Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



# Fractionated but Not Single-Dose Radiotherapy Induces an Immune-Mediated Abscopal Effect when Combined with Anti–CTLA-4 Antibody

M. Zahidunnabi Dewan,[1] Ashley E. Galloway,[1] Noriko Kawashima,[1] J. Keith Dewyngaert,[3] James S. Babb,[2] Silvia C. Formenti,[3] and Sandra Demaria[1]

**Abstract**

**Purpose:** This study tested the hypothesis that the type of dose fractionation regimen determines the ability of radiotherapy to synergize with anti–CTLA-4 antibody.

**Experimental Design:** TSA mouse breast carcinoma cells were injected s.c. into syngeneic mice at two separate sites, defined as a "primary" site that was irradiated and a "secondary" site outside the radiotherapy field. When both tumors were palpable, mice were randomly assigned to eight groups receiving no radiotherapy or three distinct regimens of radiotherapy (20 Gy × 1, 8 Gy × 3, or 6 Gy × 5 fractions in consecutive days) in combination or not with 9H10 monoclonal antibody against CTLA-4. Mice were followed for tumor growth/regression. Similar experiments were conducted in the MCA38 mouse colon carcinoma model.

**Results:** In either of the two models tested, treatment with 9H10 alone had no detectable effect. Each of the radiotherapy regimens caused comparable growth delay of the primary tumors but had no effect on the secondary tumors outside the radiation field. Conversely, the combination of 9H10 and either fractionated radiotherapy regimens achieved enhanced tumor response at the primary site ($P < 0.0001$). Moreover, an abscopal effect, defined as a significant growth inhibition of the tumor outside the field, occurred only in mice treated with the combination of 9H10 and fractionated radiotherapy ($P < 0.01$). The frequency of CD8+ T cells showing tumor-specific IFN-γ production was proportional to the inhibition of the secondary tumor.

**Conclusions:** Fractionated but not single-dose radiotherapy induces an abscopal effect when in combination with anti–CTLA-4 antibody in two preclinical carcinoma models. (Clin Cancer Res 2009;15(17):5379–88)

**Authors' Affiliations:** Departments of [1]Pathology, [2]Radiology, and [3]Radiation Oncology, New York University School of Medicine and New York University Langone Medical Center, New York, New York

Received 2/2/09; revised 5/5/09; accepted 5/16/09; published OnlineFirst 8/25/09.

**Grant support:** NIH grant R01 CA113851, American Cancer Society Research Scholar grant RSG-05-145-01-LIB, and The Chemotherapy Foundation (S. Demaria); Department of Defense Center of Excellence grant BC030282 and The Breast Cancer Research Foundation (S. C. Formenti); Molecular Oncology and Immunology Training grant T32 CA009161-33-34 (M.Z. Dewan); and NIH grant 5P30CA016087-27 (New York University Cancer Institute).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** S. C. Formenti and S. Demaria share senior authorship.

**Requests for reprints:** Sandra Demaria, Department of Pathology, MSB-504, New York University Langone Medical Center, 550 First Avenue, New York, NY 10016. Phone: 212-263-7308; Fax: 212-263-8211; E-mail: demars01@med.nyu.edu or Silvia Formenti, Department of Radiation Oncology, Clinical Cancer Center, New York University Langone Medical Center, 160 34th Street, New York, NY 10016. Phone: 212-263-2601; Fax: 212-263-8211; E-mail: silvia.formenti@med.nyu.edu.

© 2009 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-09-0265

Ionizing radiation therapy is an effective tool for local tumor control, and plays an important role in the treatment of breast and other cancers. In the setting of metastatic disease, however, the role of radiotherapy is generally limited to palliation of symptoms. We have previously proposed a partnership between local radiation and immunotherapy in the treatment of cancer (1). Recent evidence that radiation induces an immunogenic tumor cell death and alters the tumor microenvironment to enhance recruitment of antitumor T cells supports the hypothesis that radiation can enhance both the priming and the effector phase of the antitumor immune response (2–5). Clinical observations consistent with this hypothesis, however, are very rare. One such observation is known as the "abscopal effect" and refers to tumor regression seen outside the field of radiation, implying an indirect antitumor effect induced by local radiotherapy (6–9). The paucity of evidence that radiotherapy can promote therapeutically effective antitumor immunity is not surprising, considering that successful vaccination often does not translate into clinical tumor responses (10). Development



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



### Translational Relevance

Therapeutics targeting immunomodulatory molecules to enhance antitumor immunity, such as the CTLA-4 inhibitory receptor on T cells, are being tested in clinical trials. When used as single agents in metastatic disease, their activity is generally limited to a small fraction of patients, prompting testing in combination with other treatment modalities.

We have previously shown that local radiotherapy combined with anti–CTLA-4 antibody induces effective systemic antitumor responses (abscopal effect). Importantly, the preclinical definition of optimal dose and fractionation of radiotherapy when used in combination with anti–CTLA-4 antibody is an important step to inform the correct design of a clinical trial that translates this experience to patients. The findings reported here indicate that the specific radiotherapy regimen used is a critical determinant of the success of the combined treatment.

of tolerance and immunosuppression in tumor-bearing hosts have been identified as major obstacles to the success of immunotherapy in general (11) and may also impair the immune-mediated abscopal effect induced by radiation (12).

With the use of 4T1, a syngeneic mouse breast cancer model that spontaneously metastasizes systemically soon after implantation, we have previously shown that local radiotherapy to the primary s.c. tumor induces a CD8 T-cell–mediated immune response inhibiting lung micrometastases when combined with a strategy of blocking the CTLA-4 receptor to overcome T-cell tolerance (13). In this model, CTLA-4 blockade as single modality did not significantly inhibit lung metastases or extend mice survival. The strength of the CD8 antitumor T-cell response triggered by radiotherapy and CTLA-4 blockade was regulated by invariant natural killer T cells and, in a fraction of the mice, was sufficient to cause the complete regression of the well-established primary irradiated tumor (14). The latter was facilitated by radiation-induced release of CXCL16, a chemokine enhancing the recruitment of activated CD8 cells to the irradiated tumor site (5).

Before translating these findings to the clinic, we elected to explore whether different dose-fractionation regimens have an influence on the abscopal effect observed. We chose the TSA breast cancer and the MCA38 colon cancer mouse models to test whether radiotherapy to one tumor nodule in combination with CTLA-4 blockade can induce an immune-mediated abscopal effect in a second, palpable tumor nodule outside the radiation field. A single large dose and two fractionated radiotherapy regimens had similar ability to control tumor growth at the irradiated primary site but had no effect on the secondary tumor outside the treatment field. CTLA-4 blockade as single modality did not have any effect on either tumor. However, when CTLA-4 blockade was combined with radiotherapy, there was enhanced inhibition of the primary as well as the secondary (abscopal effect) tumors. Regression of the secondary tumor was proportional to the frequency of tumor-specific T cells, consistent with an immune-mediated effect. Surprisingly, in either model, the abscopal effect was seen with either fractionated, but not with the single-dose radiotherapy regimen. Overall, these data support testing the combination of radiotherapy with immunomodulatory antibodies in patients with metastatic disease, and suggest that the schedule and dose per fraction of radiotherapy may be critical determinants of its ability to synergize with immunotherapy.

## Materials and Methods

***Mice.*** Six-to-eight-week-old BALB/c and C57BL/6 mice were obtained from Taconic Animal Laboratory (Germantown, NY), and maintained under pathogen-free conditions in the animal facility at New York University Langone Medical Center. All animal experiments were done according to protocols approved by the Institutional Animal Care and Use Committee of New York University.

***Cell line and reagents.*** TSA is a BALB/c mouse–derived poorly immunogenic mammary carcinoma cell line (15) and MCA38 is a C57BL/6 mouse–derived poorly immunogenic colon carcinoma (16). TSA and MCA38 cells were cultured in DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, $2.5 \times 10^{-5}$ mol/L 2-mercapthoethanol, and 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA; complete medium). These cells were found to be free of contamination by *Mycoplasma* by a *Mycoplasma* detection kit (Roche Diagnostics, Chicago, IL). Anti–CTLA-4 hamster monoclonal antibody (mAb) 9H10 was purified as previously described (13).

***Tumor challenge and treatment.*** BALB/c and C57BL/6 mice were injected s.c. with $1 \times 10^5$ TSA and $5 \times 10^5$ MCA38 cells, respectively, in the right flank on day 0 (primary tumor) and in the left flank on day 2 (secondary tumor). Perpendicular tumor diameters were measured with a vernier caliper, and tumor volumes were calculated as length × width$^2$ × 0.52. On day 12, when both tumors were palpable (average volume for TSA: 32 and 21 mm$^3$ for primary and secondary tumors, respectively; average volume for MCA38: 50 and 25 mm$^3$ for primary and secondary tumors, respectively), animals were randomly assigned to various treatment groups as indicated. Radiotherapy was given as previously described (13), with some modifications. Briefly, all mice (including mice receiving mock radiation) were lightly anesthetized by i.p. injection of Avertin (240 mg/kg); mice were positioned on a dedicated plexiglass tray, and the whole body was protected by lead shielding except for the area of the tumor to be irradiated. Radiotherapy was delivered to a field, including the tumor, with 5-mm margins through the use of a Clinac 2300 C/D linear accelerator (Varian Medical Systems, Palo Alto, CA) fitted with a 25-mm radiosurgery conical collimator (BrainLAB AG, Feldkirchen, Germany), which is designed to deliver very sharp and limited radiation dose fields. Superflab bolus (1.5 cm tissue equivalent material) was placed over the tumor, and a source-to-skin distance of 100 cm was set. Radiation was delivered at 600 cGy/min with 6 MV X-rays. Mice received a single dose of 20 Gy, three fractions of 8 Gy, or five fractions of 6 Gy in consecutive days (Fig. 1). CTLA-4–blocking mAb 9H10 or vehicle (PBS) was given i.p. at a dose of 200 μg/mouse (10 mg/kg) on days 14, 17, and 20. In some experiments, 9H10 was given on days 12, 15, and 18; days 14, 17, and 20; or days 16, 18, and 21, as indicated in the figure legends. Tumor growth was evaluated every 2 to 3 d until day 35. All mice were sacrificed on day 35, and the tumors harvested and weighed.

***Immunostaining of tumor sections.*** Tumors from treated and untreated mice were harvested on day 35 post tumor inoculation, fixed for 1 h at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in optimum cutting temperature medium. Sections (8 μm) were incubated with 0.1% Tween 20 and 0.01% Triton X-100 for 20 min, followed by 4% rat serum in 4% bovine serum albumin/PBS for an additional 30 min. Sections were stained with PE-Texas Red–conjugated rat anti-mouse CD4 or Phycoerythrin (PE)-conjugated rat anti-mouse CD8α (Caltag, Carlsbad, CA), and counterstained with 5 μg/mL 4′,6-diamidino-2-phenylindole (Sigma).



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



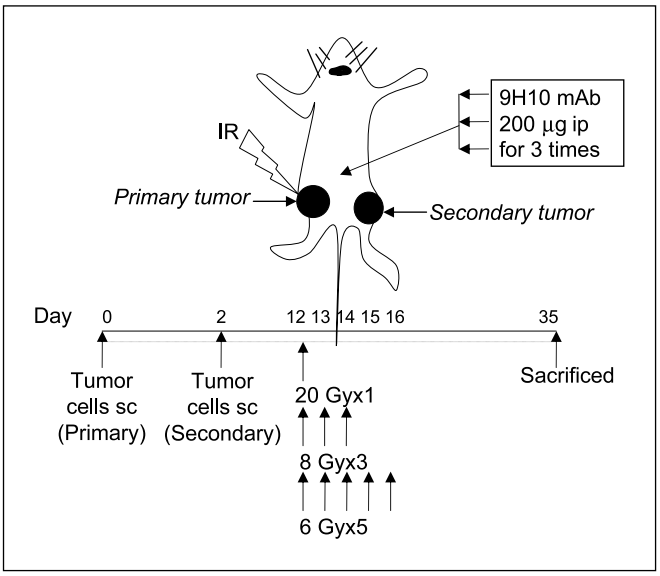



**Fig. 1.** Tumor model and treatment schedule. Immunocompetent mice were injected s.c. with syngeneic TSA cells ($1 \times 10^5$) into the right (defined as "primary" tumor) and left (defined as "secondary" tumor) flank on days 0 and 2, respectively. Ionizing radiation was given locally, exclusively to the primary tumor with the rest of the body shielded, in a single dose or multiple fractions given in consecutive days starting on day 12. CTLA-4–blocking mAb 9H10 was given i.p every 3 d, thrice starting on day 12, 14, or 16 as indicated. Primary and secondary tumor volumes were measured until day 35, at which time mice were sacrificed and the tumors weighed.

Images were obtained with the use of a Nikon Eclipse 800 deconvolution microscope. The number of CD4 and CD8 T cells was counted in three randomly selected (×20) fields in each tumor.

**Ex vivo *production of IFN-γ by spleen cells.*** Spleen cells ($1 \times 10^6$) from TSA tumor-bearing mice were cultured in 24-well tissue culture plates with $2.5 \times 10^5$ irradiated (20 Gy) TSA cells for 24 h in 1 mL fresh RPMI 1640 supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μmol/L 2-mercapthoethanol, and 10% fetal bovine serum (T-cell medium). The supernatants were collected and stored at -80°C. IFN-γ was measured in cell-free supernatants of duplicate wells by ELISA (Diaclone Tepnel, Lifecodes Corp., Stamford, CT). Tumor-specific IFN-γ production was calculated by subtracting the background values measured in supernatants of spleen cells cultured with medium alone.

***Flow cytometry analysis of IFN-γ–producing CD8 T cells.*** For *in vitro* restimulation, $3.5 \times 10^6$ spleen cells from TSA tumor-bearing mice were cultured with the TSA-derived immunodominant CD8 epitope AH1 peptide (SPSYVYHQF; 1 μmol/ml; ref. 15), whereas spleen cells from MCA38 tumor-bearing mice were cultured with $1 \times 10^6$ irradiated (50 Gy) MCA38 cells. After 5-d culture in 24-well tissue culture plates in 2 mL T-cell medium supplemented with 10 U/mL human recombinant interleukin 2 (provided by the National Cancer Institute Biological Resources Branch Preclinical Repository), the percentage of CD8 T cells producing IFN-γ was determined. Briefly, T cells were cultured for 16 h with irradiated TSA or MCA38 target cell, or with irrelevant target RMA-S Ld cells preloaded with mouse cytomegalovirus peptide (17) at 1:1 ratio in the presence of 1 μL/mL Brefaldin A, washed and incubated with rat anti-mouse CD16/CD32 mAb (2.4G2) to block nonspecific binding, and then stained with CD8α-PE-Cy5 and IFN-γ-FITC, or control antibodies according to the manufacturer's instructions (BD Pharmingen). Cells were analyzed with the use of a FACScan flow cytometer and FlowJo version 8.7.1 (Tree Star, Ashland, OR).

***Statistical analysis.*** Random regression coefficients were used to model log tumor volume and log tumor weight as functions of elapsed time from treatment onset and to compare treatment regimens with respect to tumor growth rate. Separate analyses were conducted to assess the effect of treatment on the growth of primary and secondary tumors. The logs of tumor weight and of tumor volume were used in place of the observed data to better satisfy underlying distributional assumptions, and because changes over time in tumor volume and weight were well approximated as log-linear. The use of random regression coefficients permits a separate tumor growth curve to fit the data from each animal. The treatments are then compared on the basis of aggregate tumor growth models; for a given treatment, the aggregate growth model is a single curve describing the average change in tumor volume among animals receiving the treatment. The models for predicting log tumor weight or volume each included the level of radiotherapy exposure, and the variable identifying whether the animal received PBS or 9H10 as fixed classification factors and as terms representing the interaction of these factors. The models also included time from treatment onset as a numeric factor and terms representing the interaction of time with treatment. To account for statistical dependencies among data derived for a single animal, the covariance structure was modeled by assuming the observations to be correlated only when acquired from the same animal. All reported *P*-values are two-sided and were declared statistically significant at the 5% level. The statistical computations were carried out with the use of SAS version 9.0 for Windows (SAS Institute, Cary, NC).

## Results

***Fractionated but not single-dose radiotherapy synergizes with anti–CTLA-4 antibody in the TSA breast cancer model.*** We have previously shown in the 4T1 mouse model of metastatic breast cancer that local radiotherapy in combination with CTLA-4 blockade induces an antitumor immune response inhibiting systemic growth of micrometastases (13). To determine whether the induced antitumor immune response could be effective against larger "metastatic" tumor nodules, we used the TSA mouse mammary carcinoma cells injected at two separate sites, as illustrated in Fig. 1. Similar to 4T1, TSA is a poorly immunogenic carcinoma with ability to shed spontaneous metastases. In contrast to 4T1, however, TSA cells metastasize with a delay of a few weeks from initial implantation (18), providing a window during which the potential effects of the spontaneously shed tumor cells on the growth of the two s.c. implanted tumors are negligible. To mimic the clinical setting in which radiotherapy is applied to the largest (symptomatic) nodule, the site designated as "primary" and receiving local radiation was injected 2 days earlier than the "secondary" site outside the field of radiation. On day 12, when both tumors were palpable, mice were randomly assigned to eight treatment groups receiving mock radiation, one dose of 20 Gy, three fractions of 8 Gy, or five fractions of 6 Gy to the primary tumor (Fig. 1). CTLA-4–blocking mAb 9H10 was given to half of the mice in each radiation group thrice, on days 14, 17, and 20.

In the absence of radiotherapy, 9H10 administration did not have any effect on either primary or secondary tumors (Fig. 2). Radiotherapy as single modality caused significant growth delay in the primary tumor that was comparable with all regimens used but had no effect on secondary tumors (Fig. 2A). Radiotherapy and 9H10 showed a significant interaction ($P < 0.001$) on the primary tumor growth only when given in three fractions of 8 Gy and five fractions of 6 Gy, causing enhanced tumor inhibition in comparison with radiation alone and complete regression in the majority of mice (Fig. 2B, *left*). Importantly, growth of the secondary tumors was significantly inhibited ($P < 0.01$) only in mice treated with fractionated but not single-dose radiotherapy in combination with 9H10, and



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



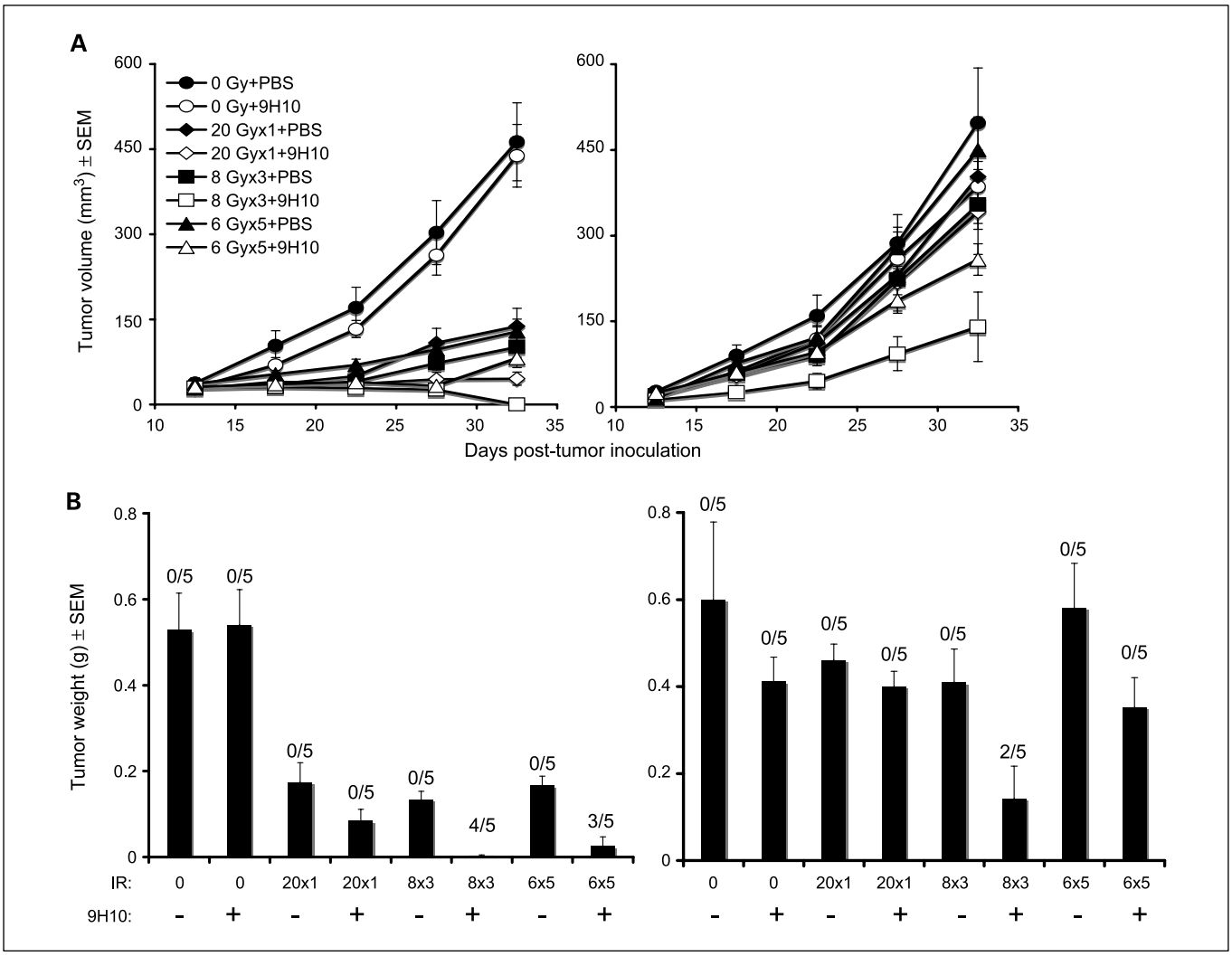



**Fig. 2.** The abscopal effect is induced in TSA tumor-bearing mice by fractionated radiation in combination with anti–CTLA-4 antibody. *A,* tumor growth delay of primary irradiated tumors (*left*) and secondary nonirradiated tumor (*right*) in mice treated with PBS (*closed circles*), 9H10 (*open circles*), 20 Gy × 1 + PBS (*closed diamonds*), 20 Gy × 1 + 9H10 (*open diamonds*), 8 Gy × 3 + PBS (*closed squares*), 8 Gy × 3 + 9H10 (*open squares*), 6 Gy × 5 + PBS (*closed triangles*), or 6 Gy × 5 + 9H10 (*open triangles*). 9H10 was given on days 14, 17, and 20. Data, mean ± SE of five mice per group. *B,* tumor weight of primary (*left*) and secondary (*right*) tumors at day 35. Data, mean ± SE from one of two independent experiments with similar results. The number of mice with complete tumor regression over the total number of mice per group is indicated.

in two mice treated with three fractions of 8 Gy, the secondary tumor completely regressed (Fig. 2B, *right*).

These data indicate that different radiation regimens causing similar direct effects in terms of growth inhibition of the irradiated tumor convey a different propensity to induce an abscopal effect in combination with CTLA-4 blockade.

***Effect of anti–CTLA-4 antibody administration schedule on TSA tumor inhibition in mice treated with radiotherapy.*** To determine whether the time of administration of 9H10 mAb relative to radiotherapy could play a role in its ability to induce an abscopal effect, 9H10 treatment was started on different days. As single modality, administration of 9H10 starting on day 12 did not show any effect on TSA tumor growth, similarly to what was observed when 9H10 was started on day 14 (Figs. 2 and 3A). Importantly, administration of 9H10 on days 12, 15, and 18 did not enhance the inhibition of either the primary or secondary tumors by a single 20-Gy dose, yielding results similar to the delayed administration on days 14, 17, and 20 (Fig. 3A). Consistent with these results, no significant interaction between 20 Gy and 9H10 started on day 12 was observed ($P = 0.21$ and $P = 0.42$ for primary and secondary tumors, respectively). Therefore, the observed differential ability of single-dose and fractionated radiotherapy to induce an abscopal effect (Fig. 2) cannot be explained by the 2-day interval between the 20-Gy irradiation and the beginning of immunotherapy.

Next, we tested whether starting 9H10 mAb treatment 2 days before the conclusion of fractionated radiotherapy (day 12), at conclusion (day 14), or 2 days later (day 16) affected the inhibition of tumor growth observed in mice treated with three fractions of 8 Gy. Primary tumor inhibition was significantly enhanced by administration of 9H10 on days 12, 15, and 18, or days 14, 17, and 20 compared with radiotherapy alone ($P < 0.001$ for both schedules; Fig. 3B). Although five of six primary tumors completely regressed with three fractions of 8 Gy plus 9H10 started at day 14, and only three of six completely regressed when 9H10 was started at day 12, this difference was



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



not statistically significant ($P = 0.08$). Likewise, the growth of secondary tumors was significantly inhibited in both groups of mice ($P < 0.05$ compared with control mice), and there was no significant difference when 9H10 mAb administration was started on day 12 or 14 ($P = 0.9$; Fig. 3B). Delaying administration of 9H10 mAb until day 16 reduced the therapeutic effect, with only one of six primary tumors showing complete regression and a reduced growth inhibition of the secondary tumors (Fig. 3B). This suggests that delaying immunotherapy may reduce its potential benefit. Of note, however, is the fact that complete regression of one secondary tumor was obtained in mice receiving fractionated radiotherapy to the primary tumor even when CTLA-4 blockade was started on day 16, whereas in mice receiving single-dose radiotherapy to the primary tumor, early administration of 9H10 on day 12 did not induce a significant abscopal effect.

Overall, the data indicate that the schedule of administration of 9H10 mAb relative to radiotherapy influences the therapeutic efficacy of this combination treatment. However, the radiotherapy regimen chosen is a fundamental determinant of the ability of the combination treatment to induce an abscopal effect.

***Three fractions of 8 Gy are more effective than five fractions of 6 Gy in inducing antitumor immunity in combination with anti–CTLA-4 antibody.*** The data described above (Fig. 2) suggested

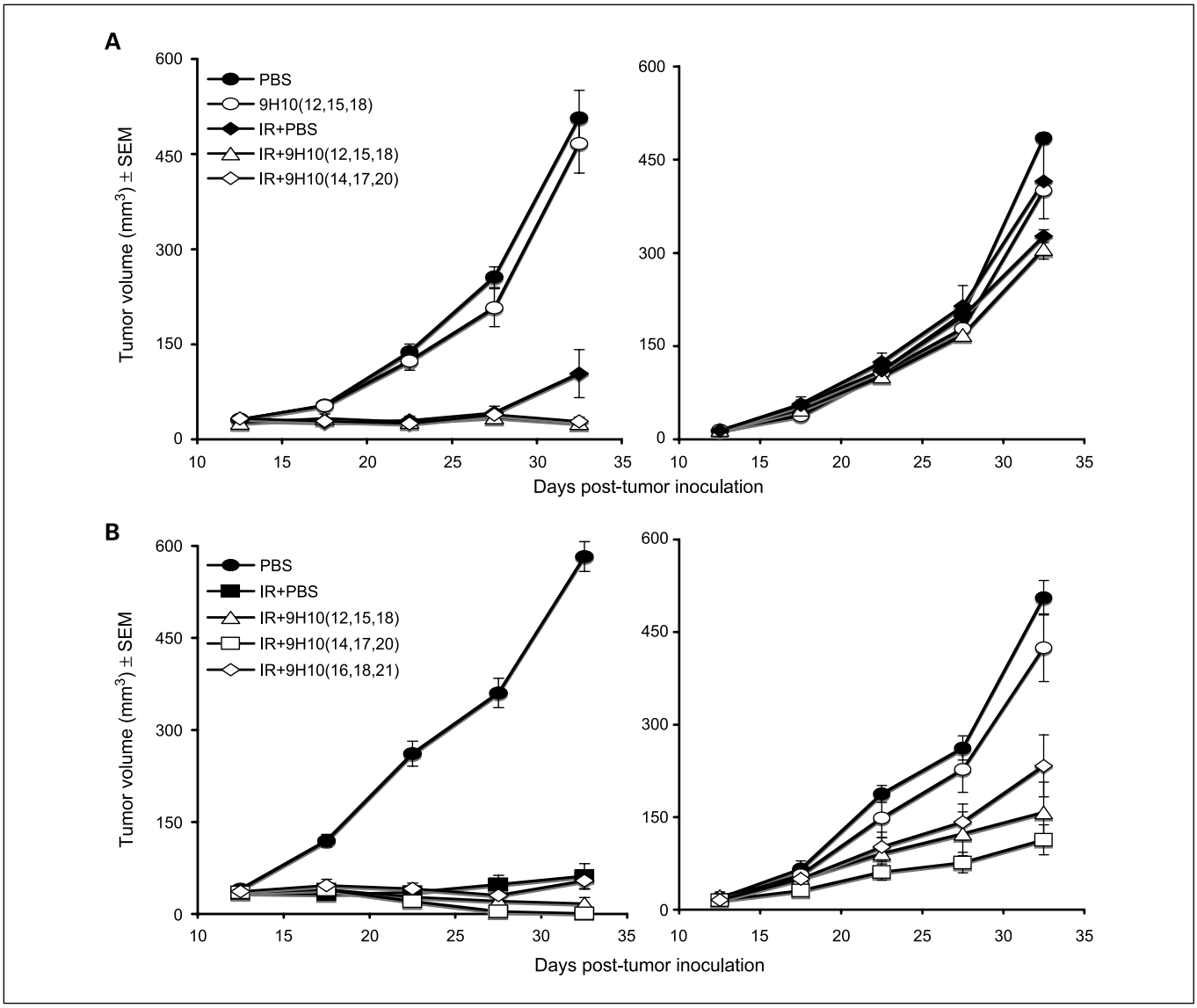



**Fig. 3.** Effect of time of administration of anti–CTLA-4 antibody on the abscopal effect induced by radiotherapy in TSA tumor-bearing mice. *A,* tumor growth delay of primary irradiated tumors (*left*) and secondary nonirradiated tumor (*right*) in mice treated with PBS (*closed circles*), 9H10 given on days 12, 15, and 18 (*open circles*), 20 Gy × 1 + PBS (*closed diamonds*), 20 Gy × 1 + 9H10 given on days 12, 15, and 18 (*open triangles*), or 20 Gy × 1 + 9H10 given on days 14, 17, and 20 (*open diamonds*). Data, mean ± SE of five mice per group. No complete regression of either primary or secondary tumors was observed in any of the treatment arms. *B,* tumor growth delay of primary irradiated tumors (*left*) and secondary nonirradiated tumor (*right*) in mice treated with PBS (*closed circles*), 8 Gy × 3 + PBS (*closed squares*), 8 Gy × 3 + 9H10 given on days 12, 15, and 18 (*open triangles*), 8 Gy × 3 + 9H10 given on days 14, 17, and 20 (*open squares*), and 8 Gy × 3 + 9H10 given on days 16, 18, and 21 (*open diamonds*). Data, mean ± SE of six mice per group. Complete regression was seen in three of six primary and one of six secondary tumors in mice treated with 8 Gy × 3 + 9H10 given on days 12, 15, and 18; in five of six primary and one of six secondary tumors in mice treated with 8 Gy × 3 + 9H10 given on days 14, 17, and 20; and in one of six primary and one of six secondary tumors in mice treated with 8 Gy × 3 + 9H10 given on days 16, 18, and 21.



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



that among the two radiotherapy fractionation regimens, the three fractions of 8 Gy protocol was the most effective for induction of the abscopal effect in combination with CTLA-4 blockade. To confirm this and to examine the immunologic mechanisms of the abscopal effect, mice bearing two separate TSA tumors were mock treated or given three fractions of 8 Gy, or five fractions of 6 Gy to the primary tumor, in combination with 9H10 mAb given on days 14, 17, and 20. Radiotherapy plus 9H10 was very effective at inhibiting the growth of the irradiated ($P < 0.0001$ compared with mock-treated mice for both regimens) as well as nonirradiated ($P < 0.0001$ for 8 Gy × 3; $P = 0.015$ for 6 Gy × 5 compared with mock-treated mice) tumor (Fig. 4A). However, 8 Gy × 3 was significantly more effective than 6 Gy × 5 at inhibiting the growth of both the irradiated ($P = 0.038$) and nonirradiated ($P = 0.014$) tumors, and complete regression of primary and secondary tumors was observed more frequently in mice receiving 8 Gy × 3 (Fig. 4B), supporting a superior therapeutic effect of this regimen when combined with CTLA-4 blockade.

Analysis of secondary tumors for the presence of tumor-infiltrating lymphocytes showed that, whereas in mice treated with radiotherapy and 9H10 as single modalities there was a minimal increase in the number of CD4+ and CD8+ tumor-infiltrating lymphocytes, treatment with 8 Gy × 3 and 9H10 caused a significant ($P < 0.05$ compared with all other groups) increase in CD4+ and CD8+ tumor-infiltrating lymphocytes (Fig. 5A and B), suggesting that cell-mediated immunity was responsible for the abscopal effect. Consistent with this interpretation, *ex vivo* tumor-specific production of IFN-γ by spleen cells was elevated only in mice that were effectively rejecting the secondary tumor (Fig. 5C). The frequency of CD8+ T cells showing tumor-specific IFN-γ expression after *in vitro* restimulation with

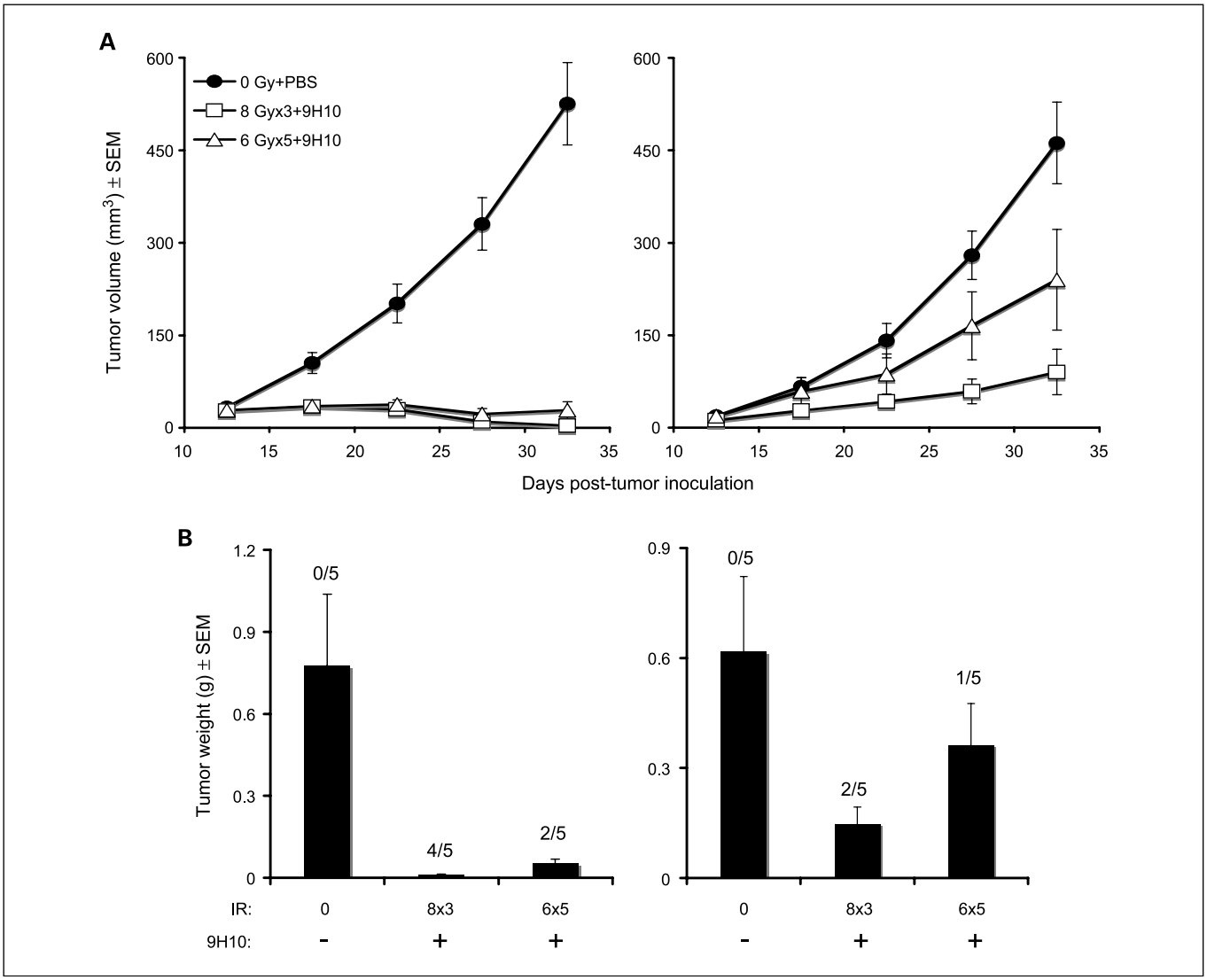



**Fig. 4.** Fractionated radiotherapy given to TSA tumor-bearing mice in three doses of 8 Gy is more effective than five doses of 6 Gy at synergizing with anti–CTLA-4 antibody. *A,* tumor growth delay of primary irradiated tumors (*left*) and secondary nonirradiated tumor (*right*) in mice treated with PBS (*closed circles*), 8 Gy × 3 + 9H10 (*open squares*), or 6 Gy × 5 + 9H10 (*open triangles*). 9H10 was given on days 14, 17, and 20. Data, mean ± SE of five mice per group. *B,* tumor weight of primary (*left*) and secondary (*right*) tumors at day 35. Data, mean ± SE. The number of mice with complete tumor regression over the total number of mice per group is indicated.



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



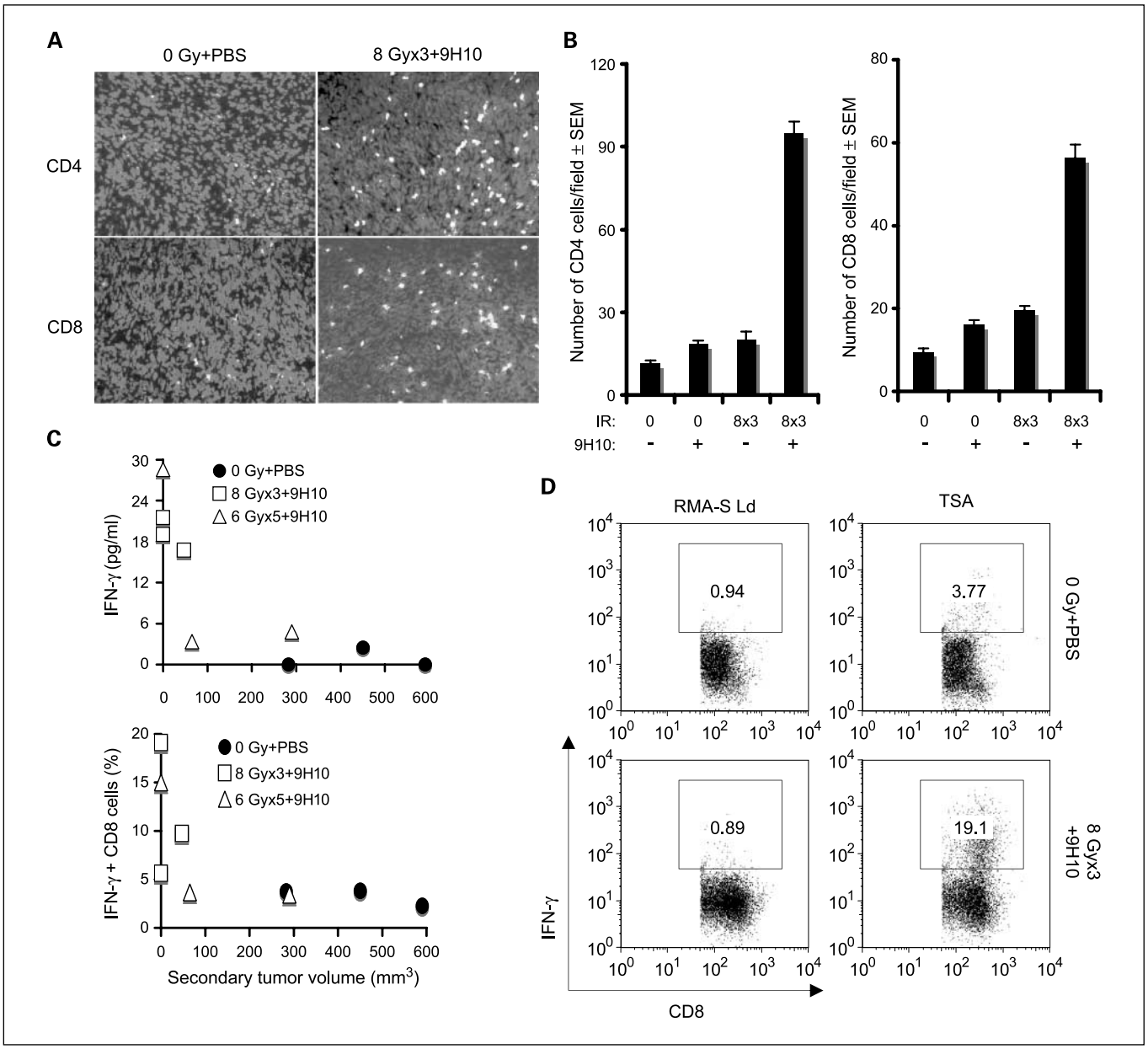



**Fig. 5.** The combination of fractionated radiotherapy with anti–CTLA-4 antibody enhances TIL in secondary TSA tumors and tumor-specific T cells producing IFN-γ. *A* and *B,* secondary tumors were excised at day 35 and analyzed by fluorescence microscopy for the presence of CD4+ and CD8+ T cells. *A,* representative fields showing CD4+ (*top*) and CD8+ (*bottom*) T cells (*white*) infiltrating secondary TSA tumors in mice treated as indicated. Nuclei were stained with DAPI (*light gray*). *B,* mean number ± SE of CD4+ and CD8+ TILs in three mice per group. Both CD4+ and CD8+ TILs were significantly increased in mice treated with the combination of 8 Gy × 3 + 9H10 ($P < 0.05$ compared with all other groups), whereas radiation and 9H10 as single modalities did not have a significant effect. *C* and *D,* analysis of tumor-specific IFN-γ production by spleen cells harvested at day 35 from mice in the various treatment groups. *C,* IFN-γ concentration in supernatants of total spleen cells isolated from mice treated with 0 Gy + PBS (*closed circles*), 8 Gy × 3 + 9H10 (*open squares*), or 6 Gy × 5 + 9H10 (*open triangles*), and cultured overnight with irradiated TSA cells were plotted against the volume of the secondary tumor (*top*). The percentage of CD8+ T cells expressing IFN-γ when exposed to TSA cells as determined by intracellular staining (*D*) after *in vitro* restimulation with the TSA-derived immunodominant CD8 epitope AH1 was plotted against the volume of the secondary tumor (*bottom*). Symbols, as above; each represents one animal. *D,* representative histograms showing the percentage of CD8+ T cells positive for IFN-γ by intracellular staining and flow cytometry in response to TSA cells or the irrelevant target RMA-S-Ld. Samples were gated on CD8+ T cells. TIL, tumor-infiltrating lymphocytes; DAPI, 4′,6-diamidino-2-phenylindole.

a CTL epitope known to be an immunodominant antigen in TSA cells (15) was also increased in treated mice that rejected secondary tumors but not in those that did not (Fig. 5C and D).

Collectively, these results show that treatment with fractionated radiotherapy and CTLA-4 blockade induces tumor-specific T-cell responses that, when sufficiently strong, are associated with complete rejection of tumors outside the radiation field.

***Fractionated radiotherapy synergizes with anti–CTLA-4 antibody in the MCA38 colon cancer model.*** To determine whether the same effects of radiotherapy in combination with 9H10 would be seen in a different tumor type growing in mice of a different genetic background, we used the MCA38 mouse colon carcinoma cells injected at two separate sites into C57BL/6 mice. On day 12, when both tumors were palpable, mice were



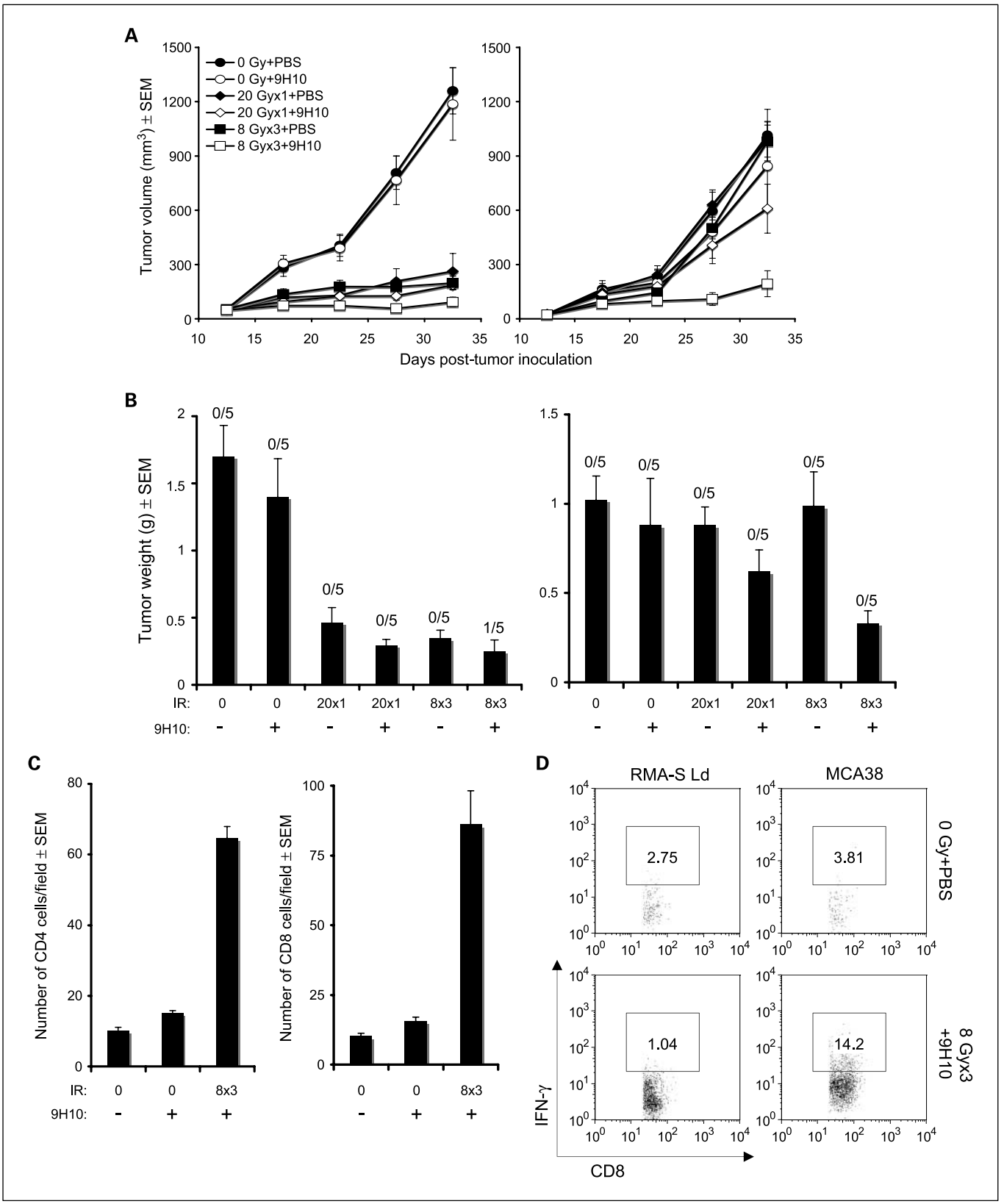
Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



randomly assigned to receive mock radiation, a single 20-Gy dose, or three fractions of 8 Gy to the primary tumor as described above (Fig. 1), and 9H10 was given to half of the mice in each treatment group on days 14, 17, and 20.

Similar to what observed in the TSA model, 9H10 administration as single modality did not have any effect on growth of primary or secondary MCA38 tumors (Fig. 6A). Radiation alone caused a significant ($P < 0.0001$) growth delay of primary






Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



irradiated tumors that was similar at 20 Gy × 1 and 8 Gy × 3, but had no effect on secondary tumors. Addition of 9H10 treatment to mice treated with 8 Gy × 3 significantly improved growth inhibition of primary tumors compared with radiation alone ($P = 0.0049$) and caused a marked inhibition of secondary tumors ($P = 0.0001$) (Fig. 6A and B). The combination of 9H10 with radiation at 20 Gy × 1 failed to significantly enhance the inhibition of primary tumors ($P = 0.145$) and, although it modestly reduced the growth of secondary tumors, the effect was significantly less than that observed with 8 Gy × 3 + 9H10 ($P < 0.0001$; Fig. 6A and B).

Therefore, in the MCA38 model, fractionated more than single-dose radiation triggered an abscopal effect when combined with CTLA-4 blockade.

Administration of 9H10 as single modality did not significantly enhance CD4+ and CD8+ tumor-infiltrating lymphocytes in secondary MCA38 tumors, whereas tumor-infiltrating lymphocytes were markedly increased in mice treated with 8 Gy × 3 + 9H10 ($P < 0.05$ for both CD4+ and CD8+ T cells compared with control and 9H10 alone; Fig. 6C). The frequency of CD8+ T cells showing tumor-specific IFN-γ expression after *in vitro* restimulation with MCA38 cells was also increased in mice treated with 8 Gy × 3 and 9H10, confirming the development of tumor-specific immunity after treatment (Fig. 6D).

## Discussion

In this study, we show in breast and colon carcinoma models that fractionated local radiotherapy to one palpable tumor can synergize with CTLA-4 blockade to induce antitumor T-cell immunity and inhibit a second palpable tumor outside the radiation field. This abscopal effect was not seen with radiotherapy alone. Although localized tumor irradiation by itself has been shown to enhance the generation of tumor-specific T cells in both preclinical models and patients, the therapeutic effects of this response remain undetermined (3, 19). Clearly, irradiation to the primary tumor was required in our models to induce growth inhibition of the secondary tumors outside the field because CTLA-4 blockade by itself was ineffective (Figs. 2 and 6). This is consistent with the hypothesis that radiation-induced immunogenic tumor cell death as well as its induction of danger signals contribute to generate an *in situ* vaccine (20–22). Whereas the response generated is not sufficient to be therapeutically significant, additional immunotherapeutic interventions might enable it to result in meaningful antitumor immunity.

Clinically, radiotherapy is usually given in multiple fractions to identify a compromise that achieves tumor control while enabling repair of damage to normal tissues within the field. Modern technologies enable better visualization and targeting of tumors, with selective concentration of dose distributions to achieve a therapeutic advantage (23). Our data indicate that a large single dose of 20 Gy was as effective as the two fractionation regimens of 8 Gy × 3 and 6 Gy × 5 at controlling the growth of the irradiated tumor (Fig. 2). However, the degree to which radiation by itself achieved local tumor control did not predict its ability to synergize with CTLA-4 blockade. Addition of 9H10 mAb to mice receiving 20 Gy did not significantly improve the response, whereas a dramatic improvement in control of both primary and secondary tumors was seen when 9H10 was given to mice treated with either of the two fractionated radiotherapy regimens tested (Fig. 2). Importantly, the regimen of 8 Gy × 3 was superior to 6 Gy × 5 in the induction of the abscopal effect and of tumor-specific T cells (Figs. 4 and 5C), suggesting that a specific therapeutic window exists for the optimal use of fractionated radiotherapy in combination with CTLA-4 blockade.

With the use of the B16 mouse melanoma model, Lugade et al. have shown that a single dose of 15 Gy irradiation resulted in priming of tumor-specific T cells in the draining lymph node that was at least comparable with that achieved after a regimen of 3 Gy × 5 fractions (3). We have previously shown in the 4T1 mouse breast cancer model that, although two fractions were better, a single dose of 12 Gy did also synergize with CTLA-4 blockade and induce antitumor CD8 cells capable of inhibiting lung micrometastases (13). It is conceivable that single-dose radiotherapy could promote cross-priming but that the magnitude of the elicited immune response is insufficient at controlling "bulky" palpable tumors, such as the tumors outside the radiation field in the current study.

The mechanisms underlying our finding that single-dose and fractionated radiation differ in their ability to synergize with CTLA-4 blockade warrant further investigation. Interestingly, a recent report analyzing gene expression profiles of breast, prostate, and glioma tumor cells exposed to single-dose (10 Gy) versus fractionated (2 Gy × 5) radiation showed marked differences in the molecular response of these cells to the two regimens both *in vitro* and *in vivo* (24). Among the genes selectively induced by fractionated radiation in all three tumor cell lines were several IFN-related genes, including signal transducers and activators of transcription 1, but their role in promoting inflammation versus radioresistance remains to be clarified (24).

Antibodies targeting immunomodulatory molecules on T cells to induce or enhance antitumor immunity are finding their way into the clinic. Among them, two CTLA-4–blocking mAbs (ipilimumab and tremelimumab) are at a more advanced stage of testing and have shown some promising results (25). CTLA-4 blockade has activity as single treatment in melanoma,

**Fig. 6.** The abscopal effect is induced in MCA38 tumor-bearing mice by fractionated radiation in combination with anti–CTL-4 antibody. C57BL/6 mice were injected with syngeneic MCA38 colon carcinoma cells ($5 \times 10^5$) s.c. into the right and left flanks as outlined in Fig. 1. *A,* tumor growth delay of primary irradiated tumors (*left*) and secondary nonirradiated tumors (*right*) in mice treated with PBS (*closed circles*), 9H10 (*open circles*), 20 Gy × 1 + PBS (*closed diamonds*), 20 Gy × 1 + 9H10 (*open diamonds*), 8 Gy × 3 + PBS (*closed squares*), or 8 Gy × 3 + 9H10 (*open squares*). 9H10 was given on days 14, 17, and 20. Data, mean ± SE of five mice per group. *B,* tumor weight of primary (*left*) and secondary (*right*) tumors at day 35. Data, mean ± SE. The number of mice with complete tumor regression over the total number of mice per group is indicated. *C,* secondary tumors were excised at day 35 and analyzed by fluorescence microscopy for the presence of CD4+ and CD8+ T cells. Data, mean ± SE of CD4+ and CD8+ TILs in three mice per group. Both CD4+ and CD8+ TILs were significantly increased in mice treated with the combination of 8 Gy × 3 + 9H10 ($P < 0.05$ compared with all other groups). *D,* analysis of tumor-specific IFN-γ production by spleen cells harvested at day 35 from treated and untreated mice, and restimulated *in vitro* with irradiated MCA38 cells. Histograms, percentage of CD8+ T cells positive for IFN-γ by intracellular staining and flow cytometry in response to MCA38 cells or the irrelevant target RMA-S-Ld. Samples were gated on CD8+ T cells. Spleen cells from three mice in each treatment group were pooled.



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265



but the rate of complete response, disease control, and overall survival was improved when it was given together with a cytotoxic agent (26). No data is currently available on the clinical use of radiotherapy with CTLA-4 blockade, whereas local radiation has been tested in combination with other immunotherapies (27–29). The results of these studies are consistent with preclinical predictions and support the hypothesis that local radiation can synergize with immunotherapy to promote antitumor immunity (1).

The data presented indicate that, in tumors that are refractory to treatment with CTLA-4 blockade alone, the combination with radiotherapy to one tumor site can induce systemic tumor control and, in some cases, complete regression. Importantly, the dose fractionation of radiation can determine the overall efficacy of the combination treatment, an invaluable information in designing the clinical translation of this work.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank the personnel of the New York University Cancer Institute Flow Cytometry and Experimental Pathology (Histopathology) core facilities, and of the Department of Radiation Oncology for expert assistance.

## References

1. Demaria S, Bhardwaj N, McBride WH, Formenti SC. Combining radiotherapy and immunotherapy: a revived partnership. Int J Radiat Oncol Biol Phys 2005;63:655–66.
2. Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 2007;13:1050–9.
3. Lugade AA, Moran JP, Gerber SA, Rose RC, Frelinger JG, Lord EM. Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. J Immunol 2005;174:7516–23.
4. Demaria S, Formenti SC. Sensors of ionizing radiation effects on the immunological microenvironment of cancer. Int J Radiat Biol 2007;83:819–25.
5. Matsumura S, Wang B, Kawashima N, et al. Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. J Immunol 2008;181:3099–107.
6. Ehlers G, Fridman M. Abscopal effect of radiation in papillary adenocarcinoma. Br J Radiol 1973;46:220–2.
7. Robin HI, AuBuchon J, Varanasi VR, Weinstein AB. The abscopal effect: demonstration in lymphomatous involvement of kidneys. Med Pediat Oncol 1981;9:473–6.
8. Ohba K, Omagari K, Nakamura T, et al. Abscopal regression of hepatocellular carcinoma after radiotherapy for bone metastasis. Gut 1998;43:575–7.
9. Wersall PJ, Blomgren H, Pisa P, Lax I, Kalkner KM, Svedman C. Regression of non-irradiated metastases after extracranial stereotactic radiotherapy in metastatic renal cell carcinoma. Acta Oncol 2006;45:493–7.
10. Rosenberg SA, Sherry RM, Morton KE, et al. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. J Immunol 2005;175:6169–76.
11. Herber DL, Nagaraj S, Djeu JY, Gabrilovich DI. Mechanism and therapeutic reversal of immune suppression in cancer. Cancer Res 2007;67:5067–9.
12. Demaria S, Ng B, Devitt M-L, et al. Ionizing radiation inhibition of distant untreated tumors (abscopal effect) is immune mediated. Int J Radiat Oncol Biol Phys 2004;58:862–70.
13. Demaria S, Kawashima N, Yang AM, et al. Immune-mediated inhibition of metastases following treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clin Cancer Res 2005;11:728–34.
14. Pilones KA, Kawashima N, Yang AM, Babb JS, Formenti SC, Demaria S. Invariant natural killer T cells regulate breast cancer response to radiation and CTLA-4 blockade. Clin Cancer Res 2009;15:597–606.
15. Rosato A, Santa SD, Zoso A, et al. The cytotoxic T-lymphocyte response against a poorly immunogenic mammary adenocarcinoma is focused on a single immunodominant class I epitope derived from the gp70 Env product of an endogenous retrovirus. Cancer Res 2003;63:2158–63.
16. Thurnherr N, Deschner EE, Stonehill EH, Lipkin M. Induction of adenocarcinomas of the colon in mice by weekly injections of 1,2-dimethylhydrazine. Cancer Res 1973;33:940–5.
17. Alexander-Miller MA, Burke K, Koszinowski UH, Hansen TH, Connolly JM. Alloreactive cytotoxic T lymphocytes generated in the presence of viral-derived peptides show exquisite peptide and MHC specificity. J Immunol 1993;151:1–10.
18. Cavallo F, DiCarlo E, Butera M, et al. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. Cancer Res 1999;59:414–21.
19. Schaue D, Comin-Anduix B, Ribas A, et al. T-cell responses to survivin in cancer patients undergoing radiation therapy. Clin Cancer Res 2008;14:4883–90.
20. Obeid M, Panaretakis T, Joza N, et al. Calreticulin exposure is required for the immunogenicity of γ-irradiation and UVC light-induced apoptosis. Cell Death Differ 2007;14:1848–50.
21. Formenti SC, Demaria S. Local control by radiotherapy: is that all there is? Breast Cancer Res 2008;10:215.
22. Apetoh L, Ghiringhelli F, Tesniere A, et al. The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy. Immunol Rev 2007;220:47–59.
23. Verellen D, Ridder MD, Linthout N, Tournel K, Soete G, Storme G. Innovations in image-guided radiotherapy. Nat Rev Cancer 2007;7:949–60.
24. Tsai MH, Cook JA, Chandramouli GV, et al. Gene expression profiling of breast, prostate, and glioma cells following single versus fractionated doses of radiation. Cancer Res 2007;67:3845–52.
25. Fong L, Small EJ. Anti-cytotoxic T-lymphocyte antigen-4 antibody: the first in an emerging class of immunomodulatory antibodies for cancer treatment. J Clin Oncol 2008;26:5275–83.
26. Hersh EM, Weber JS, Powderly JD, et al. Disease control and long-term survival in chemotherapy-naive patients with advanced melanoma treated with ipilimumab (MDX-010) with or without dacarbazine [abstract 9022]. J Clin Oncol 2008;26:485s.
27. Gulley JL, Arlen PM, Bastian N, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. Clin Cancer Res 2005;11:3353–62.
28. Chi KH, Liu SJ, Li CP, et al. Combination of conformal radiotherapy and intratumoral injection of adoptive dendritic cell immunotherapy in refractory hepatoma. J Immunother 2005;28:129–35.
29. Lechleider RJ, Arlen PM, Tsang KY, et al. Safety and immunologic response of a viral vaccine to prostate-specific antigen in combination with radiation therapy when metronomic-dose interleukin 2 is used as an adjuvant. Clin Cancer Res 2008;14:5284–91.



Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.

Published OnlineFirst August 25, 2009; DOI: 10.1158/1078-0432.CCR-09-0265

AACR American Association for Cancer Research

Clinical Cancer Research

# Fractionated but Not Single-Dose Radiotherapy Induces an Immune-Mediated Abscopal Effect when Combined with Anti–CTLA-4 Antibody

M. Zahidunnabi Dewan, Ashley E. Galloway, Noriko Kawashima, et al.

*Clin Cancer Res* 2009;15:5379-5388. Published OnlineFirst August 25, 2009.

**Updated version** Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-09-0265

**Cited articles** This article cites 29 articles, 17 of which you can access for free at: http://clincancerres.aacrjournals.org/content/15/17/5379.full#ref-list-1

**Citing articles** This article has been cited by 82 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/15/17/5379.full#related-urls

**E-mail alerts** Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions** To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions** To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/15/17/5379. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

Downloaded from clincancerres.aacrjournals.org on November 24, 2020. © 2009 American Association for Cancer Research.