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Absence of scavenger receptor A promotes dendritic cell-mediated cross-presentation of cell-associated antigen and antitumor immune response

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Abstract

Given the primary expression of scavenger receptor A (SRA) or CD204 on antigen presenting cells, we investigate the immune-regulatory activities of SRA/CD204 in the context of cross-presentation of cell-associated antigen and the immunogenicity of dying tumor cells. Immunization with dying prostate cancer cells results in profoundly increased control of subsequently inoculated tumors in SRA/CD204 knockout mice. Using OVA-expressing RM1 prostate tumor line (RM1-OVA), we show for the first time that SRA absence greatly enhances dendritic cells (DCs)-mediated cross-presentation of OVA antigen-derived from dying RM1 cells. While the phagocytic ability of DCs is not significantly impacted by the lack of SRA/CD204, DCs deficient in SRA/CD204 display increased expression of inflammatory cytokines and chemokines, as well as co-stimulatory molecules upon interaction with dying RM1 cells, implicating a suppressive regulation of the functional activation of DCs by SRA/CD204. Further, SRA/CD204 deficient DCs pulsed with dying RM1-OVA cells are more effective than wild-type counterparts in priming antigen-specific T-cell responses, resulting in improved control of RM1 tumor growth in both prophylactic and therapeutic settings. Our findings suggest that the increased immunogenicity of dying tumor cells in SRA/CD204 knockout mice is attributed to the altered functions of DCs in the absence of SRA/CD204, which underscores the important role of SRA/CD204 in host immune homeostasis. Selective downregulation or blockade of this

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immunoregulatory molecule may lead to enhanced potency of DC-based vaccines capable of breaking immune tolerance against cancer.

Keywords

dendritic cells; antigen presentation; immune response; CD204; scavenger receptor

Introduction

Scavenger receptor (SRA), also termed CD204 or macrophage scavenger receptor, is the prototypic member of scavenger receptor family. It was the first receptor identified and cloned in macrophages (Mφ) for modified low density lipoprotein (LDL), such as acetylated low density lipoprotein (LDL) and oxidized LDL that are pertinent to the development of vascular disease [1], [2]. The role of SRA/CD204 in atherosclerosis has been extensively studied because it mediates cholesterol uptake from modified lipoproteins and the resultant lipid laden macrophages closely resemble the macrophage derived foam cells which are a central feature of the pathology of atherosclerosis [3]-[5].

It has been recognized that SRA/CD204 functions as a pattern recognition receptor (PRR) with a broad ligand-binding specificity. SRA/CD204 is known to interact with modified or altered molecules, pathogen associated molecular patterns (PAMPs) (e.g., lipopolysaccharide), and extracellular stress proteins [6]-[9]. Several lines of studies have documented the roles of SRA/CD204 in host defense through pathogen recognition and clearance [3], [10]-[12] as well as in endotoxic shock [13], [14]. Indeed, it was recently suggested that SRA/CD204 may act as an innate inhibitory receptor to limit proinflammatory responses stimulated by foreign pathogen molecules or PAMPs [15], [16]. Despite the recognition of the important functions of SRA/CD204 in the innate immunity against pathogen, the contribution of this receptor to T-cell priming and adaptive immunity remains less defined [17].

We recently reported that SRA/CD204 is capable of attenuating immunostimulatory adjuvant induced antigen-specific cytotoxic T-cell response [17] and antitumor immunity [18]. Given the enhanced immunogenicity of dying tumor cells in $SRA^{-/-}$ mice and preferential expression of SRA/CD204 on antigen-presenting cell (APCs), such as dendritic cells (DCs), we have investigated the impact of SRA/CD204 on the cross-presentation of cell-associated antigen derived from dying tumor cells. In the present study, we provide the first evidence that SRA/CD204 suppresses DC-mediated cross-presentation of tumor cell-derived antigen and subsequent T-cell priming. The lack of SRA/CD204 in DCs greatly enhances T-cell responses and antitumor efficacy augmented by DC-based vaccination, which provides supporting evidence for a critical role of SRA/CD204 in the functional regulation of DCs and adaptive immunity.

Masteries and Methods

Mice and cell lines

Wild-type (WT) C57BL/6 mice were obtained from National Cancer Institute (Bethesda, MD). SRA/CD204 knockout mice ($SRA^{-/-}$) on C57BL/6 background and OT-I mice bearing Vα2Vβ5 TCR specific for the $OVA_{257–264}$ peptide (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. TRAMP-C2, RM1 and RM1-OVA cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Antibodies and reagents

Mouse monoclonal antibodies (mAbs) to CD8 (53-6.7), CD11c (HL3), IFN-γ (XMG1.2) and isotype control rat immunoglobulin IgG2b (RTK4530) were purchased from BioLegend (San Diego, CA). CD86 (PO3.1) was purchased from eBioscience (San Diego, CA). SRA/CD204 polyclonal antibodies for immunoblotting and monoclonal antibodies (2F8) for immune staining were purchased from R&D Systems (Minneapolis, MN) and AbD Serotec (Raleigh, NC). iTag MHC tetramer (OVA, SIINFEKL) was purchased from Beckman Coulter (Fullerton, CA). Recombinant human interleukin (IL)-2 was purchased from Novartis Pharmaceuticals (Emeryville, CA). CellTrace™ 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation kit was purchased from Molecular Probes (Eugene, OR).

Immunization and tumor studies

For immunization with dying tumor cells, cells were irradiated at 120 Gy using Gammacell 40 Exactor Research Irradiator (Best Theratronics, Canada) and injected into mice s.c. Mice received immunization twice at weekly intervals. One week after the second immunization, mice were challenged with 2×10^6 TRAMP-C2 cells or 3×10^5 RM1 cells. For immunization with dying tumor cells loaded DCs, bone marrow (BM)-DCs were incubated with dying RM1 cells at 1:1 ratio for 24 h. Mice received 1×10^6 DCs subcutaneously. twice at weekly intervals. Spleens were collected at indicated time points for analysis of OVA-specific T-cell responses. For tumor treatment study, mice were first established with tumors by injecting 1×10^5 RM1 cells, followed by administration of DCs pulsed with dying RM1 cells. One week after last treatment, splenocytes were prepared and subjected to immunological assays.

In vitro cross-presentation and T-cell priming

BM-DCs were generated by culture of mouse BM cells in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) as described previously [17]. BM-DCs were pulsed with dying RM1-OVA cell lysates (1:1 ratio) for 3 h. After washing, serially diluted DCs were incubated with 5×10^4 OT-I cells in 200 μL of RPMI 1640 medium in a round-bottom 96-well microtiter plate. Cells were cultured for 60 h and pulsed with ^{3}H-thymidine (^{3}H-TdR, 0.5 μCi/well) during the last 16 hours of culture period. T-cell proliferation was assessed by ^{3}H-TdR incorporation assays. For some experiments, OT-I cells were labeled

with 2.5 μM CFSE before co-culturing with DCs. T-cell proliferation was measured by fluorescence-activated cell sorting (FACS) analysis. Levels of IL-2 in the supernatants were determined using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience).

Real time PCR analysis

Total RNA was extracted from BM-DCs using TRIzol reagent (Invitrogen, Carlsbad, CA). For reverse transcription, 1 μg of total RNA and 50 ng of oligo-dT primer were used for 20 μL reaction volumes with Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed on the ABI 7900HT Fast Real-time PCR System (Applied Biosystem, Foster City, CA, USA) using TaqMan® Universal PCR Master Mix and TaqMan® Gene Expression Assays probe and primer mix (Applied Biosystem). The Assay Identification number for *il6* is Mm99999064_m1, for *ip10* is Mm99999072_m1 and for *il-12p35* is Mm00434169_m1. Amplification reactions in triplicate for each sample were performed and the results were normalized to the ACTB gene expression level. An analysis of relative gene expression data was performed, using the $2^{-\Delta\Delta CT}$ method. The fold change in studied gene expression, normalized to endogenous control, was calculated using: $RQ = 2^{-\Delta\Delta CT}$.

Phagocytosis assay

UV irradiated TRAMP-C2 cells were labeled with 2 μM CFSE in PBS at 37°C for 5 min. Unbound dye was quenched by incubation with an equal volume of fetal bovine serum at 37°C for 30 min. Cells were washed extensively and co-cultured with BM-DCs at a 2:1 ratio for 6 h, followed by staining with anti-CD11c-PE antibodies. Phagocytosis was quantified by FACS using a FACScalibur flow cytometer (BD Biosciences) as the percentage of double positive staining cells.

OVA tetramer staining

A total of 5×10^5 peripheral blood lymphocytes (PBLs) was stained with phycoerythrin-conjugated H-2Kb/OVA tetramer and FITC-labeled anti-CD8 antibodies at 4°C for 40 min. Cells were washed twice with PBS before propidium iodide (PI) staining to exclude dead cells and analyzed by FACS. The frequency of OVA-specific T-cell precursors was calculated as the number of tetramer-positive cells divided by the number of $CD8^+$ cells

ELISPOT and intracellular cytokine staining

For enzyme-linked immunosorbent spot (ELISPOT) assay, splenocytes were stimulated with 1 μg/mL $OVA_{257-264}$ peptide in the presence of IL-2 (20 U/mL) for 2 days to determine antigen-specific IFN-γ production as previously described [17]. For intracellular IFN-γ staining, splenocytes were stimulated with $OVA_{257-264}$ peptide (1 μg/mL) for 72 h. Cells were treated with PMA (phorbol 12-myristate-13-acetate, 50 ng/mL) plus ionomycin (1 μg/mL) in the presence of brefeldin A (5 μg/mL, BD GolgiPlug; BD Biosciences) for 3 h at 37°C. Cells were then stained with anti-CD8-FITC antibodies, followed by fixation, permeabilization (BD Cytofix/Cytoperm; BD Biosciences) and staining with anti-IFN-γ-PE antibodies (BD Biosciences). Cells were subjected to FACS analysis gating on $CD8^+$ T-cells.

Statistical analysis

Differences between groups within experiments were tested for significance with analysis of Student *t* test using GraphPad Prism software (GraphPad, San Diego, CA). *p* values less than .05 were considered statistically significant.

Results

Dying tumor cells exhibit increased immunogenicity in SRA/CD204 knockout mice

Given the role of SRA/CD204 as a potential negative regulator of antitumor immunity [18], we initially assessed the immunogenicity of dying prostate cancer cells. WT or $SRA^{-/-}$ mice were immunized with ionizing radiation-treated RM1 tumor cells, followed by tumor challenge with live RM1 cells. RM1 tumors grew aggressively in untreated WT and $SRA^{-/-}$ mice. Upon immunization with irradiated RM1 cells, tumor growth was inhibited more profoundly in $SRA^{-/-}$ mice compared to WT counterparts (Fig. 1a). Similar observations were made when a different prostate tumor line TRAMP-C2 was used (data not shown). Immunization of $SRA^{-/-}$ mice with ultraviolet (UV)-treated TRAMP-C2 cells also resulted in increased protection against subsequent tumor challenge (Fig. 1b). In addition, the therapeutic efficacy of vaccination with irradiated TRAMP-C2 cells was then assessed. Treatment of $SRA^{-/-}$ mice pre-established with TRAMP-C2 tumors led to a greater control of tumor growth, whereas treatment of tumor-bearing WT mice had no effect (Fig. 1c).

SRA/CD204 absence does not alter phagocytic capability of antigen-presenting cells

FACS analysis showed that both bone marrow (BM)-derived DCs and Mφ express SRA/CD204 (Fig. 2a), which agrees with our previous observation of the preferential expression of SRA/CD204 in antigen-presenting cells (APCs) [18]. We hypothesized that SRA/CD204 was involved in the regulation of APC functions, leading to altered immunogenicity of dying tumor cells. When BM-DCs were co-cultured with irradiated, dying tumor cells, it was seen that SRA/CD204 expression was up-regulated in $CD11c^{+}$ BM-DCs, as indicated by both FACS (Fig. 2b, left) and immunoblotting (Fig. 2b, right) analyses.

SRA/CD204 has been implicated in clearance of apoptotic cells by in vitro experimentation [19] and the impairment of apoptotic/dying cell phagocytosis can cause the breakdown of self-tolerance [20], [21]. We compared the phagocytic capability of BM-DCs (Fig. 2C) and BM-Mφ (data not shown) from WT and $SRA^{-/-}$ mice. The quantification of phagocytic uptake by detecting $CD11c^{+}$ DCs that also contained CFSE-labeled dying cells showed that the phagocytic capability of DCs from $SRA^{-/-}$ mice is similar to that of WT mice.

SRA/CD204 absence enhances DC-mediated cross-presentation of cell associated antigen

To determine the impact of SRA/CD204 absence on DC activities in the context of cross-presentation of cell-associated antigen, we established RM1 cells line stably transduced with a model antigen OVA, which allows effective immune monitoring of an antigen-specific response in vitro and in vivo. The expression of OVA was confirmed by standard PCR (Fig. 3a). For cross-presentation assays, WT DCs or $SRA^{-/-}$ DCs were pulsed with dying RM1-OVA cells, and co-cultured with OVA-specific OT-I cells. It was seen that $SRA^{-/-}$ DCs were much more effective in driving OT-I cell proliferation, as indicated by increased

incorporation of ^{3}H-thymidine into OT-I cells (Fig. 3b). Similar result was obtained when DCs were used to stimulate CFSE-labeled OT-I cells as responder cells, followed by FACS analysis (Fig. 3c). In addition, ELISA assays showed that higher levels of IL-2 were present in the supernatant when OT-I cells were co-cultured with SRA$^{-/-}$ DCs than WT DCs (Fig. 3d).

SRA/CD204 absence promotes inflammatory activation of DCs pulsed with dying RM1 cells

Cell injury has been shown to release endogenous adjuvant for immune stimulation and generation of T-cell responses [22]. We therefore examined the effect of SRA/CD204 absence on the inflammatory response in DCs when pulsed with dying tumor cells. BM-DCs from WT or SRA$^{-/-}$ mice were co-cultured with dying RM1 cell lysates, followed by real-time PCR analysis of mRNA levels of inflammatory mediators in DCs. SRA$^{-/-}$ DCs displayed significantly increased gene transcription of *il6, ip10* and *il12p35* compared with WT DCs (Fig. 4a). As expected, SRA$^{-/-}$ DCs also produced more IL-6, IP10 and IL-12 proteins after pulsing with dying RM1 cells, as measured by ELISA assays of culture media (Fig. 4b).

We also examined the surface expression of co-stimulatory molecules by FACS analysis of CD11c^{+} cells after exposure to dying cells. Following co-culture with dying RM1 cells, SRA$^{-/-}$ DCs displayed more efficient up-regulation of CD86 compared to WT DCs, as shown by percentage increase of CD86 expressing cells as well as fold increase of mean fluorescence intensity (MFI) (Fig. 4c and d).

SRA/CD204 suppresses immunostimulatory capability of DCs in vivo

Given that SRA/CD204 absence enhanced the immunostimulatory activities of DCs during the cross-presentation of cell-associated antigen in vitro, we next assessed whether SRA/CD204-expressing DCs may play a role in the increased immunogenicity of dying tumor cells. WT DCs and SRA$^{-/-}$ DCs were pulsed with dying RM1-OVA cells and used to immunize WT mice, followed by immunological assays of OVA-specific T-cell responses. Tetramer staining of PBLs showed a higher percentage of tetramer positive CD8^{+} T cell population in SRA$^{-/-}$ DC-vaccinated mice than in WT DC vaccinated mice (Fig. 5a). In addition, splenocytes derived from DC immunized mice were also collected and stimulated with OVA$_{257\text{-}264}$ peptide. The number of OVA$_{257\text{-}264}$ specific CD8^{+} T cells producing IFN-γ was determined by intracellular cytokine staining (Fig. 5b) and ELISPOT (Fig. 5c). CD8^{+} T cells from SRA$^{-/-}$ DC immunized mice showed more robust IFN-γ production than those from WT DC immunized mice.

Immunization with SRA$^{-/-}$ DCs induces enhanced anti-tumor efficacy

We determined whether increased T-cell response elicited by immunization with dying tumor cell-loaded SRA$^{-/-}$ resulted in improved antitumor activity against RM1 tumors, Following loading of DCs with dying RM1 cells, mice were immunized with WT DCs or SRA$^{-/-}$ DCs, and subsequently challenged with live RM1 cells. It was seen that vaccination with WT DCs only modestly delayed RM1 tumor growth, whereas SRA$^{-/-}$ DCs provided a much stronger tumor-protective effect (Fig. 6a).

We also assessed the ability of dying RM1 cell loaded DCs to control prostate tumors in a therapeutic setting. WT mice were established with RM1 tumors, followed by administration of WT DCs or SRA$^{-/-}$ DCs pulsed with dying RM1 cells. While treatment with WT DC failed to inhibit RM1 tumor growth, vaccination with SRA$^{-/-}$ DC resulted in more effective suppression of tumor growth, indicating increased antitumor efficacy of DCs in the absence of SRA/CD204 (Fig. 6b). We next examined whether the improved antitumor efficacy was correlated with enhanced immune responses. Splenocytes cells from treated or untreated tumor-bearing mice were stimulated with $OVA_{257-264}$ or dying RM1 tumor cells. Splenocytes from SRA$^{-/-}$ DC treated mice showed increased proliferation and more robust Th1 cytokine (IL-2 and IFN-γ) production compared to those from WT DC treated mice (Fig. 6c).

Discussion

The roles of SRA/CD204 in host response against pathogen have been well documented. However, its involvements in adaptive immunity are poorly defined. In the present study, we demonstrate for the first time that SRA$^{-/-}$ DCs are more effective than WT counterparts in priming antigen-specific T-cells in the context of cross-presentation of cell-associated antigen derived from prostate cancer cells. Further, the increased immunostimulatory capability of SRA$^{-/-}$ DCs, indicated by enhanced T-cell priming and antitumor response, appears to involve the SRA/CD204 absence promoted activation of DCs. Our studies provide an explanation for the enhanced immunogenicity of dying tumor cells in SRA$^{-/-}$ mice and extend our previous finding showing that SRA/CD204 attenuates a CTL response following immunization with a soluble protein antigen in the presence of an adjuvant engaging pathogen-sensing toll like receptor (TLR) signaling [17].

Although SRA/CD204 has been implicated in clearance of apoptotic cells [19], no obvious phagocytic deficiency in vivo was observed in SRA$^{-/-}$ mice under normal and enhanced apoptotic cell load [23]. No significant difference between WT and SRA$^{-/-}$ DCs was observed in the uptake of dying tumor cells, suggesting that the presence of other redundant endocytic receptors on the cell surface may have compensated for the loss of SRA/CD204 [23], [24]. Therefore, it is likely that SRA/CD204-mediated immune suppression of OVA-derived from dying RM1 tumor cells may not primarily involve the phagocytic activity of SRA/CD204. Interestingly, the lack of SRA/CD204 renders DCs more responsive to stimulation by dying RM1 cells, as indicated by increased production of several inflammatory mediators as well as up-regulation of co-stimulatory molecule CD86. These findings are in line with our previous observation [17] and report by others [15], [16], [25] in the context of TLR-mediated recognition of PAMPs, suggesting that SRA/CD204 may act as a negative regulator for DC maturation and inflammatory response upon stimulation with both endogenous and foreign inflammatory molecules.

DCs are sentinels distributed around the body that sense ‘dangers’ though PRRs and play essential roles in immune initiation. The current work provides the first evidence that SRA/CD204 appears to block the activation of DCs upon encountering dying tumor cells, therefore, suppressing subsequent T-cell priming. Identities of the molecules released from damaged or dying tumor cells that are able to provide inflammatory stimulatory signals or

adjuvant activities remain to be determined [22]. We speculate that endogenous alarmin or 'danger' molecules, such as stress/heat shock proteins [26], the high-mobility-group box 1 (HMGB1) [27] or uric acid [28], may be involved. These endogenous damage-associated molecular patterns (DAMPs) have also been shown to engage TLR signaling [27], [29], which is required for DCs to efficiently process and cross-present antigen from dying tumor cells [30]. Given the involvement of SRA/CD204 in inflammatory responses induced by both foreign and endogenous self molecules, our studies underscore an important role that SRA/CD204 plays in maintenance of immune homeostasis.

Although our studies using both in vitro and in vivo models support a regulatory role of SRA/CD204 in DC-mediated cross-presentation of cell-associated antigen in the context of anti-tumor immunity, a recent report by Tewalt *et al* was not able to show the SRA/CD204 effect in the context of an anti-viral response and gp96-peptide complexes-elicited immune response [31]. In their studies, immunizations were performed either by infecting mice with viruses or gp96-peptide complexes-pulsed DCs. Compared to the gp96-peptide loaded DCs, it is likely that DCs exposed to dying tumor cells may receive additional inflammatory or stimulatory signals released during cell injury, which is clearly indicated by the increased cytokine production presented in our study. The ability of SRA/CD204 to attenuate inflammatory activation of DCs, as shown in our earlier studies, [17] may result in the different results seen in these two studies. In addition, we delivered DC vaccines subcutaneously, whereas Tewalt *et al* administrated viruses and DCs intravenously or intraperitoneally. Given that many cell types and mechanisms have been implicated in the cross priming pathway, it is possible that different experimental models and delivery routes may also have contributed to the observed discrepancy.

In the present study, we demonstrate that prostate cancer cell-based vaccination results in a greater protection against tumor challenge in $SRA^{-/-}$ mice compared to WT mice, which is consistent with our previous findings of SRA/CD204 absence promoted immunogenicity of dying tumor cells [18]. The role of SRA/CD204 in immune tolerance is supported by a recent report showing that depletion of scavenger receptors was associated with higher autoantibody responses against self-antigens [32]. In our studies, immunization with $SRA^{-/-}$ DCs loaded with dying RM1-OVA cells generated a more potent OVA-specific immune response than did WT DCs, as indicated by higher antigen-specific $CD8^+$ T-cell frequency and increased IFN-γ production in OVA-specific T cells. Further, the absence of SRA/CD204 in DCs significantly enhanced antitumor efficacy augmented by tumor lysate-pulsed DCs, which correlates with increased immune responses against tumors and tumor-associated antigens. Based on the action of SRA/CD204 in DC-mediated cross-presentation of cells-associated antigen, we propose that increased immunogenicity of dying tumor cells is attributed to the altered APC functions in the absence of SRA/CD204, and that SRA/CD204 in APCs, such as DCs, may function as an immune inhibitory molecule involved in T-cell priming and antitumor immunity. Efficient cross-presentation of tumor-associated antigens is critical for determining host response to tumors and treatment outcomes of cancer immunotherapy. Given the promising results that DC-based vaccines have produced in both basic research and clinical trials [33], manipulation or selective targeting of SRA/

CD204 on APCs could lead to rationally designed and improved DC vaccines and cancer immunotherapeutic approaches.

Together, we have demonstrated that SRA/CD204 exhibits important immunoregulatory properties in the functional activation of DCs and represents one of key factors that determinate the immunogenicity of dying tumor cells. These results increase our understanding of SRA/CD204 as a multi-functional innate PPR in host homeostasis and immune tolerance, which also have important implications for vaccines, autoimmunity and inflammation.

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Abbreviations

SRA	scavenger receptor
DC	dendritic cell
PRR	pattern recognition receptor

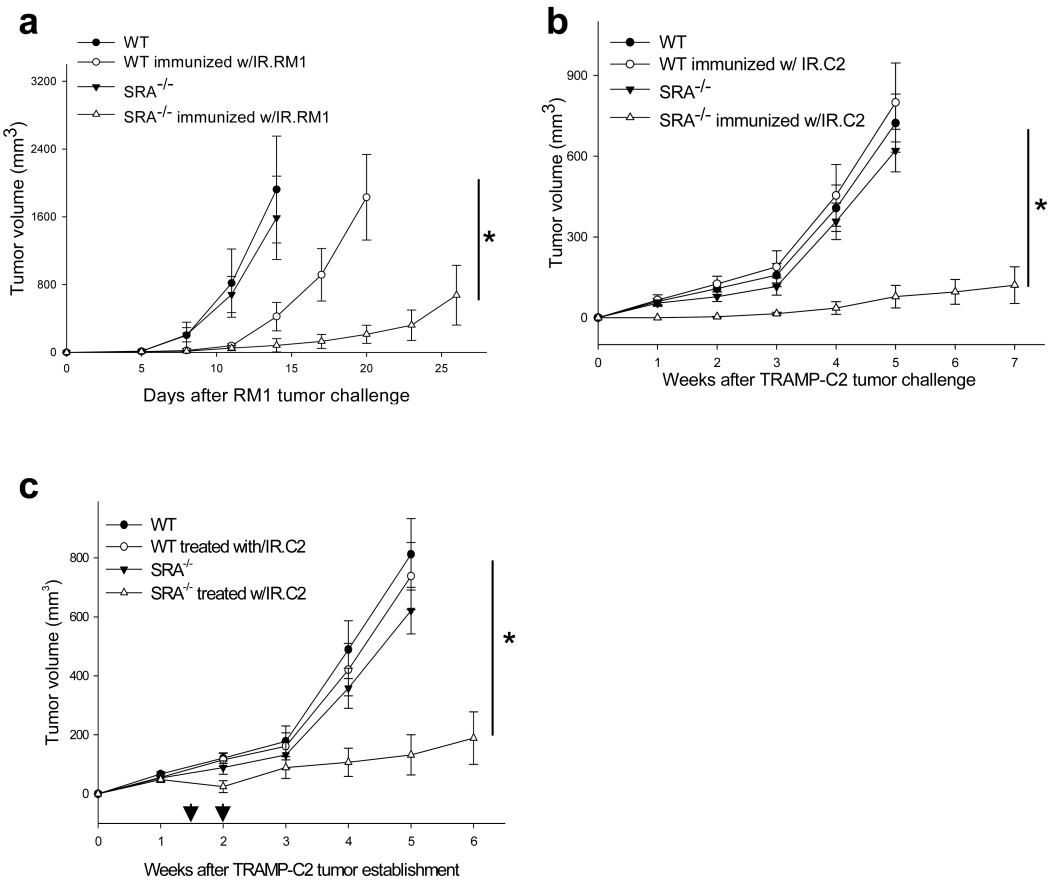

Figure 1. SRA/CD204 absence enhances the immunogenicity of dying prostate cancer cells

(**a-b**) Protective immunity induced by dying tumor cells. WT and SRA$^{-/-}$ mice (n=5) were immunized with ionizing radiation-treated RM1 cells (IR.RM1, **a**) or UV-treated TRAMP-C2 cells (IR.C2, **b**). Mice were then challenged with RM1 or TRAMP-C2 tumor cells, respectively. (**c**) Therapeutic efficacy of tumor cell-based vaccination. SRA$^{-/-}$ mice (n=5) established with TRAMP-C2 tumors were treated with IR.C2 cells on days indicated. * $p<$ 0.001. Values are mean ± SD. The results shown represent three independent experiments.

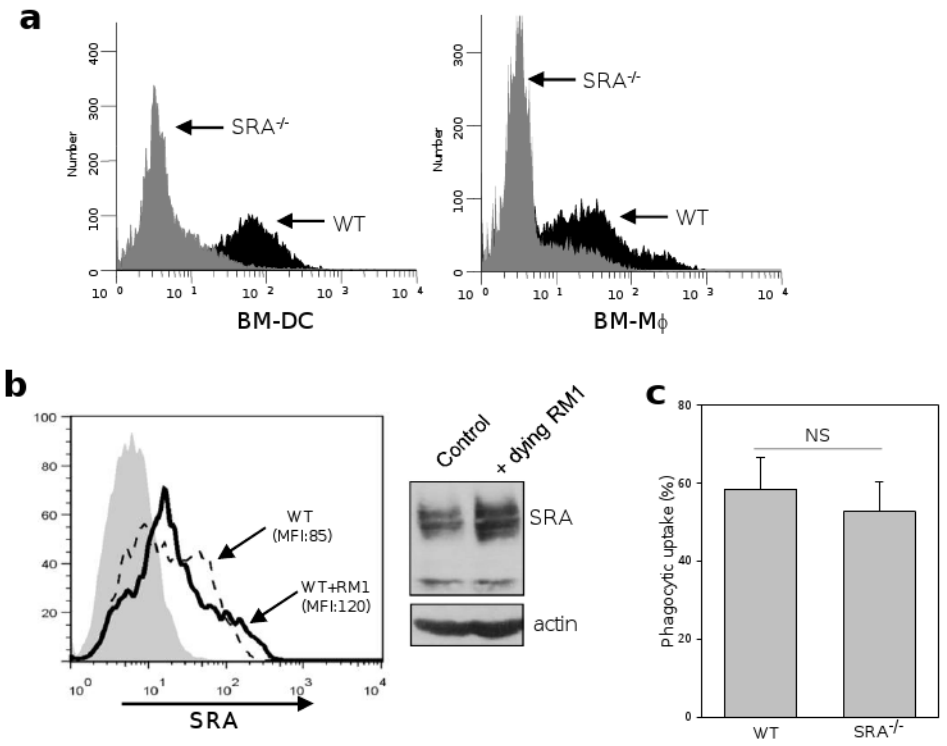

Figure 2. SRA/CD204 absence does not result in deficiency on DC phagocytosis

(**a**) Expression of SRA/CD204 in APCs. BM-DCs and BM-M were stained with anti-SRA/CD204 antibodies and subjected to FACS analysis. Cells derived from SRA$^{-/-}$ mice serve as controls. (**b**) BM-DCs were co-cultured with dying RM1 cells at 1:1 ratio for 24 h. Cells were stained with CD11c and SRA/CD204 antibodies and analyzed by flow cytometry (left panel). Filled grey histogram, isotype control; Dot lines, WT DCs; solid lines, WT DCs pulsed with dying RM1 cells. In addition, the increased SRA/CD204 expression in dying cell-pulsed DCs was confirmed by immunoblotting analysis using anti-SRA/CD204 antibodies (right panel). (**c**) BM-DCs from both WT and SRA$^{-/-}$ mice efficiently engulf apoptotic/dying cells. CFSE labeled, UV irradiated TRAMP-C2 cells were co-cultured with BM-DCs for 4 h. Cells were stained with anti-CD11c-PE antibodies, and phagocytosis was quantified by FACS as the percentage of double positive staining cells. Values are mean ± SD. * $p > 0.05$, the results representative of two independent experiments are shown.

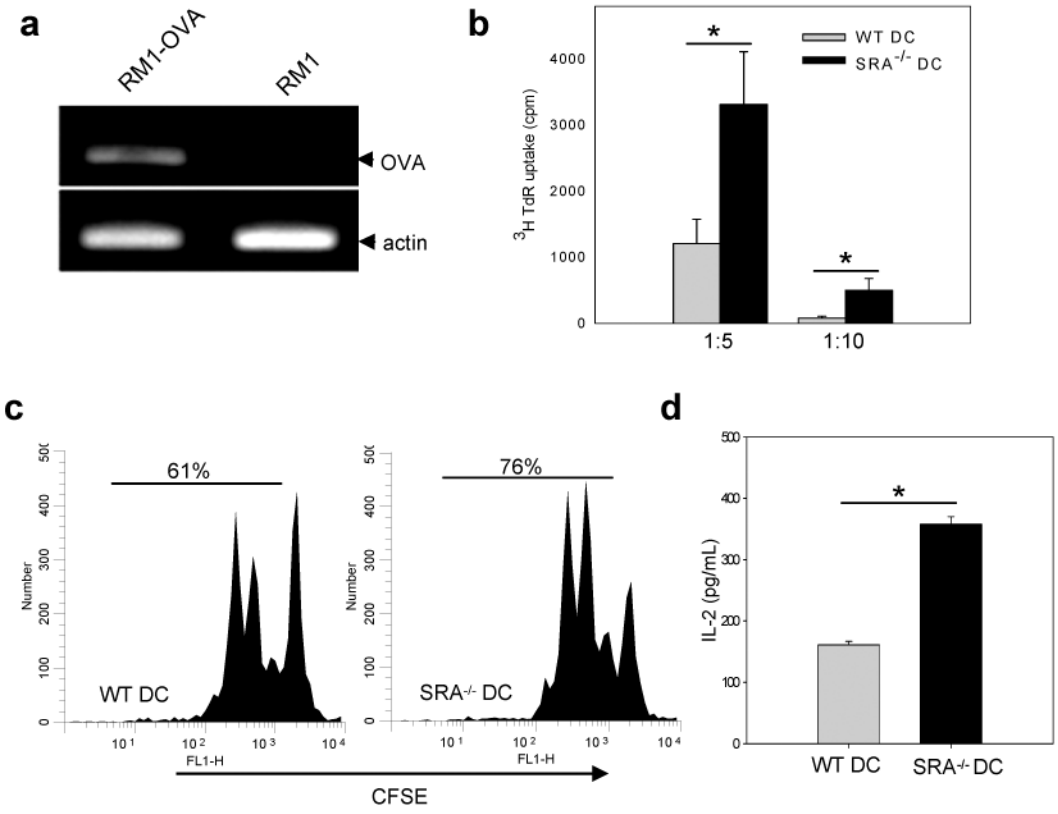

Figure 3. Lack of SRA/CD204 in DCs enhances cross-presentation of cell associated antigen and T-cell activation in vitro

(**a**) Establishment of RM1 prostate tumor cell line stably expressing OVA (RM1-OVA). RM1 cells were transduced with pcDNA-OVA using FuGENE transfection reagent and selected in G418-containing medium. Expression of OVA was analyzed using RT-PCR. (**b**) WT or SRA$^{-/-}$ DCs were pulsed with dying RM1-OVA cell lysates for 5 h, and co-cultured with OT-I cells for 72 h. OT-I cell proliferation was assessed by ^{3}H-thymidine incorporation assay. (**c**) WT or SRA$^{-/-}$ DCs pulsed with dying RM1-OVA cells were co-cultured with CFSE labeled OT-I cells. T-cell proliferation was assessed 48 h later by FACS analysis based on the dilution of CFSE intensity. (**d**) 48 h after co-culture, IL-2 concentrations in the culture media was determined by ELISA. Values are mean ± SD. * $p < 0.01$, the results representing at least three independent experiments are shown.

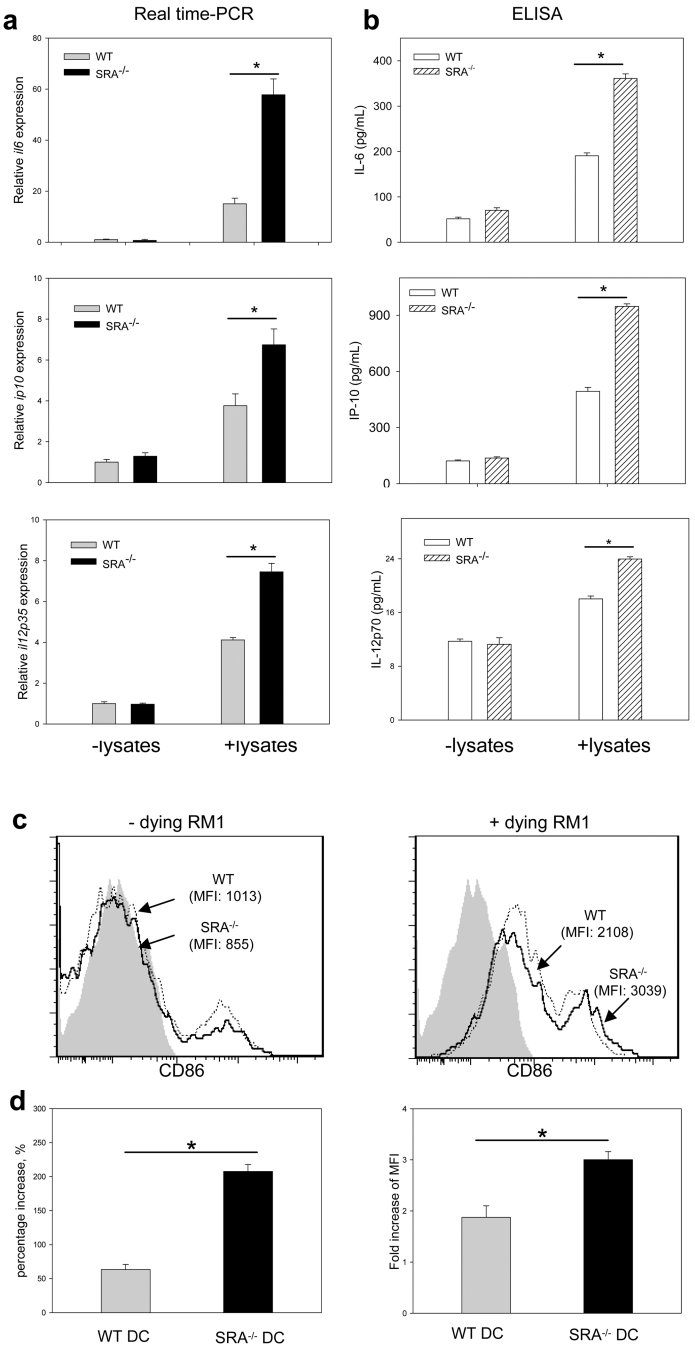

Figure 4. SRA/CD204 absence promotes an inflammatory response in BM-DCs pulsed with dying RM1 cells

(**a-b**) WT DCs and SRA$^{-/-}$ DCs were incubated with dying RM1 cells, followed by RNA preparations from DCs. Transcriptional levels of cytokines and chemokines were analyzed by quantitative real-time PCR using specific primers (**a**). β-actin is used as an internal control. In addition, protein levels of IL-6, IP-10 and IL-12 in the culture media were determined by ELISA (**b**). Values are mean ± SD. * $p< 0.01$, the results representing three independent experiments are shown. (**c**) WT and SRA$^{-/-}$ DCs were co-cultured with dying

RM1 cells at 1:1 ratio for 24 h and stained with antibodies for CD11c and CD86. Representative histogram profiles show CD86 expression in DCs with or without dying RM1 cell pulsing. Filled grey histogram, isotype control; Dot lines, WT DCs; solid lines, $SRA^{-/-}$ DCs. (**d**) Percentage increase and fold increase of MFI in CD86 expressing $CD11c^{+}$ cells. Values are mean ± SD. * $p < 0.01$.

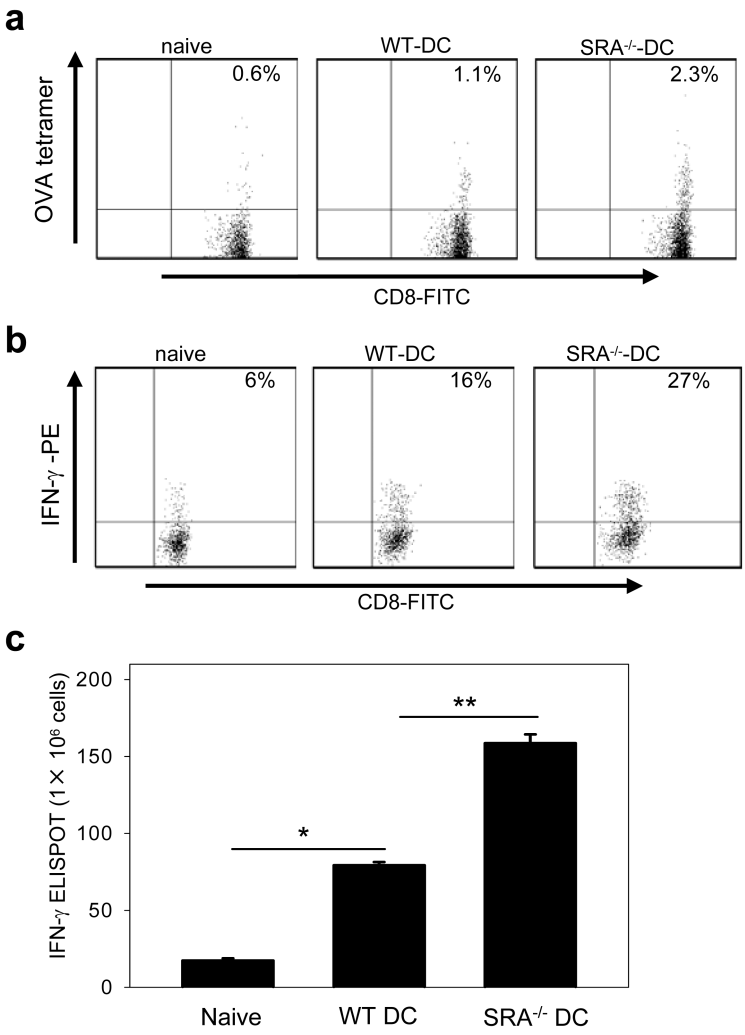

Figure 5. Immunization with SRA$^{-/-}$ DCs pulsed with dying RM1-OVA cells results in increased OVA-specific T-cell responses

(**a**) Mice were immunized with WT DCs or SRA$^{-/-}$ DCs pulsed with dying RM1-OVA cells. One week later, PBLs were stained with phycoerythrin-conjugated H-2Kb/OVA tetramer and FITC-labeled anti-CD8 antibodies, followed by FACS analysis for the frequency of OVA-specific CD8$^+$ T cells. (**b**) Following DC vaccination, splenocytes were stimulated with $OVA_{257-264}$ and the frequency of antigen-specific IFN-γ producing CD8$^+$ T cells were determined by intracellular cytokine staining. (**c**) ELISPOT assays were performed to assess the IFN-γ production in OVA-specific T-cells using splenocytes from immunized mice. Values are mean ± SD. *, ** $p < 0.01$. The results shown represent three independent experiments.

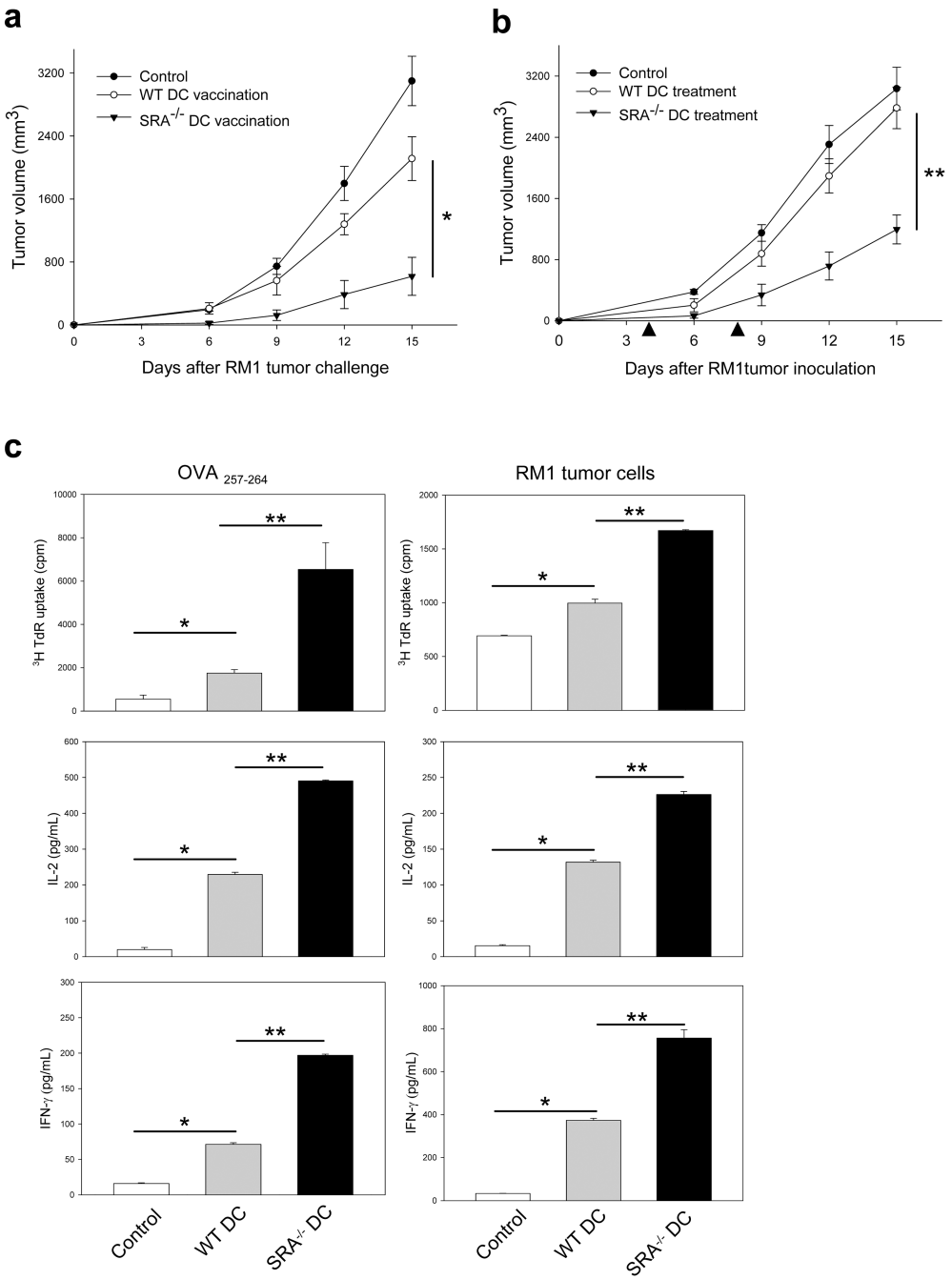

Figure 6. SRA absence promotes antitumor efficacy induced by vaccination with dying RM1 cells loaded DCs

(**a**) C57BL/6 mice were immunized with RM1 cell lysates loaded WT DCs or SRA$^{-/-}$ DCs twice at week intervals. One week later, mice were challenged with live RM1 cells. (**b**) C57BL/6 mice were inoculated with RM1 cells on day 0. On days 4 and 8, mice were treated with RM1 cell lysates loaded WT DCs or SRA$^{-/-}$ DCs. Values are mean ± SD. *, ** $p < 0.01$, the results representative of three independent experiments are shown. (**c**) RM1-OVA tumor-bearing mice cells were treated with as described. Splenocytes were stimulated

with $OVA_{257-264}$ or dying RM1 cells. Cell proliferation using measured using ^{3}H-thymidine incorporation assays. IL-2 and IFN-γ levels in the culture media were assayed using ELISA. Values are mean ± SD. *, ** $p < 0.01$, the results representative of two independent experiments are shown.