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CD204 Suppresses Large Heat Shock Protein-Facilitated Priming of Tumor Antigen gp100-Specific T Cells and Chaperone Vaccine Activity against Mouse Melanoma

Jie Qian,*†‡§ Huanfa Yi,*†‡§ Chunqing Guo,*†‡§ Xiaofei Yu,*†‡§ Daming Zuo,*†‡§ Xing Chen,‖ John M. Kane, III,‖ Elizabeth A. Repasky,‖ John R. Subjeck,‖ and Xiang-Yang Wang*†‡§

We previously reported that scavenger receptor A (SRA/CD204), a binding structure on dendritic cells (DCs) for large stress/heat shock proteins (HSPs; e.g., hsp110 and grp170), attenuated an antitumor response elicited by large HSP-based vaccines. In this study, we show that SRA/CD204 interacts directly with exogenous hsp110, and lack of SRA/CD204 results in a reduction in the hsp110 binding and internalization by DCs. However, SRA/CD204 was silenced in DCs using short hairpin RNA-encoding lentivirus. In addition, hsp110-stimulated SRA/CD204−/− DCs produced more inflammatory cytokines associated with increased NF-κB activation, implicating an immunosuppressive role for SRA/CD204. Immunization with the hsp110-gp100 vaccine resulted in a more robust gp100-specific CD8+ T cell response in SRA/CD204−/− mice than in WT mice. Lastly, SRA/CD204 absence markedly improved the therapeutic efficacy of the hsp110-gp100 vaccine in mice established with B16 melanoma, which was accompanied by enhanced activation and tumor infiltration of CD8+ T cells. Given the presence of multiple HSP-binding scavenger receptors on APCs, we propose that selective scavenger receptor interactions with HSPs may lead to highly distinct immunological consequences. Our findings provide new insights into the immune regulatory functions of SRA/CD204 and have important implications in the rational design of protein Ag-targeted recombinant chaperone vaccines for the treatment of cancer. *The Journal of Immunology, 2011, 187: 000–000.

Extensive studies have documented that stress proteins or heat shock proteins (HSPs) are capable of integrating both innate and adaptive immune responses and may be used as immunostimulatory adjuvants to develop novel immunotherapeutic approaches (1–3). Large HSPs (e.g., hsp110 and grp170) represent two members of the hsp70 superfamily (4). They are highly conserved molecular chaperones, which are much more efficient in binding to and stabilizing client proteins during heat shock (4–7). We exploited this superior chaperoning property of large HSPs to create targeted cancer vaccines by heating recombinant large HSPs in the presence of clinically relevant protein tumor Ags (e.g., gp100, HER-2/neu), causing them to form chaperone complexes (8–11). We demonstrated that this chaperone/tumor protein Ag complex can effectively generate Ag-specific tumor immunity in various preclinical murine models (8–11).

A number of studies suggested that the immunostimulating potency of exogenously delivered HSP-based vaccines is generated via specific interaction of HSPs with various receptors on APCs. Over the last few years, a major emphasis has been placed on the identification of HSP-binding receptors and characterization of their roles in HSP-facilitated Ag cross-presentation and immune activation. Indeed, several receptors on highly specialized APCs (e.g., dendritic cell [DC]), including CD91 (12, 13), LOX-1 (14), SRA/CD204 (15, 16), SREC (17–19), FEEL-1 (20), CD14 (21), CD40 (22, 23), and TLR2/4 (24, 25), were shown to participate in HSP-mediated Ag uptake, as well as immunostimulatory activities. However, the precise contribution of these receptors to HSP-promoted adaptive T cell responses in vivo and antitumor immunity has not been well defined.

Interestingly, scavenger receptors, such as SRA/CD204, LOX-1, SREC, and FEEL-1, seem to be major players among the HSP-binding receptors. SRA/CD204 is the first cloned member of the structurally diverse scavenger receptor family (26, 27). Our recent finding of SRA/CD204 as a binding receptor for large HSPs (16) prompted initial study of whether SRA/CD204 participated in large HSP-augmented antitumor effects. Strikingly, we observed...
that large HSP (i.e., gp170)-induced tumor-protective response remained intact and was profoundly enhanced in SRA/CD204 knockout mice (28). In light of the primary expression of SRA/CD204 on APCs, as well as DCs, we investigated the effect of large stress protein–SRA/CD204 interaction on recombiant chaperone vaccine-promoted activation of Ag (i.e., gp100)-specific CD8+ T cells and resultant antitumor activity in melanoma-bearing mice. Our results confirmed that SRA/CD204 acts as a bona fide binding receptor for hsp110 on the surface of DCs. However, the presence of SRA/CD204 greatly reduced hsp110-mediated immunostimulation of DCs, as well as activation of CD8+ T cells reactive with melanoma-associated Ag gp100. Furthermore, the magnitude and quality of chaperone vaccine-induced CD8+ T cell responses were substantially enhanced in SRA/CD204 knockout mice compared with wild-type (WT) mice, leading to improved tumor control in a therapeutic setting. Our observations suggested that receptors involved in binding/uptake of exogenous HSPs on APCs do not necessarily or always facilitate the cross-presentation of HSP-shuttled Ags or CD8+ T cell activation. Given the presence of multiple HSP-binding scavenger receptors on APCs, our findings provide the rationale for selective targeting of functionally distinct scavenger receptors to achieve maximum therapeutic activities of recombiant chaperone vaccines.

Materials and Methods

Mice and cell lines

C57BL/6 mice were purchased from the National Institutes of Health (Bethesda, MD). SRA/CD204 knockout mice (SRA−/−) and pmel transgenic mice carrying TCR transgene specific for the mouse homolog (pnel-17) of human gp100 (29) were purchased from the Jackson Laboratory (Bar Harbor, ME). Melanoma cell line B16-gp100 was maintained in DMEM, supplemented with 10% heat-inactivated FBS (Life Technologies, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All experimental procedures were conducted according to the protocols approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (Richmond, VA).

Reagents and Abs

Recombinant proteins, including hsp110, gp170, and gp100, were expressed in a BacPak baculovirus-expression system (BD Biosciences Clontec, Palo Alto, CA), as previously described (7, 8). All glassware was depyrogenated for 4 h at 250°C to avoid or reduce endotoxin contamination as much as possible. Endotoxin levels in the recombiant HSP preparations are always <10–15 EU/mg protein, measured using a Limulus Ameobocyte Lysate Kit (BioWhittaker, Walkersville, MD). In some experiments, hsp110 was preincubated with polymyxin B (1 μg/ml), incubated with proteinase K (50 μg/ml) at 50°C for 1 h, or boiled for 5 min before incubation with DCs.

Immunoblotting and immunoprecipitation

Immunoblotting and immunoprecipitation assay buffer (50 mM Tris-Cl [pH 7.4], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 μg/ml each of aprotinin and leupeptin, and 1 mM Na2VO3). For immunoprecipitation, 1 mg cell extracts was incubated with 2 μg Abs for 2 h at 4°C, followed by incubation with 40 μl Protein A/G-plus agarose (Santa Cruz Biotechnology) overnight at 4°C. The beads were washed with radio-immunoprecipitation assay buffer, and immune complexes were eluted by boiling in 2× SDS Laemmli loading buffer for 5 min. For immunoblotting, 20–50 μg protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose mem-

In vitro stimulation of T cells

For preparation of protein Ag chaperone complexes, recombinant gp100 protein and HSPs (1:1 molar ratio) were incubated at 50°C for 30 min, followed by incubation at 37°C for 30 min, as previously described (8). Bone marrow-derived DCs (BMDCs) were generated from bone marrow cells in the presence of mouse GM-CSF from PeproTech (Rocky Hill, NJ) (28). BMDCs were pulsed with gp100 protein (30 μg/ml) or hsp110-gp100 complexes (30 μg/ml) for 3 h. DCs were washed and incubated with 5×106 pmel cells in 200 μl RPMI 1640 medium in a round-bottom 96-well microtiter plate. Cells were cultured for 60 h and pulsed with [3H]thymi-
dine ([3H]Tdr; 0.5 μCi/well) during the last 16 h of culture. T cell proliferation was assessed using [3H]Tdr-incorporation assays. Levels of cytokine IL-2 or IFN-γ in the culture supernatant were determined using ELISA kits from eBioscience (San Diego, CA).

Lentivirus-mediated gene silencing

Lentiviruses (LVs) encoding mouse SRA/CD204 short hairpin RNA (shRNA) or scramble shRNA were packaged using Phoenix cells co-transfected with pLKO.1 constructs and pMD.G and pCMVΔR8.91. Day-6 BMDCs were infected with LV-scramble shRNA or LV-SRA shRNA in the presence of 4 μg/ml polybrene and 20 ng/ml GM-CSF. Cells were collected 48 h later, washed extensively, and used for immunoblotting or T cell-priming assays.

NF-κB–activation assays

For luciferase assays, DC1 cells (3×106 cells/well) in a 24-well plate were transiently transfected with pCMV-SRA or empty vector, together with different amounts of pGL3–NF-κB–Luc using Fugene HD reagent from Roche (Indianapolis, IN). The plasmid pRL-TK was used as an internal control. Twenty hours after transfection, cells were stimulated with hsp110 or BSA (80 μg/ml) for 12 h. Luciferase activity was determined using a Glomax luminometer from Promega (Madison, WI). For endogenous NF-κB activation, nuclear extracts were prepared using a Nuclear Extract kit from Active Motif (Carlsbad, CA). p65 NF-κB transcriptional activity was measured using an ELISA-based TransAM NF-κB p65 transcription factor assay kit (Active Motif). Colorimetric reactions were developed and measured using a Microplate reader at 450 nm, with a reference wave-

Adoptive cell transfer

Lymph node cells from pmel mice were prepared in a single-cell suspension, and 2×106 pmel cells were transferred into recipient mice by tail vein injection. The next day, mice were immunized i.p. with gp100-gp100 complexes (30 μg/gp100 mixed with 42 μg hsp110 at 1:1 molar ratio). Spleen and lymph nodes were harvested 5 d later, and erythrocytes were removed by hypotonic lysis or Ficol-gradient separation. Pmel cell prolif-
eration was measured by staining cells with CD90.1 and CD8 Abs, followed by FACS analysis. Intracellular IFN-γ staining was performed using the Cytofix/Cytoperm kit from BD Biosciences (San Jose, CA), according to the manufacturer’s instructions.

Immunization and T cell-functional assays

Mice were immunized s.c. twice at 2-week intervals with 30 μg gp100 complexed with hsp110 or gp170 at a 1:1 molar ratio. One week after immunization, splenocytes or lymph node cells were stimulated with gp100 (3×105) peptide (1 μg/ml) or gp100 protein (20 μg/ml) at 37°C for 48 h. For intracellular IFN-γ staining, cells were treated with brefeldin A (5 μg/ml, BD GolgiPlug; BD Biosciences) for 3 h at 37°C. In some cases, cells were stimulated with PMA (50 ng/ml) plus ionomycin (1 μg/ml). Cells were stained with anti–CD8–FITC Abs, followed by fixation, perme-

Immunoblotting and immunoprecipitation

Cells were washed with ice-cold PBS and lysed in modified radio-
immunoprecipitation assay buffer (50 mM Tris-Cl [pH 7.4], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSE, 1 μg/ml each of aprotinin and leupeptin, and 1 mM Na2VO3). For immunoprecipitation, 1 mg cell extracts was incubated with 2 μg Abs for 2 h at 4°C, followed by incubation with 40 μl Protein A/G-plus agarose (Santa Cruz Biotechnology) overnight at 4°C. The beads were washed with radio-immunoprecipitation assay buffer, and immune complexes were eluted by boiling in 2× SDS Laemmli loading buffer for 5 min. For immunoblotting, 20–50 μg protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose mem-

ELISA assays (8). To measure cytokolytic activity of effector T cells, splenocytes were stimulated with mitomycin C-treated mouse GM-CSF (10 IU/ml) and subjected to ELISA assays (8). To measure cytokolytic activity of effector T cells, splenocytes were stimulated with mitomycin C-treated tumor cells and then cocultured with 35Cr-labeled B16 cells at varying E:T ratios, as previously described (8, 30).

Tumor studies

For the tumor-prevention study, mice were immunized with hsp110-gp100 protein complexes twice at a 1 wk interval. One week later, mice were challenged s.c. with 4×106 B16-gp100 tumor cells. For the tumor-therapy study, mice were established with tumors by injection of 2×106 B16-gp100 cells on day 0. Tumor-bearing mice were treated with
hsp110-gp100 protein complexes on days 4, 7, and 10. Tumor growth was monitored by measuring perpendicular tumor diameters using an electronic digital caliper. The tumor volume was calculated using the formula $V = \frac{\text{shortest diameter} \times \text{longest diameter}}{2}$. Collected tumor tissues were digested and analyzed using FACS analysis, as described previously (11).

### Statistical analysis

Comparisons between two groups were performed using the Student t test. Comparisons among multiple groups were carried out using ANOVA. A $p$ value $<0.05$ was considered statistically significant.

### Results

**SRA/CD204 directly interacts with exogenous hsp110**

To examine the direct interactions between hsp110 and SRA/CD204, we performed reciprocal immunoprecipitation studies using BMDCs after coculturing with recombinant His-tagged hsp110 protein (Hisp110). It was noted that exogenous hsp110 was associated with endogenous SRA/CD204 pulled down by the specific Abs (Fig. 1A, top panels). Similarly, SRA/CD204 was bound to hsp110 precipitated by anti–His-tag Abs (Fig. 1A, bottom panels). In addition, His-hsp110, but not His-tagged gp100 protein (His-gp100) that was prepared similarly using the baculovirus-insect system, was able to bind to SRA/CD204 (Fig. 1B), which rules out the potential effect of His-tag on hsp110 binding to SRA/CD204. Moreover, excess of His-gp100 did not interfere with association of biotinylated His-hsp110 with SRA/CD204, whereas excess “cold” His-hsp110 effectively blocked the binding of biotinylated His-hsp110 to SRA/CD204 (Fig. 1C, left panels). Lastly, binding of recombinant His-hsp110 to SRA/CD204 could be efficiently inhibited by autologous hsp110 that was purified from spleens, as previously described (31) (Fig. 1C, right panels). These results confirmed our previous observation that SRA/CD204 on DCs serves as a binding structure for exogenous large HSPs (16).

We next examined the effect of SRA/CD204 on binding/internalization of exogenous hsp110 using WT and SRA$^{-/-}$ DCs. Following incubation with His-hsp110 at 4°C, cells were washed to remove unbound hsp110 and continued to be cultured at 37°C. Cell lysates were prepared at different times and analyzed for the presence of His-tagged hsp110 by immunoblotting. Lack of SRA/CD204 resulted in reduced binding of hsp110 by SRA$^{-/-}$ DCs at 4°C (Fig. 1D). However, we did not see the significant difference in His-hsp110 levels between WT and SRA$^{-/-}$ cells after DCs were moved to 37°C, probably due to the less-optimal binding of hsp110 at the low temperature and the rapid degradation of hsp110 protein at 37°C. We next performed the uptake study by incubating DCs with His-hsp110 for 30 min at 37°C. Cells were then washed and continued to be cultured at 37°C for

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** SRA/CD204 absence reduces binding and internalization of hsp110. A, SRA/CD204 directly interacts with exogenous hsp110. WT BMDCs were incubated with His-hsp110 at 4°C for 30 min and then washed extensively with PBS. Cell lysates were immunoprecipitated with Abs for SRA/CD204 (top panels) or His-tag (bottom panels). The immunocomplexes were subjected to immunoblotting analyses using Abs for His-tag (1:4000; top panels) or SRA/CD204 (1:3000; bottom panels). IgG served as a negative control. B, BMDCs were incubated with His-hsp110 or His-gp100 protein. SRA/CD204 was immunoprecipitated and analyzed for its association with hsp110 or gp100 using His-tag Abs. C, Competition assays. DCs were incubated with biotinylated His-hsp110 (40 μg/ml) in the presence of 3× or 9× excess of His-gp100 or His-hsp110. Immunocomplexes pulled down by SRA/CD204 Abs were analyzed by immunoblot using avidin-HRP or anti-SRA Abs (left panels). Alternatively, DCs were incubated with His-hsp110 in the presence of BSA or autologous hsp110-purified from mouse spleen, followed by immunoblotting analysis of His-hsp110 association with immunoprecipitated SRA/CD204 (right panels). D and E, SRA/CD204 absence results in reduced hsp110 binding and internalization. WT and SRA$^{-/-}$ BMDCs were incubated with His-hsp110 for 30 min at either 4°C (D) or 37°C (E). Cells were washed and cultured at 37°C for the times indicated. Total-cell lysates were prepared and analyzed for the presence of His-hsp110 using His-tag Abs. Immunoblots were quantified by densitometry analysis using ImageJ. The data are presented as fold of change in protein expression for each sample compared with WT DCs at 0 min. The ratio of His-hsp110 to β-actin in WT DCs at 0 min is set to 1. Representative results from three independent experiments with similar results are shown. *$p < 0.01$. 


SRA/CD204 suppresses hsp110-facilitated T cell stimulation in vitro

In vitro T cell-stimulation assays were carried out to assess the effect of SRA/CD204 on the cross-presentation of hsp110-associated tumor protein Ag gp100. WT DCs or SRA/CD204 DCs were pulsed with hsp110 alone, gp100 alone, or hsp110-gp100 chaperone complexes and subsequently used to stimulate naïve gp100-specific CD8+ T cells (i.e., pmel cells) derived from the pmel-transgenic mice. As expected, complexing gp100 to hsp110 greatly enhanced the efficiency of DCs to stimulate the proliferation of pmel cells (Fig. 2A). However, SRA/CD204 DCs were much more effective than were WT DCs in driving pmel cell proliferation, as indicated by increased incorporation of [³H]TdR (Fig. 2A). ELISA assays showed that higher levels of IL-2 were present in the supernatant of pmel cells cultured with SRA/CD204 DCs compared with those cultured with WT DCs (Fig. 2B). An irrelevant protein control (i.e., BSA) was included in pmel cell-stimulation assays. In contrast to the distinct effects of SRA/CD204 on gp100 chaperone complex-treated DCs, WT and SRA/CD204 DCs pulsed with BSA-gp100 activated pmel cells in a comparable manner (Fig. 2C). We also compared the general Ag-presenting ability of WT and SRA/CD204 DCs by introducing model Ag OVA protein into the cytosol of DCs. FACS analysis showed similar levels of H2Kb-OVA257–264 (SIINFEKL) peptide complexes on the cell surface, suggesting that the loss of SRA/CD204 did not affect the presentation of endogenous Ag to MHC class I (Supplemental Fig. 1). The enhanced DC immunogenicity was also observed when DCs were pulsed with gp100 complexed with grp170, another large HSP and chaperone molecule resident in the endoplasmic reticulum (Fig. 2D).

To validate the suppressive activity of SRA/CD204 in hsp110-facilitated T cell activation, LVs encoding shRNA were used for SRA/CD204 silencing in BMDCs. Scramble shRNA or SRA/CD204 shRNA-transduced DCs were incubated with hsp110-gp100 complexes prior to coculture with gp100-specific CD8+ T cells. SRA/CD204-silenced DCs were more efficient than were scramble shRNA-treated cells in stimulating pmel cell proliferation (Fig. 2E). Similar results were seen in DCs loaded with gp100 chaperone complexes reconstituted in the presence of grp170 (Fig. 2F). In addition, gp100-specific pmel cells produced much more IFN-γ after coculture with SRA/CD204-silenced DCs that had been exposed to hsp110-gp100 or grp170-gp100 complexes (Fig. 2G), suggesting that SRA/CD204 is directly involved in the negative regulation of large chaperone vaccine-facilitated T cell activation.

SRA/CD204 attenuates hsp110-induced inflammatory activation of DCs

Given that HSPs, including large HSPs, have been shown to act as danger signals that stimulate an innate immune response, we...
examined the effect of SRA/CD204 on the production of inflammatory cytokines in response to hsp110 stimulation. ELISA assays showed that SRA−/− DCs secreted more TNF-α, IL-6, and IL-12p40 after treatment with hsp110 (Fig. 3A). However, WT and SRA−/− DCs displayed similar responses upon stimulation with TNF-α, which is not a ligand of SRA/CD204, as indicated by a comparable production of cytokine IL-6 (Supplemental Fig. 2).

To rule out the possibility of endotoxin contamination in the hsp110 preparations, we examined hsp110 activity in the presence of proteinase K and endotoxin inhibitor polymyxin B. Proteinase K treatment of hsp110 completely abolished the effect of hsp110 on IL-6 production by DCs, whereas the effect of LPS remained intact (Fig. 3B). In addition, polymyxin B treatment effectively blocked the stimulatory activity of LPS but not hsp110 protein (Fig. 3B).

We also examined surface expression of costimulatory molecules by FACS analysis of CD11c+ cells after exposure to hsp110 in vitro. There was no significant difference in the levels of CD80 and CD86 between WT and SRA−/− cells (data not shown). However, the percentage of CD40-expressing SRA−/− DCs seemed to be modestly higher than WT DCs after treatment (Fig. 3C), which is consistent with an earlier report of increased CD40 expression in SRA−/− DCs in response to inflammatory stimulus (32). Boiling of hsp110 completely abolished the stimulatory activity of hsp110 (Fig. 3C), implicating a protein-mediated effect.

NF-κB is a master transcription factor that regulates many inflammatory genes involved in immunity. We prepared nucleic protein lysates from hsp110-stimulated BMDCs and measured p65 NF-κB transcriptional activity using an ELISA-based TransAM NF-κB–transcription assay. The increased nuclear translocation and phosphorylation of p65 subunit of this transcription factor were shown in hsp110-stimulated SRA−/− DCs compared with WT cells (Fig. 3D). We also examined the potential effect of SRA/CD204 on the levels of IκBα, an inhibitor that can sequester NF-κB in the cytoplasm. Thirty minutes after treatment with hsp110, SRA−/− DCs displayed lower levels of total IκBα than did WT DCs (Fig. 3E). Furthermore, we assessed the ability of SRA/CD204 to interfere with hsp110-induced NF-κB–dependent luciferase activity in DCs. Cells were transfected with pCMV-SRA together with a luciferase reporter construct controlled by a NF-κB promoter (pGL3-5×NF-κB). Transfection of SRA/CD204 reduced NF-κB–dependent luciferase activity effectively after hsp110 stimulation compared with mock-treated

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**FIGURE 3.** Lack of SRA/CD204 promotes inflammatory cytokine production by DCs upon stimulation with hsp110. A, WT or SRA−/− DCs (1×10⁶ cells) in a 24-well plate were incubated with hsp110 (80 μg/ml) for 24 or 48 h; levels of TNF-α, IL-6, and IL-12p40 in the culture media were determined by ELISA. Values are mean ± SD. The results shown represent three independent experiments. B, Immunostimulatory activity of hsp110 is not due to endotoxin contamination. BMDCs were stimulated or not with hsp110 treated with proteinase K or polymyxin B. IL-6 levels were assayed using ELISA 48 h later. C, BMDCs were treated with hsp110 or boiled hsp110 for 48 h and subjected to FACS analysis using anti-CD11c Abs (N418). D, Enhanced translocation of phosphorylated NF-κB p65 in SRA−/− DCs. Nuclear extracts were prepared after hsp110 treatment. NF-κB activation was determined by ELISA assays, which specifically measures the level of phosphorylated NF-κB p65 bound to its consensus sequence 5′-GGGACTTTCC-3′. Values are mean ± SD. Results shown are from three independent experiments. E, Enhanced IκBα degradation in SRA−/− DCs. Cell lysates were prepared after hsp110 treatment and subjected to immunoblotting using anti-IκBα Abs. β-actin served as a loading control. Immunoblots were quantified by densitometry analysis using ImageJ. The data are presented as fold change in protein expression relative to WT DCs at 0 min. F, Overexpression of SRA/CD204 blocks NF-κB–dependent luciferase activities. DC line was transfected with a 5× NF-κB luciferase reporter construct together with SRA/CD204 expression plasmid or control vector. Cells were then stimulated with hsp110 or BSA for 12 h. Hsp110 was also used to stimulate DCs after boiling for 5 min or in the presence of polymyxin B. Luciferase activities were determined by a dual-luciferase reporter assay, normalized by Renilla luciferase activity and expressed as fold induction relative to the activity in unstimulated cells. Values are mean ± SD. Results shown are representative of two independent experiments. *p < 0.01.
cells (Fig. 3F). Disruption of hsp110 protein functions by boiling abolished the hsp110 effect on luciferase activity, whereas the presence of polymyxin B had no effect (Fig. 3F).

**SRA/CD204 inhibits large chaperone vaccine-induced activation of adoptively transferred gp100-specific CD8+ T cells**

To determine the effect of SRA/CD204 on chaperone complex vaccine-activated CD8+ T cell responses, gp100-specific CD90.1+ T cells from pmel-transgenic mice were transferred to WT or SRA-/- mice, followed by immunization with hsp110-gp100 complexes. Although there was little proliferation of pmel cells in nonimmunized mice, CD90.1+CD8+ pmel cells in spleens and lymph nodes displayed a robust expansion in SRA-/- mice compared with WT mice (4.45 ± 0.36% versus 2.12 ± 0.18% in spleens; 10.85 ± 0.93% versus 5.12 ± 0.58% in lymph nodes) (Fig. 4A). These pmel cells in WT and SRA-/- mice similarly expressed an activated phenotype, with high expression of CD44 and CD69 and reduced expression of CD62L (data not shown). We also assessed the effect of SRA/CD204 on the functional status of gp100-specific CD8+ T cells in the immunized mice using intracellular cytokine staining. Hsp110-gp100 complex-immunized SRA-/- mice showed a marked increase in the levels of gp10025–33-specific, IFN-γ-producing CD8+ T cells compared with WT mice (Fig. 4B, 3- and 1.8-fold increase in spleen and lymph node, respectively). Additionally, we examined the SRA/CD204 effect on T cell activation following immunization with gp100 in the presence of the widely used Alum adjuvant. Gp100-specific pmel T cells displayed similar proliferation in WT and SRA-/- mice after vaccination with gp100-Alum, suggesting that SRA-/- mice are functionally equivalent to WT mice in the context of immunization with Alum adjuvant (Supplemental Fig. 3).

**SRA/CD204 suppresses chaperone vaccine-induced activation of endogenous gp100-specific CD8+ T cells**

To further determine the effect of SRA/CD204 on the hsp110-gp100 complex-augmented CD8+ T cell response against gp100, WT and SRA-/- mice were immunized with gp100 chaperone vaccine, and lymphoid tissues were subjected to intracellular IFN-γ-staining analysis. Vaccinated SRA-/- mice showed a higher percentage of IFN-γ-expressing CD8+ T cells than did WT mice in both spleens and lymph nodes (Fig. 5A), which is consistent with the data from the adoptive cell-transfer study. Chromium release assay was performed to assess effector T cell functions after vaccination. Compared with WT counterparts, T cells from immunized SRA-/- mice exhibited increased cytolytic activities against B16-gp100 cells (Fig. 5B). The enhanced T cell response in the absence of SRA/CD204 was also observed after mice were immunized with the grp170-gp100 chaperone complexes. Upon stimulation with either gp10025–33 peptide or gp100 protein, splenocytes from SRA-/- mice produced more IFN-γ than did those from WT mice (Fig. 5C). A higher frequency of IFN-γ-expressing cells was seen in gp100 protein-stimulated splenocytes, probably due to the stimulation of T cells capable of recognizing multiple T cell epitopes derived from gp100 protein chaperoned by the grp170 (Fig. 5C).

**SRA/CD204 downregulates the antitumor efficacy of the hsp110-gp100 chaperone vaccine**

To assess the impact of SRA/CD204 on chaperone vaccine-augmented antitumor immunity, mice were immunized with the
hsp110-gp100 complexes, followed by challenge with B16-gp100 tumor cells. Hsp110-gp100 vaccine provided an enhanced tumor-protective effect in SRA<sup>−/−</sup> mice compared with WT mice (Fig. 6A).

We next examined whether the improved antitumor efficacy observed in the absence of SRA/CD204 was caused by enhanced immune responses. Tumor-draining lymph node cells from treated or untreated tumor-bearing mice were stimulated with gp100<sub>25–33</sub> peptide and analyzed by intracellular cytokine staining. A significant increase in the percentage of IFN-γ–producing CD8<sup>+</sup> T cells was seen in treated tumor-bearing SRA<sup>−/−</sup> mice compared with corresponding WT mice (Fig. 7A). In addition, FACS analysis showed increased tumor infiltration of CD8<sup>+</sup> cells in treated SRA<sup>−/−</sup> mice (Fig. 7B). Upon stimulation with B16-gp100 tumor cells, splenocytes from SRA<sup>−/−</sup> mice produced higher levels of IFN-γ than did those from WT mice, indicating an elevated immune reactivity with tumor cells in the chaperone vaccine-treated SRA<sup>−/−</sup> mice (Fig. 7C).

We also examined the gp100-specific Ab response in the chaperone vaccine-treated mice. There was no significant difference in the IgG levels against gp100 in WT and SRA<sup>−/−</sup> mice (Fig. 7D).

**Discussion**

In light of our unexpected observations that lack of SRA/CD204, a previously identified binding receptor for large HSPs, significantly promoted tumor-protective activities of large-chaperone (e.g., grp170) vaccines (28), we investigated the impact of SRA/CD204 suppression on the antitumor efficacy of hsp110-gp100 chaperone vaccine. A. Increased IFN-γ production by gp100-specific CD8<sup>+</sup> T cells in SRA<sup>−/−</sup> mice. Mice were immunized with the hsp110–gp100 complex. Splenocytes (SP) and lymph node (LN) cells were stimulated with gp100<sub>25–33</sub> and analyzed by intracellular cytokine staining.

**FIGURE 5.** SRA/CD204 suppresses the activation of endogenous gp100-specific CD8<sup>+</sup> T cells induced by the hsp110-gp100 chaperone vaccine. A, Increased IFN-γ production by gp100-specific CD8<sup>+</sup> T cells in SRA<sup>−/−</sup> mice. Mice were immunized with the hsp110–gp100 complex. Splenocytes (SP) and lymph node (LN) cells were stimulated with gp100<sub>25–33</sub> and analyzed by intracellular cytokine staining. B, Increased cytolytic activity of effector CD8<sup>+</sup> T cells in SRA<sup>−/−</sup> mice. Splenocytes were stimulated with gp100<sub>25–33</sub> in the presence of IL-2 for 5 d, and chromium-release assays were carried out using B16-gp100 cells as targets. Data shown as mean ± SD are representative of two experiments. C, Following immunization with gp170–gp100 complexes, splenocytes were stimulated with gp100<sub>25–33</sub> peptide or gp100 protein and assessed for IFN-γ production using ELISPOT assays. Representative results from three independent experiments are shown. *p < 0.01.

**FIGURE 6.** SRA/CD204 inhibits the antitumor efficacy of hsp110-gp100 chaperone vaccine. A, Increased tumor-protective activity of chaperone vaccine in SRA<sup>−/−</sup> mice. WT and SRA<sup>−/−</sup> mice (n = 5) were immunized with the hsp110–gp100 complexes and then challenged with 2 × 10<sup>6</sup> B16-gp100 tumor cells. Results are representative of two independent experiments. B, Enhanced therapeutic activity of chaperone vaccine in tumor-bearing SRA<sup>−/−</sup> mice. Mice (n = 5) were established with B16-gp100 tumors on day 0 and treated with the hsp110-gp100 vaccine on days 4, 7, and 10. Values are mean ± SD. Results representative of three independent experiments are shown. *p < 0.01.
CD204 on the immunogenicity of DCs in the context of large HSP-facilitated activation of CD8+ T cells specific for melanoma protein Ag gp100. Using both in vitro and in vivo systems, we demonstrated that SRA/CD204 represents an immune regulatory HSP-binding receptor that is able to attenuate large HSP-promoted CD8+ T cell activation, as well as gp100 chaperone vaccine-augmented therapeutic efficacy against established mouse melanoma.

The immunological function of HSPs that has been the greatest focus of attention is their ability to shuttle associated Ags into the endogenous-presentation pathway of APCs through interaction with surface receptors. To our knowledge, the current study provides the first biochemical evidence that SRA/CD204 serves as a binding structure on APCs and directly interacts with exogenous molecules and secretion of proinflammatory cytokines or chemokines. The present study showed that hsp110-promoted SRA−/− DCs display increased NF-κB activation and produce more proinflammatory cytokines than do WT DCs. Indeed, several independent studies reported that SRA/CD204 acts as a scavenger of inflammatory responses that limits the activation of macrophages or DCs (32, 37–40). In addition, we observed that hsp110 treatment resulted in an increase in SRA−/− DCs expressing CD40 compared with WT cells, which agrees with an earlier report by Platt and colleagues (32) showing that lack of SRA/CD204 promoted TLR-induced CD40 expression. It is of interest to note that CD40 on DCs was shown to be an HSP-binding receptor (22, 23, 34) and participate in the tolerance-breaking activity of hsp70 (41).

SRA/CD204, originally recognized for its ability to internalize or “scavenge” modified lipoproteins, has largely been studied in relation to atherosclerosis, as well as innate immunity against pathogen infection (42). Like other scavenger receptors, SRA/CD204 binds to a broad spectrum of ligands or macromolecules, including nonself (e.g., LPS), altered-self (e.g., oxidized low-density lipoprotein), and self (e.g., HSPs) components; therefore, internalized molecules could play an essential role in determining the subsequent immune consequences. As a scavenging molecule, SRA/CD204 might be able to target bound immunostimulatory molecules (e.g., hsp110) for degradation, and the absence of SRA/CD204 would lead to the prolonged presence of the intracellular hsp110–gp100 complex. However, immunoblotting analysis of the levels of internalized His-hsp110 at various time-points did not seem to support this possibility. More studies are necessary to define the molecular mechanisms by which SRA/CD204 downregulates the adjuvant activities of hsp110.

It has been well documented that APC–HSP interaction also promotes phenotypic and functional activation of APCs, such as DCs or monocytes, resulting in upregulation of costimulatory molecules and secretion of proinflammatory cytokines or chemokines (21, 34–36). The present study showed that hsp110-stimulated SRA−/− DCs display increased NF-κB activation and produce more proinflammatory cytokines than do WT DCs. Indeed, several independent studies reported that SRA/CD204 acts as a suppressor of inflammatory responses that limits the activation of macrophages or DCs (32, 37–40). In addition, we observed that hsp110 treatment resulted in an increase in SRA−/− DCs expressing CD40 compared with WT cells, which agrees with an earlier report by Platt and colleagues (32) showing that lack of SRA/CD204 promoted TLR-induced CD40 expression. It is of interest to note that CD40 on DCs was shown to be an HSP-binding receptor (22, 23, 34) and participate in the tolerance-breaking activity of hsp70 (41).

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Interestingly, Mule¨ and colleagues (49) recently reported that induction of an Ag-specific CTL response and antitumor immunity. stimulating capability of APCs–DCs in particular, thereby affecting an important role that SRA/CD204 plays in modulating the T cell- marker for M2 macrophages (47) and is suggested to participate as polyinosinic-polycytidylic acid and CpG oligodeoxynucleotides, of SRA/CD204 are not limited to large HSPs. Additional studies of different origins suggests that immune-regulatory activities ening Ag-specific adaptive immunity augmented by adjuvants nization (46). That SRA/CD204 seems to be capable of damp-

References

The authors have no financial conflicts of interest.

Disclosures

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Supplementary figure 1. Lack of SRA does not affect the presentation of endogenous antigen to MHC I. OVA protein (500 ng) was introduced into the cytosol of WT and SRA−/− DCs using FugeneHD, followed by cell staining using 25-D1.16 antibodies. FACS analysis was used to examine the levels of H2Kb-SIINFEKL peptide complexes on the cell surface. The data shown represent two independent experiments.
Supplementary figure 2. SRA absence does not alter DC response to TNF-α stimulation. WT and SRA−/− DCs were stimulated with TNF-α (10 ng/ml) for 4 h. mRNA levels of IL-6 were assayed using qRT-PCR. NS. Not significant. The data shown represent two independent experiments.
Supplementary figure 3. SRA/CD204 does not affect Alum adjuvant induced gp100-specific T cell activation. WT and SRA\(^{-/-}\) mice were injected i.v. with \(1 \times 10^7\) pmel cells on day -1, and immunized s.c. with gp100 (25 μg) emulsified in 100 μl Imject\textsuperscript{®} Alum. 5 days later, proliferation of pmel CD8\(^+\) T cells in spleen and lymph nodes was analyzed by FACS following cell staining with CD8 and CD90.1 antibodies. The data shown represent three independent experiments with similar results.