

## LPS activation of Toll-like receptor 4 signals CD11b/CD18 expression in neutrophils

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**Zhou, Ximing, Xiao-Pei Gao, Jie Fan, Qinghui Liu, Khandaker N. Anwar, Randall S. Frey, and Asrar B. Malik.** LPS activation of Toll-like receptor 4 signals CD11b/CD18 expression in neutrophils. *Am J Physiol Lung Cell Mol Physiol* 288: L655–L662, 2005. First published November 24, 2004; doi:10.1152/ajplung.00327.2004.—We identify herein a novel signaling function of the Toll-like receptor-4 (TLR4), the lipopolysaccharide (LPS) receptor mediating the innate immune response, in inducing the expression of CD11b/CD18 integrin in polymorphonuclear leukocytes (PMNs). Studies were made in PMNs isolated from TLR4-deficient (TLR4<sup>-/-</sup>) and C57BL/6 [wild-type (WT)] mice. We observed increased CD11b expression in WT PMNs within 3 h after LPS challenge, whereas CD11b was not expressed in TLR4<sup>-/-</sup> PMNs above basal levels. TLR4-activated CD11b expression was cycloheximide sensitive and involved the activation of transcription factors, NF- $\kappa$ B and c-Jun/PU.1. TLR4<sup>-/-</sup> PMNs challenged with LPS were functionally defective as the result of the impaired CD11b expression in that they failed to adhere and did not migrate across endothelial cells in response to N-formylmethionyl-leucyl-phenylalanine. TLR4 also promoted increased binding of LPS to PMNs on the basis of expression of CD11b. Thus TLR4 signaling activates synthesis and upregulation of CD11b and is essential for PMN adhesion and transmigration. Our data suggest an important role of TLR4-activated CD11b expression in the mechanism of the PMN host-defense response to LPS.

CD11b/CD18 integrin; Toll-like receptor; lipopolysaccharide; polymorphonuclear leukocyte adhesion

BACTERIA-INDUCED GENE EXPRESSION regulated through Toll-like receptors (TLRs) is an essential component of the host-defense response (15). TLR4 promotes the cross talk between polymorphonuclear leukocytes (PMNs) and endothelial cells that is dependent on PMN adhesion to the endothelium (7). Challenge of PMNs with LPS activates several mitogen-activated protein kinase (MAPK) signaling pathways, the extracellular signal-regulated kinase (ERK), c-Jun NH<sub>2</sub>-terminal kinase (JNK), and p38 mitogen-activated protein kinase, which in turn induce gene transcription (11). LPS also induces the activation of nuclear transcription factors, nuclear factor  $\kappa$ B (NF- $\kappa$ B), activator protein-1 (AP-1), PU.1, and interferon regulatory factor-1 in PMNs and lungs (3, 11, 18, 19). Although TLR4 is considered to be the primary receptor for lipopolysaccharide (LPS) (25), the question of how TLR4 regulates PMN adhesion and migration remains unclear. It is also not known whether TLR4 is capable of activating other LPS receptors such as the CD11b/CD18 integrin (47). In the present study, we addressed the possibility of an interaction between TLR4 and CD11b

such that TLR4 signaling plays an important role in the mechanism of CD11b expression.

PMN adhesion and transmigration to the site of microbial infection are essential for innate immunity (31). Recognition of LPS requires that LPS bind to the LPS-binding protein, which in turn induces the transfer of LPS to the membrane-bound CD14 receptor (38). TLR4 and CD11b/CD18 are the other key receptors that bind to LPS and activate the intracellular signaling pathways in PMNs (40). Activation of TLR4 by LPS, a member of IL-1/Toll receptor family, has been identified as being critical for the regulation of innate immune response (16, 48). CD11b is a member of  $\beta_2$ -integrin family, which also includes CD11a, CD11c, and CD11d. CD11b combines with the common subunit (CD18) to form the heterodimer, CD11b/CD18 complex, on the leukocyte surface (49). CD11b serves an important function both as an adhesion molecule and a receptor for LPS (10, 22, 37, 46). The adhesive functions of CD11a and CD11b appear to be distinct in that the binding of CD11a/CD18 to ICAM-1 expressed on the endothelium triggers the initial capture of PMNs by reducing PMN rolling velocity and causing PMN adhesion, whereas engagement of CD11b/CD18 to ICAM-1 promotes firm PMN adhesion and subsequent PMN transmigration (20, 22, 29, 51).

TLR4 activates the NF- $\kappa$ B-inducing kinase, a member of the mitogen-activated protein kinase kinase kinase family, and the pathway leading to activation of I $\kappa$ B kinases (IKK $\alpha$  and IKK $\beta$ ), which in turn phosphorylate I $\kappa$ B and promote its degradation (32). I $\kappa$ B degradation leads to the release of NF- $\kappa$ B subunit enabling NF- $\kappa$ B to translocate to the nucleus and initiate gene transcription (14). TLR4 signaling also leads to the activation of ERK and JNK signaling pathways (14). In addition, the transcription factor PU.1 is involved in the response to LPS in specific cell types such as macrophages (39) and may regulate signaling downstream of TLR4. Although PU.1 binding activity in nuclei is increased in response to LPS challenge (36, 45), the contribution of PU.1 in LPS-induced gene expression through TLR4 in PMNs is not well understood.

In the present study, we tested the hypothesis that TLR4 activates CD11b such that both TLR4 and CD11b may function to optimize antimicrobial defenses. Our data show that PMNs from TLR4-deficient (TLR4<sup>-/-</sup>) mice failed to induce CD11b synthesis and expression in response to LPS challenge, indicating the essential role of TLR4 in mediating CD11b expression. Because TLR4<sup>-/-</sup> PMNs did not express CD11b in response to LPS, these PMNs also failed to normally adhere

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and migrate across endothelial cells. We showed that CD11b expression induced by TLR4 was mediated by the activation of transcription factors, c-Jun/PU.1 and NF- $\kappa$ B.

## METHODS

**Animals.** TLR4<sup>-/-</sup> mice were provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan), and wild-type (WT) C57BL/6 mice (obtained from Jackson Laboratory, Bar Harbor, ME) were used as controls. Mice (weighing 22–26 g, 8–10 wk old) were housed in pathogen-free conditions with free access to food and water in the University of Illinois Animal Care Facility. Studies were made in accordance with institutional and National Institutes of Health guidelines after approval was obtained from the Institutional Review Board.

**Reagents and antibodies.** Antibodies against mouse CD11a, CD11b, and CD11c and horseradish peroxidase (HRP)-labeled secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FITC label kit and FITC-conjugated LPS (*Escherichia coli* serotype 055:B5) were purchased from Molecular Probes (Eugene, OR). LPS (serotype 0111:B4) used to challenge cells, N-formylmethionyl-leucyl-phenylalanine (fMLP), and cycloheximide were purchased from Sigma (St. Louis, MO). Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was purchased from Roche Applied Science (Indianapolis, IN). SuperScript First-Strand Synthesis System RT-PCR kit was purchased from Invitrogen (Carlsbad, CA). The inhibitors SP-600125 (for JNK), PP2 (for Src kinase), and UO126 (for MEK) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA), and IKK-Nemo binding domain peptide (NBD), to block NF- $\kappa$ B activation) was obtained from Dr. Sankar Ghosh (Yale University, New Haven, CT). The primer pair for CD11b was synthesized by Integrated DNA Technologies (Corallville, IA). GAPDH PCR primer pair was purchased from R&D Systems (Minneapolis, MN). T4 polynucleotide kinase was purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

**PMN isolation and flow cytometry.** PMNs were isolated from whole mouse blood as described (6, 43) using NIM-2 leukocyte isolation medium (Cardinal Associates, Santa Fe, NM). PMN purity was >96%. PMN cell surface expression of CD11a, CD11b, and CD11c was assessed by the flow cytometric assay. Briefly, TLR4<sup>-/-</sup> PMNs isolated from TLR4<sup>-/-</sup> mice and WT PMNs isolated from WT C57BL/6 mice were incubated with LPS (0.5  $\mu$ g/ml) in endothelial cell basal medium 2 (EBM-2) containing 10% FBS at 37°C for 1 h. Antibodies (1:500) against mouse CD11a, CD11b, or CD11c labeled with FITC were added to the PMN suspension and incubated for 30 min at 4°C. Matched FITC-conjugated isotype IgG was used as the control. We then immediately analyzed the samples by measuring fluorescence from the gated leukocyte population using the Coulter EPICS Elite ESP (Coulter, Miami, FL) with the laser set at 530 nm. The forward and side light scatter profiles were adjusted to gate for the PMN population. The flow cytometer was triggered by FITC fluorescence, and the fluorescence parameters were collected using four decade logarithmic amplification.

**PMN adhesion assay.** PMNs (1  $\times$  10<sup>4</sup>/well) were added to fibronectin (10  $\mu$ g/ml)-coated 96-well microplates and then incubated with LPS (0.5  $\mu$ g/ml) in EBM-2 containing 10% FBS or TNF- $\alpha$  (20 ng/ml) in the presence or absence of inhibitors or antibodies at 37°C for 30 min followed by washing with HBSS. To quantify PMN adhesion, myeloperoxidase activity was measured at 490 nm as described (27).

**Transendothelial PMN migration assay.** PMN transmigration was measured as described with modifications (41). In brief, Costar Transwells (polycarbonate filter, 3- $\mu$ m pore size) were coated with human pulmonary arterial endothelial cells for 36 h at 37°C in EBM-2 medium (EBM-2, 10% FBS). PMNs (2  $\times$  10<sup>6</sup>) added to the upper chamber were challenged with LPS (0.5  $\mu$ g/ml), and fMLP (10<sup>-7</sup> M), the chemotaxin, was added simultaneously to the bottom chamber. Control experiments were made in the absence of fMLP. Cells were

incubated at 37°C for 3 h, subsequently collected, and counted microscopically.

**LPS binding and PMN aggregation assays.** PMNs (2  $\times$  10<sup>6</sup>) were isolated from mouse whole blood, treated with the FITC-conjugated LPS (0.5  $\mu$ g/ml) at 37°C for 3 h, and then washed with culture medium. LPS binding to PMNs as well as PMN aggregation were determined by fluorescent microscopy. RT-PCR was performed to correlate CD11b mRNA expression with LPS binding and PMN aggregation.

**Western blotting.** PMNs were isolated from mouse blood and incubated with LPS (0.5  $\mu$ g/ml) or TNF- $\alpha$  (20 ng/ml) in EBM-2 containing 10% FBS for various times. Cell lysates were separated by 10% SDS-PAGE under nonreducing conditions. The separated proteins were electroblotted onto polyvinylidene difluoride membrane and blocked for 1 h at room temperature with Tris-buffered saline containing 1% BSA. The membranes were then probed with a 1:1,000 dilution of the purified polyclonal IgG against mouse CD11b at room temperature for 1 h. After being washed, CD11b was detected by HRP-conjugated secondary antibodies followed by ECL according to the manufacturer's instructions (Amersham, Arlington Heights, IL). Equal protein was loaded in each lane, and actin was used as a reference to normalize the CD11b protein.

**Reverse transcription and PCR.** Isolated mouse PMNs (4  $\times$  10<sup>6</sup>) were incubated with LPS (0.5  $\mu$ g/ml, 1 h, 37°C), cycloheximide (10  $\mu$ M, 30 min, 37°C), IKK-NBD peptide (100  $\mu$ M, 15 min, 37°C), UO126 (10  $\mu$ M, 15 min, 37°C), SP-600125 (100  $\mu$ M, 15 min, 37°C), and PP2 (0.05  $\mu$ M, 15 min, 37°C) in EBM-2 containing 10% FBS. Total RNA from PMN was extracted using the TRI reagent following the manufacturer's instructions. cDNA was synthesized through reverse transcription using the SuperScript Preamplification kit (Invitrogen) from total RNA. PCR was then performed using the cDNA as the template and oligonucleotides specific for CD11b (5'-AGC CCA AGA TCA CAT G-3' and 5'-TGC AGA AGC ATA ACC C-3') as primers. PCR products were then separated using 1.2% agarose gel and identified by ethidium bromide staining.

**Nuclear protein extraction.** Nuclear protein extracts were prepared from isolated mouse PMNs as described (4). PMNs (1  $\times$  10<sup>7</sup>) were homogenized in a Dounce tissue homogenizer with 4 ml of solution A (0.6% Nonidet P-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, and 0.5 mM PMSF). Debris was pelleted by centrifuging at 2,000 rpm for 30 s. The supernatant was incubated on ice for 5 min followed by centrifugation for 10 min at 5,000 rpm. Nuclear pellets were then resuspended in 300  $\mu$ l of solution B (25% glycerol, 20 mM HEPES, pH 7.9, 420 mM NaCl, 1.2 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 2 mM benzamidine, 5  $\mu$ g/ml pepstatin, 5  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin) and incubated on ice for 20 min. The mixture was then centrifuged at 14,000 rpm for 1 min. Supernatants containing nuclear proteins were stored at -70°C.

**Electrophoretic mobility shift assay.** The probes for electrophoretic mobility shift assay (EMSA) were a 21-bp double-stranded oligonucleotide containing the PU.1 consensus binding sequence (5'-ATG GGC TCA GGA AAC AAC TGA-3') and a 24-bp double-stranded construct of the NF- $\kappa$ B consensus binding sequence (5'-AGG-GACTTTCCGCTGGGACTTTCC-3'). [<sup>32</sup>P]ATP-labeled oligonucleotides were purified on a Sephadex G-50 M column (Pharmacia Biotech, Piscataway, NJ). An aliquot of 5  $\mu$ g of nuclear protein was incubated with the labeled double-stranded probe (~50,000 cpm) in the presence of 5  $\mu$ g of nonspecific blocker, poly(dI-dC), in binding buffer (10 mM Tris·HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM DTT) at 25°C for 20 min. Specific competition was performed by adding unlabeled double-stranded oligonucleotide (100 $\times$ ), whereas for nonspecific competition, the unlabeled double-stranded mutant oligonucleotide (100 $\times$ , 5'-AGCT-CAATCTCCCTGGGACTT-3'), which does not bind PU.1 and NF- $\kappa$ B, was added. To detect supershift, mouse anti-PU.1 monoclonal antibody (2  $\mu$ g) was incubated with nuclear extracts for 15 min at 4°C before addition of the probe. The mixture was separated by electro-

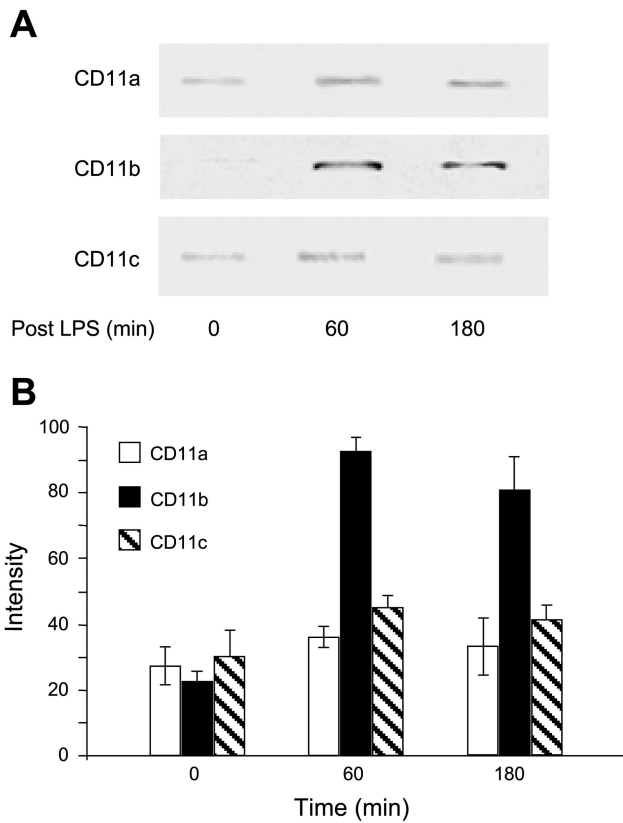


Fig. 1. LPS induces CD11b upregulation in polymorphonuclear leukocytes (PMNs). Time course of CD11a, CD11b, and CD11c expression as assessed by Western blotting using whole cell lysates of PMNs with or without challenge with LPS (0.5  $\mu\text{g}/\text{ml}$ ) at 37°C. The results show that only CD11b expression increased between 1 and 3 h after LPS stimulation. Results are representative 3 independent experiments. Bar graphs indicate means  $\pm$  SE ( $n = 3$ ).

phoresis on a 5% polyacrylamide gel in 1 $\times$  Tris glycine EDTA buffer. Gels were vacuum dried and subjected to autoradiography and phosphorimager analysis.

*Statistical analysis.* Data are presented as means  $\pm$  SE. Differences in mean values were compared by ANOVA and were considered significant at  $P < 0.05$ .

## RESULTS

*LPS induces CD11b integrin upregulation in PMNs via TLR4.* To determine the role of LPS in the regulation of CD11/CD18 expression, we investigated the expression of CD11a, CD11b, and CD11c after LPS stimulation of WT PMNs. Western blotting showed that CD11b expression was significantly increased within 60–180 min after LPS challenge (with peak fourfold increase seen at 60 min), whereas expression of CD11a and CD11c was unchanged (Fig. 1). We next determined the effects of LPS in mediating the upregulation of CD11a, CD11b, and CD11c in PMNs obtained from WT and TLR4<sup>-/-</sup> mice. Flow cytometric analysis showed that 1 h of LPS challenge failed to induce CD11b expression in TLR4<sup>-/-</sup> PMNs in contrast to the robust response in WT PMNs (Fig. 2). There was no significant effect of LPS in inducing the expression of CD11a and CD11c in WT PMNs as indicated in the Western blotting data (Fig. 2). The basal CD11b expression was less ( $P < 0.05$ ) in TLR4<sup>-/-</sup> PMNs than WT PMNs (Fig. 2).

*TLR4 deletion inhibits LPS-induced PMN adhesion and transmigration.* Because CD11b/CD18 is involved in PMN adhesion and transendothelial PMN migration following LPS exposure (9, 41), we addressed the possibility that failure of CD11b expression in TLR4<sup>-/-</sup> PMNs could interfere with both responses. As shown in Fig. 3A, LPS induced 5.6-fold increase in adhesion of WT PMNs compared with the control group, but no increase was observed in TLR4<sup>-/-</sup> PMNs. In

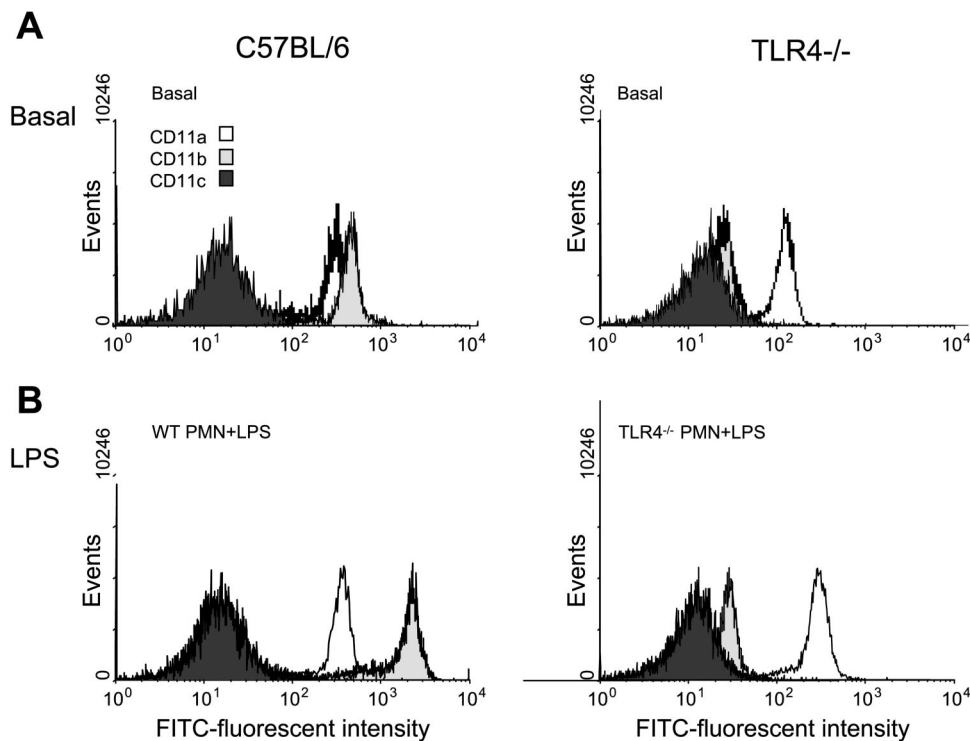


Fig. 2. Toll-like receptor (TLR) 4 dependence of LPS-induced CD11b expression in PMNs. We determined using flow cytometry the expression of CD11a, CD11b, and CD11c in PMNs from wild-type (WT) and TLR4<sup>-/-</sup> mice after 1-h LPS challenge (0.5  $\mu\text{g}/\text{ml}$ ). In the basal condition, CD11b expression in TLR4<sup>-/-</sup> PMNs was less than in WT PMNs ( $P < 0.05$ ), whereas expression of CD11a and CD11c was similar in both types of PMNs (A). CD11b was upregulated by LPS in WT PMNs but not in TLR4<sup>-/-</sup> PMNs (B). Results are representative of 4 independent experiments.

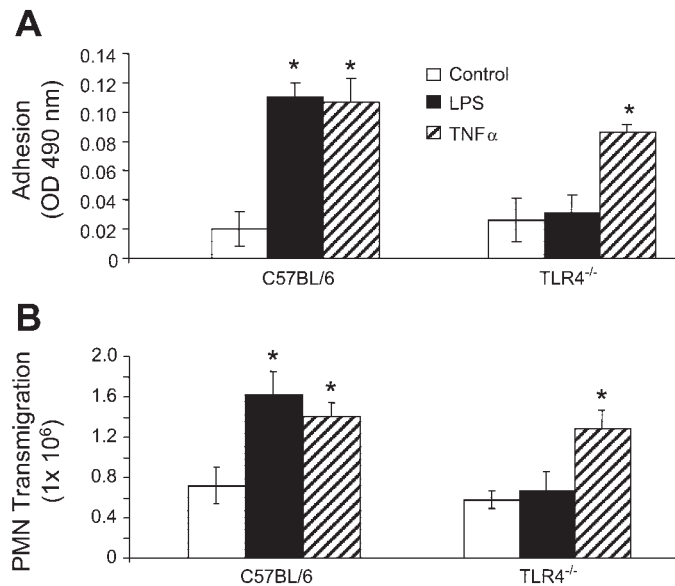


Fig. 3. Defective LPS-induced PMN adhesion and transendothelial PMN migration in TLR4<sup>-/-</sup> PMNs. PMNs isolated from mouse blood were treated with LPS (0.5  $\mu$ g/ml) at 37°C for 3 h or TNF- $\alpha$  (20 ng/ml) at 37°C for 3 h. PMN transmigration and adhesion assays were performed as described in METHODS. *A*: increased adhesion of TLR4<sup>-/-</sup> PMNs induced by TNF- $\alpha$  stimulation but impaired adhesion of TLR4<sup>-/-</sup> PMNs induced by LPS. \*Increase in PMN adhesion ( $P < 0.05$ ,  $n = 4$  experiments). OD, optical density. *B*: increased transendothelial migration of TLR4<sup>-/-</sup> PMNs induced by TNF- $\alpha$  stimulation but inhibited transmigration of TLR4<sup>-/-</sup> PMNs in response to N-formylmethionyl-leucyl-phenylalanine (fMLP). \*Increase ( $P < 0.05$ ,  $n = 4$  experiments). Bar graphs indicate means  $\pm$  SE.

contrast, increases in adhesion of WT and TLR4<sup>-/-</sup> PMNs in response to TNF- $\alpha$  challenge (a positive control) were the same. Figure 3B shows that the number of WT PMNs transmigrating was significantly increased from basal value of  $0.72 \pm 0.18 \times 10^6$  to  $1.62 \pm 0.23 \times 10^6$  PMNs after LPS stimulation ( $P < 0.05$ ) but remained unchanged in the case of TLR4<sup>-/-</sup> PMNs [basal value of  $0.58 \pm 0.09 \times 10^6$  to  $0.67 \pm 0.19 \times 10^6$  after LPS challenge ( $P > 0.05$ )]. TNF- $\alpha$  also increased the transmigration of WT PMNs, but in contrast to

LPS challenge, the response to TNF- $\alpha$  was not blocked in the TLR4<sup>-/-</sup> PMNs (Fig. 3B).

*Impaired LPS binding and aggregation of TLR4<sup>-/-</sup> PMNs.* To address the possible role of the TLR4-mediated expression of CD11b in altering LPS interaction with PMNs, we monitored binding of FITC-conjugated LPS as well as PMN aggregation at 37°C. Results showed reductions in binding of FITC-conjugated LPS to TLR4<sup>-/-</sup> PMNs and aggregation of TLR4<sup>-/-</sup> PMNs compared with WT PMNs (Fig. 4).

*TLR4 induces CD11b expression in PMNs by a protein synthesis-dependent mechanism.* Because the CD11b expression in response to LPS was delayed (Fig. 1), we next addressed the possibility that TLR4 signaling mediates de novo synthesis of CD11b. Total RNA was isolated from PMNs and reverse-transcribed followed by PCR with a CD11b-specific primer. In the basal state, CD11b transcription was not detected in TLR4<sup>-/-</sup> PMNs but was seen at low levels in WT PMNs (Fig. 5A). CD11b transcription in WT PMNs increased twofold after LPS stimulation; however, LPS failed to induce CD11b mRNA expression in TLR4<sup>-/-</sup> PMNs (Fig. 5A), paralleling the absence of protein expression (Fig. 5B). Cycloheximide blocked CD11b expression induced by LPS in WT PMNs and had no effect in TLR4<sup>-/-</sup> PMNs (Fig. 5B).

*Role of JNK/PU.1 and NF- $\kappa$ B in TLR4-induced CD11b expression and PMN adhesion.* To address the signaling pathways regulating LPS-induced CD11b expression, we used *Src* kinase inhibitor PP2 (5), JNK inhibitor SP-600125 (28), NF- $\kappa$ B inhibitor IKK-NBD peptide (35), and MEK inhibitor UO126 (44). RT-PCR analysis showed that only SP-600125 and IKK-NBD peptide prevented the LPS-induced CD11b mRNA upregulation (Fig. 6A). We also examined the possible signaling pathways mediating the LPS-induced CD11b protein expression compared with TNF- $\alpha$ -induced CD11b expression (Fig. 6B). Inhibitors of JNK and NF- $\kappa$ B pathways abrogated the LPS-induced CD11b protein expression consistent with the RT-PCR data (Fig. 6A), indicating that JNK and NF- $\kappa$ B signaling pathways were dominant in mediating the LPS/TLR4-induced CD11b expression. In contrast, all inhibitors interfered with TNF- $\alpha$ -induced CD11b protein expression (Fig. 6B),

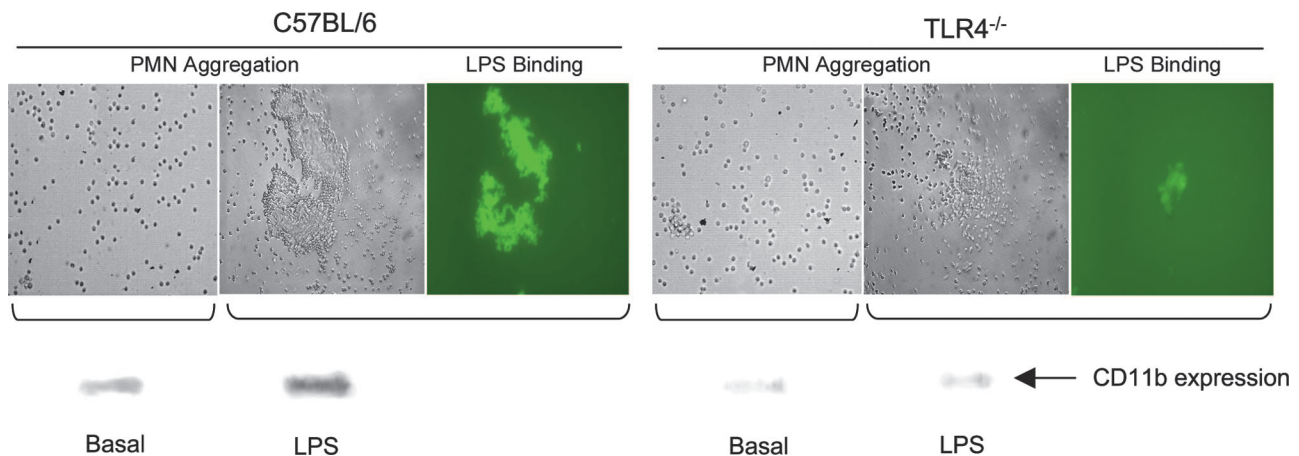


Fig. 4. Defective LPS binding and aggregation in TLR4<sup>-/-</sup> PMNs. PMNs isolated from mouse whole blood (2,000,000 cells/ml) were incubated with FITC-conjugated LPS (0.5  $\mu$ g/ml) at 37°C for 3 h and washed with culture medium. LPS binding to PMNs and PMN aggregation were visualized under fluorescent microscopy. RT-PCR was performed to assess CD11b mRNA expression in PMNs. TLR4<sup>-/-</sup> PMNs displayed less fluorescent intensity (binding of FITC-LPS) as shown in the green fluorescence image and formed much fewer aggregates in response to LPS challenge compared with WT PMNs. The results are representative of 3 experiments.

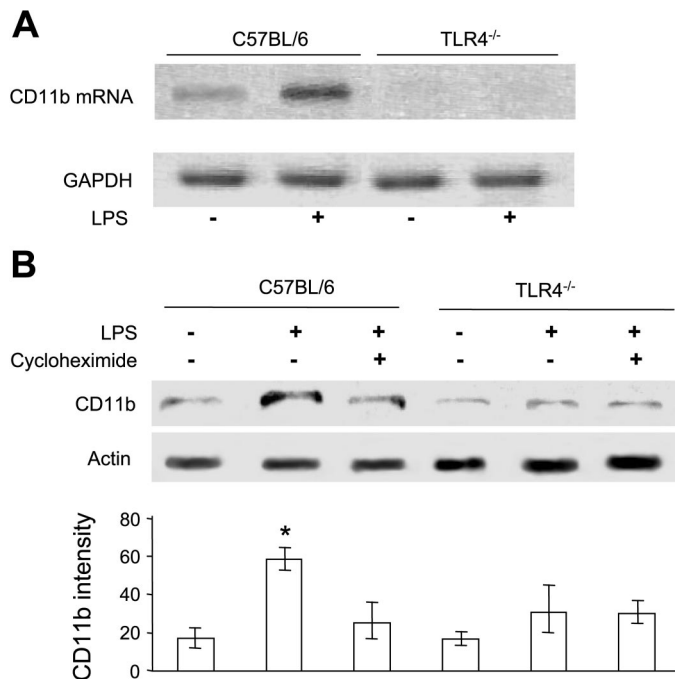


Fig. 5. TLR4 dependence of LPS-induced CD11b mRNA and protein expression in PMNs. **A:** RT-PCR for CD11b mRNA expression in PMNs. PMNs were incubated with LPS (0.5  $\mu\text{g/ml}$ ) at 37°C for 3 h, and total RNA was isolated, reversed-transcribed, and amplified with a primer pair for CD11b and GAPDH as described in METHODS. GAPDH was used as reference for normalization. CD11b mRNA was upregulated in response to LPS in WT PMNs, but not in TLR4<sup>-/-</sup> PMNs. Results are representative of 3 experiments. **B:** Western blot showing CD11b expression in PMNs. WT or TLR4<sup>-/-</sup> PMNs were treated with cycloheximide (10  $\mu\text{M}$ ) at 37°C for 30 min and then incubated with LPS (0.5  $\mu\text{g/ml}$ ) at 37°C for 3 h. CD11b expression was assessed by Western blotting using whole cell lysates. Cycloheximide prevented ( $*P < 0.05$ ) CD11b expression in response to LPS in WT PMNs. CD11b expression in TLR4<sup>-/-</sup> PMNs was low under basal conditions and did not increase significantly after LPS challenge. Corresponding actin is shown as evidence of comparable loading. Bar graphs indicate means  $\pm$  SE;  $n = 3$  experiments.

although only the NF- $\kappa\text{B}$  activation inhibitor blocked the TNF- $\alpha$  response to the same extent as the LPS response. Thus the responses of the two mediators appeared to be differentially regulated. Adhesion of WT PMNs to endothelial cells induced by LPS was also blocked by inhibitors of both NF- $\kappa\text{B}$  (IKK-NBD peptide) and JNK (SP-600125), and not by the *Src* kinase (PP2) and MEK (UO126) inhibitors (Fig. 6C).

**TLR4 regulation of LPS-induced PU.1 and NF- $\kappa\text{B}$  activation.** As the CD11b promoter has PU.1 DNA binding sites (2, 13) and the transcription factor c-Jun activated by JNK associates with the Ets domain of PU.1 to induce gene expression (30), we carried out EMSA using the PU.1 consensus sequence as the probe. LPS failed to increase the nuclear binding of PU.1 in TLR4<sup>-/-</sup> PMNs, whereas binding of PU.1 was significantly increased in WT PMN with the maximum response observed at 1 h (Fig. 7A). We also observed that the LPS-induced NF- $\kappa\text{B}$  (p50/p65) nuclear translocation in PMNs was TLR4 dependent since it was blocked in TLR4<sup>-/-</sup> PMNs (Fig. 7B).

## DISCUSSION

In this study, we demonstrate that LPS activation of TLR4 induces the de novo synthesis and expression of CD11b in

PMNs via JNK/c-Jun/PU.1 and NF- $\kappa\text{B}$  signaling pathways. We also show that the TLR4 upregulation of CD11b is required for the LPS-induced PMN adhesion and transendothelial PMN migration responses. Thus our results point to the crucial role of LPS ligation of TLR4 in mediating the upregulation of CD11b and thereby in regulating the antimicrobial activity of PMNs.

Previous studies showed that in human monocytes both CD11a and CD11b were expressed in response to LPS,

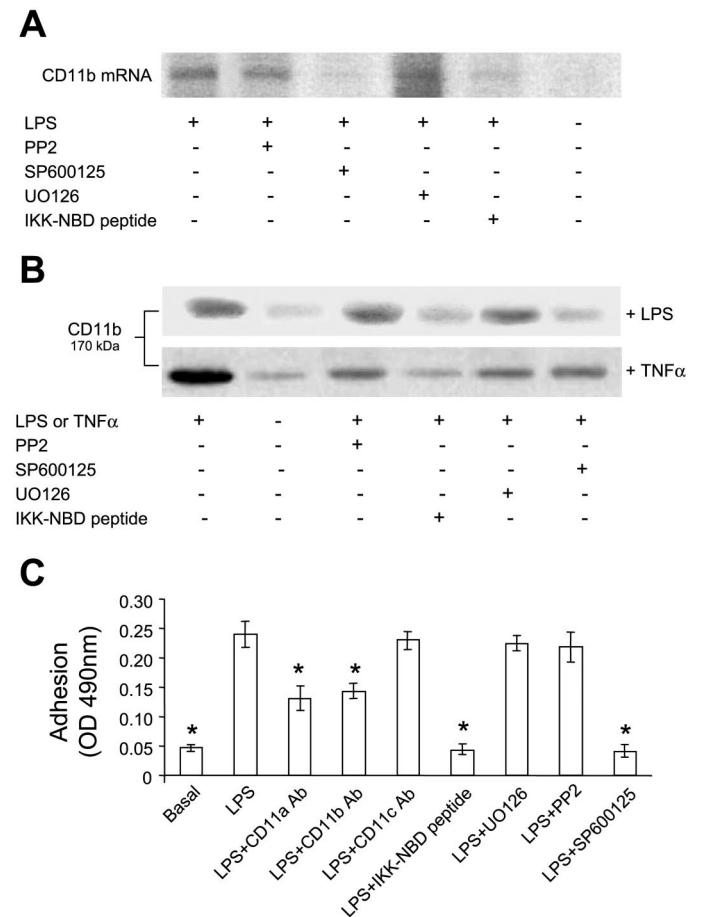


Fig. 6. Role of JNK/c-Jun/PU.1 and NF- $\kappa\text{B}$  pathways in TLR4-mediated expression of CD11b and induction of PMN adhesion. **A:** WT PMNs were pretreated with inhibitors PP2 (0.05  $\mu\text{M}$ ), SP-600125 (100  $\mu\text{M}$ ), UO126 (10  $\mu\text{M}$ ), or I $\kappa\text{B}$  kinase-Nemo binding domain (IKK-NBD) peptide (100  $\mu\text{M}$ ) at 37°C for 15 min and then challenged with LPS (0.5  $\mu\text{g/ml}$ ); this was followed by RT-PCR for analysis of CD11b expression. JNK and NF- $\kappa\text{B}$  inhibitors (SP-600125 or IKK-NBD peptide) blocked CD11b mRNA expression. Results are representative of 3 experiments. **B:** Western blotting for CD11b expression analysis was carried out in WT PMNs treated with inhibitors at 37°C for 15 min followed by incubation with LPS (0.5  $\mu\text{g/ml}$ ) or TNF- $\alpha$  (20 ng/ml) at 37°C for 3 h. Only the inhibitors of JNK and NF- $\kappa\text{B}$  (SP-600125 or IKK-NBD peptide) prevented the LPS-induced CD11b protein expression. In contrast, all of the above inhibitors reduced CD11b upregulation induced by TNF- $\alpha$ . Results are representative of 3 experiments. **C:** anti-CD11b or anti-CD11a monoclonal antibody prevented  $\sim 50\%$  of the increase in adhesion of WT PMNs induced by LPS. Anti-CD11c antibody or a nonspecific isotype matched antibody failed to inhibit PMN adhesion (data not shown). Inhibitors of either NF- $\kappa\text{B}$  (IKK-NBD peptide) or JNK (SP-600125) blocked PMN adhesion induced by LPS. Pretreatment period was 30 min for all inhibitors or antibodies before PMNs were incubated by LPS (0.5  $\mu\text{g/ml}$ ) at 37°C for 1 h. Results are presented as mean of 4 experiments. Bars indicate  $\pm$  SE. \*Different from LPS challenge of control WT PMNs ( $P < 0.05$ ).

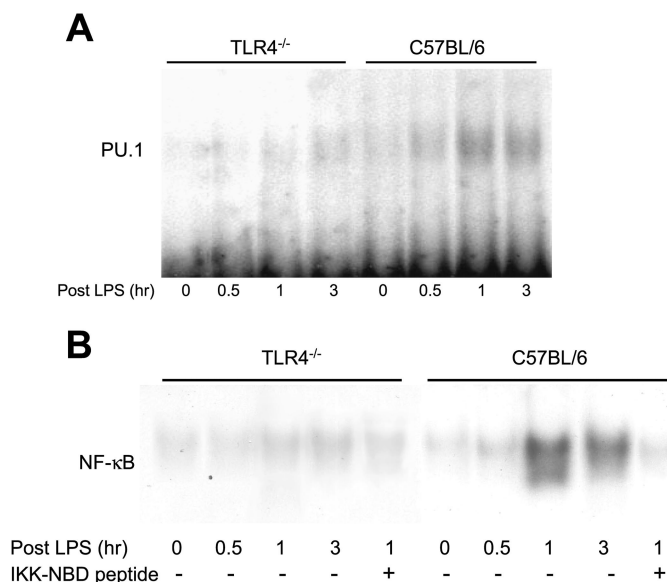


Fig. 7. TLR4 regulation of LPS-induced PU.1 and NF- $\kappa$ B activation. *A*: EMSA showing LPS-induced PU.1 nuclear translocation in WT and TLR4<sup>-/-</sup> PMNs. PU.1 nuclear translocation reached highest level at 1 h after LPS stimulation in WT PMNs, whereas nuclear translocation did not occur in TLR4<sup>-/-</sup> PMNs. Results are representatives of 3 experiments. *B*: EMSA was carried out in PMNs treated with IKK-NBD peptide (100  $\mu$ M) at 37°C for 15 min followed by challenge with LPS (0.5  $\mu$ g/ml). LPS induced NF- $\kappa$ B nuclear translocation in WT PMNs but not in TLR4<sup>-/-</sup> PMNs. The response in WT PMNs was blocked by the IKK-NBD peptide. Results are representative of 3 experiments.

whereas in PMNs CD11b was upregulated and CD11a was unaffected following LPS challenge (8, 12). Therefore, anti-CD11a antibody had no effect on the transmigration of PMNs, whereas it blocked monocyte migration (26). Studies also showed that PMN TLR4 could regulate the interaction of PMNs with endothelial cells and that subsequent endothelial activation was dependent on PMN adhesion (6). In the present study, we showed using PMNs from TLR4<sup>-/-</sup> mice that CD11b is the primary  $\beta_2$ -integrin upregulated by LPS activation of TLR4 and that this process requires the engagement of the CD11b transcriptional machinery.

Studies have shown that activation of TLR4 induced by low concentrations of LPS is necessary for the expression of a repertoire of genes: TNF- $\alpha$ , cyclooxygenase-2, interferon- $\gamma$ -inducible protein, IL-12 p40, and IL-12 p35 (47). Moreover, TLR4 functioned in conjunction with CD11b/CD18 and CD14 to elicit the full gene expression profile in response to LPS (17, 29). Although these findings suggest that LPS receptors may act synergistically to optimize the PMN antimicrobial defenses, little is known about a possible interaction between individual LPS receptors, such as between TLR4 and CD11b/CD18. Our study demonstrates that LPS activation of TLR4 induces expression of CD11b. We also observed augmented LPS binding to PMNs and PMN aggregation consistent with enhanced binding of LPS to the expressed CD11b. This response did not occur in TLR4<sup>-/-</sup> PMNs, suggesting that TLR4 may be capable of amplifying the response to LPS through CD11b upregulation.

In contrast to LPS-induced CD11b expression that was fully dependent on TLR4, the TNF- $\alpha$ -induced CD11b expression was differentially mediated. This is likely the result of activa-

tion of different receptors and signaling pathways by LPS vs. TNF- $\alpha$  even though the end result in both cases is CD11b/CD18 expression. We observed that the basal level of CD11b expression in TLR4<sup>-/-</sup> PMNs was less than in WT PMNs, indicating that the deficiency in TLR4 was also capable of downregulating the constitutive PMN CD11b transcriptional machinery.

The PMN adhesion molecules, L-selectin, and the  $\beta_2$ -integrins, CD11a, CD11b, and CD11c, are essential for recruitment of PMNs to site of infection (1, 9, 50, 51). We observed that PMN adhesion and transendothelial PMN migration were markedly defective in TLR4<sup>-/-</sup> PMNs, probably as the result of the impaired CD11b expression in these cells. CD11b/CD18 is known to be the key  $\beta_2$ -integrin on the PMN surface regulating both PMN adhesion and transmigration to microbial infection (30). In contrast, the TNF- $\alpha$ -induced adhesion and migration of TLR4<sup>-/-</sup> PMNs were unaffected, indicative of a separate mechanism mediating these TNF- $\alpha$  responses.

We addressed the pathways mediating the LPS/TLR4-induced expression of CD11b. Signaling via JNK/c-Jun/PU.1 and NF- $\kappa$ B was shown to be crucial for CD11b expression and the resultant PMN adhesion response. This was evident by the studies in which only inhibitors of JNK and NF- $\kappa$ B prevented CD11b expression and PMN adhesion induced by LPS; in contrast, inhibitors of *Src* kinase and MEK had no effect. The LPS-induced CD11b expression was different from the TNF- $\alpha$ -induced CD11b expression in that all of the above inhibitors reduced to varying degrees the TNF- $\alpha$  response.

We observed that PMN adhesion was fully blocked in TLR4<sup>-/-</sup> PMNs, whereas it was partially reduced in WT PMNs by either anti-CD11a or anti-CD11b antibody, consistent with the role of each integrin in contributing to a component of the response. This finding suggests TLR4 may regulate PMN adhesion by other mechanisms in addition to mediating the expression of CD11b. Although we did not detect by flow cytometry increased expression of CD11a in WT PMNs following LPS challenge, it is possible that CD11a is qualitatively activated by a conformation change (21) in a TLR4-dependent manner. This could account for the inhibition of adhesion of TLR4<sup>-/-</sup> PMNs.

LPS-induced gene expression via TLR4 may be the result of activation of IKK NF- $\kappa$ B and MAPK signaling pathways and the downstream transcription factors AP-1 (c-Jun) and NF- $\kappa$ B (p50/p65) (11, 24). Studies show that LPS activated the transcription factor PU.1 via JNK and thereby induced gene expression in macrophages (3). Studies also show that PU.1 was required for the expression of TLR4 gene in myeloid cells (33, 36). The role of JNK in signaling TLR4-activated responses is consistent with evidence that the JNK-interacting protein 3 associates with TLR4 cytoplasmic domain and mediates JNK activation (23). TLR4 was shown to activate PU.1, which in turn translocated to the nucleus where the Ets domain of PU.1 formed a complex with the basic domain of c-Jun (30, 33). The PU.1/c-Jun complex then binds to CD11b promoter to activate gene transcription (30, 33). In the present study, we observed that LPS stimulation of TLR4 induced activation of PU.1 and its translocation to PMN nuclei. In addition, NF- $\kappa$ B was activated by LPS in a TLR4-dependent manner. We observed that inhibition of JNK and NF- $\kappa$ B pathways prevented the TLR4-activated CD11b upregulation, indicating that TLR4 activation of both JNK/c-Jun/PU.1 and NF- $\kappa$ B

pathways is likely responsible for signaling CD11b expression. In contrast, inhibitors of MEK or *Src* kinase had no effect on TLR4-induced CD11b expression.

In summary, we have identified the essential role of LPS activation of TLR4 in signaling the expression of CD11b in PMNs through the activation of JNK/c-Jun/PU.1 and NF- $\kappa$ B pathways. TLR4 upregulation of CD11b was responsible for activation of PMN adhesion and transendothelial migration responses; thus TLR4-induced upregulation of CD11b may serve an important role in the innate immune response. In pathological conditions associated with increased PMN adhesion and transvascular PMN migration such as sepsis-induced acute lung injury, TLR4 and downstream JNK/c-Jun/PU.1 and NF- $\kappa$ B signaling pathways may be important in controlling the host-defense function of PMNs.

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