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 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 InVivogen (San Diego, CA) were maintained in DMEM supplemented with 10% FBS, 10 μg/ml basicfusidic acid, and 100 μg/ml hygromycin B. Human monocytic cell line THP-1 cells were maintained in RPMI1640 medium with 10% FBS. Murine DC lines DC1.2 (SRA/CD204-expressing) and its subclone DC1 (SRA/CD204-deficient) were kindly provided by Dr. Kenneth C. Will (Boston, MA) and Dr. T.-C. Wu (John Hopkins University, Baltimore, MD), respectively, and maintained in RPMI1640 medium with 10% FBS. Murine DC lines DC1.2 (SRA/CD204-expressing) and its subclone DC1 (SRA/CD204-deficient) were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Center, Worcester, MA) and Dr. T.-C. Wu (John Hopkins University, Baltimore, MD), respectively, and maintained in RPMI1640 with 10% FBS. Immortalized WT and MyD88−/− macrophages were provided by Dr. Douglas Golenbock (University of Massachusetts) and maintained in DMEM with 10% FBS and 10 ng/ml macrophage colony-stimulating factor. Mouse BMDCs were prepared as previously described (12). The purity of CD11c⁺ MHCI⁺ was consistently >90% at days 8–9, as assessed by flow cytometry.

*Reagents and Antibodies—*Ultrapure LPS was purchased from InVivoGen (San Diego, CA). Cytochalasin D and chloroquine were obtained from Sigma-Aldrich. U0126 (MEK1/2 inhibitor) was obtained from Cell Signaling Biotechnology (Beverly, MA). Ro106-9920 (1×B ubiquitination inhibitor) and control were obtained 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-AcGFp1-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 Supernscript II reverse transcriptase (Invitrogen). A quantitative transcription profile of Tnfα 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 predeveloped 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 2ΔΔCT 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-CD14-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-kB 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 × 10^6 cpm) double-stranded NF-kB oligonucleotide (5′-AGTT-GAGGGGACTTTCCCAGG-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-deoxycytidylic 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-kB transcriptional activity was measured using an ELISA-based TransAM NF-kB 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_0011067979, amino acids 1–55) were cloned into pBAX-P-AHis (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 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.
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 $Tnfa$ after 4 h of stimulation 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 these inflammatory factors than did WT controls (Fig. 1E), indicating that SRA/CD204 is directly involved in the restriction of the TLR4-mediated inflammatory response.

**SRA/CD204 Inhibits LPS-induced NF-κB Activation**—Given that NF-κB is a master transcription factor that controls an array of genes involved in inflammation and immunity, we assessed the effect of SRA/CD204 on LPS-induced NF-κB activity. Upon LPS stimulation, SRA $^{-/-}$ DCs displayed higher levels of IκBα phosphorylation, increased IκBα degradation, and a longer period of IκBα loss compared with WT cells (Fig. 2A). SRA/CD204 deficiency also increased LPS-stimulated 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 $Tnfa$ 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 and degradation of IκBα 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).

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 $Tnfa$ (Fig. 2D) and $Il6$ (Fig. 2E) was only
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.

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 Tnfα 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 Tnfα (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.

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).
immunoprecipitation assay using anti-TRAF6 antibodies (supplementary 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<sup>GFP</sup> and TRAF6<sup>RFP</sup> exhibited punctated staining throughout the cytoplasm. Upon LPS stimulation, they co-lo-
calized 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.
suggesting that endosomal functions may be essential for SRA/CD204-mediated signaling-regulatory activities.

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 overexpressed 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 plus His-tagged full-length TRAF6 or various truncated TRAF6 deletion mutants. Immunoprecipitation was performed 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.

(Fig. 4G, bottom), suggesting that endosomal functions may be essential for SRA/CD204-mediated signaling-regulatory activities.

**FIGURE 5. TRAF6 binds to SRA/CD204 via TRAF-C domain.** A, ICD region of SRA/CD204 interacts with TRAF6. GST- or GST-ICD-conjugated Sepharose beads were incubated with protein lysates prepared from DC1.2 cells transfected with HisGly-tagged TRAF6. After washing, precipitated proteins were analyzed by immunoblot (IB). Endogenous TRAF6 and exogenous His-tagged TRAF6 were pulled down by GST-ICD as bait. B, suppression of NF-κB activity by ICD of SRA/CD204. HEK293-TLR4/MD2-CD14-TRAF6 cells were co-transfected with a 5×NF-κB luciferase reporter construct with V5-tagged SRA/CD204 (SRA-FL) or HisGly-tagged intracellular domain (SRA-ICD) constructs (*, p < 0.01; **, p < 0.05). C, schematic diagram of full-length TRAF6 and its deletion mutants. D, TRAF-C domain is essential for the binding of TRAF6 to SRA/CD204. HEK293-TLR4/MD2-CD14 cells were transfected with V5-tagged SRA/CD204 plus His-tagged full-length TRAF6 or various truncated TRAF6 deletion mutants. Immunoprecipitation was performed 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. IκB kinase) 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−/− cells (Fig. 6B). Given that dimerization of TRAF6 is a prerequisite for its E3 ligase activity, we next asked whether SRA/CD204 could interfere with LPS-induced TRAF6 dimer formation. Immunoblotting analysis of native gels showed that SRA/CD204 absence enhanced LPS-stimulated dimer formation of TRAF6 (Fig. 6C). Interestingly, the majority of intracellular SRA/CD204 is present as a trimer, and LPS stimulation modestly induced trimerization of SRA/CD204 (Fig. 6C). 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−/− BMDCs upon LPS stimulation (Fig. 7A). However, an increase in the TRAF6 recruitment to IRAK1 was seen in LPS-stimulated SRA−/− 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
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−/− 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-κB 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 adap-
tor 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 recep-
tors may sufficiently compensate for the loss of SRA/CD204.
Indeed, the activated phenotype of myeloid cells, not the phag-
ocytic capability, was recently shown to contribute to the
higher levels of proinflammatory cytokines in pathogen-in-
fected SRA/CD204−/− mice (23), suggesting that SRA/CD204-
dependent phagocytosis and inhibition of cytokine production
may be controlled independently (24). Interestingly, immuno-
fluorescence staining in our studies clearly shows the existence
of an intracellular pool of SRA/CD204 in DCs. Its broad distri-
bution 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
NF-κB phosphorylation. Treatment with cytochalasin D reduced
the phosphorylation of IκBα, suggesting that initial ligand
binding/uptake activities of endocytic receptors may actually
amply the signaling and contribute to the pathogen recogni-
tion. 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-depend-
ent 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 ubi-
quitination by SRA/CD204 ex vivo also provides important
evidence for the role of SRA/CD204 in TLR4 signaling modula-
tion. Last, the SRA/CD204 deficiency does not alter LPS-
stimulated ubiquitination and degradation of IRAK1, an adap-
tor 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 regula-
tion 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 acti-
vation 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 recogni-
tion 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 signal-
ing. 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 initi-
at ing an inflammatory response and facilitating pathogen rec-
ognition, 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 in-
cludes 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 interac-
tions between SRA/CD204 and TLR4 signaling pathways are
also important for the DC immunogenicity. It has been docu-
mented 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-in-
duced 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 posi-
tively involved in the regulation of dsRNA sensing (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 of 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.

Acknowledgments—We gratefully thank Drs. Kenneth Rock and Douglas Golenbock (University of Massachusetts) for kindly providing DC1.2 and MyD88−/− cell lines. We thank Dr. T.-C. Wu (Johns Hopkins University) for generously providing the DC1 cell line. We thank Dr. Paul Graves for critically reading the manuscript.

REFERENCES

1. Murphy, J. E., Tedbury, P. R., Homer-Vanvassinkam, S., Walker, J. H., and Ponnambalam, S. (2005) Atherosclerosis 182, 1–15
2. Suzuki, H., Kurihara, Y., Takeya, M., Kamada, N., Kataoka, M., Jishage, K., Ueda, O., Sakaguchi, H., Higashi, T., Suzuki, T., Takashima, Y., Kawabe, Y., Cynsh, O., Wada, Y., Honda, M., Kurihara, H., Aburatani, H., Doi, T., Matsumoto, A., Azuma, S., Noda, T., Toyoda, Y., Itakura, H., Yazaki, Y., and Kodama, T. (1997) Nature 386, 292–296
3. Areschoug, T., and Gordon, S. (2009) Cell Microbiol. 11, 1160–1169
4. Thomas, C. A., Li, Y., Kodama, T., Suzuki, H., Silverstein, S. C., and El Khoury, J. (2000) J. Exp. Med. 191, 147–156
5. Haworth, R., Platt, N., Keshav, S., Hughes, D., Darley, E., Suzuki, H., Kurihara, Y., Kodama, T., and Gordon, S. (1997) J. Exp. Med. 186, 1431–1439
6. Cotena, A., Gordon, S., and Platt, N. (2004) J. Immunol. 173, 6427–6432
7. Józefowski, S., Arredouani, M., Sulahian, T., and Kobzik, L. (2005) J. Immunol. 175, 8032–8041
8. Medzhivet, R. (2007) Nature 449, 819–826
9. Kawai, T., and Akira, S. (2010) Nat. Immunol. 11, 373–384
10. Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. I. (2000) Cell 103, 351–361
11. Liew, F. Y., Xu, D., Brint, E. K., and O’Neill, L. A. (2005) Nat. Rev. Immunol. 5, 446–458
12. Li, Y., Yu, X., Gao, P., Wang, Y., Baek, S. H., Chen, X., Kim, H. L., Subjeck, J. R., and Wang, X. Y. (2009) Blood 113, 5819–5828
13. Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002) J. Clin. Invest. 109, 1541–1550
14. Livak, K. J., and Schmittgen, T. D. (2001) Methods 25, 402–408
15. Moffat, J., Grueneberg, D. A., Yang, X., Kim, S. Y., Klopfer, A. M., Hinkle, G., Piqani, B., Eisenhaure, T. M., Luo, B., Grenier, J. K., Carpenter, A. E., Foo, S. Y., Stewart, S. A., Stockwell, B. R., Hacohen, N., Hahn, W. C., Lander, E. S., Sabatini, D. M., and Root, D. E. (2006) Cell 124, 1283–1298
16. Park, J., Easton, D. P., Chen, X., MacDonald, I. J., Wang, X. Y., and Subjeck, J. R. (2003) Biochemistry 42, 14893–14902
17. Puntervoll, P., Linding, R., Gemünd, C., Chabanas-Davidson, S., Matting-sdal, M., Cameron, S., Martin, D. M., Ausiello, G., Brannetti, B., Costantini, A., Ferrè, F., Maselli, V., Via, A., Cesareni, G., Diella, F., Superti-Furgi, G., Wyrwicz, L., Ramu, C., McGuigan, C., Gudavalli, R., Letunic, I., Bork, P., Rychlewski, L., Küster, B., Helmer-Citterich, M., Hunter, W. N., Aasland, R., and Gibson, T. J. (2003) Nucleic Acids Res. 31, 3625–3630
18. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., and Chen, Z. J. (2001) Nature 412, 346–351
19. Sly, L. M., Rauh, M. J., Kalesnikhoff, I., Song, C. H., and Krystul, G. (2004) Immunology 111, 227–239
20. Ravetch, J. V., and Bolland, S. (2001) Annu. Rev. Immunol. 19, 275–290
21. Józefowski, S., and Kobzik, L. (2004) J. Leukoc. Biol. 76, 1066–1074
22. Van Amersfoort, E. S., Van Berkel, T. J., and Kuiper, I. (2003) Clin. Microbiol. Rev. 16, 379–414
23. Hollifield, M., Bou Ghanem, E., de Villiers, W. J., and Garvy, B. A. (2007) Infect. Immun. 75, 3999–4005
24. Amiel, E., Acker, J. L., Collins, R. M., and Berwin, B. (2009) Infect. Immun. 77, 4567–4573
25. Husebye, H., Halaas, Ø., Stenmark, H., Tunheim, G., Sandanger, Ø., Bogen, B., Brench, A., Latz, E., and Espevik, T. (2006) EMBO J. 25, 683–692
26. Li, Q., Harraz, M. M., Zhou, W., Zhang, L. N., Ding, W., Zhang, Y., Eggleston, T., Yeaman, C., Banfi, B., and Engelhardt, J. F. (2006) Mol. Cell Biol. 26, 140–154
27. Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003) J. Exp. Med. 197, 1107–1117
28. Hoebe, K., Georger, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sotvath, S., Shamel, L., Hartung, T., Zähringer, U., and Beutler, B. (2005) Nature 433, 523–527
29. Jeannin, P., Bottazzi, B., Sironi, M., Doni, A., Rusnati, M., Presta, M., Maina, V., Magistrelli, G., Haeuw, J. F., Heirman, C., Vaeremans, E., Thielemans, K., Goldschmidt, B., Brech, A., Latz, E., and Espevik, T. (2006) Nature 441, 275–290
30. Seimon, T. A., Obstfeld, A., Moore, K. J., Golenbock, D. T., and Tabas, I. (2003) J. Clin. Invest. 112, 649–654
31. Andreakos, E., Williams, R. O., Wales, J., Foxwell, B. M., and Feldmann, M. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 19794–19799
32. Andrekos, E., Williams, R. O., Wales, J., Foxwell, B. M., and Feldmann, M. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 14459–14464
33. Lind, E. F., Ahonen, C. L., Wasiuk, A., Kosaka, Y., Becher, B., Bennett, K. A., and Noelle, R. J. (2008) J. Immunol. 181, 354–363
34. Moore, F., Buonocore, S., Aksoy, E., Oleed-Haddou, N., Goriely, S., Lazaro, E., Paulart, F., Heirman, C., Vaeremans, E., Thielemans, K., Goldman, M., and Flamand, V. (2007) J. Immunol. 178, 1301–1311
35. Józefowski, S., Sulahian, T. H., Arredouani, M., and Kobzik, L. (2006) J. Leukoc. Biol. 80, 870–879
36. DeWitte-Orr, S. J., Collins, S. E., Bauer, C. M., Bowdish, D. M., and Mossman, K. L. (2010) PLoS Pathog. 6, e1000829
37. Platt, N., and Gordon, S. (2001) J. Clin. Invest. 108, 649–654