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Radiotherapy Promotes Tumor-Specific Effector CD8⁺ T Cells via Dendritic Cell Activation

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Radiotherapy is an important treatment for cancer. The main mode of action is thought to be the irreversible damage to tumor cell DNA, but there is evidence that irradiation mobilizes tumor-specific immunity, and recent studies showed that the efficacy of high-dose radiotherapy depends on the presence of CD8⁺ T cells. We show in this study that the efficacy of radiotherapy given as a single, high dose (10 Gy) crucially depends on dendritic cells and CD8⁺ T cells, whereas CD4⁺ T cells or macrophages are dispensable. We show that local high-dose irradiation results in activation of tumor-associated dendritic cells that in turn support tumor-specific effector CD8⁺ T cells, thus identifying the mechanism that underlies radiotherapy-induced mobilization of tumor-specific immunity. We propose that in the absence of irradiation, the activation status of dendritic cells rather than the amount of tumor-derived Ag is the bottleneck, which precludes efficient anti-tumor immunity. *The Journal of Immunology*, 2012, 189: 558–566.

It is now well accepted that the immune system plays a role in controlling the growth of tumors. For instance, there is a positive correlation between infiltration by effector T cells and survival (1, 2), the risk to develop cancer is increased in immunosuppressed patients, and dormant tumors are kept in check by the adaptive immune system (3). Furthermore, cancer patients often develop spontaneous humoral and/or cellular immune responses to tumor-associated Ags (4–7). Nevertheless, complete rejection of tumors by the immune system is rare, and there is evidence that local events inhibit tumor-specific effector function (8). Boosting the immune system in a way that supports the development of tumor-specific effector function locally is therefore a promising therapeutic strategy, which is illustrated by the clinical benefit seen in patients who received immunotherapy (9).

Ionizing radiation, or radiotherapy, is used as a primary treatment modality for cancer, and the induction of damage to the tumor or to tumor stroma is thought to be its major mode of action (10). However, several studies showed that local irradiation of tumors has immunomodulatory effects, and, although there is some evi-

dence for radiotherapy-induced immunosuppression (11), most studies showed stimulation of tumor-specific immunity (12–14). The recent advances in radiotherapy allow the use of high-dose irradiation as part of stereotactic radiotherapy while minimizing adverse side effects. Clinical evidence shows that patients treated with one to two high doses of radiotherapy respond better than those treated with fractionated low-dose radiotherapy, and the involvement of other mechanisms than direct killing of tumor cells has been suggested (15–18). It was shown recently that single high-dose radiotherapy but not fractionated low-dose radiotherapy increased accumulation of CD8⁺ T cells in the tumor (19), crucially depended on CD8⁺ T cells (20), and resulted in the generation of tumor-specific CTLs (21). Very recent evidence showed that radiotherapy induced local production of IFN- β , which improved the capacity of tumor-infiltrating dendritic cells (DCs) to cross-present (22). This is in line with the observation that a self-limiting tumor is not controlled in mice in which DCs cannot respond to type I IFN (23). Because these recent findings may change our understanding of how radiotherapy works and may thus have consequences for future treatment of cancer patients, we embarked to dissect the immunological events associated with local high-dose radiotherapy and their importance for therapeutic efficacy.

In accordance with previous findings, we found that the therapeutic efficacy of high-dose radiotherapy depends on the presence of DCs and CD8⁺ T cells, but not CD4⁺ T cells or macrophages, during or immediately after radiotherapy. Our results identify the activation of tumor-associated DCs as the mechanism through which local high-dose radiotherapy supports the function of local, tumor-specific CD8⁺ effector T cells. On the basis of our findings, we propose that the maturation status of tumor-associated DC—and not the amount of tumor-derived Ag that is presented or the availability of tumor-specific CD8⁺ T cells—is the bottleneck for immunological control of tumors.

Materials and Methods

Mice

C57BL/6, TCR327 CD45.1 (24), and CD11c-DTR (25) mice were obtained from the Institute of Laboratory Animal Science (University of Zurich)

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; LCMV, lymphocytic choriomeningitis virus; MHC-II, MHC class II; TAM, tamoxifen; Treg, regulatory T cell.

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and were bred and maintained under specific pathogen-free conditions. TCR327 CD45.1 mice carry a transgenic TCR that recognizes the lymphocytic choriomeningitis virus (LCMV) gp-derived epitope 33–41 in the context of H-2D^b. CD11c-DTR mice allow diphtheria toxin (DT)-mediated deletion of CD11c⁺ cells. DIETER mice are double transgenic mice generated on a C57BL/6 background that express two LCMV-derived epitopes including gp33–41 and one β -galactosidase-derived epitope specifically in CD11c⁺ DCs upon injection of tamoxifen (TAM) (26). Experiments were performed with age- and sex-matched mice in accordance with the Swiss federal and cantonal laws on animal protection or with permission of the Landesuntersuchungsamt Rheinland-Pfalz (Germany).

Peptide

LCMV gp33–41 (KAVYNFATC, H-2D^b) was purchased from NeoMPS (Strasbourg, France) in immunograde quality.

Bone marrow chimeras

To allow chronic depletion of DCs by DT in CD11c-DTR mice (25) or to increase the number of age- and sex-matched double transgenic DIETER mice, CD11-DTR \rightarrow C57BL/6 and DIETER \rightarrow C57BL/6 bone marrow chimeras were generated as described (26). Briefly, age- and sex-matched C57BL/6 recipient mice were lethally irradiated with 10 Gy with a ¹³⁷Cs source and received 2×10^6 to 3×10^6 bone marrow cells from age- and sex-matched donor mice on the same day by i.v. injection. Mice were rested 8 wk before use in experiments.

Induction of syngeneic tumors

C57BL/6 mice were injected s.c. with 2×10^6 MC-38 (methylcholanthrene-induced murine colon carcinoma) (27), 2×10^6 LLC (Lewis lung carcinoma) (28), or 2×10^5 B16gp (melanoma) cells. B16gp cells are B16F10 cells stably transfected to express LCMV-derived gp (29). Cells were injected in Matrigel (BD Biosciences, San Jose, CA) in a total volume of 100 μ l. Tumor size was measured with a caliper and documented as length \times width (surface, mm²).

B16gp-DTR cells are B16gp melanoma cells that were stably transfected to express the human diphtheria toxin receptor (DTR) (25). Briefly, hDTR (plasmid kindly donated by Natalio Garbi, Deutsches Krebsforschungszentrum, Heidelberg, Germany) was cloned in pcDNA6 (Invitrogen), and B16gp cells were transfected with the pcDNA6-hDTR using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected with 50 μ g/ml Basticidin and subcloned under selection. DTR expression was confirmed by PCR (forward primer, 5'-GCC ACC ATG AAG CTG CTG CCG-3'; reverse primer, 5'-TCA GTG GGA ATT AGT GCC-3') and susceptibility to DT-induced death in vitro.

Treatment of mice

Local radiotherapy. Local radiotherapy of the tumors was started when tumors were \sim 12–16 mm² in size (usually 11–13 d after inoculation). Using a customized shielding device, mice were given a strictly locoregional radiotherapy of 1×10 Gy with a Gulmay 200 kV X-ray unit at 100 cGy/min at room temperature.

Depletion of CD4⁺ or CD8⁺ cells. CD4⁺ or CD8⁺ T cells were depleted by i.p. injection of 2 mg monoclonal rat anti-mouse CD4 (YTS 191.1) or rat anti-mouse CD8 (YTS169.4), respectively, at day -1 relative to irradiation. Both hybridomas (30) were originally obtained from H. Waldmann (Department of Surgery, University of Cambridge, Cambridge, U.K.).

Depletion of macrophages. Liposomes were prepared as described earlier (31). One hundred microliters corresponding to 2 mg was injected i.v. 1 d before irradiation. To confirm depletion of marginal zone macrophages and metallophilic macrophages, spleens were isolated 3 d after injection of clodronate liposomes or empty liposomes, and 5- μ m cryosections were stained with rat anti-marginal zone macrophage (ERTR-9) and with rat anti-metallophilic macrophage (MOMA-1) (Biomedicals, Augst, Switzerland) (data not shown).

Depletion of CD11c⁺ cells. To enable long-term depletion of CD11c⁺ cells (32), bone marrow chimeras were generated: C57BL/6 mice were lethally irradiated (10 Gy) with a ¹³⁷Cs source. Bone marrow was isolated from femurs and tibias of age- and sex-matched CD11c-DTR donor mice, and 2×10^6 to 3×10^6 bone marrow cells were injected i.v. Mice were rested for 8 wk before use. CD11c⁺ cells were depleted from CD11c-DTR \rightarrow C57BL/6 chimeras by i.p. injection of 4 ng DT per gram body weight (Sigma Chemical Co., St. Louis, MO) in 100 μ l PBS at days -1 , $+1$, $+3$, and $+5$ relative to irradiation or with PBS as control. This regimen depleted

CD11c⁺ cells from spleen, lymph nodes, and tumor for the remaining course of the experiment. Because CD11c expression is upregulated on effector CD8⁺ T cells (33), we adoptively transferred 2×10^6 TCR327 CD45.1 splenocytes, which resulted in 1% TCR transgenic cells of total CD8⁺ T cells, to avoid possible depletion of activated CD8⁺ T cells by DT at the time of injection of B16gp cells.

Induction of Ag presentation by DCs in DIETER mice. Cre recombinase activity resulting in Ag presentation of transgenic epitopes was induced in DIETER \rightarrow C57BL/6 BM chimeras by a single i.p. injection of 2 mg TAM in a volume of 0.1 ml. TAM was suspended in 96% ethanol. Nine volumes of olive oil were added, and TAM was dissolved at 37°C during 1 h. Control-treated mice were injected with 0.1 ml olive oil i.p. To induce DC maturation, mice were injected i.v. with 30 μ g of agonistic FGK45.5 Ab directed to CD40 (34). TAM and anti-CD40 were given at the same time.

Blockade of CD70–CD27 interactions in vivo. One day before and 2 d after radiotherapy, mice were injected i.p. with 1 mg and 0.5 mg, respectively, of the blocking but nondepleting monoclonal rat anti-mouse CD70 Ab (FR70) (35, 36) or with control rat IgG (Southern Biotech, Birmingham, AL) in PBS.

In vitro activation and adoptive transfer of TCR327 CD45.1 cells

TCR327 CD45.1 splenocytes (5×10^6) were activated for 48 h with 2×10^{-8} M of the relevant LCMV-gp33–41 peptide (KAVYNFATC) in RPMI-1640 plus 10% FCS plus 10^{-5} M 2-mercaptoethanol plus antibiotics in 24-well plates at 2 ml/well. Cells were harvested, and 2×10^7 cells, which corresponded to 2×10^6 activated TCR 327 transgenic cells, were adoptively transferred into B16gp tumor-bearing C57BL/6 mice immediately after local irradiation with 10 Gy.

Flow cytometry

Blood was collected in FACS buffer (PBS plus 2% FCS plus 40 mM EDTA plus 0.05% Na₃N), and spleens were mechanically homogenized to obtain a single-cell suspension. Tumors were cut into small pieces and subsequently digested with 1.5 mg/ml collagenase plus 100 μ g/ml DNase for 1 h at 37°C followed by filtration through a 50- μ m cell strainer. Single-cell suspensions were stained with the following Abs: CD45.2 PerCP, CD45.2 allophycocyanin, CD45.2 FITC, CD8 allophycocyanin, CD3 Pacific blue, CD4 PE, MHC class II (MHC-II) Pacific blue, CD11c allophycocyanin, CD8b PE, and IFN- γ allophycocyanin. PE-labeled tetrameric complexes of biotinylated murine MHC class I H-2D^b or H-2D^d H chain, β_2 -microglobulin, and relevant peptide were prepared as described (37) and used in combination with allophycocyanin-labeled CD8 α mAb 53-6.7. Abs were obtained from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), and BioLegend (San Diego, CA). For intracellular staining to detect IFN- γ , cells were incubated for 6 h at 37°C with 10^{-6} M peptide (gp33–41, KAVYNFATC) or with medium alone in the presence of 5 μ g/ml brefeldin A and 5 μ g/ml monensin. Cells were surface stained with CD8 α mAb, fixed with 2% paraformaldehyde, permeabilized with permeabilization buffer (FACS buffer plus 0.1% saponin), and stained intracellularly for IFN- γ . Anti-CD45.2 or anti-CD45.1 (to identify leukocytes or TCR327 cells, respectively) was included in every staining. Samples were measured with a FACSCalibur (Becton Dickinson, Mountain View, CA) or with a CyAn ADP9 (Beckman Coulter, Brea, CA) and analyzed using FlowJo analysis software (Tree Star, Ashland, OR).

Statistical analysis

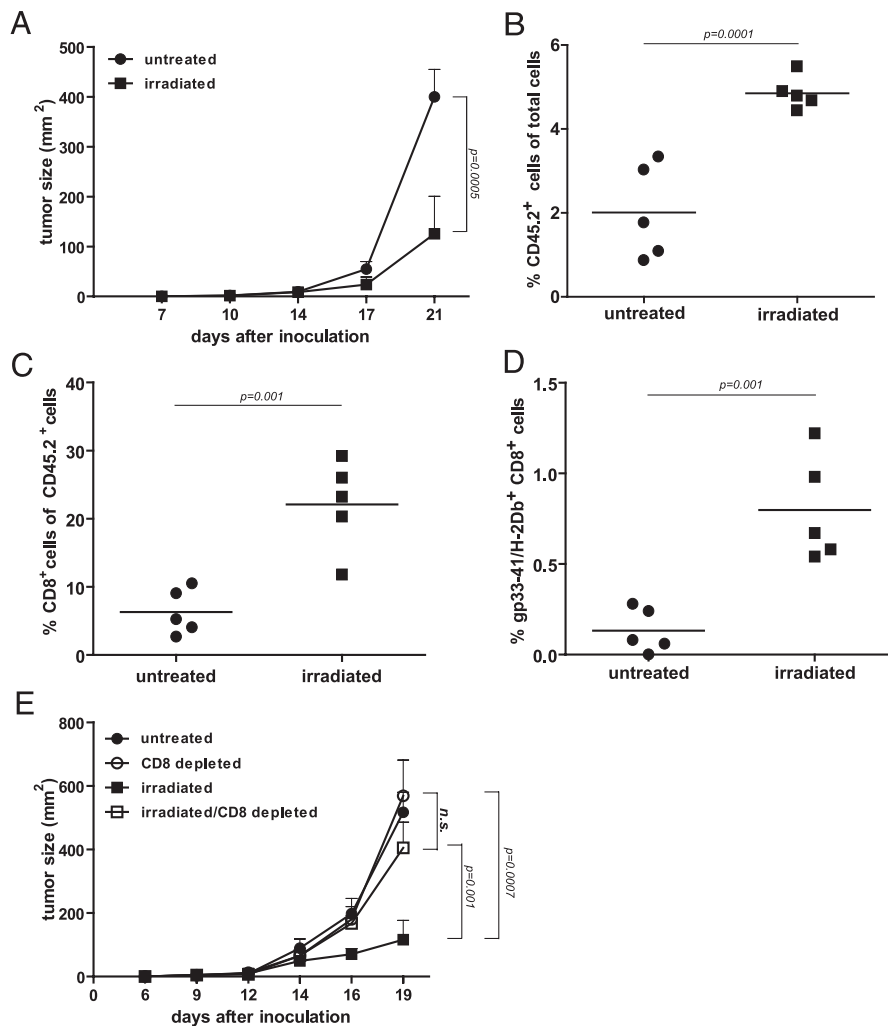
Statistics were done using an unpaired Student two-tailed *t* test. Error bars represent SD. The *p* values <0.05 were considered significant.

Results

Local high-dose radiotherapy enhances accumulation of tumor-specific CD8⁺ T cells

C57BL/6 mice bearing B16gp melanoma tumors were locally irradiated with a single dose of 10 Gy when tumors were \sim 12–16 mm² in size, which resulted in a significant reduction in tumor growth (Fig. 1A). B16gp cells are B16 melanoma cells, which are stably transfected to express the LCMV-derived gp and thus allow the monitoring of tumor-specific CD8⁺ T cell responses. Upon flow cytometric analysis of digested tumors 7 d after irradiation, we found a striking increase in the percentage of infiltrating CD45⁺ leukocytes (Fig. 1B), CD8⁺ T cells (Fig. 1C), and tumor-

FIGURE 1. Local high-dose irradiation increases accumulation of tumor-specific CD8⁺ T cells, which are crucial for therapeutic success. C57BL/6 mice were injected s.c. with 2×10^5 B16gp melanoma cells in Matrigel. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of ~ 12 – 16 mm² (days 11–13), whereas control mice received no radiotherapy. One week after radiotherapy, mice were euthanized, and tumor infiltrate was analyzed by flow cytometry. **(A)** Tumor growth. **(B)** Percentage of CD45.2⁺ cells within the tumor after gating on live cells. **(C)** Percentage of CD8⁺ cells within the tumor after gating on live CD45.2⁺ cells. **(D)** Percentage of gp33–41/H-2D^b-specific cells within the tumor as stained by tetramer and after gating on live CD45.2⁺CD8⁺ cells. **(E)** One day before radiotherapy, CD8⁺ T cells were depleted in two of four experimental groups (irradiated or not irradiated), and the growth of B16gp tumors is shown. Data are representative of four independent experiments. Experimental groups consisted of five mice.



specific CD8⁺ T cells as determined by staining with gp33–41/D^b tetramers (Fig. 1D) compared with that of untreated tumors.

CD8⁺ T cells are crucial for the therapeutic effect of local high-dose radiotherapy, whereas CD4⁺ T cells or macrophages are dispensable

To examine whether the increased accumulation by CD8⁺ T cells is relevant for the therapeutic effect of radiotherapy, we depleted CD8⁺ T cells from mice bearing B16gp tumors 1 d before radiotherapy. Depletion of CD8⁺ T cells completely abolished the therapeutic effect of radiotherapy (Fig. 1E) showing that the presence of CD8⁺ T cells during or immediately after irradiation is crucial for therapeutic efficacy. To exclude the possibility that the CD8 dependence of radiotherapy is a phenomenon unique to the B16 melanoma model, we performed a similar experiment using LLC or MC-38 tumors. The data were comparable to those obtained with B16gp tumors such that CD8 depletion significantly abolished the therapeutic effect of radiotherapy (Supplemental Fig. 1). To exclude that the CD8 dependence of radiotherapy is a peculiarity of C57BL/6 mice, we repeated the experiments described above using CT26 (mouse colon carcinoma) tumors in BALB/c mice and obtained similar results (data not shown).

Next we examined whether the therapeutic effect of local radiotherapy required the presence of CD4⁺ T cells or macrophages during or immediately after irradiation. We depleted CD4⁺ T cells using Abs or macrophages using clodronate liposomes (31) 1 d before irradiation. In addition, we performed depletion

in tumor-bearing mice that did not receive radiotherapy. All six groups were included in one and the same experiment; the data are split into two graphs (Fig. 2A, 2B) for reasons of clarity. We found that all irradiated groups had similar tumor growth curves, no matter if they were depleted of CD4⁺ T cells, macrophages, or not (Fig. 2A), which we interpret such that radiotherapy does not require the presence of macrophages or CD4⁺ T cells (in contrast to CD8⁺ T cells) to be effective. Fig. 2B shows all non-irradiated groups; here we found that mere depletion of CD4⁺ T cells also resulted in significantly retarded tumor growth, presumably as a result of CD4⁺CD25⁺ regulatory T cell (Treg) depletion. Although not statistically significant, also the depletion of macrophages from tumor-bearing mice seemed to result in reduced tumor progression (Fig. 2B), which fits with the negative impact of tumor-associated macrophages on tumor control (38, 39).

Thus, the therapeutic effect of local high-dose radiotherapy crucially depends on the presence of CD8⁺ T cells during or immediately after radiotherapy, which suggests that high-dose radiotherapy results in the recruitment of tumor-specific CD8⁺ T cells. Our findings are in line with recently published data (20).

Tumor-specific effector CD8⁺ T cells are sufficient to control tumor growth

The above-mentioned data suggest that irradiation supports tumor-specific effector CD8⁺ T cells. To address the question whether the presence of tumor-specific effector CD8⁺ T cells is sufficient to control the tumor or whether a local event that promotes accu-

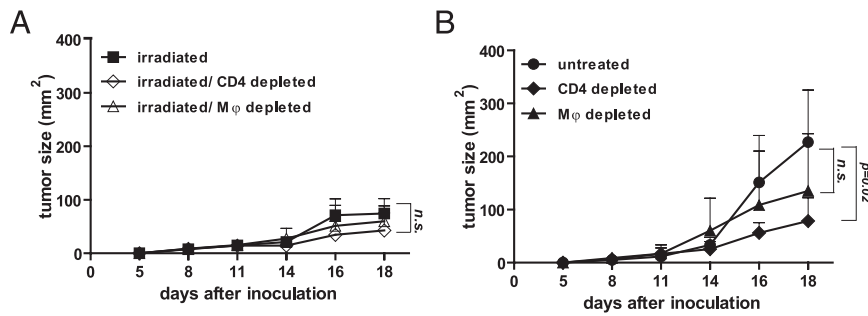


FIGURE 2. The therapeutic effect of local high-dose radiotherapy does not require CD4⁺ T cells or macrophages. C57BL/6 mice were injected s.c. with 2×10^5 tumor cells in Matrigel. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of $\sim 12\text{--}16\text{ mm}^2$ (days 11–13), whereas control mice received no radiotherapy. One day before radiotherapy, CD4⁺ T cells or macrophages were depleted in some experimental groups. **(A)** Growth of B16gp tumors in irradiated mice. **(B)** Growth of B16gp tumors in untreated mice. Data are representative of two independent experiments. Experimental groups consisted of five mice.

mulation is required in addition, we adoptively transferred tumor-specific effector CD8⁺ T cells into B16gp tumor-bearing mice immediately after irradiation or in untreated mice as control. To generate tumor-specific CTLs, we incubated splenocytes from TCR327 CD45.1 mice (which carry a transgenic TCR specific for LCMV-derived gp33–41/D^b) in vitro for 48 h with 2×10^{-8} M gp33–41 peptide and adoptively transferred 2×10^7 cells (corresponding to 2×10^6 tumor-specific CD8⁺ T cells). Adoptive transfer of effector TCR327 transgenic CD8⁺ T cells significantly reduced the growth of B16gp tumors, suggesting that the presence of tumor-specific CD8⁺ effector T cells is sufficient to control the tumor, even without a local event such as irradiation (Fig. 3A). The control of tumor growth by transferred effector CTLs alone was similar to that observed after local high-dose radiotherapy (Fig. 3A). Moreover, we observed a further reduction in tumor growth in those mice that received a combination of local high-dose radiotherapy plus effector CD8⁺ T cells (Fig. 3A), suggesting that local irradiation supports the anti-tumor effect of tumor-specific CTLs, presumably by enhancing accumulation and/or by supporting effector function. To address this point specifically, we analyzed tumors 6 d after irradiation by flow cytometry. Indeed, we observed a significant increase of tumor-infiltrating TCR327 cells (CD45.1⁺CD8⁺) (Fig. 3B, right) and endogenous CD8⁺ T cells (CD45.2⁺CD8⁺) (Fig. 3B, left) in irradiated mice compared with untreated animals. We then performed kinetic analysis of tumors and draining lymph nodes. We observed very few TCR327 cells in tumors 1 d after irradiation, but their number steadily increased on day 3 and even more on day 5 in irradiated tumors. Although the accumulation of TCR327 cells of non-irradiated tumors was similar to that of irradiated tumors at days 1 and 3, at day 5 this increased respective to that on day 3 in irradiated tumors, whereas it decreased in nontreated tumors (Fig. 3C, left panel). In addition, the capacity to produce IFN- γ was steadily decreasing over time in nontreated tumors, whereas this was maintained in irradiated tumors (Fig. 3C, right panel). These observations were not mirrored in the draining lymph nodes (data not shown), suggesting that the radiotherapy-induced support of tumor-specific CD8⁺ T cells is a very local phenomenon. Thus, effector CD8⁺ T cells infiltrate and control tumors independent of irradiation, although irradiation supports accumulation, maintenance of effector function, and tumor control.

Activation of DCs in tumor-bearing mice is sufficient for the induction of tumor-specific CD8⁺ T cells and subsequent tumor control

Because radiotherapy will result in the release of tumor-associated Ags and presumably in enhanced activation of tumor-specific CD8⁺

T cells, we aimed to investigate whether increased presentation in the absence of radiotherapy per se is sufficient for tumor control. We therefore chose two different approaches: DIETER mice and B16gp-DTR tumors.

The first approach took advantage of DIETER mice, in which injection of TAM plus agonistic anti-CD40 Abs induces presentation of gp33–41 by activated CD11c⁺ DCs (26), which results in protective CD8⁺ T cell-mediated immunity. We thus treated B16gp tumor-bearing DIETER mice with TAM plus anti-CD40 (presentation of gp33–41 by 3–5% of DCs, all of which are activated), with anti-CD40 (activation of all DCs) alone, or left them untreated. Induced presentation of gp33–41 by activated DCs resulted in the control of B16gp tumors, and surprisingly the protective effect was not different from that in the group in which we only activated DCs (Fig. 4A), suggesting that the activation status of DCs rather than the amount of Ag presented in tumor-bearing mice was limiting tumor control. To support this further, we measured the amount and effector function of endogenously primed gp33–41-specific CD8⁺ T cells in all three groups of mice. We detected no tumor-specific CD8⁺ T cells by tetramer staining in the spleens of untreated, tumor-bearing DIETER mice, whereas $\sim 5\%$ of CD8⁺ T cells were gp33–41 specific in TAM plus anti-CD40-treated tumor-bearing DIETER mice (Fig. 4B). The observation that tumor control upon injection of anti-CD40 (DC activation) was comparable to that upon injection of TAM plus anti-CD40 (increased Ag presentation by activated DCs) is similar despite a substantial difference in the numbers of gp33–41-specific CD8⁺ T cells in the spleen of both groups may seem controversial at first sight. However, TAM plus anti-CD40 results in gp33–41 presentation by mature DCs all over the body (including the spleen), and anti-CD40 results in gp33–41 presentation only in tissues that are drained by the tumor (most like not the spleen), which explains the higher responses seen in the spleen of TAM plus anti-CD40-treated animals. Notably, we also detected gp33–41-specific CD8⁺ T cells in the spleen of anti-CD40-treated tumor-bearing DIETER mice (Fig. 4B), which shows that the tumor itself generates sufficient Ag for T cell priming, provided the DCs are activated. This is further supported by the fact that we never detected gp33–41-specific CD8⁺ T cells in anti-CD40-treated DIETER mice without B16gp tumors [Ref. 26 and data not shown]. Although substantially less than in TAM plus anti-CD40-treated tumor-bearing mice, the gp33–41-specific CD8⁺ T cells primed in tumor-bearing DIETER mice after injection of anti-CD40 killed specific targets in vivo (Fig. 4C).

In the second approach, we aimed to increase artificially the amount of released Ag in the absence of further inflammatory signals. We therefore stably transfected B16gp cells with the DTR,

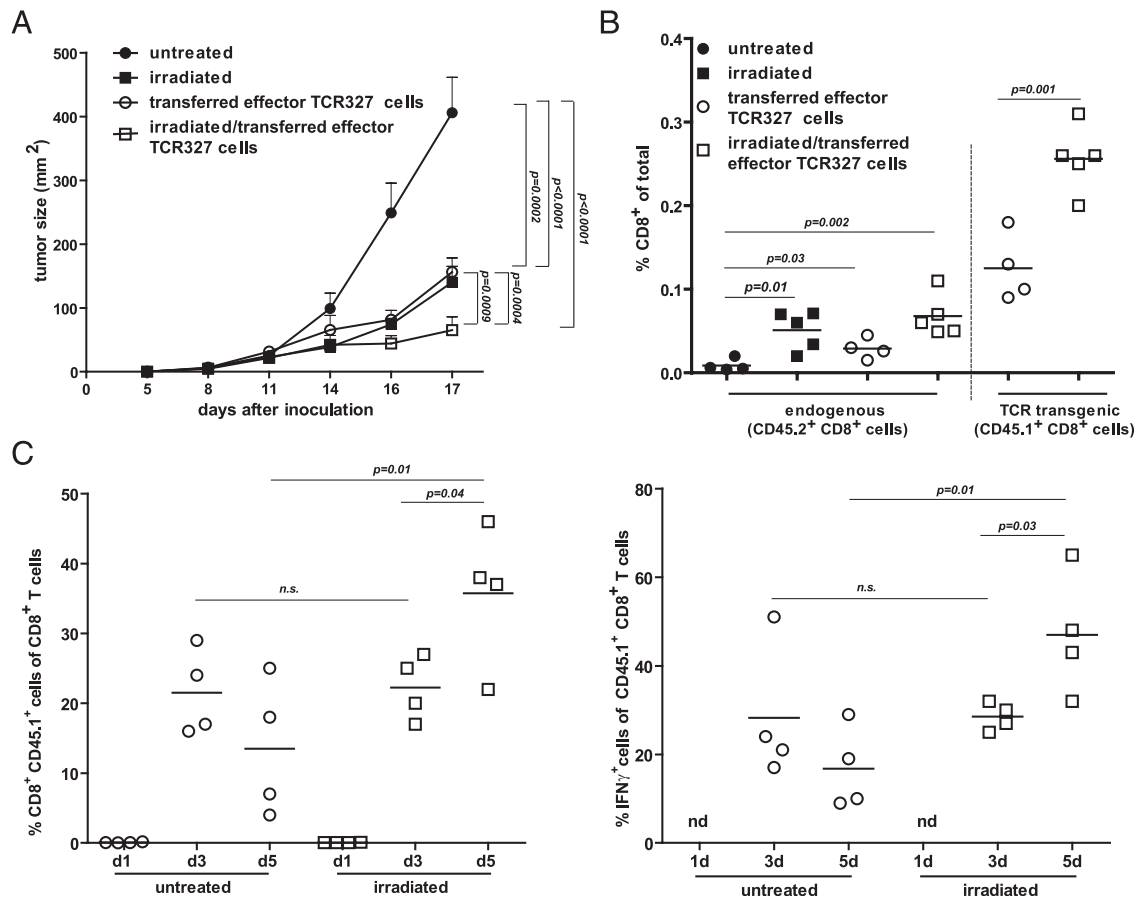


FIGURE 3. Adoptive transfer of tumor-specific effector CD8⁺ T cells controls B16gp tumors. C57BL/6 mice were injected s.c. with 2×10^5 tumor cells in Matrigel. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of ~ 12 – 16 mm² (days 11–13), whereas control mice received no radiotherapy. Immediately after radiotherapy, 2×10^6 in vitro-activated tumor-specific TCR327 CD45.1 effector cells (gp33–41/H-2D^b specific) were adoptively transferred into some experimental groups. **(A)** Growth of B16gp tumors. **(B)** Percentage tumor-infiltrating CD8⁺ T cells 6 d after irradiation as determined by flow cytometry after gating on live CD45.2⁺CD8⁺ (endogenous CD8) or on live CD45.1⁺CD8⁺ (transferred TCR327) cells. **(C)** Percentage of tumor-specific TCR327 (live CD45.1⁺CD8⁺) cells in tumor (*left panel*) and percentage of IFN- γ -producing TCR327 cells in tumor (*right panel*) as determined by intracellular staining for IFN- γ after a 6-h in vitro incubation with 10^{-6} M of the antigenic peptide gp33–41 or with media. The analysis was performed at day 1, day 3, and day 5 after radiotherapy (or no treatment as control). Each symbol represents an individual mouse.

which allowed the induction of tumor cell death through injection of DT. Mice bearing B16gp-DTR tumors were given 10 ng DT per gram body weight at days 9, 12, and 15 or PBS as control. Upon

injection of DT, tumors declined (Supplemental Fig. 2). This, however, was not sufficient to increase the percentage or function of infiltrating TCR327 effector cells within the tumor (Supplemental

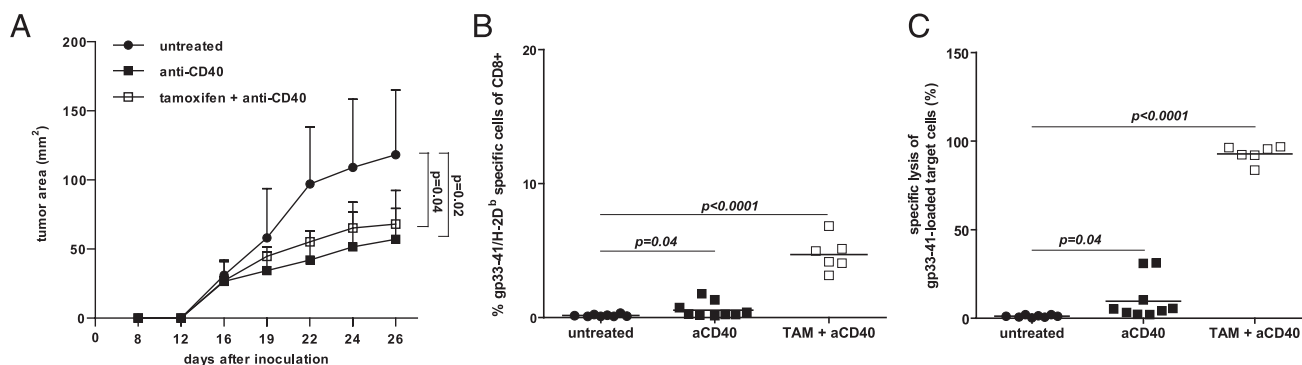


FIGURE 4. Activation of DCs in tumor-bearing mice results in priming and has a therapeutic effect. DIETER \rightarrow C57BL/6 bone marrow chimeras were injected s.c. with 2×10^5 B16gp cells in Matrigel. Mice were injected with 30 μ g anti-CD40 (DC activation) or with 30 μ g anti-CD40 plus 2 mg TAM (presentation of gp33–41 by $\sim 5\%$ of activated DCs) or were left untreated when tumors had reached a size of ~ 12 – 16 mm² (day 16). Eight days later (day 24), mice were injected i.v. with 4×10^{-6} M CFSE^{high} gp33–41 loaded plus 0.4×10^{-6} M CFSE^{low} C57BL/6 splenocytes. Spleens were removed 24 h later for quantitative and functional analysis of gp33–41/H-2D^b-specific CD8⁺ T cells. **(A)** Growth of B16gp tumors. **(B)** Percentage gp33–41/H-2D^b-specific of total CD8⁺ T cells in the spleen after gating on live CD45.2⁺CD8⁺ cells. Each symbol represents an individual mouse. **(C)** The percentage of specific killing was calculated as follows: (number of CFSE^{high}/number of CFSE^{low}) $\times 100\%$ after gating on live CD45.2⁺ cells. Each symbol represents an individual mouse.

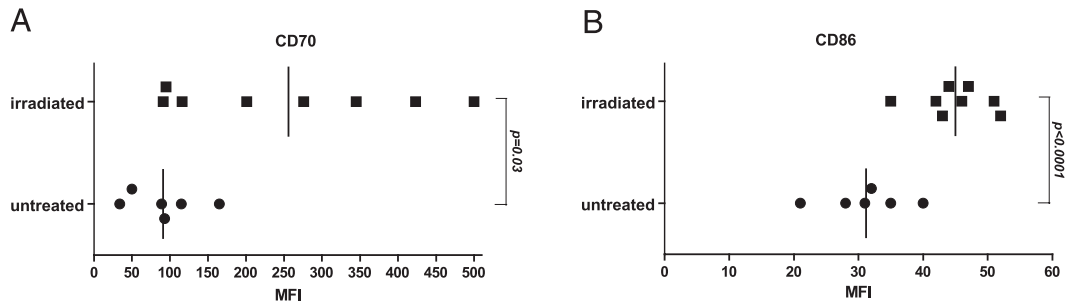


FIGURE 5. Local high-dose radiotherapy results in the activation of tumor-infiltrating DCs. C57BL/6 mice were injected s.c. with 2×10^5 tumor cells in Matrigel. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of $\sim 12\text{--}16\text{ mm}^2$ (day 12), whereas control mice did not receive radiotherapy. Mice were euthanized 48 h after irradiation, and the expression of activation markers on tumor-infiltrating DCs was measured by flow cytometry after gating on live CD45.2⁺CD11c^{high}I-A^b high cells. **(A)** Surface expression of CD70. **(B)** Surface expression of CD86. Each symbol corresponds to an individual mouse.

Fig. 2). Thus, the amount of Ag derived from B16gp tumors is sufficient to induce expansion and development of effector function of tumor-specific CD8⁺ T cells when DCs are activated. In contrast, the same or higher (after DT) amount of tumor-derived Ag does not result in detectable priming in the spleen or in tumor control under steady-state conditions.

Local high-dose radiotherapy results in the activation of tumor-associated DCs in vivo

Having shown that the limiting factor for the induction of tumor-specific effector cells that control tumor growth is apparently not the amount of tumor-derived Ag but the activation status of (local) DCs (Figs. 4, 5) and that local irradiation results in CD8⁺ T cell-dependent tumor control, we investigated the possibility that high-dose irradiation results in the maturation of tumor-associated DCs. We locally irradiated B16gp tumor-bearing C57BL/6 mice with a single dose of 10 Gy and analyzed digested tumors 48 h later by flow cytometry. We found that the expression of two costimulatory molecules that are crucially involved in T cell priming—CD70 (35) and CD86 (40)—was significantly upregulated on live CD45⁺CD11c^{high} MHC-II^{high} cells upon irradiation (Fig. 5A, 5B). We found no difference in the expression level of MHC-II (data not shown). The significant increase in leukocyte accumulation, which we observed 7 d after irradiation (Fig. 1), was not seen after 48 h (data not shown). This suggests that irradiation results in an inflammatory process that activates local DCs, which then promotes the recruitment and activation of tumor-specific CD8⁺ T cells. In contrast to high-dose radiotherapy, induced Ag release through injection of mice with B16gp-DTR tumors with DT did not change the percentage of tumor-associated DCs or the expression of maturation markers (Fig. 6).

The therapeutic effect of local high-dose radiotherapy depends on DCs and on CD70

To strengthen the point that high-dose radiotherapy acts through DC activation, we used CD11c-DTR mice, which allow DT-mediated depletion of DCs (25). To avoid toxicity after multiple DT injections, we generated CD11c-DTR → C57BL/6 bone marrow chimeras (41). In addition, to avoid potential depletion of CD8⁺ effector T cells due to their transient expression of CD11c (33), we adoptively transferred DTR-negative, naive TCR327 CD45.1 splenocytes 1 d before injecting the B16gp tumors. This experimental setup ensures that transferred transgenic CD8⁺ T cells and endogenous CD8⁺ T cells experience no difference with respect to the amount and context of tumor-derived Ag they encounter during tumorigenesis. We started DC depletion of B16gp-bearing CD11c-DTR → C57BL/6 chimeras 1 d before irradiation and gave DT every second day for the remaining duration of the experiment. Control mice were left untreated, only depleted for DCs, or only irradiated. Whereas local high-dose irradiation resulted in significantly smaller tumors, tumors in irradiated plus DC-depleted mice did not differ significantly in size from untreated or DC-depleted-only mice (Fig. 7A). The negative impact of DC depletion on the therapeutic efficacy of irradiation was reflected by the degree of accumulation of CD8⁺ T cells, as the percentage of tumor-specific CD8⁺ T cells was only significantly increased in DC-proficient irradiated mice (Fig. 7B).

To investigate whether the mere presence of DCs during radiotherapy or their concomitant maturation is crucial for the therapeutic efficacy of radiotherapy, we blocked interactions of the costimulatory molecule CD70 with its ligand CD27. We chose CD70 because the expression of this molecule was upregulated on

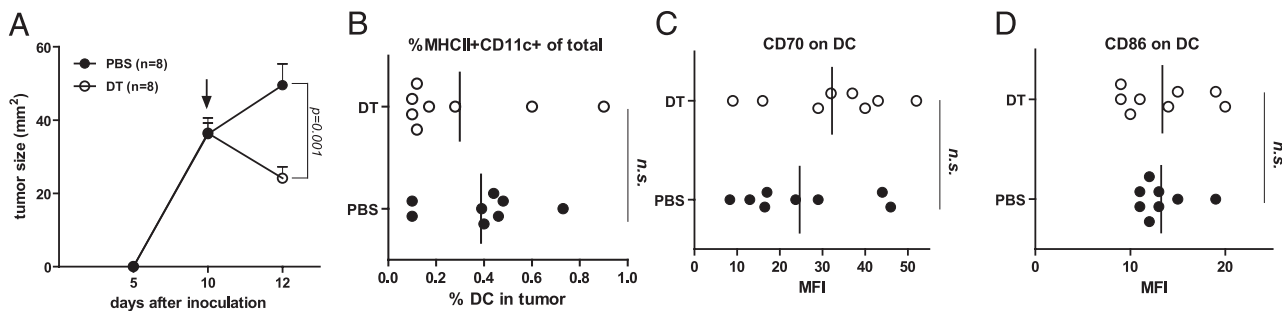


FIGURE 6. Increased Ag release does not result in higher percentage of tumor-associated DCs or in their activation. C57BL/6 mice were injected s.c. with 2×10^5 B16gp-DTR tumor cells in Matrigel. Mice were injected with 10 ng DT per gram body weight when tumors had reached a size of $\sim 12\text{--}16\text{ mm}^2$ (day 9), whereas control mice received PBS. Three days later, tumor-associated DCs were analyzed. **(A)** Tumor growth. **(B)** The percentage of MHC-II^{high}CD11c^{high} cells in the tumor. **(C)** Surface expression of CD70 on MHC-II^{high}CD11c^{high} cells in the tumor. **(D)** Surface expression of CD86 MHC-II^{high}CD11c^{high} cells in the tumor. Each symbol corresponds to an individual mouse.

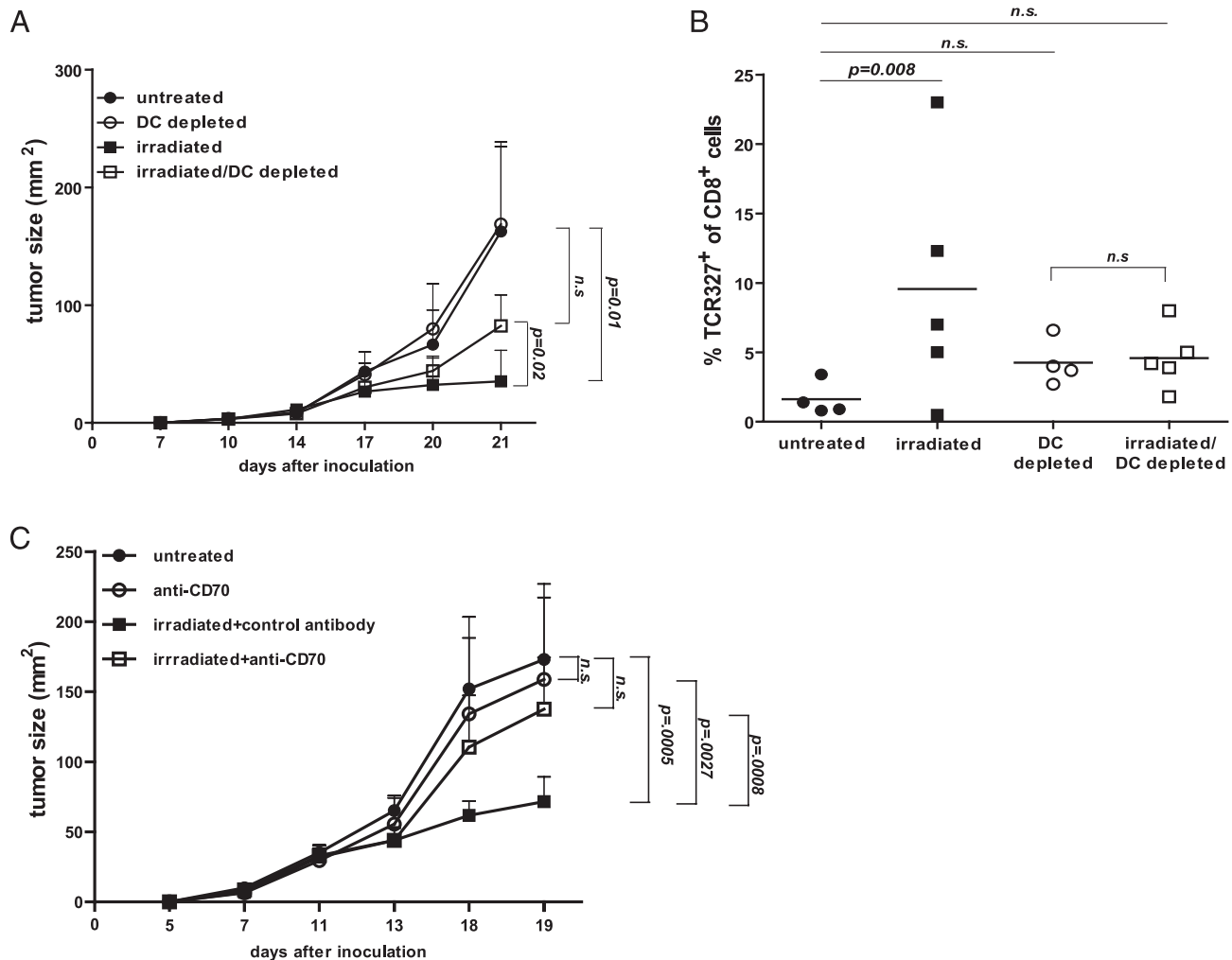


FIGURE 7. The therapeutic effect of local high-dose radiotherapy depends on DCs and CD70. CD11c-DTR → C57BL/6 bone marrow chimeras were injected s.c. with 2×10^5 tumor cells in Matrigel. On the same day, all mice received 2×10^6 naive TCR327 CD45.1 cells i.v. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of $\sim 12\text{--}16\text{ mm}^2$ (day 15), whereas control mice received no radiotherapy. One day before radiotherapy, CD11c^{high} cells (DCs) were depleted in some experimental groups by injection of 100 ng DT then later on every second day until the end of the experiment. **(A)** Growth of B16gp tumors. **(B)** Percentage tumor-infiltrating tumor-specific TCR327 cells 7 d after irradiation as determined by flow cytometry after gating on live CD45.1⁺CD8⁺ cells. **(C)** C57BL/6 mice were injected s.c. with 2×10^5 B16gp tumor cells in Matrigel. In two of four experimental groups, tumors were irradiated with a single dose of 10 Gy when they had reached a size of $\sim 12\text{--}16\text{ mm}^2$ (day 11), whereas two groups received no radiotherapy. One day before radiotherapy, irradiated groups were injected either with 1 mg FR70 to block CD70 interactions or with the control Ab, whereas one of the non-irradiated groups received 1 mg FR70. Two days after radiotherapy, an additional 0.5 mg FR70 or control Ab was injected to the respective groups. Depicted is the tumor growth as the mean \pm SD of all four groups. Each group consisted of seven to eight mice.

DCs upon radiotherapy and because we previously showed that CD70 plays a crucial role in the decision between priming versus tolerance induction of CD8⁺ T cells in the context of infections and cancer (35, 42). We thus injected FR70, a blocking but non-depleting Ab against CD70 (35, 36), 1 d before and 2 d after radiotherapy and found that this treatment significantly reduced the therapeutic effect of radiotherapy (Fig. 7C). These data show that the efficacy of radiotherapy depends on the presence of DCs during radiotherapy and on the concomitant upregulation of CD70 and maybe other maturation-associated molecules.

Discussion

Local high-dose radiotherapy, given as a single dose of 10 Gy to tumor-bearing C57BL/6 mice, inhibited the growth of syngeneic B16gp melanoma, MC-38 colon carcinoma, or LLC lung carcinoma and resulted in a concomitant accumulation of leukocytes, especially of CD8⁺ T cells. At least a proportion of these CD8⁺ T cells are tumor specific as shown by tetramer staining, and at

least a proportion of tumor-specific CD8⁺ T cells were primed by the tumor per se, as we observed the presence of those cells in the blood of tumor-bearing mice even before irradiation (data not shown). Depletion of CD8⁺ T cells just before radiotherapy completely abolished the therapeutic effect in the B16gp model and significantly reduced the therapeutic effect of high-dose radiotherapy in three other models (MC-38 and LLC in C57BL/6, CT26 in BALB/c).

Opposed to CD8⁺ T cells, the presence of macrophages and CD4⁺ T cells during and/or immediately after radiotherapy is not required for therapeutic success of high-dose radiotherapy. In contrast, depletion of CD4⁺ T cells or to a lesser extent macrophages resulted in reduced tumor growth even without further treatment. These findings are not unexpected because the immunosuppressive function of tumor-associated macrophages and myeloid-derived suppressor cells is well documented (8, 43). The beneficial effect of CD4⁺ depletion is presumably due to the depletion of Tregs, which are known to contribute to local immune

subversion in cancer patients (44) and in animal models (45, 46). Indeed, we found similar beneficial effects in B16gp-bearing DEREK mice upon depletion of Foxp3⁺ Tregs (47) (data not shown). Although the majority of our experiments were performed using a transfected cell line (B16gp), which may therefore be more immunogenic, we obtained similar results using two additional, non-transfected cell lines in C57BL/6 (LLC, MC-38) and in BALB/c (CT26) mice. More importantly, we confirmed the increased infiltration upon radiotherapy using paired samples from human sarcoma patients (48) suggesting that our observations are not a peculiarity to transfected cell lines or mouse models but rather are of clinical relevance.

Our interpretation that the therapeutic efficacy of high-dose radiotherapy crucially depends on CD8⁺ T cells during or immediately after radiotherapy is well in line with previously published findings (19–21). A recent publication showed that fractionated radiotherapy given within a time frame of 6 h and a total dose of 7.5 Gy had immunostimulatory effects as well (49). Therefore, it may be so that a high dose of radiotherapy must not necessarily be given as a single dose but may be given in fractions in a short time. Several mechanisms by which high-dose radiotherapy supports local, tumor-specific CD8⁺ T cells are possible. For example, radiotherapy may promote infiltration of the tumor by leukocytes through effects on the vascular endothelium or through the local production of chemoattractants. In addition, radiotherapy may induce increased Ag release from dying tumor cells causing increased presentation of tumor-derived Ag; however, deliberate Ag release in the absence of additional stimuli (DT-induced tumor cell death) did not support the presence or function of tumor-infiltrating CD8⁺ T cells, nor did it affect tumor-infiltrating DCs. These results also suggest that the released tumor-derived Ag in the absence of radiotherapy is not limiting local tumor-specific immunity. Furthermore, radiotherapy may support the survival of CD8⁺ T cells in the tumor.

Adoptively transferred tumor-specific effector CD8⁺ T cells controlled tumor growth and infiltrated the tumor also without radiotherapy, indicating that the sole presence of appropriately activated effector CD8⁺ T cells is sufficient. The fact that tumor control does not often happen spontaneously suggests that tumor-specific CD8⁺ effector T cells fail to develop or cannot maintain their function upon infiltration of a tumor. We observed that adoptively transferred naive tumor-specific CD8⁺ T cells do not control the tumor (data not shown), suggesting that defective differentiation into effector cells rather than a too-low precursor frequency of tumor-specific CD8⁺ T cells is responsible for failure to control the tumor. This vision is also well in line with our finding that DC activation through agonistic anti-CD40 Abs is sufficient for tumor control: both the endogenous CD8⁺ T cell frequency and the amount of available Ag are sufficient for the development of tumor-specific CD8⁺ effector T cells provided the DCs are mature.

Besides inducing tumor cell death and concomitant Ag release, radiotherapy results in acute, local inflammatory reactions (50) that may significantly contribute to infiltration and the quality of local APCs (13, 22). Specifically, a recent publication showed that local radiotherapy resulted in the production of IFN- β , which acted on DCs and improved the capacity to cross-present Ags of the latter (22), an effect of type I IFNs that was recognized before (51). Other recent publications showed that the efficacy of tumor-specific CD8⁺ T cells to control a tumor crucially depends on type I IFN and on the DCs responding to this cytokine (23, 52); again, the improved capacity of DCs to cross-present was found to be of central importance. Notably, IFN- α was shown to upregulate PD-1 on CD8⁺ T cells in the context of TCR engagement (53).

Together, these data suggest that an innate inflammatory response promotes CD8⁺ T cell-mediated immunity but also attenuates such responses when inflammation is sustained. This may also explain why repeated radiotherapy is less immuno-supportive than a single dose (15–20).

Taken together, we propose that local high-dose radiotherapy results in direct or indirect activation of tumor-associated DCs, which in turn is crucial for the development and/or maintenance of effector function of tumor-specific CD8⁺ T cells and for the therapeutic efficacy of local high-dose radiotherapy.

Our findings have important implications for the way scientists and physicians think about radiotherapy and should especially evoke a reconsideration of the use of single, high-dose radiotherapy instead of fractionated, low-dose radiotherapy. The latter presumably antagonizes the immunostimulatory effect of radiotherapy by acting directly on expanding T cells, but also by upregulating PD-1 on tumor-specific CD8⁺ T cells. Furthermore, our findings may offer a scientific fundament to explore further the combination of radiotherapy and immunotherapy such as PD-1 blockade in the treatment of cancer and optimally to exploit the synergism between these two treatment modalities.

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Disclosures

The authors have no financial conflicts of interest.

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