Osteopontin (OPN) is a secreted glycoprotein that is expressed by various immune cells and serves as a modulator in immune responses, inflammation, tumor growth, metastasis, bone formation, and remodeling. It is widely accepted that OPN acts as a cytokine, integrins, and CD44 variants, to activate PI3K/Akt, NIK/NF-κB, or IKKB/NF-κB signal pathways that activate distinct patterns of cytokine/chemokine expression and specific immune responses. It has been reported in macrophages that interaction between sOPN and integrin receptors stimulated IL-12 expression, whereas interaction between sOPN and CD44 inhibited IL-10 expression.

Recently, TLR9-induced T-bet-dependent expression of iOPN in plasmacytoid dendritic cells (pDCs) was found to mediate IFN-α expression by selectively coupling TLR9 signaling with activation of IRF7 (14), whereas iOPN expression in conventional dendritic cells (cDCs) has a key role in promoting Th17-cell responses by suppressing the expression of IL-27 (15).

Macrophages express and secrete OPN constitutively. LPS can further up-regulate OPN expression in macrophages (16). Previously, we demonstrated OPN transcription and promoter activity are significantly up-regulated in response to nitric oxide (NO) in LPS-stimulated RAW264.7 murine macrophages through heterogeneous nuclear ribonucleoprotein proteins (hnRNP)-A/B and hnRNP-U proteins (16, 17). The stimulation of TLR4 by LPS induces 1kb kinase (IKKs), mitogen-activated protein kinase (MAPK), and PI3K, ultimately leading to activa-
tion of NF-κB and AP-1 resulting in the production of various proinflammatory mediators (18, 19). A consensus AP-1 binding site (TGACACA between nt −69 and nt −75) has been identified in the murine OPN promoter and is responsible for OPN expression in several cell lines (20, 21), suggesting that LPS-induced AP-1 activation may play an important role in LPS-stimulated OPN expression in macrophages.

In the present study, we investigated the molecular mechanisms of TLR-induced OPN expression in macrophages. The results indicate that TLR stimulation can induce OPN expression through TLR-induced PI3K, JNK, ERK, and AP-1 activation. Importantly, the intracellular form of OPN (iOPN), but not the secreted form of OPN (sOPN), is induced following TLR stimulation in macrophages in vivo and in vitro. In addition, TLR-induced iOPN expression negatively regulates interferon-β production in murine macrophages.

EXPERIMENTAL PROCEDURES

Mice and Reagent—C57BL/6 mice were obtained from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were undertaken in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Shandong University, Jinan, Shandong, P. R. China. LPS (Escherichia coli, 055:B5), LTA and poly(I: C) were purchased from Sigma, and LPS was re-purified as described (22). LY294002, a specific inhibitor of PI3K, PD98059, a specific inhibitor of JNK, SP600125, a specific inhibitor of p38 kinase, and the specific inhibitor of ERK, U0126 were purchased from Abcam (Cambridge, MA). The cell culture medium (DMEM) was purchased from Life Technologies (Paisley, U.K.), and fetal calf serum (FCS) (Invitrogen). After centrifugation, the supernatant was stored at −80°C.

Plasmid Constructs—The iOPN reporter plasmid (−882/+79) was kindly provided by Dr. David T. Denhardt (Rutgers University, New Brunswick, NJ). The mouse OPN promoter plasmid with the AP-1 binding site mutation was constructed by two-step PCR and described previously (20). The ISRE cis-reporting plasmid was kindly provided by Dr. Hongbing Shu (Wuhan University, China). The expression plasmids for the secreted form of OPN (sOPN) was constructed by RT-PCR with primers: OPN-F, 5′-GAGATTGGCAGTGATTTG-3′; OPN-R, 5′-CGCGAATTCCAT-TGTTACCAACTGGGAC-3′; the 885-bp fragment was then inserted into the mammalian expression vector pcDNA3.1; pcDNA3.1 was used as control cells.

Cell Culture—Female C57BL/6 mice (5–6 weeks old) were used for the preparation of primary mouse macrophages, and thioglycolate-elicited mouse peritoneal macrophages were prepared as described (27). The cells were cultured in endotoxin-free Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) (Invitrogen). After 1 h, nonadherent cells were removed. On the next day, the cells were treated with TLR agonists. Mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA) and cultured at 37°C under 5% CO2 in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. LPS, poly(I: C), and LTA were used at a final concentration of 100 ng/ml, 10 μg/ml, and 1 μg/ml, respectively.

Quantitative Real-time PCR (qRT-PCR)—Total RNA was extracted with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). A LightCycler (ABI PRISM® 7000) and a SYBR RT-PCR kit (Takara) were used for quantitative real-time RT-PCR analysis. Specific primers used for RT-PCR assays were 5′-GCTGTGTTTGGCATTGCCTCCTC-3′ (sense), 5′-CACCAGTCTCTGCGGCAAAG-3′ (antisense) for osteopontin, and 5′-GTTTACACTGGGACGACA-3′ (sense), 5′-CTGGGTCAATTTTACACGCT-3′ (antisense) for β-actin. Data were normalized to β-actin expression in each sample.
natants were measured by ELISA kits (R&D Systems, Minneapolis, MN). For cytokine quantitation from peritoneal lavage fluid and serum, peritoneal lavage fluid and serum were prepared as above, and secreted osteopontin and TNF-α were measured by ELISA kits (R&D Systems, Minneapolis, MN).

Western Blot—Cells were lysed with M-PER Protein Extraction reagent (Pierce) supplemented with a protease inhibitor ‘mixture’; protein concentrations in the extracts were measured with a bicinchoninic acid assay (Pierce). OPN dephosphorylation and deglycosylation were performed using bovine alkaline phosphatase and peptide N-glycosidase F, respectively as described previously by Christensen et al. Equal amounts of extracts were separated by SDS-PAGE, then were transferred onto nitrocellulose membranes for immunoblot analysis as described previously (22).

Chromatin Immunoprecipitation (ChIP) Assay—RAW264.7 macrophages were stimulated with 100 ng/ml LPS for 2 h or left unstimulated. Chromatin from macrophages was fixed and immunoprecipitated using the ChIP assay kit as recommended by the manufacturer (Upstate Biotechnology, Inc.). The purified chromatin was immunoprecipitated using 2 μg of anti-c-Jun and c-Fos or 2 μg of irrelevant antibody (anti-actin). The input fraction corresponded to 0.1 and 0.05% of the chromatin solution before immunoprecipitation. After DNA purification, the presence of the selected DNA sequence was assessed by PCR. The primers were 5′-CTCATGTTAGTTCTTGGC-TATTA-3′, 5′-TCTCATCCTT-AGCAAGGAAAAAG-3′ for OPN promoter (−156~+6) and 5′-GTC-CAAAATAGACCTTTACT-3′, 5′-TAAGCAGAAGAATTCCCCAG-3′ for OPN promoter (−136~−136). The PCR program was 94 °C × 4 min; followed by 94 °C × 45 s, 55 °C × 45 s, and 72 °C × 45 s for a total of 28 cycles; and then 72 °C × 7 min. PCR products were resolved in 10% acrylamide gel electrophoresis. The average size of the sonicated DNA fragments subjected to immunoprecipitation was 500 bp as determined by ethidium bromide gel electrophoresis.

Assay of Luciferase Reporter Gene Expression—Assay of luciferase reporter gene expression was performed as previously described (27). Briefly, RAW 264.7 cells (1.5 × 10^4 cells/well) were seeded onto 96-well plates 24 h before co-transfection with 100 ng of various reporter plasmids and 25 ng of Renilla-TK plasmid using Jet-PEI transfection reagent (Polyplus). In some cases, iOPN expression plasmid was cotransfected; the total amount of transfected plasmid was equalized by empty vector pcDNA3.1. Twenty-four hours after transfection, the cells were left untreated or treated with LPS or IFN-γ for 6 h. Luciferase activities were measured using Dual-Luciferase Reporter Assay system (Promega) on a LMAXII luminometer (Molecular Devices) according to the manufacturer’s instructions. Firefly luciferase activity was normalized against Renilla luciferase activity.

Statistical Analysis—All data are presented as means ± S.E. of three or four experiments. Analysis was performed using a Student’s t test. Values of p < 0.05 were considered significant.

RESULTS

TLR-induced OPN Gene Expression in Macrophages—We first examined OPN gene expression in macrophages stimulated with LPS (TLR4 ligand) for various times by RT-PCR and real-time quantitative PCR. As shown in Fig. 1A, constitutive expression of OPN mRNA was found in unstimulated control RAW264.7 murine macrophages. Upon stimulation with LPS, a
significant increase in expression of OPN mRNA was detected in RAW264.7 macrophages. Expression of OPN mRNA in RAW264.7 macrophages reached the peak level after stimulated with LPS for 4 h (Fig. 1A). To further confirm LPS-induced OPN mRNA expression in macrophages, thioglycolate-elicited mouse primary peritoneal macrophages were used. In a similar fashion, expression of OPN mRNA was up-regulated by LPS treatment (Fig. 1B); however, OPN mRNA expression reached the peak level in these cells 8 h after LPS treatment (Fig. 1B).

To confirm whether other TLR agonists can also induce OPN mRNA expression in macrophages, LTA (TLR2 ligand) and poly(I:C) (TLR3 ligand) were used to stimulate macrophages. Similarly, stimulation with both LTA and poly (I:C) greatly enhanced OPN mRNA expression 4 h after stimulation in both RAW 264.7 macrophages and peritoneal macrophages (Fig. 1, C and D). Taken together, these data indicate that TLR signaling is associated with significantly increased OPN mRNA expression in macrophages.

**OPN Protein Expression and Secretion in TLR-stimulated Macrophages**—Two isoforms of OPN, secreted form of OPN (sOPN) and intracellular form of OPN (iOPN), have been identified. Immunoblot analysis and ELISA were used to determine iOPN protein expression in the cell lysate and sOPN secretion in TLR-stimulated macrophages, respectively. Unstimulated RAW264.7 macrophages expressed and secreted detectable levels of OPN (Fig. 2, A and D), consistent with the constitutive expression of OPNmRNA as shown in Fig. 1A. Consistent with the LPS-induced expression of OPN mRNA as shown in Fig. 1A, OPN protein level in the cell lysate was greatly increased by LPS stimulation (Fig. 2D). In contrast, the level of secreted OPN remained the same as that of unstimulated macrophages during a 24-h period of LPS stimulation (Fig. 2A). As a control, TNF-α secretion was greatly increased by LPS stimulation (Fig. 2A), indicating LPS stimulation and the secretory machinery are not impaired in these LPS-stimulated macrophages.

Similarly, primary peritoneal macrophages expressed and secreted low detectable levels of OPN, and LPS further increased OPN protein expression in the cell lysate (Fig. 2E). But, the level of secreted OPN remained unchanged and showed the same expression as that of unstimulated macrophages during a 24-h period of LPS stimulation (Fig. 2B). As a
control, TNF-α secretion was greatly increased by LPS stimulation (Fig. 2B) in peritoneal macrophages.

TLR2 ligand LTA and TLR3 ligand poly (I:C) were used to stimulate primary peritoneal macrophages, and OPN protein expression in the cell lysate and OPN secretion were measured as above. Immunoblot showed that OPN protein expression in the cell lysate was greatly induced in both RAW264.7 macrophages (Fig. 2D) and peritoneal macrophages (Fig. 2E), consistent with the increased OPN mRNA after stimulation (Fig. 1, C and D). Similar to the LPS stimulation, the level of secreted OPN displayed a comparable level to that of unstimulated control peritoneal macrophages (Fig. 2C). TNF-α secretion was also greatly increased by LTA and poly(I:C) stimulation (Fig. 2C).

Finally, immunoblot analysis was performed using OPN 2A1 Ab that has been used successfully to detect both forms of OPN (6). As shown in Fig. 2F (upper panel), two protein bands were detected in LPS-stimulated peritoneal macrophages. Importantly, the upper band (∼60 kDa), corresponding to the secreted OPN, remained unchanged after LPS stimulation, whereas, the lower band (∼54 kDa), corresponding to the intracellular form of OPN, was greatly increased by LPS stimulation. OPN is a glycosylated phosphoprotein. OPN from control and LPS-stimulated cells was dephosphorylated and deglycosylated using bovine alkaline phosphatase and peptide N-glycosidase F, respectively. After dephosphorylation and deglycosylation, two protein bands (∼50 kDa and ∼44 kDa) can still be detected with OPN 2A1 Ab (Fig. 2F, lower panel), suggesting that the two protein bands are not the full-length protein whose post-translational modifications have been altered by TLR signaling.

Collectively, these data suggest that macrophages differentially express both forms of OPN, sOPN is expressed constitutively in macrophages, while iOPN is expressed in TLR-activated macrophages. TLR signaling can further increase iOPN protein expression.

**Differential Expression of sOPN and iOPN in Vivo**—To further confirm differential OPN expression in macrophages, we measured OPN secretion in blood and peritoneal fluid in a mouse model of endotoxemia by intraperitoneal injection of LPS. Female C57BL/6J mice (4 weeks old) were treated with 100 ng/ml LPS as indicated. Secreted OPN in serum or peritoneal fluid was measured by ELISA. As shown in Fig. 3A, serum OPN level was greatly increased as early as 4 h after LPS injection, and continued to reach the peak level at 24 h after LPS injection. In contrast, OPN secretion was also greatly increased by LPS in peritoneal lavage (Fig. 3B).

**FIGURE 3. TLRs induce OPN expression in vivo.** A, female C57BL/6J mice (4 weeks old) were intraperitoneally injected with thioglycolate to elicit peritoneal macrophages. After 3 days, the mice were treated with PBS or 1.8 mg/kg LPS intraperitoneal administration (1.8 mg/kg) for the indicated time period. Secreted OPN and TNF-α production in the serum were detected by ELISA. B, female C57BL/6J mice (4 weeks old) were treated with PBS or 1.8 mg/kg LPS intraperitoneal administration (1.8 mg/kg) for the indicated time period. Secreted OPN in peritoneal lavage were detected by ELISA. C, female C57BL/6J mice (4 weeks old) were treated with PBS or 1.8 mg/kg LPS intraperitoneal administration (1.8 mg/kg) for the indicated time period. Secreted OPN in peritoneal lavage was detected by ELISA. Actin was used as a cytoplasmic protein loading control. Data are shown as mean ± S.D. (n=3) of one representative experiment.
level in peritoneal fluid remained unchanged compared with that of PBS-simulated mouse (Fig. 3B). As a control, TNF-α secretion in both serum and peritoneal fluid was greatly increased in LPS-challenged mice (Fig. 3, A and B). LPS-induced OPN expression was induced by LPS intraperitoneal administration in the peritoneal macrophages at various time points after LPS stimulation. As shown in Fig. 3C, intraperitoneal OPN protein expression in iOPN protein expression in peritoneal macrophages at various time points after LPS stimulation was significantly decreased by JNK inhibitor SP60012 treatment and ERK inhibitor PD98089 treatment (Fig. 3C). Similarly, LPS-induced OPN protein expression at 4 and 8 h after LPS stimulation was also greatly decreased by JNK inhibitor SP60012 treatment and ERK inhibitor PD98089 treatment (Fig. 3C). These data indicate that LPS-induced MAP kinases JNK and ERK activation is associated with significantly increased OPN mRNA and iOPN protein expression in macrophages.

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).

**MAP Kinases JNK and ERK Are Involved in LPS-induced iOPN**

**Expression in Macrophages**—The stimulation of TLR4 by LPS induces activation of MAP kinases including JNK, ERK, and p38 leading to NF-κB activation (23). Similarly, LPS-induced OPN expression in RAW264.7 cells was significantly decreased by JNK inhibitor PD98089 treatment and ERK inhibitor PD98089 treatment, but not by p38 kinase inhibitor SB203580 treatment (Fig. 4A). To confirm these results, two sets of macrophages were isolated and stimulated in vitro with LPS for the indicated time periods. Expression level of OPN mRNA was examined by both RT-PCR (23) and quantitative PCR (8). The production of intracellular OPN was detected by Western blotting (C). Data are shown as mean ± S.D. (n = 3) of one representative experiment (**) (p < 0.01).
Differential Expression of OPN Isoforms in Macrophages

AP-1 binds specifically to the AP-1 cis element. To confirm the functional relevance of AP-1 in the setting of OPN promoter activation and OPN protein expression with LPS stimulation, constructs with point mutations in the AP-1 core sequence were used (Fig. 6B). WT OPN promoter construct demonstrated a 4-fold increase in luciferase activity by LPS stimulation. In contrast, mutation of the AP-1 binding site completely ablated this increase in luciferase activity (p < 0.01), suggesting the AP-1 site is essential for LPS-induced OPN promoter activation.

To assess the functional relevance of AP-1 in the setting of LPS-induced OPN expression, c-Jun and c-Fos siRNA was designed to inhibit c-Jun and c-Fos expression. C-Jun and c-Fos siRNA greatly decreased both c-Jun and c-Fos expression in RAW264.7 macrophages as measured by immunoblot analysis (Fig. 6C, left). Then, OPN protein expression in LPS-stimulated RAW264.7 macrophages was measured by immunoblot analysis. Transfection of c-Jun and c-Fos siRNA significantly decreased LPS-induced OPN expression (Fig. 6C, right). These results suggest that the AP-1 site (nt −69 and nt −75) of the OPN promoter is a binding site for AP-1, which is essential for OPN promoter activation and OPN protein expression with LPS stimulation.

**TLR-induced iOPN Expression Negatively Regulates Interferon-β Production**—TLR9-induced expression of iOPN was found to mediate IFN-α expression in pDCs (14). To investigate the role of TLR-induced iOPN expression in Type I interferon production in macrophages, RAW264.7 macrophage stable cell lines with high expression and knockdown of iOPN were constructed. The overexpression and knockdown of iOPN was confirmed by immunoblot analysis (Fig. 7, A and C). IFN-β production from OPN knockdown stable cells was greatly increased by ~50% compared with that of control siRNA-transfected cells (p < 0.01) after LPS stimulation (Fig. 7B).

Exogenous addition of recombinant OPN protein to the culture medium of OPN knockdown stable cells did not reverse the increase of IFN-β production (data not shown). In contrast, as shown in Fig. 7D, after transfection of His-tagged iOPN expression into RAW264.7 cells, LPS-induced IFN-β production was significantly decreased by ~50% compared with that of control plasmid transfected cells (p < 0.01). Then cotransfection experiments with IFN-β promoter reporter plasmid and iOPN expression plasmid were performed (Fig. 7E). Cotransfection of iOPN expression plasmid inhibited LPS-induced IFN-β reporter gene activity by ~40% (p < 0.01). These data indicate that iOPN is a negative regulator for LPS-induced IFN-β expression and secretion in macrophages.

LPS-induced IFN-β production can activate specific target genes through ISREs in macrophages. To confirm the function of iOPN mediated down-regulation of IFN-β production, transient transfection assays were performed using the ISRE cis-reporting vector together with iOPN expression plasmid (Fig. 7E). LPS induced ISRE-dependent luciferase activity was significantly lower by ~80% in iOPN expression plasmid–transfected cells compared with that of control vector-transfected cells (p < 0.01). Taken together, these data indicate that LPS-induced iOPN expression negatively regulates INF-β production and subsequent INF-β signaling.

**DISCUSSION**

OPN is a ~298-amino acid secreted phosphoprotein and contributes to diverse physiological and pathological processes including immune responses, inflammation, tumor growth, and metastasis, bone formation, and remodeling (5, 25). Its expression is tissue-specific and subject to regulation by many transcription factors (2). Macrophages constitutively express and secret low levels of OPN. Its expression can be further up-regulated by LPS stimulation (16). But, the molecular mechanisms of LPS-mediated induction of OPN expression and
Differential Expression of OPN Isoforms in Macrophages

A number of transcription factors have been found to control OPN expression in immune cells. For example, OPN expression in activated T cells and pDCs depends on the transcription factor T-bet (14, 23), an essential factor in Th1 lineage commitment. But, transcription factors controlling OPN expression in macrophages remain to be determined. AP-1 is often activated in TLR-stimulated macrophages downstream of the MAP kinase and PI3K. A variety of inflammatory genes are under the control of AP-1-dependent signaling pathways. CHIP assays revealed AP-1 binding to the consensus site in the OPN promoter after LPS stimulation in macrophages. The importance of this AP-1 site in LPS-induced OPN promoter activity was further demonstrated by a complete loss of inducible OPN promoter activity after site-directed mutagenesis of this AP-1 site. The transcription factor AP-1 consists of a variety of dimers composed of members of the Jun and Fos families of proteins (28). The Jun proteins can both homo- and heterodimerize with Fos members to form transcriptionally active complexes. Consistently, c-Jun and c-Fos were found to bind to OPN promoter in CHIP assays, and RNAi knockdown of c-Jun and c-Fos may involved in TLR-induced OPN expression. We speculated that NO-independent and PI3K/JNK/ERK-dependent mechanism mediate early expression of OPN (1–8 h of stimulation), whereas NO-dependent mechanism can mediate late expression of OPN (8–24 h of stimulation). Consistent with this speculation, OPN transcription can be detected during a period to 24 h of LPS stimulation.

Prior to this work, we demonstrated a nitric oxide (NO)-dependent mechanism to control OPN transcription in LPS-stimulated macrophages through heterogeneous nuclear ribonucleoprotein proteins (hnRNPs) A/B and hnRNPs U proteins (16, 17). However, macrophage NO production was initially detected following 8 h of LPS treatment; peak NO production was noted after 12 h of stimulation (data not shown). We detected OPN transcription as early as 1 h following LPS simulation with the peak expression after 4 h of stimulation (Fig. 1A). In addition, inhibition of NO synthesis by pretreatment with L-NAME can’t ablate LPS-induced OPN expression (data not shown). Therefore, a NO-independent mechanism may be present to regulate LPS-induced OPN expression in macrophages.

TLR signaling through ligation with TLR agonists induces various kinases including IκB kinase (IκKs), mitogen-activated protein kinase (MAPK), and PI3K, resulting in the production of various proinflammatory mediators and type I interferon (18, 19). The transcription of proinflammatory mediators such as TNF-α and IL-6 is very rapid and transient after TLR stimulation. Therefore, a NO-independent mechanism may be involved in TLR-induced OPN expression. We found that treatment with the JNK specific inhibitor SP60012, ERK specific inhibitor PD98059, and PI3K specific inhibitor LY294002 significantly decreased OPN expression to a level equivalent to that of unstimulated controls. PI3K involvement in OPN expression has been shown in melanoma and breast cancer cells (26, 27). LPS-induced JNK and ERK activation can be inhibited by PI3K specific inhibitor LY294002, but not by JNK specific inhibitor SP60012, indicating LPS-induced OPN expression is controlled by sequential JNK and ERK activation, which are involved in LPS-induced OPN expression. We speculated that NO-independent and PI3K/JNK/ERK-dependent mechanism mediate early expression of OPN (1–8 h of stimulation), whereas NO-dependent mechanism can mediate late expression of OPN (8–24 h of stimulation). Consistent with this speculation, OPN transcription can be detected during a period to 24 h of LPS stimulation.

whether other TLR agonists can induce OPN expression are not well defined.

Previously, we demonstrated a nitric oxide (NO)-dependent mechanism to control OPN transcription in LPS-stimulated macrophages through heterogeneous nuclear ribonucleoprotein proteins (hnRNPs) A/B and hnRNPs U proteins (16,17). However, macrophage NO production was initially detected following 8 h of LPS treatment; peak NO production was noted after 12 h of stimulation (data not shown). We detected OPN transcription as early as 1 h following LPS simulation with the peak expression after 4 h of stimulation (Fig. 1A). In addition, inhibition of NO synthesis by pretreatment with L-NAME can’t ablate LPS-induced OPN expression (data not shown). Therefore, a NO-independent mechanism may be present to regulate LPS-induced OPN expression in macrophages.

TLR signaling through ligation with TLR agonists induces various kinases including IκB kinase (IκKs), mitogen-activated protein kinase (MAPK), and PI3K, resulting in the production of various proinflammatory mediators and type I interferon (18, 19). The transcription of proinflammatory mediators such as
expression ablated LPS-induced OPN expression. These data indicate that heterodimers composed of c-Jun and c-Fos can bind to OPN promoter and regulate OPN expression in LPS-stimulated macrophages.

Two forms of OPN have been identified. One is the full-length OPN with the signal peptide that targets OPN for secretion (sOPN), whereas another is the intracellular form of OPN lacking the OPN signal sequences by alternative translation (6). It was proposed that different immune cells show differential expression of the two forms of OPN. APCs including DCs and macrophages express high levels of iOPN but low levels of sOPN, whereas T cells display a reversed expression pattern (7).

In accordance with this proposal, we found that both forms of OPN are expressed in macrophages. Macrophages express a low level of secreted form of OPN (sOPN) constitutively. TLR signaling can further up-regulate OPN expression in macrophages and the main form of TLR-induced OPN is iOPN. The differential expression of sOPN and iOPN was further confirmed by measuring OPN secretion in blood and peritoneal fluid in a mouse model of endotoxemia by intraperitoneal injection of LPS. Differential expression of sOPN and iOPN in immune cells suggests sOPN and iOPN have different functions in the immune responses. Indeed, sOPN has been shown to interact with integrins and LCD44 to regulate cell migration (29), cell activation and apoptosis (32, 33), and proinflammatory cytokine production (4, 34). Whereas, iOPN has been shown to modulate cell motility (9, 35) and cytokine expression (10). It was found to increase IFN-α production (36). Both type I IFN and TLR signaling ablate iOPN expression in cDCs, we found that TLR-induced iOPN is a negative regulator for IFN-α production and subsequent cell activation in macrophages. This feedback regulation may be important to further increase iOPN expression through attenuation of the suppression effect of type I IFN in macrophages. The functions of iOPN especially the cross-regulation between iOPN and type I IFN in macrophages are currently being investigated.

In conclusion, our results demonstrate that TLR-induced OPN expression in macrophages requires TLR-induced PI3K, JNK, ERK and AP-1 activation. Macrophages express a low level of secreted form of OPN constitutively. TLR signaling can further up-regulate iOPN expression in macrophages and increased iOPN expression can attenuate IFN-β production in TLR-stimulated macrophages. Therefore, our findings delineate a new molecular mechanism involved in the transcriptional regulation of OPN and clarify the forms and functions of OPN produced by macrophages.

Acknowledgments—We thank Dr. David T. Denhardt and Dr. Hongbing Shu for the mouse OPN reporter plasmid and the ISRE reporter plasmid, respectively.

REFERENCES
1. Denhardt, D. T., Noda, M., O’Regan, A. W., Pavlin, D., and Berman, J. S. (2001) *J. Clin. Invest.* **107**, 1055–1061
2. Wai, P. Y., and Kuo, P. C. (2004) *J. Surg. Res.* **121**, 228–241
3. Chabas, D., Baranzini, S. E., Mitchell, D., Bernard, C. C., Rittling, S. R., Denhardt, D. T., Sobel, R. A., Lock, C., Karpuj, M., Pedotti, R., Heller, R., Oksenberg, J. R., and Steinman, L. (2001) *Science* **294**, 1731–1735
4. Ashkar, S., Weber, G. F., Panoutsakopoulou, V., Sanchirico, M. E., Jansson, M., Zawaideh, S., Rittling, S. R., Denhardt, D. T., Glimcher, M. J., and Cantor, H. (2000) *Science* **287**, 860–864
5. Wang, K. X., and Denhardt, D. T. (2008) *Cytokine Growth Factor Rev.* **19**, 333–345
6. Shinohara, M. L., Kim, H. J., Kim, J. H., Garcia, V. A., and Cantor, H. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 7235–7239
7. Cantor, H., and Shinohara, M. L. (2009) *Nat. Rev. Immunol.* **9**, 137–141
8. Weber, G. F., Ashkar, S., Glimcher, M. J., and Cantor, H. (1996) *Science* **271**, 509–512
9. Zhu, B., Suzuki, K., Goldberg, H. A., Rittling, S. R., Denhardt, D. T., McCulloch, C. A., and Sodek, J. (2004) *J. Cell. Physiol.* **198**, 155–167
10. Lin, Y. H., and Yang-Yen, H. F. (2001) *J. Biol. Chem.* **276**, 46024–46030
11. Philip, S., and Kundu, G. C. (2003) *J. Biol. Chem.* **278**, 14487–14497
12. Helluin, O., Chan, C., Vilaire, G., Mousa, S., DeGrado, W. F., and Bennett, J. S. (2000) *J. Biol. Chem.* **275**, 18337–18343
13. Weiss, J. M., Renkl, A. C., Maier, C. S., Kimmig, M., Liaw, L., Ahrens, T., Kon, S., Maeda, M., Hotta, H., Uede, T., and Simon, J. C. (2001) *J. Exp. Med.* **194**, 1219–1229
14. Shinohara, M. L., Lu, L., Bu, J., Werneck, M. B., Kobayashi, K. S., Glimcher, L. H., and Cantor, H. (2006) *Nat. Immunol.* **7**, 498–506
15. Shinohara, M. L., Kim, J. H., Garcia, V. A., and Cantor, H. (2008) *Immunity* **29**, 68–78
16. Gao, C., Guo, H., Wei, J., Mi, Z., Wai, P., and Kuo, P. C. (2004) *J. Biol. Chem.* **279**, 11236–11243
17. Gao, C., Guo, H., Mi, Z., Wai, P. Y., and Kuo, P. C. (2005) *J. Immunol.* **175**, 523–530
18. Kawai, T., and Akira, S. (2007) *Semin. Immunol.* **19**, 24–32
19. Liew, F. Y., Xu, D., Brint, E. K., and O’Neill, L. A. (2005) *Nat. Rev. Immunol.* **5**, 446–458
20. Mi, Z., Guo, H., Wai, P. Y., Gao, C., Wei, J., and Kuo, P. C. (2005) *J. Biol. Chem.* **279**, 46659–46667
21. Ogawa, D., Stone, J. F., Takata, Y., Blaschke, F., Chu, V. H., Towler, D. A., Law, R. E., Hsueh, W. A., and Bruemmer, D. (2005) *Circ. Res.* **96**, e59–67
22. Zhao, W., An, H., Zhou, J., Xu, H., Yu, Y., and Cao, X. (2007) *Immunol. Lett.* **108**, 137–142
23. Shinohara, M. L., Jansson, M., Hwang, E. S., Glimcher, L. H., and Cantor, H. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 17101–17106
24. Fukao, T., Tanabe, M., Terauchi, Y., Ota, T., Matsuda, S., Asano, T., Kadokawa, T., Takeuchi, T., and Koyasu, S. (2002) *Nat. Immunol.* **3**, 875–881
25. Rangaswami, H., Bulbule, A., and Kundu, G. C. (2006) *Trends Cell Biol.* **16**, 79–87
26. Packer, L., Pavey, S., Parker, A., Stark, M., Johansson, P., Clarke, B., Pollock, P., Ringner, M., and Hayward, N. (2006) *Carcinogenesis* **27**, 1778–1786
27. An, H., Xu, H., Zhang, M., Zhou, J., Feng, T., Qian, C., Qi, R., and Cao, X. (2005) *Blood* **105**, 4685–4692
28. Rochum, W., Passegué, E., and Wagner, E. F. (2001) *Oncogene.* **20**, 2401–2412
29. Weber, G. F., Zawaideh, S., Hikita, S., Kumar, V. A., Cantor, H., and Ashkar, S. (2002) *J. Leukoc. Biol.* **72**, 752–761
30. Yu, Y., Wang, M. I., Sudhir, P. C., Chen, G. D., Chi, C. W., and Chen, J. Y. (2007) *Cancer Res.* **67**, 2734–2742
31. Hur, E. M., Chung, H. S., Chan, C., Chiu, Y. S., Yoon, J. J., and Yang-Yen, H. F. (2002) *J. Biol. Chem.* **277**, 2734–2742
32. Khan, S. A., Lopez-Chua, C. A., Zhang, J., Fisher, L. W., Sørensen, E. S., and Denhardt, D. T. (2002) *J. Cell. Biochem.* **85**, 728–736
33. Renkl, A. C., Wussler, J., Ahrens, T., Kon, S., Uede, T., Martin, S. F., Simon, J. C., and Weiss, J. M. (2005) *Blood* **106**, 946–955
34. Zohar, R., Suzuki, N., Suzuki, K., Arora, P., Glogauer, M., McCulloch, C. A., and Sodek, J. (2000) *J. Cell. Physiol.* **184**, 118–130