Pattern Recognition Scavenger Receptor CD204 Attenuates Toll-like Receptor 4-induced NF-κB Activation by Directly Inhibiting Ubiquitination of Tumor Necrosis Factor (TNF) Receptor-associated Factor 6*¶

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The collaboration and cross-talk between different classes of innate pattern recognition receptors are crucial for a well-coordinated inflammatory response and host defense. Here we report a previously unrecognized role of scavenger receptor A (SRA; also known as CD204) as a signaling regulator in the context of Toll-like receptor 4 (TLR4) activation. We show that SRA/CD204 deficiency leads to greater sensitivity to LPS-induced endotoxic shock. SRA/CD204 down-regulates inflammatory gene expression in dendritic cells by suppressing TLR4-induced inflammatory response and host defense. Here we report a previously unrecognized role of scavenger receptor A (SRA; also known as CD204) as a signaling regulator in the context of Toll-like receptor 4 (TLR4) activation. We show that SRA/CD204 deficiency leads to greater sensitivity to LPS-induced endotoxic shock. SRA/CD204 down-regulates inflammatory gene expression in dendritic cells by suppressing TLR4-induced activation of the transcription factor NF-κB. For the first time, we demonstrate that SRA/CD204 executes its regulatory functions by directly interacting with the TRAF-C domain of TNF receptor-associated factor 6 (TRAF6), resulting in inhibition of TRAF6 dimerization and ubiquitination. The attenuation of NF-κB activity by SRA/CD204 is independent of its ligand-binding domain, indicating that the signaling-regulatory feature of SRA/CD204 can be uncoupled from its conventional endocytic functions. Collectively, we have identified the molecular linkage between SRA/CD204 and the TLR4 signaling pathways, and our results reveal a novel mechanism by which a non-TLR pattern recognition receptor restricts TLR4 activation and consequent inflammatory response.

The scavenger receptors (SRs) constitute a large family of structurally diverse pattern recognition receptors (PRRs) (1). Scavenger receptor A (SRA), also termed CD204, is a prototypic member of the growing SR family. The role of SRA/CD204 in atherosclerosis has been extensively studied because it was the first receptor identified for modified lipoproteins (e.g. oxidized or acetylated low density lipoproteins) that are pertinent to the development of vascular disease (2). As a PRR primarily expressed on myeloid cells, such as dendritic cells (DCs) and macrophages, SRA/CD204 binds not only to altered or modified self macromolecules but also to a wide range of pathogen-associated molecular patterns, including lipopolysaccharide (LPS), bacterial CpG DNA, and double strand RNA (3). SRA/CD204-deficient mice are significantly more susceptible than their wild type (WT) counterparts to infection with Listeria monocytogenes (2) and Staphylococcus aureus (4). Loss of SRA/CD204 expression led to an increased mortality in Bacillus Calmette-Guérin primed animals, which has been partially attributed to the overproduction of proinflammatory cytokines by macrophages rather than impaired LPS clearance in vivo (5). Several lines of evidence suggest that SRA/CD204 on myeloid cells functions as a suppressor that can limit an inflammatory response (6, 7). However, the molecular basis underlying the SRA/CD204-mediated regulation of inflammation and production of inflammatory cytokines remains unexplored.

The Toll-like receptors (TLRs) represent a family of evolutionarily conserved PRRs and are believed to play central roles in the induction of innate as well as adaptive immunity to pathogen infection (8). Binding of the microbial pattern molecules (i.e. pathogen-associated molecular patterns) by TLRs stimulates intracellular signaling cascades, leading to the production of inflammatory cytokines (9). TLR activation triggers the recruitment and interaction of several adaptor molecules. These include TNF receptor-associated factor 6 (TRAF6), a RING-domain-containing E3 ubiquitin protein ligase, which can catalyze formation of a polyubiquitin chain on IκB kinase complex and also on TRAF6 itself (10). Ubiquitination-dependent activation of the TRAF6 leads to activation of IκB kinase and the mitogen-activated protein kinase (MAPK) cascade. The transcription factor NF-κB is sequestered in the cytoplasm through its association with the inhibitor IκB in resting cells. Phosphorylation of IκB by the IκB kinase complex targets this inhibitor for degradation, thereby allowing NF-κB to translocate to the nucleus and activate target genes involved in inflammation and immunity. As a result, TLR activation generates collaborative cellular responses, including production of an
array of inflammatory cytokines, maturation of antigen-presenting cells, or initiation of an adaptive immune response against pathogens. TLR signaling must be tightly regulated because prolonged and excessive activation of TLRs can cause uncontrolled inflammation detrimental to the host, leading to the pathogenesis of inflammatory and infectious diseases or autoimmunity (11).

Our recent studies showed that SRA/CD204 is capable of dampening the immunogenicity of DCs and subsequent adaptive immune responses driven by TLR4 activation, suggesting that SRA/CD204 is involved in the modification of TLR4-triggered immune responses (12). Despite the observations suggesting that SRA/CD204 attenuates TLR4 signaling-induced inflammatory responses, very little is known about the biochemical nature of such interactions, which are likely to be essential for the immunoregulatory activities of SRA/CD204 in vivo. Here we show for the first time that SRA/CD204 down-regulates LPS-stimulated NF-κB activation via interaction with TRAF6, a critical signal transducer in TLR4-mediated signaling cascades. Our results reveal that SRA/CD204, a classic endocytic PPR, can also execute signaling-regulatory functions by directly modifying the magnitude of TLR4 signaling, providing a molecular view into the functional interplay between these two classes of PRRs in host immune homeostasis.

**EXPERIMENTAL PROCEDURES**

**Mice and Cells**—C57BL/6 mice and SRA/CD204 knock-out mice were obtained from Jackson Laboratory (Bar Harbor, ME). SRA−/− mice have been back-crossed to C57BL/6 mice for at least 10 generations (2). Mice were bred and maintained under pathogen-free conditions. All experiments have been reviewed and approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Phoenix cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 1 mM sodium pyruvate, and 10 mM HEPES at 37 °C with 5% CO2. The HEK293-TLR4/MD2-CD14 cells purchased from Calbiochem. For some experiments, inhibitors or vehicle control (0.1% DMSO) were added to cell cultures 1 h before the treatments. Antibodies against phospho-Erk (Thr202/Tyr204), phospho-IκBα (Ser32/36), Erk1/2, and IκBα were from Cell Signaling Biotechnology (Beverly, MA). Antibodies against TRAF6 (H-274), ubiquitin (FL-76, 6C1) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 antibody was from Sigma. Anti-HisGly and anti-V5 antibodies were from Invitrogen. Polyclonal antibody for SRA/CD204 was purchased from R&D Systems (Minneapolis, MN). Alexa Fluor 488 and Alexa Fluor 594 were from Invitrogen.

**Cloning and Plasmid Constructions**—cDNA sequences encoding human SRA/CD204, TRAF6, and ubiquitin were obtained by PCR using mRNAs derived from THP-1 cells. V5-tagged human SRA/CD204 was cloned into the pCDNA3.1(+) vector. TRAF6 and its deletion mutants were cloned into pCMV-HisGly vector. Ubiquitin was cloned into pCMV-Tag2B vector. Mouse SRA/CD204 and TRAF6 were cloned from DC1.2 cells and inserted into the pLVX-AcGFPI-N1 vector and pLVX-DsRed-Monomer-N1 vector, respectively. DsRed-tagged Rab7 construct was purchased from Addgene (Cambridge, MA) (13). All of the plasmids used in this study were purified using the Endofree plasmid maxi K from Qiagen (Valencia, CA).

**Cytokine Assays**—For quantitative PCR analysis, total RNA was isolated using RNeasy mini K from Qiagen (Valencia, CA). RNA was treated with RNase-free DNase I (Invitrogen) and quantified by using an Ultra-Spec spectrophotometer (Amerham Biosciences). For reverse transcription, 1 μg of total RNA and 50 ng of oligo(dT) primer were used for a 20-μl reaction volume with Superscript II reverse transcriptase (Invitrogen). A quantitative transcription profile of *Tnfa* and *Il6* was evaluated by TaqMan quantitative PCR on an ABI prism 7900HT sequence detection system using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Primers and FAM-labeled probe sets were obtained as predesigned assay reagents from Applied Biosystems. The PCR was started with 2 min at 50 °C and an initial 10-min denaturation at 94 °C, followed by a total of 40 cycles of 15-s denaturation at 94 °C, and 1 min of annealing and elongation at 60 °C. Gene expression was quantified relative to the expression of β-actin and normalized to that measured in control BMDCs by standard 2−ΔΔCT calculation as described previously (14). Conditioned media from BMDCs were harvested 20 h after LPS treatment. Serum was collected from LPS-challenged animals at different time points. Cytokine levels were assayed using a Luminex 100 analyzer (Luminex Corp., Austin, TX) or ELISA as described previously (12).

**Luciferase Assay**—HEK293-TLR4/MD2-C14-TRAF6 cells were generated by transfecting the cells with HisGly-tagged TRAF6 and selected with G418. The stable TRAF6-expressing cells were cultured in 24-well plates and then transiently transfected with pCMV-V5-hSRA or empty vector together with pGL3-NF-κB-Luc or an IP-10 promoter luciferase reporter plasmid using Fugene HD reagent from Roche Applied Science. The plasmid pRL-TK (10 ng) was used as an internal control. In all cases, the total amount of DNA was kept constant by adding various concentrations of the appropriate control plasmids.
20 h later after transfection, cells were stimulated with or without LPS for 4 h. The cells were then prepared with passive lysis buffer, and luciferase activity was determined using a Glomax luminometer from Promega (Madison, WI).

**NF-κB Activation Assays**—Nuclear extracts were prepared using a nuclear extract kit from Active Motif (Carlsbad, CA) and used in an electrophoretic mobility shift assay (EMSA). Briefly, nuclear extract was incubated with γ-32P-labeled (1 × 10^5 cpm) double-stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTCCCAGGC-3′) in 20 μl of binding solution containing 10 mM HEPES (pH 7.9), 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 μg/ml poly(deoxyinosinic-deoxy-cytidylic acid). DNA-protein complexes were then resolved on a 6% polyacrylamide gel under nondenaturing conditions at 140 V for 2 h at 4°C. Gels were dried and subjected to autoradiography.

**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 VERSA microplate reader from Molecular Devices (Sunnyvale, CA) at 450 nm with a reference wavelength of 655 nm.

**Lentivirus Production and Transduction**—pLKO.1-puro lentiviral vectors expressing scrambled control shRNA and mouse SRA/CD204 shRNA were purchased from Open Biosystems (Huntsville, AL). Phoenix cells were co-transfected by the pLKO.1 backbone vectors together with pMD.G and pCMVΔR.89 vectors using Fugene HD as described (15). For stable silencing of SRA/CD204 in DC1.2 cells, single cell colonies were isolated and screened by limited dilution.

**Immunoblotting and Immunoprecipitation**—Cells were washed with ice-cold PBS and lysed in modified RIPA buffer (50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml each of aprotinin and leupeptin, and 1 mM Na3VO4). For immunoblotting, 20–50 μg of protein were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes. Native PAGE for detecting protein trimers or dimers was prepared without SDS. Membranes were immunoblotted with primary antibodies followed by HRP-conjugated secondary antibodies. Reactions were visualized by enhanced chemiluminescence reagents (Amersham Biosciences). For immunoprecipitation, 1 mg of cell extracts was incubated with 2 μg of antibodies for 2 h at 4°C, followed by incubation with 40 μl of Protein A/G Plus-Sepharose beads (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The beads were washed with RIPA buffer, and immune complexes were eluted by boiling in 2× SDS Laemmli loading buffer for 5 min.

**GST Pull-down Assay**—The cDNAs encoding the full length and the intracellular domain (ICD) of mouse SRA/CD204 (GenBank™ accession number NP_001106797, amino acids 1–55) were cloned into pBAC-P-AK-His (Clontech) and pGEX-2TK (GE Healthcare) vector, respectively. His-tagged full-length SRA/CD204 protein was expressed and prepared as described previously (16). GST-tagged protein was expressed in *Escherichia coli* BL21 (DE3) and purified using glutathione-Sepharose 4B beads. For GST pull-down assays, GST-mSRA-ICD (10 μg) or GST-conjugated glutathione-Sepharose beads were incubated with 1 mg of total protein lysates in 1 ml of RIPA buffer at 4°C overnight. Beads were washed with modified RIPA buffer, and bound protein was subjected to immunoblotting.

**Immunofluorescence and Confocal Microscopy**—BMDCs were stimulated with LPS for 20 min and fixed in 2% paraformaldehyde for 10 min at room temperature, permeabilized with 0.1% Triton X-100 for 5 min, blocked with 10% goat serum for 1 h, and stained with diluted primary antibodies in 1% goat serum overnight at 4°C overnight. After incubation with Alexa Fluor-conjugated secondary antibodies (Invitrogen), slides were mounted in Vectashield mounting medium. To visualize the co-localization of SRA/CD204 and TRAF6 in HEK293-TLR4/MD2/CD14 cells, the cells were co-transfected with plasmid encoding GFP-tagged SRA/CD204 and RFP-tagged TRAF6. 24 h post-transfection, cells were treated with 100 ng/ml LPS for 45 min and fixed with 2% paraformaldehyde. Images for GFP and RFP were taken using a confocal microscope excited at 488 and 543 nm, respectively. All of the images were acquired with a ×63 oil immersion objective on a Leica TCS SP2 spectral confocal microscope and processed using Photoshop software (Adobe Systems Inc.), and the adjustments of brightness and contrast were applied to the whole image.

**In Vitro Ubiquitination Assay**—TRAF6 was used as ubiquitin E3 ligase/substrate was immunopurified with anti-TRAF6 antibodies and protein A/G-Sepharose beads from 1 mg of DC1 cell lysates. The autoubiquitination assay was performed in a 20-μl reaction volume using the ubiquitin conjugation reaction buffer kit (Boston Biochem, Cambridge, MA). Briefly, the immunoprecipitated TRAF6 was preincubated with recombinant SRA/CD204 protein, which was prepared using a baculovirus insect cell expression system or the irrelevant luciferase control protein on ice for 30 min, and then 0.15 μg of recombinant E1-activating enzyme, 0.6 μg of Ubc13/Uev1a-conjugating complex, and 2.5 μg of ubiquitin were added. 20 mM ATP was added to initiate the reaction, and the mixtures were incubated at 30°C for 2 h with gentle agitation. The beads were washed four times with RIPA buffer and subjected to immunoblotting with the indicated antibodies.

**Statistical Analysis**—Data are expressed as mean ± S.D. values. Statistical significance between groups within experiments were determined by Student’s *t* test or an analysis of variance test, with a value of *p* < 0.05 considered to be statistically significant.

**RESULTS**

SRA/CD204 Suppresses LPS-induced Inflammatory Response—Based on our previous finding that SRA/CD204 was able to attenuate TLR4 activation-induced immune responses (12), we used the LPS-induced endotoxic shock model to assess the *in vivo* function of SRA/CD204 in TLR4 activation. On day 4 after LPS challenge, the lower dose (10 mg/kg) had killed 70% of the SRA−/− mice but only 10% of the WT mice (Fig. 1A). A similar observation was made in animals challenged with a higher dose of LPS (30 mg/kg) (Fig. 1B). Upon LPS challenge, the levels of tumor necrosis factor α (TNF-α) were significantly elevated in the sera of SRA−/− mice compared with WT mice (Fig. 1C), suggesting that an aberrant cytokine response to LPS contributed to the increased mortality in SRA/CD204-deficient mice.
Fine Tuning of TLR4 Signaling by SRA/CD204

Given our previous observation of SRA/CD204 up-regulation on myeloid cells (e.g. DCs and macrophages) in LPS-challenged mice (12), these results indicate that SRA/CD204 plays an important role in regulation of TLR4 activation-induced inflammatory response.

BMDCs from WT and SRA−/− mice were obtained in similar numbers and had similar expression of surface activation markers, such as major histocompatibility complex class I/II, CD40, and B7.1/CD86 (12), suggesting that SRA/CD204 is not required for normal differentiation of bone marrow cells. The similar characteristics of the BMDCs allowed us to compare cellular responses to a TLR4 ligand or agonist, such as LPS.

Compared with WT cells, SRA−/− BMDCs displayed considerably higher levels of mRNA transcripts for Tnfα and Il6 after overnight treatment, as assayed by quantitative RT-PCR (Fig. 1D). ELISA analysis of the media showed that SRA−/− DCs produced substantially higher levels of inflammatory cytokines Tnfα and Il6, which agrees with the data obtained from SRA−/− BMDCs. In light of our previous studies showing that SRA/CD204 silencing of SRA/CD204 resulted in enhanced phosphorylation of Tnfα and Erk1/2 after MPL treatment (supplemental Fig. 2). Additionally, we established stable SRA/CD204 knockdown cells by infecting DC1.2 cells with lentiviruses encoding shRNA for SRA/CD204, in which the protein levels of SRA/CD204 were reduced by more than 90% compared with the scrambled control (Fig. 2C). The silencing of SRA/CD204 resulted in enhanced phosphorylation of Tnfα and Erk1/2 signaling in SRA−/− DCs. In light of our previous studies showing that SRA/CD204 deficiency promotes the immunostimulatory adjuvant activities of MPL, a chemically modified LPS (12), we assessed the effect of SRA/CD204 on MPL-stimulated NF-κB signaling in WT and SRA−/− BMDCs. As expected, the absence of SRA/CD204 led to increased phosphorylation of IkBα after MPL treatment (supplemental Fig. 2).

The signaling pathway-specific inhibitors were used to gain more insights into the signaling pathways involved in the SRA/CD204 effect. Intriguingly, the SRA/CD204 absence-enhanced gene transcription of Tnfα (Fig. 2D) and Il6 (Fig. 2E) was only phosphorylation of Erk1/2 (Fig. 2A), a signaling molecule in the TLR4 activated MAPK signaling pathway.

To validate the suppressive effect of SRA/CD204, we subsequently transduced the SRA/CD204 expression construct into the mouse dendritic cell DC1.2 line. Enforced overexpression of SRA/CD204 profoundly attenuated LPS-induced phosphorylation of IκBα and Erk1/2 (Fig. 2B). Induction of inflammatory genes, such as Tnfα and Il6, was also suppressed at the transcriptional level (supplemental Fig. 1). Additionally, we established stable SRA/CD204 knockdown cells by infecting DC1.2 cells with lentiviruses encoding shRNA for SRA/CD204, in which the protein levels of SRA/CD204 were reduced by more than 90% compared with the scrambled control (Fig. 2C). The silencing of SRA/CD204 resulted in enhanced phosphorylation of Tnfα and increased Erk1/2 phosphorylation (Fig. 2C), which agrees with the data obtained from SRA−/− DCs. In light of our previous studies showing that SRA/CD204 deficiency promotes the immunostimulatory adjuvant activities of MPL, a chemically modified LPS (12), we assessed the effect of SRA/CD204 on MPL-stimulated NF-κB signaling in WT and SRA−/− BMDCs. As expected, the absence of SRA/CD204 led to increased phosphorylation of IκBα after MPL treatment (supplemental Fig. 2).

FIGURE 1. Increased susceptibility to LPS-induced endotoxic shock in SRA−/− mice is associated with enhanced production of inflammatory cytokines in SRA−/− BMDCs. Survival of mice (n = 10) after administration intraperitoneally with 10 mg/kg (A) or 30 mg/kg (B) LPS (p < 0.01, analysis of variance). C, TNF-α levels in sera pooled from LPS-treated mice (n = 5), as measured by ELISA (*, p < 0.01). Data are representative of three separate experiments. D, BMDCs were stimulated with 100 ng/ml LPS and mRNA levels of inflammatory genes (Tnfα and Il6) were assessed using quantitative RT-PCR. The results were presented as -fold induction of treated over untreated WT samples. E, TNF-α and IL-6 levels in culture media were determined using ELISA (*, p < 0.05; **, p < 0.01). Data are means ± S.D. (error bars) (n = 3) and are representative of three experiments.
blocked by Ro106-9920, a specific IκBα ubiquitination inhibitor, but not U0126, a highly selective inhibitor of MEK1/2.

Given the SRA/CD204-mediated suppression of LPS-induced IκBα phosphorylation and degradation in SRA−/− DCs after LPS treatment by immunoblot analysis, B, reduced IκBα and Erk1/2 phosphorylation in SRA/CD204-overexpressing DCs. Cell lysates prepared from LPS-stimulated control (empty vector) or His-tagged SRA/CD204-transfected DC1.2 cells were blotted with antibodies as indicated. IκBα, enhanced IκBα phosphorylation and degradation in SRA/CD204-silenced DCs compared with scrambled control. D and E, NF-κB inhibitor diminishes the SRA/CD204 absence-enhanced up-regulation of Tnf and Il6. BMDCs were pretreated with 20 μM Ro106–9920 or 10 μM U0126 for 1 h, followed by LPS stimulation for 4 h. The expression of Tnfa (D) and Il6 (E) was assessed by quantitative RT-PCR. The results are presented as -fold induction over untreated WT samples (*, p < 0.01; **, p > 0.05). Data are means ± S.D. (error bars) (n = 3) and are representative of three experiments.

**FIGURE 2.** SRA/CD204 attenuates LPS-stimulated production of inflammatory factors via the NF-κB pathways in DCs. A, enhanced IκBα phosphorylation and degradation in SRA−/− DCs after LPS treatment by immunoblot analysis. B, reduced IκBα and Erk1/2 phosphorylation in SRA/CD204-overexpressing DCs. Cell lysates prepared from LPS-stimulated control (empty vector) or His-tagged SRA/CD204-transfected DC1.2 cells were blotted with antibodies as indicated. C, enhanced IκBα phosphorylation and degradation in SRA/CD204-silenced DCs compared with scrambled control. D and E, NF-κB inhibitor diminishes the SRA/CD204 absence-enhanced up-regulation of Tnf and Il6. BMDCs were pretreated with 20 μM Ro106–9920 or 10 μM U0126 for 1 h, followed by LPS stimulation for 4 h. The expression of Tnfa (D) and Il6 (E) was assessed by quantitative RT-PCR. The results are presented as -fold induction over untreated WT samples (*, p < 0.01; **, p > 0.05). Data are means ± S.D. (error bars) (n = 3) and are representative of three experiments.

**FIGURE 3.** SRA/CD204 inhibits LPS-stimulated NF-κB activity in HEK293-TLR4/MD2-CD14-TRAF6 cells. A, SRA/CD204 inhibits NF-κB activity in a dose-dependent manner. B, EMSA was performed using a radiolabeled NF-κB-specific probe and nuclear extracts from LPS-stimulated DCs. SRA-overexpressing DCs displayed greater DNA binding activity for NF-κB than WT counterpart cells (Fig. 3C). Results from an ELISA also showed an increased nuclear translocation and phosphorylation of p65, a subunit of the NF-κB, in LPS-stimulated SRA−/− DCs compared with that in WT cells (Fig. 3D).

Intracellular SRA/CD204 Co-localizes and Associates with TRAF6—The computing software from the Eukaryotic Linear Motif (ELM) resource (available on the World Wide Web) (17) predicts that SRA/CD204 contains several functional protein-binding consensus motifs, including motifs for TRAF2/6. Given that TRAF6 is a pivotal signal transducer in TLR4 signaling-induced NF-κB activation, we sought to investigate whether SRA/CD204 may negatively regulate TLR4 signaling via interaction with TRAF6. Co-immunoprecipitation assays showed that, upon LPS stimulation, there was a transient increase of TRAF6 binding to endogenous SRA/CD204 (Fig. 4A). The association of these two proteins was confirmed by a reciprocal
immunoprecipitation assay using anti-TRAF6 antibodies (supplemental Fig. 3A). We further validated the association using V5-tagged SRA/CD204 and HisGly-tagged TRAF6 in HEK293-TLR4/MD2-CD14 cells (supplemental Fig. 3B). LPS stimulation of the transduced HEK293-TLR4/MD2-CD14 cells similarly induced the recruitment of TRAF6 to SRA/CD204 (Fig. 4B). The same observation was made in LPS-stimulated macrophages differentiated from human THP-1 cells (Fig. 4C). To address the question of whether SRA/CD204 may also directly interact with TLR4, we performed additional co-immunoprecipitation assays. Interestingly, SRA/CD204 association with TLR4 or other signaling adaptor molecules, including MyD88 and IRAK1, was not detected in LPS-stimulated BMDCs (supplemental Fig. 3C), suggesting that these two PRRs (i.e. SRA/CD204 and TLR4) do not physically interact with each other.

To study the compartmentalization of these two molecules, we co-expressed GFP-tagged SRA/CD204 and RFP-tagged TRAF6 in HEK293-TLR4/MD2-CD14 cells. In resting cells, both SRA\textsuperscript{GFP} and TRAF6\textsuperscript{RFP} exhibited punctated staining throughout the cytoplasm. Upon LPS stimulation, they co-lo-
localized extensively at the cytoplasmic perinuclear region (Fig. 4D). SRA/CD204 was also seen to co-localize with Rab7, a marker of late endosome/lysosome (Fig. 4E). In addition, immunofluorescence staining of primary BMDCs showed that SRA/CD204 was predominantly localized in the cytoplasm with a small amount of protein present on the plasma membrane. LPS stimulation led to a significant increase in the perinuclear accumulation and co-localization of SRA/CD204 and TRAF6 (indicated by the yellow overlap in Fig. 4F). Subcellular fractionation studies showed that both SRA/CD204 and TRAF6 were present in the same fractions that were also positive for Rab5 and LAMP-1 (i.e. endosomal/lysosomal makers) (supplemental Fig. 3D).

We next assessed whether endocytic trafficking pathways were involved in the observed SRA/CD204 effect using different inhibitor molecules. Although cytochalasin D (an inhibitor of endocytosis/phagocytosis) decreased LPS-stimulated IκBα phosphorylation in WT and SRA−/− DCs, the phosphorylation levels of IκBα remained significantly higher in SRA−/− DCs (Fig. 4G, top). In contrast, treatment with chloroquine (an inhibitor of endosome functions) dramatically impaired the SRA/CD204 impact on LPS-induced IκBα phosphorylation.

**FIGURE 4.** SRA/CD204 co-localizes and interacts with signaling transducer TRAF6. A, TRAF6 is recruited to SRA/CD204 in response to LPS stimulation. BMDCs were treated with LPS, and equal amounts of protein were used for immunoprecipitation (IP) with the SRA/CD204 antibodies. The immunoprecipitates were probed for the presence of TRAF6 and SRA/CD204, respectively. Immunoblot (IB) was also carried out for TRAF6 using whole cell lysate (WCL). B, HEK293-TLR4/MD2-CD14 cells were transfected with V5-tagged SRA/CD204 and HisGly-tagged TRAF6 plasmids. Cell lysates were immunoprecipitated with anti-HisGly antibodies, followed by immunoblotting with anti-TRAF6 or anti-V5 antibodies. C, LPS-induced association of SRA/CD204 and TRAF6 in human macrophages. SRA/CD204 was pulled down from PMA-differentiated THP-1 cells and analyzed for the binding of TRAF6. D, co-localization of SRA/CD204 and TRAF6. HEK293-TLR4/MD2-CD14 cells transfected with GFP-tagged SRA/CD204 (green) and RFP-tagged TRAF6 (red) were analyzed by confocal microscopy. E, the presence of SRA/CD204 in the endosomal compartment was examined by imaging of HEK293-TLR4/MD2-CD14 cells expressing GFP-tagged SRA/CD204 (green) and RFP-tagged Rab7 (red) following LPS stimulation. F, co-localization of endogenous SRA/CD204 (red) and TRAF6 (green) in BMDCs. Cells were permeabilized and immunostained with antibodies against SRA/CD204 and TRAF6. Cells were counterstained with DAPI (blue). The arrows indicate representative co-localization between SRA/CD204 and TRAF6. Bars (D–F), 10 μM. G, endosomal functions are involved in the SRA/CD204 effect. BMDCs were pretreated with 10 μM cytochalasin D (Cyt-D) or 200 μM chloroquine (CQ) before LPS stimulation. IκBα phosphorylation was determined by immunoblot. Results represent at least three independent experiments.
The TRAF-C Domain of TRAF6 Is Necessary for Association with SRA/CD204—SRA/CD204 consists of an N-terminal ICD and extracellular domain, which includes an α-helical coiled-coil region, a collagenous region, and a cysteine-rich globular head. To determine whether TRAF6 could directly interact with the ICD of SRA/CD204, we prepared a recombinant GST-ICD fusion protein and performed in vitro GST pull-down assays. Whole cell lysates from DC1.2 cells transfected with HisGly-tagged TRAF6 were incubated with either GST- or GST-ICD-conjugated Sepharose beads. The GST-ICD fusion protein, but not GST protein, was found to bind to both over-expressed HisGly-TRAF6 and endogenous TRAF6, indicating that TRAF6 interacts directly with the ICD of SRA/CD204 (Fig. 5A). To determine the functional effect of SRA-ICD, we assessed NF-κB-dependent luciferase activity in the SRA-ICD-transduced HEK293-TLR4/MD2-CD14-TRAF6 cells. The presence of ICD significantly inhibited LPS-stimulated luciferase activity (Fig. 5B), suggesting that the SRA/CD204 effect on TLR4-induced NF-κB activation can occur independent of the extracellular ligand-binding domain of SRA/CD204.

TRAF6 is a multifunctional protein with a RING finger domain and zinc finger domain that can mediate downstream signaling events and a TRAF-C domain for interaction with upstream receptors and other signaling molecules. In order to map the region of TRAF6 responsible for the association with SRA/CD204, a series of TRAF6 truncation constructs were generated based on the domain structure of TRAF6 and then cloned into expression vectors in frame with the HisGly tag-encoding sequence (Fig. 5C). HEK293-TLR4/MD2-CD14 cells were co-transfected with V5-tagged SRA/CD204 and various TRAF6 truncation plasmids, followed by immunoprecipitation analysis using anti-HisGly antibodies. The presence of co-precipitated SRA/CD204 was detected by immunoblot using anti-V5 antibodies. Expression of SRA/CD204 and TRAF6 truncation mutants was confirmed by immunoblot. Representative results from three independent experiments are shown. Error bars, S.D.
SRA/CD204 interacts with the TRAF6 via its TRAF-C domain.

SRA/CD204 Suppresses the Dimerization and Autoubiquitination of TRAF6—Upon LPS stimulation, the E3 ligase activity of TRAF6 is activated after dimerization and targets itself or other molecules (e.g. IKK/β) for Lys63-linked polyubiquitination (18). Therefore, the regulatory function of SRA/CD204 may have the potential to interfere with TRAF6 ubiquitination. When HisGly-tagged TRAF6 and FLAG-tagged ubiquitin were transfected together into HEK293-TLR4/MD2-CD14 cells, ubiquitination of TRAF6 was readily detected, as reported previously (18). Expression of V5-tagged SRA/CD204 together with TRAF6 resulted in reduced TRAF6 ubiquitination (Fig. 6A). In addition, ubiquitination levels of immunoprecipitated endogenous TRAF6 were significantly elevated in LPS-stimulated SRA/CD204 deficient cells (TRAF6 was immunoprecipitated from LPS-stimulated WT and SRA/CD204 deficient cells). BMDCs were stimulated with LPS, and dimerization of TRAF6 was examined using native gel fractionation and immunoblot with anti-TRAF6 antibodies. The levels of TRAF6 and SRA/CD204 under denatured conditions were also measured. C, endogenous TRAF6 was immunoprecipitated from DC1 cells and subjected to in vitro ubiquitination assays in the presence or absence of SRA/CD204 protein. The modification of TRAF6 was detected by immunoblotting with anti-ubiquitin antibodies. Luciferase protein was used as a non-relevant control. Representative results from three independent experiments are shown. IB, immunoblot; WCL, whole cell lysate.

To assess the ability of SRA/CD204 to interfere with TRAF6 autoubiquitination directly, we performed ex vivo ubiquitination assays using immunoprecipitated TRAF6. The presence of recombinant SRA/CD204 protein greatly reduced the ubiquitination of TRAF6 (Fig. 6D).

MyD88-dependent Signaling Is Required for the SRA/CD204-TRAF6 Interaction—To determine whether the negative signaling regulation by SRA/CD204 may occur upstream of TRAF6, we examined the ubiquitination and degradation of IRAK1, an essential upstream signal adaptor that is recruited to TLR4-MyD88 complex prior to TRAF6 activation (9). The ubiquitination levels of IRAK1 were not altered in the absence of SRA/CD204, and IRAK1 protein levels decreased to a similar extent in WT and SRA/CD204 DCs upon LPS stimulation (Fig. 7A). However, an increase in the TRAF6 recruitment to IRAK1 was seen in LPS-stimulated SRA/CD204 DCs compared with WT cells (Fig. 7B). These results indicate that SRA/CD204 selectively regulates TRAF6 activation downstream of IRAK1 and that a general ligand competition effect does not appear to be a major contributor to the SRA/CD204 absence-enhanced NF-κB activity. Last, we examined LPS-stimulated interactions between SRA/CD204 and TRAF6 in the absence of MyD88. The binding of TRAF6 with SRA/CD204 was significantly

FIGURE 6. SRA/CD204 suppresses LPS-induced TRAF6 oligomerization and ubiquitination. A, reduced TRAF6 ubiquitination in SRA/CD204-overexpressing cells. HEK293-TLR4/MD2-CD14 cells were co-transfected with HisGly-tagged TRAF6 and FLAG-tagged ubiquitin constructs together with or without V5-tagged SRA/CD204. 48 h later, cell lysates were subjected to immunoprecipitation (IP) with anti-HisGly antibodies and analyzed using anti-FLAG antibodies. B, enhanced TRAF6 ubiquitination in SRA/CD204-deficient cells. TRAF6 was immunoprecipitated from LPS-stimulated WT and SRA/CD204 deficient cells. C, enhanced TRAF6 dimerization in the absence of SRA/CD204 following LPS stimulation. BMDCs were stimulated with LPS, and dimerization of TRAF6 was examined using native gel fractionation and immunoblot with anti-TRAF6 antibodies. The levels of TRAF6 and SRA/CD204 under denatured conditions were also measured. D, endogenous TRAF6 was immunoprecipitated from DC1 cells and subjected to in vitro ubiquitination assays in the presence or absence of SRA/CD204 protein. The modification of TRAF6 was detected by immunoblotting with anti-ubiquitin antibodies. Luciferase protein was used as a non-relevant control. Representative results from three independent experiments are shown. IB, immunoblot; WCL, whole cell lysate.
reduced in MyD88 \(^{-/-}\) cells compared with WT cells (Fig. 7C), suggesting that the MyD88-dependent signaling contributes to or is required at least partially for the recruitment of TRAF6 to SRA/CD204 in response to LPS stimulation.

Several TLR4 signaling suppressors have been shown to participate in the induction and maintenance of LPS tolerance, a state refractory to further LPS-induced response (19). We therefore examined the potential involvement of SRA/CD204 in endotoxin tolerance in vivo and in vitro. WT and SRA/CD204 \(^{-/-}\) DCs became tolerant to the second dose of LPS, and both strains of mice were resistant to the secondary challenge with a lethal dose of LPS (supplemental Fig. 4), suggesting that SRA/CD204-mediated inhibition of TLR4 signaling functions independently of the endotoxin tolerance mechanism.

**DISCUSSION**

Although it has been recognized that certain endocytic receptors can perform dual functions (i.e. internalizing ligand and triggering signaling transduction cascades) (20), the roles of SRA/CD204 in regulating TLR4 signaling pathways in myeloid cells and inflammatory responses has remained largely unknown. Using loss- and gain-of-function approaches, we have established SRA/CD204 as a signaling suppressor of TLR4-induced NF-\(\kappa\)B activity, inflammatory cytokine production, and LPS-induced endotoxic shock, underscoring the importance of this multifunctional molecule in immune homeostasis. Our findings are consistent with several previous reports suggesting that SRA/CD204 serves as a negative regulator of an inflammatory response (5–7, 21).
Importantly, here we provide the first biochemical evidence that the signaling-regulatory feature of SRA/CD204 involves direct interference of dimerization/ubiquitination of TRAF6, a key adaptor molecule downstream of TLR4 signaling cascades, and can be uncoupled from its previously described ligand-binding properties.

The linkage of elevated NK-κB activity with SRA/CD204 deficiency or silencing raises the question as to whether this enhanced activation is caused by a direct effect of SRA/CD204 as a *bona fide* signaling regulator or an indirect effect that is manifested through scavenging or removing TLR4 agonist (i.e. LPS). The lack of SRA/CD204 does not impair LPS clearance *in vivo* (5, 22), indicating that other redundant endocytic receptors may sufficiently compensate for the loss of SRA/CD204. Indeed, the activated phenotype of myeloid cells, not the phagocytic capability, was recently shown to contribute to the higher levels of proinflammatory cytokines in pathogen-infected SRA/CD204−/− mice (23), suggesting that SRA/CD204-dependent phagocytosis and inhibition of cytokine production may be controlled independently (24). Interestingly, immunofluorescence staining in our studies clearly shows the existence of an intracellular pool of SRA/CD204 in DCs. Its broad distribution throughout the cytoplasm also implies that SRA/CD204 probably has unrecognized functions beyond traditional ligand binding and uptake on the cell surface.

Although endocytic pathways have been reported to regulate TLR4 signaling (25), the inhibition of endo-/phagocytosis in our studies does not abolish SRA/CD204 absence-enhanced IκBα phosphorylation. Treatment with cytochalasin D reduced the phosphorylation of IκBα, suggesting that initial ligand binding/uptake activities of endocytic receptors may actually amplify the signaling and contribute to the pathogen recognition. Importantly, the intracellular domain of SRA/CD204 that lacks a ligand-binding region is capable of not only directly interacting with TRAF6 but also suppressing NF-κB-dependent luciferase activity induced by LPS, indicating that ligand recognition and binding are not essential for the regulatory effect of SRA/CD204. The direct suppression of TRAF6 ubiquitination by SRA/CD204 *ex vivo* also provides important evidence for the role of SRA/CD204 in TLR4 signaling modulation. Last, the SRA/CD204 deficiency does not alter LPS-stimulated ubiquitination and degradation of IRAK1, an adaptor molecule upstream of TRAF6, further supporting the notion that the signaling regulatory ability of SRA/CD204 can be dissociated from its widely recognized endocytic feature.

We have identified TRAF6, an E3 ubiquitin ligase, as the molecular link responsible for SRA/CD204-mediated regulation of NF-κB activation in DCs. Intriguingly, SRA/CD204 does not appear to associate directly with TLR4 and MyD88 or IRAK1, which are signaling receptor/adaptor molecules upstream of TRAF6. Increased TRAF6 dimerization and ubiquitination and its recruitment to IRAK1 observed in LPS-stimulated SRA/CD204-deficient DCs, together with SRA/CD204-mediated interference with TRAF6 ubiquitination as shown in *ex vivo* ubiquitination assays, provide compelling evidence that SRA/CD204 interactions with TRAF6 is capable of altering the activation status of TRAF6, thereby impacting the LPS-induced NF-κB activation and subsequent inflammatory responses. The present study also shows that disruption of endosomal function abolishes the SRA/CD204 loss-enhanced NF-κB activation, consistent with an earlier report that TRAF6 is recruited to the endosomal compartment in the context of IL-1β-mediated NF-κB activation (26). However, definition of the molecular events involved in SRA/CD204 and TRAF6 recruitment and the subcellular context in which the interaction occurs is needed to understand the precise role of SRA/CD204 in fine tuning the TLR4 signaling.

It has been documented that certain non-TLR pattern recognition receptors, including SRs, serve as adaptor molecules or co-stimulatory receptors for TLR2 activation (27–29). Our results, however, reveal that SRA/CD204 represents a major regulatory receptor involved in the suppression of TLR4 signaling. Indeed, LPS challenge-induced up-regulation of SRA/CD204 on myeloid cells (12) argues for an intimate as well as complex interplay of endocytic and signaling PRRs (e.g. SRA/CD204 and TLR4) under stress conditions. In addition to initiating an inflammatory response and facilitating pathogen recognition, TLR4 activation can trigger a regulatory mechanism involving physical interactions of SRA/CD204 with TRAF6 for signaling attenuation, as exemplified in the present study (Fig. 7D). The SRA/CD204-mediated negative feedback loop includes the direct interference with TLR4-NF-κB signaling by suppressing TRAF6 ubiquitination and possibly conventional ligand internalization for removal as well. It is conceivable that engaging these two mechanisms simultaneously will limit the TLR4-induced inflammatory signaling more efficiently. As a general stress sensor, both intracellular and cell surface SRA/CD204, in the form of a trimer, can be mobilized in response to LPS stimulation and actively participate in the interactions with TRAF6. Loss of the SRA/CD204 would result in dysregulation of the LPS-induced inflammatory response in myeloid cells, which contributes to the increased susceptibility of SRA/CD204-deficient mice to endotoxin-induced lethal shock that has been reported here and by others (5). In support of our results, Tabas and co-workers (30) recently showed that SRA/CD204 suppresses the IRF-3–IFN-β signaling branch, resulting in an altered functional outcome of TLR4 signaling in ER-stressed macrophages.

Given the critical role of DCs in sensing “dangers” and as a major target for TLR agonists as adjuvants during vaccination or immunotherapies, the biochemical and functional interactions between SRA/CD204 and TLR4 signaling pathways are also important for the DC immunogenicity. It has been documented that NF-κB activation can have a significant adjuvant effect on DC functions (31), such as enhanced antigen cross-presentation (32) and up-regulation of co-stimulatory signals (33). Indeed, therapeutic implications of the present study in vaccine design have been highlighted in our recent finding that down-regulation of SRA/CD204 promotes TLR4 activation-induced T-cell priming (12).

Our studies also raise the question as to whether SRA/CD204 affects other TLR signaling. Indeed, Kozik and co-workers (34) showed that SRA/CD204 mediates negative regulation of macrophage responses to CpG-ODN, an agonist of TLR9. Intriguingly, a recent report showed that SRA/CD204 is positively involved in the regulation of dsRNA signaling (35).
Fine Tuning of TLR4 Signaling by SRA/CD204

Therefore, the regulatory functions or biological specificity of SRA/CD204 appears to be determined by the nature of the stimulus or signals.

Accumulating evidence has shown that the multifunctional SRA/CD204 actively participates in a variety of biological processes (e.g. phagocytosis and adhesion), pathological conditions (e.g. atherosclerosis and Alzheimer disease), innate immunity, and host defense (36). Here we provide the first molecular basis that establishes an endo-/phagocytosis-independent signaling-regulatory feature of SRA/CD204 and uncover a novel biochemical mechanism underlying the fine control of LPS-induced TLR4-NF-κB signaling by SRA/CD204. Better understanding of the underappreciated roles of SRA/CD204 in inflammation and immunity will lead to new opportunities for therapeutic intervention in infectious, inflammatory, and malignant diseases of clinical importance.

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