Inducible binding of PU.1 and interacting proteins to the Toll-like receptor 4 promoter during endotoxemia

Tetyana V. Pedchenko, Gye Young Park, Myungsoo Joo, Timothy S. Blackwell, and John W. Christman

Division of Allergy, Department of Medicine, Pulmonary and Critical Care Medicine, Vanderbilt University Medical Center; and Department of Veterans Affairs, Nashville, Tennessee

Submitted 24 January 2005; accepted in final form 5 May 2005

Pedchenko, Tetyana V., Gye Young Park, Myungsoo Joo, Timothy S. Blackwell, and John W. Christman. Inducible binding of PU.1 and interacting proteins to the Toll-like receptor 4 promoter during endotoxemia. Am J Physiol Lung Cell Mol Physiol 289: L429–L437, 2005; doi:10.1152/ajplung.00046.2005.—We hypothesized that PU.1 and PU.1 interacting proteins (PIP) binding to the Toll-like receptor 4 (TLR4) promoter is involved in endotoxin-induced upregulation of TLR4 gene expression. Our results employing chromatin immunoprecipitation assays indicate that PU.1 binds to the murine TLR4 promoter both in macrophage cells and, most importantly, in whole lung tissue. Treatment of RAW 264.7 cells with endotoxin induced the association of PU.1 and the TLR4 promoter in a time-dependent manner, and this was closely tied to interactions between the TLR4 promoter and the PIP interferon regulatory factors (IRF)4 and IRF8. PU.1 binding was related to increases in steady-state TLR4 mRNA and total TLR4 protein in RAW cells. Endotoxemia in animals caused the similar inducible interaction between PU.1 and IRF4 and the TLR4 promoter in lung tissue of mice that was treated with a single intraperitoneal injection of endotoxin. PU.1 binding to the TLR4 promoter was not enhanced in the lung tissue of endotoxin-resistant C3H/HeJ mice in response to endotoxemia. Transient transfection studies in RAW cells indicate that inducible binding of PU.1 to the TLR4 promoter is abrogated by a Ser148 to Ala mutation in PU.1. These data suggest that induction of PU.1/PIP binding to the TLR4 promoter is involved in endotoxin response in vivo and may mediate transcriptional changes in TLR4 gene expression.

PU.1 interacting proteins

BACTERIAL LPS or endotoxin, the major structural component of the outer wall of gram-negative bacteria, is a potent activator of macrophages. Recognition of gram-negative endotoxin by the Toll-like receptor 4 (TLR4) complex leads to rapid secretion of inflammatory cytokines and chemokines that mediate the pathophysiology of severe sepsis and the acute respiratory distress syndrome. TLR4 is a pattern recognition molecule on the surface of macrophages that is essential, but not entirely sufficient, for mediation of the endotoxin response (5). Several laboratories have shown that mice that lack functional TLR4 are highly resistant to endotoxin (16, 37, 49). Monocytes/macrophages are intrinsically more responsive to endotoxin than neutrophils, basophils, or eosinophils (43) due to abundant expression of TLR4. It is possible that modulation of TLR4 gene expression in pulmonary macrophages is a critical determinant of the intensity and/or duration of the response to endotoxin.

Both human and mouse TLR4 gene contain multiple purine-rich sequence motifs that are recognized by transcription factors of the Ets family, including myeloid-specific factor PU.1. Several lines of evidence suggest that PU.1 is critical for both macrophage maturation and transcriptional regulation of TLR4 in macrophages. Mice homozygous for a disruption in the PU.1 DNA binding domain prematurely die of severe sepsis because of loss of myeloid lineages (24, 25). Although PU.1 is involved in multiple myeloid lineages, including macrophages, B-lymphocytes, and neutrophils, only high levels promote differentiation to fully competent mature macrophages (8). A recent in vitro study that employed an EMSA in RAW cells indicates that PU.1 protein binds to a cognate sequence in the TLR4 promoter, and mutation of this site impairs expression of a promoter-reporter construct (40). Finally, granulocyte/macrophage colony-stimulating factor (GM-CSF) null macrophages that lack detectable TLR4 are induced to express abundant TLR4 when transfected with PU.1 (46), indicating that induction of PU.1 may mediate the effects of GM-CSF on mature macrophage function.

PU.1 is involved in combinatorial regulation of gene expression through interaction with coregulatory partners. Two of them, Pip/interferon regulatory factor (IRF)4 and interferon consensus sequence-binding protein (ICSBP)/IRF8, are members of the IRF family, which are specifically expressed in immune cells. Both factors could be recruited to a composite Ets/IRF DNA binding site in target genes through interaction with PU.1 (7, 10, 23, 26). Functional studies show that phosphorylation of PU.1 at serine/threonine residues can enhance PU.1-dependent transcription in endotoxin-stimulated cells by promoting interaction between PU.1 and PU.1 interacting proteins (PIP). Formation of a PU.1/PIP transcriptional complex is dependent on conformational changes in the PEST domain of PU.1 that is mediated by serine phosphorylation (34, 40). PU.1 is potentially phosphorylated on five separate serine residues (Ser41, 45, 132, 133, and 148) that are within consensus sites for casein kinase II (CKII); however, phosphorylation of Ser148 appears to regulate transaction (21) and is necessary for maximal expression of the human TLR4 promoter. Based on this literature, we hypothesized that PU.1/PIP binding to the TLR4 promoter regulates TLR4 gene and protein expression in the pulmonary macrophages during endotoxemia. In these studies, we employed a novel chromatin immunoprecipitation (ChiP) assay to investigate PU.1/PIP interactions with the murine TLR4 promoter in RAW cells and in whole lung tissue in response to treatment with endotoxin.

MATERIALS AND METHODS

Cell culture. A murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was maintained in...
DMEM (Cellgro) containing 10% FBS (Hyclone), penicillin-streptomycin (Invitrogen), and 2 mM glutamine (Sigma).

Bone marrow-derived macrophages. Bone marrow was collected from the femurs of mice ranging from 8 to 16 wk of age with a 25-gauge needle and unlabelled DMEM. Cells were then centrifuged and suspended in a macrophage differentiation medium. This medium consisted of DMEM, 10% heat-inactivated FBS, 4 mM d-glutamine (GIBCO, Grand Island, NY), 100 units of penicillin (GIBCO)/ml, 0.1 mg of streptomycin (GIBCO)/ml, and 10% conditioned medium from L929 mouse fibroblasts (conditioned L929 medium contains GM-CSF, necessary for macrophage differentiation and maturation). After 5–7 days, macrophage precursors divide and differentiate into mature macrophages. Cells were cultured for 7 days at 37°C and 5% CO2. After being incubated overnight with medium that contained only 1% FBS, cells were treated with endotoxin (1 μg/ml of highly purified LPS from Escherichia coli, serotype R515, Alexis Biochemicals).

After treatment, cells were used in ChIP assay as described further.

Animal model. Male and female C57Bl/6, C3H/HeJ and BALB/c mice weighing 20–28 g were used for experiments. Highly purified endotoxin was given as a single intraperitoneal injection of 2 μg/g in normal saline. Control animals were injected with saline instead of endotoxin. Mice were killed by CO2 asphyxiation at different times after being incubated overnight with medium that contained only 1% FBS, cells were treated with endotoxin (1 μg/ml of highly purified endotoxin for different periods of time).

Bone marrow was collected from the femurs of mice ranging from 8 to 16 wk of age with a 25-gauge needle and unlabelled DMEM. Cells were then centrifuged and suspended in a macrophage differentiation medium. This medium consisted of DMEM, 10% heat-inactivated FBS, 4 mM d-glutamine (GIBCO, Grand Island, NY), 100 units of penicillin (GIBCO)/ml, 0.1 mg of streptomycin (GIBCO)/ml, and 10% conditioned medium from L929 mouse fibroblasts (conditioned L929 medium contains GM-CSF, necessary for macrophage differentiation and maturation). After 5–7 days, macrophage precursors divide and differentiate into mature macrophages. Cells were cultured for 7 days at 37°C and 5% CO2. After being incubated overnight with medium that contained only 1% FBS, cells were treated with endotoxin (1 μg/ml of highly purified LPS from Escherichia coli, serotype R515, Alexis Biochemicals).

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Flow cytometric analysis. RAW 264.7 cells (~10^6/sample) were incubated in serum-depleted medium overnight and stimulated with 1 μg/ml of highly purified endotoxin for the different periods of time. Cells were washed twice with cold 0.1% Na2-PBS and harvested. After being suspended in 1 ml of cold 0.1% Na2-PBS, each sample was divided into two 500-μl aliquots. One aliquot was used to detect the cell surface expression of TLR4 and the other one to detect total cellular expression. To evaluate the cell surface TLR4/MD-2 expression, cells were suspended in 250 μl of 1% FBS-0.1% Na2-PBS and incubated with 2.5 μl of TLR4/MD-2 phycoerythrin-conjugated antibody (MDS510, Santa Cruz) on ice for 45 min. To detect total cellular expression of TLR4/MD-2 complex, samples were fixed with 1% paraformaldehyde-PBS and permeabilized with 0.1% saponin (wt/vol)-1% FBS-0.1% Na2-PBS buffer. After being incubated with antibody, cells were washed and resuspended in 1% paraformaldehyde-0.1% NaN3-PBS. Cells incubated with an isotype-matched irrelevant antibody (mouse IgG2a) were used as a control. Staining with 7-amino-actinomycin D (cat. no. A-1310, Molecular Probes) was performed to exclude dead cells. Thereafter, cells were subjected to flow cytometric analysis on a BD-FACScan analyzer (BD Biosciences).

Western blotting for TLR4 in membrane fraction of RAW cells. Cells were incubated in serum-depleted medium overnight and treated with 1 μg/ml of LPS for 2 h. Cells were washed with cold PBS and lysed in lysis buffer (20 mM Tris, 5 mM EDTA, protease inhibitors cocktail added) by freezing-thawing (3 cycles) using dry ice. Sonication, total cell lysate was ultracentrifuged at 100,000 g for 1 h at 4°C. The supernatant was collected as cytosol fraction. The pellet was resuspended in lysis buffer with 1% Nonidet P-40 added. Supernatant was collected as a membrane fraction after additional centrifugation at 100,000 g for 1 h at 4°C. Proteins of membrane fraction were separated by SDS-PAGE and were transferred to polyvinylidene difluoride membrane. The membrane was probed with anti-TLR4 antibody (H-80, Santa Cruz). Protein detection was carried out using ECL system.

ChIP. ChIP was performed by using a modified protocol from Upstate Biotechnology (Lake Placid, NY). Cells were grown to 60–70% confluence in 10-cm dishes. Cells were then cultured in the serum-depleted media overnight before endotoxin stimulation. Proteins were cross-linked to DNA by adding the formaldehyde directly to culture medium to a final concentration of 1% for 10 min. After being rinsed three times with ice-cold PBS, the cells were resuspended in SDS-lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors). Sonication of cell lysate was performed three times for 12 s each, followed by centrifugation at 4°C for 10 min. Supernatants were collected and diluted 1:10 with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 0.1% Triton X-100, and protease inhibitors). A portion of diluted supernatant (1%) was kept to estimate the amount of DNA present in different samples. It is referred to as “input” sample. After preclearing the extracts with 60 μl of salmon sperm DNA-saturated protein A- or G-Sepharose beads 50% slurry (Zymed, San Francisco, CA) for 30 min at 4°C, the immunoprecipitation was carried out overnight at 4°C with 2–5 μg of specific antibody. The antibodies used were: rabbit-polyclonal α-Pu.1 (Santa Cruz, CA), goat polyclonal α-IRF4/IRFp, α-IRF8/ICSBP (Santa Cruz, CA), mouse M2 α-FLAG (Sigma), or irrelevant IgG (Santa Cruz, CA). After immunoprecipitation, 40 μl of salmon sperm DNA-saturated protein A- or G-Sepharose beads 50% slurry were added, and samples were incubated for 1 h followed by brief centrifugation.

PCR primers were produced for 398 and 352 bp, respectively.
at 72°C for 7 min. PCR for the input was performed with five times less template amount. The PCR products were analyzed on 1% agarose gel or 8% polyacrylamide gel. Primers for the promoter region of the mouse TLR4 gene were 5'-CAG AAG ACA CGGCAA CTG AT-3' (sense) and 5'-TCG CAG GAG GGA AGT TAG AA-3' (antisense). This primer set flanks the TLR4 promoter sequence region -359 to -35 containing putative PU.1 binding sites (40, 41) and is predicted to yield a 325-bp PCR product.

**Lung ChIP assay.** The lungs were perfused with 15 ml of PBS through the heart with incision in the left ventricle. The tissue was then fixed with 2 ml of 2% formaldehyde delivered to the lung intratracheally. The lungs were dissected and incubated in 1% formaldehyde for ~10 min. After that, the lungs were harvested and transferred into cold PBS solution. After being washed in PBS, the lung tissue (~60–80 mg) was homogenized with 1 ml of SDS lysis buffer (with protease inhibitors cocktail) using Polytron homogenizer. The lysate was incubated on ice for 20 min and sonicated (12 s × 4 times). Thereafter, the ChIP assay was performed as described for the cells.

**Coimmunoprecipitation of PU.1 and IRF4.** After the PU.1 immunoprecipitation step in ChIP assay described above, coimmunoprecipitated protein was analyzed by IRF4 immunoblotting. Samples (A/antibody/proteins/DNA complex) were boiled for 10 min with Laemmli buffer, resolved on 10% gel, and an immunoblot procedure was performed using anti-IRF4 antibody (M-17).

**Statistical analysis.** For comparison among groups, unpaired t-tests and unpaired ANOVA tests were used (with the assistance of InStat; GraphPad Software, San Diego, CA). *P* values < 0.05 were considered significant.

**RESULTS**

**TLR4 mRNA and protein expression in macrophages in response to treatment with endotoxin.** Initially, we determined the time course for the expression of steady-state TLR4 mRNA in RAW 264.7, a macrophage-like cell line, in response to in vitro treatment with endotoxin. TLR4 mRNA was detected by RT-PCR and normalized to HPRT mRNA expression. As shown in Fig. 1A, treatment of RAW cells with endotoxin resulted in a substantial increase in steady-state TLR4 mRNA between 2 and 4 h. Increased TLR4 mRNA was detected as early as 30 min of endotoxin treatment, increased until 4 h, and decreased steadily before approaching preinduction level at 24 h following stimulation.

We next determined whether the TLR4 mRNA expression profile in RAW cells treated with LPS reflects response of pulmonary macrophages to endotoxemia in vivo. Mice were injected intraperitoneally with endotoxin, and TLR4 mRNA was measured in bronchoalveolar lavage cells collected from mice at 4 and 24 h following the injection (Fig. 1B). Expression of mRNA was increased by 4 h in adherent bronchoalveolar lavage cells, and it was decreased by 24 h following treatment with endotoxin.

To investigate TLR4 protein production, first we have showed the decrease of TLR4 protein level in the RAW cell membrane fraction after 2 h of treatment with LPS (Fig. 2A). Then we cultured RAW cells with endotoxin for various periods of time and evaluated both cell surface and total cell expression of TLR4/MD-2 complex using fluorescent antibody and fluorescence-activated cell sorting (FACS) analysis. The TLR4/MD-2 antibody was selected because a specific murine TLR4 antibody could be obtained that measures the functional protein complex capable to bind LPS. Our results demonstrate a time-dependent decrease of TLR4/MD-2 expression on the RAW cell surface in response to treatment with endotoxin, with a return to the untreated control level by 8 h (Fig. 2A).

In contrast, FACS analysis of permeabilized cells showed upregulation of total antigenic TLR4/MD-2 (Fig. 2B), which remains elevated at the 8-h time point. Presumably, the difference between intracellular pool and presented at cell surface pool of...
TLR4/MD-2 is the result of TLR4 internalization in response to interaction with endotoxin. We interpret these data as indicating that there is a small increase in total antigenic TLR4/MD-2 in macrophages in response to treatment with endotoxin that is not available on the cell surface.

Endotoxin stimulation results in an inducible interaction between PU.1 and TLR4 promoter in vivo. Because our initial data indicated that endotoxin resulted in an increase in both TLR4 mRNA and TLR4/MD-2 protein complex production in RAW cells, we employed a novel ChIP to evaluate an interaction between PU.1 and the TLR4 promoter. RAW cells were treated with endotoxin for various lengths of time and fixed with formaldehyde to cross-link protein to DNA to determine binding of specific transcription factors to the TLR4 promoter that regulate gene expression. A ChIP assay was performed, using α-Pu.1 antibody for DNA-protein complex immunoprecipitation and the primers, flanking both potential Pu.1 binding sites on murine TLR4 promoter, for PCR amplification. Immunoprecipitation with an irrelevant immunoglobulin, instead of specific antibody, was used as a control for the assay specificity. A weak band was detected initially indicating a basal interaction between Pu.1 and TLR4 promoter in the absence of stimulation (Fig. 3A). However, there was a marked increase in the interaction between Pu.1 and the TLR4 promoter, and this was sustained at 4 h.

To reveal changes in binding of Pu.1 to the TLR4 promoter that result from endotoxemia, we adapted the ChIP assay to determine the interaction between Pu.1 and the TLR4 promoter in whole lung tissue. Mice were injected with 2 μg/g of endotoxin intraperitoneally, and the protein-DNA cross-link in the lung tissue was performed by intratracheal formaldehyde delivery to the lung at different periods of time following injection. Lung was harvested, and whole lung homogenate was processed further similar to cell lysate before immunoprecipitation with α-Pu.1 and detection of the TLR4 promoter by PCR. These data show (Fig. 3B) that there is slight binding of Pu.1 to the TLR4 promoter in the lung tissue of untreated animals. Similar to the finding in RAW cells, endotoxin treatment markedly induced Pu.1 binding activity in a time-dependent manner. The maximum activation was observed at 2 h. By 16 h following endotoxin injection, binding activity returned to basal level. Although this assay is done with whole lung tissue, the interaction between Pu.1 and the TLR4 promoter is specific for Pu.1 containing cells that in lung are presumed to be macrophages.

PU.1 interaction with TLR4 promoter is a part of lung response to endotoxin. Data shown in Fig. 3 indicate that the interaction between Pu.1 and the TLR4 promoter was induced in a time-dependent manner following treatment with endotoxin. An inducible interaction between Pu.1 and the TLR4
promoter could have a physiological role in regulating TLR4 gene expression during endotoxemia. To confirm that this interaction between PU.1 and the TLR4 promoter is induced by treatment with endotoxin, we examined PU.1 binding to the TLR4 promoter in whole lung tissue from C3H/HeJ mice treated with endotoxin. These mice are known to carry the point mutation (proline to histidine at position 712) in the intracellular domain of TLR4 receptor, which makes them unresponsive to endotoxin (16, 34). We employed the lung tissue ChIP assay to investigate the interaction of PU.1 and TLR4 promoter in this animal model. Before this experiment, we demonstrated by Western blot analysis that TLR4 mutation does not affect total PU.1 protein level (data not shown). Next, we performed ChIP analysis on a whole lung from both wild-type and TLR4 mutant mice (Fig. 4). PU.1 binding to TLR4 promoter was assessed at 2 h after endotoxin stimulation since this was shown to be a peak of activation in previous experiments (Fig. 3B). This ChIP assay did not detect a change in the interaction between PU.1 and the TLR4 promoter in the lung tissue from the C3H/HeJ mice in response to treatment with endotoxin. In contrast, there was a substantial increase in the interaction between PU.1 and the TLR4 promoter in the lung tissue of the wild-type mice. These data support the conclusion that the interaction between PU.1 and the TLR4 promoter is a part of response to treatment with endotoxin in vivo.

PU.1 phosphorylation at Ser148 is critical for the interaction with TLR4 promoter. The activation of PU.1 in response to endotoxin is presumed to be regulated at the posttranslational level, since there were no changes in PU.1 protein levels in response to treatment with endotoxin (data not shown). Stimulation of RAW 264.7 cells with endotoxin has been shown to induce PU.1 phosphorylation at Ser148 through the induction of CKII, resulting in enhanced transactivation function (21). Based on these data, we expected that endotoxin could increase TLR4 gene expression by inducing phosphorylation of Ser148, and, therefore, PU.1 activity. To address this possibility, RAW cells were transiently transfected with plasmids expressing either mutant PU.1 S148A, where Ser148 is replaced with alanine preventing phosphorylation, or wild-type PU.1. To distinguish between transfected and endogenous PU.1, constructs were FLAG-tagged. Equivalent efficiency of mutant and wild-type PU.1 transfection was confirmed by Western blotting analysis using anti-FLAG antibody M2 showing equal expression of the transgene (data not shown). Next, transfected RAW cells were treated with endotoxin for 2 h, and ChIP assay was performed using M2 anti-FLAG antibody for immunoprecipitation. As shown in Fig. 5, lack of Ser148 phosphorylation abolished endotoxin-inducing PU.1 binding to TLR4 promoter. This finding suggests that endotoxin induces a PU.1 posttranslational modification related to phosphorylation of Ser148 that is
IRF4 is involved in TLR4 transcriptional regulation in vivo during endotoxemia. There is growing information indicating the importance of PU.1 phosphorylation for its interaction with PIP, especially with members of the IRF family. It has been shown that short region of TLR4 promoter (only 75 bp) was sufficient in transient transfection to induce maximal luciferase activity in THP-1 cells, and it contains composite interferon response factor/Ets motif (40). Both the human and murine TLR4 promoters contain composite IRF/PU.1 binding motif (40). For the murine TLR4 promoter disrupting the PU.1-binding sites by site-directed mutagenesis completely abolished (distal site) or strongly reduced (proximal site) basal luciferase activity (41). All these data, together with our finding about the important role of Ser148 phosphorylation for PU.1 interaction with endogenous TLR4 gene promoter, prompted us to address two questions: does IRF4 bind to the murine TLR4 promoter in vivo and, if so, is it involved in endotoxin signaling? To test the ability of IRF4 to bind elements within TLR4 promoter, we used lung tissue ChIP assay. Mice were treated intraperitoneally with 2 μg/g of endotoxin for indicated periods of time, and after formaldehyde fixation, lungs were harvested from control and treated animals and then subjected to ChIP analysis. The immunoprecipitation of protein-DNA complex was done with anti-IRF4 antibody. PCR amplification was done with the same set of primers used for PU.1 ChIP assay because they also flank the possible PU.1/IRF composite sites. These data show that there is an inducible interaction between IRF4 and the TLR4 promoter, with peak activity at 2 h following endotoxin injection, returning to the basal, almost undetectable level, at 24 h (Fig. 6).

PU.1 can also interact with another member of the IRF family, IRF8 (ICSBP), at IRF/PU.1 composite element (9, 11, 22, 23, 26). IRF4 has been reported to interact physically with members of the IRF family, IRF8 (ICSBP), at IRF/PU.1 composite element (9, 11, 22, 23, 26). IRF4 has been reported to interact physically with IRF8/ICSBP. To examine the possibility of formation of heterocomplex for TLR4 transcriptional regulation in macrophages during endotoxemia, we assessed binding of all three factors, PU.1, IRF4, and IRF8, to the TLR4 promoter in bone marrow-derived macrophages. Formaldehyde cross-linking was performed on cells treated with endotoxin for 2 h or were left untreated. Nuclear extract from each sample was divided in three aliquots and subjected to ChIP assay with either PU.1 or IRF4 or IRF8 immunoprecipitation followed by PCR amplification of TLR4 promoter sequence containing PU.1/IRF composite sites. The inducible binding activity was demonstrated for all three factors (Fig. 7), which suggests that endotoxin signaling involves formation of complex of PU.1/IRF4/IRF8 for TLR4 transcriptional regulation in macrophages. The possibility of PU.1/IRF complex formation on the TLR4 promoter in vivo was supported by demonstrating PU.1 and IRF4 immunoprecipitation from bone marrow-derived cells and from whole lung tissue of animals treated with LPS. After PU.1 immunoprecipitation step in ChIP assay, the samples were subjected to Western blot analysis with IRF4 antibody (Fig. 8).

DISCUSSION

We found that treatment with endotoxin accentuates PU.1 binding to the endogenous TLR4 promoter in vivo in a time-dependent manner in RAW cells, and this is associated with...
increases in steady-state TLR4 mRNA and in TLR4 protein production. We also found that two members of IRF family, IRF-4/Pip and IRF-8/ICSBP, interact with PU.1 and the TLR4 promoter in response to treatment with endotoxin.

Reports of TLR4 expression during endotoxemia are contradictory, and our results are both consistent with and in contrast to some reports. For example, endotoxin increases TLR4 mRNA expression in human monocytes and polymorphonuclear leukocytes (27). In recovered murine alveolar macrophages, endotoxin has been shown to decrease TLR4 mRNA expression (13), but no change in TLR4 mRNA expression was reported for alveolar macrophage cell line MH-S (33). The level of TLR4 mRNA in the mouse whole lung has been shown to remain unchanged after intranasal instillation of LPS in one report (33), but it was decreased after intratracheal administration of endotoxin through a mechanism that involves reduced mRNA stability according to another report (13). There are also reports that endotoxin treatment reduces the expression of TLR4 mRNA in RAW 264.7 cells (35, 50).

Previous studies have shown that expression of TLR4 receptor mRNA is not closely related to protein levels (29). After showing, by Western blot analysis, decreased TLR4 protein expression in membrane fraction of RAW cells during LPS treatment, we employed flow cytometry using an antibody against the TLR4/MD-2 protein complex. TLR4 binds endotoxin only in contact with the accessory protein MD-2. In the absence of MD-2, TLR4 is retained in Golgi apparatus (20, 28, 47), and MD-2 is required for TLR4 glycosylation at Asn526 and Asn575 and expression on the cell surface (30, 38). Thus FACS analysis with antibody against TLR4/MD-2 allows evaluation of the biologically active TLR4/MD-2 protein complex. Our data are consistent with other reports that there is down-regulation of TLR4/MD-2 on the cell surface of RAW cells that are treated with endotoxin (1, 29). In contrast to expression of antigenic TLR4/MD-2 on the cell surface, we observed a time-dependent increase in total cellular TLR4/MD-2 expression in RAW cells in response to treatment with endotoxin.

The TLR4 gene has a typical myeloid type promoter, and full human TLR4 promoter activity is retained by a 75-bp proximal TLR4 promoter construct that contains four putative protein binding sites, an inner and outer PU.1 site that flanks an octamer motif and an interferon factor binding site (40). PU.1 has been shown to be involved in murine macrophage expression of TLR4 (19, 41). The investigation of the role of PU.1 in gene expression has been mostly carried out by employing an EMSA and transient transfection assay using promoter-reporter constructs. In contrast to EMSA, which examines the interaction between the extracted protein and double-stranded DNA oligonucleotides, ChIP allows investigation of specific interactions between transcriptional factors and cellular DNA in the context of cells or tissue. We have employed ChIP assay to explore the interaction between endogenous PU.1, interacting proteins, and the TLR4 promoter in vivo to capture molecular events during endotoxemia. To elucidate whether it reflects the real events occurring in whole organism, we have developed a novel implication of ChIP assay in an animal model. We observed that treatment with endotoxin results in an increase in PU.1 binding to the TLR4 promoter in RAW cells and in lung tissue. Because PU.1 is predominantly located in macrophages, our results that employ the ChIP assay in whole lung tissue are likely reflective of an association of PU.1 with TLR4 promoter in lung macrophages, although we cannot exclude these data may also reflect an interaction in B-lymphocytes since small numbers may also be present in lung tissue.

The interaction between PU.1 and the TLR4 promoter was enhanced by treatment with endotoxin. When the endotoxin signaling through TLR4 receptor was impaired in C3H/HeJ mice), there was no enhancement of PU.1 binding in response to treatment with endotoxin. PU.1 is potentially phosphorylated on five serine residues (41, 45, 132, 133, and 148) that are within consensus sites for CKII. Treatment of the RAW macrophages with endotoxin induces phosphorylation of Ser148 through induction of CKII, resulting in enhanced transactivating activity (21). Although it would be expected from these data that endotoxin could increase TLR4 gene expression by inducing phosphorylation of Ser148 and enhancement of PU.1 trans-activation function, this has not yet been reported. After performing transient transfection with plasmid vectors that express either wild-type or mutant PU.1 (Ser148 is replaced by alanine), we subjected RAW cell lysates to ChIP assay. The prevention of Ser148 phosphorylation abrogated PU.1 binding activity in endotoxin-stimulated macrophages. Therefore, we showed that phosphorylation of PU.1 is involved in the process of recruitment to endogenous TLR promoter by endotoxin stimulation.

Functional studies showed that phosphorylation of PU.1 can enhance PU.1-dependent transcription in endotoxin-stimulated cells, presumably by promoting interaction between PU.1 and other transcriptional regulatory factors (17, 21). Furthermore, phosphorylation of Ser148 in PU.1 PEST domain is known to be important for physical interaction with IRF4, also called Pip, an essential cofactor in B cells (10, 32, 36) and macrophages (22, 42). IRF was recognized as a part of ternary complex between PU.1 and a domain of the Ig light chain enhancer (34). PU.1 and IRF4 can bind as a complex to a composite PU.1/IRF4 DNA-binding element, which has been identified in a number of genes (9, 11, 15, 22, 31, 36). EMSA analysis revealed that murine macrophages contained both IRF4/PU.1 and ICSBP/PU.1 complexes (22). PU.1 and ICSBP, also known as IRF8, were reported to participate in the basal regulation of human TLR4 in myeloid cells (40).

Our data show that endotoxin stimulation recruits endogenous IRF4 (Pip) to TLR4 promoter in murine lung in a time-dependent manner very similar to PU.1 binding profile. The difference is that a basal binding activity observed with ChIP for PU.1 was almost undetectable in the case of IRF4. We demonstrated by coinmunoprecipitation the interaction between PU.1 and IRF4 in both bone marrow-derived cells and whole lung tissue during endotoxemia. This finding is in agreement with a previously suggested mechanism of PU.1/IRF interaction: IRF4 (Pip) and IRF-8 (ICSBP) bind very weakly to DNA containing an IRF site but can be recruited via interactions with other transcriptional factors, such as PU.1 and Spi-B, to the composite Ets/IRF element (7, 10, 12, 35). Our data indicate that IRF8 (ICSBP) is also involved in formation of PU.1/IRF transcriptional complex on TLR4 promoter in bone marrow-derived macrophages.

In conclusion, our data indicate that the macrophage response to endotoxin involves activation of PU.1 binding to the TLR4 promoter followed by modulation of both TLR4 mRNA levels and protein expression. Endotoxin stimulation of macrophages results in phosphorylation of PU.1 followed by in-
interaction with IRF4 via their PEST domains and thus enhances the transcriptional activation of TLR4 promoter. TLR4 transcriptional regulation in murine lung during endotoxins involves formation of heterocomplex between PU.1 and IRF family members. The lung ChIP assay developed in our laboratory is a unique tool to reveal the real-time interactions in an animal model between transcriptional factors and regulated gene promoters.

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