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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 tumor 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 evidence 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—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)
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) gp33-derived epitope 33–41 in the context of H-2D^d. 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 (KAVYNFAC, H-2^d) 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 → C57BL/6 and DIETER → C57BL/6 bone marrow chimera 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 5 × 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 × 10^6 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 μl. Tumor size was measured with a caliper and documented as length × width (surface, mm^2).

1640 plus 10% FCS plus 100 U/ml penicillin, 100 μg/ml streptomycin, 25 μg/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 ∼12–16 mm^2 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 Gutman 2000 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

Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Transfected cells were selected with 50 μg/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.

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 ∼12–16 mm^2 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-
specific CD8+ T cells as determined by staining with gp33–41/Db 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-
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 TCR272 CD45.1 mice (which carry a transgenic TCR specific for LCMV-derived gp33–41 peptide) 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 TCR272 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 TCR272 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 TCR272 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 TCR272 cells in 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 ∼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,
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 3.

ADOPTIVE TRANSFER OF TUMOR-SPECIFIC EFFECCTOR 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 mm^2 (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-2Db 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.

FIGURE 4. Activation of DCs in tumor-bearing mice results in priming and has a therapeutic effect. DIETER → C57BL/6 bone marrow chimeras were injected s.c. with 2 × 10^5 B16gp cells in Matrigel. Mice were injected with 30 μg anti-CD40 (DC activation) or with 30 μg anti-CD40 plus 2 mg TAM (presentation of gp33–41 by ∼5% of activated DCs) or were left untreated when tumors had reached a size of ∼12–16 mm^2 (day 16). Eight days later (day 24), mice were injected i.v. with 4 × 10^{-6} M CFSE<sup>high</sup> gp33–41 loaded plus 0.4 × 10^{-6} M CFSE<sup>low</sup> C57BL/6 splenocytes. Spleens were removed 24 h later for quantitative and functional analysis of gp33–41/H-2Db-specific CD8+ T cells. (A) Growth of B16gp tumors. (B) Percentage gp33–41/H-2Db-specific 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<sup>high</sup>/number of CFSE<sup>low</sup>) × 100% after gating on live CD45.2+CD8+ cells. Each symbol represents an individual mouse.
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+CD11chigh MHC-IIhigh 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
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 responses.

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 $2 \times 10^5$ tumor cells in Matrigel. On the same day, all mice received $2 \times 10^6$ naive TCR327 CD45.1 cells i.v. Tumors were irradiated with a single dose of 10 Gy when they had reached a size of 12–16 mm$^2$ (day 15), whereas control mice received no radiotherapy. One day before radiotherapy, CD11c$^{DTR^+}$ 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 $2 \times 10^5$ 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 12–16 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 ± SD of all four groups. Each group consisted of seven to eight mice.
subversion in cancer patients (44) and in animal models (45, 46).
Indeed, we found similar beneficial effects in B16gp-bearing
DEREG 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 addi-
tional, 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 im-
mediately after radiotherapy is well in line with previously pub-
lished findings (19–21). A recent publication showed that frac-
tionated 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 radio-
therapy 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; how-
ever, 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
primary CD8+ T cells. 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 dif-
ferentiation into effector cells rather than a too-low precursor fre-
cquency 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 1 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 sin-
gle dose (15–20).
Taken together, we propose that local high-dose radiotherapy
results in direct or indirect activation of tumor-associated DCs,
which in tumor 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 pre-
sumably 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 combina-
tion 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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