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Proteomic Analysis of ABCA1-Null Macrophages Reveals a Role for Stomatin-Like Protein-2 in Raft Composition and Toll-Like Receptor Signaling*[S]

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Lipid raft membrane microdomains organize signaling by many prototypical receptors, including the Toll-like receptors (TLRs) of the innate immune system. Raft-localization of proteins is widely thought to be regulated by raft cholesterol levels, but this is largely on the basis of studies that have manipulated cell cholesterol using crude and poorly specific chemical tools, such as β-cyclodextrins. To date, there has been no proteome-scale investigation of whether endogenous regulators of intracellular cholesterol trafficking, such as the ATP binding cassette (ABC)A1 lipid efflux transporter, regulate targeting of proteins to rafts. *Abca1*$^{-/-}$ macrophages have cholesterol-laden rafts that have been reported to contain increased levels of select proteins, including TLR4, the lipopolysaccharide receptor. Here, using quantitative proteomic profiling, we identified 383 proteins in raft isolates from *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ macrophages. ABCA1 deletion induced wide-ranging changes to the raft proteome. Remarkably, many of these changes were similar to those seen in *Abca1*$^{+/+}$ macrophages after lipopolysaccharide exposure. Stomatin-like protein (SLP)-2, a member of the stomatin-prohibitin-flotillin-HflK/C family of membrane scaffolding proteins, was robustly and specifically increased in *Abca1*$^{-/-}$ rafts. Pursuing SLP-2 function, we found that rafts of SLP-2-silenced macrophages had markedly abnormal composition. SLP-2 silencing did not compromise ABCA1-dependent cholesterol efflux but reduced macrophage responsiveness to multiple TLR ligands. This was associated with reduced raft levels of the TLR co-receptor, CD14, and defective lipopolysaccharide-induced recruitment of the common TLR adaptor, MyD88, to rafts. Taken together, we show that the lipid transporter ABCA1 regulates the protein repertoire of rafts and identify SLP-2 as an ABCA1-dependent regulator of raft composition and of the innate immune response. *Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045179, 1859–1870, 2015.*

Lipid rafts are cholesterol-enriched membrane microdomains, thought to be present in all cells, that concentrate and organize cell-surface signal transduction events in several signaling cascades, including those of the Toll-like receptors (TLRs) (1). The selectivity of rafts for particular proteins, and, consequently, the signal strength of pathways initiating from ligated raft-resident receptors, are thought to derive in large part from the high cholesterol content of raft microdomains (2–4). *In vitro*, altering raft cholesterol of living cells downward or upward with chemical tools (*e.g.* cyclodextrins) leads to parallel changes in raft protein abundance (3, 4). The relevance of cholesterol-driven alterations in the raft proteome to disease is suggested by reports that hypercholesterolemia cholesterol-loads macrophage rafts and amplifies their responsiveness to lipopolysaccharide (LPS) (3, 4). Proteomic strategies have recently been applied to raft isolates from a variety of cell types, aiming to better understand the identity of proteins tonically present in rafts, as well as proteins dynamically recruited to rafts upon cell stimulation (2, 5–8). To date, however, most reports have used cell lines of uncertain physiological relevance. In addition, although raft cholesterol levels are regulated *in vivo* by intracellular cholesterol trafficking (1), no reports to date have sought to define how the raft proteome is physiologically regulated by cholesterol trafficking proteins.

ATP binding cassette (ABC) A1, a member of the ABC transporter superfamily, plays a key role in regulating levels of

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cholesterol in macrophages and other cells via promoting efflux of cellular cholesterol to extracellular acceptors, in particular lipid-free apolipoprotein (apo) A-I (9). The importance of ABCA1[1] to human health is clearly illustrated by Tangier disease, a rare ABCA1 mutation syndrome typified by severe HDL deficiency, widespread macrophage foam cells, and premature atherosclerosis (10). In addition, the large number of common ABCA1 polymorphisms that have been associated with human cardiovascular disease (10) suggest a broad-spanning impact of ABCA1 on human health. It remains somewhat controversial whether ABCA1-effluxed cholesterol derives from raft or extra-raft membranes (11). Nonetheless, both human Tangier disease cells and ABCA1-null murine macrophages have been shown to have greatly expanded lipid rafts that contain increased cholesterol and increased TLR4 (12, 13). These changes are associated with enhanced responsiveness to LPS that can be reversed by cholesterol depletion (13–15). Collectively, these findings indicate that ABCA1 may regulate the raft proteome and innate immune response through control of raft cholesterol. However, no proteomic analysis of rafts from ABCA1-deficient cells has been reported to date.

Herein, we report a proteomic analysis of raft isolates from naive and LPS-stimulated *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ primary murine macrophages. Unexpectedly, we found that ABCA1 deletion and LPS stimulation induced many similar changes in the raft proteome. Stomatin-like protein 2 (SLP-2), a lesser known member of the stomatin-prohibitin-flotillin-HflK/C (SPFH) family of membrane scaffolding proteins, was unique among SPFH proteins in being robustly up-regulated in rafts of unstimulated *Abca1*$^{-/-}$ cells compared with *Abca1*$^{+/+}$ counterparts. We found that rafts of SLP-2 knockdown cells were abnormal, displaying increased binding of cholera toxin subunit B—a probe for the raft-specific ganglioside GM1—but markedly decreased protein, including flotillins-1 and -2, and CD14. Whereas SLP-2 silencing did not compromise ABCA1-dependent cholesterol efflux, it reduced macrophage responsiveness to LPS and multiple additional TLR ligands. Taken together, we report that ABCA1 regulates the macrophage raft proteome and identify SLP-2 as a novel ABCA1-dependent regulator of raft composition that controls the innate immune response.

EXPERIMENTAL PROCEDURES

Reagents—*Escherichia coli* 0111:B4 LPS, penicillin, and streptomycin were from Sigma (St. Louis, MO). The Bio-Rad protein assay (Hercules, CA) and BCA protein assay (Pierce) were used. Pam3CSK4, heat-killed Listeria monocytogenes (HKLM), and imiquimod were from Invivogen (San Diego, CA).

Macrophage Culture and Treatment—Myeloid-specific (LysM-Cre) *Abca1*$^{-/-}$ mice and littermate controls were generated and bred as previously described (13, 15). All experiments were performed in accordance with the Animal Welfare Act and the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals after review by the Animal Care and Use Committee of Wake Forest School of Medicine. Bone-marrow-derived macrophages were prepared and cultured in DMEM containing 20% (v/v) FBS and 30% (v/v) L cell-conditioned medium for 5 days as previously described (13, 15). RAW 264.7 macrophages (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% heat-inactivated endotoxin-free fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin under a humidified 5% CO_2 atmosphere at 37 °C. Macrophages were stimulated for 2–24 h with buffer (control), LPS (0.1–100 ng/ml), Pam3CSK4 (2 ng/ml), HKLM (1×10^7/ml), or imiquimod (2 μg/ml).

Macrophage Transfections and Transductions—Peritoneal macrophages were collected 4 days after injection of 1 ml of 10% brewer's thioglycolate into the peritoneal cavity of 9–15 week-old mice. Following overnight culture in antibiotic-free complete media, 50 nM of control or SLP-2 smart pool siRNA was transfected with DharmaFECT reagent and incubated for 72 h according to the manufacturer's protocol (Dharmacon, Lafayette, CO). siRNA-treated macrophages were then stimulated with 100 ng/ml LPS for 6 h before cell culture supernatant was collected for analysis of cytokine expression by ELISA. Stable macrophage lines were produced using a set of lentiviral shRNAs against murine SLP-2 from Open Biosystems/ThermoFisher (Huntsville, AL). Lentiviral packaging was achieved by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) to transiently transfect HEK293T/17 cells (ATCC) with shRNA in a pLKO.1 vector together with vesicular stomatitis virus G glycoprotein and packaging plasmids. Supernatant was collected 48 h posttransfection and concentrated by centrifugation (50,000 $\times$ *g*, 2 h). Pellets were resuspended in PBS and used for infection. Titers were determined by performing quantitative PCR to measure the number of lentiviral particles that integrated into the host genome. In addition, biological titration of viruses that coexpressed fluorescent moieties was determined by flow cytometry. RAW 264.7 macrophages were infected at multiplicity of infection of 100 and selected 48 h postinfection with 10 μg/ml puromycin (Calbiochem) for ~9 days. The puromycin-selected stable cells (passage 10–16) were used for all further experiments after replating in puromycin-free media. The sequences of the scrambled shRNA and the two shRNAs that were found to effectively silence SLP-2 are shown in Table S2.

Detergent-Resistant Membrane (DRM) preparation—DRMs were prepared as previously reported (13). Raft fractions were detected by immunoblotting for raft (flotillin-1) and non-raft (transferrin receptor) markers and were identified as flotillin-1-positive, transferrin receptor-negative. Fractions were then pooled, sedimented (20,000 $\times$ *g*, 1h, 4 °C centrifugation after fourfold dilution in MES-buffered saline [25 mM MES, pH 6.5, 150 mM NaCl]), and an equal protein mass from each condition resolved by SDS-PAGE.

Protein Digestion—In-gel digestion was carried out for the four lipid raft samples. Each SYPRO-Ruby stained gel lane was cut into 24 equal slices and digested in a 96-well tray using a Progest robotic digester (Genomic Solutions, Inc., Ann Arbor, MI). In brief, gel slices were minced and placed in one well of a 96-well plate. Common in-gel digestion protocol was used. Briefly, the gel bands were soaked with 50 μl of 25 mM ammonium bicarbonate for 15 min and dehydrated with 50 μl of acetonitrile. These steps were performed two times before digestion was started with trypsin. Digestion of protein was carried out using 250 ng of trypsin in each well, and the solution was

[1] The abbreviations used are: ABCA1, ATP binding cassette transporter A1; CtB, cholera toxin B subunit; DRM, detergent-resistant membrane; LPS, lipopolysaccharide; MyD88, myeloid differentiation primary response 88; SLP-2, stomatin-like protein 2; SPFH, stomatin-prohibitin-flotillin-HflK/C; TLR, Toll-like receptor.

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allowed to incubate for 8 h at 37 °C. Proteins were extracted from the gel using 10% formic acid and acetonitrile as well. Supernatants were pooled, lyophilized, and resuspended in 40 μl of 0.1% formic acid. Samples were kept at −80 °C before LC/MS analysis was performed.

Nano- LC-MS/MS and Bioinformatics Analysis—Nano LC-MS/MS analysis was performed using an Agilent 1100 nanoLC system online with an Agilent XCT Ultra ion trap mass spectrometer equipped with the Chip Cube interface. Peptides were separated by reverse-phase LC utilizing a trapping column and nanoflow analytical column (Agilent Technologies, Santa Clara, CA) and analyzed both in full MS scan and CID MS/MS modes. Briefly, 20 μl of peptide digest was loaded into an Agilent C18 chip followed by a 15 min wash with 5% (v/v) acetonitrile, 0.1% formic acid (v/v). Peptides were eluted with a linear gradient of increasing concentration of acetonitrile (in 0.1% v/v formic acid) as follows: 0–45 min, 5–50% (v/v) acetonitrile; 45–50 min, acetonitrile increased to 95% (v/v), and 50–60 min acetonitrile maintained at 95%. Mass spectrometer was used in positive ion mode, standard enhanced mode, and included setting of a mass range from 200–2,200 *m/z*. MS/MS data were acquired using a data-dependent acquisition format, and six more abundant ions were targeted for MS/MS from each MS scan. The automated switching for MS/MS required a threshold of 5,000 counts. Dynamic ion exclusion of two spectra or 1.0 min ion trap resident time was used during MS/MS data acquisition. Tandem mass spectra were extracted by Spectrum Mill version (ReV A.03.03.078). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were also analyzed using Spectrum Mill (Agilent). Spectrum Mill was set up to search the NCBInr.rodent database (selected for RODENT, unknown version, 14,227,560 entries) using the digestion enzyme trypsin. Spectrum Mill was searched with a fragment ion mass tolerance of 0.70 Da and a parent ion tolerance of 2.5 Da. Oxidation of methionine was specified in Spectrum Mill as a variable modification. Scaffold (version Scaffold 3 00 08, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80.0% probability as specified by the Peptide Prophet algorithm (Keller, A *et al.* Anal. Chem. 2002;74(20):5383–92). Protein identifications were accepted if they could be established at greater than 80.0% probability and contained at least two identified peptides per protein. Annotated spectra and associated information are shown in Fig. S3 for two proteins that were identified on the basis of high-quality data from single peptides; for both proteins, additional distinct peptides were identified in independent sample runs. Protein probabilities were assigned by the Protein Prophet algorithm (16). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. For high and low confidence identification, these criteria were relaxed or tightened (~90% protein and peptide probability) and specified in the respective data analysis in the manuscript and the respective figures. For protein identifications, Table S1 shows spectral counts, Table S3 shows peptide counts, and Table S4 shows percentage amino acid coverage.

NF-κB Activity Assays—Activation of the p65 component of NF-κB in the nuclear fraction from cells (NE-PER kit; Pierce) was quantified by use of a sandwich ELISA (p65 TransAM™ kit, Active Motif, Carlsbad, CA) upon equalized nuclear protein input (BCA protein assay).

Cholera Toxin B, Filipin, and Rhodamine-Phalloidin Staining and Cell Imaging—GM1 was stained using Alexa Fluor® 488-Cholera Toxin B (CtB)(Invitrogen/Molecular Probes), a raft probe with high affinity for the raft ganglioside GM1. Cells in growth medium were incubated in AF488-CtB (1 μg/ml, 10 min, room temperature), washed, and cross-linked with anti-CtB antibody provided by the manufacturer (200-fold dilution, 15 min, room temperature). Cells were fixed in chilled 1x PBS containing 4% paraformaldehyde (15 min, room temperature) and then washed several times with 1x PBS. Cholesterol was imaged by staining with filipin III (Sigma). Briefly, cells were fixed (4% paraformaldehyde, 20 min, room temperature), quenched (50 mM NH_4Cl, 10 min, room temperature), washed, and then stained with 100 μg/ml Filipin III (20 min, room temperature), either with or without prior detergent permeabilization, with similar results. Actin was imaged by staining with rhodamine-phalloidin. Cells were fixed as above, washed, permeabilized with PBS containing 0.1% Triton X-100 (15 min, room temperature), washed, and then stained with 5 U/ml rhodamine-phalloidin (Molecular Probes, Eugene, OR)(20 min, room temperature). Cells were washed and mounted with ProLong Antifade Kit (Molecular Probes). Imaging was performed with a Zeiss 710 LSM microscope with a 40x objective/1.30 oil and ZEN software. For AF488-CtB, optical filters provided excitation/emission at 501 nm/566 nm; for filipin, a UV 375 nm filter set with a 385 nM long pass filter and a 364 nm laser was used; for rhodamine-phalloidin, a rhodamine filter set with a 458/561 dichroic beam splitter and a 561 nm laser was used. For analysis of fluorescence intensity, at least 10 region of interest (which included a minimum of 6–10 cells) from 2–3 individual experiments were analyzed using MetaMorph software.

Cytokine Analysis—Cytokines were quantified by ELISA (Biolegend, San Diego, CA).

Cholesterol Efflux Assay—Cholesterol efflux to BSA or apoA-I was quantified as previously reported (15).

Western Blotting—An equal protein mass (BCA assay) of whole cell lysate or DRMs was resolved on 4–20% Tris-HCl gels (BioRad), transferred to nitrocellulose (Amersham Biosciences), and probed with primary antibodies. Rabbit anti-MyD88 (1:100) was from EMD/Millipore, and mouse anti-flotillin-1, mouse anti-flotillin-2, and rabbit anti-CD14 (all 1:1,000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Membranes were washed in Tween Tris buffered saline (TTBS), and exposed to species-specific HRP-conjugated secondary antibody (GE Healthcare; 1:5,000; 60 min) in 5% milk/TTBS. After further TTBS washes, signal was detected with HyGlo (Quick Spray) chemiluminescent HRP antibody detection reagent, followed by film exposure (HyBlot CL, Denville Scientific).

Statistical Analysis—Analysis was performed using GraphPad Prism statistical software (San Diego, CA). Data are represented as mean ± S.E. Two-tailed student's *t* test was applied for comparisons of two groups, and analysis of variance (ANOVA) for comparisons of >2 groups. For all tests, $p < .05$ was considered significant.

RESULTS

Proteins Detected in Macrophage Raft Isolates—To define the effect of ABCA1 on the raft proteome of resting and LPS-exposed macrophages, we cultured bone-marrow-derived macrophages from myeloid-specific *Abca1*$^{-/-}$ mice (13) and littermate *Abca1*$^{+/+}$ controls. In four independent experiments, *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ macrophages were left unstimulated or exposed to *E. coli* 0111:B4 LPS (100 ng/ml, 30 min). Following this, detergent-resistant membranes (DRMs), a widely accepted surrogate for rafts (1, 4), were isolated using standard sucrose density gradient centrifugation methods (4). Gradient fractions containing DRMs were identified by immunoblotting to detect presence of the raft-specific protein flotillin-1, and absence of the non-raft marker transferrin receptor (Fig. S1). DRM fractions were pooled and sedimented by ultracentrifugation, resolved by 10% SDS-PAGE, and imaged with SYPRO Ruby protein stain (Fig. S2). In-gel tryptic digest was then performed on the gel lanes, followed by

protein identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with spectral counting.

As shown in Table S1, 383 unique proteins were collectively identified (0.1% protein false discovery rate, 0.7% peptide false discovery rate, ≥2 peptides) in raft isolates from at least one of the conditions across four experiments. Many raft proteins previously reported by our group and others (2, 5, 6, 8, 17) were detected, including flotillins-1 and -2, stomatin, CD18, CD44, CD36, aminopeptidase N, syntaxins, annexins, SNAREs, various heterotrimeric G-protein subunits, and Rab, Ras, and Rap family members. Of interest, we detected three proteins that regulate intracellular trafficking of TLRs but that have not previously been reported in rafts to our knowledge. Niemann-Pick C1, a protein that regulates ABCA1-dependent cholesterol efflux (18) and TLR4 trafficking to the plasma membrane (19), was noted in all ABCA1 genotype and LPS conditions, without evident alteration in abundance as judged by spectral count. Rab10, a protein that promotes TLR4 signaling by regulating TLR4 recycling to the plasma membrane (20), was detected in rafts from all experimental conditions but without notable change across them. Last, Unc93b1, a protein that regulates intracellular trafficking of several TLRs other than TLR4 (21), was found in rafts from all four experimental conditions, with a notable increase in unstimulated *Abca1*$^{-/-}$ rafts (detected in four of four experiments) as compared with unstimulated *Abca1*$^{+/+}$ rafts (detected in one of four experiments).

Several previously reported components of the LPS receptor complex were detected, including CD14, HSP70, HSP90, and moesin (3, 22), albeit without any consistent change in spectral abundance across conditions. RP105, a TLR4 homologue that inhibits LPS signaling by directly inhibiting TLR4 binding of LPS (23), was detected in both unstimulated *Abca1*$^{+/+}$ and unstimulated *Abca1*$^{-/-}$ rafts in two of four experiments but was not detected in LPS-stimulated *Abca1*$^{+/+}$ rafts in any of the four experiments, perhaps suggesting that LPS promotes its exclusion from macrophage rafts. By contrast, although TLR7 and TLR13 were detected in individual experiments, we did not detect spectra for the LPS receptor itself, TLR4. As we have previously confirmed TLR4 in *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ bone-marrow-derived macrophage rafts by immunoblotting (13) but have not detected it in RAW 264.7 rafts by LC-MS/MS (17), we speculate that this nondetection by MS reflects low protein abundance and/or poor peptide ionizability.

Impact of ABCA1 Deletion and LPS Exposure on the Macrophage Raft Proteome—Depiction of the abundance of individual raft proteins across experimental conditions and replicate experiments in the format of a heat map (Fig. 1*A*) demonstrated a significant degree of biological variation, a challenge that has been previously noted in proteomic analysis of primary cells (24). Initial overlay of proteins detected in the various ABCA1 and LPS conditions in the form of Venn diagrams nevertheless revealed a significant number of proteins both common and unique to the various conditions, indicating evident effects of both ABCA1 deletion and LPS exposure on the macrophage raft proteome (Fig. 1*B*). An initial spectral count-based evaluation of quantitative changes induced in raft proteins by ABCA1 deletion and LPS (*i.e.* ratio of spectral count in unstimulated *Abca1*$^{-/-}$ or LPS-stimulated *Abca1*$^{+/+}$ rafts over that in unstimulated *Abca1*$^{+/+}$ rafts) yielded modest (*i.e.* less than twofold) changes in a short list of proteins as well as considerable interexperiment variability (data not shown). Given this, we focused our efforts on using qualitative protein changes (*i.e.* proteins that were not detected in unstimulated *Abca1*$^{+/+}$ rafts but that were detected in unstimulated *Abca1*$^{-/-}$ and/or in LPS-stimulated *Abca1*$^{+/+}$ rafts) as an initial screen for identifying raft proteins that were more robustly impacted by ABCA1 and LPS.

We examined proteins that were not detected in rafts from unstimulated *Abca1*$^{+/+}$ cells but that were detected in rafts from unstimulated *Abca1*$^{-/-}$ cells (13 proteins; Fig. 2*A*) or in rafts from LPS-stimulated *Abca1*$^{+/+}$ cells (11 proteins; Fig. 2*B*). Of interest, comparison of the two protein lists shows them to be nearly identical. Nine of the 11 proteins not detected in unstimulated *Abca1*$^{+/+}$ rafts but detected in LPS-stimulated *Abca1*$^{+/+}$ rafts were also detected in rafts of unstimulated *Abca1*$^{-/-}$ cells. Nine of the 13 proteins not detected in unstimulated *Abca1*$^{+/+}$ rafts but detected in unstimulated *Abca1*$^{-/-}$ rafts were also detected in LPS-stimulated *Abca1*$^{+/+}$ rafts. Taken together, these results indicate a striking degree of convergence in the changes induced in the raft proteome by LPS and ABCA1 deletion. While the full significance of this convergence of protein expression changes is uncertain, given that LPS down-regulates ABCA1 in macrophages (25, 26), it may interestingly suggest that a significant proportion of alterations induced in the raft proteome by LPS occur secondary to its repression of ABCA1.

In order to validate proteins not detected by LC-MS/MS in unstimulated *Abca1*$^{+/+}$ rafts but detected in unstimulated *Abca1*$^{-/-}$ rafts and/or LPS-stimulated *Abca1*$^{+/+}$ rafts, we next immunoblotted raft isolates. We confirmed up-regulation of Cdc42 by both LPS exposure and ABCA1 deletion, up-regulation of L-plastin by ABCA1 deletion, and up-regulation of SLP-2 by ABCA1 deletion, as well as, more modestly, by LPS stimulation (Fig. 2*C*). Generally consistent with the spectral count data (Table S1), immunoblotting revealed prohibitin-2 to be modestly reduced with respect to its levels in unstimulated *Abca1*$^{+/+}$ rafts, in both LPS-stimulated *Abca1*$^{+/+}$ rafts and unstimulated *Abca1*$^{-/-}$ rafts, whereas flotillin-1, another member of the SPFH family of membrane scaffolding proteins, was unchanged. Similarly, CD14 was unchanged across ABCA1 and LPS conditions. Notably, immunoblotting of whole cell lysates for all of these targets revealed unchanged whole-cell expression, indicating that the differences reflect distributive changes to lipid rafts. Although the qualitative raft protein changes as identified by LC-MS/MS and quantitative raft protein changes as confirmed by

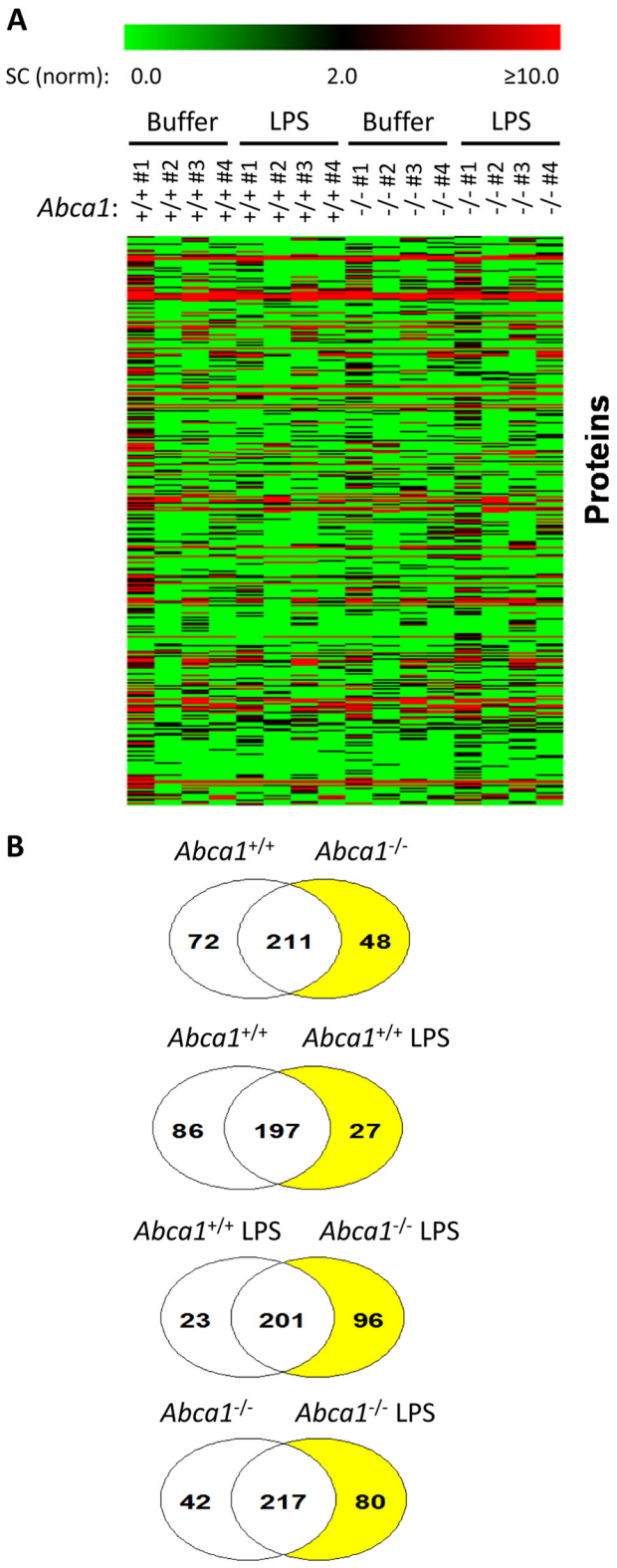

FIG. 1. **LPS- and ABCA1-dependent changes in the macrophage raft proteome.** (*A*) *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ murine bone-marrow-derived macrophages were treated with buffer (control) or LPS in four independent experiments, after which detergent-resistant membranes (DRMs) were isolated and profiled for their proteome by LC-MS/MS. The heat map depicts expression in the form of normalized spectral counts (SC) (quantitative value, calculated by Scaffold [Proteome Software, Portland, OR]) for unique proteins (rows) identified in all conditions (0.1% protein false discovery rate, 0.7% peptide false discovery rate, minimum two peptides per protein for identification). (*B*) Venn diagrams indicating the number of common and unique proteins identified in DRM preparations from the indicated experimental conditions.

immunoblotting are intriguing, these results must be considered as at most interesting leads that will require future quantitative and functional validation.

SLP-2 Silencing Reduces Macrophage Response to TLR Ligands—SPFH proteins are characterized by a conserved SPFH (also called "prohibitin homology") domain, and, while poorly understood, are thought to serve structural/organizational roles in rafts through targeting proteins to rafts, linking rafts to the actin cytoskeleton, and/or promoting nucleation of raft lipid species (27). Among family members, SLP-2 is unique in not possessing an N-terminal hydrophobic domain, instead being more loosely associated with rafts via myristoylation and palmitoylation (28, 29). Our attention was drawn to SLP-2, as, unlike SPFH members flotillins-1 and -2, prohibitins-1 and -2, and erlins-1 and -2, all of which were decreased or unchanged in *Abca1*$^{-/-}$ rafts (Table S1, Fig. 2), we found SLP-2 to be dramatically increased (Fig. 2*C*). Moreover, recent work indicates that SLP-2 plays a role in membrane microdomain organization in the mitochondrion and also in the plasma membrane, where it associates with the T cell receptor and promotes T cell receptor signaling (30–33). Given this, we hypothesized that SLP-2 might serve an important role in macrophage raft function, perhaps either promoting the enhanced TLR responses of *Abca1*$^{-/-}$ macrophages, or regulating ABCA1-dependent cholesterol efflux.

In order to define SLP-2 function in macrophages, we used siRNA to silence it in *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ murine macrophages (Fig. 3*A*). As previously reported by us and others (13, 15), *Abca1*$^{-/-}$ macrophages (transfected with control, nontargeting siRNA) were hyperresponsive to LPS, producing elevated IL-6 (Fig. 3*B*). SLP-2 silencing equivalently reduced LPS-induced IL-6 in both *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ macrophages, indicating that it is required for intact TLR4 signaling but suggesting that it does not account for the enhanced TLR4 response of the latter genotype. We next tested the effect of SLP-2 silencing on apoA-I-induced cholesterol efflux, the hallmark function of ABCA1 (15), in wild-type murine macrophages. As shown in Fig. 3*C*, apoA-I-induced cholesterol efflux was unchanged by SLP-2 silencing, suggesting that SLP-2 does not appreciably impact ABCA1 efflux function. SLP-2 silencing also did not impact diffusional cholesterol efflux to bovine serum albumin.

Aiming to further validate and define the role of SLP-2 in TLR4 signaling, we generated two stable SLP-2 knockdown cell lines via lentiviral shRNA transduction of RAW 264.7

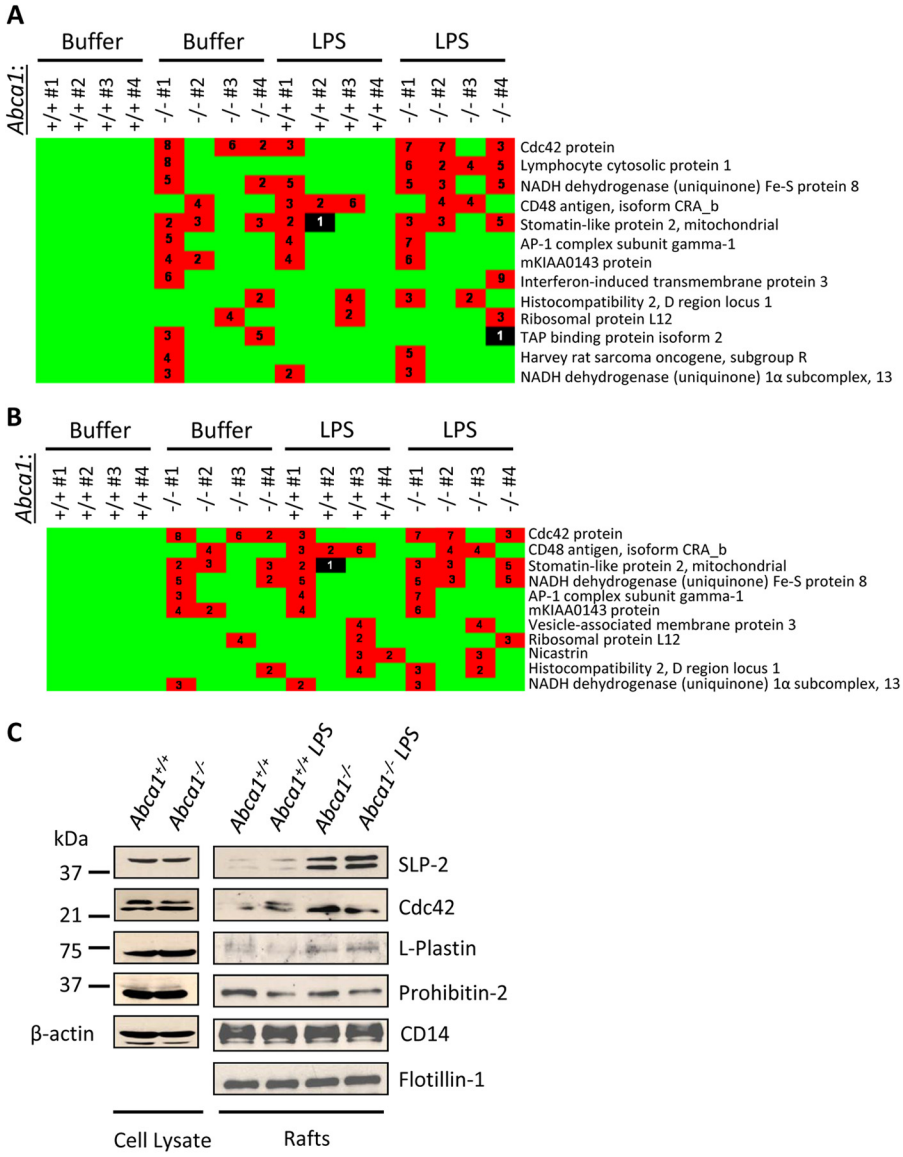

FIG. 2. **LPS treatment and ABCA1 deletion induce specific changes in raft-localization of proteins in the macrophage.** (*A*) The datasets were queried to identify proteins absent in untreated *Abca1*$^{+/+}$ rafts but detected in unstimulated *Abca1*$^{-/-}$ rafts, including both high-confidence (≥2 peptides/dataset; 95% protein probability) and lower confidence (≥1 peptide; 80% confidence) proteins. Among these proteins, only those that had ≥8 spectral counts (SCs) across all datasets were further considered. The heat map displays SC values for these proteins across all conditions in four independent experiments. Green = 0 SCs (nondetected); black = 1 SC; red = 2 or more SCs. (*B*) The datasets were queried to identify proteins absent in untreated *Abca1*$^{+/+}$ rafts but detected in LPS-stimulated *Abca1*$^{+/+}$ rafts, using the same criteria as applied in panel *A*. Color scheme is as described for *A*. (*C*) Detergent-resistant membranes (DRMs) were isolated from cell/treatments as indicated, and equal protein loads immunoblotted for the indicated protein targets. In parallel, whole cell lysates from *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ macrophages were immunoblotted for select targets displaying abundance changes in the DRM preparations. Representative of two to three independent experiments.

macrophages with two alternate constructs (Fig. 4*A*). We then exposed these SLP-2-silenced lines, along with a scrambled (nontargeting) shRNA control line, to LPS. As shown in Fig. 4*B*, the two SLP-2 knockdown macrophage lines produced lower TNFα than the control line at two concentrations of LPS, in general agreement with our siRNA results in primary murine macrophages. Lipid rafts are thought to regulate signaling by virtually all TLRs. Given this, we next evaluated the effect of SLP-2 silencing upon macrophage responses to a broader panel of ligands to both plasma membrane-localized (TLR1, -2) and endosomal (TLR7) TLRs. Both, Pam_3CSK_4 (TLR2/TLR1) and heat-killed *Listeria monocytogenes* (TLR2) induced less TNFα in SLP-2 knockdown macrophages than in controls (Fig. 4*C*). In addition, imiquimod, a TLR7 agonist, induced reduced TNFα in SLP-2 knockdown macrophages. Similar to our findings in SLP-2-silenced primary murine macrophages (Fig. 3), SLP-2 knockdown also reduced IL-6 output from macrophages stimulated with LPS, Pam_3CSK_4, or imiquimod (Fig. 4*D*). LPS induces TNFα and other proinflammatory cytokines via rapid activation of the transcription factor, NF-κB. Consistent with its attenuation of LPS-induced cytokines, SLP-2 silencing significantly reduced LPS-induced activation of NF-κB (Fig. 4*E*). Taken together, these findings indicate that SLP-2 is required for intact signaling from a broad array of both plasmalemmal and endosomal TLRs.

SLP-2 Regulates Raft Composition in Macrophages—Given that rafts regulate TLR signaling, (3, 4, 13) and SLP-2 is reported to play a role in membrane microdomain organization in T cells (30, 33), we next sought to determine whether SLP-2 is required for normal raft composition in macrophages. GM1 is a raft-specific ganglioside that is commonly used (via imaging with the high-affinity ligand, cholera toxin B subunit [CtB]) as a marker of rafts (15) but is itself reported to regulate raft function. Cell surface GM1, as assessed by CtB,

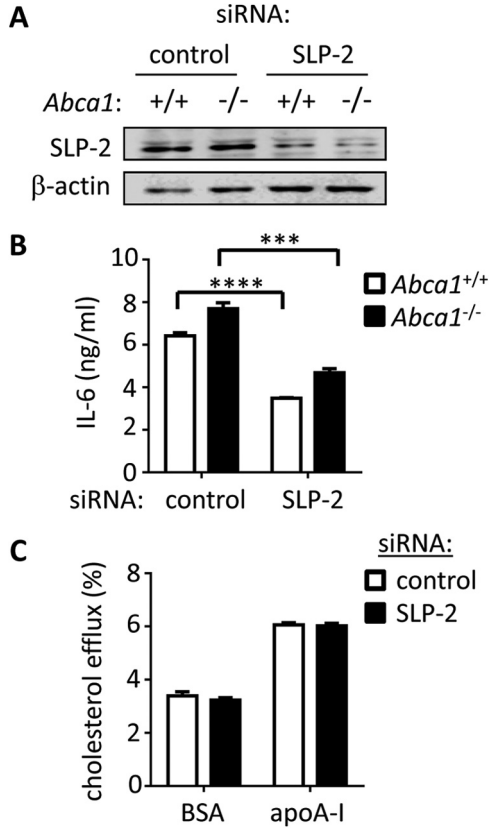

FIG. 3. **SLP-2 regulates the LPS response of macrophages.** (*A*) *Abca1*$^{+/+}$ and *Abca1*$^{-/-}$ murine bone marrow-derived macrophages were transfected with control non-targeting siRNA or SLP-2 siRNA and then immunoblotted as shown. (*B*) Cells under the same conditions as in panel *A* were stimulated with LPS, and then cell media IL-6 was quantified by ELISA. Representative of two independent experiments performed in triplicate. ****, $p < .0001$; ***, $p < .001$. (*C*) *Abca1*$^{+/+}$ macrophages were transfected with control nontargeting siRNA or SLP-2 siRNA. Cholesterol efflux to bovine serum albumin or apoA-I was then quantified. Representative of three independent experiments. BSA = bovine serum albumin; apoA-I = apolipoprotein A-I.

is increased in *Abca1*$^{-/-}$ macrophages (15). GM1 content in rafts is also up-regulated during cell stimulation (34) and in disorders characterized by abnormal raft signaling (*e.g.* lupus (35)). Of interest, we found that, compared with scrambled controls, SLP-2 knockdown macrophages bound dramatically increased levels of CtB (Figs. 5*A* and 5*B*), consistent with increased cell surface GM1. By contrast, staining with filipin, a fluorescent probe that binds cholesterol in lipid rafts and also in internal membranes, revealed no significant distributive or quantitative differences between scrambled control and SLP-2 knockdown cells in cholesterol, either in the plasma membrane, or internally (Fig. 5*C*). This is generally consistent with our finding that SLP2 silencing did not modify apoA-I-induced cholesterol efflux (Fig. 3*C*), and together suggests that SLP-2 does not modify intracellular cholesterol trafficking. As a screen for possible effects of SLP-2 silencing on the actin cytoskeleton, we also stained with the F-actin probe rhodamine-phalloidin. Phalloidin staining revealed no distributive or quantitative differences between scrambled control and SLP-2 knockdown cells either in the unstimulated state (Fig. 5*D*) or following stimulation with LPS or the chemotactic formylated tripeptide fMLP (data not shown), arguing against significant effects of SLP-2 on cytoskeletal remodeling.

Pursuing the effect of SLP-2 silencing upon raft protein composition, we next exposed SLP-2 knockdown and scrambled macrophage lines to LPS (or left them unexposed) and then isolated, pooled, and sedimented rafts. Notably, total protein mass in rafts from unstimulated SLP-2-silenced macrophages was significantly lower than that in unstimulated control cells (Fig. 5*E*), suggesting a global effect of SLP-2 on raft protein integrity. An equal protein mass from all four experimental conditions was next processed for immunoblotting. Although rafts from SLP-2 knockdown macrophages excluded the non-raft marker transferrin receptor as expected (not depicted), they were dramatically depleted of the hallmark raft proteins flotillin-1 and flotillin-2 and also had a significant reduction in CD14, a raft-localized protein that serves as a co-receptor for signaling by TLR4 and other TLRs (3) (Fig. 5*F*). Myeloid differentiation primary response 88 (MyD88), an adaptor protein required for signaling from all TLRs other than TLR3, is rapidly recruited to rafts in response to LPS, where it associates with TLR4 (3). An LPS-induced increase in MyD88 in rafts was observed in control macrophages but not in SLP-2-silenced macrophages (Fig. 5*F*). Taken together, these findings indicate that SLP-2 is required for lipid/protein integrity of macrophage rafts and for the raft-compartmental signaling that underlies the innate immune response.

DISCUSSION

The protein repertoire of rafts has been thought to be regulated by raft cholesterol (1, 2). However, to date, there has been no proteome-scale investigation of whether/how cells physiologically regulate the composition of their rafts. In the present report, we identify for the first time a suite of proteins whose abundance in macrophage rafts is modified by deletion of the cholesterol efflux transporter ABCA1. We find, remarkably, that LPS exposure induces many of the same changes in the raft proteome that are induced by ABCA1 deletion. This suggests the interesting possibility that LPS, a known repressor of ABCA1 (26), may modify the raft proteome, at least in part, via ABCA1 down-regulation.

Several proteins we identified in DRMs in this study have been previously localized to mitochondria. Multiple prior reports across a variety of cell types, including one by our group in RAW 264.7 macrophages (17) have identified several "mitochondrial" proteins in raft preparations (5–7, 36–38). While some have suggested that raft-like domains exist in mitochondria (39) or that these proteins represent technical artifact (40), stringent *in situ* approaches such as microscopy and

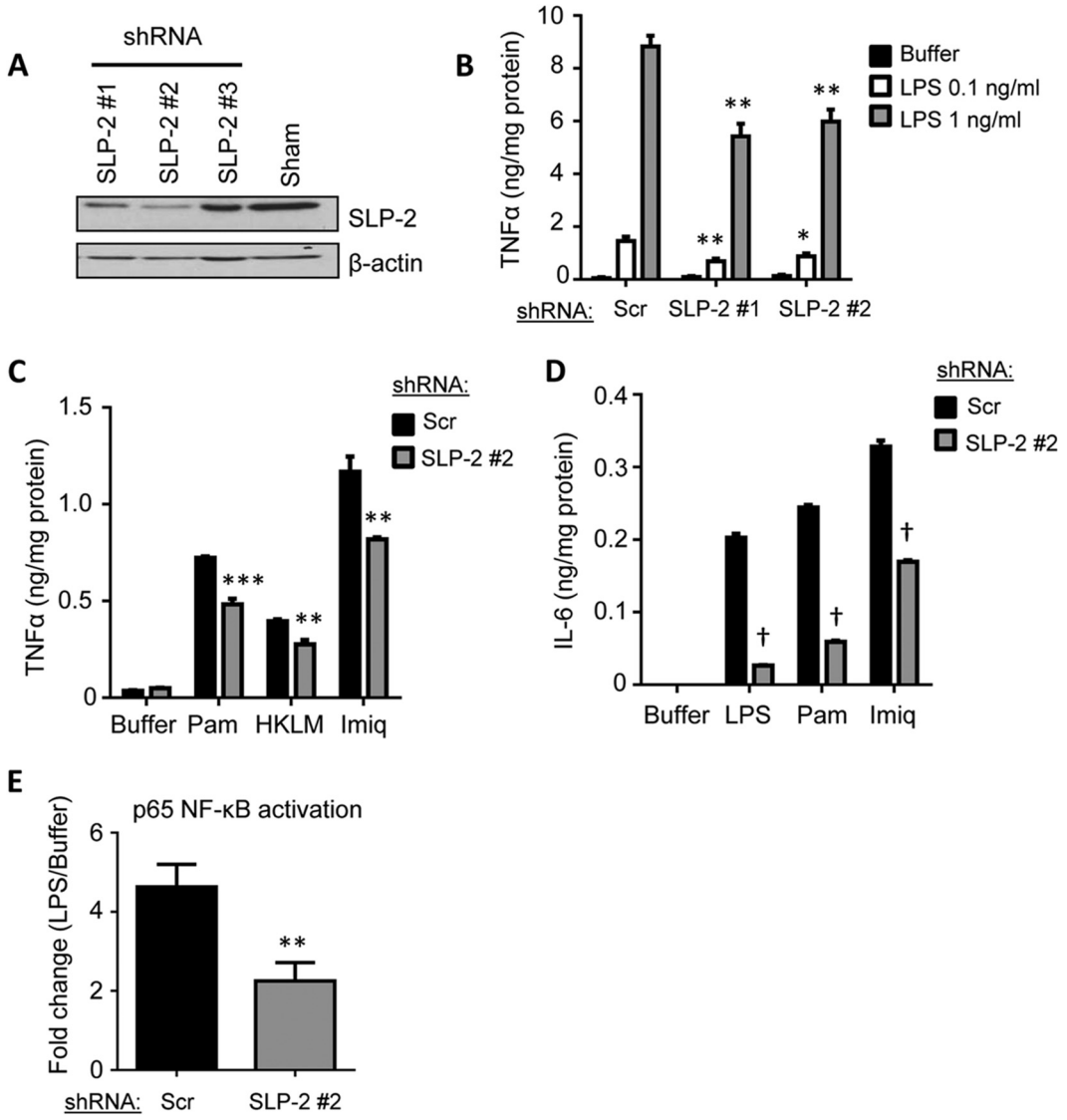

FIG. 4. **SLP-2 regulates the innate immune response triggered by multiple TLRs.** (*A*) Stable SLP-2 lentiviral shRNA knockdown RAW 264.7 macrophage lines were generated and then probed for SLP-2 and β-actin (loading control) as shown. Significant SLP-2 silencing was achieved with lentiviral constructs #1 and #2, whereas #3 was not effective. (*B*) Stable RAW 264.7 macrophage lines transduced with a scrambled (scr; *i.e.* nontargeting) vector or with SLP-2 #1 or #2 constructs as in panel *A* were exposed to buffer or to two concentrations of LPS for 2 h, after which TNFα was quantified by ELISA in the cell media and normalized to cell protein. Representative of three independent experiments. *, $p < .05$; **, $p < .01$. (*C*) Scrambled or SLP-2 #2 knockdown RAW 264.7 macrophages as in panel *B* were exposed for 2 h to buffer, Pam3CSK4 (Pam), heat-killed *L. monocytogenes* (HKLM), or imiquimod (Imiq), after which cell media TNFα was quantified. Representative of three independent experiments. **, $p < .01$; ***, $p < .001$. (*D*) Macrophages as in panel c were exposed for 24 h to buffer, LPS, Pam3CSK4, or imiquimod, after which cell media IL-6 was quantified. Representative of three independent experiments. †, $p < .0001$. (*E*) Macrophages as in panels *C* and *D* were exposed to buffer or LPS for 30 min, after which DNA binding of p65 NF-κB was quantified in nuclear isolates. Fold-change p65 activation in LPS as compared with buffer is shown. Representative of six biological replicates. **, $p < .01$. All statistical comparisons are to the corresponding "scr" exposure control.

cell-surface labeling have convincingly demonstrated "ectopic" plasma membrane localization of several of these proteins, including prohibitin, porin, NADH dehydrogenase, ubiquinol-cytochrome C reductase, and ATP synthase (36, 37).

Several putative mitochondrial proteins were detected by LC-MS/MS in *Abca1*$^{-/-}$ but not *Abca1*$^{+/+}$ DRMs, suggesting they are increased in the former. These include SLP-2, NADH dehydrogenase Fe-S protein 8, NADH dehydrogenase 1 alpha subcomplex subunit 13, NADH dehydrogenase subunit 1, cytochrome C oxidase subunit 7C, mitochondrial trifunctional protein β-subunit (Hadhb), and electron transferring flavoprotein alpha polypeptide (supplemental Table S1). By contrast, prohibitins-1 and -2, which play key roles in mitochondrial function, were relatively reduced in *Abca1*$^{-/-}$ rafts. ABCA1 deficiency was recently reported to reduce mitochondrial-

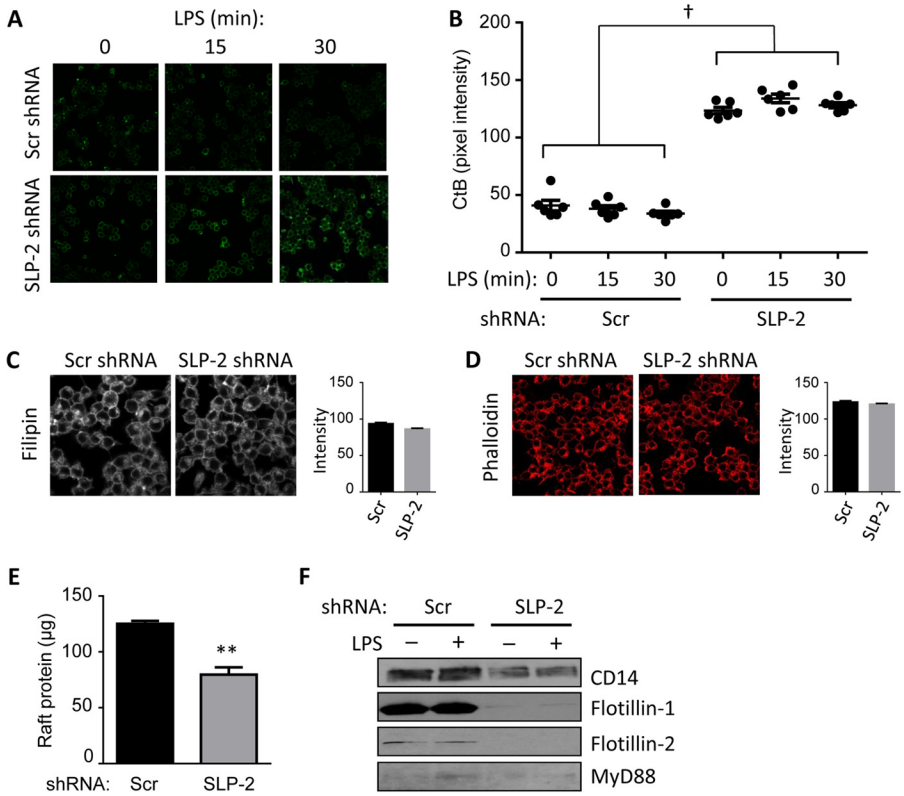

FIG. 5. **SLP-2 silencing dysregulates raft composition.** (*A*) RAW 264.7 macrophage lines stably transduced with either scrambled (scr; *i.e.* non-targeting) or SLP-2 lentiviral shRNA (SLP-2 #2 shRNA was used throughout this figure) were left untreated or treated with LPS (10 ng/ml), and then stained with Alexa488-cholera toxin B (CtB), and imaged using a Zeiss 710 LSM microscope (40x objective). A representative experiment of three is depicted. (*B*) Alexa488-CtB signal was quantified across three independent experiments as described in panel *A*. †, $p <$ .0001. (*C*) Cells were stained with the cholesterol probe filipin and imaged using a 63X oil objective with a further 1.5X zoom. Overall intensity was quantified using Metamorph software, as shown at right (p = NS). (*D*) Cells were stained with the actin probe rhodamine-phalloidin and imaged using a 63X oil objective. Overall intensity was quantified using Metamorph software, as shown at right (p = NS). (*E*) Rafts were isolated from unstimulated SLP-2-silenced and scrambled shRNA control macrophages, and raft protein mass quantified as shown. Normalized data across three independent experiments is shown. **, $p <$ .01. (*F*) SLP-2 lentiviral shRNA macrophages and scrambled shRNA controls were left unstimulated, or exposed to LPS. Raft fractions were then isolated, pooled, and sedimented for each of the four conditions, and an equal protein mass (35 μg) immunoblotted for the targets shown. Data are representative of 2–3 independent experiments.

mediated death in cancer cells by increasing mitochondrial cholesterol (41), and it has been proposed that this finding could underlie other recent reports linking ABCA1 deficiency to cancer (42, 43). Given this, some of the targets we found increased in *Abca1*$^{-/-}$ DRMs may possibly underlie the cancer cell survival advantage recently associated with ABCA1 deficiency. Of interest in this regard, SLP-2 up-regulation has recently been associated with worse outcomes in human cancers (44–46). Mitochondrial reactive oxygen species and other intermediates have also recently been shown to play a key role in the LPS response of cells (47). Thus, whether the enhanced LPS response of *Abca1*$^{-/-}$ macrophages reported by us and others is in part driven by altered mitochondrial function is also an important question worthy of further investigation.

SLP-2, previously found by us and others in raft preparations from a variety of cell types (6, 7, 17), is thought to reside in both mitochondrial and plasma membrane pools (31). Rigid topographic distinctions between mitochondria and plasma membrane may actually be unnecessary in this context, as it was recently reported that net exchange of membrane material between the two may occur during T cell activation, when mitochondria, plasma membrane, and increased SLP-2 coalesce at the immunological synapse (31). This is consistent with additional past reports documenting translocation of mitochondria to the plasma membrane (48, 49).

Roles for SLP-2 as an organizer of membrane microdomains have been proposed in both the plasma membrane and mitochondrial inner membrane. In the former site, SLP-2 is recruited to lipid rafts of T cells upon cell activation, where it associates with T cell receptor signalosome components and the actin cytoskeleton and is required for T cell receptor signaling, consistent with a scaffolding function (31, 32). In the mitochondrion, SLP-2 promotes formation of cardiolipin-enriched membrane domains in which respiratory chain components and prohibitins are optimally assembled and stabilized and enhances mitochondrial biogenesis and ATP production (28, 30, 33, 50, 51). We are unaware of prior

reports of SLP-2 in relation to macrophages, ABCA1, or TLR signaling.

In this report, we show that, unlike other SPFH proteins, SLP-2 was robustly and specifically increased in DRMs of *Abca1*$^{-/-}$ macrophages. Given this, we speculate that SLP-2 may subserve a key compensatory role in raft organization of ABCA1-deficient cells and, moreover, that this may be relevant to both Tangier disease and more common functional ABCA1 polymorphisms found in humans. Consistent with a fundamental role for SLP-2 in raft organization, we found SLP-2-silenced cells to have markedly abnormal rafts, with dramatically increased cell-surface ganglioside as assessed by CtB, reduced raft protein mass, including reductions in key raft proteins, including the flotillins and CD14, but unchanged raft cholesterol as indicated by filipin.

Moreover, we found that SLP-2 impacts LPS signaling in macrophages, albeit somewhat modestly. The LPS response has previously been shown by us and others to require raft integrity, as TLR4 and its proximal adaptors, including MyD88, translocate to the vicinity of CD14 in raft microdomains (3). We speculate that the effects of SLP-2 silencing on TLR signaling are most likely indirect (*i.e.* a secondary consequence of changes to lipid rafts). Thus, although our findings suggest that SLP-2 "regulates" the innate immune response in a general sense (*i.e.* TLR signaling is attenuated in SLP-2-depleted cells), this should not be construed to indicate that SLP-2 necessarily plays a direct regulatory role in the underlying signaling cascade. Of some interest, we found that SLP-2 silencing led to a relatively larger reduction in IL-6 than TNFα in response to TLR2, TLR4, and TLR7 stimulation (Figs. 4*B*-4*D*). While the specific underlying mechanism is not clear, this may suggest a somewhat selective effect of SLP-2 on downstream proinflammatory pathway output by TLRs.

Although GM1 has widely been treated as a simple surrogate measure for rafts, several papers have shown that GM1 levels can be dissociated from levels of raft proteins and raft cholesterol (52–54). Indeed, GM1 up-regulation has itself been linked to mislocalization of raft proteins, reduced membrane fluidity, and abnormal raft-dependent signaling (35, 55, 56). Exogenous GM1 inhibits LPS signaling (57), and a naturally occurring GM1^{hi} human monocyte subset has recently been shown to display a reduced LPS response compared with GM1^{lo} counterparts (58). It is thus possible that SLP-2 deficiency may attenuate LPS signaling in rafts via leading to increased GM1 in raft microdomains.

In summary, we report that ABCA1 regulates the macrophage raft proteome as assessed through DRM preparations and identify a suite of proteins whose abundance in rafts is up- or down-regulated in the absence of ABCA1. Among these, we submit that SLP-2, robustly and specifically up-regulated in *Abca1*$^{-/-}$ rafts, represents a promising new target in studies of the molecular pathogenesis of ABCA1 deficiency, lipid raft structural organization, and the innate immune response. Future studies employing advanced quantitative proteomic techniques, such as stable isotope labeling by amino acids in cell culture (SILAC), AQUA, and/or isobaric tags for relative and absolute quantification (ITRAQ) may foreseeably identify additional important effects of ABCA1 deletion upon the raft proteome with enhanced precision and sensitivity.

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Ⓢ This article contains supplemental material Tables S1 to S4 and Figs. S2 to S3.

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