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Radiotherapy Potentiates the Therapeutic Efficacy of Intratumoral Dendritic Cell Administration

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ABSTRACT

We examined whether radiotherapy (RT) could enhance the efficacy of dendritic cell (DC)-based immunotherapy of cancer. Mice bearing s.c. D5 melanoma or MCA 205 sarcoma tumors were treated with intratumoral (i.t.) injections of bone marrow-derived unpulsed DCs in combination with local fractionated tumor irradiation. DC administration alone slightly inhibited D5 tumor growth and had no effect on MCA 205. RT alone caused a modest inhibition of both tumors. DC administration combined with RT inhibited D5 and MCA 205 tumor growth in an additive and synergistic manner, respectively. In both tumor models, RT intensified the antitumor efficacy of DC administration independent of apoptosis or necrosis within the tumor mass. Combination treatment of i.t. DCs plus RT was superior to s.c. injections of tumor lysate-pulsed DCs plus interleukin 2 in inhibiting D5 tumor growth and prolonging survival of mice. Splenocytes from mice treated with i.t. DCs plus RT contained significantly more tumor-specific, IFN-γ-secreting T cells compared with control groups. Moreover, adoptive transfer of these splenocytes mediated significant tumor regression in mice bearing established pulmonary metastases. Combined treatment followed by resection of residual s.c. tumor conferred protective immunity against a subsequent i.v. tumor challenge. Furthermore, i.t. DC plus RT treatment of s.c. tumor in mice bearing concomitant pulmonary metastases resulted in a significant reduction of lung tumors. i.t. DC administration combined with RT induces a potent local and systemic antitumor response in tumor-bearing mice. This novel regimen may be beneficial in the treatment of human cancers.

INTRODUCTION

It is now well established that DCs[3] primed with tumor antigens have the capacity to elicit antitumor immune responses *in vitro* as well as mediate effective tumor regression *in vivo* in various murine models (1, 2). Numerous strategies have been devised to load DCs with TAAs, which include pulsing DCs with whole tumor cell lysates, with apoptotic tumor bodies, with tumor RNA, with tumor-derived exosomes, and with peptides of defined TAAs and creating tumor-DC fusion (3–8). However, implementing these methods to a large-scale clinical setting might prove difficult. Potential obstacles include a limited supply of TAAs as in the case of autologous tumor, undefined TAAs in many human malignancies, uncertainty regarding the function of defined TAAs as tumor rejection antigens, and elaborate laboratory techniques mandating prolonged *ex vivo* manipulation of DCs. Nevertheless, due to promising results obtained in preclinical studies, pilot and Phase I clinical trials of DC-based tumor vaccines in patients with advanced malignancies have been conducted (9–15). These trials have shown this mode of treatment to be feasible and nontoxic. Moreover, tumor-specific T-cell responses and regression of metastatic disease have been reported. However, despite these encouraging results, the overall therapeutic efficacy has been limited. Developing methods to enhance the antitumor activity of DC-based immunotherapy of cancer are definitely warranted.

RT is currently applied in the treatment of a wide array of human cancers. Recent evidence indicates that besides exerting direct toxic effects on tumor cells, ionizing radiation also exhibits various immunomodulatory effects (16). Inflammatory responses are triggered within irradiated tissues. These, in turn, discharge danger/alarm signals (17) that recruit DCs to sites of inflammation. At the site, DCs acquire antigens, undergo maturation, and then migrate to the draining lymph node, where they present processed antigens to T cells (18). Thus radiation, via induction of inflammation, engenders antigen-specific cellular immunity. Animal studies suggest radiation can mediate modulation of tumor-specific immunity (16). In addition, irradiated tumor cells have been shown to serve effectively as a source of TAAs to elicit specific T-cell responses *in vitro* when processed and presented by DCs (19).

In light of these observations, we explored the capacity of ionizing radiation to augment the therapeutic efficacy of DC-based tumor vaccines. We chose to inject unpulsed DCs directly into irradiated tumors. This approach might prove advantageous in many aspects (20, 21). First, it alleviates the need to perform *in vitro* loading of DCs with tumor antigens. Second, it delivers DCs directly to the tumor site, thus overcoming trafficking concerns that might arise on other routes of administration. Third, it might allow DCs to acquire, process, and present multiple relevant tumor antigens in the context of appropriate co-stimulation provided by an inflammatory milieu. We postulated that this technique of *in vivo-in situ* priming of DCs could elicit tumor-specific reactive T cells. In this report, we show that RT augments the therapeutic efficacy of DC administration in two histologically distinct syngeneic murine tumor models. Furthermore, the combined treatment generates a potent local and systemic antitumor response leading to regression of established tumors.

MATERIALS AND METHODS

Mice. Female C57BL/6 (denoted henceforth as B6) mice were purchased from Harlan Inc. (Indianapolis, IN) and housed in specific pathogen-free conditions at the Animal Maintenance Facility of the University of Michigan Medical Center. The mice were used for experiments at 8 weeks of age or older.

Tumors. The D5 melanoma is a poorly immunogenic subclone of the B16-BL6 tumor of spontaneous origin in the B6 strain. This tumor has been characterized previously in our laboratory (22). Tumor cells were maintained by *in vitro* culture in CM. CM consisted of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 μg/ml streptomycin, 100 units/ml penicillin, 50 μg/ml gentamicin, 0.5 μg/ml Fungizone (all from Life Technologies, Inc., Grand Island, NY), and 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO).

MCA 205 is a 3-methylcholanthrene-induced fibrosarcoma syngeneic to B6 mice. This tumor has been previously characterized to be weakly immunogenic (23). Tumors were maintained *in vivo* by serial s.c. transplantation in B6 mice

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[3] The abbreviations used are: DC, dendritic cell; TAA, tumor-associated antigen; RT, radiotherapy; CM, complete medium; TP-DC, tumor lysate-pulsed dendritic cell; IL, interleukin; i.t., intratumoral; FACS, fluorescence-activated cell-sorting; PI, propidium iodide; CFSE, 5,6-carboxy-fluorescein succinimidyl ester; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

and were used within the eighth transplantation generation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in 40 ml of HBSS (Life Technologies, Inc.) containing 40 mg of collagenase, 4 mg of DNase I, and 100 units of hyaluronidase (Sigma) with constant stirring for 3 h at room temperature. The resulting suspension was passed through a 70 μm cell strainer. All tumor cells were washed in PBS (Life Technologies, Inc.) three times before administration to animals.

EL-4 is a T-cell thymoma syngeneic to B6 mice. This tumor was used as a specificity control in some experiments. MBL-2 is a murine T-cell lymphoma that was used as a positive control.

Ionizing Radiation. Mice were placed in plastic constrainers to ensure immobilization. Local tumor irradiation was delivered in five consecutive daily fractions using the PANTAK Therapax DXT 300 Model X-Ray Unit (Cast Haven, CT). The total radiation dose administered was 42.5 Gy (8.5 Gy $\times$ 5).

Generation of Bone Marrow-Derived DCs and TP-DCs. Erythrocyte-depleted bone marrow cells from flushed marrow cavities of femurs and tibias of naive syngeneic mice were cultured in CM supplemented with 10 ng/ml granulocyte macrophage colony-stimulating factor and 10 ng/ml IL-4 (Pepro Tech, Inc., Rocky Hill, NJ) at 1×10^6 cells/ml. On day 5, DCs were harvested by gentle pippetting and enriched by 14.5% metrizamide (Sigma)-CM gradients. The low density interface was collected by gentle pipette aspiration. The DCs were washed twice with PBS and resuspended at 1×10^6 cells/0.05 ml PBS for i.t. injection.

For preparation of tumor lysate, D5 melanoma cells were suspended in PBS and subjected to four cycles of rapid freeze (liquid N_2)/thaw (37°C water bath) exposures and then spun at 1000 rpm at 4°C for 5 min to remove cellular debris. In some experiments, DCs were incubated with tumor lysates at a ratio of 3 tumor cell equivalents:1 DC in CM. After 18 h, TP-DCs were harvested, washed twice with PBS, and resuspended at 1×10^6 cells/0.1 ml PBS for s.c. injection.

Treatment Protocols. B6 mice were inoculated s.c. in the mid-right flank with 2×10^6 D5 or 3×10^6 MCA 205 tumor cells on day 0. Unpulsed DCs (10^6) were administered i.t. on days 6, 11, 14, and 18 or on days 3, 10, 13, and 17 for the melanoma and sarcoma tumors, respectively. D5 and MCA 205 tumors were locally irradiated on days 7–11 or 6–10, respectively. These schedules of treatment were designed so that RT would commence on the day mean tumor sizes reached 25 mm^2 and DC injections would be given before RT, 3–4 h, 72 h, and 7 days after the last dose of radiation. Control groups of mice received either no treatment (i.t. PBS), i.t. DCs alone, or RT plus i.t. PBS. Tumors were measured in a blinded coded fashion three times per week in the largest perpendicular diameters using vernier calipers, and the size was recorded as tumor area (in mm^2). Data are reported as the average tumor area $\pm$ SE of five or more mice/group.

In some experiments, mice bearing 6-day s.c. D5 tumors received three s.c. injections of 1×10^6 D5 TP-DCs at 7-day intervals. TP-DC immunizations were given s.c. in the left flank, whereas the s.c. tumor was established in the right flank. IL-2 (Chiron Corp., Emeryville, CA) was given i.p. twice daily at 60,000 IU in 0.5 ml of PBS for 5 consecutive days after each immunization. Tumor size was measured and recorded as described above. Survival was followed and recorded as the percentage of surviving animals over time (in days) after tumor inoculation.

In some experiments, IL-2 was added to treatment with DCs plus RT. After each i.t. injection of DCs, 60,000 IU of IL-2 were given i.p. twice a day for 3 consecutive days.

ELISPOT Assay. MultiScreen Filtration Plates (96-wells/plate; Millipore, Bedford, MA) were coated with 100 μl/well of 4 μg/ml purified antimouse IFN-γ monoclonal antibody (clone R4-6A2; PharMingen, San Diego, CA) overnight at 4°C and then incubated for 90 min at room temperature with 150 μl/well 1% BSA (Sigma) in PBS. Erythrocyte-depleted splenocytes harvested from treated or control mice 13 days after tumor inoculation or from age-matched naive mice (5×10^4 splenocytes in 100 μl of CM) were placed into each well and incubated for 24 h at 37°C, 5% CO_2 in the absence or presence of irradiated (60 Gy) D5 or EL-4 tumor cells (5×10^3 cells in 100 μl of CM). Plates were then incubated overnight at 4°C with 100 μl/well of 4 μg/ml biotinylated rat antimouse IFN-γ monoclonal antibody (clone XMG1.2; PharMingen) followed by a 90-min incubation with 100 μl/well anti-biotin alkaline phosphatase (Vector Laboratories, Inc., Burlingame, CA) diluted 1:1000. Spots were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase substrate and counted using the ImmunoSpot analyzer (Cellular Technology, Ltd., Cleveland, OH). Data are reported as the average number of spots per 5×10^4 responders $\pm$ SE of triplicate samples.

Adoptive Transfer Model. Mice received 1×10^5 D5 tumor cells i.v. via the lateral tail vein to establish experimental pulmonary metastases. On day 3 after tumor inoculation, 15×10^6 or 20×10^6 splenocytes were administered i.v. Splenocytes used for adoptive transfer were harvested 21 days after s.c. tumor inoculation from mice treated with DCs + RT. Control groups of mice received either no treatment, splenocytes from naive mice, splenocytes from untreated (i.t. PBS) s.c. D5 tumor-bearing mice, splenocytes from s.c. D5 tumor-bearing mice treated with DCs alone, or splenocytes from s.c. D5 tumor-bearing mice treated with RT + i.t. PBS. On day 16 after i.v. tumor inoculation, mice were euthanized, and lungs were insufflated and fixed with Fekette's solution. Pulmonary metastases were enumerated in a blinded, coded fashion. Data are reported as the mean number of metastases $\pm$ SE of eight or more mice per group.

Protective Immunity Model. On day 21 after D5 tumor inoculation, mice treated with DCs + RT underwent resection of residual s.c. tumors. To perform tumor resection, mice were anesthetized by i.p. injection of 10 μl/g body weight ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine [Phoenix Scientific, Inc., St. Joseph, MO (6.5 mg and 1 mg/ml PBS, respectively)]. The skin was closed using Tevdek 3-0 sutures. After 24 h, mice were rechallenged i.v. with 1×10^5 D5 tumor cells. Naive mice served as a control group because tumors in the control treatment groups were unresectable. Sixteen days after tumor rechallenge, lungs were harvested for enumeration of metastases as described above. There were at least 12 mice/group.

On day 14 after D5 tumor inoculation, untreated mice underwent resection of s.c. tumors as described above. After 24 h, mice were rechallenged i.v. with 1×10^5 D5 tumor cells. Naive mice served as a control group. Sixteen days after tumor rechallenge, lungs were harvested for enumeration of metastases as described above. There were at least 7 mice per group.

Concomitant Pulmonary Metastases and s.c. Tumor Model. B6 mice were inoculated s.c. with 2×10^6 D5 tumor cells on day 0. Then, on day 4 after tumor inoculation, the mice received i.v. injection with 1×10^5 D5 tumor cells. DCs + RT or control treatments were administered as detailed above. On day 20 after s.c. tumor inoculation, lungs were harvested for enumeration of metastases as described above. There were at least 6 mice/group.

Detection of Apoptotic and Necrotic Cells. D5 tumor cells in culture were exposed to a single radiation dose of 10 Gy (PANTAK Therapax X-Ray Unit). After 6, 12, 24, 48, and 72 h, cells were harvested using trypsin-Versene Mixture (BioWhittaker, Walkersville, MD). Untreated cells served as a negative control. Cells exposed to UVB light (Gel Doc 2000; Bio-Rad, Hercules, CA) for 20 min (equal to 200 mJ/cm^2) followed by a 4-h culture served as a positive control. EL-4 and MBL-2 cells, harvested 48 h after exposure to 10 Gy, served as an additional positive control. Tumor cell suspensions were analyzed for apoptotic and necrotic cells using a standard FACS assay (R&D Systems, Inc., Minneapolis, MN) that detects binding of annexin V-fluorescein and exclusion or inclusion of PI (24, 25).

B6 mice bearing s.c. MCA 205 and D5 tumors were treated with RT as described above. Tumors were harvested 6, 24, 48, and 72 h and 7 days after the last dose of radiation. Control tumors received no treatment. Suspensions of MCA 205 tumor cells were prepared and analyzed as described above. In addition, paraffin-embedded tissues were prepared for both tumors. Slides were deparaffinized and stained using TUNEL method with ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Serological Corp., Temecula, CA), according to the manufacturer's instructions. MCA 205 sections were stained with 3,3′-diaminobenzidine, and D5 sections were stained with vector VIP.

CFSE Staining. D5 tumor cells were washed twice in PBS and resuspended in PBS at 10×10^6 cells/ml. The cells were incubated with 1 μM CFSE (Molecular Probes, Inc., Eugene, OR) for 10 min at room temperature in the dark. Then, cells were washed twice with PBS and cultured in CM. After 24 h, cells were exposed to a single radiation dose of 10 Gy (PANTAK Therapax X-Ray Unit). Cells were harvested using trypsin-versene mixture after an additional 24 h of incubation. The tumor cells were washed in PBS and then fixed with 500 μl of 2% paraformaldehyde (Sigma) in PBS. Control cells were either unstained, fixed immediately after labeling, fixed 24 h after labeling, or not irradiated. Analysis of stained cells was performed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Statistical Analysis. Data were evaluated by Fisher's protected least significant difference (PLSD) analysis of variance (ANOVA) test using StatView software. *P*s < 0.05 were considered statistically significant. No mice were excluded from the statistical evaluations.

RESULTS

RT Enhances the Antitumor Efficacy of i.t. DC Administration. We examined whether RT could augment the local therapeutic efficacy of i.t. DC administration in the poorly immunogenic D5 melanoma tumor model. D5 tumors in untreated mice rapidly progressed (Fig. 1*A*). DC administration alone caused a modest, albeit significant, inhibition of tumor growth that reached 27.9% on day 21 after tumor inoculation. Radiation alone inhibited D5 tumor growth by 38.8% compared with nontreated tumors. DC administration combined with radiation resulted in an additive inhibition of D5 tumor growth. This inhibition reached 65.9% on the last day of follow-up ($P < 0.005$ *versus* all other groups). In no treated mouse was autoimmune depigmentation observed.

Next we sought to validate these results in another, histologically distinct, tumor model. Thus, we assessed the impact of the combined treatment in the weakly immunogenic MCA 205 sarcoma. DCs alone had no effect on MCA 205 tumor growth (Fig. 1*B*). Attempts to induce an antitumor effect by increasing the dose of DCs injected i.t. or pulsing DCs with a foreign helper protein, keyhole limpet hemocyanin, before i.t. injection proved futile (data not shown). Radiation alone moderately inhibited MCA 205 tumor growth. DC administration combined with RT inhibited MCA 205 tumor growth in a synergistic manner ($P < 0.03$ *versus* all other groups). These findings indicate that even tumors that are resistant to DC immunizations can respond to combined treatment with DCs plus radiation.

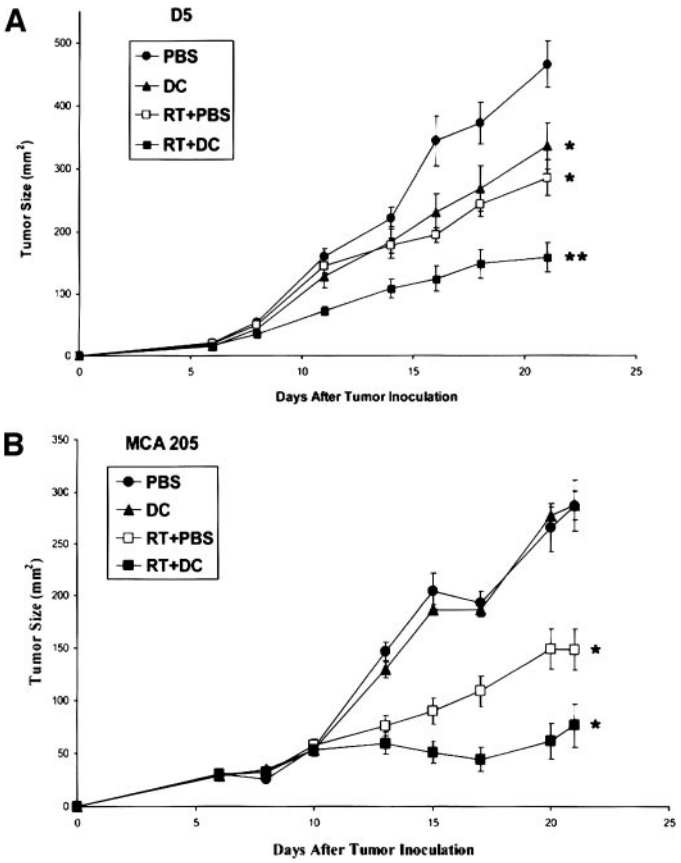

Fig. 1. i.t. DC administration combined with RT inhibits D5 melanoma (*A*) and MCA 205 sarcoma (*B*) tumor growth in an additive and synergistic manner, respectively. B6 mice were inoculated s.c. with 2×10^6 D5 (*A*) or 3×10^6 MCA 205 (*B*) tumor cells on day 0. DCs were administered i.t. on days 6, 11, 14, and 18 (*A*) or on days 3, 10, 13, and 17 (*B*). Tumors were locally irradiated on days 7–11 (*A*) or 6–10 (*B*). Control groups received either no treatment (i.t. PBS), i.t. DCs alone, or RT plus i.t. PBS. Data are reported as the average tumor area ± SE of five or more mice per group. Experiments were repeated three times with similar results. *A*: ∗, $P < 0.03$ *versus* PBS; ∗∗, $P < 0.005$ *versus* all other groups. *B*: ∗, $P < 0.03$ *versus* all other groups.

i.t. DC Administration Combined with RT Is More Potent than Immunization with TP-DCs Plus IL-2 in Mediating Tumor Regression. Numerous studies in a variety of tumor models have shown that DCs pulsed with whole tumor cell lysates can mediate antitumor immune responses and tumor regression *in vivo* (3, 26). Furthermore, it has been reported that systemic administration of IL-2 can enhance the therapeutic efficacy of DC-based tumor vaccines (27, 28). Thus, using the D5 melanoma tumor model, we compared the antitumor efficacy of s.c. immunization with TP-DCs with or without IL-2 *versus* the i.t. administration of DCs plus radiation. As shown in Fig. 2*A*, treatment of established 6-day s.c. D5 tumors with TP-DCs (three s.c. injections at 7-day intervals) with or without IL-2 (60,000 IU i.p. twice a day for 5 consecutive days after each immunization) did not cause significant inhibition of tumor growth. On the other hand, i.t. DC administration combined with radiation induced a potent antitumor response ($P < 0.0001$ *versus* all other groups). In the same study, TP-DCs with or without IL-2 did not significantly prolong survival of D5 tumor-bearing mice compared with untreated mice (Fig. 2*B*). However, treatment with DCs plus radiation resulted in a significant survival benefit ($P < 0.0001$). These findings demonstrate that DCs combined with RT is superior to TP-DCs plus IL-2 as a treatment protocol for established s.c. tumors.

We have also examined whether exogenous systemic administration of IL-2 could intensify the therapeutic effect of DCs combined with RT. D5 tumor-bearing mice that were treated with DCs plus RT received after each i.t. injection of DCs 60,000 IU of IL-2 i.p. twice a day for 3 consecutive days. IL-2 did not enhance the therapeutic activity of the combined treatment as measured by s.c. tumor size (Fig. 2*C*) and survival (data not shown) of treated animals.

i.t. DC Administration Combined with RT Confers Systemic Antitumor Immunity. Because both components of the combined treatment, namely DCs and radiation, are delivered directly to the tumor, we investigated whether this protocol could induce a systemic antitumor response. We and others have shown previously that *in vitro* tumor-specific IFN-γ production by host-derived T cells correlated with systemic antitumor immunity *in vivo* (29, 30). Thus, using ELISPOT assays, we evaluated whether treatment of D5 tumor-bearing mice with DCs plus RT could elicit tumor-specific IFN-γ secreting T cells. Fig. 3 shows that splenocytes retrieved on day 13 after tumor inoculation from mice subjected to the combined therapy contained significantly more tumor-specific IFN-γ-secreting cells compared with splenocytes from control groups ($P < 0.0001$ *versus* all other groups). This response was immunologically specific to D5 tumor cells because it was not detected spontaneously or in response to EL-4 tumor cells, a nonrelevant tumor syngeneic to B6 mice. EL-4, rather than MCA 205, was used as a specificity control because the latter probably shares TAAs with D5 (31, 32).[4] In addition, we found that local tumor irradiation alone induced tumor-specific IFN-γ-secreting cells in the spleens of treated mice. ELISPOT assays performed on splenocytes harvested on day 21 after tumor inoculation from all treated groups generated data similar to those disclosed in Fig. 3 (data not shown). Splenocytes retrieved from treated mice contained few, if any, detectable tumor-specific IL-10-secreting cells (data not shown).

[4] Unpublished observations.

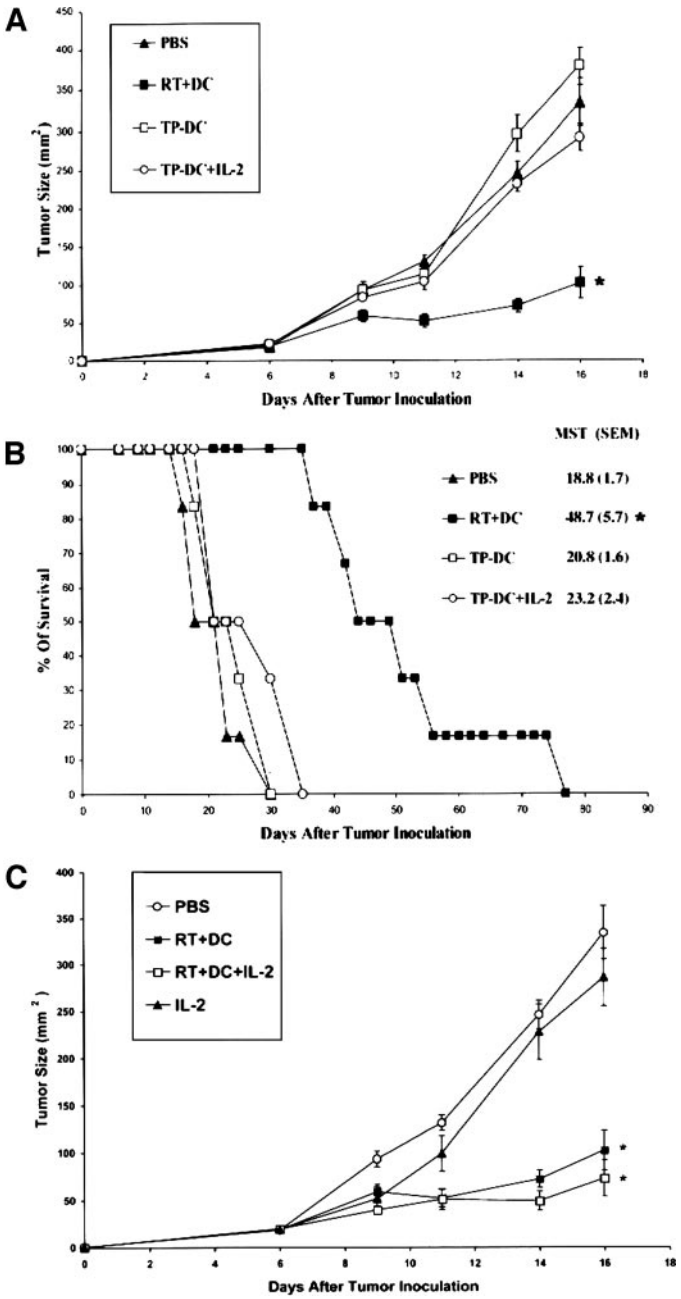

Fig. 2. i.t. DC administration combined with RT is superior to s.c. injections of TP-DCs plus IL-2 in inhibiting D5 tumor growth (*A*) and prolonging survival of mice (*B*). B6 mice were inoculated s.c. with 2×10^6 D5 tumor cells on day 0. TP-DCs were injected s.c. on days 6, 13, and 20. IL-2 was given i.p. twice daily at 60,000 IU for 5 consecutive days after each immunization. Control groups received either no treatment (*PBS*), TP-DCs without IL-2, or DCs combined with RT as described in Fig. 1*A*. *A*, data are reported as the average tumor area ± SE of five or more mice per group. *, $P < 0.0001$ *versus* all other groups. *B*, survival was monitored over time after tumor inoculation, and the median survival time (*MST*; in days) was determined. *, $P < 0.0001$ *versus* all other groups. *C*, IL-2 does not enhance the antitumor efficacy of DCs combined with RT. D5 tumor-bearing mice treated with RT plus DCs were given 60,000 IU of IL-2 i.p. twice a day for 3 consecutive days after each i.t. injection of DCs. Control groups received either no treatment (*PBS*), IL-2 alone, or DCs plus RT without IL-2. Data are reported as the average tumor area ± SE of five or more mice per group. *, $P < 0.0006$ *versus* PBS and IL-2.

We evaluated the antitumor immune function of these splenocytes in an adoptive transfer model. Splenocytes harvested from treated mice on day 21 after tumor inoculation were transferred i.v. to mice bearing day 3 pulmonary metastases. Splenocytes from naive mice or from PBS-treated D5 tumor-bearing mice had no antitumor efficacy compared with no treatment (Table 1). Adoptive transfer of splenocytes from D5 tumor-bearing mice treated with DC administration alone caused a significant reduction in the number of pulmonary metastases. Splenocytes of mice subjected to RT alone mediated inhibition of lung nodules as well. Thus, the frequency of IFN-γ tumor-responsive cells correlated with *in vivo* antitumor activity. The most pronounced reduction in lung metastases was detected after adoptive transfer of splenocytes retrieved from mice treated with DCs plus RT ($P < 0.05$ *versus* all groups after adoptive transfer of 20×10^6 splenocytes). FACS analysis of the splenocytes used for adoptive transfer disclosed no significant difference in CD3, CD4, CD8, or B220 phenotype between the various treatment groups (data not shown).

Another way to assess whether or not a treatment confers systemic immunity is to determine its capacity to induce protective immunity against a subsequent tumor challenge. Therefore, D5 tumor-bearing mice were treated with DCs plus RT. On day 21 after tumor inoculation, residual s.c. tumors were resected, and then the mice were challenged i.v. with 1×10^5 D5 tumor cells. Sixteen days later, lungs were harvested for enumeration of metastases. Because tumors in the control groups were unresectable on day 21 after s.c. tumor inoculation, naive mice served as a control group. As shown in Fig. 4*A*, lungs harvested from control mice harbored >300 metastases. On the other hand, most lungs from mice treated with DCs combined with RT before tumor rechallenge were free of disease ($P < 0.0001$). We have shown previously that the D5 tumor clone is poorly immunogenic (22). Thus, establishment of D5 tumor in the host should not confer protection against subsequent tumor challenge. Nevertheless, to verify that naive mice are a valid control for this experiment, we have demonstrated that there was no significant difference in the number of pulmonary metastases between naive mice injected i.v. with D5 tumor cells and s.c. D5 tumor-bearing mice that were rechallenged i.v. with the same number of tumor cells (Fig. 4*B*). It is of interest to note that RT alone could not mediate protective immunity against a subsequent tumor challenge (data not shown).

Taken together, our results demonstrate that DCs combined with RT of a solitary tumor confers protection against tumor rechallenge. Translated to a clinical setting, this might imply that treatment of a local tumor may prevent the outgrowth of micrometastatic disease. In an attempt to simulate this scenario, mice bearing both s.c. tumors and experimental pulmonary micrometastases were subjected to treatment of the s.c. tumor only. Table 2 displays the impact of local therapy of a s.c. tumor on concomitant pulmonary micrometastases. RT alone led to a significant reduction in the number of pulmonary nodules. To show this finding was immune mediated and not due to direct toxic effect of scattered radiation to the lungs, an additional control group of mice, harboring lung metastases with no s.c. tumors, received local radiation to the right flank. Scattered radiation could not fully account for the reduction in lung tumors observed due to RT (data not shown). The most favorable outcome was detected in mice treated with DCs combined with RT; however, this response did not reach statistical significance compared with treatment with DCs alone. Collectively, our data indicate that DC administration combined with RT induces tumor antigen-specific cellular-mediated immune responses in tumor-bearing mice.

RT Augments the Antitumor Efficacy of DC Administration Independent of Tumor Apoptosis or Necrosis Induction. DCs are capable of acquiring antigens from apoptotic or necrotic cells and eliciting an effective immune response (25, 33). Furthermore, it has been reported that i.t. injection of unpulsed DCs into tumors with a high level of baseline apoptosis inhibits tumor growth (21). Thus we hypothesized that radiation might induce apoptosis or necrosis within the tumor mass. This in turn, would render the tumor more susceptible

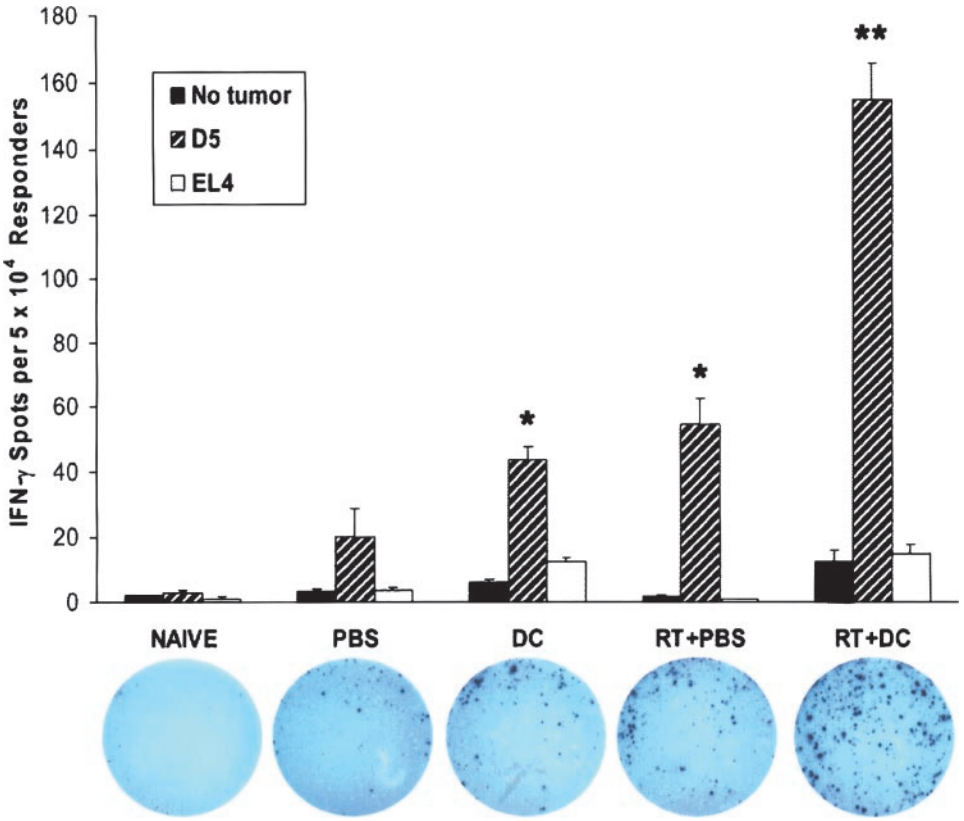

Fig. 3. i.t. DC administration combined with RT induces tumor-specific IFN-γ-secreting cells in the spleens of treated mice. Mice were injected with D5 cells and treated as detailed in Fig. 1*A*. Thirteen days after tumor inoculation, splenocytes from treated, control, and naive mice were incubated with or without specific or irrelevant irradiated tumor cells in an IFN-γ ELISPOT assay. Data are reported as the average number of spots per 5×10^4 responders ± SE of triplicate samples. *, $P < 0.05$ *versus* PBS; **, $P < 0.0001$ *versus* all other groups. Representative wells from ELISPOT plate are shown *below* the graph. This experiment was repeated three times with similar results.

to immunotherapy with direct DC administration. To test this hypothesis, we first studied the effect of radiation on D5 cells *in vitro*. This eliminated the effects of infiltrating nontumor cells, the presence of nonviable cells due to tumor hypoperfusion, and concerns regarding loss of cells during tumor digestion. D5 tumor cells were harvested 6, 12, 24, 48, and 72 h after a single radiation dose of 10 Gy and analyzed for apoptotic or necrotic cells using the annexin V/PI FACS assay. Exposure of D5 cells to UVB served as a positive control. Exposure of EL-4 thymoma and MBL-2 lymphoma cells to the same irradiation dose lead to a prominent, 2-fold increase in the percentage of nonviable cells after 48 h in culture (Fig. 5*A*). However, at all time points evaluated, we could not detect a significant increase in the percentage of apoptotic or necrotic cells in irradiated D5 cells compared with untreated cells (Fig. 5*B*). Applying a single radiation dose of 5 Gy yielded similar results (data not shown).

Table 1 *Splenocytes from treated D5 tumor-bearing mice mediate antitumor immune responses in vivo*

Source of splenocytes used for adoptive transfer[b]	Mean no. of lung metastases (SE)[a]	
	Expt. 1[c]	Expt. 2[d]
None	279 (13)	247 (6)
Naive mice	273 (12)	236 (8)
Untreated (i.t. PBS) D5-bearing mice	271 (14)	236 (11)
D5-bearing mice treated with DC	190 (7)[e]	154 (12)[e]
D5-bearing mice treated with RT + PBS	154 (6)[e]	145 (8)[f]
D5-bearing mice treated with RT + DCs	140 (16)[g]	105 (13)[e]

[a] Lungs were harvested for enumeration of metastases 16 days after i.v. tumor inoculation.
[b] Splenocytes harvested on day 21 after s.c. D5 tumor inoculation from treated, control, or age-matched naive mice were administered i.v. to mice harboring day 3 D5 pulmonary metastases.
[c] Mice received 15×10^6 splenocytes (at least 8 mice/group).
[d] Mice received 20×10^6 splenocytes (at least 9 mice/group).
[e] $P < 0.05$ *versus* all previous groups.
[f] $P < 0.05$ *versus* all previous groups except DC.
[g] $P < 0.05$ *versus* all previous groups except RT + PBS.

In an *in vivo* model, we evaluated the impact of radiation on the MCA 205 sarcoma tumor. We analyzed dispersed cells from s.c. tumors that had been irradiated *in vivo* according to our fractionated treatment protocol (8.5 Gy × 5). This would allow us to detect indirect toxic effects occurring solely *in vivo* (*e.g.*, damage of endothelial cells leading to i.t. vascular thrombosis and secondary tumor necrosis) as well as direct toxic effects of radiation on tumor cells. At 6, 24, 48, and 72 h and 7 days after the last dose of radiation, we could not demonstrate a significant increase in the level of apoptosis or necrosis within the irradiated tumor masses compared with control nonirradiated tumors (Fig. 5*C*).

In situ TUNEL staining of irradiated *versus* unirradiated D5 and MCA 205 tumor sections at 6, 24, 48, and 72 h and 7 days after the last dose of radiation confirmed these results (Fig. 5*D*). Thus, we concluded that RT enhanced the therapeutic efficacy of DC administration independent of apoptosis or necrosis induction.

In light of these findings, we examined the biological effect of radiation on cell proliferation. D5 tumor cells were labeled with CFSE. After 24 h in culture, the cells were exposed to 10 Gy of ionizing radiation. The cells were harvested 24 and 48 h after radiation and analyzed by FACS assay. As shown in Fig. 6, unstained cells (*far left curve*) served as a negative control. The *far right curve* depicts the CFSE intensity of tumor cells immediately after labeling. Cells grown in culture for 24 h after labeling (*second curve from the right*) serve as a positive control because this time point correlates with the time radiation was administered. The leftward shift of this curve along the *X* axis illustrates division of tumor cells that is proportional to loss of CFSE intensity. After an additional 24 h had passed, the curve of nonirradiated cells (*second curve from the left*) shifted further to the left, reflecting ongoing cell proliferation. On the other hand, the curve

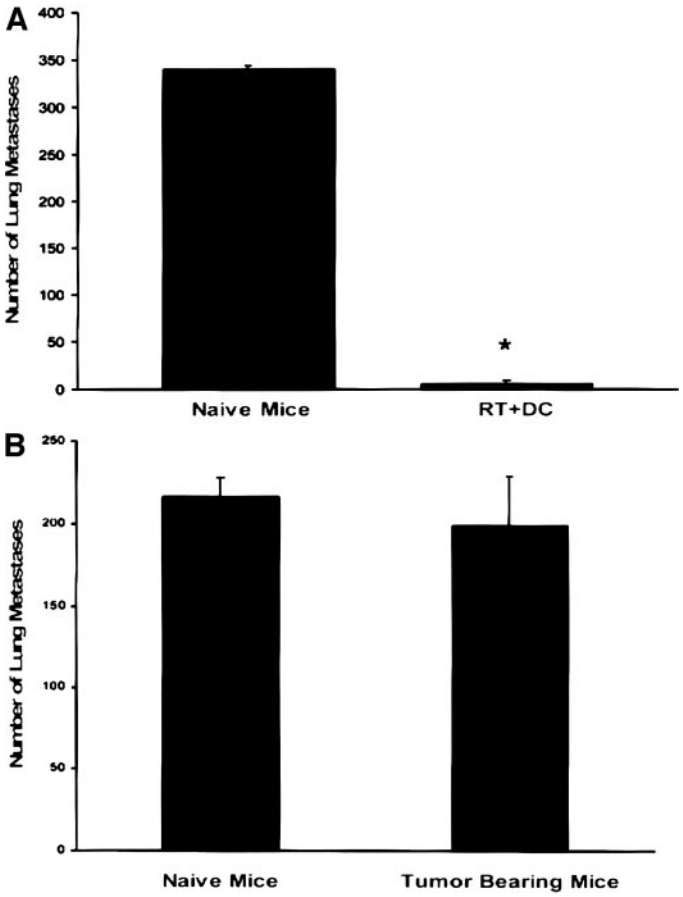

Fig. 4. *A*, i.t. DCs combined with RT confer protection against tumor rechallenge. B6 mice were inoculated s.c. with 2×10^6 D5 tumor cells on day 0. Combined treatment with DCs and RT was administered as described in Fig. 1*A*. On day 21 after tumor inoculation, residual s.c. tumors were resected. After 24 h, mice were rechallenged i.v. with 1×10^5 D5 tumor cells. Naive mice served as a control group. Sixteen days after tumor rechallenge, lungs were harvested for enumeration of metastases. Data are reported as the mean number of metastases ± SE. This experiment was repeated three times with similar results. *, $P < 0.0001$. *B*, establishment of D5 tumor in B6 mice does not confer protection against subsequent tumor challenge. Mice were inoculated s.c. with 2×10^6 D5 tumor cells on day 0. On day 14, s.c. tumors were resected. After 24 h, mice were rechallenged i.v. with 1×10^5 D5 tumor cells. Naive mice served as a control group. Sixteen days after tumor rechallenge, lungs were harvested for enumeration of metastases.

representing irradiated cells (*third curve from the left*) shifted leftward to a lesser extent. This indicates that radiation inhibits the division of tumor cells. Data obtained 48 h after radiation support this notion (data not shown).

DISCUSSION

DCs can be found within solid human malignancies *in vivo*, and the degree of DC infiltration has been associated with improved prognosis (34). This association may reflect the induction of an effective immune response. By contrast, other investigators have found that mature DCs from tumor-bearing hosts can have defects in antigen presentation function (35, 36). In addition, human cancer cells have been shown to release soluble factors that can inhibit the maturation of DCs (37). Nevertheless, DCs derived from precursors obtained from tumor-bearing hosts can be effective antigen delivering vehicles for tumor immunotherapy (36, 38). The immunization strategy we describe here could circumvent tumor-induced antigen-presenting cell dysfunction by using functional DCs grown from DC precursors *in vitro*. In this regard, Nikitina and Gabrilovich (39) have demonstrated that induction of an antitumor immune response in tumor-bearing mice treated with combination of radiation and DC administration (s.c. plus i.v.) requires presentation of TAAs by *ex vivo*-generated DCs. Endogenous DCs can enhance T-cell responses elicited by DC vaccines (40) but are insufficient to initiate them. As we have reported previously, functional human DCs can be obtained readily by *in vitro* culture of peripheral blood-derived precursors (9).

The use of unpulsed DCs in the tumor vaccine approach reported here abrogates the need to load DCs with TAAs *in vitro*. Pulsing of DCs *in vivo-in situ* with irradiated tumors as the source of TAAs has the potential to stimulate immunity against multiple relevant tumor antigens while alleviating the need to obtain autologous tumor tissue or identify shared TAAs for specific cancers that would be limited to individuals expressing a particular corresponding MHC allele. Furthermore, because the immunization is patient specific, it could induce immunity against uniquely expressed tumor antigens that may be an important component of an effective antitumor response.

Direct i.t. injection of DCs ensures their accumulation within the desired site for antigen(s) uptake while overcoming potential obstacles related to trafficking into solid tumor masses that might arise on other routes of administration (*i.e.*, s.c., intradermal, and i.v.). Nishioka *et al.* (20) have shown, using the MCA 205 murine sarcoma model, that numerous i.t. injected DCs migrate to the draining lymph node within 24 h. We believe this approach to be clinically applicable in many cancer patients, particularly under the guidance of ultrasound imaging, and it might allow DC dose reduction without compromising antitumor efficacy. A pilot clinical study in patients with metastatic melanoma and breast carcinoma demonstrated that i.t. injection of DCs generated *in vitro* is feasible and well tolerated (41). Regressions of injected tumors (6 of 10 patients) and satellite noninjected tumors (2 of 10 patients) were observed; however, no clinical responses were detected in metastases distant from the injection site.

Candido *et al.* (21) have reported that direct local administration of naive syngeneic unpulsed DCs into a murine breast carcinoma, but not into chemically induced sarcomas, causes significant inhibition of tumor growth. Because DCs can efficiently acquire antigen from apoptotic cells and induce MHC class I-restricted, antigen-specific $CD8^+$ CTLs (25), they attributed the failure of those sarcomas to respond to treatment with i.t. DC injection to the low level of baseline apoptosis (~4–8%) within those tumors. They went on to demonstrate that increasing the apoptotic index within the breast carcinoma tumor (from 27% to 68%) by systemic administration of tumor necrosis factor α leads to a greater DC-mediated antitumor effect. Other studies have documented that i.t. injection of DCs, in combination with systemic chemotherapy that induces apoptosis of tumor cells, enhances the therapeutic efficacy (42, 43). Our results corroborate these findings in that D5 melanoma, which responded to treatment with i.t. DC administration, was found to have a relatively high level of baseline apoptosis (~30%; data not shown), whereas MCA

Table 2 *Treatment of s.c. D5 tumor mediates regression of concomitant pulmonary metastases*

Treatment[b]	Mean no. of lung metastases (SE)[a]		
	Expt. 1[c]	Expt. 2[d]	Combined
PBS	372 (36)	276 (26)	324 (24)
RT + PBS	236 (39)[e]	182 (18)[e]	209 (22)[e]
DC	138 (34)[e]	168 (42)[e]	156 (28)[e]
RT + DC	109 (20)[f]	106 (23)[f]	108 (15)[f]

[a] Lungs were harvested for enumeration of metastases 16 days after i.v. tumor inoculation.
[b] D5 cells were injected into mice s.c. on day 0 and i.v. on day 4 (2×10^6 and 1×10^5, respectively). Treatment was administered as described in Fig. 1*A*.
[c] At least 6 mice/group.
[d] At least 9 mice/group.
[e] $P < 0.05$ *versus* PBS.
[f] $P < 0.05$ *versus* all groups except DC.

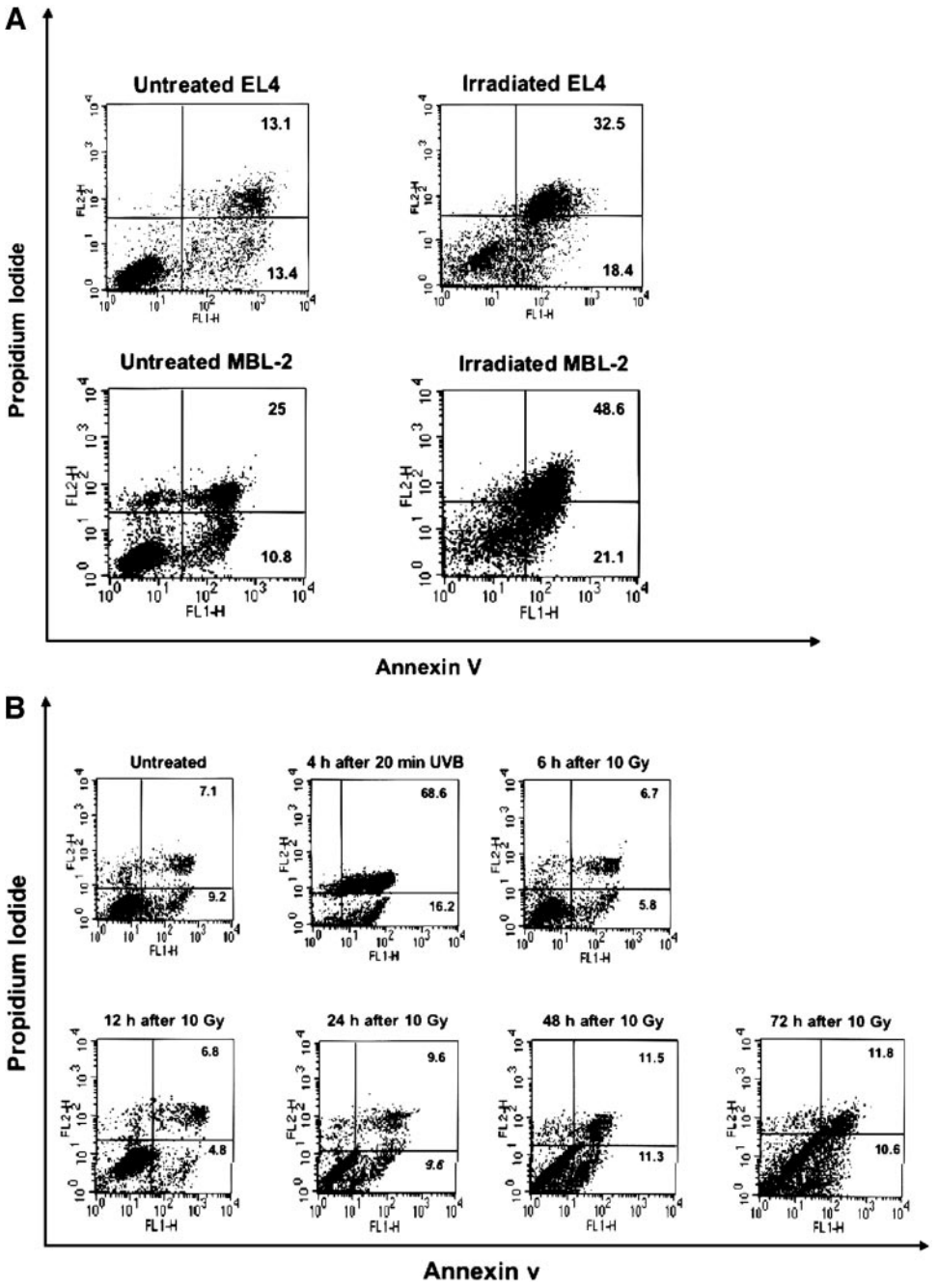

Fig. 5. *A*, radiation induces apoptosis and/or necrosis of murine thymoma and lymphoma cells. EL-4 and MBL-2 cells were harvested 48 h after a single radiation dose of 10 Gy and analyzed for apoptotic and necrotic cells using the annexin V/PI FACS assay. Untreated cells served as a negative control. The *bottom left quadrant* represents viable cells, the *bottom right quadrant* depicts cells in early apoptosis, and the *top right quadrant* represents necrotic and late apoptotic cells. *B*, radiation does not induce significant apoptosis or necrosis in D5 cell culture. D5 cells were harvested 6, 12, 24, 48, and 72 h after exposure to 10 Gy and analyzed as described above. Untreated cells served as a negative control. Exposure of cells to UVB served as a positive control. *C*, radiation does not induce significant apoptosis or necrosis within the MCA 205 tumor mass. Mice were inoculated s.c. with 3×10^6 MCA 205 cells on day 0. RT was delivered on days 6–10 (8.5 Gy $\times$ 5). Tumors were harvested 6, 24, 48, and 72 h and 7 days after the last dose of radiation. Control tumors received no treatment. Tumor cell suspensions were prepared as described in "Materials and Methods" and analyzed as detailed above. *D*, *in situ* TUNEL staining of irradiated (*B* and *D*) *versus* unirradiated (*A* and *C*) MCA 205 (*A* and *B*) and D5 (*C* and *D*) tumor sections 72 h and 6 h after the last dose of radiation, respectively. Representative fields are shown (magnification, $\times$40). Apoptotic cells are stained *brown* in MCA 205 sections and *purple* in D5 sections.

205 sarcoma, which was resistant to treatment with DCs alone, exhibited a low apoptotic index (~15%; data not shown). Furthermore, the data reported herein demonstrate that combining i.t. DC administration with RT can mediate effective inhibition of tumor growth in tumors with a high apoptotic index as well as those with a low apoptotic index. Because radiation is known to induce apoptosis in some tumor cell lines (44, 45), we initially hypothesized that RT increases the level of apoptosis within D5 and MCA 205 tumor masses. However, quantification of the level of apoptosis after radiation by FACS analysis using the annexin V/PI assay did not confirm this hypothesis. Recently, it has been shown that necrotic tumor cells can serve as a source of multiple TAAs to pulse DCs as effectively as apoptotic tumor cells (46). However, radiation did not significantly increase the level of necrosis within the tumor mass either. Rather, it seems that, at least in the D5 tumor model, radiation mediates tumor inhibition by suppressing cell proliferation. Celluzzi *et al.* (47) have shown that DCs cocultured with viable tumor cells can induce tumor-specific $CD8^+$ cytotoxic T cells and mediate regression of established tumors *in vivo*. Moreover, a recent report indicated that DCs pulsed with irradiated viable tumor cells were superior to TP-DCs in conferring protective immunity against tumor challenge (19). In accordance with our findings, radiation (100 Gy) did not induce apoptosis or necrosis of tumor cells in their model. Thus, it is possible that radiation augments the antitumor efficacy of DC administration by modifying tumor cells to become more immunogenic. This would allow *ex vivo*-generated functional DCs to acquire TAAs from irradiated tumor cells more efficiently than they would acquire them from nonirradiated tumor cells. Additional studies aimed at exploring this possibility are currently being conducted in our laboratory.

Another possible mechanism underlying our findings is related to

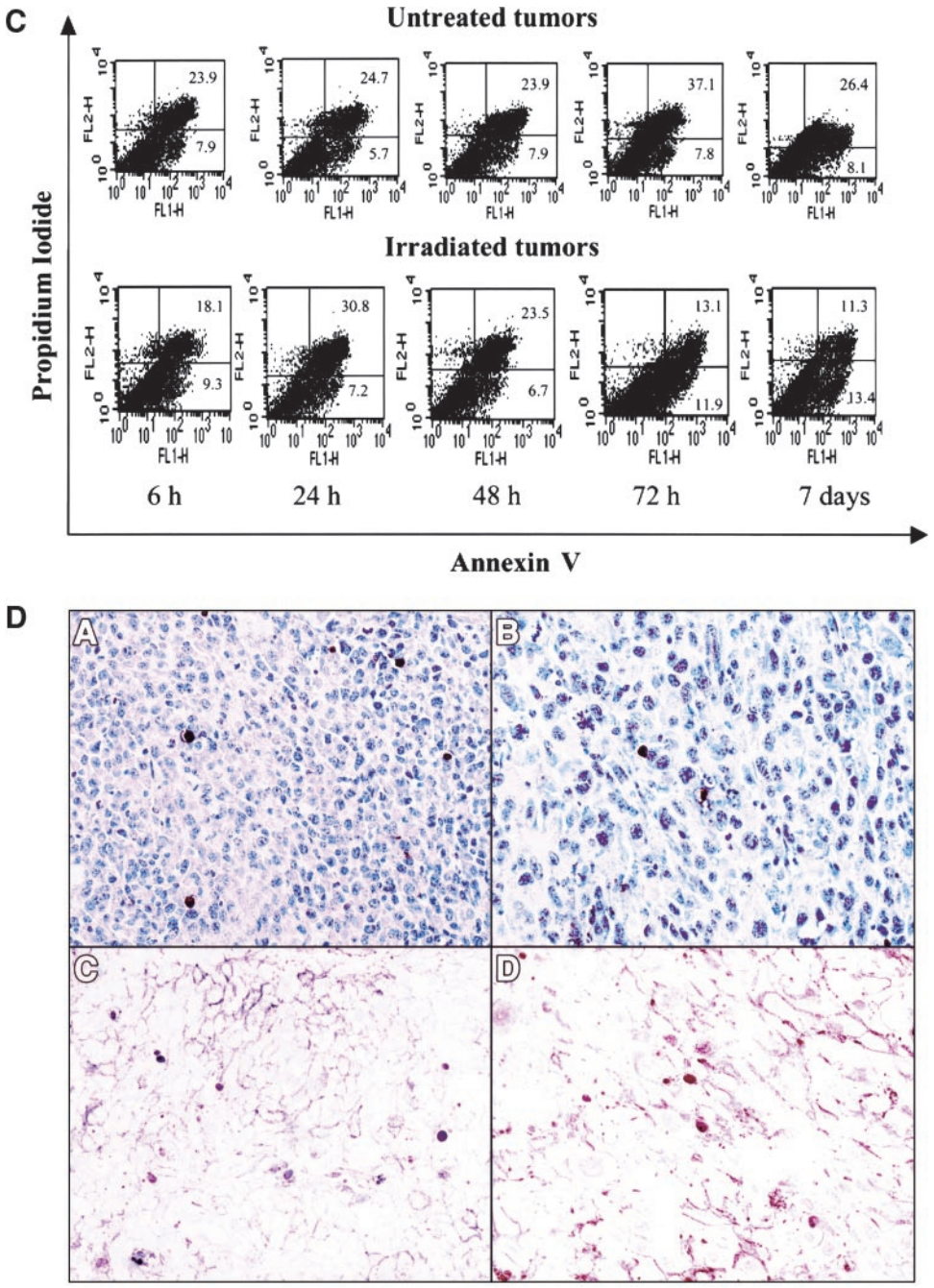

Fig. 5. *Continued.*

the induction of inflammation within irradiated tissues. One of the hallmarks of inflammation is an increase in the permeability of the local vasculature that leads to recruitment of circulating leukocytes into surrounding tissues. In a similar fashion (48, 49), radiation facilitates the extravasation of both antigen-presenting cells (39) and effector T cells (49) into solid tumors. In this regard, Ganss *et al.* (50) have shown that radiation before adoptive transfer of activated tumor-specific T lymphocytes allows the effector T cells to infiltrate solid tumors and leads to their eradication. Moreover, the activation of DCs, which enables them to offer co-stimulatory signals and thus to initiate immune responses, can be induced by endogenous danger signals released by tissues in distress, including inflamed tissues. Some recently identified endogenous danger signals are heat shock proteins, nucleotides, reactive oxygen intermediates, extracellular matrix breakdown products, neuromediators, and cytokines such as tumor necrosis factor α (51). Radiation has been reported to induce cytokines, chemokines, and inflammatory mediator release and to upregulate the expression of adhesion molecules, co-stimulatory molecules, and heat shock proteins in tumor, stromal, and endothelial cells (16). Thus, the proinflammatory microenvironment within irradiated tumors could provide DCs with maturation-inducing stimuli critical for eliciting effective antigen presentation. Another approach to enhance antitumor T-cell responses utilizes i.t. administration of genetically modified DCs (20, 52).

Tumor regression due to local administration of unpulsed DCs has been shown to be dependent on host-derived $CD8^+$ T cells (21). This might explain the capacity of splenocytes from mice subjected to DC treatment with or without RT to mediate, upon adoptive transfer, regression of established pulmonary metastases. Additional studies are currently under way in our laboratory to characterize the effector

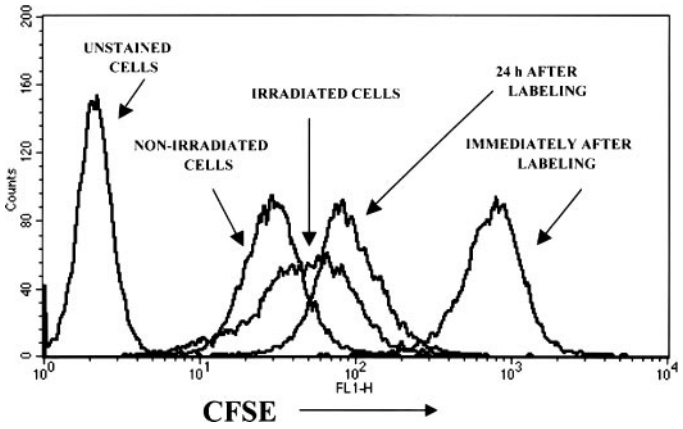

Fig. 6. Radiation inhibits the proliferation of D5 tumor cells *in vitro*. D5 cells were labeled with CFSE and then cultured. After 24 h, the cells were exposed to 10 Gy of radiation. The cells were harvested 24 h after radiation and analyzed by FACS assay. Control cells were either unstained, fixed immediately after labeling, fixed 24 h after labeling, or not irradiated. All experiments were repeated twice with similar results.

cells responsible for mediating systemic immunity after radiation therapy alone.

The data presented in this report indicate that i.t. DC administration combined with RT mediates potent inhibition of local tumors. In addition, the combined treatment induces a systemic antitumor immune response that is at least as effective as that generated by DCs alone. Thus, this novel regimen may be beneficial in the treatment of patients with advanced metastatic disease as well as in the neoadjuvant setting before resection of tumors known to have a high recurrence rate.

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Radiotherapy Potentiates the Therapeutic Efficacy of Intratumoral Dendritic Cell Administration

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