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Radiotherapy Combined with Intratumoral Dendritic Cell Vaccination Enhances the Therapeutic Efficacy of Adoptive T-Cell Transfer

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Abstract

Treatment of C57BL/6 mice with cyclophosphamide (100 mg/kg) and fludarabine (200 mg/kg) induced non-myeloablative lymphodepletion without inhibiting D5 melanoma tumor growth. Utilizing this model, we found that induction of lymphopenia prior to adoptive transfer of *ex vivo* anti-CD3/CD28 activated and IL-2 expanded D5-G6 tumor draining lymph node cells enhanced the anti-tumor efficacy of the infused cells in both pulmonary metastases and subcutaneous D5 bearing mice. However, induction of lymphopenia did not promote intratumoral or extratumoral proliferation or accumulation of the infused cells. We have previously shown that radiotherapy enhances the therapeutic efficacy of intratumoral unpulsed dendritic cell vaccination in subcutaneous murine tumor models by augmenting the induction of anti-tumor cellular immune responses. Here, we confirmed this finding in a murine metastatic melanoma liver tumor model. Furthermore, local tumor irradiation combined with intratumoral dendritic cell administration significantly enhanced the therapeutic efficacy of tumor-reactive T cell adoptive transfer in this lymphodepleted liver tumor model. This was evident by reduced liver tumor size, decreased incidence of spontaneous intra-abdominal metastasis, and prolonged survival, resulting in 46% of mice cured. This enhanced anti-tumor activity was associated with a selective increase in proliferation, accumulation, and function of $CD4^+$ rather than $CD8^+$ infused cells. This multimodality regimen may have translational applications for the treatment of human cancers.

Keywords

Radiation; Dendritic Cell; Adoptive Transfer; Cancer Vaccine; Immunotherapy

INTRODUCTION

Cancer mortality is predominantly due to the outgrowth of metastatic disease. The liver constitutes a particularly frequent metastatic site for various human malignancies, including: gastro-intestinal, breast, lung, and melanoma (1). The survival rate for advanced-stage melanoma hasn't changed over the past 30 years, and melanoma patients with liver metastases

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have an average life span of only 4 to 8 months (2). Experimental anti-tumor therapies are frequently tested for efficacy in subcutaneous (s.c.) tumor models since they provide convenient access to tumor induction and follow up. However, s.c. tumors may not adequately mirror orthotopic tumors, particularly in regard with response to immunological strategies. Whereas antigen presentation in s.c. tissues tends to elicit immunity, antigen presentation in the liver tends to induce tolerance (3,4). This may be related to the physiologic functions of the liver which involve clearance of toxic metabolic products and foreign antigens derived from the gastrointestinal tract. To accommodate these functions, the microenvironment within the liver is characterized by local release of immunosuppressive mediators such as IL-10, prostaglandin E_2, and transforming growth factor β. Therefore, compared with the skin, the liver represents a less permissive environment for induction of an effective anti-tumor immune response.

Adoptive cellular immunotherapy aims to eradicate metastatic cancer by transferring tumor-reactive T cells derived from the tumor-bearing host using either tumor-infiltrating lymphocytes, tumor-primed lymph node cells, or *in vitro* sensitized peripheral blood lymphocytes (5). Since this approach has shown promising results in animal models and in clinical trials, a major research effort has focused on designing strategies to enhance the anti-tumor activity of the infused cells (6). Part of this research effort has been devoted to optimizing *in vitro* methods used for generation of tumor-reactive T cells for adoptive transfer (AT) (7–9). Another approach has focused on modulating the immunological environment within the tumor-bearing host. One of the earliest examples is the exogenous administration of interleukin 2 (IL-2) after adoptive T-cell transfer which augments therapeutic efficacy (10). More recently, induction of lymphopenia prior to AT has been shown to enhance the anti-tumor activity of the infused cells (11,12). In the clinical setting, non-myeloablative lymphodepletion is induced by administration of chemotherapy, of which cyclophosphamide plus fludarabine have been most frequently used. Most animal studies, on the other hand, have utilized either total body irradiation or genetically deficient mice. This enhancement in the therapeutic efficacy of AT has been attributed to several factors, including: homeostatic proliferation of the infused cells (13,14), elimination of host regulatory T cells (15), enhanced availability of homeostatic cytokines (16), and removal of competition at the surfaces of antigen presenting cells (17). The effector function and the persistence of adoptively transferred tumor-reactive T cells within the tumor-bearing host are major factors contributing to treatment outcome.

Dendritic cells (DCs) loaded *in vitro* with tumor-derived antigens have the capacity to prime naïve T cells (18) as well as to stimulate activated, tumor-reactive T cells (19). Intratumoral (i.t.) vaccination with unpulsed DCs is based on *in vivo-in situ* priming of DCs. This approach offers the potential advantage of eliciting immunity against multiple relevant host-specific tumor antigens without major histocompatibility complex allele restrictions, while alleviating the need to obtain and load DCs with tumor-antigens. Utilizing various s.c. tumor models, we have previously shown that local tumor irradiation augments the capacity of unpulsed i.t. administered DCs to induce tumor-specific cellular immune responses resulting in enhanced therapeutic efficacy (20–23). In a s.c. melanoma model, radiotherapy combined with i.t. DC vaccination proved superior to tumor lysate pulsed-DC immunization in inhibiting tumor growth and prolonging survival (20). In this study, we examined whether radiotherapy could also enhance the therapeutic efficacy of DC administration in a metastatic melanoma liver tumor model. Furthermore, utilizing this liver tumor model, we evaluated whether the combination of radiotherapy and i.t. DC vaccination could stimulate adoptively transferred tumor-reactive T cells *in vivo* within a lymphodepleted host leading to improved anti-tumor efficacy of the infused cells.

To this end, we transferred T cells derived from *ex vivo* activated and expanded tumor draining lymph node (TDLN) cells. This source of effector cells has been studied extensively in animal

models (7–9,24–26), and its clinical counterpart, vaccine primed lymph node, has been evaluated in various clinical trials (27–30). TDLNs contain *in vivo* sensitized pre-effector cells that can be stimulated *in vitro* to generate tumor-specific $CD8^+$ and $CD4^+$ cells, both of which mediate tumor regression upon AT (31,32).

MATERIALS AND METHODS

Mice

Female C57BL/6 mice with CD45.2 or CD45.1 phenotype were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in specific pathogen-free conditions at the Animal Maintenance Facility of the University of Michigan Medical Center and used for experiments at 8 weeks of age or older. The University of Michigan Committee on Use and Care of Animals reviewed and approved all animal protocols.

Tumor Cells

D5 melanoma is a poorly immunogenic subclone of the B16-BL6 tumor of spontaneous origin in the C57BL/6 strain (24). D5-G6 is a D5 clone, transduced to express murine granulocyte macrophage colony-stimulating factor (24). Tumor cells were cultured in complete medium (CM) (20).

Depletion of Host Immune Elements

To induce lymphopenia, mice received two consecutive daily doses of cyclophosphamide (50 mg/kg; Sigma, St. Louis, MO) and fludarabine (100 mg/kg; Sicor, Irvine, Canada) intraperitonealy (i.p.).

In Vivo D5 Tumor Models

To induce lung metastases, mice were inoculated intravenously (i.v.) with 1×10^5 D5 cells. To establish subcutaneous tumors, mice were inoculated s.c. in the right flank with 2×10^6 D5 cells. To induce solitary metastatic liver tumors, mice were inoculated intrahepatically with 1×10^5 D5 cells. Tumor cells were injected subcapsularly into the ventral-caudal aspect of the left lateral lobe so that established tumors would be located ~1 cm caudal to the xiphoid tip and along the mid-line. This procedure was performed under isoflurane anesthesia via mini-laparotomy.

Generation of T cells for AT

To induce TDLNs, mice were inoculated s.c. in bilateral flanks with 1×10^6 D5-G6 cells. After 9 days, inguinal lymph nodes were harvested. TDLN cells (1×10^6/ml CM) were activated in 6-well culture plates with immobilized anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) (BD Biosciences, San Diego, CA) for 2 days. Culture plates were pre-coated with 1 μg of each mAb/ml PBS (3 ml/well) overnight at 4°C. After activation, cells (1×10^5/ml) were expanded in CM containing human recombinant IL-2 (60 IU/ml; Chiron, Emeryville, CA) for 3 days. Activated and expanded D5-G6 TDLN cells were injected i.v. to D5 tumor-bearing mice 7 days after tumor inoculation. Following AT, mice received IL-2 (i.p. 42,000 IU × 2/day for 8 doses). In some experiments, prior to AT, cells were stained with 5-(and 6-) Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) as previously described (20).

Flow Cytometric Analysis

Blood samples were drawn via the lateral saphenous vein. Spleens were harvested and disrupted to single cell suspensions. Erythrocytes in blood and spleens were depleted. After sacrificing mice, lungs were flushed prior to harvesting by injecting PBS into the right ventricle of the heart. Tumor-bearing lungs and s.c. tumors were mechanically disaggregated into single cell

suspensions using a Medimachine (DakoUSA, Carpinteria, CA) as previously described (21, 33). All tissue samples were collected, processed, and analyzed individually.

All mAbs were purchased from BD Biosciences. Fc receptors were blocked, and tissue-derived single cell suspensions were stained as previously described (21). Cell staining was performed using fluorochrome-conjugated mAbs against CD3, CD4, CD8, CD45, CD45.1, and matching isotype control mAbs recommended by the manufacturer. Analysis was performed using a FACSCalibur cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). To determine absolute number of cells in samples, a known quantity of 15 μm polystyrene microbeads (Bangs Laboratories, Fishers, IN) was added to each sample (21,33). To determine the proliferation state of adoptively transferred cells within collected tissues, the geometrical mean of CFSE intensity of $CD45.1^+CD8^+$ and $CD45.1^+CD8^-$ cells was recorded.

To assess IFNγ secretion in response to tumor cell stimulation, erythrocyte-depleted peripheral blood cells (1×10^6/ml CM) from tumor-bearing mice were placed into 24-well culture plates and stimulated with D5 cells (1×10^5/ml CM). After 4 hours, Brefeldin A (GolgiPlug, 1 μl/ml, BD Biosciences) was added. Cells were cultured for an additional 16 hours and then stained for CD4 and CD8. Intracellular staining for IFNγ was performed according to the manufacturer's recommendations.

Tumor Irradiation

Liver tumors were irradiated by the Experimental Irradiation Core at the Cancer Center. Mice were placed in individual plastic restraints to ensure immobilization. Radiation was directed to the tumor site with the rest of the mouse being shielded. Radiation was delivered in 3 consecutive daily fractions of 4 Gy on days 3–5 after tumor inoculation, using an X-ray unit (Pantak Therapax DXT 300 Model; Pantak, East Haven, CT) at a dose rate of ~3 Gy/min.

Generation of Bone Marrow-Derived DCs

DCs were prepared as previously described (20) and suspended at 1×10^6 cells/0.05 ml PBS for i.t. injection. DC vaccination was administered on day 6 and 10 after intrahepatic tumor inoculation via mini-laparotomy.

Monitoring Anti-Tumor Responses

On day 15 or 16 after induction of pulmonary metastases, mice were euthanized, and lungs were insufflated and fixed in Fekette's solution. Metastases were enumerated under a magnifying glass. S.c. tumors were measured three times per week in the largest perpendicular diameters. Liver tumors were measured via exploratory laparotomy performed on day 14 after tumor inoculation. In some experiments, hepatic tumors were measured on day 19 by ultrasound imaging. This procedure was performed by the Center for Integrative Genomics under anesthesia using the high frequency-high resolution Vevo 770 ultrasound machine (VisualSonics, Toronto, ON, Canada) designed for imaging of mice (34). Images were acquired in sagittal and transverse planes. Three perpendicular tumor diameters were measured in the largest cross-section frame. Tumor volumes were calculated using the formula for the volume of an ellipsoid. Our preliminary studies and published reports (35,36) established good correlation between liver tumor measurements obtained via ultrasound in mice and those obtained under direct vision. The abdominal cavity was explored for metastatic disease during laparotomy and at autopsy. Mice survival was monitored and recorded. Mice were euthanized when metastatic disease precluded peritoneal closure during laparotomy or once they have reached a moribund state as defined by the University Committee on Use and Care of Animals policy for end-stage illness and humane endpoints.

Statistical Analysis

Data were evaluated by unpaired t test (2 cohorts) or one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test for multiple comparisons (>2 cohorts). When data sets did not show a normal distribution, groups were compared using the Kruskal-Wallis test. Survival curves were compared using the log-rank test. P values < 0.05 were considered statistically significant.

RESULTS

Lymphodepletion augments the therapeutic efficacy of adoptive T-cell transfer without promoting proliferation or accumulation of the infused cells

Treatment of C57BL/6 mice with cyclophosphamide (100 mg/kg) and fludarabine (200 mg/kg) mediated a profound, yet transient, reduction in total white blood cell (CD45) and T cell (CD3) counts in peripheral blood samples (Fig. 1A). CD45 and CD3 cell counts decreased by 90% and 80%, respectively, compared with pre-treatment values 4 days after initiation of treatment and gradually reverted to normal over a period of 67 days. Importantly, administration of this chemotherapy regimen to mice bearing 3-day D5 pulmonary metastases or s.c. tumors did not significantly inhibit tumor growth or prolong mice survival (Fig. 1B and C).

We utilized this clinically relevant murine model to examine the effects of a lymphopenic environment on AT of activated, tumor-reactive T cells. Our previous studies have shown that AT of *ex vivo* antibody-activated and IL-2-expanded D5-G6 TDLN cells followed by IL-2 administration mediates regression of 4-day D5 pulmonary metastases (24). Therefore, in this study, the anti-tumor efficacy of these effector cells was evaluated in a 7-day D5 lung metastases model and in a 7-day s.c. D5 tumor model. As shown in Fig. 2A, infusion of tumor-reactive T cells to mice bearing 7-day D5 pulmonary metastases followed by IL-2 treatment led to a modest reduction of lung tumors compared with control mice ($P<0.02$ for all three doses of infused cells). However, induction of lymphopenia prior to AT significantly enhanced therapeutic efficacy at all cell doses tested. AT of tumor-reactive T cells induced tumor regression in a dose dependent manner in both wild type and lymphodepleted hosts, but this trend reached statistical significance only in the latter group ($P<0.002$ comparing infusion of 30×10^6 versus 10×10^6 cells after administration of chemotherapy). Growth of 7-day s.c. D5 tumors was not inhibited by AT of 3×10^7 cells (Fig. 2B). In contrast, administration of chemotherapy before T cell infusion caused significant tumor growth inhibition and significantly prolonged mice survival (Fig. 2C).

To assess *in vivo* accumulation and proliferation of the infused cells, CFSE-labeled CD45.1 tumor-reactive T cells were injected to CD45.2 tumor-bearing mice. In the D5 pulmonary metastases model, during the first 5 days after AT, accumulation of infused CD45.1 T cells within spleens, blood samples and lung tumors did not differ significantly between lymphodepleted and wild type mice (Fig. 3A). On day 1 and 3 after AT, the mean percentage of CD45.1 cells in spleens of mice treated with chemotherapy plus AT was significantly increased compared with mice treated with AT alone. However, this finding is probably due to depletion of host immune elements by the administration of chemotherapy since the absolute number of CD45.1 cells in the spleen did not differ significantly between the two treatment groups. Previous studies have established that 24 hours after AT, most of infused cells in the lung are localized within tumor nodules (33). Thus, evaluation of blood-flushed tumor-bearing lungs reflects intratumoral accumulation of the infused cells. Based on dye dilution within the above mentioned tissues, i*n vivo* proliferation of CD45.1 infused cells occurred to the same extent in lymphopenic versus wild type mice (Fig. 3B, C, and data not shown). Similar results were observed in the s.c. D5 tumor model (data not shown). These findings confirm that a

lymphopenic environment augments the anti-tumor efficacy of adoptively transferred tumor-reactive T cells. However, in this tumor model, lymphodepletion failed to facilitate proliferation and accumulation of the infused cells within tumor-bearing hosts.

Radiotherapy enhances the therapeutic efficacy of DC vaccination in a liver tumor model

Utilizing s.c. tumor models, we have previously demonstrated that tumor irradiation augments the therapeutic efficacy of i.t. unpulsed DC administration (20,22). To validate these results in a metastatic melanoma liver tumor model, mice were inoculated intrahepatically with D5 cells on day 0. Tumor irradiation was delivered on days 3–5. DCs were administered i.t. on days 6 and 10. Measurement of liver tumors on day 14 after tumor inoculation showed that both radiation therapy alone and DC vaccination alone significantly inhibited tumor growth compared with untreated mice (Fig. 4A). However, combining radiation with DC vaccination resulted in enhanced tumor regression ($p < 0.01$ versus all other groups). Mice were evaluated for intra-abdominal metastasis on day 14, 19, and after death. In all untreated mice, D5 hepatic tumors metastasized spontaneously into the peritoneal cavity by day 14 (Fig. 4B). Radiation therapy alone and DC vaccination alone slightly, albeit significantly, prolonged metastasis-free survival. Compared with both mono-therapies, radiation plus DC administration significantly inhibited the incidence of metastatic spread. Radiation alone, as well as DC vaccination alone, significantly prolonged overall survival (Fig. 4C). Further increase in mice survival was observed in response to the combined treatment ($p <0.01$ versus all other groups). These results suggest that an effective anti-tumor immune response can be elicited against liver tumors. However, despite the significant enhancement in therapeutic efficacy, all mice treated with radiotherapy plus DC vaccination eventually developed disseminated metastatic disease and succumbed to melanoma.

Radiotherapy combined with DC vaccination enhances the therapeutic efficacy of adoptive T-cell transfer

We hypothesized that tumor irradiation combined with DC administration might stimulate adoptively transferred T cells *in vivo* within a lymphodepleted host leading to improved anti-tumor efficacy of the infused cells. To test this hypothesis, D5 hepatic tumors were induced in mice and then treated with radiation plus DCs as described above. In addition, mice received chemotherapy on days 3 and 4. AT was given on day 7, followed by IL-2. Exploratory laparotomy, performed on day 14 after tumor inoculation, demonstrated that compared with control mice, chemotherapy plus AT plus IL-2 (Chemo+AT+IL-2) significantly inhibited tumor growth (Fig. 5A). However, in mice treated with radiation plus DC plus chemotherapy plus AT plus IL-2 (RT+DC+Chemo+AT+IL-2) tumors underwent further or complete regression. This reduction in mean tumor size reached statistical significance versus all other groups except RT+DC. Therefore, liver tumors in these two groups were further evaluated on day 19 via ultrasound imaging. Mean tumor volume in mice treated with RT+DC+Chemo+AT +IL-2 was significantly reduced (Fig. 5B).

The presence of intra-abdominal metastasis was assessed during laparotomy on day 14, and after death. All untreated mice and mice treated with Chemo+IL-2 had disseminated metastatic disease on day 14 after tumor inoculation (Fig. 5C). RT+DC and Chemo+AT+IL-2 significantly prolonged metastasis-free survival. However, treatment of mice with RT+DC +Chemo+AT+IL-2 further increased metastasis-free survival ($p<0.002$ versus all other groups). RT+DC and Chemo+AT+IL-2 increased overall survival (Fig. 5D). Compared with these two treatment groups, RT+DC+Chemo+AT+IL-2 further prolonged survival and resulted in 46% of mice reaching long-term survival (>90 day). These findings demonstrate that in a lymphodepleted tumor-bearing host, radiotherapy combined with DC vaccination augments the therapeutic efficacy of adoptive T-cell transfer.

Radiotherapy combined with DC vaccination promotes proliferation, accumulation, and cytokine secretion of CD4+ cells

To elucidate the mechanisms by which radiation plus DC therapy enhances the anti-tumor activity of adoptively transferred T cells, we infused CFSE-labeled CD45.1 effector cells to CD45.2 liver tumor-bearing mice. Analysis of peripheral blood samples, using flow cytometry, disclosed a significantly higher number of $CD45.1^+$ cells per ml of blood in mice treated with RT+DC+Chemo+AT+IL-2 compared with mice treated with Chemo+AT+IL-2 (Fig. 6A). This increase in the total number of donor cells was evident on day 5 and 10, but not 2, after AT. T cells used for AT consisted of ~70% $CD8^+$ cells and ~30% $CD4^+$ cells (data not shown). Further examination revealed that the number of $CD45.1^+CD8^+$ cells was not significantly elevated (Fig. 6B). In contrast, the number of $CD45.1^+CD4^+$ cells was markedly increased. At all time points tested, $CD4^+$ donor cells detected in blood samples proliferated to a greater extent in mice treated with RT+DC+Chemo+AT+IL-2 versus mice treated with Chemo+AT +IL-2 (Fig. 6C). On day 2 after AT, $CD8^+$ donor cells exhibited a similar trend. At later time points, dye dilution in both groups exceeded the detection limit precluding data interpretation.

To evaluate whether this enhanced proliferation and accumulation of $CD4^+$ infused cells correlates with augmented *in vivo* anti-tumor function, we examined the incidence of tumor-reactive IFNγ-producing $CD4^+$ cells in blood samples of individual mice. This study, performed 5 days after AT, measured the percentage of donor and recipient $CD4^+$ cells that produced IFNγ in response to *in vitro* D5 tumor stimulation. Blood samples of mice treated with Chemo+IL-2 contained a higher percentage of $IFN\gamma^+CD4^+$ cells versus untreated mice (Fig. 6D). However, this finding is probably due to the induction of lymphodepletion and therefore is unlikely to reflect an absolute increase in the number of IFNγ-producing cells. The increased incidence of $IFN\gamma^+CD4^+$ cells detected in blood samples of mice treated with RT +DC attests to the capacity of this combined modality to prime naïve T cells. Chemo+AT+IL-2 increased the incidence of $IFN\gamma^+CD4^+$ cells, but the difference did not reach statistical significance versus Chemo+IL-2. In contrast, RT+DC+Chemo+AT+IL-2 induced the highest level of circulating tumor-reactive IFNγ-producing $CD4^+$ cells ($p<0.02$ versus all other groups). Using the same approach, we also examined the incidence of tumor-reactive $IFN\gamma^+CD8^+$ cells in peripheral blood samples of individual mice. As shown in the lower panel of Fig. 6D, treatment of mice with RT+DC induced a significant increase in the percentage of circulating $IFN\gamma^+CD8^+$ cells compared with untreated mice. The mean incidence of $IFN\gamma^+CD8^+$ cells in blood samples of mice treated with RT+DC+Chemo+AT+IL-2 was higher than that detected in mice treated with Chemo+AT+IL-2, but the difference between these two groups was not statistically significant. These results show that the enhancement in therapeutic efficacy of adoptive T-cell transfer mediated by radiation combined with DC vaccination is associated with a preferential increase in proliferation and accumulation of the $CD4^+$ infused cells. Furthermore, this approach appears to augment the anti-tumor function of both endogenous and transferred $CD4^+$ cells.

DISCUSSION

To the best of our knowledge, this is the first report to demonstrate that radiotherapy combined with DC vaccination enhances the therapeutic efficacy of adoptive T-cell immunotherapy of cancer. The enhancement in therapeutic efficacy was manifested by increased regression of tumors at the primary site as well as decreased incidence of metastatic spread leading to a pronounced benefit in survival. These findings indicate that this novel approach offers better control of both local and systemic disease.

The experimental design of this animal study was meant to enhance the clinical relevancy and applicability of the presented data. The identity of target antigens on tumor cells remains undefined when i*n vivo* priming of DCs and T cells with whole tumor-derived antigens is

implemented. This strategy, however, particularly in the clinical setting, provides the potential advantage of eliciting immunity against a broad and unique repertoire of tumor associated antigens that is characteristic of each individual host. A phase I clinical trial conducted in patients with advanced hepatoma demonstrated that administration of radiotherapy plus i.t. DCs is feasible, safe, and capable of eliciting tumor-specific immune responses as well as clinical responses (37). Therefore, addition of this combined treatment to adoptive T-cell transfer is unlikely to increase toxicity. The major morbidity associated with this approach is related to induction of lymphopenia (12). Further studies evaluating the capacity of radiation plus DCs to enhance the anti-tumor efficacy of AT in lymphodepleted versus wild type tumor-bearing hosts may address this concern.

Lou *et al.* (19) reported that immunization of s.c. B16-bearing mice with DCs pulsed with human gp100-derived peptide improves the anti-tumor efficacy of adoptively transferred pmel-1 cells. Park *et al.* (38) expanded this concept to a carcinoembryonic-expressing colon adenocarcinoma model. Recently, dendritic-tumor hybrid vaccination has been shown to augment the therapeutic efficacy of tumor-reactive T-cell AT (39,40). Koike *et al.* (41) demonstrated that induction of lymphodepletion in tumor-bearing hosts does not enhance the anti-tumor efficacy of ovalbumin peptide-pulsed DC vaccination, but in this setting naïve T-cell AT induces tumor regression.

In this study, adoptively transferred T cells consisted of $CD8^+$ and $CD4^+$ cells, both of which have previously been shown to independently mediate tumor regression upon AT (31,32). At the same time, DCs were loaded *in vivo* with the wide spectrum of whole tumor-derived antigens. This allowed us to directly compare the *in vivo* stimulatory effects of radiation plus DC vaccination on $CD8^+$ versus $CD4^+$ infused cells. Surprisingly, radiation plus DC vaccination preferentially increased expansion of $CD4^+$, rather than $CD8^+$, transferred cells. This pronounced proliferation and accumulation of $CD4^+$ cells correlated with enhanced tumor-reactive IFNγ secretion. Although $CD8^+$ CTLs are the cell subset known to mediate direct tumor cell kill (42), $CD4^+$ T cells control the effector function, memory, and maintenance of these $CD8^+$ T cells (15). Therefore, future studies will be aimed at elucidating the extent and mechanisms by which $CD4^+$ tumor-reactive T cells contribute to the enhanced therapeutic efficacy of combined therapy with radiation, DC vaccines, and adoptive T-cell transfer.

Radiotherapy is already applied in the treatment of a wide array of human cancers due to its direct cytotoxic effect. However, accumulating data indicates that local tumor irradiation can also enhance the anti-tumor efficacy of DC-based immunotherapy by various pathways. Besides serving as an excellent source of tumor antigens (43), irradiated dying tumor cells have been shown to release the high-mobility-group box 1 (HMGB1) alarmin (44). Extracellular HMGB1 has been reported to act as a chemoattractant and activator of DCs (45). Binding of HMGB1 to Toll-like receptor 4 expressed on DCs was found to induce efficient processing and cross-presentation of tumor-derived antigens (44). We have reported that tumor irradiation augments the capacity of exogenously i.t. administered DCs to capture tumor antigens, home to the draining lymph node, and present processed antigens to T cells (21). Down-regulation of CCL21 gene expression within irradiated tumors may account for the facilitated migration of i.t. administered DC to the draining lymph node (21). Tumor irradiation may promote recruitment of effector T cells by inducing release of CXCL16 from cancer cells (46), and by facilitating normalization of the vasculature (47). Ionizing radiation may favorably alter the immunosuppressive microenvironment within solid tumors (48) leading to increased accumulation and improved function of DC-elicited and DC-stimulated tumor-reactive T cells. Exposure of tumor cells to radiation may render the cells more susceptible to T-cell killing by up-regulating cell surface expression of Fas (49). Tumor irradiation has also been shown to sensitize cancer stroma cells for efficient destruction by CTLs (50). These recent intriguing findings shed new light on the immunomodulatory capacity of ionizing radiation, and suggest

that gaining better insight into the effects of radiotherapy on the tumor environment may allow future harnessing of the full potential of this modality to the combat against human cancer.

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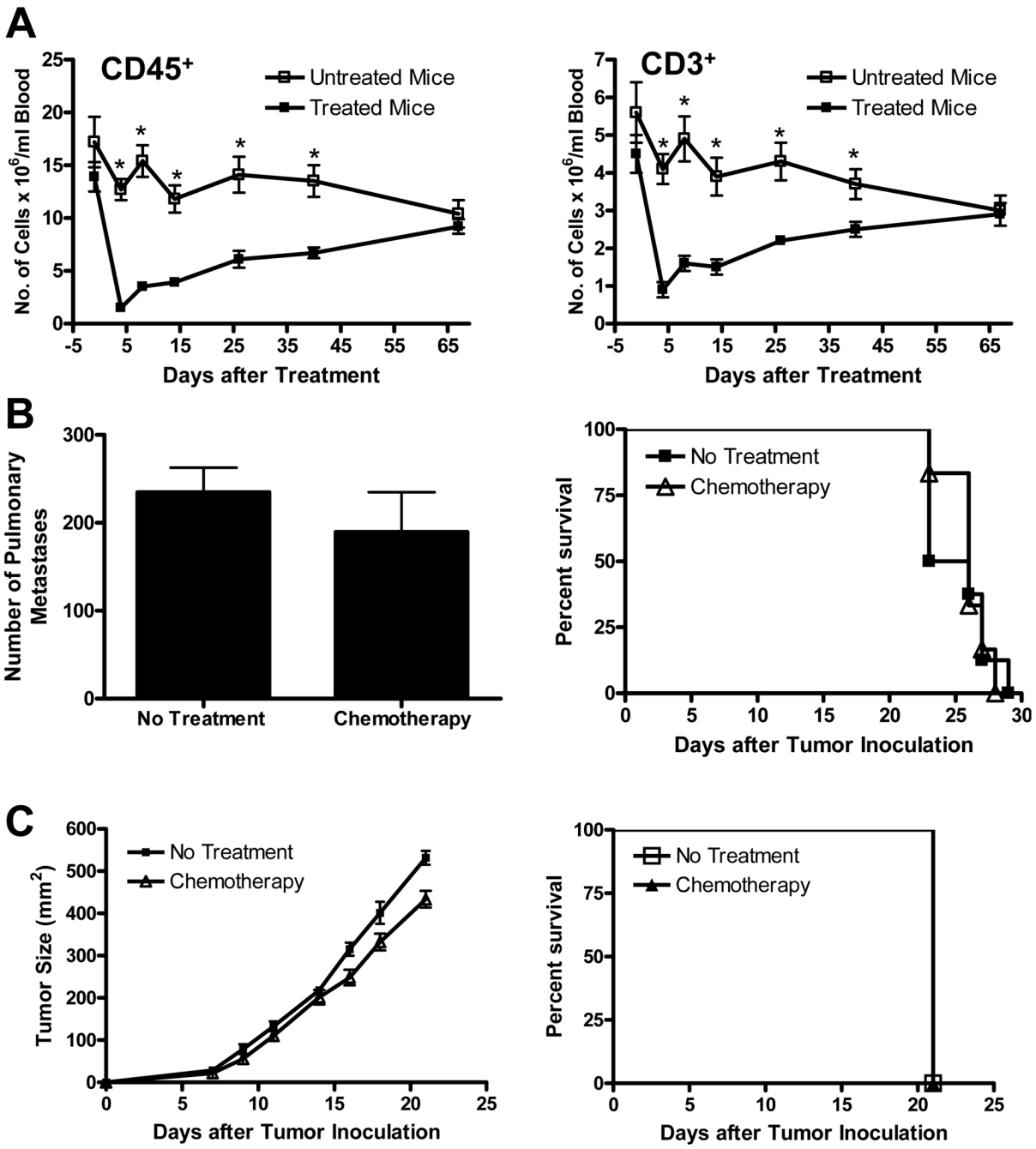

FIGURE 1.

Treatment of mice with cyclophosphamide and fludarabine induces a profound, yet transient, reduction in the number of $CD45^+$ and $CD3^+$ cells in peripheral blood samples without inhibiting D5 tumor growth or prolonging mice survival. A, Naïve mice received chemotherapy on days 0 and 1. Blood samples, drawn at the designated time points, were analyzed for $CD45^+$ and $CD3^+$ cells. Data represents mean ± SE (n=5). *P<0.02. B and C, Mice were inoculated i.v. (B) or s.c. (C) with D5 cells on day 0. Chemotherapy was administered on days 3 and 4. Anti-tumor responses were evaluated by enumerating pulmonary metastases on day 15 (B left), measuring s.c. tumor size (C left), and monitoring mice survival (B and C right). (n=6). All experiments were repeated two times with similar results.

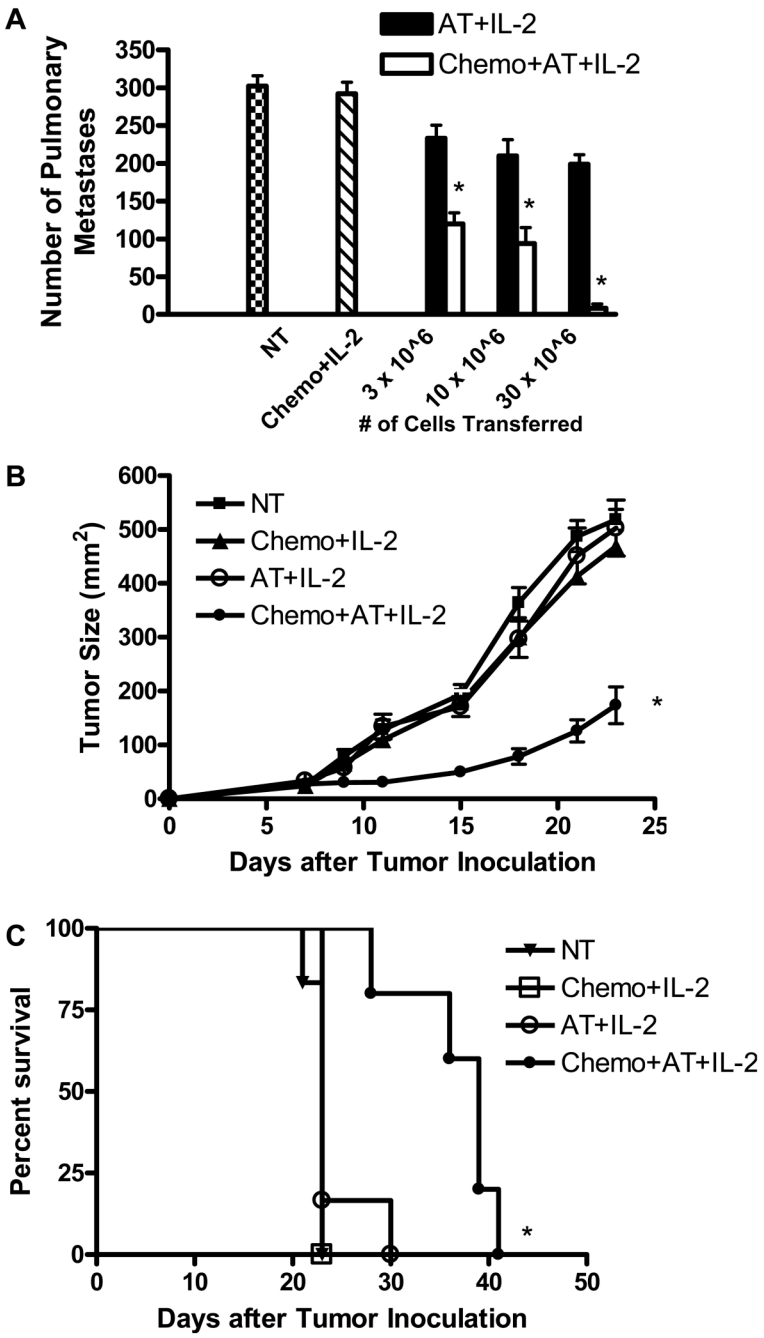

FIGURE 2.

Induction of lymphopenia prior to adoptive transfer (AT) enhances the therapeutic efficacy of the infused cells. D5 pulmonary metastases (A) or s.c. tumors (B, C) were established in mice on day 0. Chemotherapy (chemo) was administered on days 3 and 4. AT (3×10^7 cells, unless otherwise specified) was given on day 7, followed by IL-2. Anti-tumor responses were evaluated by enumerating lung metastases on day 16 (A), measuring tumor size (B), and monitoring mice survival (C). Data represents mean ± SE (n=5). A,*P<0.001 comparing equal number of cells transferred with or without chemotherapy. B and C,*P<0.005 versus all other groups. All experiments were repeated two times with similar results. NT, no treatment.

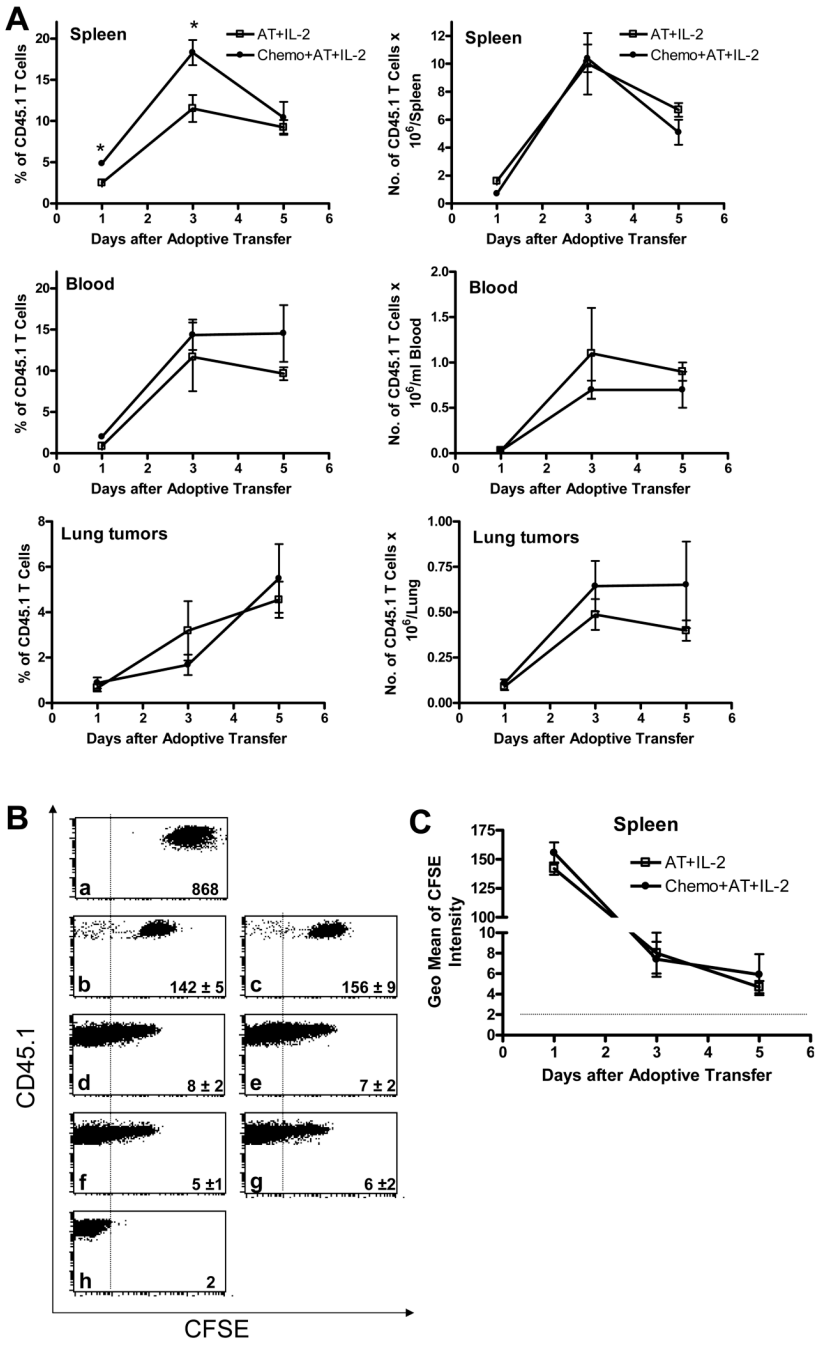

FIGURE 3.

Induction of lymphopenia prior to AT does not enhance accumulation (A) or proliferation (B and C) of infused cells. Pulmonary metastases were induced in CD45.2 mice and treated as described in Fig. 2A. CFSE-labeled CD45.1 cells (3×10^7) were used for AT. Spleens, blood samples, and lungs were collected 1, 3, and 5 days after AT. A, Tissue-derived single cell suspensions were analyzed for $CD45.1^+$ cells. Data are presented as mean percentage (left panels) or mean absolute number (right panels) of CD45.1 T cells ± SE (n=5). *P<0.02. B, Dot plots of CD45.1 T cells with (a) or without (h) CFSE labeling before adoptive transfer. Representative dot plots (gated on $CD45.1^+$ cells) of spleen cells obtained 1 (b and c), 3 (d and e), and 5 (f and g) days after AT from mice treated with AT+IL-2 (b, d, and f) or Chemo+AT

+IL-2 (c, e, and g). Numbers in dot plots designate mean ± SE of the geometrical mean of CFSE intensity (n=5). Dotted lines indicate lower limit of detection. C, The geometrical mean of CFSE intensity of $CD45.1^+$ cells in spleen samples was recorded. Data represent mean ± SE (n=5). Dotted line indicates lower limit of detection based on unstained cells.

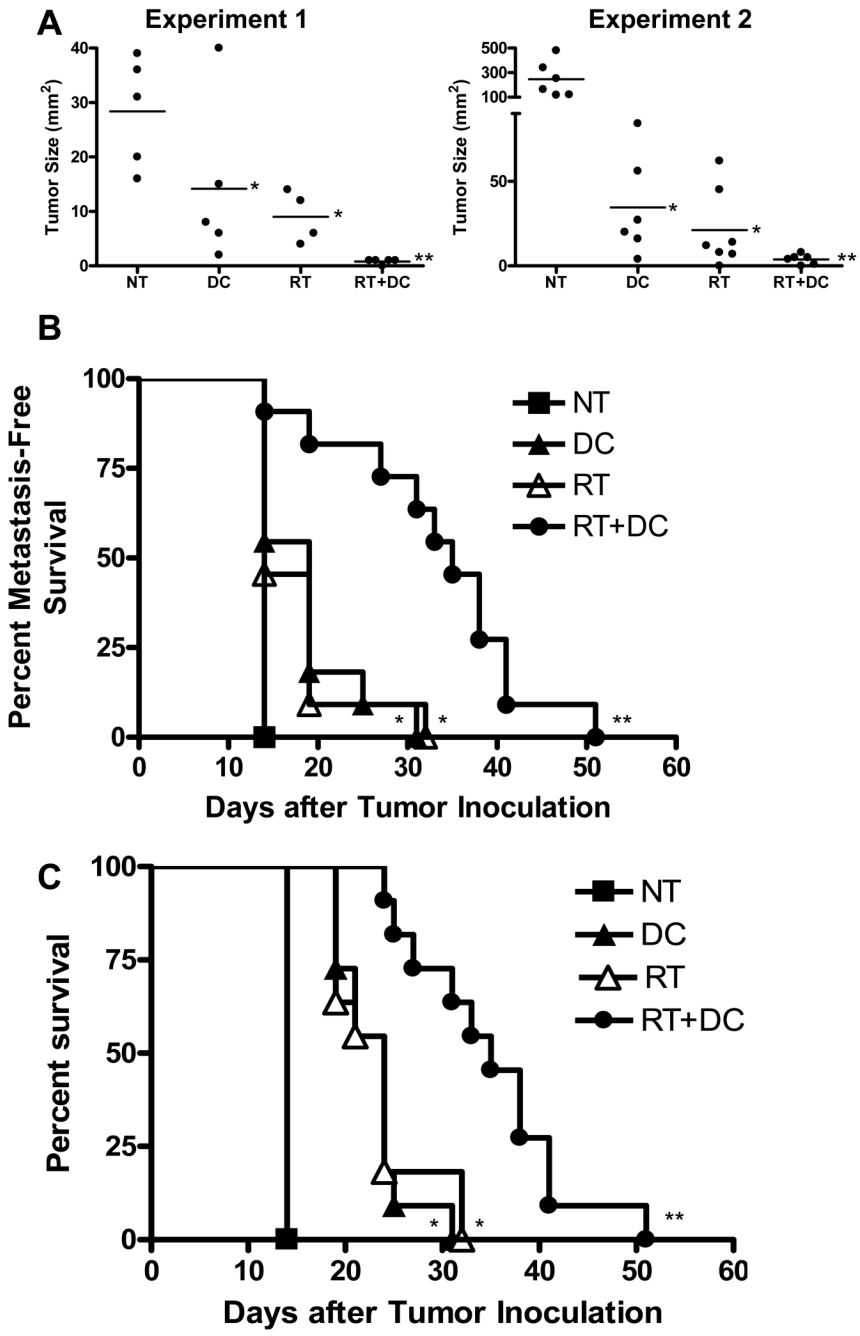

FIGURE 4.

Radiotherapy enhances the therapeutic efficacy of dendritic cell (DC) vaccination in a liver tumor model. D5 hepatic tumors were induced in mice and then treated with radiation (RT) plus i.t. DC injections. Anti-tumor responses were evaluated by measuring tumor size on day 14 (A), assessing for intra-abdominal metastatic spread on day 14, 19, and after death (B), and monitoring mice survival (C). Each group consisted of a total of 11 mice, treated in 2 independent experiments. A, Each data point represents an individual mouse; bars depict mean. B and C, Survival curves present cumulative data of 2 experiments. *P<0.02 versus no treatment (NT), **P<0.01 versus all other groups. Statistical analysis compared groups across the 2 experiments.

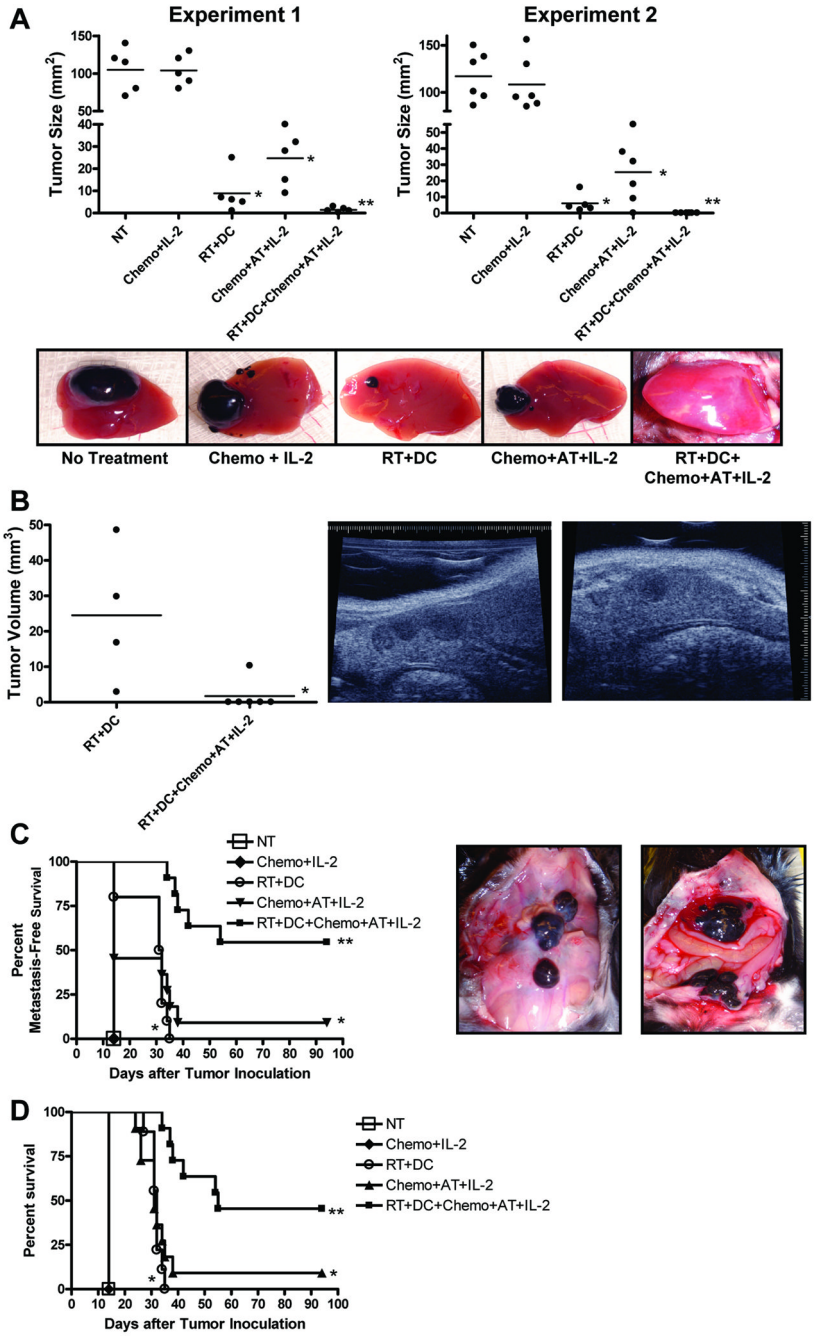

FIGURE 5.

Radiotherapy (RT) plus dendritic cell (DC) vaccination enhances the therapeutic efficacy of adoptive transfer (AT). D5 hepatic tumors were induced in mice and then treated with RT+DC +Chemo+AT+IL-2. Anti-tumor responses were evaluated by measuring tumor size on day 14 via laparotomy (A), measuring tumor volume on day 19 via ultrasound imaging (B), assessing intra-abdominal metastatic spread on day 14 and after death (C), and monitoring mice survival (D). Each group consisted of a total of 11 mice, treated in 2 independent experiments. A, Each data point represents an individual mouse; bars depict mean. *P<0.0001 versus no treatment (NT) and chemotherapy plus interleukin-2 (Chemo+IL-2), **P<0.05 versus all other groups except RT+DC. Pictures of a representative D5 liver tumor from each group taken on day 14

are shown. B,*P<0.03. A sagittal (middle) and transverse (right) view of a representative ultrasound image of a D5 liver tumor from RT+DC are shown. C and D, Survival curves present cumulative data of 2 experiments. C,*P<0.02 versus NT and Chemo+IL-2, **P<0.002 versus all other groups. Representative pictures of disseminated intra-abdominal metastatic disease taken on day 14 from an untreated mouse (middle) and a mouse treated with Chemo+IL-2 (right) are shown. D,*P<0.0001 versus NT and Chemo+IL-2, **P<0.002 versus all other groups.

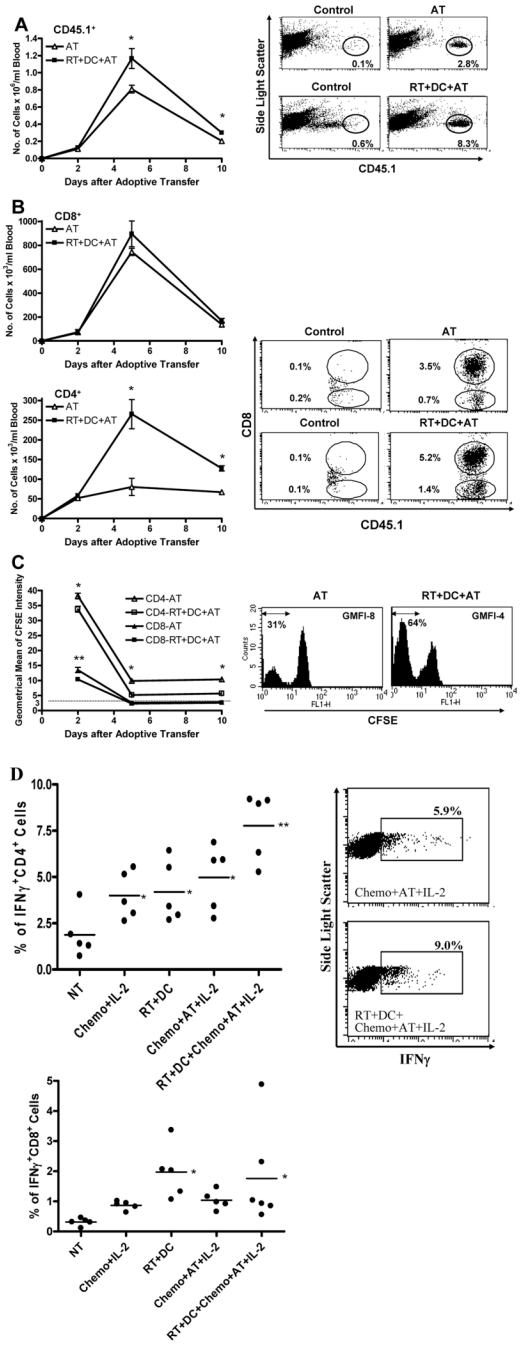

FIGURE 6.

Radiotherapy (RT) plus dendritic cell (DC) vaccination promotes proliferation, accumulation, and IFNγ secretion of $CD4^+$ cells. Liver tumors were induced in CD45.2 mice and then treated as described in Fig. 5. CFSE-labeled CD45.1 cells were used for adoptive transfer (AT). A, B, and C, All groups received chemotherapy plus IL-2 in addition to designated treatment. Erythrocyte-depleted peripheral blood cells, collected on days 2, 5, and 10 after AT, were analyzed for $CD45.1^+$ (A), $CD45.1^+CD8^+$ (B), and $CD45.1^+CD8^-$ (B) cells. The geometrical mean of CFSE intensity (GMFI) of $CD45.1^+CD8^+$ and $CD45.1^+CD8^-$ cells was recorded (C). Dotted line indicates lower limit of detection based on mean value of unstained cells. Data on left represents mean ± SE (n=6). A, *P<0.02. B, *P<0.002. C, *P<0.01 versus CD4-RT+DC

+AT, **P<0.02 versus CD8-RT+DC+AT. D, Blood samples were analyzed for IFNγ production of $CD4^+$ and $CD8^+$ cells in response to *in vitro* D5 tumor stimulation. Data points represent individual mice; bars depict mean. *P<0.05 versus no treatment (NT), ** P<0.02 versus all other groups. Representative dot plots or histograms acquired on day 5 after AT are shown in the right panels (A, no gate; B, gated on $CD45.1^+$ cells; C, gated on $CD45.1^+CD8^-$ cells; D, gated on $CD4^+$ cells).