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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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Abstract

Given the complexity of prostate cancer progression and metastasis, multimodalities that target different aspects of tumor biology, e.g., radiotherapy (RT) 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 (DCs) failed to synergize with fractionated RT. 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 radio-immunotherapeutic 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 RT combined with *in situ* DC vaccination by promoting a robust

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systemic antitumor immunity. Further studies are warranted to test this novel combinatorial approach for translating into improved clinical outcomes in prostate cancer patients.

Keywords

Prostate cancer; radiotherapy; dendritic cell; T cell; tumor immunity; IFN-γ; CD204; scavenger receptor A

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 definite therapy, e.g., radiotherapy (RT), perhaps due to occult metastatic disease. Androgen deprivation therapy in addition to RT improves overall survival of patients at high risk for recurrence (2). This approach, however, is associated with significant side effects. Given that prostate cancer patients often present with low tumor burden, one potential treatment is the incorporation of immunotherapy into a standard RT protocol (3). With recent approval of an antigen-presenting cell vaccine sipuleucel-T (Provenge) by the US 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 RT 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., DCs) in the context of the costimulatory or “danger” signals, resulting in effective T cell activation (7). RT also renders tumor cells more susceptible to recognition and attack by tumor-specific CTLs (8–10). Gulley *et al.* recently reported clinical trials in which RT combined with a PSA-targeted vaccine displayed a favorable toxicity profile and generated significant T cell responses in prostate cancer patients (11, 12). Indeed, several phase II/III clinical trials of RT in combination with therapeutic cancer vaccines, e.g., 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 demonstrated 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 RT-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 RT-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 RT 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 $OVA_{257–264}$ (SIINFEKL) were purchased from The Jackson Laboratory (Bar Harbor, ME). All experiments and procedures involving mice were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Androgen-refractory mouse prostate cancer cell lines, RM1 (21) kindly provided by Dr. TC Thompson (Baylor College of Medicine, TX, USA) and TRAMP-C2 (22) were used. RM1 cells-expressing OVA (17) or luciferase were generated in our laboratory. All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM 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 were conducted by the authors.

Short hairpin (sh) RNA–mediated gene silencing in DCs

Lentiviral (LV) vector encoding mouse SRA/CD204 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, BM cells were cultured in media containing recombinant mouse GM-CSF (20 ng/ml, Preprotech, Rocky Hill, NJ). Day 3 BM-DCs were infected with lentiviruses in the presence of 8 μg/mL polybrene for 3 hours. Cells were then re-suspended and plated in 12-well plates at 1×10^6 cells/mL. Day 8 DCs were collected and used for studies.

Tumor treatment and immunization protocols

Mice were injected with 1×10^5 RM1 tumor cells or 2×10^6 TRAMP-C2 tumor cells subcutaneously (*s.c.*) in the right hind leg. When tumors reached 4–5 mm in diameter, mice were anesthetized using ketamine (80 mg/kg)/xylazine (10 mg/kg), and locally irradiated with a Picker ^{60}Co source at 1 Gy/min. Mice received a single dose of 10 Gy on each of three consecutive days. For *in situ* DC immunization, mice were injected with DCs (2×10^6) into the tumors 24 and 72 hours after the last radiation treatment. In some experiments, $CD4^+$, $CD8^+$ T cells or NK cells were depleted by intraperitoneal (*i.p.*) injection of GK1.5, 2.43 or PK136 monoclonal antibodies (mAbs), respectively, as previously described (16).

Experimental lung metastases

C57BL/6 mice bearing *s.c.* RM1 tumors were established with lung metastases by intravenously (*i.v.*) injecting 5×10^4 RM1-luciferase cells in PBS (without Ca^{2+} or Mg^{2+}). Only tumors in the flank were treated. Mice were injected *i.p.* with D-luciferin (150 mg/kg, Caliper Life Sciences, Hopkinton, MA) and examined using a Xenogen IVIS Imaging System. Lung metastatic nodules were also enumerated. In the case of TRAMP-C2 tumor, 1×10^6 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 5×10^4 OT-I cells in a round-bottom 96-well microtiter plate. Cells were cultured for 60 hours and pulsed with ^{3}H-thymidine (0.5 μCi/well) during the last 16 hours of culture period. T cell proliferation was assessed based on ^{3}H-thymidine incorporation. Levels of IL-2 in the supernatants were determined using Enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked immunosorbent spot (ELISPOT) 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 ELISPOT assays as previously described (19). For intracellular cytokine staining, cells were stimulated with $OVA_{257–264}$ peptides (AnaSpec Inc., San Jose, CA) for 3 days, followed by treatment with brefeldin A (BD GolgiPlug; BD Biosciences). Cells were then stained with Abs for CD8 and IFN-γ, and subjected to FACS analysis (19).

Cytotoxicity assays

An *in vivo* CTL assay to determine antigen-specific cytolytic activity of $CD8^+$ T cells was performed as previously described (18). Briefly, naïve splenocytes that were labeled with 12.5 μM CFSE (i.e., $CFSE^{high}$ cells) using CellTrace™ 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester cell proliferation kit (Molecular Probes, Eugene, OR), and pulsed with $OVA_{257–264}$ served as targets. Control cells were labeled with 0.5 μM CFSE (i.e., $CFSE^{low}$ cells) and pulsed with irrelevant peptides. A mixture of these cell populations (total 5×10^6 cells) at 1:1 ratio was injected *i.v.* into treated mice, followed by FACS analysis of splenocytes 16 hours later.

Statistical analysis

Data are presented as mean ± SD. Differences between groups within experiments were analyzed using two-tailed unpaired Student *t* test. Animal survival data were analyzed using log-rank test. *p* values < .05 were considered statistically significant.

Results

SRA/CD204 dampens the therapeutic effectiveness of conventional RT

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 RT. As previous reported, RM1 tumors grew aggressively at a similar rate in WT and $SRA^{-/-}$ mice before treatment (17). However, RM1 tumor growth was profoundly inhibited by RT in $SRA^{-/-}$ mice compared with WT mice (Fig. 1), suggesting that SRA/CD204 ablation sensitizes RM1 tumors to RT.

In situ vaccination using SRA/CD204-deficient DCs enhances the effectiveness of RT 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 RT (Fig. 2A). Our initial studies showed that fractionated RT (i.e., 3 consecutive 10 Gy) delayed tumor growth (Fig. 2B, left). Direct injections of WT or $SRA^{-/-}$ DCs without RT 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 RT resulted in increased tumor suppression compared with RT alone or RT plus WT DCs (Fig. 2B, *middle*). The enhanced tumor control in mice treated with RT plus $SRA^{-/-}$ DCs was also associated with significantly improved animal survival (Fig. 2B, *right*).

To determine whether the increased tumor control by combined RT and $SRA^{-/-}$ DC vaccine correlated with enhanced T cell activation, RM1 tumors expressing a non-"self" model antigen OVA (RM1-OVA) was used to facilitate monitoring of antigen-specific T cells. The frequency of IFN-γ-producing $CD8^+$ cells reactive with $OVA_{257–264}$ epitope increased in mice treated with RT plus WT DC. However, $SRA^{-/-}$ DCs were much more efficient than WT DCs in generating OVA-specific $CD8^+$ T cells when combined with RT (Fig. 2C).

Tumor immune evasion often involves a complex array of immune suppressive 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 co-cultured with naïve OT-I cells in RM1 tumor-conditioned media, $OVA_{257–264}$-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 caused upregulation of SRA/CD204 protein levels (data not shown).

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

BM-DCs were genetically modified by infection with lentiviral vectors (LV) 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 *ifnβ*) similarly increased in DC-SRA shRNA (Supplementary Fig. S1A–C). Elevation of IL-6 expression was further confirmed by intracellular cytokine 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). Additionally, 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 3D).

RT 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 RT 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 RT-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 RT 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 one day before the implantation of *s.c.* RM1 tumors. Treatment of *s.c.* tumors with RT alone or RT plus DC-scram vaccine partially inhibited lung metastases. However, RT plus DC-SRA shRNA was far more effective than other treatment modalities in controlling metastases

(Fig. 4C and 4D, *left*). The result was also validated using the metastasis model of TRAMP-C2 tumor (Fig. 4D, *right*). Mice receiving RT 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).

RT 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 $OVA_{257–264}$ peptide, splenocytes from RM1-OVA tumor-bearing mice treated with RT plus DC-SRA shRNA exhibited increased proliferation (Fig. 5A, *top*) and IL-2 production (Fig. 5A, *bottom*) compared to those from mice treated with RT plus DC-scram. Mice receiving RT 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 six-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 RT protocol effectively primed $CD8^+$ T cells recognizing $mSTEAP_{326–335}$ (Fig. 5C, *left*) or RM1 tumor cells (Fig. 5C, *right*). T cell functions assays showed that RT-treated RM1-OVA tumor-bearing mice receiving DC-SRA shRNA vaccine developed a stronger cytolytic response against $OVA_{257–264}$-pulsed targets *in vivo* (Fig. 5D) and RM1-OVA tumor cells *in vitro* (Supplementary Fig. S2B) compared to other treatment modalities.

SRA/CD204 silencing enhanced antitumor efficacy is dependent on IFN-γ-producing $CD8^+$ T cells

TUNEL assays showed that RT plus DC-SRA shRNA induced more RM1 tumor cell death than RT alone or RT plus DC-scram. In parallel, RT 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 *ex vivo* genetically engineered DCs, because the majority of DCs injected post-RT migrated out of the tumor site within 24 hours (data not shown).

ELISA analysis of tumor tissues showed that the levels of IFN-γ, a cytokine critical for effective antitumor immunity, were significantly higher in mice receiving RT 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-γ, 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 RT and DC-SRA shRNA displayed markedly increased expression of IFN-γ (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-γ^+CD8^+ cells compared to IFN-γ^+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). Lastly, blocking IFN-γ using neutralizing antibodies also abrogated the therapeutic efficacy of the combinatorial therapy (Fig. 6D), suggesting that IFN-γ-producing $CD8^+$ cells represents a key factor mediating *SRA/CD204* silencing-promoted antitumor immunity.

Discussion

Given that RT is the primary treatment option for high-risk localized prostate cancer and the biochemical relapse rate of many of these patients is high, combining RT 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 RT 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 RT-treated tumors, but also limits experimental metastatic outgrowth. Our studies establish the feasibility and efficacy of this novel combinatorial radio-immunotherapy in the two 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 RT has also been reported to provide additional antitumor benefits (27). The failure of WT DCs or DC-scram to synergize with local RT for controlling established RM1 tumor in our initial study was unexpected, which may be attributed to its poorly immunogenic nature or the immune suppression 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 immune suppressor capable of attenuating DC functions (16–20, 28).

Local RT is accompanied by the release of tumor antigen and endogenous ‘danger’ signals (29), such as stress proteins (30), oxidized LDL (31) and high-mobility-group box 1 protein (HMGB1) (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., RT or chemotherapy) augment a TLR4-dependent immune response (35, 36), which has been attributed to an essential role of TLR4 signaling 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 may also enable DCs more resistant to tumor-mediated immune suppression, as shown in the current study and a recent report that SRA/CD204 contribute to DC dysfunction in tumor-bearing mice and cancer patients (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 RT. Considering the immunomodulating effects of RT, e.g., sensitizing cancer cells to T cell-mediated attack (8–10), and promoting T cell trafficking (38), we expect that RT and conventional DC vaccination with antigen-

loaded, SRA/CD204-silenced DCs should also display enhanced synergistic antitumor efficacy.

Combining RT 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 $CD8^+$ cells is achieved following the combinatorial therapy, which also positively correlates with increased tumor cell death. The supporting evidence also derives from the result showing that antitumor efficacy of RT 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 RT.

Our findings contrast to the previous study by Teitz-Tennenbaum *et al* (39), in which enhanced therapeutic efficacy of adoptively transferred T cells following RT and DC administration was associated with a selective increase in proliferation and function of $CD4^+$ T cells. Several differences between these two studies may contribute to this discrepancy. While our research is centered on SRA 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., RT plus genetically engineered DCs) is also different from the one employed by Teitz-Tennenbaum *et al*. (DC vaccination following RT 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.

RT is commonly combined with androgen deprivation therapy (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-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., RT plus ADT), leading to effective eradication of recurrence and metastases.

In summary, we have demonstrated that selective downregulation of SRA/CD204 in DCs can overcome immune tolerance and enhance antitumor potency of RT combined with *in situ* DC-based vaccination. Strategically combining RT 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 RT, SRA/CD204-targeted vaccines and/or other novel approaches that effectively break tumor-mediated immune regulatory mechanisms (41) can be successfully translated into improved clinical outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

RT	Radiotherapy
SRA	scavenger receptor A
DC	dendritic cell
CTL	cytotoxic T lymphocytes

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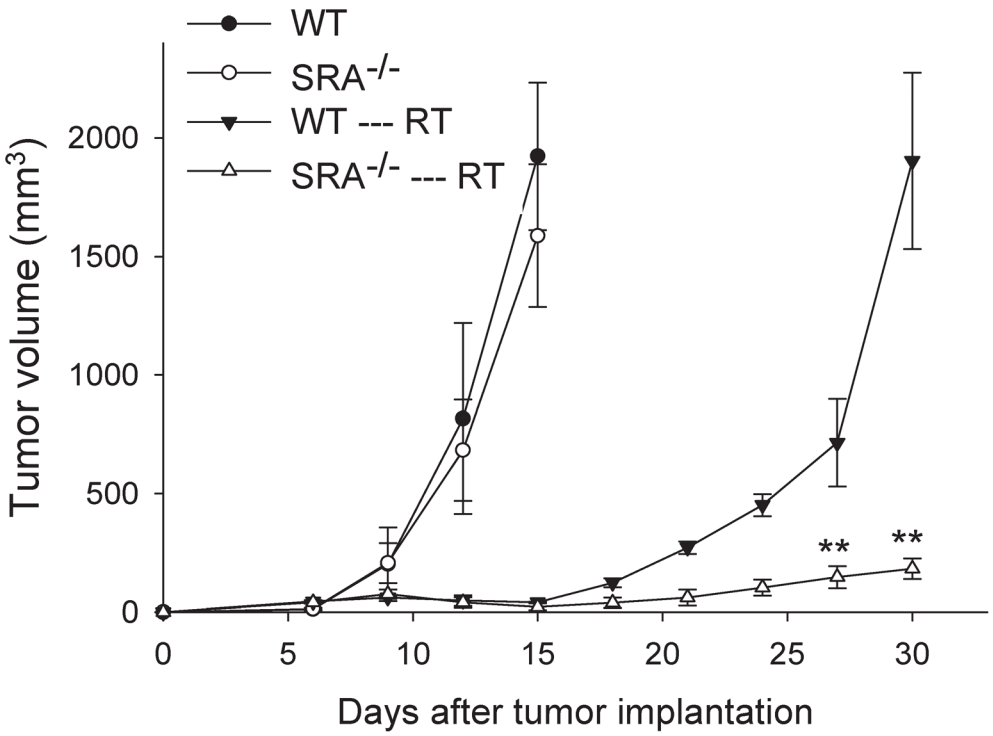

Figure 1. SRA/CD204 ablation improves the effectiveness of RT against established prostate tumors

RM1 tumor-bearing WT and $SRA^{-/-}$ mice (n=5) were treated with or without RT. ** $p < 0.01$. The results shown are representative of at least three independent experiments.

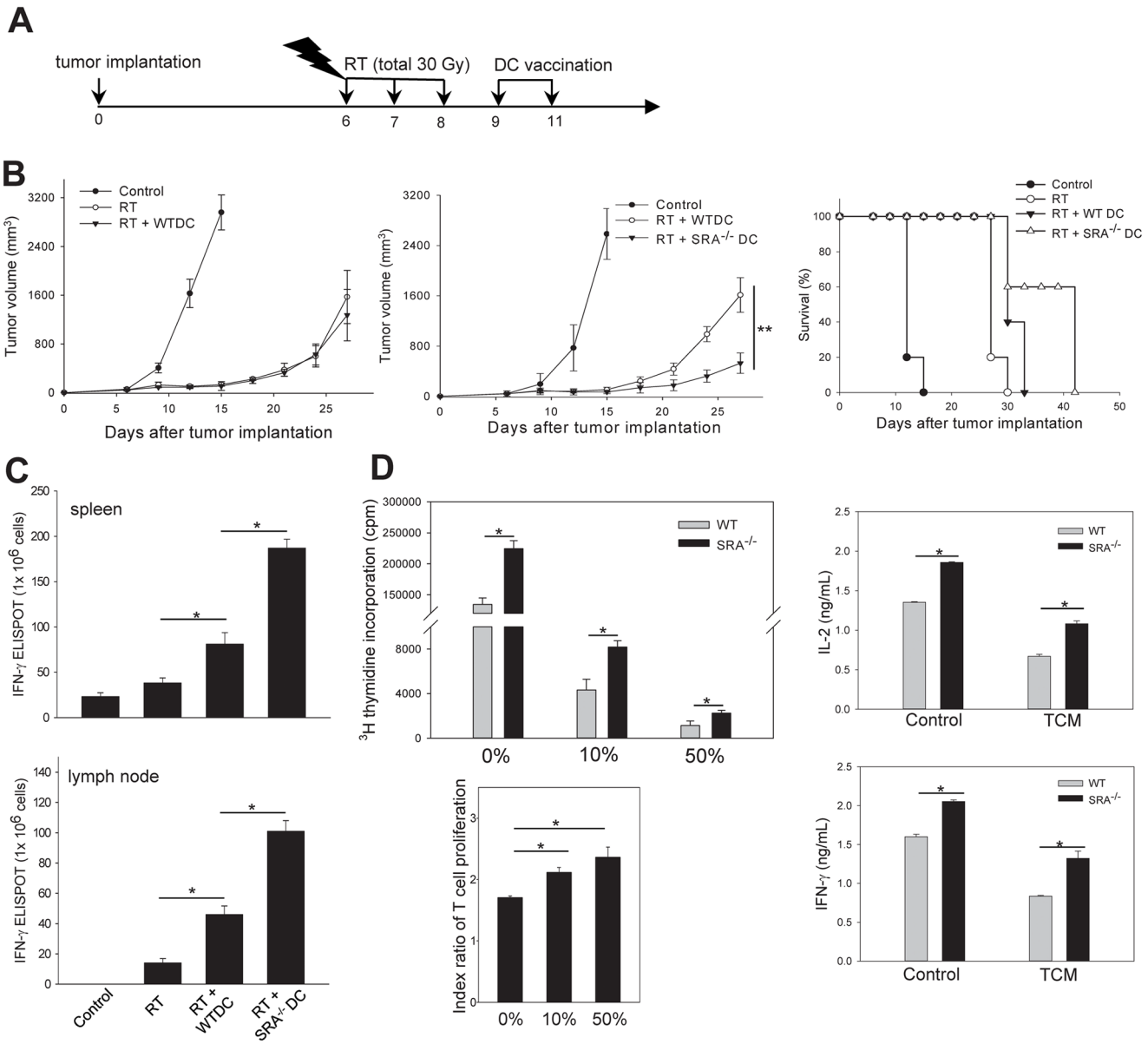

Figure 2. SRA/CD204 absence in DCs enhances the antitumor efficacy of RT combined with DC vaccination

A. Scheme for the combined RT and *in situ* DC vaccination. **B.** RM1 tumor-bearing mice (n=5) were treated with RT alone, RT plus WT DCs or left untreated. WT DC vaccine failed to enhance tumor inhibition following RT (*left*). In contrast, administration of $SRA^{-/-}$ DC vaccine combined with RT led to improved tumor control (*middle*) and survival rate (*right*). ** $p < 0.01$, RT + WT DC *vs* RT + $SRA^{-/-}$ DC. **C.** Enhanced T cell activation by RT and $SRA^{-/-}$ DC vaccination. Splenocytes (*top*) and lymph node cells (*bottom*) from treated RM1-OVA tumor-bearing mice were stimulated with $OVA_{257–264}$ 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. $OVA_{257–264}$-loaded DCs were co-cultured with OT-I cells in the presence of RM1 tumor conditioned media (TCM) at indicated concentrations. T cell proliferation was assessed using ^{3}H-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 (* $p < 0.01$).

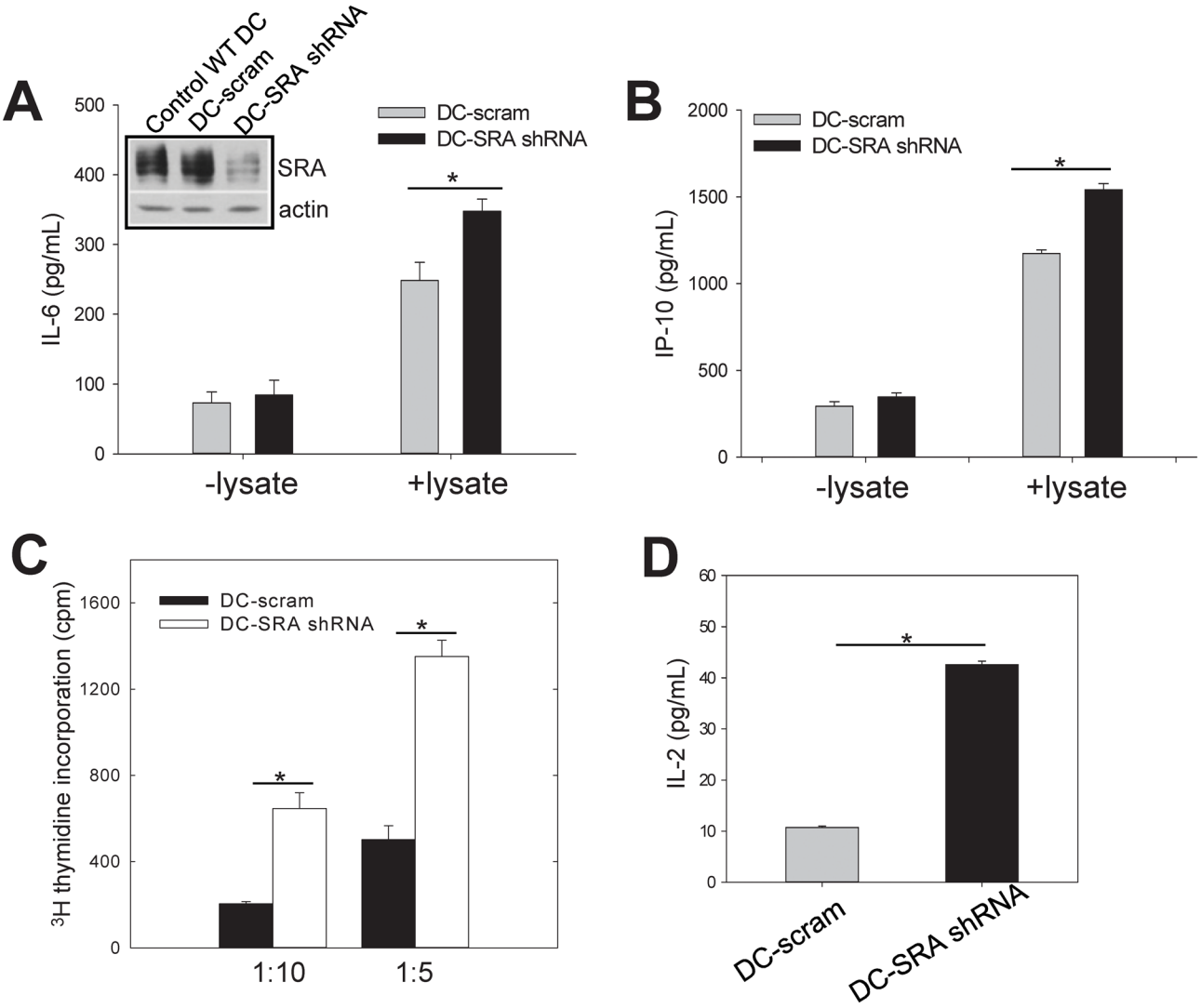

Figure 3. *SRA/CD204* silencing in DCs promotes cross-presentation of RM1 tumor cell-associated antigens

A–B. SRA/CD204 downregulation renders DCs more responsive to stimulation with dying tumor cells. DCs were infected with lentiviruses encoding scrambled shRNA or SRA shRNA, and co-cultured with RM1 cell lysates prepared after ionizing radiation. Protein levels of IL-6 (**A**) and IP-10 (**B**) in the media were determined using ELISA. The efficiency of SRA/CD204 silencing in DCs was assessed using immunoblotting (A, *insert*). **C–D.** Increased $CD8^+$ T cell activation by *SRA/CD204*-silenced DCs. DCs were pulsed with RM1-OVA cell lysates, and co-cultured with naïve OT-I cells. T cell proliferation was assessed using ^{3}H-thymidine incorporation assays (**C**). The IL-2 concentration in the supernatant was determined using ELISA (**D**). * $p < 0.01$. The results shown represent three experiments.

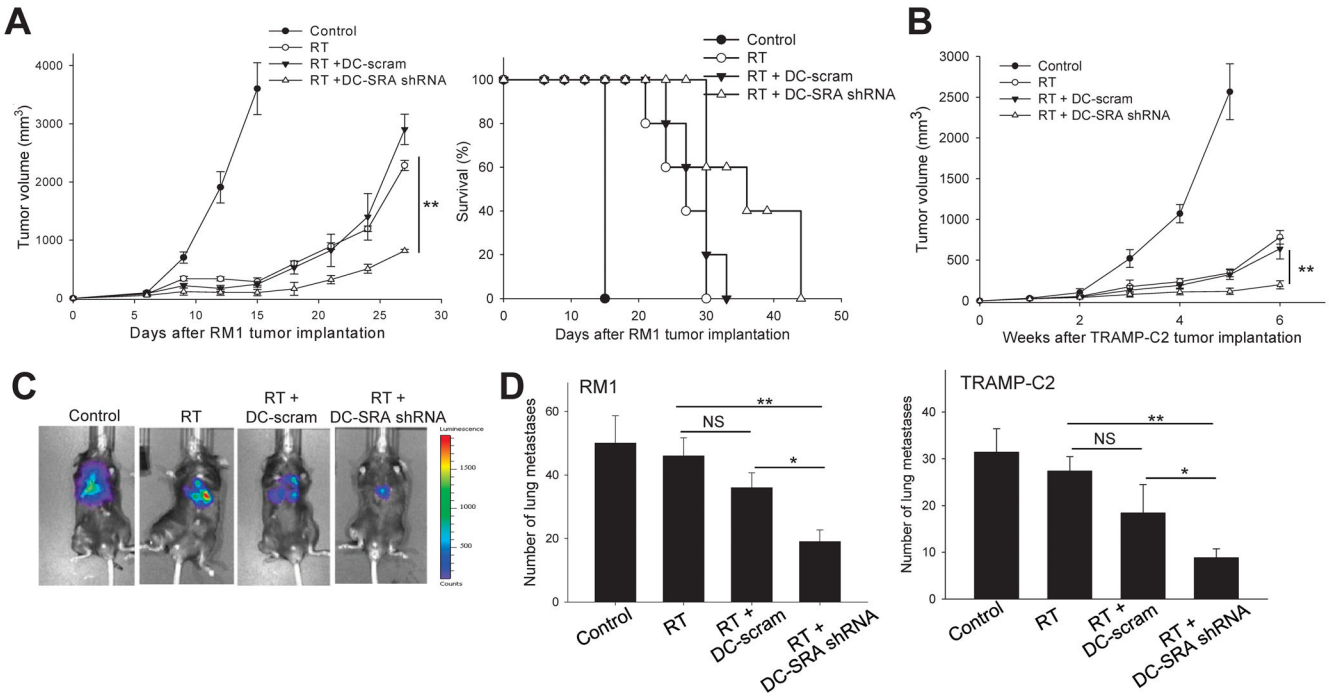

Figure 4. *In situ* vaccination with *SRA/CD204*-silenced DCs enhances the antitumor efficacy of RT

A. RM1 tumor-bearing mice (n=5) received RT alone, RT plus DC-scram or DC-SRA shRNA, or left untreated. Tumor growth (*left*) and survival of RM1 tumor-bearing mice (*right*) were monitored. ** $p<0.01$, RT+DC-SRA shRNA *vs* RT+DC-scram. **B**. Enhanced suppression of TRAMP-C2 prostate tumor by SRA/CD204-targeted combinatorial therapy. * $p < 0.01$. **C–D.** Treatment of local prostate tumors with RT plus DC-SRA shRNA reduces distant metastases. Mice (n=5) were simultaneously established with *s.c.* 'primary' RM1 tumors in the flank and lung metastases through *i.v.* injection of RM1-luciferase cells. Tumors in the flank were subjected to treatment only. The metastases in the lungs were assessed using bioluminescence imaging (**C**). Lung tissues were collected from RM1-tumor (*left*) or TRAMP-C2 tumor (*right*)-bearing mice following treatment of *s.c.* tumors, and metastatic tumor nodules in the lungs were counted (**D**). * $p < 0.05$, ** $p < 0.01$. Data are representative of two experiments with similar results.

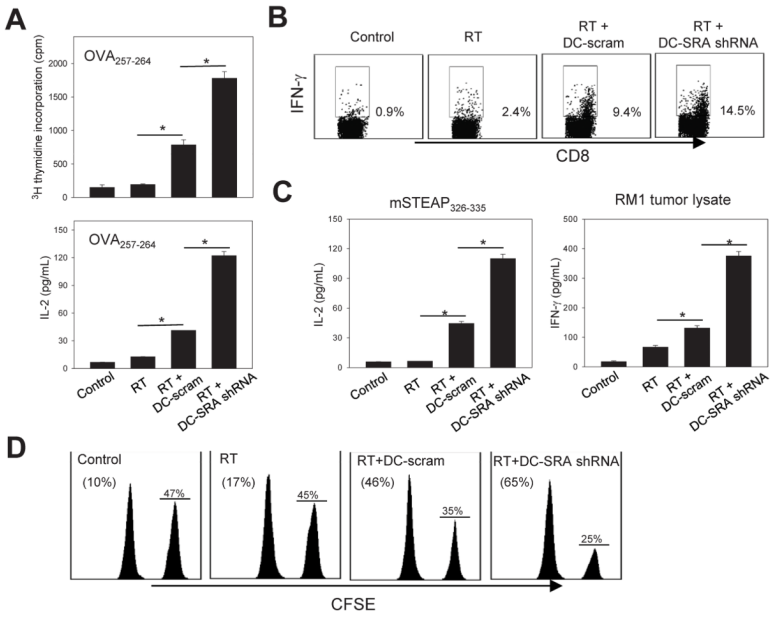

Figure 5. *SRA/CD204* silencing enhances tumor-specific T cell responses after RT combined with *in situ* DC vaccination

A. Increased activation of antigen-specific $CD8^+$ T cells. RM1-OVA tumor-bearing mice were treated with RT alone or RT plus DC vaccines. Splenocytes were stimulated with $OVA_{257–264}$ and analyzed for T cell proliferation (*top*) and IL-2 production (*bottom*). **B**. The percentage of IFN-γ-producing $CD8^+$ T cells was assessed using intracellular cytokine staining assays. **C.** Enhanced immune cell recognition of prostate tumor antigen STEAP and RM1 tumor cells. Splenocytes were stimulated with $mSTEAP_{326–335}$ (*left*), or irradiated RM1 cells (*right*). IL-2 or IFN-γ production was assessed using ELISA. * $p < 0.01$. Data are representative of two experiments in which at least of 3 mice of each group were analyzed. **D.** Increased cytolytic activity of effector T cells. One week after DC vaccination, RM1-OVA tumor-bearing mice (n=3) were injected *i.v.* with $OVA_{257–264}$-pulsed, $CFSE^{high}$ splenocytes mixed with $CFSE^{low}$ splenocytes pulsed with irrelevant peptides. Spleens were analyzed 16 hours later using flow cytometry. The percentage of killing of antigen-positive target is shown in parentheses. Representative histograms from three experiments with similar results are shown.

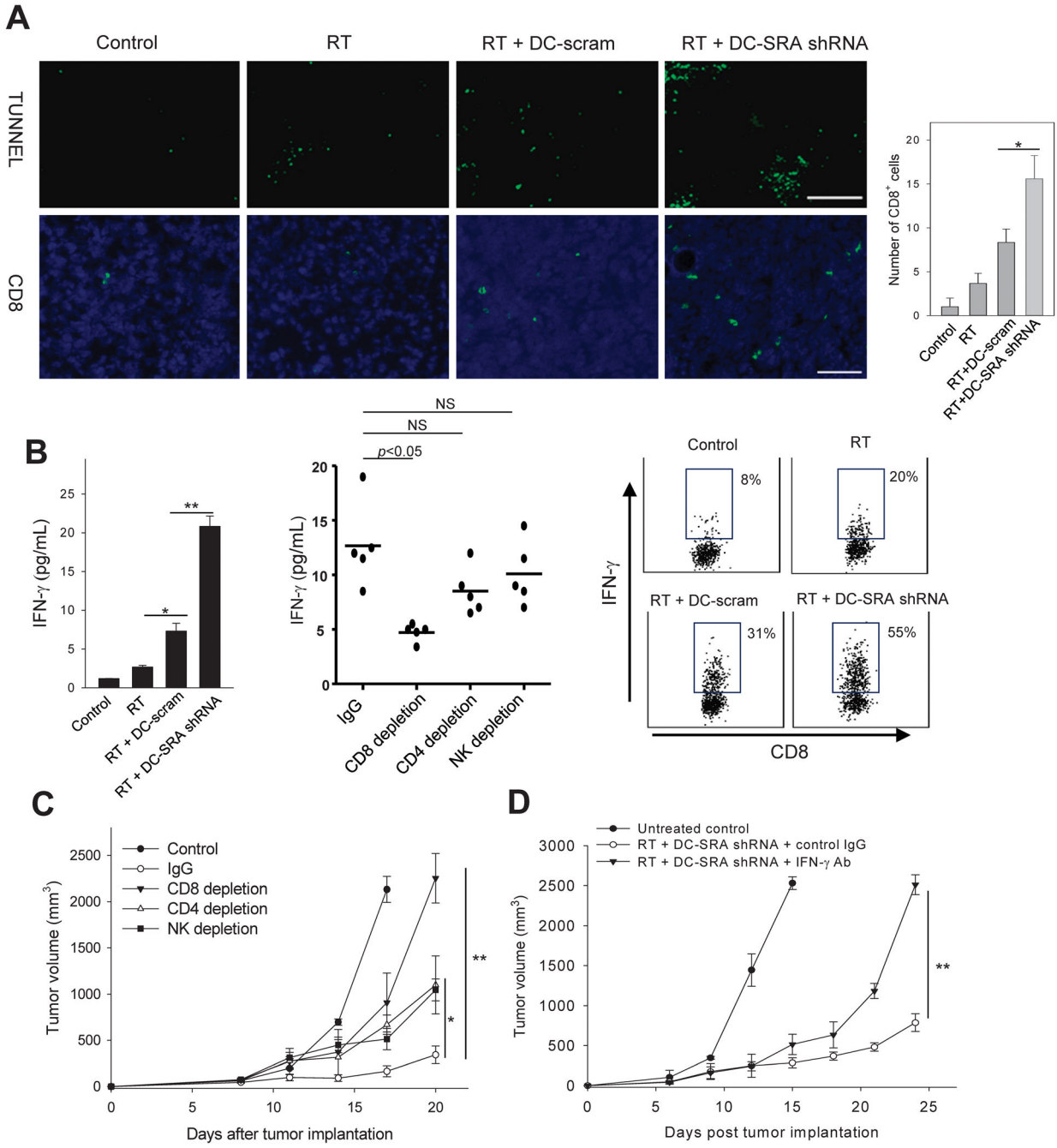

Figure 6. SRA/CD204 silencing-enhanced RM1 tumor control depends on IFN-γ production by CD8$^+$ T cells

A. RM1 tumor cell death is associated with increased tumor-infiltrating CD8$^+$ cells. One week after DC vaccination, tumor cell death was examined by TUNNEL assays. Nuclei were counterstained with DAPI. Cryosections of tumor tissues were stained with Abs for CD8 (green). Scale bar indicates 50 μm. Quantitative analysis of infiltrating cells was conducted by counting positive cells in at least five randomly selected fields of view (*right*). * $p < 0.05$. **B.** CD8$^+$ cells primarily contribute to IFN-γ production. IFN-γ levels increased in RM1 tumor tissues after treatment with RT plus DC-SRA shRNA, as determined using ELISA (*left*). Depletion of CD8 cells prior to combined therapy reduced IFN-γ levels in

tumor tissues (*middle*). In addition, tumor-infiltrating $CD8^+$ cells were analyzed for IFN-γ expression with intracellular cytokine staining assays (*right*). **C.** $CD8^+$ cells are required for *SRA/CD204* silencing-enhanced tumor inhibition. RM1 tumor-bearing mice (n=5) were depleted of effector cell subsets before the combinatorial therapy. **D**. IFN-γ is critical for *SRA/CD204* silencing-facilitated antitumor activity. RM1 tumor-bearing mice (n=5) receiving RT plus DC-SRA shRNA were administrated with IFN-γ neutralizing Abs or control IgG. NS, not significant; * $p < 0.05$; ** $p < 0.01$. Data shown are representative of two experiments.