Osteopontin and Protein Kinase C Regulate PDLIM2 Activation and STAT1 Ubiquitination in LPS-treated Murine Macrophages*

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The molecular pathways regulating signal transducer and activator of transcription 1 (STAT1) levels in states of inflammation are incompletely understood. The suppressor of cytokine signaling, protein inhibitor of STAT, and SHP-1/2 tyrosine phosphatases ultimately regulate activity of STAT molecules. However, these mechanisms do not degrade STAT proteins. In this regard, signal transducer and activator of transcription 1 (STAT1) is an essential activator of LPS and/or pro-inflammatory cytokine-mediated iNOS transcription in murine, rat, and human cells (4). All mammalian iNOS promoters contain several sites with homologies to the interferon (IFN)-γ-regulated STAT1α-binding sites (GAS). STAT signaling is tightly regulated and several mechanisms have been proposed to account for this control (5). Post-translational modifications of STAT proteins are regulated mainly by transcription factors, pre-mRNA-dependent pathways, and protein ubiquitination (6). Ubiquitination plays a major role in regulating many cellular processes by marking proteins, including transcription factors, for degradation through the 26 S proteasome-dependent pathway. Tanaka et al. (6) were the first to demonstrate that IFN-γ activated STAT1 levels may be regulated by the ubiquitin-proteasome pathway. This article has been retracted by the publisher. The same data were reused to represent different experimental conditions. Specifically, lanes 2-4 and 6-8 of the actin immunoblot in Fig. 3A were duplicated. Additionally, the nuclear P-STAT1 immunoblot from Fig. 3A was reused as P-STAT1 in Fig. 3B. The same data were reused to represent different experimental conditions. Specifically, lanes 2-4 and 6-8 of the actin immunoblot in Fig. 3A were duplicated. Additionally, the nuclear P-STAT1 immunoblot from Fig. 3A was reused as P-STAT1 in Fig. 3B.

EXPERIMENTAL PROCEDURES

Materials—N-Carbobenzoxyl-t-leucinyl-i-norleucinal (MG132), M2 anti-FLAG (Asp-Tyr-Lys-Asp-Asp-Asp-Lys)-agarose, and β-actin antibody were purchased from Sigma. The following PKC inhibitors were ordered from Calbiochem: bisindolylmaleimide I (G06850); 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-c)-carbazole (G06976); rottlerin; and Myr-SIYRRGARRWKL-OH, myristoylated protein kinase Cζ pseudosubstrate inhibitor (PKCζ inhibitor). Anti-mouse His antibody was purchased from Invitrogen. Antibodies against Stat1 p84/p91 were obtained from Santa Cruz Biotechnology.

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2 The abbreviations used are: iNOS, inducible nitric-oxide synthase; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitor of STAT; STAT, signal transducer and activator of transcription; OPN, osteopontin; GAS, IFN-γ-regulated STAT1α-binding site; BMM, bone marrow macrophage; IP, immunoprecipitation; Ab, antibody; CLP, cecal ligation and puncture.

Macrophage inducible nitric-oxide synthase (iNOS) expression is central to many of the systemic effects associated with endotoxin (LPS) stimulation (1). Utilizing both in vivo and in vitro murine models of endotoxin (LPS) stimulation, we have previously demonstrated that osteopontin (OPN) is a potent NO feedback-regulated trans-repressor of iNOS expression (2, 3). However, the underlying molecular pathway has not been fully characterized. We have previously examined the OPN-dependent effector arm of this NO-regulated negative feedback loop, focusing on iNOS transcriptional regulation. In this regard, signal transducer and activator of transcription 1 (STAT1) is an essential activator of LPS and/or pro-inflammatory cytokine-mediated iNOS transcription in murine, rat, and human cells (4). All mammalian iNOS promoters contain several sites with homologies to the interferon (IFN)-γ-regulated STAT1α-binding sites (GAS). STAT signaling is tightly regulated and several mechanisms have been proposed to account for this control (5). Post-translational modifications of STAT proteins are regulated mainly by transcription factors, pre-mRNA-dependent pathways, and protein ubiquitination (6). Ubiquitination plays a major role in regulating many cellular processes by marking proteins, including transcription factors, for degradation through the 26 S proteasome-dependent pathway. Tanaka et al. (6) were the first to demonstrate that IFN-γ activated STAT1 levels may be regulated by the ubiquitin-proteasome pathway. This article has been retracted by the publisher. The same data were reused to represent different experimental conditions. Specifically, lanes 2-4 and 6-8 of the actin immunoblot in Fig. 3A were duplicated. Additionally, the nuclear P-STAT1 immunoblot from Fig. 3A was reused as P-STAT1 in Fig. 3B. The same data were reused to represent different experimental conditions. Specifically, lanes 2-4 and 6-8 of the actin immunoblot in Fig. 3A were duplicated. Additionally, the nuclear P-STAT1 immunoblot from Fig. 3A was reused as P-STAT1 in Fig. 3B.
**Osteopontin and PDLIM2**

from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-mouse PDLIM2 serum has been described previously (8, 10). Anti-mouse iNOS antibody was obtained from BD Biosciences. Anti-phosphoserine antibody was purchased from Zymed Laboratories Inc.. Hemagglutinin (HA) monoclonal antibody (3F10) was obtained from Roche Applied Science. Protein kinase Ca was obtained from Calbiochem.

**Plasmid Constructs**—pCMV-FLAG-Stat1 was described previously (11). HA-ubiquitin vector was kindly provided by Dr. Bohmann (University of Rochester, Rochester, NY). PathDetect GAS cis-reporting vector was purchased from Stratagene (La Jolla, CA). Wild-type PDLIM2 was described previously (8). S137A-PDLIM2, S137D-PDLIM2, and T138A-PDLIM2 designate mutant His-PDLIM2 expression vectors, in which amino acids were mutated to an alanine or aspartic acid residue. All mutant vectors were generated by using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) using primers as follows: S137A, sense, 5'-CTTCGCCACCCCACCCCA

**Construction and Characterization of AdV-PDLIM2 Expression Virus**—AdV-PDLIM2 cDNA was inserted into the multiple cloning site of pShuttle-CMV vector (Stratagene, La Jolla, CA) to obtain pShuttle-CMV-PDLIM2. pShuttle-CMV-PDLIM2 and pAdEasy-1 vector (Stratagene, La Jolla, CA) were cotransfected into BJ5183 cells for homologous recombination. The recombinants were screened to obtain pAdV-PDLIM2. The verification of the AdV-PDLIM2 genome was carried out by DNA sequencing. The verified recombinant AdV-PDLIM2 was propagated in HEK 293 cells and then purified and stored at -80 °C until use. Functional characterization was done by monitoring PDLIM2 expression using Western blot analysis.

**Cell Lines**—RAW264.7, 293, and COS-1 monkey kidney cells were maintained at 37 °C under 5% CO2 in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. LPS (50 ng/ml) was used to treat RAW264.7 cells; after incubation at 37 °C for 1 h, the cells were harvested with lysate buffer.

**Phosphopeptide Mapping of PDLIM2**—WT-PDLIM2 was isolated from cell lysates and separated by PAGE. The resolving gel was stained; the WT-PDLIM2 band was excised and stored at -80 °C until use. The gel slices were subjected to in-gel tryptic and chymotryptic digestions after reduction and carboxamidomethylation. The resultant digests were pooled just prior to liquid chromatography MS/MS (tandem MS) injection. The phosphorylated peptide sequence analyses were performed at Harvard Microchemistry Facility, Cambridge, MA, by micro-capillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQ DeCa XP plus quadrupole ion trap mass spectrometer.

**Western Blot Analysis**—Cells were lysed in buffer (0.8% NaCl, 0.02% KCl, 1% SDS, 0.1% Triton X-100, 0.5% sodium deoxycholic acid, 0.144% NaH2PO4, 0.024% KH2PO4, and 2 mm phenylmethylsulfonyl fluoride, pH 7.4), put on ice for 30 min, homogenized, and centrifuged at 12,000 × g for 10 min at 4 °C. The protein concentration was determined by the protein assay kit (Bio-Rad). The protein samples were separated by 4–20% SDS-PAGE and electrotransferred onto polyvinylidine difluoride membranes (Millipore, MA) by semi-dry transfer. The membranes were blocked with 5% skim milk in PBS, 0.05% Tween for 1 h at room temperature and probed using primary antibodies directed against PDLIM2. After washing with PBS, the membranes were incubated at room temperature, using appropriate horseradish peroxidase-conjugated secondary antibodies. Membrane was developed as described previously (12). Cells were cultured in cold PBS and lysed in IP buffer (10 mmol of Tris-Cl, pH 7.5, 3 mmol of EGTA, 20 mmol of NaCl, 0.02% Triton X-100, 1× protease inhibitors mixture, 0.2 mmol of dithiothreitol, 1 mmol of phenylmethylsulfonyl fluoride). After homogenization, cell lysates were centrifuged at 13,000 rpm for 20 min at 4 °C. Aliquots (500 μg) were preclared by addition of 1.0 μg of normal IgG and 20 μl of appropriate agarose conjugate for 1 h at 4 °C. After centrifugation, the supernatant was collected and incubated with 2 μg of corresponding Ab for 1 h at 4 °C. A total of 20 μl of appropriate agarose conjugate was then added, and the mixture was incubated overnight. After washing five times with lysis buffer, immunoblotting was performed as described above.

**Assay of NO Production**—RAW264.7 cells were plated on a 24-well plate and allowed to grow for 24 h before transfection. DNA transfections were carried out in 24-well plates using JetPEI macrophage reagent (Genesee, San Diego). At least 24 h later, the medium was changed, and the cells were stimulated with 50 ng/ml LPS for 12 h. To control transfection efficiency between groups, 0.1 μg of pRL-TK vector was added to each well. Cells were harvested in 0.1 ml of reporter lysis buffer (Promega, Madison, WI), and Dual-Luciferase reporter assays were performed using the manufacturer’s protocol (Promega, Madison, WI).

**In Vitro Phosphorylation Assay**—His-tagged PDLIM2, mutant His-tagged PDLIM2-S137A, and mutant His-tagged PDLIM2-S137D vectors were expressed in COS-1 cells. The recombinant proteins were purified with MagneHis protein purification system (Promega, Madison, WI). Prior to phos-
phorylation, recombinant proteins were dialyzed (24 h, 4 °C) against 10 mM Tris-HCl, pH 7.5, containing 1 mM DTT and 100 mM NaCl. The reaction for PKC activity was carried out in a solution containing 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 0.25 mM EDTA, 0.4 mM CaCl₂, 0.3 mg/ml phosphatidyserine, 0.03 mg/ml diacylglycerol, 0.1 mg/ml BSA, and 10 μg of recombinant PDLIM2 protein, 5 μCi of [γ-³²P]ATP, and 0.1 unit of PKCα (Calbiochem). A negative control was performed in the absence of PKC. After 30 min at 30 °C, reactions were stopped with 2× SDS-loading buffer, and the proteins were separated by SDS-PAGE. Gels were dried and subjected to autoradiography.

**In Vitro Ubiquitination Assay—**The in vitro ubiquitination assay was described previously (9). Recombinant wild-type PDLIM2, mutant His-tagged PDLIM2, or empty His vector was expressed in RAW264.7 cells. Following LPS stimulation for 6 h, recombinant proteins were purified with the MagneHis protein purification system (Promega, Madison, WI) as the source of E3 ligase. Presence of PDLIM2 was confirmed by rabbit anti-PDLIM2 Ab. FLAG-tagged P-STAT1 was expressed in LPS-treated RAW264.7 cells; the cells were lysed, and whole cell extracts were subjected to immunoprecipitation with anti-STAT1 or M2 anti-FLAG-agarose as the source of substrate for in vitro ubiquitination. Anti-FLAG STAT1 immuneprecipitates were washed five times with lysis buffer and once with ubiquitination buffer (50 mM Tris-Cl, pH 7.5, 2 mM MgCl₂, 1 mM ATP, and 1 mM DTT) and incubated in 40 μl ubiquitination buffer with 5 μg of Ub, 100 ng of Ub-activating enzyme (E1), and 100 ng of GST-UbCh5a (Calbiochem) in the presence or absence of recombinant PDLIM2. In vitro ubiquitination, all samples were washed three times with SDS-PAGE analysis buffer, eluted with SDS-sample buffer, resolved by SDS-PAGE, and analyzed with anti-HA antibody using Western blot analysis.

**In Vivo Ubiquitination Assay—**Ubiquitination assays were carried out in RAW264.7. Plasmids were cotransfected with HA-ubiquitin and FLAG-STAT1 expression vectors into cells using JetPEI macrophage reagent (Genesee, San Diego). After 24 h, RAW264.7 cells were treated with 10 μM MG132 for 2 h and incubated for 6 h with 50 ng/ml LPS prior to harvest. Cells lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4 °C, and the supernatant was precleared with protein G-agarose for 1 h at 4 °C. The lysates were subsequently immunoprecipitated using anti-STAT1 or M2 anti-FLAG-agarose at 20 min at 4 °C. Immunoprecipitated proteins were subjected to SDS-PAGE analysis and analyzed with anti-HA antibody using Western blot analysis.

**Chromatin Immunoprecipitation/Real Time-PCR—**Chromatin was fixed and immunoprecipitated using the chromatin immunoprecipitation (ChIP) assay kit (Upstate Biotechnology, Billerica, MA) as recommended by the manufacturer. Sequences were identified for the mouse iNOS promoter (GenBank™ accession number L09126). Purified chromatin was immunoprecipitated using anti-STAT1 (Santa Cruz Biotechnology) or 5 μl of rabbit nonimmune serum. Eluted DNA fragments were purified to serve as templates. The input fraction corresponded to 0.1 and 0.05% of the chromatin solution before immunoprecipitation. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis. After DNA purification, the presence of the selected DNA sequence was quantified by real time-PCR. ChIP assays for activated Stat1 binding to its IFN-γ-regulated transcription factor STAT1α (GAS) site on the iNOS promoter used primers ACACGAGCTGAGCTGACCTT and CACACATGGCATGGAATT, resulting in a 186-bp fragment. Serial dilutions of genomic DNA were used to generate standard curves for RT-PCR using the corresponding primer sets. Copy numbers of the eluted promoter were normalized against the copy numbers in the corresponding inputs and expressed in arbitrary units.

**Murine Cecal Ligation and Puncture Model of Sepsis—**Wild-type (WT) and OPN(−/−) 129 male mice (12–16 weeks old) were utilized. OPN(−/−) animals were a gift from David Denhardt, Rutgers University. They were maintained on sterile standard laboratory chow and water ad libitum in individual ventilated cages under specific pathogen-free conditions in the animal facility at Duke University. All animal experiments were approved by the Duke University Institutional Animal Care and Use Committee and conducted under guidelines for the humane care and use of animals. Animals were euthanized, and bone marrow macrophages (BMM) were isolated.

**Isolation of BMM—**BMM were isolated by lavage of excised cecums, washed in PBS, plated in DMEM with 0.2% BSA into two 100-mm tissue culture dishes, and allowed to adhere for 2 h at 37 °C. After washing out nonadherent cells, cells were cultured in DMEM with 10% FBS and 20% L cell-conditioned medium (as a source of M-CSF) to generate confluent BMM. Primary BMM were maintained at 37 °C under 5% CO₂ in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. After incubation for the designated time period at 37 °C in 5% CO₂, the cells or media were harvested for assays.

**Isolation of Peritoneal Macrophages—**Mice were sacrificed, and macrophages were harvested by peritoneal lavage. Physiological saline (10 ml) was injected into the peritoneal cavity. The peritoneum was then massaged for ~2 min to bring cells into suspension, and the fluid was extracted using a hypodermic needle. Cells were then centrifuged at 200 × g and pooled into RPMI 1640 culture medium supplemented with 10% FBS, 2 mM L-glutamine, 20 mM HEPES, penicillin, and streptomycin at 2 × 10⁶ cells/ml. The cell suspension was then seeded into polystyrene Petri dishes (10 ml per dish) and incubated overnight in an atmosphere of 5% (v/v) CO₂ in air at 37 °C to allow selective macrophage adherence. Contaminating nonadherent cells were removed by thoroughly washing each dish with phosphate-buffered saline (without Ca²⁺ and Mg²⁺) at pH 7.4. Purified macrophages were then removed by incubation with 0.5% (w/v) trypsin, 0.2% (w/v) EDTA at 37 °C.
Statistical Analysis—All data are presented as mean ± S.D. Analysis was performed using the Student’s t test. Values of \( p < 0.05 \) were considered significant.

RESULTS

Identification of LPS-dependent Serine/Threonine Phosphorylation Sites in PDLIM2—In the setting of LPS stimulation of RAW264.7 macrophages, OPN increases STAT1 ubiquitination and degradation through PDLIM2. Phosphopeptide mapping was performed to identify the specific PDLIM2 amino acid phosphorylation targets in the setting of LPS exposure. Sequence analysis using PhosphoScan-site 2.0 indicates the potential presence of 28 serine, 8 threonine, and/or 2 tyrosine phosphorylation sites. To determine whether LPS and OPN induce PDLIM2 phosphorylation, we treated RAW264.7 cells with LPS (50 ng/ml) for 6 h and performed IP to measure the extent of phosphorylated serine, threonine, and tyrosine in PDLIM2 (Fig. 1). Controls consisted of untreated cells and LPS + siRNA OPN-treated cells. The results demonstrate that LPS increased PDLIM2 serine phosphorylation compared with untreated controls and LPS + siRNA OPN. Repletion of OPN to LPS + siRNA OPN cells restored serine phosphorylation in PDLIM2. An adenovirus PDLIM2 expression vector pAdV-PDLIM2 was constructed. RAW264.7 cells were infected at a 1000 multiplicity of infection by AdV-PDLIM2. After 24 h, cells were treated with LPS or LPS + siRNA OPN for 6 h, and PDLIM2 protein was purified by PAGE and submitted to the Harvard Microchemistry and Proteomics Analysis Facility for characterization. Untreated cells and cells infected with empty pAdV served as additional controls. When compared with control and LPS + siRNA OPN, LPS induces two potentially unique serine/threonine phosphorylations sites, Ser-137 and Thr-138, within the sequence RPCSPFSTPP. Otherwise, there were no additional phosphorylated serine, threonine, or tyrosine residues noted under any of the treatment conditions. To determine the potential functional relevance of these phosphorylation sites, each was mutated to a phosphoresistant (Ser/Thr to Ala) or phosphomimetic (Ser to Asp) amino acid, expressed, isolated, and tested in subsequent studies.

E3 Ligase Function and PDLIM2 Mutants—Three PDLIM2 phosphorylation mutants, S137A, T138A, and S137D, were created and examined for E3 ligase activity using \textit{in vivo} and \textit{in vitro} ubiquitination assays (Fig. 2). Recombinant His-tagged PDLIM2, His-tagged PDLIM2 mutants, or empty His vector were transfected and expressed in RAW264.7 cells and purified to use as the source of E3 ligase. In selected instances, cells were stimulated with LPS (50 ng/ml) for 6 h. FLAG-tagged STAT1 was expressed in RAW264.7 cells and used as the source of substrate for \textit{in vitro} ubiquitination. After \textit{in vitro} ubiquitination, all samples were resolved by SDS-PAGE and transferred electrophoretically to PVDF membrane. The blot is representative of three experiments. In selected instances, cells were cotransfected with HA-Ub and FLAG-STAT1 vectors. After 24 h, cells were treated with MG132 (10 \textmu M) and in selected instances and stimulated with LPS (50 ng/ml) for 6 h. The Ub-STAT1 complex was immunoprecipitated and subjected to Western blot analysis using anti-HA Ab. The blot is representative of three experiments.
was expressed in RAW264.7 cells and used as the source of substrate for in vitro ubiquitination. (Fig. 2a) A Ub-STAT1 ladder was found in LPS + WT-PDLIM2 and LPS + T138A but was absent in the LPS + S137A. Similarly, Ub-STAT1 was also found in the control S137D setting.

We then performed in vivo ubiquitination assays in which RAW264.7 cells were cotransfected with WT-PDLIM2 or mutant forms of PDLIM2 with HA-Ub and FLAG-STAT1 vectors. After 24 h, cells were treated with MG132 (10 μM) and in selected instances were stimulated with LPS (50 ng/ml) for 6 h (Fig. 2b). The Ub-STAT1 complex was immunoprecipitated and subjected to Western blot analysis using anti-HA Ab. Ub-STAT1 was found in LPS + WT-PDLIM2, LPS + T138A, and LPS + T138D cells, but it was absent in the LPS + S137A. Ub-STAT1 was not found in the control S137D setting, secondary to the absence of activated STAT1. These data suggest that phosphorylation of PDLIM2 at Ser-137 is required for OPN-dependent formation of ubiquitinated P-STAT1 in LPS-treated macrophages.

**OPN-dependent PDLIM2 Ser-137 Phosphorylation and STAT1**—Given its defined role as a STAT1 E3 ligase, the functional role of LPS-dependent PDLIM2 Ser-137 phosphorylation was then determined in the context of STAT1 degradation using PDLIM2-S137A as an antagonist or PDLIM2-S137D as an agonist (8, 9). Using RAW cells, total cellular and phosphorylated nuclear STAT1s (P-STAT1) were measured in the presence and absence of LPS (Fig. 3a). In selected instances, cells were transfected with PDLIM2-S137A, PDLIM2-S137D, and/or siRNA OPN prior to LPS stimulation. In the setting of LPS stimulation, P-STAT1 was readily detected compared with unstimulated control cells did not alter the P-STAT1 profile in comparison to LPS alone; OPN added to LPS or LPS + OPN prior to LPS stimulation. Blot is representative of three experiments. b, IP studies were performed to assess the extent of Ub-associated STAT1 in the presence of cycloheximide (10 μg/ml) and MG132 (10 μM). Cell lysates (500 μg) were immunoprecipitated with P-STAT1 Ab and immunoblotted (IB) with anti-Ub Ab. In selected instances, cells were transfected with PDLIM2-S137A, PDLIM2-S137D, and/or siRNA OPN prior to LPS stimulation. Blot is representative of three experiments. b, IP studies were performed to assess the extent of Ub-associated P-STAT1 in the presence of cycloheximide (10 μg/ml) and MG132 (10 μM). Cell lysates (500 μg) were immunoprecipitated with P-STAT1 Ab and immunoblotted (IB) with anti-Ub Ab. In selected instances, cells were transfected with PDLIM2-S137A, PDLIM2-S137D, and/or siRNA OPN prior to LPS stimulation. Blot is representative of three experiments.

**OPN Signal Transduction and PDLIM2 Phosphorylation**—The amino acid sequence surrounding Ser-137 was submitted for protein kinase substrate analysis by NetPhosK 1.0 (13). Based upon these results, we utilized pharmacological inhibitors for PKC, p38, P6K, and MEK1/2 in LPS-stimulated RAW264.7 cells and performed IP for serine-phosphorylated PDLIM2 (Fig. 4a). Administration of the general PKC inhibitor, G06850, was associated with significantly decreased serine-

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**Figure 3.** OPN-dependent PDLIM2 Ser-137 phosphorylation and STAT1. A, Western blot analysis of LPS-dependent PDLIM2 Ser-137 phosphorylation was performed using RAW cells to measure total cellular and phosphorylated nuclear STAT1 (P-STAT1) in the presence and absence of LPS. In selected instances, cells were transfected with PDLIM2-S137A, PDLIM2-S137D, and/or siRNA OPN prior to LPS stimulation. Blot is representative of three experiments. B, IP studies were performed to assess the extent of Ub-associated P-STAT1 in the presence of cycloheximide (10 μg/ml) and MG132 (10 μM). Cell lysates (500 μg) were immunoprecipitated with P-STAT1 Ab and immunoblotted (IB) with anti-Ub Ab. In selected instances, cells were transfected with PDLIM2-S137A, PDLIM2-S137D, and/or siRNA OPN prior to LPS stimulation. Blot is representative of three experiments.
Osteopontin and PDLIM2

Figure 4. Protein kinase C and PDLIM2 phosphorylation. a, Western blot analysis of PKC, p38, P38, and MEK1/2 were incubated in the presence and absence of classical PKC inhibitors, G06850 (general PKC inhibitor), G06976 (PKCα inhibitor), and pseudosubstrate peptide (PKCβ inhibitor), were incubated with LPS-stimulated RAW264.7 cells. IP was performed for PDLIM2 and then blotted with anti-phospho-PKCMAb. The blot is representative of three experiments. b, in vitro phosphorylation assay. RAW264.7 cells were cotransfected with WT-PDLIM2, HA-Ub, and FLAG-STAT1 vectors. The PKC pharmacological inhibitors, G06850 (general PKC inhibitor), G06976 (PKCα and PKCβ inhibitor), rottlerin (PKCδ and PKCθ inhibitor), and pseudosubstrate peptide (PKCζ inhibitor), were utilized. After 24 h, cells were treated with MG132 (10 μM) and stimulated with LPS (50 ng/ml) for 6 h. The Ub-STAT1 complex was immunoprecipitated and subjected to Western blot analysis using anti-HA Ab. The blot is representative of three experiments. c, in vivo ubiquitination assay. RAW264.7 cells were cotransfected with PDLIM2, HA-Ub, and FLAG-STAT1 vectors. The PKC pharmacological inhibitors, G06850 (general PKC inhibitor), G06976 (PKCα and PKCβ inhibitor), rottlerin (PKCδ and PKCθ inhibitor), and pseudosubstrate peptide (PKCζ inhibitor), were utilized. After 24 h, cells were treated with MG132 (10 μM) and stimulated with LPS (50 ng/ml) for 6 h. The Ub-STAT1 complex was immunoprecipitated and subjected to Western blot analysis using anti-HA Ab. The blot is representative of three experiments. d, in vitro phosphorylation assay. PDLIM2, S137A, and S137D were immunoprecipitated from RAW cells and incubated in the presence and absence of classical PKCα. S137A and S137D were used as controls. Blot is representative of two experiments.

Phosphorylated PDLIM2. This was not reversed by addition of exogenous OPN. We then used several PKC inhibitors to identify the roles of PKC isozymes in LPS-induced PDLIM2 phosphorylation (Fig. 4b). RAW264.7 cells were transfected with AdV-PDLIM2 for 24 h and then pretreated with G06850 (general PKC inhibitor), G06976 (PKCα and PKCβ inhibitor), rottlerin (PKCδ and PKCθ inhibitor), and pseudosubstrate peptide (PKCζ inhibitor). DMSO treatment served as a control; cells were stimulated with LPS. We found that LPS-induced PDLIM2 phosphorylation was not altered by a pseudosubstrate peptide inhibitor of PKCζ, but it was significantly suppressed by G06976 and G06850; also, it was slightly inhibited by rottlerin, which has reduced potency for inhibiting PKCα and PKCβ. In the presence of cycloheximide and MG132, we then performed in vivo ubiquitination assays for Ub-STAT1 in LPS-treated cells using the various PKC inhibitors (Fig. 4c). Again, LPS induced a Ub-STAT1 ubiquitination ladder; this was significantly diminished in the presence of G06850 and G06976, suggesting significantly decreased PDLIM2 activity in the presence of these PKC inhibitors.

In vitro phosphorylation assays were performed (Fig. 4d). PDLIM2 was immunoprecipitated from RAW cells and incubated in the presence of classical PKCα. S137A and S137D were used as controls. PKC isoform phosphorylated only wild-type PDLIM2. In contrast, we saw no phosphorylation of the S137A or S137D mutants. Total PDLIM2 protein in the immunoprecipitates, detected by immunoblot analysis, showed similar protein levels for all of the mutants tested. These results implicate serine 137 as the only PKC-dependent PDLIM2 phosphorylation site in the presence of LPS. These results indicate that PKCα plays a significant role in PDLIM2 phosphorylation and P-STAT1 ubiquitination in LPS-treated macrophages.

PDLIM2 and STAT1 Function—A GAS luciferase reporter construct was then used to assess the potential impact of PDLIM2 function on P-STAT1 transcriptional activity (Fig. 5a). Transient transfection assays were repeated using a pGL3 luciferase reporter plasmid construct bearing a quadruple repeat of the GAS enhancer element, agtttcaTTACTCTAA. LPS stimulation significantly increased STAT1-dependent luciferase activity in both LPS and LPS + PDLIM2 when compared with unstimulated controls. OPN knockdown in LPS + PDLIM2 + siRNA OPN resulted in an additional 2.2-fold increase in luciferase activity. When PDLIM2-S137A was added to LPS cells, luciferase activity was significantly increased compared with LPS + PDLIM2 and LPS alone. In LPS + S137D, luciferase activity was 75% that found in LPS + PDLIM2; in LPS + T138A, luciferase activity was not statistically different from LPS + PDLIM2 and LPS alone.

We next measured nitrite levels, an NO metabolite, as an endpoint of P-STAT1 transactivation of the iNOS promoter (Fig. 5b). The murine iNOS promoter contained two NF-κB-binding sites, NF-κB1 (nucleotides −1044 to −1034) and NF-κB2 (nucleotides −114 to −104), and a single STAT1-binding site (GAS; nucleotides −942 to −934), deemed critical for iNOS
expression (9). RAW264.7 cells were transfected using empty vector, wild-type PDLIM2, PDLIM2-S137A, PDLIM2-S137D, or PDLIM2-T138A; after 24 h, cells were treated with LPS and nitrite measured in the culture medium following a 6-h incubation. Compared with wild-type PDLIM2, PDLIM2-S137A significantly increased nitrite levels, reflecting increased P-STAT1. Ablation of OPN with siRNA also resulted in significantly increased nitrite in the culture medium. PDLIM2-S137D and PDLIM2-T138A did not significantly alter nitrite production. These data suggest that increased levels of P-STAT1 functionally translate into increased NO production. We then proceeded to perform ChIP/RT-PCR assays to determine the extent of LPS-mediated STAT1 binding to the iNOS promoter (Fig. 5c). These results demonstrate significantly increased STAT1 binding in the presence of S137A and siRNA to OPN, reflecting increased STAT1 as the result of decreased Ub-dependent degradation.

**PDLIM2 Phosphorylation in a Murine CLP Model of Sepsis**

To determine the in vivo relevance of these observations, we utilized a CLP model of sepsis utilizing WT and OPN(-/-) mice. Animals were sacrificed after 24 h, and BMMs were studied (Fig. 6). PDLIM2 was immunoprecipitated and Western-blotted for serine phosphorylation. In WT animals, CLP was associated with significantly increased PDLIM2 serine phosphorylation. In OPN(-/-), serine-phosphorylated PDLIM2 was essentially absent in CLP. Western blot analysis in BMM was then performed to determine iNOS, P-STAT1, and OPN expression in CLP in WT and OPN null animals at 0 and 24 h. WT expression of iNOS and P-STAT1 in BMM was significantly (10-fold) than that of OPN(-/-) (p < 0.01 for iNOS and P-STAT1). These results show that iNOS and P-STAT1 were significantly increased in the absence of OPN in a CLP model of sepsis. WT-sham and OPN(-/-) sham expression of iNOS, P-STAT1, and OPN at 0 and 24 h was statistically equivalent to the expressions seen in WT-CLP and OPN null...
Osteopontin and PDLIM2

CLP at 0 h for both BMM and liver, respectively (data not shown). To address the role of OPN in ubiquitination of P-STAT1 in this CLP model, P-STAT1 was immunoprecipitated to determine Ub-P-STAT1 expression in BMM at 24 h.

In BMM from WT animals, CLP was associated with ubiquitination of P-STAT1; in OPN(−/−), Ub-P-STAT1 was significantly less than that noted in WT (p < 0.01; WT versus OPN (−/−)), although overall P-STAT1 levels were increased in the OPN(−/−) animals. These in vivo studies indicate that OPN is necessary for PDLIM2 serine phosphorylation and STAT1 ubiquitination to regulate downstream STAT1-dependent functions such as iNOS expression.

We then addressed PDLIM2 serine phosphorylation, P-STAT1 formation, and nitrite expression in primary murine peritoneal macrophages (Fig. 7). Serine-phosphorylated PDLIM2 was readily detected on IP with LPS treatment; in the presence of PDLIM2-S137A or G06850, LPS-induced serine-phosphorylated PDLIM2 was significantly decreased (p < 0.01 LPS versus LPS + S137A or LPS + G06850). Nuclear P-STAT1 was also significantly increased in the presence of LPS with PDLIM2-S137A or G06850. Nitrite production in BMM and peritoneal macrophages was measured to support functional biological significance. LPS-induced NO was significantly increased with exogenous PDLIM2 and, conversely, decreased with PDLIM2-S137A (p < 0.01 LPS versus LPS + PDLIM2 or LPS + PDLIM2-S137A).

In summary, our results indicate that OPN functions as a feedback inhibitor of iNOS expression by PKC-mediated activation of the E3 ligase PDLIM2, which targets STAT1 for degradation by the proteasome.

DISCUSSION

Evidence indicates that LPS-mediated iNOS gene transcription is regulated by a redundant signal transduction pathway, with counter-regulatory pathways that repress or inhibit macrophage iNOS expression in the context of endotoxemia and sepsis. Previously, using a system of LPS-treated RAW264.7 macrophages, we demonstrated that OPN regulates ubiquitin-dependent degradation of STAT1 through the activity of PDLIM2 as the ubiquitin E3 ligase (9). This regulation of STAT1 degradation underlies the effect of OPN as an inhibitor of iNOS gene transcription. These results define OPN as a unique and as yet incompletely characterized trans-activator of STAT1 degradation by the 26 S proteasome.

STAT signaling is tightly regulated, and several mechanisms have been proposed to account for this control (14). The suppressor of cytokine signaling (SOCS) and protein inhibitor of STAT (PIAS) families of proteins have been shown to bind to and inhibit either the cytokine receptor-associated Janus kinase (JAK) or activated STAT molecule, respectively. SOCS proteins are induced following cytokine stimulation, bind to JAK kinases, and inhibit activated JAK kinases from further phosphorylation of STAT proteins (15, 16). As a result of inhibition by SOCS proteins, STAT signaling becomes a transient response, and levels of phosphorylated STAT pro-
that down-regulates STAT signaling involves tyrosine phosphatases, such as SHP-1 and SHP-2. These tyrosine phosphatases have been shown to down-regulate the activity of STAT1, STAT3, or STAT5 following activation by IFN-γ, leukemia inhibitory factor, or IL-2 either by dephosphorylating STAT proteins directly or through dephosphorylation of JAK kinases (17–19). Like the SOCS and PIAS mechanisms, the down-regulation through tyrosine phosphatases also does not lead to reduced levels of STAT proteins.

PDLIM2 (also known as Mystique and SLIM) is a postsynaptic density-95 (PSD-95)/discs large (DLG)/zonula occludens-1 (ZO-1)–Lin-11, Isl-1, Mec-3 (PDZ-LIM) domain protein that was identified in cells transformed by overexpression of the insulin-like growth factor-I receptor (20). It is a member of the actinin-associated LIM protein family of proteins that contains a single N-terminal PDZ and C-terminal LIM domain. PDLIM2 contains a PDZ domain and a LIM domain and interacts in the nucleus with tyrosine-phosphorylated STAT molecules (21).

The relationship between OPN and PDLIM2 has not been previously characterized. OPN is a secreted glycoprotein that is rich in aspartate and sialic acid residues and contains functional domains for calcium binding, phosphorylation, glycosylation, and extracellular matrix adhesion (23). OPN appears to mediate cell-matrix interactions and cellular signaling through binding with integrin, primarily \(\alpha\)V\(\beta\)3, and CD44 receptors. A variety of stimuli, including phorbol 12-myristate 13-acetate, 1,25-dihydroxyvitamin D, basic fibroblast growth factor (bFGF), TNF-\(\alpha\), IL-1, IFN-\(\gamma\), and LPS appears to regulate OPN expression. OPN has multiple molecular functions that mediate cell adhesion, chemotaxis, macrophage-directed IL-10 suppression, stress-dependent angiogenesis, prevention of apoptosis, and anchorage-independent growth of tumor cells (23). Our studies have demonstrated that OPN functions as a feedback regulated trans-repressor of iNOS expression (2, 3). Our data indicate that the underlying molecular pathway is LPS- and OPN-dependent PKC-regulated phosphorylation of PDLIM2 Ser-137 with subsequent ubiquitination and proteasome degradation of STAT1. A link between OPN and PKC has been previously established. In PC-3 prostate cancer cells, Jain et al. (24) demonstrated that exogenously administered OPN induces PKCo activation with subsequent phosphorylation of NF-\(\kappa\)B p65. Similarly, Zhou et al. (25), Tuck et al. (26), Hullinger et al. (27), and Nodi (28) have each published data indic-
cating a critical role for PKCα activation in OPN-dependent migration of prostate and/or breast cancer cells. In a similar fashion, we demonstrate the critical role of PKCα/β in OPN-dependent phosphorylation of PDLIM2 in murine macrophages exposed to LPS.

Recently, PDLIM2 has been found to play a similar role in NF-κB metabolism as described for STAT1. Tanaka et al. (10) have shown that PDLIM2 negatively regulates NF-κB activity by acting as a nuclear ubiquitin E3 ligase targeting the p65 subunit of NF-κB. PDLIM2 was bound to p65, promoted p65 polyubiquitination, and targeted p65 to discrete intranuclear compartments where Ub-p65 was degraded by the 26 S proteasome. Subsequently, Healy and O’Connor (20) examined PDLIM2 in the context of phorbol 12-myristate 13-acetate-stimulated RAW264.7 cells. In a fashion reminiscent of our results, PDLIM2 exhibited retarded mobility indicative of serine phosphorylation, which could be reversed by phosphatases and by inhibition of protein kinase C. In this case, PDLIM2 was located predominantly in the cytoplasm (20). Also, PDLIM2 knock-out mice produce more of the proinflammatory cytokines IL-6 and IL-12 in response to LPS (10, 20). In summary, previous studies support our current results by demonstrating a critical relationship between OPN and PKC, while simultaneously suggesting that serine phosphorylation of PDLIM2 is critical for its E3 ligase activities. Certainly, in our setting, phosphorylation of PDLIM2 Ser-137 is required for Ub-P-STAT1 degradation by the 26 S proteasome. Subsequently, Healy and O’Connor (20) examined the critical role of PKC in OPN-dependent inflammatory responses. In a similar fashion, we demonstrate the critical role of PKC in OPN-dependent phosphorylation of PDLIM2 in murine macrophages exposed to LPS.

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