In Situ Vaccination with CD204 Gene-Silenced Dendritic Cell, not Unmodified Dendritic Cell, Enhances Radiation Therapy of Prostate Cancer

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Abstract
Given the complexity of prostate cancer progression and metastasis, multimodalities that target different aspects of tumor biology, for example, radiotherapy in conjunction with immunotherapy, may provide the best opportunities for promoting clinical benefits in patients with high-risk localized prostate cancer. Here, we show that intratumoral administration of unmodified dendritic cells (DC) failed to synergize with fractionated radiotherapy. However, ionizing radiation combined with in situ vaccination with DCs, in which the immunosuppressive scavenger receptor A (SRA/CD204) has been downregulated by lentivirus-mediated gene silencing, profoundly suppressed the growth of two mouse prostate cancers (e.g., RM1 and TRAMP-C2) and prolonged the lifespan of tumor-bearing animals. Treatment of subcutaneous tumors with this novel combinatorial radioimmuno-therapeutic regimen resulted in a significant reduction in distant experimental metastases. SRA/CD204-silenced DCs were highly efficient in generating antigen or tumor-specific T cells with increased effector functions (e.g., cytokine production and tumoricidal activity). SRA/CD204 silencing-enhanced tumor cell death was associated with elevated IFN-γ levels in tumor tissue and increased tumor-infiltrating CD8+ cells. IFN-γ neutralization or depletion of CD8+ cells abrogated the SRA/CD204 downregulation-promoted antitumor efficacy, indicating a critical role of IFN-γ-producing CD8+ T cells. Therefore, blocking SRA/CD204 activity significantly enhances the therapeutic potency of local radiotherapy combined with in situ DC vaccination by promoting a robust systemic antitumor immunity. Further studies are warranted to test this novel combinatorial approach for translating into improved clinical outcomes in patients with prostate cancer. Mol Cancer Ther; 11(11); 2331–41. ©2012 AACR.

Introduction
Prostate cancer is the most prevalent solid tumor and the second leading cause of cancer death among men in the United States (1). Although the majority of patients are currently diagnosed with clinically localized disease, many patients develop biochemical failure after local definitive therapy, for example, radiotherapy, perhaps due to occult metastatic disease. Androgen deprivation therapy (ADT) in addition to radiotherapy improves overall survival of patients at high risk for recurrence (2). This approach, however, is associated with significant side effects. Given that patients with prostate cancer often present with low tumor burden, one potential treatment is the incorporation of immunotherapy into a standard radiotherapy protocol (3). With recent approval of an antigen-presenting cell vaccine sipuleucel-T (Provenge) by the U.S. Food and Drug Administration, immunotherapy now represents an established treatment modality for metastatic prostate cancer (4, 5).

Several lines of evidence support an approach of combined radiotherapy and immunotherapy to achieve local tumor control while at the same time generating systemic immune responses (6). Local irradiation not only debulks tumor but also generates an inflammatory microenvironment. Tumor antigens released by dying tumor cells are captured and processed by specialized antigen-presenting cells [e.g., dendritic cells (DC)] in the context of the costimulatory or “danger” signals, resulting in effective T-cell activation (7). Radiotherapy also renders tumor cells more susceptible to recognition and attack by tumor-specific CTLs (8–10). Gulley and colleagues recently reported clinical trials in which radiotherapy combined with a prostate-specific antigen-targeted vaccine
displayed a favorable toxicity profile and generated significant T-cell responses in patients with prostate cancer (11, 12). Indeed, several phase II/III clinical trials of radiotherapy in combination with therapeutic cancer vaccines, for example, ProstAtakTM (NCT01436968), PSA/TRICOM (NCT00450619), L-BLP25 (Stimuvax, NCT01496131), for treatment of high-risk prostate cancer are ongoing.

Induction of immunity against self-antigens is often restricted by multiple intrinsic inhibitory checkpoints or molecules (13, 14). Knowledge of these inhibitory regulators has led to great progress in development of immunotherapeutic strategies for cancer treatment (15). We have identified an immunosuppressive pathway in DCs involving the scavenger receptor SRA/CD204, which can dampen immune responses against several cancers, including prostate cancer (16, 17). SRA/CD204 attenuates the immunostimulatory capability of DCs and subsequent activation of CTLs in the context of vaccine therapy and tumor immunity (18, 19). Recently, we showed that silencing SRA/CD204 in primary DCs markedly enhanced the potency of DC vaccines in mounting an effective antitumor T-cell response (20).

Considering the fact that SRA/CD204 absence significantly increases the immunogenicity of ionizing radiation–treated prostate cancer cells (17), we hypothesize that local radiotherapy-induced cancer cell damage in combination with in situ vaccination with SRA/CD204-downregulated DCs could achieve improved systemic antitumor efficacy. In the present study, we provide the first experimental evidence that intratumoral administration of SRA/CD204-silenced DCs profoundly enhances the control of radiotherapy-treated local prostate cancer as well as its metastases, which is mainly mediated by IFN-γ–producing CD8+ CTLs. Our data support the concept of inhibiting SRA/CD204 as a novel strategy to optimize DC-targeted immunotherapy that may be rationally combined with radiotherapy for treatment of human prostate cancer.

Materials and Methods

Mice and cell lines

Wild-type (WT) C57BL/6 mice were obtained from National Cancer Institute (Bethesda, MD). SRA/CD204 knockout mice (SRA−/−), OT-I mice bearing TCR specific for OVA257–264 (SIINFEKL) were purchased from The Jackson Laboratory. All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University (Richmond, VA). Androgen-refractory mouse prostate cancer cell lines, RM1 (21) kindly provided by Dr. TC Thompson (Baylor College of Medicine, Houston, TX) and TRAMP-C2 (22), were used. RM1 cells expressing OVA (17) or luciferase were generated in our laboratory. All cell lines were maintained in Dulbecco’s Modified Eagles’ Media supplemented with 10% FBS (HyClone), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. These cells have been screened for mycoplasma and other pathogens before in vivo use. No authentication of the cell lines was conducted by the authors.

Short hairpin RNA–mediated gene silencing in DCs

Lentiviral vector encoding mouse SRA/CD204 short hairpin RNA (shRNA) were packaged using Phoenix cells co-transfected with pLKO.1 constructs and pMD.G and pCMVΔR8.91 (20). DCs were generated by culturing mouse bone marrow (BM) cells in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF). Lentiviral infection of primary DCs was carried out as previously described (20, 23). Briefly, bone marrow cells were cultured in media containing recombinant mouse GM-CSF (20 ng/mL, Peprotech). Day 3 BM-DCs were infected with lentiviruses in the presence of 8 μg/mL polybrene for 3 hours. Cells were then resuspended and plated in 12-well plates at 1 × 106 cells/mL. Day 8 DCs were collected and used for studies.

Tumor treatment and immunization protocols

Mice were injected with 1 × 106 RM1 tumor cells or 2 × 106 TRAMP-C2 tumor cells subcutaneously in the right hind leg. When tumors reached 4 to 5 mm in diameter, mice were anesthetized using ketamine (80 mg/kg)/xylazine (10 mg/kg) and locally irradiated with a Picker 60Co source at 1 Gy/min. Mice received a single dose of 10 Gy on each of 3 consecutive days. For in situ DC immunization, mice were injected with DCs (2 × 106) into the tumors 24 and 72 hours after the last radiation treatment. In some experiments, CD4+, CD8+ T cells, or natural killer (NK) cells were depleted by intraperitoneal (i.p.) injection of GK1.5, 2.43 or PK136 monoclonal antibodies (mAb), respectively, as previously described (16).

Experimental lung metastases

C57BL/6 mice bearing s.c. RM1 tumors were established with lung metastases by intravenously injecting 5 × 104 RM1-luciferase cells in PBS (without Ca2+ or Mg2+). Only tumors in the flank were treated. Mice were injected i.p. with d-luciferin (150 mg/kg, Caliper Life Sciences) and examined using a Xenogen IVIS Imaging System. Lung metastatic nodules were also enumerated. In the case of TRAMP-C2 tumor, 1 × 108 cells were injected i.v. to generate pulmonary metastases.

In vitro T-cell stimulation

DCs were pulsed with tumor cell lysates (1:1 ratio) for 5 hours. After washing, serially diluted DCs were incubated with 5 × 105 OT-I cells in a round-bottom, 96-well microtiter plate. Cells were cultured for 60 hours and pulsed with 3H-thymidine (0.5 μCi/well) during the last 16 hours of culture period. T-cell proliferation was assessed on the basis of 3H-thymidine incorporation. Levels of interleukin (IL)-2 in the supernatants were determined using ELISA.
Enzyme-linked immunosorbent spot assay and intracellular IFN-γ staining

Splenocytes or lymph node cells from treated mice were analyzed for the frequency of antigen-specific IFN-γ-secreting CD8⁺ T cells using enzyme-linked immunosorbent spot (ELISPOT) assays as previously described (19). For intracellular cytokine staining, cells were stimulated with OVA257–264 peptides (AnaSpec Inc.) for 3 days, followed by treatment with brefeldin A (BD Golgiplug; BD Biosciences). Cells were then stained with antibodies for CD8 and IFN-γ and subjected to fluorescence-activated cell-sorting (FACS) analysis (19).

Statistical analysis

Data are presented as mean ± SD. Differences between groups within experiments were analyzed using 2-tailed unpaired Student t test. Animal survival data were analyzed using log-rank test. P < 0.05 was considered statistically significant.

Results

SRA/CD204 dampens the therapeutic effectiveness of conventional radiotherapy

We previously showed that mouse prostate tumor RM1 cells treated with ionizing radiation displayed increased immunogenicity in SRA⁻/⁻ mice (17). Therefore, we sought to determine whether absence of SRA/CD204 in tumor-bearing mice could amplify the antitumor efficacy of local radiotherapy. As previous reported, RM1 tumors grew aggressively at a similar rate in wild-type (WT) and SRA⁻/⁻ mice before treatment (17). However, RM1 tumor growth was profoundly inhibited by radiotherapy in SRA⁻/⁻ mice compared with WT mice (Fig. 1), suggesting that SRA/CD204 ablation sensitizes RM1 tumors to radiotherapy.

In situ vaccination using SRA/CD204-deficient DCs enhances the effectiveness of radiotherapy by promoting CD8⁺ T-cell activation

SRA/CD204 is expressed in antigen-presenting cells, and enhanced antitumor immunity in SRA⁻/⁻ mice has been shown to result from increased functions of SRA⁻/⁻ DCs exposed to tumor cell lysates (17) or stress proteins (19). We tested whether improved tumor control could be achieved by intratumoral delivery of SRA⁻/⁻ DCs following radiotherapy (Fig. 2A). Our initial studies showed that fractionated radiotherapy (i.e., 3 consecutive 10 Gy) delayed tumor growth (Fig. 2B, left). Direct injections of WT or SRA⁻/⁻ DCs without radiotherapy had no effect on tumor growth (data not shown). Surprisingly, administration of WT DCs to the irradiated RM1 tumors failed to provide any additional benefits (Fig. 2B, left). In contrast, in situ vaccination with SRA⁻/⁻ DCs after radiotherapy resulted in increased tumor suppression compared with radiotherapy alone or radiotherapy plus WT DCs (Fig. 2, middle). The enhanced tumor control in mice treated with radiotherapy plus SRA⁻/⁻ DCs was also associated with significantly improved animal survival (Fig. 2B, right).

To determine whether the increased tumor control by combined radiotherapy and SRA⁻/⁻ DC vaccine correlated with enhanced T-cell activation, RM1 tumors expressing a non-“self” model antigen OVA (RM1-OVA) were used to facilitate monitoring of antigen-specific T cells. The frequency of IFN-γ-producing CD8⁺ cells reactive with OVA257–264 epitope increased in mice treated with radiotherapy plus WT DCs. However, SRA⁻/⁻ DCs were much more efficient than WT DCs in generating OVA-specific CD8⁺ T cells when combined with radiotherapy (Fig. 2C).

Tumor immune evasion often involves a complex array of immunosuppressive mechanisms including inhibition of T-cell-stimulating activity of DCs in the tumor environment. We compared the capability of WT and SRA⁻/⁻ DCs to stimulate OVA-specific CD8⁺ T cells in the presence of tumor-mediated immunosuppression. When cocultured with naïve OT-I cells in RM1 tumor-conditioned media, OVA257–264-pulsed SRA⁻/⁻ DCs were consistently more effective than WT DCs in stimulating
OT-I cell proliferation and production of cytokine IL-2 and IFN-γ (Fig. 2D). Interestingly, exposure of WT DCs with tumor-conditioned media (n = 5) failed to enhance tumor inhibition following radiotherapy (left). In contrast, administration of SRA−/− DC vaccine combined with radiotherapy led to improved tumor control (middle) and survival rate (right). ††, P < 0.01, radiotherapy + WT DC versus radiotherapy + SRA−/− DCs. C, enhanced T-cell activation by radiotherapy and SRA−/− DC vaccination. Splenocytes (top) and lymph node cells (bottom) from treated RM1 tumor-bearing mice were stimulated with OVA257-264 peptides. The frequency of IFN-γ-producing CD8+ T cells was measured using ELISPOT (∗, P < 0.01). D, resistance of SRA−/− DCs to tumor-mediated immunosuppression. OVA257-264-loaded DCs were cocultured with OT-I cells in the presence of RM1 tumor-conditioned media (TCM) at indicated concentrations. T-cell proliferation was assessed using 3H-thymidine incorporation assays (top). Index ratios of T-cell proliferation driven by DCs (SRA−/− DCs/WT DCs) are also presented (bottom). In addition, the levels of IL-2 and IFN-γ in the supernatants were determined using ELISA (†, P < 0.01).

Figure 2. SRA/CD204 absence in DCs enhances the antitumor efficacy of radiotherapy (RT) combined with DC vaccination. A, scheme for the combined radiotherapy and in situ DC vaccination. B, RM1 tumor-bearing mice (n = 5) were treated with radiotherapy alone, radiotherapy plus WT DCs, or left untreated. WT DC vaccine failed to enhance tumor inhibition following radiotherapy (left). In contrast, administration of SRA−/− DC vaccine combined with radiotherapy led to improved tumor control (middle) and survival rate (right). ††, P < 0.01, radiotherapy + WT DC versus radiotherapy + SRA−/− DCs. C, enhanced T-cell activation by radiotherapy and SRA−/− DC vaccination. Splenocytes (top) and lymph node cells (bottom) from treated RM1 tumor-bearing mice were stimulated with OVA257-264 peptides. The frequency of IFN-γ-producing CD8+ T cells was measured using ELISPOT (∗, P < 0.01). D, resistance of SRA−/− DCs to tumor-mediated immunosuppression. OVA257-264-loaded DCs were cocultured with OT-I cells in the presence of RM1 tumor-conditioned media (TCM) at indicated concentrations. T-cell proliferation was assessed using 3H-thymidine incorporation assays (top). Index ratios of T-cell proliferation driven by DCs (SRA−/− DCs/WT DCs) are also presented (bottom). In addition, the levels of IL-2 and IFN-γ in the supernatants were determined using ELISA (†, P < 0.01).

SRA/CD204 silencing in DCs enhances cross-presentation of antigen from dying tumor cells

BM-DCs were genetically modified by infection with lentiviral vectors encoding shRNA specific for SRA/CD204 or scrambled shRNA as previously described (20). SRA/CD204-silenced DCs (DC-SRA shRNA) produced more pro-inflammatory cytokine IL-6 (Fig. 3A) and chemokine IP-10 (Fig. 3B) than scrambled shRNA-treated DCs (i.e., DC-scram) upon exposure to dying RM1 tumor cells. The mRNA levels of the inflammatory genes (il6, ip10 and ifnb) similarly increased in DC-SRA shRNA (Supplementary Fig. S1A–S1C). Elevation of IL-6 expression was further confirmed by intracellular cytokine

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staining (Supplementary Fig. S1D). These results are consistent with our previous observations that lack of SRA/CD204 sensitizes DCs to inflammatory signals associated with cell injury (17). In addition, DC-SRA shRNA pulsed with RM1-OVA tumor lysates were more efficient than mock cells in stimulating OVA-specific OT-I cell activation (Fig. 3C and D).

Radiotherapy combined with SRA/CD204-silenced DC vaccine results in enhanced therapeutic efficacy against local tumors and distant metastases

Using a mouse prostate cancer RM1 model, we evaluated the antitumor response augmented by radiotherapy and in situ vaccination with SRA/CD204-silenced DCs. DC-SRA shRNA, but not DC-scram, promoted growth inhibition of RM1 tumors and prolonged the survival of radiotherapy-treated mice (Fig. 4A). The similar results were also obtained in a different mouse model of prostate cancer (Fig. 4B), TRAMP-C2, which was derived from spontaneous prostate cancers arising in TRAMP mice (22).

Although local radiotherapy of the primary tumor can prevent development of subsequent systemic metastases, tumor radiation fails to control pre-existing systemic disease. We used an experimental lung metastasis model to assess the ability of the combination therapy to control distant metastases. RM1-luciferase tumor cells were injected i.v. into WT mice 1 day before the implantation of s.c. RM1 tumors. Treatment of s.c. tumors with radiotherapy alone or radiotherapy plus DC-scram vaccine partially inhibited lung metastases. However, radiotherapy plus DC-SRA shRNA was far more effective than other treatment modalities in controlling metastases (Fig. 4C and D, left). The result was also validated using the metastasis model of TRAMP-C2 tumor (Fig. 4D, right). Mice receiving radiotherapy plus DC-SRA shRNA did not display any signs of pathology or autoimmune toxicity in the prostate (Supplementary Fig. S2A) and other organs (data not shown).

Radiotherapy combined with DC-SRA shRNA vaccination enhances activation of antigen-specific CTLs

The enhanced antitumor effect observed after the combinatorial treatment using DC-SRA shRNA prompted us to examine systemic immune responses. Upon stimulation with OVA257–264 peptide, splenocytes from RM1-OVA tumor-bearing mice treated with radiotherapy plus DC-SRA shRNA exhibited increased proliferation (Fig. 5A, top) and IL-2 production (Fig. 5A, bottom) compared with those from mice treated with radiotherapy plus DC-scram. Mice receiving radiotherapy plus DC-SRA shRNA also showed increased frequency of OVA-specific, IFN-γ-expressing CD8+ T cells, as shown by intracellular IFN-γ staining assays (Fig. 5B) and ELISPOT
assays (data not shown). Considering that OVA is a non-"self" antigen and there is no pre-existing tolerance in mice, we assessed T-cell recognition of 6 transmembrane epithelial antigen of the prostate (STEAP), an antigen highly expressed in advanced human prostate cancers and mouse prostate tumors (24, 25). Integration of DC-SRA shRNA vaccine into the radiotherapy protocol effectively primed CD8\(^+\) T cells recognizing mSTEAP326–335 (Fig. 5C, left) or RM1 tumor cells (Fig. 5C, right). T-cell functions assays showed that radiotherapy-treated RM1-OVA tumor–bearing mice receiving DC-SRA shRNA developed a stronger cytolytic response against OVA257–264-pulsed targets \textit{in vivo} (Fig. 5D) and RM1-OVA tumor cells \textit{in vitro} (Supplementary Fig. S2B) compared with other treatment modalities.

**SRA/CD204 silencing enhanced antitumor efficacy is dependent on IFN-\(\gamma\)-producing CD8\(^+\) T cells**

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays showed that radiotherapy plus DC-SRA shRNA induced more RM1 tumor cell death than radiotherapy alone or radiotherapy plus DC-scram. In parallel, radiotherapy plus DC-SRA shRNA also resulted in a significant increase in tumor-infiltrating CD8\(^+\) cells (Fig. 6A). Levels of CD4\(^+\) cells in the tumor tissues were comparable among treatment groups (Supplementary Fig. S3). Interestingly, increased tumor infiltration by CD11c\(^+\) cells, presumably DCs, was also seen in mice receiving SRA/CD204-silenced DCs (Supplementary Fig. S3). These tumor-resident CD11c\(^+\) cells were unlikely to be \textit{ex vivo} genetically engineered DCs because the majority of DCs injected post-radiotherapy migrated out of the tumor site within 24 hours (data not shown).

ELISA of tumor tissues showed that the levels of IFN-\(\gamma\), a cytokine critical for effective antitumor immunity, were significantly higher in mice receiving radiotherapy plus DC-SRA shRNA vaccination than other treatment groups (Fig. 6B, left). Removal of CD8\(^+\) cells by antibody depletion substantially decreased the intratumoral levels of IFN-\(\gamma\), whereas depletion of CD4\(^+\) or NK1.1 cells only showed a slight effect (Fig. 6B, middle). Intracellular staining showed that tumor-infiltrating CD8\(^+\) cells from mice treated with radiotherapy and DC-SRA shRNA displayed markedly increased expression of IFN-\(\gamma\) (Fig. 6B, right) as well as granzyme B (Supplementary Fig. S4). Following radiation treatment, mice receiving DC-SRA shRNA showed a dramatic increase in the percentage of IFN-\(\gamma\)-CD8\(^+\) cells compared with IFN-\(\gamma\)-CD4\(^+\) cells in the spleen (Supplementary Fig. S5). Furthermore, depletion...
of CD8^+, not CD4^+ or NK1.1 cells, abolished SRA/CD204 silencing-enhanced antitumor efficacy (Fig. 6C). Finally, blocking IFN-γ using neutralizing antibodies also abrogated the therapeutic efficacy of the combinatorial therapy (Fig. 6D), suggesting that IFN-γ–producing CD8^+ cells represent a key factor mediating SRA/CD204 silencing-promoted antitumor immunity.

Discussion

Given that radiotherapy is the primary treatment option for high-risk localized prostate cancer and the biochemical relapse rate of many of these patients is high, combining radiotherapy with immunotherapy represents an attractive strategy to improve clinical outcomes. In the present study, we provide the first experimental evidence that silencing SRA/CD204 in DCs markedly enhances antitumor effectiveness of radiotherapy combined with in situ DC vaccination. The amplified systemic antitumor immunity by SRA/CD204 downregulation in DCs not only confers an additional inhibitory effect on radiotherapy-treated tumors but also limits experimental metastatic outgrowth. Our studies establish the feasibility and efficacy of this novel combinatorial radioimmunotherapy in the 2 preclinical models of prostate cancer.

DC vaccine represents a promising immunotherapeutic approach for cancer eradication (26). While DCs are often loaded ex vivo with tumor antigens or tumor lysates to induce antitumor immune responses in conventional DC vaccination, in situ DC vaccination in a setting of local radiotherapy has also been reported to provide additional antitumor benefits (27). The failure of WT DCs or DC-scram to synergize with local radiotherapy for controlling established RM1 tumor in our initial study was unexpected, which may be attributed to its poorly immunogenic nature or the immunosuppression mediated by this tumor line. However, absence or silencing of SRA/CD204 in DCs was able to effectively restore T-cell–mediated antitumor immune responses when these modified DCs were introduced to the tumors treated with ionizing radiation. This finding was also confirmed using a second mouse model of prostate cancer (i.e., TRAMP-C2), which lends further
support to the previously proposed role of SRA/CD204 as an immunosuppressor capable of attenuating DC functions (16–20, 28).

Local radiotherapy is accompanied by the release of tumor antigen and endogenous "danger" signals (29), such as stress proteins (30), oxidized low-density lipoproteins (31), and mitochondrial antigens (32). These signals facilitate the recruitment of immune cells, including tumor-infiltrating lymphocytes (TILs), into the tumor microenvironment. Recent studies have shown that the combination of radiotherapy and immune checkpoint inhibitors can further enhance antitumor immune responses by modulating T-cell function and activating CD8+ T cells (33). Indeed, our findings demonstrate that SRA/CD204 silencing in DCs enhances tumor control in vivo, and this effect is dependent on IFN-γ production by CD8+ T cells (Figure 6A).

To investigate the mechanism by which SRA/CD204 silencing enhances tumor control, we analyzed the response of tumor-infiltrating lymphocytes (TILs) before and after treatment. The number of CD8+ T cells increased significantly in tumors treated with radiotherapy (RT) plus DC-SRA shRNA compared to control groups (Figure 6B). Furthermore, depletion of CD8+ T cells before the combinatorial therapy reduced IFN-γ levels in tumor tissues (Figure 6B). This suggests that CD8+ T cells contribute to the enhanced tumor control observed with SRA/CD204 silencing (Figure 6C).

We also investigated the role of IFN-γ in the antitumor activity facilitated by SRA/CD204 silencing. RM1 tumor-bearing mice were depleted of effector cell subsets before the combinatorial therapy, and the role of IFN-γ was assessed by administering IFN-γ neutralizing antibodies or control IgG. Consistent with our previous findings, depletion of effector cell subsets before radiotherapy (RT) plus DC-SRA shRNA treatment reduced tumor growth (Figure 6D). In addition, IFN-γ neutralizing antibodies abolished the antitumor effect of the SRA/CD204 silencing-enhanced therapy, whereas control IgG had no effect. These results support the role of IFN-γ in the antitumor activity facilitated by SRA/CD204 silencing (Figure 6D).
lipoprotein (LDL; ref. 31), and high-mobility group box 1 protein (HMGB1; ref. 32). It is likely that SRA/CD204 limits the functional activation of DCs exposed to these damage-associated molecules or “danger” signals (33, 34). Out data support this hypothesis by showing that SRA/CD204 silencing renders DCs more responsive to stimulation with dying RM1 tumor cells, as evidenced by increased expression of inflammatory mediators and T-cell-stimulating capability. These results also coincide with our recent observation of significantly heightened immunogenicity of dying prostate tumor cells in SRA−/− mice (17). In addition, lack of SRA/CD204 in DCs also enhances antigen cross-presentation, CTL activation, and antitumor responses induced by stress proteins as immunostimulators (16, 19).

It was reported that dying tumor cells produced by cancer therapies (e.g., radiotherapy or chemotherapy) augment a TLR4-dependent immune response (35, 36), which has been attributed to an essential role of TLR4 in processing and cross-presentation of tumor cell–associated antigens by DCs. Recently, our molecular studies revealed that SRA/CD204 attenuated TLR4-engaged NF-κB signaling in DCs through blockade of TRAF6 ubiquitination and oligomerization (37). The similar molecular mechanism may underlie SRA/CD204-mediated immune suppression in DCs upon stimulation with “danger” molecules released by ionizing radiation–treated RM1 tumor cells. SRA/CD204 downregulation also enables DCs more resistant to tumor-mediated immune suppression, as shown in the current study and a recent report that SRA/CD204 contributes to DC dysfunction in tumor-bearing mice and patients with cancer (28). In situ DC vaccination described here can target the highly individualized tumor antigens as well as inflammatory signals released from dying cancer cells following radiotherapy. Considering the immunomodulating effects of radiotherapy, for example, sensitizing cancer cells to T-cell–mediated attack (8–10), and promoting T-cell trafficking (38), we expect that radiotherapy and conventional DC vaccination with antigen-loaded, SRA/CD204-silenced DCs should also display enhanced synergistic antitumor efficacy.

Combining radiotherapy and in situ vaccination with SRA/CD204-silenced DCs is highly effective in stimulating a systemic, antigen-specific CTL-mediated antitumor immune response. As a result, a significant elevation in the number of tumor-infiltrating CD8+ cells, the intratumoral IFN-γ levels, and the frequency of IFN-γ-expressing CD8+ T cells is achieved following the combinational therapy, which also positively correlates with increased tumor cell death. The supporting evidence also derives from the result showing that antitumor efficacy of radiotherapy and DC-SRA shRNA vaccination is abrogated after IFN-γ neutralization or in the absence of CD8+ cells. Together with the observation of diminished IFN-γ level in the tumor bed upon CD8+ cell depletion, we conclude that CD8+ T cells predominantly contribute to the improved tumor control by SRA/CD204-silenced DC vaccine following radiotherapy.

Our findings are in contrast to the previous study by Teitz-Tennenbaum and colleagues (39) in which enhanced therapeutic efficacy of adoptively transferred T cells following radiotherapy and DC administration was associated with a selective increase in proliferation and function of CD4+ T cells. Several differences between these 2 studies may contribute to this discrepancy. While our research is centered on SRA/CD204 silencing–enhanced endogenous tumor-reactive T-cell response, their study primarily focused on the activity of adoptively transferred T cells. Treatment regimens used in our study (i.e., radiotherapy plus genetically engineered DCs) are also different from the one used by Teitz-Tennenbaum and colleagues (DC vaccination following radiotherapy and lymphodepletion plus adoptive T-cell transfer). The different observations could also derive from the use of different tumor models. It should be noted that the contribution of CD4+ T cells to tumor suppression was not directly examined in their study.

Radiotherapy is commonly combined with ADT for treatment of patients with high-risk and locally advanced prostate cancer today (40). The addition of neoadjuvant and adjuvant ADT can significantly delay tumor progression and improve disease-free and overall survival. Considering that immunotherapy (e.g., SRA/CD204-targeted DC vaccine) engages a complementary antitumor mechanism by targeting the host immune system, it therefore has the potential to further promote the additive or synergistic antitumor effects of multimodality therapy (e.g., radiotherapy plus ADT), leading to effective eradication of recurrence and metastases.

In summary, we have shown that selective downregulation of SRA/CD204 in DCs can overcome immune tolerance and enhance antitumor potency of radiotherapy combined with in situ DC-based vaccination. Strategically combining radiotherapy with SRA/CD204-silenced DCs could alter or modify the tumor environment, shifting the balance in favor of immune activation. Further studies are warranted to determine whether multimodality therapy combining standard radiotherapy, SRA/CD204-targeted vaccines, and/or other novel approaches that effectively break tumor-mediated immunoregulatory mechanisms (41) can be successfully translated into improved clinical outcomes.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: G. Yang, P.B. Fisher, X.-Y. Wang
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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Guo, H. Yi, D. Zuo, B.A. Foster, P.B. Fisher, X.-Y. Wang
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*In Situ* Vaccination with *CD204* Gene-Silenced Dendritic Cell, not Unmodified Dendritic Cell, Enhances Radiation Therapy of Prostate Cancer

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