Immune-Mediated Inhibition of Metastases after Treatment with Local Radiation and CTLA-4 Blockade in a Mouse Model of Breast Cancer

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ABSTRACT

Purpose: **Ionizing radiation therapy (RT) is an important component in the management of breast cancer. Although the primary tumor can be successfully treated by surgery and RT, metastatic breast cancer remains a therapeutic challenge. Here we tested the hypothesis that the combination of RT to the primary tumor with CTLA-4 blockade can elicit antitumor immunity inhibiting the metastases.**

Experimental Design: **The poorly immunogenic metastatic mouse mammary carcinoma 4T1 was used as a model. Mice were injected s.c. with 4T1 cells, and treatment was started 13 days later when the primary tumors measured 5 mm in average diameter. Mice were randomly assigned to four treatment groups receiving: (1) control IgG (IgG), (2) RT + IgG, (3) 9H10 monoclonal antibody against CTLA-4, (4) RT + 9H10. RT was delivered to the primary tumor by one or two fractions of 12 Gy. 9H10 and IgG were given i.p. thrice after RT.**

Results: **Consistent with the fact that 4T1 is poorly immunogenic, 9H10 alone did not have any effect on primary tumor growth or survival. RT was able to delay the growth of the primary irradiated tumor, but in the absence of 9H10 survival was similar to that of control mice. In contrast, mice treated with RT + 9H10 had a statistically significant survival advantage. The increased survival correlated with inhibition of lung metastases formation and required CD8+ but not CD4+ T cells.**

Conclusions: **The combination of local RT with CTLA-4 blockade is a promising new immunotherapeutic strategy against poorly immunogenic metastatic cancers.**

INTRODUCTION

Breast cancer is often a systemic malignancy even at clinical presentation, when the tumor is resectable (1, 2). Therefore, local treatment modalities need to be combined with an effective systemic treatment to significantly impact breast cancer survival. In this respect, immunotherapy could offer an important alternative or adjunct to chemotherapy and hormonal therapy. Several experimental strategies have been used to induce antitumor immunity in mouse models of cancer, but many have been successfully tested either as prophylaxis or in the earliest stages of tumor development and/or with relatively immunogenic tumors. The choice of an animal model that closely mimics the situation encountered with breast cancer patients is crucial for evaluating new immunotherapeutic strategies. An excellent currently available model of metastatic breast cancer is the BALB/c-derived 4T1 tumor (3). Although a transplantable model, 4T1 is poorly immunogenic and shares many characteristics with human breast cancer. After s.c. inoculation in the abdominal mammary fatpad, the primary tumor grows into a nodule with the histology of a high-grade breast cancer and sheds spontaneous systemic metastases to the lungs, liver, lymph nodes, bone marrow, and central nervous system (4–6). Metastatic growth in the lungs is usually the cause of death of mice, and surgical removal of the primary tumor once it becomes palpable does not affect metastases growth (7, 8). Therefore, this model is suitable for testing the effects of experimental therapies on metastatic disease.

Ionizing radiation therapy (RT) is a powerful inducer of apoptosis of tumor cells (9) and therefore a widely used therapeutic tool in oncology. The ability to deliver a local hit without systemic toxicity to bone marrow and lymphoid compartment makes RT an attractive candidate for combination treatments aiming at triggering antitumor immunity. Accumulated evidence supports the hypothesis that RT also has the potential to induce/enhance antitumor immunity (reviewed in ref. 10). In this respect, RT may act by both freeing tumor antigens from the dying cells and facilitating the penetration of antigen-presenting cells and effector T cells into solid tumors (11–14). However, the role of RT in cancer immunotherapy has not been fully investigated.

CTLA-4 is up-regulated on the surface of T cells during the early stages of activation and has been shown to play a

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crucial role in the down-regulation of T-cell responses (reviewed in ref. 15). In physiologic conditions, CTLA-4 has a role in the maintenance of peripheral tolerance. On the other hand, in conditions of suboptimal antigen-presenting cell function such as in tumor-bearing hosts (16), CTLA-4–mediated inhibition of T-cell activation can prevent the development of antitumor T-cell responses. Indeed, blockade of this receptor leads to induction of effective cell-mediated antitumor immunity (17, 18). However, the efficacy of CTLA-4 blockade as a single treatment is limited to intrinsically immunogenic tumors (19, 20).

In this study, we tested whether the combination of local RT to the primary tumor with CTLA-4 blockade can induce therapeutically significant antitumor immunity to the poorly immunogenic metastatic mammary carcinoma 4T1. Our data confirm that, like other poorly immunogenic tumors (19, 20), 4T1 growth is not inhibited by CTLA-4 blockade alone. As expected, local tumor growth inhibition by RT does not translate into increased survival because the metastases in the lungs of the animals keep growing. However, here we show for the first time that when CTLA-4 blockade is combined with RT to the primary tumor, CD8+ T cell–dependent antitumor immunity is elicited and it can inhibit the growth of spontaneous lung metastases extending significantly the survival of the treated mice.

MATERIALS AND METHODS

Mice

Six to 8-week-old female BALB/c mice were obtained from Taconic Animal Laboratory (Germantown, NY). All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

Cells and Reagents

4T1 is a BALB/c mouse–derived mammary carcinoma cell line (provided by Fred Miller, the Michigan Cancer Center, Detroit, MI; ref. 4) and A20 is a BALB/C mouse–derived B-cell leukemia/lymphoma (21). 4T1 cells were grown in DMEM (Invitrogen Co., Carlsbad, CA) supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5×10^{-5} mol/L 2-mercapthoethanol, and 10% fetal bovine serum (Gemini Bio-Products Woodland, CA). These cells were found to be free of contamination by Mycoplasma by the Mycoplasma detection kit (Roche Diagnostics, Chicago, IL). Anti-CTLA-4 hamster monoclonal antibody (mAb) 9H10 was purified as previously described (22). Control hamster IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Purified anti-CD4 (GK1.5), anti-CD8 (53-6.7) rat mAb, and control rat IgG were purchased from BioExpress, Inc. (West Lebanon, NH).

Tumor Challenge and Treatment

Mice were injected s.c. in the right flank with 5×10^4 4T1 cells in 0.1 mL of DMEM without additives. Perpendicular tumor diameters were measured with a Vernier caliper, and tumor volumes were calculated as length $\times$ width^2 $\times$ 0.52. Treatment was started on Day 13 when tumors reached the average diameter of 5 mm ($\sim$65 mm^3 in volume). In preliminary experiments, we established that administration of control hamster IgG did not alter tumor growth and survival of mice receiving no other treatment or receiving RT. Therefore, in subsequent experiments the additional control groups (no treatment and RT without hamster IgG) were omitted. In a first set of experiments animals were randomly assigned to four treatment groups: (1) control hamster IgG, (2) RT + control hamster IgG, (3) 9H10 mAb, and (4) RT + 9H10 mAb. All mice were briefly anesthetized by i.p. injection of Avertin (240 mg/kg). Mice assigned to radiation 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. RT was delivered to a field including the tumor with 5 mm margins using a ^{60}CO radiation source by one fraction of 12 Gy. Control hamster IgG and 9H10 were given i.p. at 200 μg at 1, 4, and 7 days after RT. In a second set of experiments, RT was delivered by two fractions of 12 Gy given at 48 hours interval, and the treatment groups did not include 9H10 mAb alone. Tumor growth was evaluated every 2 to 3 days until death or sacrifice when mice seemed moribund. At death, presence of metastases in the lungs was confirmed by macroscopic and microscopic evaluation on formalin-fixed paraffin-embedded lung sections stained with H&E using standard procedures. In some experiments, all mice were sacrificed at day 32 or 35 post-tumor inoculation, and metastatic nodules in the lungs were counted after formalin fixation using a dissecting microscope.

In vivo T-Cell Subset Depletion

Depletion of CD4+ and CD8+ T-cell subsets was done by injecting GK1.5 or 53-6.7 mAb or rat IgG i.p. at 100 μg/mouse thrice (days 10, 11, and 12 post-challenge) before RT treatment (day 13), and 2 days following RT (day 15). The depletion was maintained by repeated injections of mAb every 6 days up to the day of sacrifice (day 32). Depletion was confirmed by testing spleen cells from control animals for the presence of CD4+ and CD8+ T-cells using noncross-reactive FITC-RMA4-4 and PE-anti-CD8β mAb (BD PharMingen, San Diego, CA).

Cytotoxicity Assay

Splenocytes were isolated from 4T1 tumor-bearing mice that were cured following treatment with RT and CTLA-4 blockade 2 months after rechallenge and rejection of a new inoculum of 4T1 cells. Following RBC lysis splenocytes (3×10^6 per well) were stimulated *in vitro* with irradiated (120 Gy) 4T1 cells (10^5 per well), in RPMI 1640 (Invitrogen) supplemented with 2 mmol/L L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 2.5×10^{-5} mol/L 2-mercapthoethanol, 10% fetal bovine serum, and 30 units/mL interleukin 2 (IL-2; provided by the National Cancer Institute BRB Preclinical Repository) in a 24-well plate. After 5 days, responding T cells were harvested, washed, and used as effectors against 4T1 and A20 target cells in a standard lactate dehydrogenase-release ELISA (Roche Molecular Biochemicals). Target cells were incubated at 10^4 per well in round-bottom 96-well plates with effector cells at different effector to target ratios in triplicate wells for 18 hours. At the end of the incubation, released lactate dehydrogenase activity was measured according to the manufacturer's instructions. The percentage specific lysis was calculated as: $[(E_x - S - E_f)/(T - S)] \times 100$, where E_x is lactate dehydrogenase released in the presence of effectors, S is spontaneous release

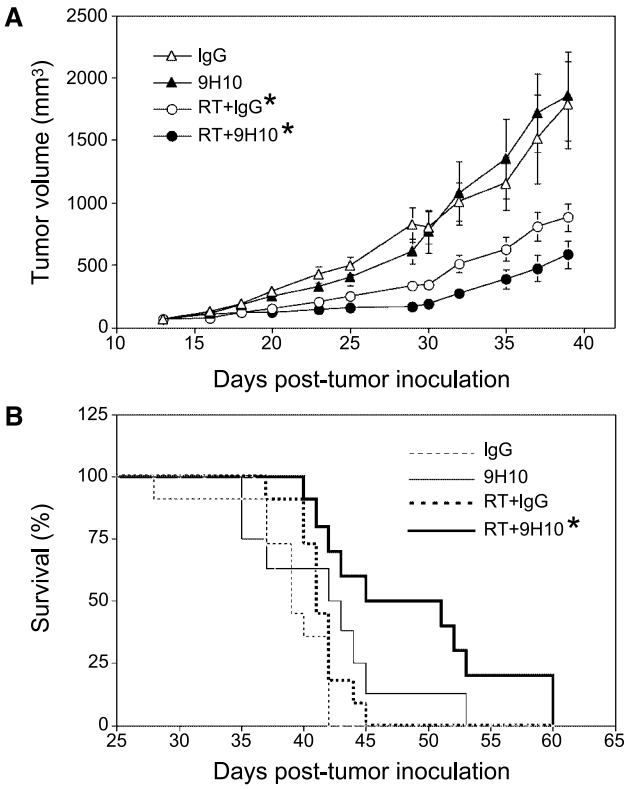

Fig. 1 The combination of local RT and anti-CTLA-4 mAb 9H10 extends the survival of mice bearing the nonimmunogenic 4T1 carcinoma. Treatment was started on day 13 after s.c. inoculation with the 4T1 mammary carcinoma in the flank. RT was delivered by a single dose of 12 Gy to the s.c. tumors. Antibodies were given i.p. 1, 4, and 7 days after RT. Data are from two combined experiments (*A*). Tumor growth delay by RT. Mice received control hamster IgG (*IgG*, △, $n = 11$), 9H10 (▲, $n = 8$), RT + IgG (○, $n = 11$), or RT + 9H10 (●, $n = 10$). Points, mean for each treatment group; bars, ±SE. Tumor volume is shown up to day 39 only because afterwards too many animals died. *, $P < 0.01$, statistically significant difference (from day 20) when compared with IgG-treated control group (*B*). Percentage of surviving mice following treatment with control IgG (*thin broken line*, $n = 11$), 9H10 (*thin line*, $n = 8$), RT + IgG (*bold broken line*, $n = 11$), or RT + 9H10 (*bold line*, $n = 10$). Survival differences between treatment groups were analyzed using a Weibull model; *, $P < 0.0005$, statistically significant difference between RT + 9H10 and all the other groups.

from targets, E_f is spontaneous release from effectors, and T is maximal release from targets lysed with 1% NP40 detergent.

Statistical Analysis

Survival differences among the treatment groups were analyzed using a Weibull model, and differences in tumor growth were evaluated by using random coefficients models to assess temporal changes in log tumor volume. Comparisons in the number of lung metastases among the treatment groups were done using the Mann-Whitney test because the distribution was not Gaussian (i.e., normal), and data are presented below as medians. All reported significant differences are based on two-sided analyses and the $\alpha < 0.05$.

RESULTS

The Combination of Local RT and CTLA-4 Blockade Extends the Survival of Mice Bearing Established 4T1 Mammary Carcinomas

The experimental mammary carcinoma 4T1 was chosen to best mimic the behavior of aggressive breast cancer with early metastatic spread. The timing and pattern of spread of 4T1 cancer cells have been described thoroughly in previous studies (4, 5). When injected s.c. in the mammary fatpad, 4T1 cells metastasized primarily by a hematogenous route and could be found in the lungs within 7 days (4), and nonmammary fatpad s.c. injection did not alter the metastatic ability of 4T1 cells (23). To facilitate localized tumor irradiation we injected 4T1 cells s.c. in the flank rather than the abdominal mammary gland and started the treatment on day 13 post-implantation when the tumors were ~5 mm in average diameter. Mice were randomly assigned to four treatment groups and followed for tumor growth and survival. As expected, given the poor immunogencity of 4T1 cells (ref. 5 and our unpublished results), CTLA-4 blockade by the 9H10 mAb did not affect the tumor growth or survival of the mice (Fig. 1*A* and *B*). RT at a single dose of 12 Gy significantly delayed the growth of the primary irradiated tumor ($P < 0.01$ from day 20 when compared with control) in the presence or absence of 9H10. Although in the presence of 9H10 there was a slightly better growth control of the primary tumor, the difference from RT + hamster IgG was not statistically significant. In contrast, the presence of 9H10 in combination with RT had a significant impact on the survival of the mice (Fig. 1*B*). Whereas no significant survival advantage was obtained with RT + hamster IgG compared with controls (means of 41 and 39 days, respectively), mice treated with RT + 9H10 lived significantly longer (mean, 49 days; $P < 0.0005$ when compared with the other groups). Because lung metastases are usually the cause of death, these results suggested that the combination treatment was inhibiting the metastases.

Inhibition of Lung Metastases after Treatment With Local RT and CTLA-4 Blockade Requires CD8+ T Cells

To directly test the effects of treatment on lung metastases, all mice were sacrificed on day 35 post-tumor implantation, and the number of surface metastases counted under a dissecting microscope. As shown in Fig. 2*A*, the median number of metastases was lower in all of the treated animals compared with controls. However, the decrease in lung metastases was significant only when RT and 9H10 were used in combination, consistent with our observation that only this treatment resulted in increased survival (Fig. 1*B*).

These results suggest that the irradiation of the 4T1 tumor is required for eliciting an effective antitumor T-cell response upon the blocking of the CTLA-4 receptor. To confirm that the inhibition of lung metastases following RT + 9H10 treatment is mediated by T cells and to identify the population involved, mice were depleted of CD4+ or CD8+ T cells starting 3 days before RT treatment, and the depletion maintained up to the day of sacrifice. The median number of metastases in mice treated with RT + 9H10 and depleted of CD8+ T cells was similar to control untreated animals (Fig. 2*B*). In contrast, the inhibition of lung metastases observed in mice treated with RT + 9H10 was not affected by CD4+ T-cell depletion. These results indicate that

CD8+ T cells play a crucial role in the antimetastatic effect of the combination treatment, although they do not exclude a role for other cell types such as natural killer cells.

Improving Local Tumor Control With Fractionated RT Affects Survival Only in Combination With CTLA-4 Blockade

The above data indicate that it is possible to induce an immune response capable of inhibiting metastases by a single RT dose (12 Gy) to the primary tumor followed by three injections of the anti-CTLA-4 mAb 9H10. However, the effect of the antitumor immune response on the primary tumor was minimal, as the tumor growth delay in mice treated with RT and in those treated with RT + 9H10 was not significantly different (Fig. 1*A*). The apparent inability of T cells to kill 4T1 cancer cells at the primary tumor site could be due to the previous irradiation, or alternatively, to the microenvironment in the larger primary tumors being highly inhibitory for T cells, as previously reported (24). To distinguish between these possibilities the effects of fractionated RT on primary tumor growth and survival of the mice were tested. Two RT fractions of 12 Gy each delivered to the primary tumor at 48 hours interval resulted in overall better local control and temporary complete tumor regression in two of seven mice treated with RT alone (Fig. 3*A*), but the survival was not improved in comparison to untreated mice with rapidly growing primary tumors (Fig. 3*C* and *D*). In contrast, in the presence of CTLA-4 blockade, complete regression of the irradiated tumor was seen in the majority of the mice and was associated with longer survival (Fig. 3*B* and *D*). In the latter group, one of seven mice was cured (no tumor recurrence for >100 days), rejected a challenge of 4T1 cells on day 111, and showed 4T1-specific cytolytic activity in the spleen 2 months after the rechallenge (Fig. 4).

These results suggest that the immune response triggered in the presence of RT and CTLA-4 blockade can be effective also against the primary tumor if the tumor environment is sufficiently altered by RT-induced death of cancer cells and/or other components of the tumor stroma. Importantly, our results indicate that, in this model, mice survival depends on lung metastases established before RT. Ablation of the primary tumor by RT, similarly to surgical removal (7, 8), does not affect the survival of the mice. However, when combined with CTLA-4 blockade, fractionated RT induces primary tumor control and therapeutically significant T cell–mediated antimetastatic effects against this poorly immunogenic carcinoma.

DISCUSSION

In this preclinical study, we tested the combination of local RT and CTLA-4 blockade for the treatment of metastatic breast cancer using a mouse model that closely mimics human disease in that cancer cells have already spread systemically by the time the primary tumor becomes palpable. This combination has never been investigated before, and presented data provide the proof of principle that it can elicit a therapeutically significant antitumor immunity and extend the survival of mice with metastatic 4T1 cancers.

The statistically significant survival advantage obtained with RT and 9H10 was limited after a single RT treatment due, at least in part, to the persistence of the primary tumors (Fig. 1). Although antitumor T cells were able to inhibit

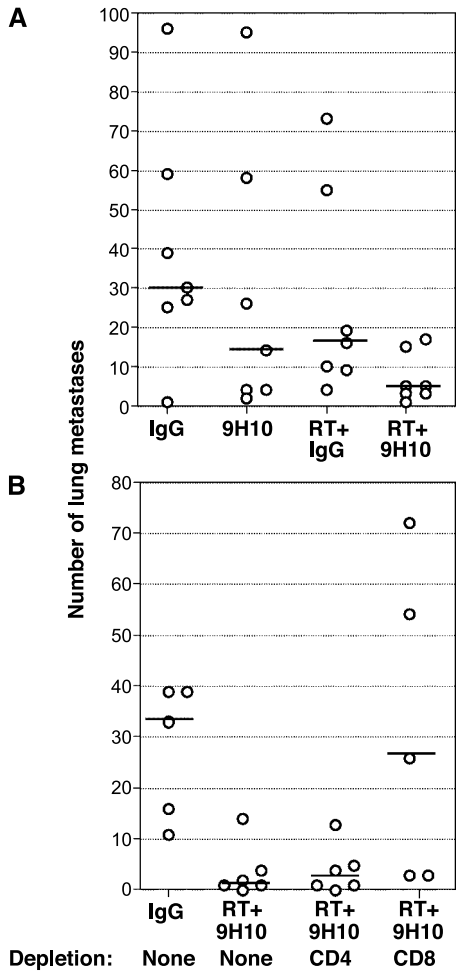

Fig. 2 Inhibition of lung metastases by RT to the primary tumor and CTLA-4 blockade requires CD8+ T cells. Treatment was started on day 13 after s.c. inoculation with the 4T1 mammary carcinoma in the flank. RT was delivered by a single dose of 12 Gy to the s.c. tumors. 9H10 and control hamster IgG were given i.p. 1, 4, and 7 days after RT. *A,* effect of treatment on lung metastases. Mice were sacrificed on day 35 and lung metastases counted. One animal in IgG, 9H10, and RT + IgG group died on days 32, 33, and 34, respectively, and lung metastases were counted at death. Symbol, single animal; horizontal lines, median number of metastases for each treatment group (n = 7 per group). The decrease in number of metastases after RT was statistically significant ($P < 0.05$) only in the presence of 9H10 according to Mann-Whitney test. *B,* depletion of CD8+ T cells abolishes the antimetastatic effect of RT + 9H10 treatment. Antibody-mediated CD4+ and CD8+ T-cell depletion was begun on day 10 and maintained up to the day of sacrifice as described in *Materials and Methods*. Mice were sacrificed on day 32 and lung metastases counted. Symbol, single animal; horizontal lines, median number of metastases for each treatment group. Each group comprised six mice, but two animals, one in control IgG and one in the group depleted of CD8+ T cells died at day 24 and were not included in the study. The difference in number of metastases between mice treated with RT + 9H10 and control IgG was statistically significant ($P < 0.02$) in the absence of T-cell depletion (*None*) and in the absence of CD4+ T cells (*CD4*), but not in the absence of CD8+ T cells (*CD8*) according to Mann-Whitney test.

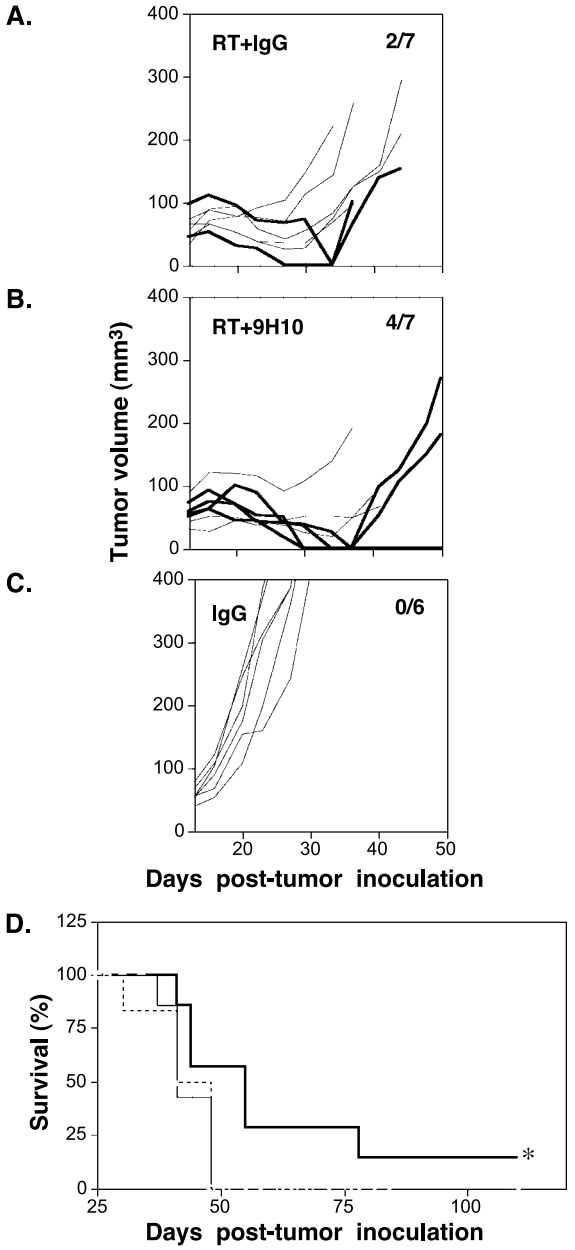

Fig. 3 Effects of two fractions of local RT in combination with CTLA-4 blockade on the primary tumor growth and survival of mice bearing the nonimmunogenic 4T1 carcinoma. Treatment was started on day 13 after s.c. inoculation with the 4T1 mammary carcinoma in the flank. RT was delivered by two fractions of 12 Gy given at 48 hour intervals to the s.c. tumors. Antibodies were given i.p. 1, 4, and 7 days after the second RT fraction. *A-C,* Primary tumor growth delay/regression by RT. Mice received RT + control hamster IgG (*A*), RT + anti-CTLA-4 mAb 9H10 (*B*), or control IgG only (*C*). Line, single mouse. Bold lines, primary tumors that showed complete temporary or permanent regression. Numbers, mice with complete tumor regression over the total number of mice treated. *D,* percentage of surviving mice after treatment with control IgG (*thin broken line*), RT + IgG (*thin line*), or RT + 9H10 (*bold line*). *, tumor cure. The survival difference between RT + 9H10 and the other groups was statistically significant ($P < 0.001$) according to analysis using a Weibull model. Representative of two similar experiments.

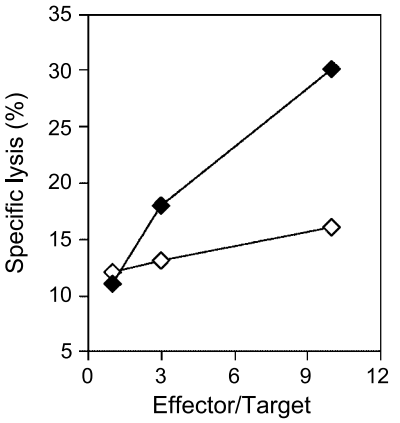

Fig. 4 Tumor cure following RT and CTLA-4 blockade is associated with development of 4T1-specific cytolytic T cells. Spleen cells isolated from a mouse cured from the 4T1 tumor and restimulated *in vitro* as described in *Materials and Methods* were tested for the ability to kill 4T1 (♦) and the A20 lymphoma (◇) cells at various effector to target ratios, as indicated. Representative of two experiments.

metastases (Fig. 2), an effect against the primary tumor was apparent only when the latter was reduced in size by fractionated RT (Fig. 3). Therefore, RT is likely to promote, rather than inhibit, infiltration of tumors by effectors T cells, as previously suggested by others (12). The fact that antitumor T cells elicited by vaccination with MHC class II- and B7.1-modified tumor cells were similarly effective against metastases but not the primary 4T1 tumors (5), suggests that large 4T1 tumors may develop an environment that inhibits T cell function (24). In support of this possibility, we have found that established primary 4T1 tumors contain Gr1 + CD11b + myeloid cells.[5] This subset of myeloid cells can inhibit CD8+ T cell function and induce their apoptotic death upon direct cell-to-cell contact (25). We are currently investigating whether the expansion of Gr1 + CD11b + myeloid cells during tumor progression plays a role in limiting the duration and potency of the antitumor CD8+ T-cell response elicited by treatment with RT + 9H10.

The survival advantage of mice treated with RT and 9H10 was improved after two RT fractions, but complete cure remained rare. This is not surprising considering that the treated mice had well-established disease, a situation more akin to cancer patients with advanced disease. It is well known that the degree of success of immunotherapeutic interventions is highly dependent on the tumor stage (18). CTLA-4 blockade has been previously tested in combination with a vaccine consisting of irradiated tumor cells modified to produce granulocyte macrophage colony-stimulating factor for the treatment of an experimental mammary carcinoma (19). This approach was successful in curing mice at the early stages of tumor development, a time when tumors would not be clinically apparent, but the efficacy against spontaneous metastases could not be tested in this model. Overall, the survival advantage

[5] S. Demaria, N. Kawashima, and A. Yang, unpublished results.

obtained with the combination of RT and CTLA-4 blockade is encouraging and comparable to that obtained in the same 4T1 model by surgical removal of the primary tumor and vaccination with MHC class II- and B7.1-modified tumor cells combined with SEB superantigen (7). Translation of the latter approach into the clinic requires the availability of sufficient tumor tissue and costly *ex vivo* manipulations. Our data suggest that local RT can be used in combination with CTLA-4 blockade as an alternative to vaccination with modified autologous tumor cells to elicit therapeutic antitumor T-cell responses.

The inhibition of lung metastases by RT + 9H10 treatment required CD8+ T cells whereas CD4+ T cells were dispensable (Fig. 2*B*). Antitumor immune responses triggered by CTLA-4 blockade have been shown to be dependent on both, CD4+ and CD8+ T cells in some but not all therapeutic protocols (17, 19, 20, 26). In mice bearing the B16 melanoma, like in our case, the therapeutic effect obtained with CTLA-4 blockade combined with a granulocyte macrophage colony-stimulating factor–producing vaccine was independent of CD4+ T cells but required CD8+ T cells (20). It is possible that RT-induced cross-presentation of tumor antigens derived from dying 4T1 cells by antigen-presenting cells is sufficient, in the presence of CTLA-4 blockade, to directly activate CD8+ T cells.

A main obstacle to the success of immune intervention in the therapeutic setting is the need to overcome the host tolerance that has been established during tumor growth (27). Therefore, the strategies for immunotherapy have to include an approach to break tolerance to the tumor, although this may involve breaking tolerance to self (28, 29). To this end, CTLA-4 blockade has proven to be successful in mouse models of melanoma, breast, and prostate carcinoma when combined with tumor vaccines transduced to produce granulocyte macrophage colony-stimulating factor (19, 20, 30). In the present study, we show that it is possible to achieve a therapeutically useful immune response against a poorly immunogenic, spontaneously metastasizing mammary carcinoma by the combination of CTLA-4 blockade and irradiation of the primary tumor *in situ*. This technically simple and relatively low cost immunotherapy approach has the advantage that it could be easily translated into the clinic. The elicited antitumor immune response was effective in the inhibition of metastases, a finding that is highly relevant to the clinical setting of breast cancer. A human CTLA-4 blocking antibody is available for clinical studies and initial results emphasize its therapeutic potential but also the risk of autoimmunity (29). We did not see any histologic evidence of autoimmunity in multiple organs, including the gastrointestinal tract, pancreas, and thyroid gland, of mice treated with RT + 9H10 (data not shown). A short treatment course with the CTLA-4–blocking mAb combined with RT to the tumor or with vaccination with irradiated tumor cells, as in previous mouse studies (19, 20, 30), may focus the immune response on the targeted tissue and be less likely to elicit autoimmune reactions to other organs that do not share the same antigens. This hypothesis needs to be carefully tested in additional preclinical studies.

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Sandra Demaria dedicates this paper to the memory of Vittorio Demaria.

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