The Proteasome as a Lipopolysaccharide-Binding Protein in Macrophages: Differential Effects of Proteasome Inhibition on Lipopolysaccharide-Induced Signaling Events

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The Proteasome as a Lipopolysaccharide-Binding Protein in Macrophages: Differential Effects of Proteasome Inhibition on Lipopolysaccharide-Induced Signaling Events

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We have developed a novel LPS probe using a highly purified and homogenous preparation of \([^{3}H]\) Escherichia coli LPS from the deep rough mutant, which contains a covalently linked, photoactivatable 4-p-(azidosalicylamido)-butylanilide group. This cross-linker was used to identify the LPS-binding proteins in membranes of the murine-macrophage-like cell line RAW 264.7. The \(\alpha\)-subunit (PSMA1 C2, 29.5 kDa) and the \(\beta\)-subunit (PSMB4 N3, 24.36 kDa) of the 20S proteasome complex were identified as LPS-binding proteins. This is the first report demonstrating LPS binding to enzymes such as the proteasome subunits. Functionally, LPS enhanced the chymotrypsin-like activity of the proteasome to degrade synthetic peptides in vitro and, conversely, the proteasome inhibitor lactacystin completely blocked the LPS-induced proteasome’s chymotrypsin activity as well as macrophage TNF-\(\alpha\) secretion and the expression of multiple inflammatory mediator genes. Lactacystin also completely blocked the LPS-induced expression of Toll-like receptor 2 mRNA. In addition, lactacystin disregulated mitogen-activated protein kinase phosphorylation in LPS-stimulated macrophages, but failed to inhibit IL-1 receptor-associated kinase-1 activity. Importantly, lactacystin also prevented LPS-induced shock in mice. These data strongly suggest that the proteasome complex regulates the LPS-induced signal transduction and that it may be an important therapeutic target in Gram-negative sepsis. The Journal of Immunology, 2003, 171: 1515-1525.

Lipopolysaccharide is the major constituent of the outer membrane of Gram-negative bacteria. The overproduction of LPS-induced cytokines such as TNF, IL-1, and IL-6 has been considered central to the pathophysiology of endotoxinemia associated with septic shock. It is generally accepted that the first step in the activation of cells by LPS is the binding of this chemically complex ligand to specific membrane receptors, which ultimately leads to induction of gene expression and the release of cytokines and other mediators of inflammation (1, 2). CD14 has been identified as a cell surface, 55-kDa glycosylphosphatidylinositol-linked protein expressed on the surface of macrophages, monocytes, and neutrophils (3, 4). Other LPS-responding cell types, i.e., lymphocytes, endothelial cells, and fibroblasts, express very low levels of or are devoid of CD14, and they use a soluble form of this molecule. CD14 is now recognized to bind molecular complexes formed between LPS and plasma LPS binding protein (LBP). Presently, it is thought that CD14 serves to “focus” the LPS-LBP complex to a functional transducing receptor, which triggers the activation of the cells through a transmembrane signaling mechanism (4).

Over the past 15 years, many cellular proteins have been reported to bind LPS and/or lipid A (5–11). However, some of these proteins have not been purified or characterized, and their biological functions remain to be fully elucidated. Recently, the type I integral membrane protein Toll-like receptor 4 (TLR4) has been identified as the major signaling receptor for enterobacterial LPS (12). A nonmembrane spanning molecule, MD-2, has also been implicated as necessary for effective LPS signaling via TLR4 (13). Several other proteins, such as the \(\beta\)-integrins (14), caveolin (15), CD55/decay accelerating factor (16), and moesin (17), have also been implicated in LPS-mediated signal transduction. Besides this pathway, LPS has been reported to also activate cells via the CD14-independent pathway (18). Despite this accumulated knowledge regarding effector molecules involved in LPS signaling, the mechanisms that facilitate the interaction of these various putative coreceptors with TLR4 remain to be unraveled.

One approach to identify molecules within an “LPS signaling complex” was first presented nearly a decade ago. A radioiodinated cross-linking agent, \([^{125}\text{I}]\)sulfosuccinimidyl-2-[\(p\)-azidosalicylamido] ethyl-1,3-dithiopropionate (SASD), which reacts with free amino groups on phosphorylethanolamine residues of LPS, was first coupled to heterogenous preparations of either LPS from...
the deep rough mutant (ReLPS) of *Salmonella minnesota* R595 or smooth *Escherichia coli* LPS (5–7). Purification of smooth LPS or ReLPS (R595) to a single species is not possible, as has been described for the ReLPS from *E. coli* D31 m4 (19). Nonetheless, Lei and Morrison (7) used this [125]I-ASD-LPS probe to label two major LPS-binding proteins in membranes of murine B cells and macrophages (i.e., 70 kDa and 38 kDa). Unfortunately, they were unable to characterize these proteins by the relatively insensitive protein sequencing techniques that were available at the time. Subsequently, the purity and specificity of the [125]I-ASD-LPS probe came under intense scrutiny when Dziarski (20) demonstrated that it preferentially cross-linked albumin that was bound to cells. Nevertheless, this same probe was very recently used to cross-link TLR4, CD14, MD-2, and TLR2 in HEK 293 cells engineered to overexpress these proteins, suggesting that these molecules may be organized within the membranes in sufficiently close proximity to be cross-linked and radiolabeled (21). However, a major caveat with such studies is that overexpressed proteins may be preferentially cross-linked, resulting in the failure to detect molecules within the complex that are not overexpressed. In addition, another potential flaw associated with using [125]I-ASD-LPS is that [125]I-SASD by itself (in the absence of LPS) was found to bind membrane proteins of 55- and 18-kDa molecular masses within the RAW 264.7 macrophage cell line (B. Jarvis, D. Morrison, and N. Qureshi, unpublished data). Thus, it would be important to continue to exercise caution in the interpretation of such studies unless very rigorous attention is directed to the preparation and characterization of LPS-cross-linking probes.

As a consequence of the problems of specificity, incomplete derivatization, and LPS heterogeneity that were identified with the [125]I-ASD-LPS probe, we sought to generate a noniodinated, but radiolabeled, homogenous LPS with a cross-linking group that would allow for greater specificity after covalent linkage to proteins within macrophage membranes from primary macrophages or a similarly LPS-responsive macrophage cell line that has not been transfected to overexpress likely components of the putative LPS signaling complex. ReLPS from *E. coli* contains charged groups as two carboxyl anions in the two Kdo units, four anionic groups in the two phosphate groups, and no free amino groups (19). The two phosphates are known to be very important for biological activity because monophosphoryl lipid A is relatively nontoxic (22). Based upon these structural features, we developed an LPS probe using a highly homogenous, purified preparation of [3H] *E. coli* ReLPS, which contains a covalently linked, novel, photoactivatable 4-[azidosalicylamido] butyramine (ASBA) group. ASBA was esterified to the carboxylic groups of the Kdo, and this cross-linker was then used to identify the LPS-binding proteins in membranes of the murine-macrophage-like cell line, RAW 264.7 cells. Using this derivatized LPS cross-linking agent, we have been able to detect binding of our radiolabeled probe to 13–18 proteins in murine macrophage membranes. Two consistently labeled LPS-binding proteins were identified by matrix-assisted laser desorption-ionization-mass spectrometry (MALDI-MS) as the α-subunit (C2) and the β-subunit (N3) of the proteasome complex. Of relevance to this finding is the fact that derivatized LPS was able to activate the chymotrypsin-like activity of bacterial and mammalian proteasomes in vitro in the absence of CD14 or LBP, further supporting the notion that LPS can bind directly to the proteasome and activate it enzymatically. Finally, lactacytin, a functional inhibitor of the proteasome, blocked LPS-induced gene expression and dysregulated phosphorylation of mitogen-activated protein kinase (MAPK), but not IL-1 receptor-associated kinase-1 (IRAK-1) activity, in macrophages. Importantly, lactacytin also prevented LPS-induced shock in mice. This is the first report establishing that bacterial products such as LPS can bind directly to and activate the proteasome complex in vitro and that proteasome inhibitors like lactacytin can selectively block this activation in vitro and prevent LPS-induced shock in mice.

### Materials and Methods

#### Reagents

Highly purified hexacycl ReLPS *E. coli* D31 m4 (19) and phenol-water-extracted *E. coli* K235 LPS (<0.008% protein) were prepared according to the methods of Qureshi et al. (19) and McIntire et al. (23), respectively. *Rhodobacter sphaeroides* diphasophoryl lipid A (RoDPLA) was prepared as described by Qureshi et al. (24, 25). ASBA and 1-ethyl-3-[3-dimethylamino-propyl]-carbodiimide hydrochloride were purchased from Pierce (Rockford, IL). Chelex 100 (Na+) and Dowex 50 (H+) were purchased from Bio-Rad (Hercules, CA). Protease inhibitors were purchased from Roche (Mannheim, Germany). Lactacytin was purchased from Calbiochem (La Jolla, CA). Protease inhibitors were purchased from Roche (Mannheim, Germany). Lactacytin was purchased from Calbiochem, and was solubilized in DMSO to form a 2 mg/ml stock solution. For treatment of macrophages, further dilutions of the stock lactacytin were made in supplemented RPMI 1640 medium or DMEM from Bio-Rad (Walkersville, MD). Heat-inactivated FCS was also purchased from Bio-Whittaker. Rabbit polyclonal anti-phospho-extracellular signal-regulated kinase 1/2 (ERK-1/2) and anti-phospho-e-Jun N-terminal kinase 1/2 (JNK-1/2) Abs were purchased from Promega (Madison, WI). Rabbit polyclonal anti-phospho-p38 MAPK Ab was purchased from New England Bioslabs (Beverly, MA), and rabbit anti-total-p38 Ab was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

#### Preparation of the LPS-ASBA derivative of LPS

[3H] *E. coli* ReLPS was prepared and purified using a DEAE-cellulose column as described previously (19, 26). The specific activity of the [3H]ReLPS was 5.5 × 10^6 dpm/µg LPS. ReLPS was weighed before derivatization. Purified [3H]ReLPS (1 mg) was dissolved in 0.1 M MES buffer (pH 6.5) and mixed with 0.6 mg of ASBA to the same buffer. ASBA is a photoactivatable carboxyl-reactive cross-linker with a spacer arm of 16.3 Å. One hundred microliters of 1-ethyl-3-[3-dimethylamino-propyl]-carbodiimide hydrochloride (10 mg/ml), a dehydrating agent, was added. The reaction mixture was incubated at 37°C for 5 h in the dark, and was extracted with chloroform:methanol 2:1. The lower organic phase was filtered and evaporated to dryness under a stream of nitrogen. The ASBA cross-linker group (Pierce) that reacts with the carboxyl anions was used in preparation of [3H]ReLPS with a representative specific activity of 5.5 × 10^6 dpm/µg. The molecular mass of ReLPS-ASBA derivative is 2470 Da (N. Qureshi, S. Lin, and R. J. Cotter, manuscript in preparation).

#### Preparation of the solubilized membranes from RAW 264.7 cells

RAW 264.7 cells (1 × 10^6) grown in DME containing 10% FCS and gentamicin were washed three times with saline. The cells were pelleted by centrifugation at 700 × g for 5 min. The pellet was suspended in lysis buffer (10 mM, 1 × 10^6 cells/ml) containing 25 mM HEPES (pH 7.3), 0.5 mM EDTA, 0.5 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM sodium fluoride, protease inhibitors, and one tablet of Complete Mini EDTA-free (Roche, Indianapolis, IN) for every 50 ml of lysis buffer. Cells were lysed by freeze-thawing three times. Cell debris was removed by centrifugation at 700 × g for 5 min at 4°C. The supernatant was centrifuged at 5,800 × g for 7 min at 4°C to remove aggregates of cytoskeletal elements. The supernatant was finally centrifuged at 100,000 × g for 1.5 h at 4°C to obtain the membrane pellet.

The membrane pellet was suspended and solubilized in lysis buffer containing 10 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (Sigma-Aldrich, St. Louis, MO). Protein determination was performed by Bio-Rad. This method of membrane preparation is a modification of that reported by Bhat et al. (27).

#### Cross-linking of ReLPS-ASBA to solubilized membranes

[3H]ReLPS-ASBA was used for binding studies to the solubilized membrane proteins from RAW 264.7 cells. Several concentrations of [3H]ReLPS-ASBA ranging from 10 to 714 µg/ml were tested. Typically, [3H]ReLPS-ASBA (free acid form, 8 µg/µl, 4.4 × 10^6 dpm, 286 µg/µl, or 16 µg/µl, 8.8 × 10^6 dpm, 572 µg/µl) was dissolved in 0.5% triethylamine solution in water and mixed with solubilized membrane proteins (100 µg/20 µl) in small glass tubes and incubated at 37°C for 1.5 h in the dark, with occasional vortexing. Similar concentrations of [3H]ReLPS
were used in a competitive LPS binding study as reported previously ([3H]ReLPS (86.66 μg/ml), CD14, LBP, and RdDPLA (666 μg/ml–6.66 mg/ml)) (26). ReLPS-ASBA-protein complexes were synthesized and irradiated with a long-wave UV lamp (model UVGL-55; Ultraviolet Products, Upland, CA) at room temperature for 30 min at a distance of 7 cm.

After irradiation, the samples (28 μl) were diluted 1:1 with SDS boiling buffer and boiled for 5 min. Two-dimensional (2-D) electrophoresis was performed according to the method of O’Farrell (28). Isoelectric focusing was conducted in a glass tube of inner diameter 2.0 mm, using 2.0% pH 4.5–9.5 ampholines (Gallard Schlesinger, Long Island, NY) for 9600 volt-hours. One microgram of an isoelectric focusing internal standard protein, tropomyosin, with lower spot of m.w. of 33,000 and p I 5.2, was added to the sample. The tube gel pH gradient plot for these ampholines was measured by a surface pH electrode. After equilibration for 10 min in Buffer “O” (10% glycerol, 50 mM DTT, 2.3% SDS, and 0.625 M Tris buffer (pH 6.8)), the tube gel was sealed to the top of the stacking gel on a 10% acrylamide slab gel (0.75 mm thick). SDS slab gel electrophoresis was conducted for 4 h at 12.5 mA/gel. The following proteins (Sigma-Aldrich) were added as m.w. standards to the agarose, which sealed the tube gel to the slab gel: myosin (220,000), phosphorylase A (94,000), catalase (60,000), actin (43,000), carbonic anhydrase (29,000), and lysozyme (14,000). These standards appear as horizontal lines on the Coomassie Brilliant Blue R 250-stained 10% acrylamide slab gel. The gel was treated with En3Hance reagent (NEN, Boston, MA) and was exposed for 30 min on X-OMAT AR film (Kodak, Rochester, NY) with exposures of 7–14 days at −70°C. The films were developed using Kodak developer and X-ray film. A duplicate gel was run and stained with silver nitrate (Kendrick Laboratories, Madison, WI). The protein spots were excised from the silver nitrate stained gel, digested with endoproteinase Lys-C, and analyzed by MALDI-MS.

**Macrophase culture**

RAW 264.7 macrophase-like cells were grown in DMEM supplemented with 10% FCS and gentamicin (10 μg/ml). Alternatively, 5- to 6-wk-old C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were infected i.p. with 3 ml of 3% fluid thioglycollate. Four days later, macrophages were harvested by peritoneal lavage with pyrogen-free saline. Cells were then pelleted and resuspended in RPMI 1640 medium supplemented with 2% FBS, 2 mM glutamine, 30 mM HEPES, 0.3% NaHCO3, 100 U/ml penicillin, and 100 μg/ml streptomycin. For RNA generation, 0.5 × 106 cells per well were cultured overnight and treated as indicated.

**Extraction of total cellular RNA and RT-PCR**

For the extraction of total cellular RNA, macrophages were pretreated with medium or medium containing lactacystin for 1 h and then with LPS for 4 h. Macrophages were then solubilized in RNA-STAT 60, and total cellular RNA was generated and reverse transcribed as detailed elsewhere (29). PCR amplifications were performed with the cDNA for the genes of interest as described previously (30, 31). Amplified products were electrophoresed on 1% agarose gels and blotted overnight onto Nitro membranes. The DNA was UV cross-linked onto the membranes and baked for 2 h at 80°C, and the amplified PCR products were detected by gene-specific oligonucleotide probes. The sequences of the primers and probes used in the detection of TNF-α, IL-12 p40, IL-12 p35, inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2), TLR4, TLR2, and GAPDH have been published previously (29). The sequences of primers and probes used in this detection of CD14 and GAPDH are as follows: CD14 sense, 5′-CT GAC TCT CGC CAC TGT GCC-3′; CD14 antisense, 5′-GCT TCG GCA GTT AAG AGC-3′; CD14 probe, 5′-ACCT CCA GGT TAC GGC CT-3′; GAPDH sense, 5′-CCAT GAG GAA GGC CCG G-3′; GAPDH antisense, 5′-CAAG TGT CAC GTG ATG ACC-3′; GAPDH probe, 5′-GGG TGT GAC AAC CAG GAA AAT-3′. The housekeeping gene GAPDH served as a control for the amount of cDNA added to each amplification reaction.

**TNF assay**

RAW 264.7 cells were plated in eight-well plates (Coming, Corning, NY) at a density of 0.6 × 106 cells/well in a final volume of 0.5 ml. After 2 h of adherence at 37°C, RAW 264.7 cells were pretreated with medium or lactacystin for 1 h, and then with medium or 2 ng/ml LPS for 1.5 h. The supernatants obtained were assayed for TNF with the Quantikine M kit (R&D Systems, Minneapolis, MN).

**SDS-PAGE and Western analysis**

Cytoplasmic extracts for analysis of MAPK phosphorylation were generated from macrophages pretreated with medium or lactacystin for 1 h and then with medium or LPS for 15 min as described previously (30). The resultant proteins were resolved on 10% SDS-PAGE gels and then transferred onto Immobilon membranes. Western blot analysis was conducted for phospho-ERK-1/2, phospho-JNK-1/2, phospho-p38, and total p38.

**Proteasome assays**

Proteasome activities of the 20S proteasomes of Methanococcus thermophila (10 μg/ml) (32), rabbit muscle proteasome (0.4 μg/ml), and macrophage proteasome were assayed with synthetic peptide substrates in 0.02 M Tris-HCl buffer (pH 7.2). The substrates used for the chymotrypsin-like, trypsin-like, and postglutaminase activity were 100 μM succinyl-Leu-Leu-Val-Tyr-aminomethyl-coumarin, Z-ARR-AMC, and Abz-GPAla-NBA, respectively (Calbiochem). LPS concentrations ranging from 4 pg/ml to 4 μg/ml were used in a total volume of 250 μl. Fluorescence was measured using an Fx 800 microplate fluorescence reader (Bio-Tek Instruments, Winoski, VT).

**Purification of macrophase proteasomes**

The RAW 264.7 cells were scraped from the plate using ice-cold PBS. After several washes with PBS, the cells were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl2 and 250 mM sucrose. The cells were broken using the freeze-thaw method, and the suspension was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was centrifuged at 100,000 × g for 1 h. Then the resulting supernatant was centrifuged at 100,000 × g for 5 h (33). The pellet was suspended in buffer and assayed for proteasome activity as described above.

**IRAK assay**

The assay for IRAK-1 activity was conducted essentially as described by Li et al. (34). Mouse macrophages were pretreated with lactacystin (25 μM) and then exposed to LPS (10 or 100 ng/ml) at the times indicated. At each time point, cells were lysed in a buffer that contained several protease and phosphatase inhibitors, and the lysates were collected by centrifugation. This kinase assay is dependent upon immunoprecipitation of IRAK-1 with anti-IRAK-1 Abs, followed by an in vitro kinase assay on the immunoprecipitates. Ab to IRAK-1 (Upstate Biotechnology, Lake Placid, NY) was added to each lysate, incubated, and followed by the addition of Protein G-agarose beads to bind specifically to the Abs and allow the separation of Ab-Ag complexes from other proteins in the lysate. The precipitate was analyzed by incubation with a substrate (myelin basic protein) in the presence of [32P]ATP. In the latter case, phosphorylation of the substrate was visualized and quantified by running the samples on an SDS-PAGE gel and exposing the gel to a phosphor imager screen. This kinase assay is dose dependent and specific to IRAK precipitates because control experiments with rabbit IgG used as precipitating Ab showed no increase in kinase activity.

**Galactosamine-sensitized mouse model**

Normal female BALB/c mice (6 wk) were obtained from The Jackson Laboratory. Animals were housed in a constant day/night regime for 2 wk before use. Mice were injected i.p. with 18 mg of galactosamine and RelPS. Lactacystin was injected i.p. 1 h before the RelPS’ galactosamine challenge. LPS-induced lethality was measured and scored over a 24- to 36-h period. All animal procedures were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals.

**Results**

**Synthesis of the [3H]ReLPS-ASBA**

A novel tritiated E. coli ReLPS-ASBA photoactive cross-linker was synthesized as described in Materials and Methods and then used to detect the LPS binding proteins in a solubilized membrane preparation of RAW 264.7 cells. The final purified cross-linking product was analyzed by soft ion mass spectrometry. These results confirmed that this coupling reaction is very efficient and that approximately one ASBA is esterified to either one of the carboxyl groups of the Kdo units of the RelPS. MALDI-MS revealed the structure of the LPS-ASBA as presented in Fig. 1, which exhibited a molecular mass of 2470 Da (N. Qureshi, S. Lin, and R. J. Cotter, The Journal of Immunology 1517 Downloaded from http://www.jimmunol.org/ by guest on April 17, 2017
manuscript in preparation). Mass spectrometry also revealed the absence of detectable free underivatized LPS in the sample, suggesting that most of the ReLPS had been chemically derivatized. The ReLPS-ASBA and the underivatized ReLPS had similar TNF-α-inducing activity in cultures of RAW 264.7 cells (data not shown), thus suggesting that the derivatization did not adversely affect the biological activity of ReLPS.

Use of [3H]ReLPS-ASBA to identify novel LPS-binding membrane proteins

Initial studies were conducted using this probe to detect novel LPS binding proteins in murine macrophage membrane preparations. For these experiments, solubilized membrane proteins obtained from RAW 264.7 cells were incubated with [3H]ReLPS-ASBA and subjected to photo-cross-linking by exposure to UV light. The photo-cross-linked complexes were boiled with SDS boiling buffer at 100°C for 5 min and then fractionated by two-dimensional electrophoresis (as described in Materials and Methods). Duplicate gels were run under identical conditions; one gel was stained with silver nitrate, whereas the other gel was stained with Coomassie blue, treated with En3Hance, dried, and analyzed by fluorography.

As anticipated, more than 100 protein species were resolved on the 2-D silver-stained gel (Fig. 2A). The replicate gel, subjected to fluorography as shown in Fig. 2B, revealed the presence of 15–18 tritium-labeled spots, indicating the presence of the cross-linked probe. For this analysis, we selected two major radioactive bands that in multiple experiments coincided with readily detectable stained protein spots delineated A and B, as observed on the silver-stained gel. Some of the radioactive signals seen in Fig. 2B were in spots that did not coincide with any detectable protein spots in the silver-stained gel, and these could not be analyzed. The major radiolabeled spots, A and B, were excised, digested with endoproteinase Lys-C, and analyzed by MALDI-MS at the Protein Chemistry Core Facility at the Howard Hughes Medical Institute/Columbia University College of Physicians and Surgeons (New York, NY). Based upon this analysis, the protein spots A and B were positively identified as proteasome components: spot A correlated with the PSMA1 C2 (α subunit, 29.5 kDa) and spot B with PSMB4 N3 (β subunit, 24.4 kDa) of the proteasome. This experiment was repeated six times and in each experiment these two proteasome subunits were consistently labeled. Proteins that were not consistently labeled were not analyzed. In competition experiments, a 5-fold or 10-fold excess of unlabeled ReLPS or RsDPLA was bound to the membrane proteins, followed by [3H]ReLPS-ASBA (286 μg/ml–572 μg/ml). In these experiments, the unlabeled ReLPS competed for binding sites, and binding of [3H]ReLPS-ASBA to macrophage proteasome subunits was not observed.

Binding of [3H]ReLPS-ASBA to the proteasome of M. thermophila

The results mentioned above strongly suggested that LPS was bound to subunits of the macrophage proteasome. To pursue this observation further, therefore, we evaluated the ability of the [3H]ReLPS-ASBA probe to bind to the commercially available

FIGURE 1. Structure of the ReLPS-ASBA. Only one ASBA group was cross-linked to the Kdo of ReLPS, as demonstrated by mass spectrometry. ASBA may be linked to either one of the Kdo and may represent a mixture of the two derivatives.

FIGURE 2. Two-dimensional SDS-PAGE analysis of the membrane proteins of RAW 264.7 (100 μg, 8.3 mg/ml) cells complexed to [3H]ReLPS-ASBA (16 μg, 8.8 × 10⁶ dpm, 572 μg/ml) in a total volume of 28 μl. The complexes were irradiated with long-wave UV and analyzed by 2-D gel electrophoresis as described in Materials and Methods. Isoelectric focusing was conducted at pH 4–8, and a 10% acrylamide slab gel was used for electrophoresis. Duplicate gels were run: one was stained with the silver nitrate stain (A), and the other was treated with Coomassie blue, En3Hance for 1 h, and then it was rehydrated in water for 30 min, dried, and fluorographed (B). Major bands A and B were labeled with [3H]ReLPS-ASBA. Several other proteins were also labeled. The arrowhead represents the internal standard tropomyosin, which has a molecular mass of 33 kDa and a pI of 5.2.
20S proteasome of *M. thermophila* (Calbiochem). In this experiment, [3H]ReLPS-ASBA was incubated with the 20S proteasome derived from *M. thermophila*, cross-linked by UV, and then analyzed as described above for the macrophage membrane preparations. Both the $\alpha$ and $\beta$ subunits of the proteasome were clearly identified in silver-stained 2-D gels after electrophoresis (Fig. 3A). Importantly, however, and consistent with the data shown in Fig. 2, [3H]ReLPS-ASBA bound predominantly to the $\alpha$ subunit (three bands, 24 kDa) and not to the $\beta$ subunit (22 kDa) (Fig. 3B). These data provide direct evidence that ReLPS-ASBA is capable of binding to the $\alpha$ subunit of this simpler *Archae* proteasome.

**LPS activates the chymotrypsin-like activity and the postglutamase activity of the proteasome**

One of the primary cellular functions of the proteasome is proteolysis of proteins. Therefore, we examined the effect of LPS on the chymotrypsin-like, trypsin-like, and postglutamase activity of the proteasome complex. For these studies, either rabbit muscle proteasome or macrophage proteasome preparations were incubated with various concentrations of LPS, and enzymatic cleavage of the

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**FIGURE 3.** Two-dimensional SDS-PAGE analysis of the proteasome of *M. thermophila* (25 $\mu$g, 0.830 mg/ml) complexed to [3H]ReLPS-ASBA (5 $\mu$g, 2.75 x 10^6 dpm, 167 $\mu$g/ml) in a total volume of 30 $\mu$l. The complexes were irradiated with long-wave UV, lyophilized, and boiled with SDS boiling buffer as described in Materials and Methods. Isoelectric focusing was conducted at pH 4–8, and a 10% acrylamide slab gel was used for electrophoresis. Slab gel was stained with silver nitrate, revealing the $\alpha$ (24 kDa) (arrow 1) and $\beta$ subunits (22 kDa) (arrow 2) of the proteasome of *M. thermophila* (A). The gel was treated with En3Hance for 1 h, rehydrated in water for 30 min, dried, and fluorographed (B).

**FIGURE 4.** Rabbit muscle proteasome (P) activity in the presence of ReLPS. A, LPS (L) or RsDPLA (R) (40 ng–4 $\mu$g) was incubated with the rabbit muscle proteasome (0.4 $\mu$g/ml) and AMC substrate III succinyl-Leu-Leu-Val-Tyr-7-amido-4-methyl coumarin (100 $\mu$M) in 96-well plates for 20 min. B, Rabbit muscle proteasome was incubated with RsDPLA (5 min), LPS (4 $\mu$g/ml), and AMC substrate III for 20 min. C, Macrophage proteasome was incubated either with LPS (black bars) or with lactacystin (30 min) and LPS (gray bars) and with AMC substrate III for 20 min. The plates were read at an absorption/emission of 360/460 nm using a microplate fluorescence reader. The data are representative of three identical experiments performed.

**FIGURE 5.** Induction of TNF-$\alpha$ in RAW 264.7 cells pretreated with the proteasome inhibitor lactacystin. RAW 264.7 cells were pretreated with the indicated concentration of lactacystin for 1 h, followed by addition of 2 ng/ml LPS for 1.5 h. The supernatants obtained were assayed for TNF-$\alpha$ with the Quantikine M kit (R&D Systems) as described in Materials and Methods. The data are representative of two identical experiments performed. M, Medium; L, LPS; and LC, lactacystin.
proteasome-specific substrate III (succinyl-Leu-Leu-Val-Tyr-7-amido-4-methyl-coumarin) was determined. LPS showed a dose-dependent activation of the rabbit muscle proteasome’s (Biomol, Plymouth Meeting, PA) chymotrypsin activity (as shown by the data in Fig. 4A) and postglutamase activity, but no activation of the trypsin-like activity (data not shown) was observed. In addition, no activation of the proteasome’s chymotrypsin-like activity was observed with the LPS antagonist (24) from RsDPLA, even at concentrations up to 4 µg/ml (Fig. 4A). These results suggest that the proteasome can be activated only by toxic LPS. RsDPLA blocked the LPS-induced proteasome activity (Fig. 4B) over a concentration range consistent with its ability to block LPS signaling in macrophages. Similar results were observed with the M. thermophila proteasome (data not shown) and the macrophage proteasome as observed in Fig. 4C, where LPS-activated chymotrypsin activity was inhibited by RsDPLA. Prior exposure of RAW 264.7 cells to lactacystin completely blocked the LPS-induced chymotrypsin activity of this proteasome in in vitro experiments.

Proteasome enzymatic inhibitors lactacystin and MG-132 block LPS-induced TNF secretion

To probe further the role of the proteasome in the LPS-induced signal transduction, we next queried whether inhibitors of the proteasome would block the activating effects of LPS on macrophages. To do these studies, RAW 264.7 cells were pretreated with specific proteasome inhibitors, lactacystin or MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), before addition of underivatized ReLPS. The supernatants were collected 1.5 h after LPS stimulation and were assayed for TNF-α. Pretreatment of RAW 264.7 cells with lactacystin (Fig. 5) or MG-132 (data not shown) resulted in a dose-dependent inhibition of TNF-α secretion with concentrations of 25 µM lactacystin or 3 µM MG-132, completely blocking secretion of LPS-induced TNF-α in these cells. Cell death, as assessed by dye exclusion test, was not observed with cells treated with these concentrations of the inhibitors. In addition, neither lactacystin nor MG-132 alone induced any TNF-α secretion.

Lactacystin blocks LPS-induced gene expression

Although the data provided thus far suggest that inhibitors of proteasome activation inhibit LPS-induced cytokine secretion, they do not provide information on either the specificity of inhibition or the point in the activation pathway at which inhibition occurs. To define the role of the proteasome in LPS-mediated gene expression, we pretreated primary cultures of murine peritoneal macrophages isolated from LPS-responsive C3H/HeOuJ mice with lactacystin before the addition of LPS. Total cellular RNA was then extracted and reverse transcribed, and gene expression analyses were performed by RT-PCR and Southern blot analysis. The panel of genes selected included a number of important macrophage activities and included cytokine genes TNF-α, IL-6, IL-12 p35, and IL-12 p40, as well as COX-2 and iNOS, two genes that encode enzymes that regulate key functions of macrophages. Expression of genes that encode LPS receptors TLR4, TLR2, and CD14 were also examined. As shown by the data in Fig. 6, LPS markedly induced the mRNA levels of all of the genes examined, with the exception of TLR4, for which the constitutive level of TLR4 mRNA was

**Figure 6.** RT-PCR analysis of LPS-induced gene expression in macrophages pretreated with the proteasome inhibitor lactacystin. Thioglycollate-elicited macrophages derived from C3H/HeOuJ mice were pretreated with lactacystin for 1 h as indicated followed by 1 ng/ml LPS for 4 h. Total cellular RNA was generated, reverse transcribed, amplified, and detected as described in Materials and Methods. The data are representative of two identical experiments performed.

**Figure 7.** Effect of lactacystin pretreatment on LPS-induced MAPK in macrophages. Macrophages from C3H/HeOuJ mice were pretreated with indicated concentrations of lactacystin for 1 h followed by 1 ng/ml LPS for 15 min. Cytoplasmic extracts were generated, and proteins were resolved on SDS-PAGE gels and immunoblotted for phospho-specific ERKs, JNKs, and p38 as described in Materials and Methods. After phospho-ERK detection, membrane was stripped and reprobed for total p38. The data are representative of two identical experiments performed.
slightly down-regulated. It is of importance that the LPS-induced expression of TLR2 mRNA was completely blocked by high concentrations of lactacystin. Interestingly, pretreatment with lactacystin blocked expression of most of the LPS-inducible genes in a dose-dependent manner, as seen in Fig. 6, whereas lactacystin alone had minimal effect on gene expression. The failure of lactacystin to block expression of the housekeeping gene GAPDH suggests that the observed inhibition is not due to a toxic effect of lactacystin on macrophages.

**Lactacystin blocks the LPS-induced phosphorylation of ERK-1 or ERK-2**

To further explore the role of the proteasome in LPS-mediated signaling, we also analyzed the effect of the proteasome inhibitor lactacystin on LPS-induced phosphorylation of the MAPK superfamily. As illustrated in Fig. 7, LPS induced phosphorylation of ERK-1,2, JNK-1,2, and p38 MAPK. Although lactacystin alone had no effect on ERK-1 or ERK-2 phosphorylation, lactacystin pretreatment resulted in a significant dose-dependent inhibition of LPS-induced ERK phosphorylation. In contrast, lactacystin itself induced phosphorylation of JNK, and pretreatment of macrophages with lactacystin before stimulation with LPS resulted in increased JNK phosphorylation. Lactacystin also increased phosphorylation of p38, particularly in the absence of LPS. IRAK-1 is a key component of the TLR4- and IL-1R-mediated signaling pathways, and it is recruited and activated after the interaction of the adaptor molecule MyD88 with the intracytoplasmic regions of TLRs or the IL-1R (35). To determine whether proteasome activ-

**FIGURE 8.** RAW 264.7 cells were treated with LPS in the absence or presence of lactacystin (25 μM). Cells were lysed at the indicated times after LPS (100 ng/ml) stimulation, and they were assayed for IRAK-1-associated kinase activity as described in Materials and Methods. A, IRAK-1 activity from a single representative experiment. B, ERK-1 phosphorylation detected by Western blot analysis in the same experiment. C, Mean ± SEM of four separate experiments in which kinase activity was measured densitometrically.

**Discussion**

The most significant finding from this study is the novel observation that LPS has the capacity to bind to selected subunits of the macrophage proteasome and that the consequences of that binding include the selective activation of specific proteases of the proteasome. Evidence that the proteasome has functional significance in the pathway of LPS-mediated macrophage signal transduction triggered by LPS derives from the observation that pretreatment of macrophages with lactacystin, a well-characterized inhibitor of proteasome activity, inhibits LPS-induced up-regulation of TLR2 and a number of proinflammatory genes in a dose-dependent fashion. Because our studies also document that lactacystin differentially regulates several of the kinases known to be involved in mediation is required for IRAK-1 activity, IRAK-1 was first immunoprecipitated from lysates of RAW 264.7 cells stimulated with LPS in the absence or presence of lactacystin. Kinase activity was measured by the incorporation of 32P into MBPs (Fig. 8, A). Fig. 8B illustrates that, although the lactacystin was effective at inhibiting IRAK-1-associated kinase activity significantly (Fig. 8, A and C).

**Lactacystin prevents LPS-induced shock in mice**

To further investigate the effect of lactacystin on LPS-induced shock, we examined its effect in galactosamine-sensitized mice challenged with toxic ReLPS (Table I). In this experiment, ReLPS challenge (0.5 g) caused 100% lethality, whereas pretreatment with lactacystin (0.4 μmoles) 30 min before challenge reduced lethality to 20%. Lactacystin alone in the absence of LPS challenge was not toxic at a dose of 0.4 μmoles. This experiment was repeated within a 2-wk time interval with similar results.

**Table I. Effect of treatment with lactacystin on lethality of galactosamine-sensitized BALB/c mice challenged with toxic ReLPS**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Pretreatment* (−30 min)</th>
<th>Challenge (0 h)</th>
<th>Survival/Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Vehicle</td>
<td>ReLPS</td>
<td>0/5</td>
</tr>
<tr>
<td>B</td>
<td>Vehicle</td>
<td>Vehicle</td>
<td>5/5</td>
</tr>
<tr>
<td>C</td>
<td>Lactacystin (0.08 μmol)</td>
<td>ReLPS</td>
<td>0/5</td>
</tr>
<tr>
<td>D</td>
<td>Lactacystin (0.4 μmol)</td>
<td>ReLPS</td>
<td>4/5</td>
</tr>
<tr>
<td>E</td>
<td>Lactacystin (0.4 μmol)</td>
<td>Vehicle</td>
<td>5/5</td>
</tr>
</tbody>
</table>

*At −30 min, mice were treated with either vehicle or lactacystin. Then at 0 h, the animals were challenged with vehicle alone or with ReLPS (1 μg/mouse) plus galactosamine (18 mg) i.p. This experiment was repeated twice with similar results. The mice were monitored for death for 24 h. \( p < 0.01 \).

**FIGURE 9.** Subunits of the human proteasome (39, 40). The mouse proteasome has essentially the same subunits. X, Y, and Z subunits (IFN-inducible subunits) exhibit chymotrypsin, peptideyl glutamase, and trypsin activities, respectively. LPS binds to the C2 and N3 subunits.
LPS-initiated signaling pathways, these findings collectively support the concept that the proteasome may well play an important and, as yet not fully appreciated, regulatory role in LPS-mediated induction of inflammation.

The original objective of these studies was to identify important and novel molecular targets in the macrophage with which LPS interacts, which might contribute to activation and secretion of inflammatory mediator molecules. The basic approach that was used was to synthesize a highly specific and chemically pure photoactivatable radiolabeled LPS probe and to examine its binding to constituents of partially purified macrophage fractions. Our expectation was that this radiolabeled LPS probe would associate with at least some of the now reasonably well-characterized membrane constituents recognized as critical to LPS signaling, including CD14, TLR4, and MD-2. Interestingly, there has been one study published in the literature that reports evidence to suggest that LPS specifically binds to TLR4 (21). However, in that study [125I]-biologically inactive lipid A antagonist, may be binding at the site of the proteasome (EC 3.4.99.46; macropain subunit C2; proteasome NU chain; multicatalytic endopeptidase complex subunit C2; molecular mass-29.5 kDa). (This structure was obtained from Protein prospector website). Table II. Spot A: proteasome component PSMA1 C2 (EC 3.4.99.46; macropain subunit C2; proteasome NU chain; multicatalytic endopeptidase complex subunit C2; molecular mass-29.5 kDa). (This structure was obtained from Protein prospector website).

<table>
<thead>
<tr>
<th>m/z</th>
<th>Position</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>3114</td>
<td>218–243</td>
<td>DLEFTTYDDDVSPPFLDGLEEERFCR</td>
</tr>
<tr>
<td>2150</td>
<td>190–208</td>
<td>HGLRALRETPARQDLFTK</td>
</tr>
<tr>
<td>1342</td>
<td>244–256</td>
<td>AQPSQADEPARK</td>
</tr>
<tr>
<td>1239</td>
<td>51–61</td>
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<td>1</td>
<td>11</td>
<td>TVWSPOCRGH</td>
</tr>
<tr>
<td>51</td>
<td>61</td>
<td>QIREAYAEMK</td>
</tr>
<tr>
<td>101</td>
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</tr>
<tr>
<td>151</td>
<td>161</td>
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</tr>
<tr>
<td>201</td>
<td>211</td>
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</tr>
<tr>
<td>251</td>
<td>261</td>
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</tr>
<tr>
<td>301</td>
<td>271</td>
<td>SQKPSQAA</td>
</tr>
</tbody>
</table>

Importantly, the consequences of interaction of LPS with proteasome subunits appear not to be inconsequential. In this regard, binding of the LPS to the M. thermophilia proteasome serves to activate its postglutamase activity and chymotrypsin-like enzymatic activity. The latter activity is also observed in partially purified rabbit muscle proteasome and murine macrophage 20S proteasome after addition of LPS. It is of note that binding of LPS to the macrophage proteasome did not result in its activation of trypsin-like activity, another enzyme activity closely associated with the activated proteasome. These findings would be consistent with the selective binding of the LPS to specific subunits as discussed above. Furthermore, the fact that the enzymatic activity generated by interaction of LPS with the macrophage proteasome could be readily blocked by lactacystin (37, 38) suggests strongly that proteolytic activity was correctly associated with the proteasome, rather than some unrelated protease activity. However, it might be noted that the available evidence would suggest that LPS does not bind to the proteasome exactly at the same site as does the peptide substrate and lactacystin (Fig. 9). We postulate that LPS activates the enzyme activity by opening up the proteasome channel to facilitate entry of the peptide substrate.
lactacystin and MG-132, which inhibit this activity in vitro, can block LPS signaling.

These observations regarding the interaction of LPS with selective subunits of the macrophage proteasome might be considered within the framework of other proteasome activators. Other studies have documented that PA28, a known proteasome activator, also binds to the C2 subunit, similar to what we have demonstrated for LPS (39, 40). This activator has recently been shown to have important consequences for Ag processing (41). Mutant mice, who fail to express PA28, are characterized by impaired processing of antigenic epitopes derived from either exogenous or endogenous Ags, consistent with the concept that compounds that function by activating the chymotrypsin activity of the proteasome are likely to be important for Ag processing. Given the fact that LPS is well recognized as a potent immunologic adjuvant that promotes immune responses to unrelated protein Ags through a macrophage-dependent pathway, it is attractive to consider LPS-dependent activation of the proteasome as a potential pathway by which potentiation of immune responses occurs.

One of the more interesting findings reported in this manuscript is the fact that LPS-induced activation of the proteasome chymotrypsin-like activity in vitro can be totally blocked by lactacystin, a potent proteasome inhibitor. Moreover, treatment of in vitro-cultured macrophages with lactacystin for short periods of time before stimulation with LPS resulted in a dose-dependent inhibition of LPS-induced TNF-α secretion. In addition, lactacystin also inhibited essentially all LPS-inducible genes measured (e.g., TNF-α, IL-6, IL-12 p40 and p35, COX-2, and iNOS). Finally, LPS treatment of macrophages leading to up-regulation of TLR2 mRNA was also blocked by lactacystin, and this inhibitor also reduced constitutive levels of TLR4 mRNA expression. Collectively, these studies would be consistent with an important functional regulatory role for the proteasome in dictating pathways of LPS-dependent signaling.

It is generally recognized that one of the key pathways up-regulated by LPS involves activation of NF-κB through dissociation and degradation of IκB. It is of interest that proteasomes have previously been shown to be involved in LPS-induced degradation of IκB (42–46). Current understanding of this pathway suggests that, after LPS activation of macrophages, a cascade of adaptors and kinases interacts with the intracytoplasmic domain of TLR4, leading to the phosphorylation and ubiquitination of IκB, which is then degraded by the proteasome. This leaves NF-κB available to translocate to the nucleus, where it can serve to promote transcription of NF-κB-dependent genes. Lactacystin and other proteasome inhibitors have been shown to block the degradation of IκB. In addition, members of the MAPK superfamily can also modulate the activity of NF-κB, and thus influence transcription of NF-κB-dependent genes (47). Recently, we have shown that MAPKs are important for LPS-induced TNF (48). Our findings reported here demonstrating that lactacystin will inhibit LPS-dependent activation of macrophages confirm that the proteasome may well be involved in the activation of ERK-1 and -2 in response to LPS.

We have also noted that lactacystin alone results in measurable increases in phosphorylation of p38 kinase and JNK, indicating that basal levels of proteasome proteolytic activity may negatively regulate basic levels of activation of these two important signaling molecules. Importantly, our studies also indicate that lactacystin is not able to diminish significantly LPS-induced levels of phosphorylation of IRAK-1, even though proteasome activity has been reported to be necessary for degradation of phosphorylated IRAK-1 (49). Taken together, these experiments would support the concept that the proteasome complex may be involved in regulation of several of the very early steps that contribute to LPS-induced signal transduction pathways, specifically at the levels of MAPK and IκB, resulting in inhibition of LPS-induced macrophage activation and gene expression of proinflammatory cytokines. This would further suggest that activation of IRAK-1 can be dissociated from the proteasome activity thought to be upstream and required for normal activation of MAPK and IκB.

We recognize that, given the intracellular location of the proteasome, it would be necessary to postulate that LPS must effectively be internalized into the macrophage in order for effective interaction with and activation of the proteasome to occur. It is possible that proteasomes may be present in the outer membrane of the cell, as has been previously reported for certain T cell sub-populations (50). Nevertheless, there is ample evidence in the literature, both from our own studies and from those of others, that LPS is rapidly internalized into macrophages. In our own published research, LPS was found to be internalized and readily detectable in the cytoplasm of macrophages within seconds of addition to macrophages (51). The internalized LPS therefore could be
rapidly made available to the proteasome by a phagosome-to-cytosol transfer pathway as originally described by Kovacsos-Bankoski and Rock (52). Through this pathway, the proteasome complex might well serve as a cytoplasmic “receptor” for LPS secondary to TLR4-mediated signaling.

The concept of LPS internalization and interaction with cytoplasmic constituents would readily encompass a scenario in which Gram-negative bacteria might be engulfed by phagocytic cells such as macrophages, enter the cytosol, and interact directly with the 20S proteasome and thus facilitate increased proteolytic activity of the proteasome. This would be expected, at a minimum, to lead to initiation of Ag presentation and triggering of a specific, acquired immune response, in addition to the potent innate immune response characterized by induction of proinflammatory cytokines. Support for this concept derives from the recent studies of Maksymowych et al. (53), who have documented that invasion of macrophages by Salmonella typhimurium rapidly induces increased expression of LMP, MECL, and PA28 proteasome genes. Finally, the data presented indicate that pretreatment of mice with lactacyclin prevents LPS-induced shock in vivo. The galactosamine-sensitized mouse model is a well-established model for LPS-induced shock, and the fact that lactacyclin blocks mortality in this model suggests that it might be important as a potential therapeutic approach for LPS-induced shock. Although additional studies will be required to fully elucidate the role of LPS-proteasome interactions in the host response to Gram-negative microorganisms, the results of the studies presented here provide support for the conclusion that such studies would be highly likely to be informative.

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