A). In addition, the cooperative effect of radiation and hu14.18K322A was not observed in FcγR-deficient mice (Fig. 2B) or mice depleted of NK cells (Fig. 2C). Collectively these results suggest that the *in vivo* interaction of radiation and hu14.18K322A is mediated, at least in part, by NK cells through ADCC.

Improved tumor control and survival with combined radiation and tumor-specific immunocytokine

We next sought to investigate whether the cooperative interaction between radiation and the antitumor immune response induced by administration of tumor-specific mAbs might be improved by substituting mAb with immunocytokine (IC)—a synthetic fusion protein consisting of a tumor-specific antibody genetically linked to an immune-stimulating cytokine. ICs exert antitumor effects by both targeting and stimulating the immune system to selectively destroy cancer cells. Hu14.18-IL2 IC consists of human IL2 genetically fused to each IgG heavy chain of the GD2 mAb, hu14.18 (23). Prior studies have demonstrated that this IC activates ADCC and additional immune pathways (28), has greater *in vivo* antitumor activity than equivalent mixtures of mAb and IL2 (29), and has clinical antitumor activity (30). Importantly, administration of IT-IC is clinically feasible with early studies suggesting promising therapeutic effects (31).

In our syngeneic B78 melanoma model, we treated mice with 12 Gy or sham radiation and 5 daily IT injections of hu14.18-IL2 or control IgG (50 μg/injection) on days 6 to 10 after radiation. The combination of radiation and hu14.18-IL2 did not appear toxic as gauged by animal appearance and weight (Supplementary Fig. S1C). We observed a strong cooperative interaction between radiation and hu14.18-IL2, resulting in enhanced tumor response and animal survival (Fig. 3A and B). For this moderate-size tumor model (~200 mm^3 at the time of radiation), we observed complete tumor regression and disease-free survival beyond 100 days in 71% (22/31) of animals compared with 14% (3/21) of those treated with radiation and



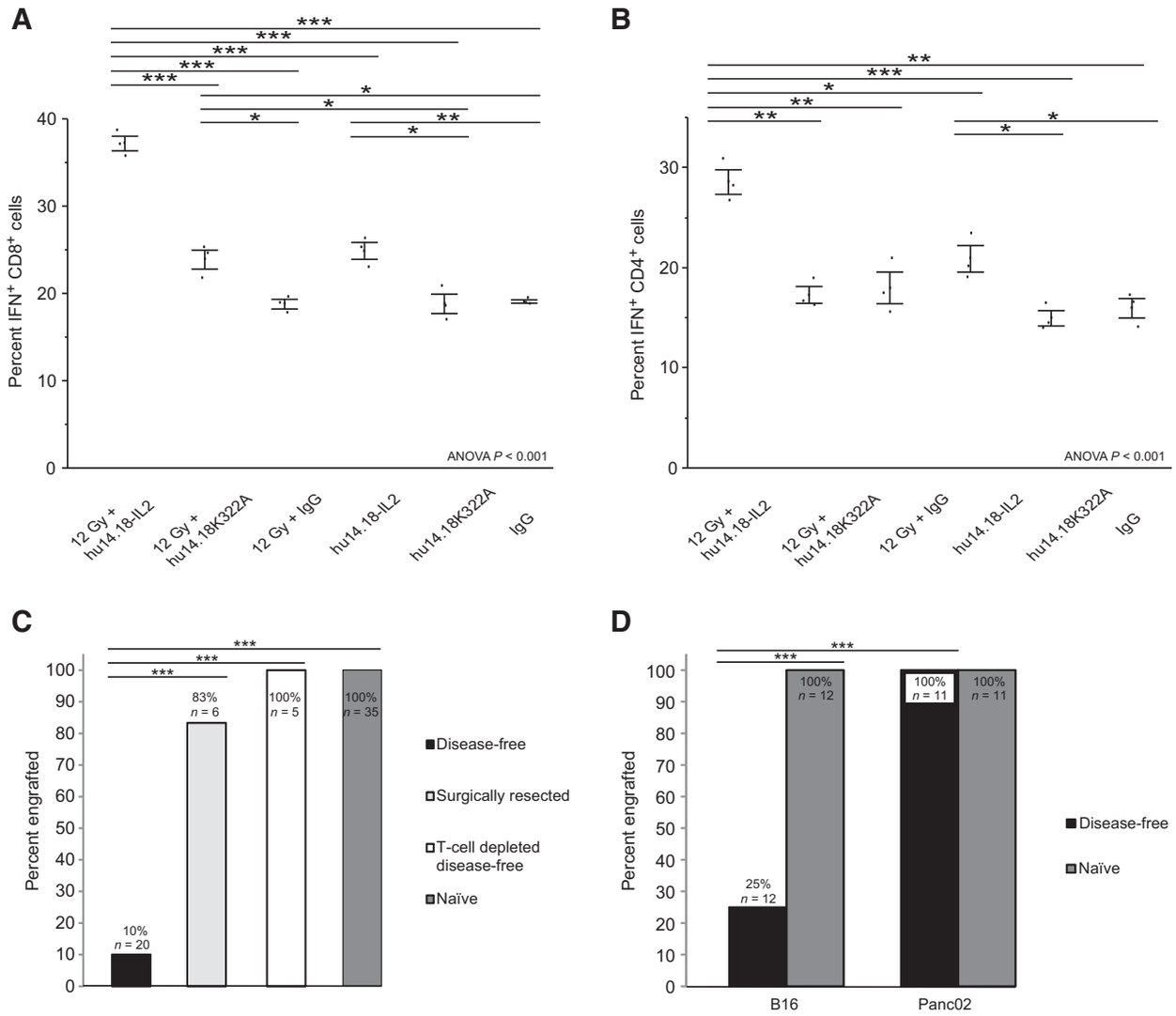


Figure 5.
Radiation and IT-IC produce an *in situ* vaccination effect, resulting in a memory T-cell response. The percent of IFNγ-positive CD8-positive (A) and CD4-positive (B) T cells was determined by flow cytometry. Means ± SEs and individual data points are shown. C, mice rendered disease-free by radiation plus hu14.18-IL2 were rechallenged by subcutaneous B78 cell injection and the percentage of developing tumors is shown. D, mice that rejected re-engraftment in C were challenged simultaneously with GD2-deficient B16 melanoma (parental to B78) and unrelated Panc02 pancreatic tumor cell injections and the percentage of developing tumors is shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

hu14.18K322A, and no mice in the other treatment groups. Hu14.18-IL2 did not affect the intrinsic sensitivity of cells to radiation *in vitro* (Supplementary Fig. S4G). As expected, hu14.18-IL2 had no effect on tumor response or animal survival following radiation in mice bearing GD2-deficient B16 melanoma (Supplementary Fig. S4E and S4F). The impact of hu14.18-IL2 on tumor growth following radiation in FcγR-deficient mice was modest, albeit significant (Fig. 3C), yet did not affect animal survival (Fig. 3D). This tumor-growth inhibition by IC (but not by mAb) in FcγR-deficient mice may reflect the capacity of hu14.18-IL2 to tether effector cells to tumor cells via IL2 receptors, as previously reported (32).

Figure 4.
Radiation and IT-IC augment tumor infiltration by NK and CD8-positive T cells and elicit a T-cell–dependent antitumor response. Immunohistochemistry was performed using NK and T-cell markers. Positively stained cells were quantified as a percent of total cells for NKG2A/C/E (A), CD8 (B), and CD4 (C). Means ± SEs and individual data points are shown. Depletion of NK cells did not prevent the impact of combined radiation and hu14.18-IL2 on tumor response (D) or animal survival (E). In nude mice, which lack mature T cells, hu14.18K322A and hu14.18-IL2 elicited comparable effects on tumor response (F) and animal survival (G) following radiation. *, $P < 0.05$; **, $P < 0.01$; NS, nonsignificant ($P \geq 0.05$).

Combined radiation and IC triggers *in situ* vaccination and a memory T-cell response

Using immunohistochemistry, we characterized tumor immune cell infiltrates following treatment with sham or 12 Gy radiation and hu14.18K322A, hu14.18-IL2, or non-specific human IgG. We observed a significant approximately 2-fold increase in tumor infiltration by NK cells following treatment with radiation (Fig. 4A, Supplementary Fig. S5A). This effect was not significantly affected by administration of hu14.18K322A. Treatment with hu14.18-IL2 alone increased tumor infiltrating NK cells, and this was not significantly increased further by combination with radiation. Neither radiation nor hu14.18K322A alone or in combination consistently affected tumor infiltration with CD8-positive T cells (Fig. 4B, Supplementary Fig. S5B). In contrast, IC alone modestly increased tumor infiltration with CD8-positive T cells compared with mAb alone whereas the combination of hu14.18-IL2 and radiation markedly increased tumor infiltration by CD8-positive T cells (Fig. 4B). No significant changes were observed in CD4-positive T-cell tumor infiltrate between the compared treatment regimens, although there was a trend toward increased infiltrate with combined 12 Gy and hu14.18-IL2 ($P = 0.14$ vs. IgG alone, Fig. 4C, Supplementary Fig. S5C).

To investigate the role of specific immune cell lineages in the response to combined radiation and mAb or IC, we examined the efficacy of treatment in immunodeficient mice. Depletion of NK cells eliminated the synergistic interaction between radiation and hu14.18K322A mAb (Fig. 2C) but did not prevent that of radiation and hu14.18-IL2 (Fig. 4D and E). On the other hand, treatment in nude mice, which lack mature T cells, did not preclude the cooperative interaction of hu14.18K322A with radiation but reduced the efficacy of radiation and hu14.18-IL2 to a level not significantly different from that of radiation and hu14.18K322A (Fig. 4F and G). This suggests that when combined with radiation in immunologically intact mice, hu14.18K322A and hu14.18-IL2 both elicit ADCC whereas only hu14.18-IL2 also generates a T-cell response that is independent of NK cells.

We quantified this T-cell response using an *ex vivo* IFN response assay. For CD8-positive T cells, we observed an increase in the percentage of IFNγ-positive cells with combined 12 Gy and hu14.18-IL2 compared with all other treatments, a modest increase with combined 12 Gy and hu14.18K322A compared with radiation or mAb alone, and a modest increase with hu14.18-IL2 compared with hu14.18K322A mAb or IgG (Fig. 5A). For CD4-positive T cells, we observed an increase in the percentage of IFNγ-positive cells with combined 12 Gy and hu14.18-IL2 compared with all other treatments and a modest increase with radiation alone compared with IgG (Fig. 5B).

Given this T-cell response, we tested whether the approximately 70% of animals rendered disease-free by combined treatment with radiation and hu14.18-IL2 developed a memory T-cell response. For this, we re-injected these animals with B78 melanoma $\geq$70 days (range, 73–105) after radiation. Nearly, all of these animals rejected this second tumor [10% engraftment (2/20), Fig. 5C] compared with 100% engraftment among age-matched control mice (35/35) and 83% (5/6) engraftment among mice rendered disease-free by surgical resection (Fig. 5C). Of the few animals rendered disease-free by combined treatment with radiation and hu14.18K322A mAb, 50% (1/2) rejected re-engraftment with B78 cells. A subset of mice rendered disease-free following initial treatment with radiation and hu14.18-IL2 was depleted of T cells and none rejected re-engraftment with B78 cells [100% engraftment (5/5), Fig. 5C].

These findings suggest a potent antitumor memory T-cell response among animals rendered disease-free by radiation and hu14.18-IL2. This memory response was tumor-specific and adaptive as 9 of 12 of mice remaining disease-free after B78 re-challenge also rejected engraftment with GD2-deficient B16 melanoma cells (parental to B78; 25% engraftment, Fig. 5D). None of these mice rejected simultaneous injection with the unrelated syngeneic Panc02 pancreatic tumor cell line [100% engraftment (12/12), Fig. 5D]. No age-matched naïve controls rejected engraftment with the same B16 or Panc02 cells [100% engraftment (11/11), Fig. 5D]. These results demonstrate that most mice rendered disease-free by combined radiation and hu14.18-IL2 developed tumor-specific immunologic memory to antigen(s) shared by B78 and B16, and thus distinct from GD2, consistent with an *in situ* vaccination effect.

Response to combined radiation and IT-IC is time-sensitive and requires FasL

The timing we have used for administering IT-IC 6 days after radiation is based on early studies we performed, which showed that the interaction of radiation and hu14.18-IL2 was strongly time dependent. IC treatment on days 6 to 10 after radiation was more effective than treatment days 1 to 5 or 11 to 15 (Fig. 6A and B). This appeared to be specific to the cooperative activity of radiation and hu14.18-IL2; there was no difference in antitumor effect when giving hu14.18K322A on days 1 to 5 versus 6 to 10 after radiation (Supplementary Fig. S6A). This suggests that the T-cell response generated by combined treatment with radiation and IC may be dependent on a delayed effect of radiation.

Prior studies show that radiation-induced DNA damage triggers a p53-dependent increase in Fas/CD95 expression (33, 34). Fas is a death receptor capable of triggering the extrinsic apoptotic pathway when engaged by its cognate ligand, FasL. Immune cell lineages express FasL and tumor cell expression of Fas has been shown to enhance antitumor ADCC (35) and T-cell responses (36). We examined the expression of Fas on cultured B78 cells surviving *in vitro* radiation and observed dose-dependent induction of expression (Supplementary Fig. S6B) that is quite time-dependent (Fig. 6C) and less sensitive to fractionation of radiation (Supplementary Fig. S6C). This time-dependence mirrors the time-sensitivity that we observe in the *in vivo* interaction between radiation and IC (Fig. 6A and B). Using a Fas-activating mAb *in vitro*, we determined that the degree of increased Fas expression induced in B78 cells at 7 days following *in vitro* radiation with 12 Gy (Fig. 6C) was sufficient to enhance the susceptibility of these cells to Fas-mediated cytotoxicity (Fig. 6D). Importantly, when B78 tumors were treated with the combination of radiation and hu14.18-IL2 in mice lacking the cognate FasL ligand, we no longer observed enhanced tumor response or animal survival compared with radiation and control IgG (Fig. 6E and F). This suggests necessity for the Fas/FasL pathway in the T-cell–dependent synergy of radiation and hu14.18-IL2, similar to the role of this pathway in T-cell cytotoxicity *in vitro* (37).

Radiation combined with IC augments response to T-cell checkpoint inhibition

Given the capacity of radiation and IT injection of hu14.18-IL2 to elicit a T-cell–dependent antitumor response, we hypothesized that this combination might augment the local and systemic

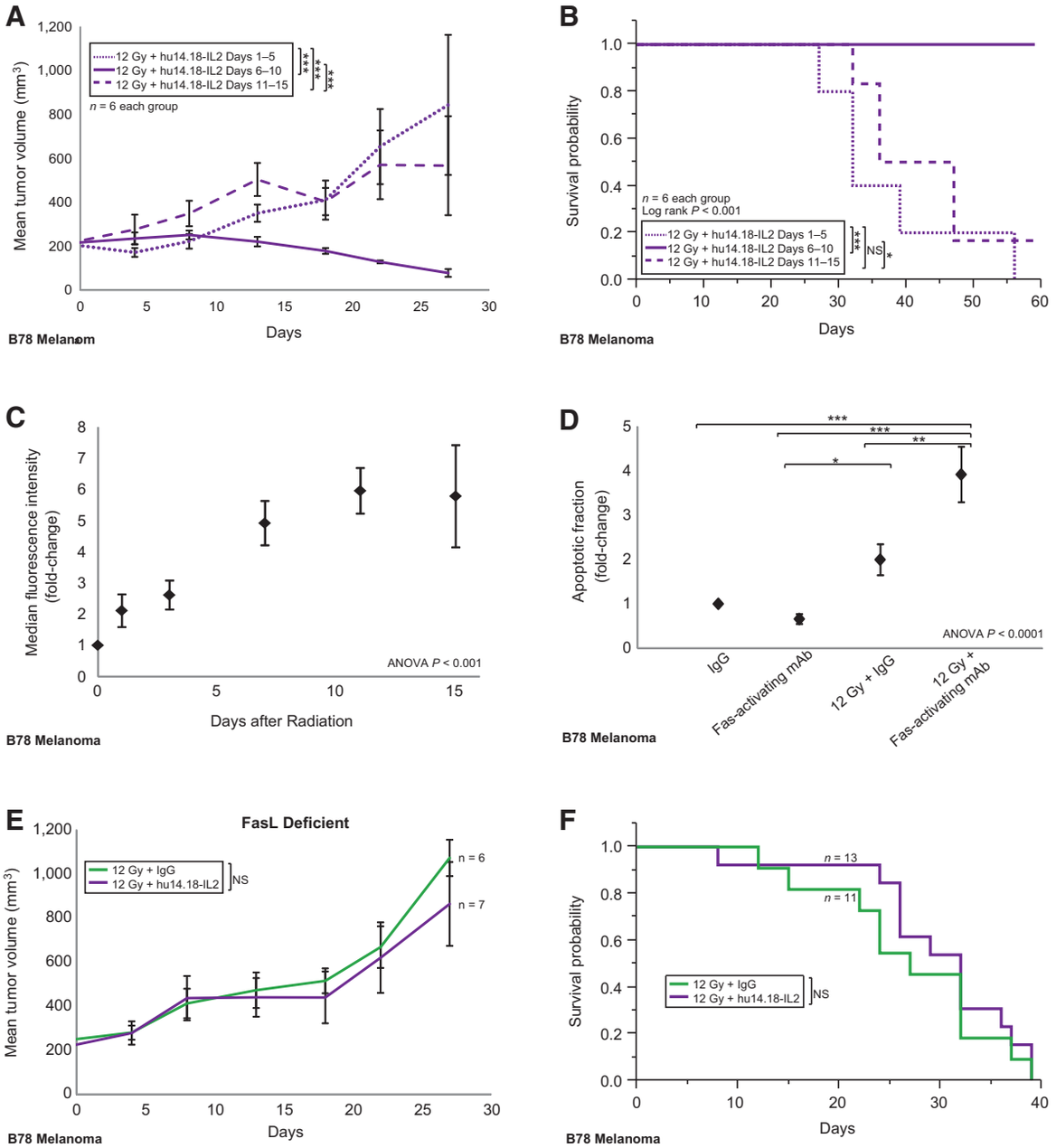


Figure 6.

B78 melanoma response to combined radiation and IC is time-sensitive and requires FasL. Hu14.18-IL2 administration on days 6 to 10 was more effective in controlling B78 melanoma tumors *in vivo* than administration on days 1 to 5 or days 11 to 15 after radiation with respect to tumor response (A) and animal survival (B). C, a similar time sensitivity is seen in the increased expression of Fas/CD95 on live B78 cells following *in vitro* radiation. Cells received 12 Gy the indicated number of days before harvest. Fas expression on live single cells was evaluated using flow cytometry. Results of triplicate experiments are expressed as mean fold-change relative to non-radiated cells ± SE. D, the degree of increased Fas/CD95 expression 7 days after *in vitro* radiation was sufficient to increase the apoptotic response to Fas-activating mAb. Cells were treated with 12 Gy or sham radiation, maintained in culture for 6 days, and then treated with Fas-activating mAb or control IgG for 18 hours. The fraction of apoptotic cells was quantified by flow cytometry. Results are displayed as mean fold-change (relative to sham RT + IgG) ± standard error. E and F, in FasL-deficient mice, *in vivo* treatment of B78 tumors with radiation and hu14.18-IL2 resulted in no significant impact on tumor response ($P = 0.362$; E) or overall survival ($P = 0.466$; F) compared radiation and IgG. Animals in A and E were engrafted and treated concurrently. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, nonsignificant ($P \geq 0.05$).

response to T-cell checkpoint blockade. Using syngeneic mice bearing large B78 melanoma tumors (~500 mm^3), we delivered combinations of single fraction (12 Gy) or sham radiation (day 1), 5 daily IT injections of hu14.18-IL2 or control IgG (days 6–10), and IP injections of anti–CTLA-4 or control IgG (days 3, 6, and 9). In this large tumor model, we observed a striking improvement in tumor control (Fig. 7A) and animal survival (Fig. 7B) with the combination of radiation, hu14.18-IL2, and anti–CTLA-4 as

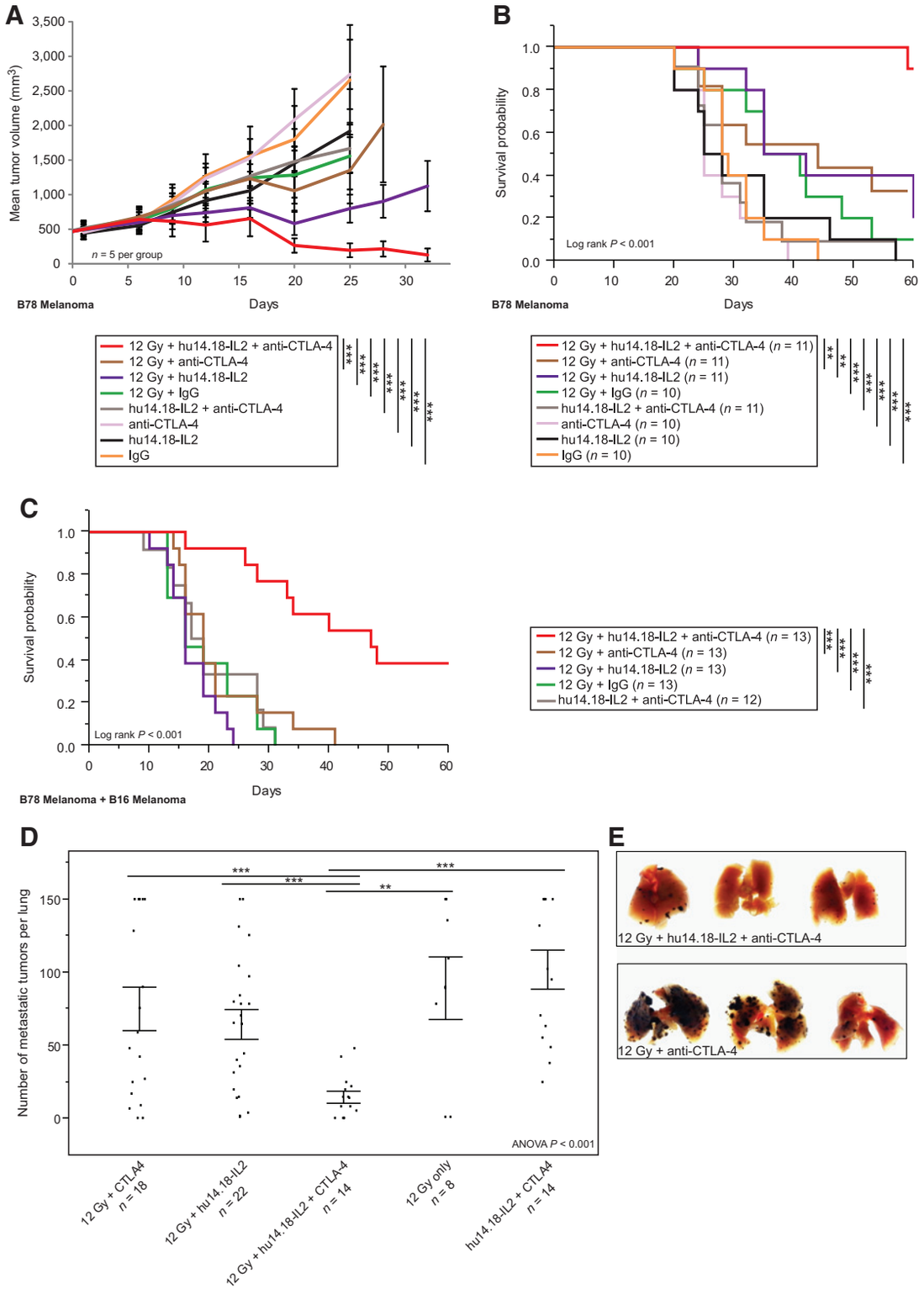


Figure 7.
Combined radiation and IT-IC augment the local and systemic response to anti–CTLA-4 T-cell checkpoint inhibition. In mice bearing a large B78 melanoma primary tumor (~500 mm^3), tumor response (A) and animal survival (B) were improved with combined radiation, IT-hu14.18-IL2, and anti–CTLA-4 as compared with mono- or dual therapy combinations. In separate experiments, to test whether IT-IC adds to the distant antitumor effect of local radiation plus systemic anti–CTLA-4, we administered B16 melanoma cells (GD2-deficient, and thus resistant to hu14.18-IL2) by i.v. injection on the day of radiation in mice bearing a large B78 tumor. In these animals, the combination of primary tumor radiation, IT-hu14.18-IL2, and anti–CTLA-4 improved survival (C) and reduced the metastatic foci per lung compared with doublet combinations or radiation alone (D). Means ± SEs and individual data points are shown. E, representative lungs are shown from animals receiving 12 Gy + hu14.18-IL2 + CTLA-4 versus 12 Gy + CTLA-4. **, $P < 0.01$; ***, $P < 0.001$.

compared with mono- or dual combinations. Complete tumor regression was observed in 73% (8/11) of animals receiving this triple combination versus 27% (3/11, $P = 0.03$) with radiation and hu14.18-IL2, 9% (1/11, $P = 0.002$) with radiation and anti–CTLA-4, and no animals in other groups.

Prior preclinical studies in murine models demonstrate an enhanced local and distant antitumor response when anti–CTLA-4 treatment is added to local radiation (38–40). On the basis of the potent tumor-specific memory demonstrated when IT-IC is added to local radiation (Fig. 5C and D), we hypothesized that adding IT-IC to the combination of anti–CTLA-4 and local radiation would further improve distant antitumor response. To test this, we generated syngeneic mice bearing a large GD2-expressing B78 melanoma "primary" tumor. To test for an adaptive immune response, these animals were given an i.v. injection of B16 melanoma cells (GD2-deficient, and thus not responsive to hu14.18-IL2) on the day of radiation. Animals were treated with combinations of single fraction (12 Gy) or sham radiation to the primary tumor (day 1), IT injection of the primary tumor with hu14.18-IL2 or control IgG (days 6–10), and IP anti–CTLA-4 or control IgG (days 3, 6, and 9). We observed cooperative activity with the combination of radiation, hu14.18-IL2, and anti–CTLA-4 resulting in improved animal survival (Fig. 7C) and reduced metastatic disease burden compared with animals treated with radiation and anti–CTLA-4 (Fig. 7D and E).

Discussion

We demonstrate a cooperative interaction between ionizing radiation and the immune response to tumor-specific mAbs delivered by IT injection. This effect seems to be generalizable, as we confirm similar findings in distinct syngeneic melanoma and neuroblastoma murine tumor models in different mouse strains and in a human HNC xenograft tumor model. Using our murine melanoma model, we determine that this cooperative interaction is enhanced by substituting tumor-specific mAb with an IC fusion protein that genetically links the IgG heavy chain of this mAb with IL2. This combination of radiation and IT-IC results in a potent T-cell response and adaptive tumor-specific immunologic memory, consistent with an *in situ* vaccination effect. In our preclinical models, this effect requires IC specificity for tumor cells and appears to be mediated at least in part through ADCC. Interestingly, two prior preclinical studies and a phase I clinical investigation suggest that cooperative activity may also be elicited through combination of radiation with ICs that exhibit specificity for tumor stromal components and do not directly mediate ADCC (41–43).

As T-cell checkpoint inhibitors become established treatments for a variety of malignancies, a persistent challenge will be to increase the rate and degree of response to these agents. *In situ* tumor vaccination approaches may be well suited to this task. A recent study suggests that radiation alone may augment the response to T-cell checkpoint inhibition by diversifying antigen recognition in an adaptive immune response (40). In a model of metastatic melanoma, we now demonstrate that delivery of radiation and IT-IC to a single tumor site may further augment both local and distant control of disease beyond that achieved with combined radiation and T-cell checkpoint inhibition. This effect is observed even with distant disease that lacks the IC target antigen. Such sites are resistant to direct treatment with radiation and IT-IC and their elimination is indicative of an adaptive immune response resulting from an *in situ* tumor vaccination effect.

Our results also suggest an opportunity for clinical investigations coordinating radiotherapy with the timing and route of delivery for tumor-specific mAbs. The interaction of radiation and tumor-specific mAb has been investigated most thoroughly in HNC. Preclinical studies demonstrate a role for i.v. cetuximab in sensitizing tumor cells to ionizing radiation, likely via the biologic effects of EGFR blockade (20, 44), and a phase III clinical study demonstrated improved survival with the addition of concurrent cetuximab to radiation in HNC patients (45). In the absence of radiation, a dual therapeutic mechanism has been proposed for EGFR-targeting antibodies whereby these agents both antagonize EGFR signal pathways and give rise to ADCC (46). Our results now suggest that radiation may enhance the *in vivo* tumor sensitivity to ADCC. Preclinical and clinical studies may be warranted to determine whether delivery of these modalities may be optimized to enhance antitumor immune response.

The interaction of radiation with mAb or IC is likely multifactorial. Following radiation, we observe a benefit to delayed (days 6–10) versus immediate (day 1–5) IC administration. Continued tumor growth during this interval indicates that radiation does not merely enable an equivalent immune response against a reduced tumor volume. Prior studies suggest that cells destined to die following radiation undergo antigenic death (1) and this may enhance T-cell response with delayed IC administration. In addition, phenotypic changes such as increased expression of Fas/CD95 on cells surviving radiation may enhance tumor immune susceptibility and subsequent immunogenicity as an *in situ* vaccine. It remains to be seen whether such changes may be useful markers for coordinating timing of radio- and immunotherapy in other settings.

Additional studies are needed to optimize radiation dose and fractionation for *in situ* vaccination. We chose a single 12 Gy fraction, as this can be clinically administered and may induce a functionally significant upregulation in Fas expression (Fig. 6D, Supplementary Fig. S6B). In the B78 melanoma model, following treatment with 12 Gy alone we do not see tumor regression *in vivo* (Fig. 1) nor evidence of increased infiltration or activation of tumor-specific CD8-positive T cells (Figs. 4B and 5A). This suggests that the time dependency of IT-IC is not merely reflecting kinetics of detectible tumor-specific T cells following radiation.

Our data are consistent with a few intriguing hypotheses. First, 12 Gy radiation causes a modest level of direct *in vivo* tumor death and increased susceptibility to effector-cell–mediated death (via ADCC and T cells). Second, the strong adaptive response to IT-IC, but not IT mAb, suggests that IC binding to radiated tumor cells facilitates antigen presentation and augmented induction of adaptive immunity. Third, this adaptive response can be expanded by anti–CTLA-4 treatment. Local depletion of regulatory T cells by radiation and anti–CTLA-4 may also play a role in this *in situ* vaccination effect. Similarly, radiation may exert a beneficial effect on immune response by interfering with myeloid-derived suppressor cells (47).

Prior clinical trials of i.v. hu14.18-IL2 in patients with melanoma and neuroblastoma demonstrated acceptable toxicity and modest antitumor activity (30, 48, 49). On the basis of the findings presented here, we are proposing clinical investigations of combined radiation, tumor-specific mAb or IC, and T-cell checkpoint inhibition in patients with melanoma and neuroblastoma.

Disclosure of Potential Conflicts of Interest

A.J. Korman has ownership interest (including patents) in BMS. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Z.S. Morris, D.M. Francis, J.A. Hank, P.M. Harari, P.M. Sondel

Development of methodology: Z.S. Morris, D.M. Francis, R.K. Yang, E.A. Armstrong, J.A. Hank, P.M. Sondel

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Z.S. Morris, E.I. Guy, D.M. Francis, M.M. Gressett, L.R. Werner, P.M. Harari

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z.S. Morris, M.M. Gressett, L.L. Carmichael, A. Korman, A.L. Rakhmilevich, P.M. Harari, P.M. Sondel

Writing, review, and/or revision of the manuscript: Z.S. Morris, E.I. Guy, D.M. Francis, M.M. Gressett, L.L. Carmichael, R.K. Yang, E.A. Armstrong, S. Huang, F. Navid, S.D. Gillies, J.A. Hank, A.L. Rakhmilevich, P.M. Harari, P.M. Sondel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Z.S. Morris, E.I. Guy, P.M. Sondel

Study supervision: P.M. Sondel

Other (created the antibody and immunocytokine used in the study): S.D. Gillies

Other (provided characterized antibody reagents): A. Korman

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