Quantitative Proteomics Analysis of Macrophage Rafts Reveals Compartmentalized Activation of the Proteasome and of Proteasome-mediated ERK Activation in Response to Lipopolysaccharide

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Lipopolysaccharide (LPS), a glycolipid component of the outer membrane of Gram-negative bacteria, is a potent initiator of the innate immune response of the macrophage. LPS triggers downstream signaling by selectively recruiting and activating proteins in cholesterol-rich membrane microdomains called lipid rafts. We applied proteomics analysis to macrophage detergent-resistant membranes (DRMs) during an LPS exposure time course in an effort to identify and validate novel events occurring in macrophage rafts. Following metabolic incorporation in cell culture of heavy isotopes of amino acids arginine and lysine ([13C6]Arg and [13C6]Lys) or their light counterparts, a SILAC (stable isotope labeling with amino acids in cell culture)-based quantitative, liquid chromatography-tandem mass spectrometry proteomics approach was used to profile LPS-induced changes in the lipid raft proteome of RAW 264.7 macrophages. Unsupervised network analysis of the proteomics data set revealed a marked representation of the ubiquitin-proteasome system as well as changes in proteasome subunit composition following LPS challenge. Functional analysis of DRMs confirmed that LPS causes selective activation of the proteasome in macrophage rafts and proteasome inactivation outside of rafts. Given previous reports of an essential role for proteasomal degradation of IκB kinase-phosphorylated p105 in LPS activation of ERK mitogen-activated protein kinase, we tested for a role of rafts in compartmentalization of these events. Immunoblotting of DRMs revealed proteasome-dependent activation of MEK and ERK specifically occurring in lipid rafts as well as proteasomal activity upon raft-localized p105 that was enhanced by LPS. Cholesterol extraction from the intact macrophage with methyl-β-cyclodextrin was sufficient to activate ERK, recapitulating the LPS-IκB kinase-p105-MEK-ERK cascade, whereas both it and the alternate raft-disrupting agent nystatin blocked subsequent LPS activation of the ERK cascade. Taken together, our findings indicate a critical, selective role for raft compartmentalization and regulation of proteasome activity in activation of the MEK-ERK pathway. Molecular & Cellular Proteomics 8:201–213, 2009.

Lipopolysaccharide (LPS),¹ a glycolipid component of the outer membrane of Gram-negative bacteria, is a potent initiator of the inflammatory response of the macrophage (1–4). LPS signals through Toll-like receptor 4 (TLR4) (5), activating a variety of signaling molecules including protein-tyrosine kinases, Rho GTPases, IκB kinase (IκK), and the MAPKs ERK, p38, and JNK (6). This results in the downstream activation of transcription factors, such as NF-κB and AP-1, and the consequent induction of proinflammatory cytokines, including tumor necrosis factor-α and interferon-β (7, 8). LPS has been linked to a number of diseases, including sepsis, acute lung injury, asthma, and atherosclerosis (9–11); thus, the signals it elicits in the host cell, although incompletely described, are of great biomedical significance.

The LPS receptor TLR4 and its proximal signaling adapter molecules (e.g. MyD88) are recruited to and assembled in the lipid raft microdomain of leukocytes following LPS exposure (12). Lipid rafts are cholesterol-enriched membrane microdomains thought to dynamically organize cellular signaling events triggered by extracellular stimuli (13, 14). Signaling receptors and other interacting molecules use the lipid raft as a platform to selectively assemble and facilitate signal transduction (12–16). The dynamic remodeling of lipid rafts during proximal LPS signaling is believed to actively recruit non-raft

¹ The abbreviations used are: LPS, lipopolysaccharide; DRM, detergent-resistant membrane; SILAC, stable isotope labeling with amino acids in cell culture; IκK, IκB kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TLR4, Toll-like receptor 4; UPS, ubiquitin-proteasome system; DMEM, Dulbecco’s modified Eagle’s medium; mβCD, methyl-β-cyclodextrin; TTBS, Tween-TBS.
proteasome-dependent signaling cascade to ERK requires
202 complex that degrades proteins that have been ubiquitin-
cation of its targets. The 26 S proteasome, a large multiprotein
induce remodeling of the proteasome in rafts and respecifi-
cation to identify novel signals in the leukocyte triggered by LPS
exposure (12, 19). In the current work, we undertook comple-
mentary qualitative and quantitative stable isotope labeling with
proteomics approaches to study macrophage DRMs during an LPS
exposure time course. We reasoned that the former approach
would globally identify both basal raft-resident proteins and
expression and this would be comple-
mentated by the identification, using the latter approach, of
quantitative enrichment of select raft-resident proteins during
LPS exposure. Unsupervised network analysis of the pro-
proteomics data set revealed marked representation of the ubiq-
uitin-proteasome system (UPS) in macrophage DRMs, includ-
ing most of the subunits of the 26 S proteasome. Expression of
several proteasome subunits and ubiquitin ligases in DRMs
was sensitive to LPS treatment, suggesting that LPS may
induce remodeling of the proteasome in rafts and respecifi-
cation of its targets. The 26 S proteasome, a large multiprotein
complex that degrades proteins that have been ubiquitin-tagged by ubiquitin ligases, is reported to regulate activation of
both ERK and NF-κB by LPS (20, 21). Generally described as
a cytoplasmic in location, a few reports have indicated that the
proteasome may be membrane-associated, although what, if any, role rafts play in compartmentalization of the
proteasome and its function remains unclear (22–25). One
group has suggested that LPS may directly bind specific
proteasome subunits in macrophage membranes and acti-
ivate the proteasome, although neither rafts nor activity of the
proteasome within membrane fractions was examined (26).

Herein in validation of our proteomics findings, we con-
firmed that LPS selectively activates the 26 S proteasome in
macrophage DRMs. To our knowledge, this is the first direct
demonstration of proteasome function in rafts. Moreover we
implicate the proteasome in raft-compartmentalized activation of
ERK by LPS through its upstream degradation of IκK-phosphorylated p105 (20, 21, 26), and we report that the
proteasome-dependent signaling cascade to ERK requires
raft integrity. Extending the importance of cholesterol-de-
pendent membrane microdomains for proteasome-depen-
dent regulation of the ERK pathway as well as the basic link
between cholesterol and function of the UPS in the macro-
phage, we furthermore demonstrate that the LPS pathway to
ERK activation can largely be recapitulated by cholesterol
extraction from the macrophage.

MATERIALS AND METHODS

Chemicals and Reagents—RAW 264.7 cells were acquired from
ATCC (Manassas, VA). Custom Dulbecco’s modified Eagle’s medium
lacking arginine and lysine and the regular Dulbecco’s modified Ega-
le’s medium (DMEM) were purchased from Invitrogen, fetal bovine
serum was purchased from Atlanta Biologicals (Atlanta, GA), and
dialyzed fetal calf serum was purchased from Sigma-Aldrich. Stable
isotope-containing amino acids, [13C6]arginine and [13C6]lysine were
purchased from Invitrogen. Escherichia coli 0111:B4 LPS was pur-
chased from List Biological Laboratories (Campbell, CA). Sequencing
grade porcine trypsin was purchased from Promega Corp. (Madison,
WI). Solvents for liquid chromatography were purchased as follows:
HPLC-grade acetonitrile (Fisher Scientific) and formic acid (Sigma-
Aldrich). Raft-disrupting agents nystatin and methyl-β-cyclodextrin
were purchased from Sigma-Aldrich. Bay 11-7082 (IκB inhibitor),
PD98059 (MEK inhibitor), MG-132 (proteasome inhibitor), and lacta-
cystin (proteasome inhibitor) were purchased from Calbiochem.
DMSO was purchased from Sigma-Aldrich.

Cell Culture and Treatment—RAW 264.7 cells were cultured in
DMEM supplemented with 10% heat-inactivated endotoxin-free fetal
bovine serum, 2 mm l-glutamine, 100 μg/ml streptomycin, and 100
units/ml penicillin under a humidified 5% CO2 atmosphere at 37 °C.
Inhibitors other than polymyxin B and methyl-β-cyclodextrin (mβCD)
were dissolved in DMSO, and cells were treated with inhibitor or
vehicle in parallel for a final vehicle concentration of 0.1% (v/v). Cells
were treated with mβCD and mβCD-cholesterol complex as de-
scribed previously (12) with the exception that endotoxin-free Hanks’
balanced salt solution was used in place of Krebs-Ringer phosphate
dextrose buffer.

Stable Isotope Labeling with Amino Acids in Cell Culture—RAW
264.7 murine macrophages were adapted for SILAC during growth in
DMEM containing “light” [13C6]arginine and [13C6]lysine or “heavy”
[15N2]arginine and [15N2]lysine supplemented with 10% dialyzed fetal
calf serum and antibiotics. Heavy arginine and lysine were used
in LPS Exposure and DRM Isolation—Heavy SILAC-labeled RAW
264.7 macrophages (107 cells) were stimulated with freshly prepared
E. coli 0111:B4 LPS (100 ng/ml), and light labeled macrophages (107
cells) were treated with endotoxin-free PBS (control). Following stim-
ulation, DRMs were isolated by using a modification to the method
described by Fessler et al. (12). Briefly cells were washed three times
with ice-cold PBS. Following the wash, cells were centrifuged
(1000 × g, 10 min, 4 °C) and resuspended in 1 ml of ice-cold lysis
buffer (25 mm MES (pH 6.5), 150 mm NaCl, 1% Triton X-100, 1 mm
PMSF, 1 mm NaF, 0.1 mm Na3VO4, 5 μg/ml leupeptin, 5 μg/ml
aprotinin (all reagents purchased from Sigma-Aldrich)). Cells
were vortexed for 15 s on ice and allowed to sit on ice for 10 min. This
step was repeated three times. High density insoluble debris were re-
moved by centrifugation (1000 × g, 10 min, 4 °C). The supernatant
(∼1 ml) was added to an equal volume of 80% sucrose in MBS (25 mm
MES (pH 6.5), 150 mm NaCl), gently mixed by vortexing, and then
centrifuged through a 5–30% continuous sucrose gradient using an
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S. W. A.1 rotor (20 h, 37,000 rpm, 4°C) as reported previously (28). Following centrifugation, 12 1-mL fractions were harvested from the bottom by puncturing the tube with an 18-gauge needle. Protein concentrations in each fraction were determined by the Bradford protein assay. 10 μg of protein from each fraction was used to screen for rafts by immunoblotting for raft (flotillin-1) and non-raft (transferrin receptor) markers. Raft fractions were then pooled, and the total protein was quantified using protein assay. Heavy and light labeled proteins were combined 1:1, resolved by SDS-PAGE, and stained with SYPRO Ruby. Each SDS-PAGE minigel lane was loaded with 100 μg of total protein (50 μg of heavy isoform labeled protein, 50 μg of light isoform labeled protein).

Protein Digestion—Each SYPRO Ruby-stained minigel lane was cut out into 24 equal slices and digested in a 96-well tray using a Progester robotic digester (Genomic Solutions Inc., Ann Arbor, MI). Briefly each gel slice was minced and placed in one of the 96 wells. During digestion, gel bands were incubated twice for 15 min in 100 μL of 25 mM ammonium bicarbonate, 50% (v/v) acetonitrile. Following this, they were dehydrated by incubation in 100 μL of acetonitrile for 20 min and dried under a nitrogen stream. Trypsin (250 ng) was added to each well, and the sample was allowed to incubate at 37°C for 8 h. The supernatants from the digests were stored at −80°C until analysis. Additionally the gel was re-extracted three times: once with 50 μL of water for 20 min and twice with 20-min incubations in 50 μL of 5% (v/v) formic acid, 50% (v/v) acetonitrile. Supernatants were pooled, lyophilized, and resuspended in 40 μL of 0.1% formic acid.

Nano-LC-MS/MS/Analysis and Database Analysis—All nano-LC-MS/MS experiments were performed on an Agilent 1100 nano-ESI system on line with an Agilent XCT Ultra ion trap mass spectrometer with the chip cube interface (Agilent Technologies) and analyzed in both the full-scan MS and in the CID MS/MS modes.

Peptides were separated by reverse-phase LC with a trapping column/analytical column nanoflow setup (HPLC-Chip cube; Agilent Technologies) and separated by reverse-phase LC with a trapping column/analytical column nanoflow setup (HPLC-Chip cube; Agilent Technologies) and separated by reverse-phase LC with a trapping column/analytical column nanoflow setup (HPLC-Chip cube; Agilent Technologies). 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%. The mass spectrometer was used in the positive ion, standard enhanced mode and included settings of 5000 counts. Dynamic ion exclusion of two spectra or 1.0-min concentrations of 1 mM DTT, 0.5 mM ATP, 0.5 mg/ml BSA, and 100 μM succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin substrate as well as a 1.0-V fragmentation amplitude (CID collision energy). MS/MS data were acquired using a data-dependent acquisition format with the six most abundant ions from each MS scan further interrogated by MS/MS. The automated switching for MS/MS required a threshold of 5000 counts. Dynamic ion exclusion of two spectra or 1.0-min concentrations of 1 mM DTT, 0.5 mM ATP, 0.5 mg/ml BSA, and 100 μM succinyl-Leu-Leu-Val-[13C6]lysine as modifications. SILAC ratios for heavy and light peptides were calculated from the area under the extracted ion chromatogram from the MS data. If an identical trypsin peptide was identified from the heavy and the light samples, it was treated as one peptide for protein identification purposes; the peptide ratios were calculated to determine the protein ratio.

Western Blot—RAW 266.7 macrophages were washed once in ice-cold 1× PBS, then lysed with 1× Laemmli buffer supplemented with 20 μL DTT, and boiled for 8 min. Lysates were resolved on 8% (for p105 detection) or 10% SDS-PAGE gels, transferred to nitrocellulose (Bio-Rad), and then probed with primary antibodies. Rabbit anti-p65-ERK1/2, rabbit anti-p38, goat anti-p105, and mouse anti-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p47-p65, rabbit anti-p47-MEK1/2, rabbit anti-p47-p38, rabbit anti-p47-JNK, rabbit anti-JNK, and rabbit anti-ERK1/2 were from Cell Signaling Technology (Danvers, MA). Mouse anti-flotillin-1 receptor (Ingenuity Systems Inc.) was used to conduct a knowledge-based bioinformatics network analysis using the data extractor feature of the SpectrumMill Proteomics Workbench Version A.03.03.078 (Agilent Technologies). The MS and MS/MS data were extracted by limiting the data search to deconvolved ions observed between 300 and 6000 Da and a retention time between 10 and 60 min. MS scans with the same precursor mass (±1.5 m/z) and retention time within 15 s were merged. Moreover of the remaining MS/MS spectra, only spectra that contained sequence tag information of four or more residues were submitted for database searching. The resulting extracted data were searched against the rodent subset of the National Center for Bio-

technology Information (NCBI) RefSeq non-redundant (nr) protein database (release date January 24, 2006 with a total of 3,229,765 protein entries) using the MS/MS Search function of SpectrumMill as the search engine. All searches allowed for partial oxidation of methionine residues, formation of glutamic acid, and allowance for one trypsin missed cleavage with mass tolerance set to ±1.5 Da for precursor mass and ±0.7 Da for product ion masses and a minimum matched spectral intensity of 70%. Confident protein identification was based on three criteria: 1) matching of ≥2 tryptic peptides, 2) a SpectrumMill score >17, and 3) a molecular weight of the protein consistent with the gel region from which the protein was identified as indicated by molecular weight standards. All peptides with a score >17 were automatically validated, whereas MS/MS spectra of all the remaining peptides were manually inspected and validated. For SILAC experiments, all analyses were carried out as described above with the inclusion of [13C6]arginine and [13C6]lysine as modifications. SILAC ratios for heavy and light peptides were calculated from the area under the extracted ion chromatogram from the MS data. If an identical trypsin peptide was identified from the heavy and the light samples, it was treated as one peptide for protein identification purposes; the peptide ratios were calculated to determine the protein ratio.
activity were determined using two-way student’s t test (Graphpad Prism). A p value <0.05 was considered statistically significant. The SILAC ratios are reported as mean ratios (heavy:light) ± S.D. calculated for the heavy and light peptide pair that contributed to the identification of the protein. The standard deviation reported for a SILAC ratio of a given protein is the S.D. in the ratio of the heavy:light peptides for that particular protein.

RESULTS

Qualitative Proteomics Analysis of Lipid Rafts before and after LPS Treatment—A descriptive proteomics approach was first undertaken to profile the lipid raft proteome in resting and LPS-treated RAW 264.7 macrophages. To test the possibility that LPS induces recruitment of non-raft proteins to the lipid raft, macrophages were treated with endotoxin-free PBS (control) or LPS (100 ng/ml) for 5 or 30 min. As maximal LPS-induced MAPK activation occurs at ~15 min in RAW 264.7 macrophages (31–33), we selected 5 min as a time point that should enrich for early, “upstream” regulatory targets and 30 min as a time point enriching for “downstream” function-regulatory proteins. Following treatment, the cells were lysed, and DRMs were isolated using ultracentrifugation over a continuous sucrose gradient (5–30%). Raft fractions were identified by immunoblotting for flotillin-1 (raft marker) and transferrin receptor (non-raft marker). Following cell lysis and sucrose density gradient ultracentrifugation, 12 1-ml fractions were collected. Equal aliquots (10 μg) were analyzed for flotillin-1 and transferrin receptor (TfR) expression with the respective antibodies.

Three LPS-treated biological samples and three corresponding, paired untreated control samples were generated for each time point, 5-min LPS exposure or 30-min LPS exposure. In total, six samples (three control and three LPS-treated) were analyzed for 5-min LPS exposure, and a similar second set of six samples was analyzed for 30-min LPS exposure. A separate set of control samples was generated for each treatment condition to minimize biological variation during sample preparation. The non-redundant proteins identified for the control-treated pair for the 5-min time point are listed in supplemental Tables S1–S6; those corresponding to the 30-min time point are listed in supplemental Tables S7–S12. In the paired 5-min analysis, a mean of 383 (±79) proteins was identified in the control sample, and a mean of 463 (±71) proteins was identified in the LPS-treated sample. Similarly in the 30-min paired analysis, a mean of 241 (±9) proteins was identified in the control sample, and a mean of 409 (±106) proteins was identified for 30-min LPS-treated samples. A tight standard deviation in the number of proteins identified between individual analytical runs indicated good analytical reproducibility in LC-MS/MS analysis. Several functional categories were represented among the raft proteins in each experimental condition (supplemental Fig. S4). The major functional groups include cell movement, cell signaling, cell-to-cell signaling and interaction, cellular assembly and organization, molecular transport, lipid metabolism, post-translational modification, and small molecule biochemistry. Representation of some functional categories was noted to change with LPS treatment; notable was a relative depletion of cellular movement proteins after 5 min of LPS (supplemental Fig. S4).

Consistently for both time points (5 and 30 min), a larger number of proteins were identified in DRMs from the LPS-treated macrophages compared with the control samples, suggesting an active recruitment of basally raft-excluded proteins to the lipid raft upon LPS treatment. This increase in number of raft proteins from base line with LPS exposure was statistically significant (p < 0.05) at 30 min (Fig. 2). Table I represents a condensed list of select proteins that were newly recruited to the lipid raft following LPS treatment. The term "de novo" is used to indicate these newly recruited proteins: "de novo 3" indicates those proteins present in all three experiments in samples from 5 or 30 min after LPS exposure but absent in all three control samples; the "de novo 2" list represents those proteins present in two of the three experiments in samples from 5 or 30 min after LPS exposure but absent in all three control samples. Using these stringent criteria, the repertoire of putative raft-resident proteins in the resting macrophage and those proteins that are newly recruited to lipid rafts upon LPS exposure were thus identified.

An unsupervised network analysis (Ingenuity Pathway Analysis 6, Ingenuity Systems Inc.) of the qualitative DRM proteomics data set indicated that the UPS was the best populated pathway with a striking representation of its components (p = 0.006). A total of 33 proteins present along the UPS pathway were identified. The overall UPS pathway and, within it, pathway members identified in the DRM analysis are mapped in supplemental Fig. S5. Specific proteasome subunits identified in DRMs are illustrated in Fig. 3. Several proteins involved in protein ubiquitination were noted to be newly
recruited into rafts upon LPS treatment (Table I). Moreover 10 distinct proteasome subunits were identified in rafts at baseline, whereas three additional proteasome subunits were newly recruited to rafts upon LPS treatment (5 and 30 min combined) (Table II and Fig. 3). Of the several proteasome subunits identified in the analysis, subunit $\alpha_9$ has been reported to bind LPS (26). Taken together, these identifications suggest a potential role for the raft-localized proteasome in LPS signaling as well as the possibility that LPS might physically and functionally remodel the proteasome and proteins targeted for it within lipid rafts.

To confirm proteasomal function in lipid rafts and to test for a possible associated modification of proteasomal activity in the setting of LPS exposure, 26 S proteasomal chymotrypsin-like activity upon the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin was measured (34) in both raft and non-raft subcellular fractions. Chymotrypsin-like activity is one of the well described prototypical specificities exercised by the proteasome upon protein substrates (34). As shown in Fig. 4, LPS induced a modest but statistically significant increase in proteasome activity in rafts in contrast to a marked reduction in proteasomal activity outside rafts. Taken together, these findings indicate that a compartmentalized effect of LPS upon proteasome function occurs within lipid rafts. To better define the LPS responsiveness of proteasome expression within rafts, we next undertook a quantitative proteomics approach to determine relative changes in expression of specific subunits within the raft upon LPS treatment.

**Quantitative Proteomics Analysis of Lipid Rafts Reveals compartmentalized remodeling and activation of the proteasome by LPS**—LPS-induced changes in the lipid raft can involve quantitative enrichment of raft-resident proteins in addition to new recruitment to rafts of proteins that are basally raft-excluded (12). Hence qualitative identification of protein presence in rafts may fail to detect more subtle, but nonetheless biologically important quantitative changes in expression. To confirm proteasomal function in lipid rafts and to test for a possible associated modification of proteasomal activity in the setting of LPS exposure, 26 S proteasomal chymotrypsin-like activity upon the fluorogenic substrate succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin was measured (34) in both raft and non-raft subcellular fractions. Chymotrypsin-like activity is one of the well described prototypical specificities exercised by the proteasome upon protein substrates (34). As shown in Fig. 4, LPS induced a modest but statistically significant increase in proteasome activity in rafts in contrast to a marked reduction in proteasomal activity outside rafts. Taken together, these findings indicate that a compartmentalized effect of LPS upon proteasome function occurs within lipid rafts. To better define the LPS responsiveness of proteasome expression within rafts, we next undertook a quantitative proteomics approach to determine relative changes in expression of specific subunits within the raft upon LPS treatment.

A SILAC-based stable isotope labeling approach (Fig. 5) was thus used to quantify LPS-induced changes in relative protein expression in rafts as reported previously (35, 36). Within this global quantitative analysis, our specific focus was to profile possible LPS-induced changes in expression of raft-resident proteasome subunits. Following culture of the macrophages in light $[^{12}\text{C}_6]$arginine and $[^{12}\text{C}_6]$lysine or heavy $[^{13}\text{C}_6]$arginine and $[^{13}\text{C}_6]$lysine medium, light labeled cells were treated with PBS (control), whereas heavy labeled cells were exposed to LPS. As in the qualitative analysis, paired buffer-LPS exposures were performed for both 5 and 30 min of LPS exposure. Each exposure was carried out in triplicate, resulting in three biological SILAC samples for each time point. Following raft isolation, the pooled raft isolates from the light and heavy labeled cell were combined 1:1, separated by SDS-PAGE, and analyzed by LC-MS/MS. The $b$- and $y$-series ions from the MS/MS fragmentation along the peptide backbone were used to identify the peptide sequence, and the light:heavy peptide ratios were determined from the MS experiment by calculating the area under the peak of the extracted ion chromatogram. A mean of 143 and 145 proteins were identified from the 0–5-min and 0–30-min SILAC experiments, respectively. During SILAC data analysis, an identical light and heavy peptide was treated as a single unique peptide for protein identification purposes; this constraint resulted in a lower number of proteins reported as identified in the SILAC experiments compared with the non-SILAC experiments described above. This is due to reduced sensitivity as a result of dividing the total amount of protein (same total protein amount analyzed in each experiment) by two.

The proteins identified in the SILAC experiments along with the calculated light:heavy SILAC ratios are summarized in the supplemental Tables S13–S18. Proteins identified from only their heavy peptide were arbitrarily assigned the largest ratio determined in that data set as reported previously (37). Similarly proteins that were identified with only the light peptide...
were arbitrarily assigned the smallest ratio determined in that data set (37). Overall modest protein ratios were observed, consistent with previous reports applying SILAC methodology to short cell exposures like those used in the present study (38). The standard deviations calculated for the heavy:light SILAC ratios were consistently tight across all six experiments, with a mean standard deviation in the ratio across all six experiments of 0.16. As in previous SILAC analyses (35, 37), a protein ratio exceeding two standard deviations above unity was defined as indicating up-regulation. Conversely a ratio lower than two standard deviations below unity was defined as indicating down-regulation. Similarity in the number of proteins identified between three different biological samples and three different technical analyses along with the calculated mean standard deviation of 0.16 in the SILAC ratios suggests good biological and analytical reproducibility.

Using these criteria, the SILAC analysis revealed statistically significant LPS-induced enrichment in rafts of LPS receptor components, including CD14 and HSP70, consistent with other reports (18, 39) (supplemental Table S19). TLR4 was not detected in resting or LPS-stimulated rafts. Also noted was LPS-induced quantitative enrichment in rafts of tyrosine kinases (lyn and hck) and adhesion molecules (CD44) reported to associate with TLR4 or its signaling (40–42). Two enzymes, acid sphingomyelinase and gp91 phox, enriched in rafts at both 5 and 30 min of LPS exposure have been reported to regulate recruitment of LPS receptor components to rafts (43, 44).

In addition, seven different proteasome subunits were detected in rafts (Table III). Six of the seven subunits were

| Protein name                                      | NCBInr accession number (gi no.) |
|---------------------------------------------------|----------------------------------|
| Calcium-activated potassium channel rsk3          | 2564072                          |
| Fatty acid synthase, isoform CRA_b                | 148702826                        |
| Integrin α3                                       | 7305189                          |
| Leucine-rich repeats and immunoglobulin-like domains 3 | 42475968                        |
| NADH dehydrogenase subunit 1                      | 111380589                        |
| N-Glycan processing α-mannosidase iix             | 108744609                        |
| Serine/threonine kinase 16                        | 31543784                         |
| E3 ubiquitin-protein ligase CBL-B                 | 88911266                         |
| Leucine-rich repeat protein 4, neuronal            | 22122701                         |
| Mitogen-activated protein kinase 1, isoform CRA_b | 148665020                        |
| Mitogen-activated protein kinase kinase 10        | 34870816                         |
| Predicted: similar to mitogen-activated kinase kinase 3 | 109460509                      |
| Predicted: similar to ubiquitin-activating enzyme E1 | 149273657                      |
| Predicted: similar to ubiquitin-protein ligase E3A isoform 2 | 109458840         |
| Rho guanine nucleotide exchange factor 7          | 125987797                        |
| Solute carrier family 25, member 35               | 21312550                         |
| Ubiquitin-associated protein 2-like, isoform CRA_e | 28893565                         |
| Ubiquitin-specific protease 32                    | 148683226                        |
| Ubiquitin-specific protease 54                    | 149031228                        |
| Ubiquitin-activating enzyme E1-like, isoform CRA_a | 148706006                        |

**Table I**

A condensed list of representative proteins newly recruited to the macrophage lipid raft upon LPS treatment

NACHT, nucleotide binding oligomerization domain; PYD, pyrin-domain; CBL-B, Cas-Br-M (murine) ecotropic retroviral transforming sequence b; CRA, sequence derived from the Celera database.

| NCBInr accession number (gi no.) | Protein name                                      |
|----------------------------------|---------------------------------------------------|
| 110625671                        | Apolipoprotein B                                  |
| 78214312                         | ATP synthase, H¹-transporting, mitochondrial F⁰ complex, subunit b, isoform 1 |
| 7514005                          | Major vault protein                               |
| 124571716                        | Solute carrier family 12, member 2                |
| 1887360                          | Toll-like receptor 7                              |
| 146325812                        | Vacuolar ATP synthase subunit d 2                 |
| 123173782                        | Voltage-gated channel like 1                      |
| 18700004                         | Acetyl-coenzyme A acyltransferase 1               |
| 148690825                        | Adaptor protein complex AP-2, α 1 subunit         |
| 27545388                         | ATP-binding cassette, subfamily A (ABC1), member 5 |
| 148702433                        | ATP-binding cassette, subfamily A (ABC1), member 6 |
| 1143305                          | Cytosolic phospholipase A₂                         |
| 3335569                          | Fatty acid transport protein 4; FATP4             |
| 148673922                        | Heat shock protein 110, isoform CRA_a             |
| 149029498                        | Lipoxigenase homology domains 1 (predicted)       |
| 148699347                        | NACHT, leucine-rich repeat, and PYD-containing 2, isoform CRA_b |
| 109458474                        | Predicted: similar to ryanodine receptor 1 (skeletal) |
| 51948448                         | RAB21, member RAS oncogene family                 |
| 56205310                         | Rho-interacting protein 3                         |
| 2144101                          | Tricarboxylate carrier                            |
| 33469031                         | Ubiquitin-specific peptidase 53                   |
| 76443681                         | Ubiquitin-specific protease 11                    |
The marked preponderance of proteasome subunits and other UPS-associated proteins in the lipid raft and the observed sensitivity of expression and function to LPS suggested that the proteasome might play an upstream regulatory role in LPS signaling at the level of the membrane. Experiments were thus next undertaken to investigate this hypothesis. Given previous reports that the proteasome regulates early, upstream events in LPS activation of the ERK MAPK pathway (20, 21, 26) we tested for a regulatory role of rafts in functional compartmentalization of these events.

Rafts Couple the Proteasome to LPS-induced Activation of ERK—The proteasome is reported to regulate LPS-induced ERK activation in macrophages (Fig. 6A) (20, 21, 26). Following LPS exposure, IxK phosphorylates p105, targeting it for ubiquitination and proteasomal degradation. Proteasomal degradation releases the inhibitory binding interaction of p105 upon the MEK kinase Tpl2, thereby allowing consequent MEK and then ERK activation. Nevertheless the subcellular localization and organizing principle of these events that involve the proteasome upstream of ERK remain undetermined. Before investigating a possible role for rafts in compartmentalization of these events, we first confirmed roles for IxK and the proteasome in LPS-induced activation of ERK in our system. Consistent with a previous report (21), LPS-induced activation of ERK and its upstream kinase MEK were attenuated by pretreatment of macrophages with the proteasome inhibitor MG-132 (Fig. 6B). Similar results were found with the proteasome inhibitor lactacystin (data not shown). By contrast, activation of the alternate MAPKs p38 and JNK was not reduced by MG-132 (Fig. 6B) or lactacystin (data not shown), suggesting a specific role for the proteasome in ERK activation. IxK inhibition with Bay 11-7082 blocked LPS-induced phosphorylation of p105, MEK, and ERK and attenuated LPS-induced degradation of the former consistent with its localization upstream of the p105-MEK-ERK cascade (Fig. 6C). Degradation of p105 following LPS was attenuated by MG-132 (Fig. 6D), also consistent with the reported role of the proteasome in p105 degradation (21). Taken together, these findings confirm previous reports (20, 21, 26) of an upstream role for the proteasome in the ERK cascade with a hierarchy of sequential events: 1) IxK phosphorylation of p105, 2) proteasomal degradation of p105, 3) MEK activation, and 4) ERK activation.

To investigate a role for rafts in the signaling events upstream of LPS-induced ERK activation and to confirm a role for the raft-localized proteasome in them, we pretreated RAW 264.7 macrophages with MG-132 (or vehicle) followed by LPS (or buffer) and then isolated DRM and non-DRM fractions for targeted immunoblotting. As shown in Fig. 7, despite expression of the majority of cellular ERK outside of rafts, activated (i.e. phosphorylated) ERK was detected only in rafts following LPS, indicating high specific activity in this compartment. Phosphorylated MEK was similarly detected only in raft fractions following LPS. Although the majority of cellular p105 was expressed outside of rafts, a minor fraction was detected in rafts. Expression of raft and non-raft p105 was enhanced following MG-132 treatment, suggesting that p105 in both

Fig. 3. An enlarged section of the ubiquitin-proteasome system canonical pathway highlighting proteasome subunits and their reported IFNγ-induced exchange in formation of the “immuno-proteasome” (65, 66). The gray-shaded subunits were identified in macrophage DRMs. PSM, proteasome subunit; THIMET, thimet oligopeptidase 1; TAP, transporter 1 ATP-binding cassette (TAP1 and TAP2); IFNG, interferon-γ; ER, endoplasmic reticulum; MHC, major histocompatibility complex. The proteasome subunits with the asterisks represent components observed in more than one biological sample, and the double lined shapes represent a group of proteins/protein complex.
compartments is a substrate for the proteasome. Moreover, raft p105 was down-regulated by LPS, consistent with promotion by LPS of p105 proteasomal degradation in rafts, as observed in cell lysates (Fig. 6, C and D). Taken together, these findings indicate that compartmentalized, proteasome-dependent activation of the ERK cascade occurs in rafts and suggest that p105 is a target for the proteasome in rafts.

To further confirm a regulatory role for rafts in organization of the p105-MEK-ERK cascade, we tested the effect of two alternate, structurally dissimilar agents that have been used to perturb/disrupt raft microdomain organization by different mechanisms, mβCD (45–47) and nystatin (48, 49). mβCD is thought to disorganize rafts through extraction of cholesterol, whereas nystatin is thought to sequester membrane cholesterol. p105 phosphorylation and degradation and ERK phos-
phosphorylation were selected as upstream and downstream readouts, respectively, to assess the effect of these raft-perturbing agents on the LPS pathway. LPS-induced p105 phosphorylation and degradation and ERK phosphorylation were all attenuated by both mβCD and nystatin (Fig. 8A). Of interest, in the absence of LPS, mβCD but not nystatin was sufficient to induce phosphorylation of p105 and ERK. This stimulating activity of mβCD on ERK was not due to LPS contamination as ERK activation by LPS but not mβCD was abolished by the LPS-sequestering agent polymyxin B (50) (Fig. 8B). Moreover consistent with mβCD acting through cholesterol extraction, its activity upon ERK was abolished by preloading with cholesterol as reported previously (12). As mβCD has been reported by us and others to recapitulate several of the features of LPS signaling (12, 51), we investigated the mechanism of its activation of ERK for comparison with that of LPS. Like LPS (Fig. 6), mβCD-induced activation of both ERK and its upstream kinase MEK was inhibited by both proteasome inhibition and IκK inhibition (Fig. 8C), indicating that the proteasome and IκK are upstream of MEK-ERK activation. Also similar to LPS, mβCD-induced phosphorylation and degradation of p105 appear to be mediated by IκK as they were both inhibited by Bay 11-7082. Finally as with LPS, mβCD-induced p105 degradation was blocked by pretreatment with MG-132, consistent with its mediation by the proteasome.

DISCUSSION

Complementary qualitative and quantitative proteomics approaches were used to probe the dynamics of the lipid raft proteome in RAW 264.7 macrophages during LPS stimulation, together allowing for both global and targeted investigations. Using strict criteria, the qualitative profiling approach revealed that during LPS exposure a substantial number of new (i.e. basally raft-excluded) proteins are recruited to rafts in a time-dependent manner. We are unaware of any previous report that has documented a stimulus-induced statistically significant global increase in the number of distinct proteins in rafts. Reproducibly distinct sets of proteins were recruited at the two time points (5 and 30 min) following LPS challenge, supporting our selection of these two post-LPS time points as biologically distinct and informative. Classification of raft proteins from resting and LPS-stimulated macrophages revealed a variety of functional categories as well as relative changes in class representation during the LPS time course; of particular note was a relative depletion of cellular movement proteins noted 5 but not 30 min following LPS exposure. A more focused network analysis of the qualitative proteomics data set then revealed the UPS to be the best represented pathway in rafts. De novo recruitment to rafts of several UPS members following LPS suggested that the UPS might be LPS-responsive, a hypothesis that was supported by quantitative proteomics measurements of proteasome subunits. In final validation, it was confirmed that LPS induces functional changes in proteasome activity specific to rafts and that the proteasome plays an important role in raft-compartmentalized activation of the ERK pathway by LPS.

Although not proving physical complex formation among the proteasome subunits identified, the data collectively provide firm evidence for the localization of proteasome subunits to rafts, their reorganization within rafts following LPS exposure, and an associated functional change in proteasomal activity specific to rafts that occurs during LPS signaling. Ubiquitination of a target protein and its subsequent proteolysis by the 26 S proteasome is a well documented process in biology. The "canonical" NF-κB pathway is one such well characterized pathway that involves the rapid ubiquitination of IκB and its degradation by the 26 S proteasome, resulting in release of NF-κB dimers into the nucleus and consequent gene transcription (52, 53). Proteasomal degradation of p105

### TABLE III

| NCBInr accession number (gi no.) | Protein name (CRA, sequence derived from the Celera database) | SILAC ratios |
|----------------------------------|---------------------------------------------------------------|--------------|
| 5-min LPS                        | Proteasome subunit, β type 1                                 | >2.42        |
| 16758370                         | Proteasome subunit, β type 1, isoform CRA_b                   | >2.27        |
| 149047100                        | Predicted: similar to 26 S proteasome non-ATPase regulatory subunit 14 |
| 109468228                        | Proteasome 26 S subunit, non-ATPase, 1                       | 2.83         |
| 74315975                         | Proteasome subunit, α type 6, isoform CRA_b                   | 0.63 (0.18)bc|
| 148704781                        | Proteasome subunit, α type 3, isoform CRA_e                   | 1.82         |
| 148704620                        | Proteasome subunit, α type 2                                 | 1.20d        |
| 38051991                         | Proteasome subunit, β type 1                                 | 1.53d        |
| 37231712                         | Proteasome subunit, β type 1                                 | >3.13        |
| 31982099                         | Proteasome subunit, β type 6                                 |              |

* a H, heavy and L, light.
* b Represents standard deviation for the calculated ratio.
* c Observed in three of three biological samples.
* d Observed in two of three biological samples.
has also been shown to occur upstream of LPS-stimulated ERK activation (54, 55). We now show the cascade of LPS-triggered events extending from proteasomal degradation of p105 down to activation of ERK to be compartmentalized to rafts. Moreover as previously reported for LPS (12, 51) and other raft-dependent signaling pathways (e.g. epidermal growth factor receptor (56)), we used m/H9252CD to show that raft “integrity” may play a role in both basal quiescence and ligand-stimulated activation of signaling cascades. Previous reports have indicated that plasma membrane remodeling induced by m/H9252CD (57) may in certain cases trigger “ligand-independent” signaling phenomena perhaps either through liberation of raft-sequestered signals or through microdomain remodeling (51). Taken together, the findings in the present report suggest that raft integrity is required for feed-forward LPS-induced activation of the I/H9260K-p105-proteasome-MEK-ERK cascade and that primary, ligand-independent lipid perturbations of this membrane compartment may nevertheless suffice to trigger these same signaling cascades.

DRMs have been studied extensively in the literature as biochemical approximations of in vivo rafts; nevertheless they are not identical to rafts in cellular membranes in vivo (58, 59). Hence the descriptive list of DRM protein identifications we provide should be considered as hypothesis-generating in

![Diagram](image-url)

**Fig. 6.** Selective regulation of LPS-ERK pathway by the proteasome through activity upon p105. A, schematic representation of the role of the proteasome in the regulation of LPS-ERK pathway. B, RAW 264.7 macrophages were pretreated with 5 μM MG-132 or 0.1% DMSO vehicle (60 min) and then exposed to buffer or 10 ng/ml LPS (15 min). Cells were then washed and lysed, and rafts were isolated as described under “Materials and Methods.” Non-raft and raft fractions were individually pooled from all conditions, and 20-μg protein aliquots (Bradford assay) from each were then subjected to SDS-PAGE, nitrocellulose transfer, and immunoblotting for the targets shown. Results are representative of two independent experiments. TIR, transferrin receptor.

![Diagram](image-url)

**Fig. 7.** LPS selectively activates the proteasome and the p105-MEK-ERK pathway in rafts. RAW 264.7 macrophages were pretreated with 5 μM MG-132 or 0.1% DMSO vehicle (60 min) and then exposed to buffer or 10 ng/ml LPS (15 min). Cells were then washed and lysed, and rafts were isolated as described under “Materials and Methods.” Non-raft and raft fractions were individually pooled from all conditions, and 20-μg protein aliquots (Bradford assay) from each were then subjected to SDS-PAGE, nitrocellulose transfer, and immunoblotting for the targets shown. Results are representative of two independent experiments.
nature. Independent approaches, such as confocal microscopy and co-immunoprecipitation, may be used to complement DRM analysis in confirmation of localization of specific proteins to rafts in less global approaches (60, 61). Nevertheless approaches like the one herein that target stimulus-induced changes in DRM protein expression and activity are more specific for the in vivo raft proteome (14, 47) particularly when coupled with confirmatory functional data using raft-perturbing agents such as mβCD and nystatin.

It has been postulated that distinct subcellular pools of the proteasome, characterized by differing subunit composition and activity profiles, may possibly subserve distinct roles in cell biology (62–64). We are unaware of any previous reports of stimulus-sensitive compartmentalization of proteasome activity to lipid rafts or of LPS-induced dynamic exchange of proteasome subunits in the macrophage. LPS has been reported to bind directly to specific proteasome subunits and to directly activate the proteasome in vitro. LPS is also thought to be “targeted” to lipid rafts in host cells in part through its interactions with raft-resident proteins (i.e. CD14). Taken together, it is interesting to speculate that these features may account for the specificity of LPS for activation of the raft-localized proteasome in our study. To what extent LPS promotes proteasomal degradation of p105 upstream of ERK through activation of the proteasome as compared with specific ubiquitin targeting of p105 is uncertain. Future efforts will be needed to investigate the means of localization of the proteasome to rafts, the functional effects specific to the proteasome subunits noted to change with LPS in this study, and additional roles for the 26 S proteasome in raft biology.

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[5] The on-line version of this article (available at http://www.mcpออนไลne.org) contains supplemental material.

Fig. 8. Raft-disrupting agents modulate the LPS-p105-MEK-ERK pathway. A, RAW 264.7 macrophages were left untreated or pretreated with mβCD (CD) (10 mM, 30 min) or nystatin (25 μg/ml, 30 min). Cells were then exposed to 10 ng/ml LPS, lysed, electrophoresed, and probed for the indicated protein targets. B, RAW 264.7 macrophages were pretreated with buffer or polymyxin B (25 μg/ml, 30 min) and then left untreated or treated with mβCD (10 mM, 30 min), LPS (10 ng/ml, 30 min), or mβCD-cholesterol complex. Lysates were probed for the targets shown. C, RAW 264.7 macrophages were pretreated for 60 min with 0.1% DMSO vehicle, 5 μM MG-132 (MG), or 10 μM Bay 11-7082 (Bay) and then either left untreated or exposed to mβCD (10 mM, 30 min). Lysates were probed for the targets shown. Results are representative of four independent experiments.

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