Toll-like receptor 4 mediated inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells

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Paik, Yong Han

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Abstract

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Bacterial lipopolysaccharide (LPS) stimulates Kupffer cells and participates in the pathogenesis of alcohol-induced liver injury. However, it is unknown whether LPS directly affects hepatic stellate cells (HSCs), the main fibrogenic cell type in the injured liver. This study characterizes LPS-induced signal transduction and pro-inflammatory gene expression in activated human HSCs. Human HSCs were isolated and activated in culture. Expressions of CD14, Toll-like receptor (TLR) 4, and MD2 mRNA were assessed by RT-PCR. IκBα and phospho-c-Jun were assessed by Western blot analysis. IκBα kinase (IKK) and c-Jun N-terminal

kinase (JNK) activity were measured by *in vitro* kinase assay using a GST-IκB(1-54) or GST-c-Jun substrates, respectively. LPS-induced NF-κB transcriptional activation was assessed by a luciferase reporter gene assay in response to various concentrations of purified LPS (1-1000 ng/ml). Nuclear translocation of NF-κB was assessed by immunofluorescent staining for p65 and electrophoretic mobility shift assay (EMSA). IL-8 and MCP-1 expression were assessed by an RNase protection assay and enzyme-linked immunosorbent assay (ELISA). ICAM-1 and VCAM-1 expression were assessed by flow cytometry. Culture-activated HSCs and HSCs isolated from patients with hepatitis C virus-induced cirrhosis express LPS-associated signaling molecules including CD14, TLR4, and MD2. Stimulation of culture-activated HSCs with LPS results in a rapid and marked activation of NF-κB. Lipid A induces NF-κB activation in a similar manner. Both LPS- and lipid A-induced NF-κB activation is blocked by preincubation with either anti-TLR4 blocking antibody (HTA125) or Polymyxin B. Lipid A induces NF-κB activation in HSCs from C3H/OuJ (TLR4-

sufficient mice) but not from C3H/HeJ (TLR4-deficient mice). LPS also activates c-Jun N-terminal Kinase (JNK). LPS upregulates gene expression and secretion of IL-8 and MCP-1. LPS-induced IL-8 secretion is completely inhibited by the IκB super-repressor (Ad5IκB) and partially inhibited by a specific JNK inhibitor, SP600125. LPS also upregulates cell surface expression of ICAM-1 and VCAM-1. Human activated HSCs utilize components of TLR4 signal transduction cascade to stimulate NF-κB and JNK, and upregulate chemokines and adhesion molecules. Thus, HSCs are a potential mediator of LPS-induced liver injury.

Key Words: lipopolysaccharide, toll-like receptor, hepatic inflammation, hepatic stellate cell, hepatic fibrosis

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I. Introduction

Lipopolysaccharide (LPS), a major constituent of the outer membrane of gram-negative bacteria, potently stimulates host innate immune responses.[1] LPS-induced activation of monocytes/macrophages leads to secretion of a number of proinflammatory cytokines such as TNF-α, IL-1, and IL-6.[2] Although upregulation of these cytokines is an important host defense mechanism to eliminate bacteria from infected site, their excessive production may results in fatal septic shock.[1] The gram-negative flora of the intestine is a substantial reservoir of

endogenous LPS. LPS is absorbed from the gut and transported in the portal vein to the liver where it is rapidly cleared by the Kupffer cells.[3] Several lines of evidence indicate that LPS plays a role in chronic liver diseases. Serum LPS levels are significantly elevated in patients with chronic hepatitis and cirrhosis.[4] Moreover, LPS has been implicated in experimental liver injury induced by alcohol, CCl_4, galactosamine, and choline deficiency.[5] An emerging concept is that LPS-induced Kupffer cell activation mediates hepatic injury in these experimental models. Acute or chronic treatment with ethanol increases gut permeability to LPS,[6] resulting in increased level of LPS in serum, which activates Kupffer cells to produce proinflammatory mediators such as TNF-α.[5,7]

Hepatic stellate cells (HSCs) mediate fibrosis and inflammation in the injured liver. HSCs regulate leukocyte trafficking and activation through secretion of chemokines such as monocyte chemotactic protein-1 (MCP-1)[8,9] and interleukin (IL)-8.[10] HSCs express CD40, which activates NF-κB and JNK and upregulates chemokine secretion. Therefore, cross-talk between HSCs and immune effector cells is likely to occur.[11] LPS induces secretion of

chemokines including MCP-1 and macrophage inflammatory protein-2 (MIP-2) in activated rat HSCs.[12,13] Cytokines such as TNF-α or IL-1β induce NF-κB and upregulate adhesion molecules such as ICAM-1 in activated HSCs.[14] However, the direct effect of LPS on the proinflammatory response in human HSCs has not been studied.

Recent studies have begun to clarify the molecular basis for LPS intracellular signaling. Activation of LPS-responsive cells, such as monocytes and macrophages, occurs after LPS interacts with circulating LPS-binding protein (LBP).[15] After binding with LBP, LPS-induced cell activation depends on the presence of three proteins: CD14, TLR4, and MD2 comprising the LPS receptor complex.[16] CD14 is a 55-kDa-sized glycophosphatidylinositol-linked protein expressed on the surface of macrophages and monocytes.[17] However, because CD14 lacks a transmembrane domain, it cannot transduce a signal intracellularly.[17] The human TLRs are mammalian homologues of the *Drosophila* Toll protein, which plays a critical role in the establishment of dorsoventral polarity and the antifungal response in adult flies.[18] Human TLR families consist of at least 10 members, which are involved in the

recognition of pathogen-associated molecular patterns.[19] TLR2 and TLR4 confer responsiveness to bacterial products.[20,21] Recent evidence suggests that TLR4 is involved in LPS signaling,[22] whereas TLR2 responds to peptidoglycans, lipoteichoic acids and lipoptroteins.[23,24] Human MD2 is a novel accessory molecule expressed on the cell surface that associates with TLR4 and confers LPS responsiveness.[25] LPS is brought into close proximity to TLR4 only when CD14 and TLR4 are co-expressed with MD2.[16]

Activation of TLR4 by LPS triggers several crucial intracellular signaling pathways, including stress-activated mitogen activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK) and p38, as well as NF-κB by utilizing components of the IL-1 pathway, such as MyD88, IRAK, and TNF receptor-associated factor (TRAF)-6 in monocyte, macrophage, and endothelial cell lines.[26] Interestingly, alcohol-induced liver injury and TNF-α expression are reduced in C3H/HeJ mice compared to C3H/OuJ mice.[27] However, the molecular mechanism of LPS signal transduction in HSCs is unknown.

This study characterized TLR4-mediated LPS signaling associated with proinflammatory gene expression in activated

HSC. The activated human HSCs expressed LPS recognizing receptors such as CD14, TLR4, and MD2. Low concentration of LPS induced activation of NF-κB and JNK and expression of chemokines and adhesion molecules in activated human HSCs. The direct regulation of HSC gene expression by LPS represents a novel mechanism for hepatic injury and fibrosis.

II. Materials and Methods

1. Reagents

LPS from *E. Coli* serotype O127:B8 (phenol extracted and then chromatographically purified by gel filtration, protein content <1%) was purchased from Sigma (St.Louis, MO, USA). Synthetic lipid A from *Salmonella Minnesota* R595 LPS was obtained from ICN Biomedicals (Costa Mesa, CA, USA). Human recombinant TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). Polymyxin B and phorbol myristate acetate (PMA) were purchased from Sigma. Mouse anti-human TLR4 blocking Ab (HTA 125) was obtained from eBioscience (San Diego, CA, USA), and isotype $IgG_{2\alpha}$ Ab was purchased from BD

PharMigen (San Diego, CA, USA). SP600125, a selective JNK inhibitor, was obtained from Celgene Inc. (San Diego, CA, USA).

2. Isolation and culture of human HSCs

HSCs were isolated by a two-step collagenase perfusion from surgical specimens of six nontumorous normal portion of human livers with metastatic carcinoma or two HCV-induced human cirrhotic livers as described previously.[11] All tissues were obtained by qualified medical staff, with donor consent and the approval of the Institutional Ethical Committee. HSC purity was assessed microscopically and by using the autofluorescence property of the stored retinoids in the HSC. Cell viability was determined by Trypan blue dye exclusion. More than 96% of the cells were identified as HSCs and viable. Isolated HSCs were seeded on uncoated plastic tissue culture dishes and cultured in DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat inactivated FCS and standard antibiotics in 95% air and 5% CO_2 humidified atmosphere at 37°C. After 2 days in culture, HSCs had a quiescent phenotype, and after 14 days in culture, the HSCs had an activated phenotype as described.[14] The

culture-activated human HSCs isolated from normal portion of human liver from passage 3 to 9 were used in all experiments. The purity of activated HSCs were assessed by immunohistochemistry using mouse anti-human smooth muscle α-actin Ab (DAKO, Carpinteria, CA, USA). Activated HSCs were >99% pure. We used HSCs isolated from the same patient in each different assays to avoid individual variations. The human onocytic cell line THP-1 (from American Type Culture Collection) were cultured in RPMI medium with 10% FCS. THP-1 cells were differentiated by adding 10ng/ml of PMA for 18 h.

3. Animals

C3H/HeJ and C3H/OuJ mice were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). HSCs were isolated by *in situ* perfusion of the livers with collagenase and pronase, followed by arabinogalactan gradient ultracentrifugation and then cultured in DMEM with 10% FCS.[14] All animal procedures were performed in compliance with institutional guidelines.

4. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from quiescent or *in vitro* culture-activated human HSCs (from normal portion of human liver), *in vivo*-activated human HSCs (from HCV-induced cirrhotic liver) and differentiated THP-1 cells by the TRIzol method (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using dT_{15}-oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA) in 25μl. One microliter of the reverse transcriptase reaction was subjected to PCR to measure the mRNA of CD14, TLR4, MD2, and β-actin. PCR amplification was performed with *Taq* polymerase (Qiagen, Valencia, CA, USA) for 35 cycles at 92 °C for 45 s, 56 °C for 45 s, and 72°C for 2 min. PCR primers for CD14 were 5'-GGTGCCGCTGTGTAGGAAAGA sense and 5'-GGTCCTCGAGCGTCAGTTCCT antisense. PCR primers for TLR4 were 5'-TGTCCCTGAACCCTATGAAC sense and 5'-GCCTTTTGAGAGATTTGAGT antisense. PCR primers for MD2 were 5'-GAAGCTCAGAAGCAGTATTGGGTC sense and 5'-

GGTTGGTGTAGGATGAC AAACTCC antisense. PCR primers for β-actin were 5'- CCAACCGCGAGAAGATGACC sense and 5´-GATCTTCATGAGGTAGTCAGT antisense.

5. Western blot analysis

Whole cell extracts were prepared from activated human HSCs by using Triton lysis buffer containing protease and phosphatase inhibitors as described.[11] One hundred μg of protein (for CD14 and TLR4) or 20 μg of protein (for IκBα , phospho-c-Jun, and phospho-IκBα) were electrophoresed on 10% SDS-polyacrylamide gels. The gels were then blotted onto the nitrocellulose membrane. For immunoreaction of CD14, TLR4, IκBα, phospho-c-Jun, and phospho-IκBα, rabbit anti-human CD14 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human TLR4 Ab (eBioscience, San Diego, CA, USA), rabbit anti-human IκBα, mouse anti-human phospho-c-Jun antibody (Santa Cruz Biotechnology) and rabbit anti-human phospho-IκBα (Cell Signaling Technology, Beverly, MA, USA), all diluted 1:1000 were used, respectively, and the enhanced chemiluminescence light (ECL) detecting kit (Amersham Pharmacia Biotech,

Piscataway, NJ, USA) was used as described.[11]

6. Kinase assays

Activated HSCs were lysed in Triton lysis buffer containing protease and phosphatase inhibitors after treatment with LPS (100 ng/ml), TNF-α (10 ng/ml), IL-1β (5 ng/ml) for various times. IKK and JNK kinase assays were performed as previously described.[11] For IKK assays, 100 μg protein was immunoprecipitated with 2 μl anti-IKKγ antibody (Santa Cruz Biotechnology) for 2 h, followed by 20 μl protein A/G agarose (Santa Cruz Biotechnology) for 1 h. The kinase reaction was performed for 30 min at 30°C using GST-IκBα (amino acid 1-54). For JNK assays, 25 μg protein was incubated with 1μl GST-c-Jun bound to reduced glutathione beads, washed, and subjected to a kinase reaction for 30 min at 30°C. Supernatant from the kinase reactions was analysed on 10% SDS-acrylamide gel. Coomassie staining was used to demonstrate equal protein loading. Phosphate incorporation was determined by autoradiography and quantitated by phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA, USA).

7. Recombinant adenoviral infection of HSCs

Recombinant adenoviral vectors expressing either a luciferase reporter gene driven by NF-κB transcriptional activation (Ad5NF-κBLuc),[29] a dominant negative mutant form (S32A/S36A) of IκB (Ad5IκB),[30] or a green fluorescent protein (Ad5GFP)[31] were used for functional studies. Infection of activated HSCs by adenovirus was evaluated using different multiplicities of infection (MOI) of Ad5GFP and subsequent checking GFP expression by fluorescent microscopy. Increasing MOI efficiently infected HSCs in a dose-dependent manner, Based on this data, the lowest MOI required to achieve 100% transduction rate (MOI=500) was used for Ad5NF-κBLuc infection. Incubation of Polymyxin B did not affect the infection of HSCs by Ad5GFP (data not shown). HSCs were infected with Ad5NF-κBLuc (MOI 500) and/or Ad5IκB (MOI 1000), Ad5GFP (MOI 1000) for 12 h in DMEM containing 0.5% FCS.[28] After infection the medium was changed to fresh medium with 0.5% FCS and the culture was continued for an additional 8 h before performing the individual experiments.

8. NF-κB responsive luciferase assay

The luciferase assay system with Luciferase Cell Lysis Buffer (BD Pharmigen, San Diego, CA, USA) was used to measure NF-κB-mediated transcriptional induction according to the manufacturer's protocol. All measurements of luciferase activity (relative light units) were normalized to the protein concentration.

9. Immunofluorescent staining of p65

Twenty thousand human HSCs were plated onto uncoated 6-well plate, and treated as described. After 1 h of LPS (100 ng/ml) treatment or 15 min of IL-1β (5 ng/ml) treatment, the cells were fixed in ice-cold methanol for 10 min, washed 3 times with PBS, and blocked with 10% normal goat serum in PBS. For the detection of p65, the cells were incubated with rabbit anti-human p65 (Rockland, Gilbertsville, PA, USA) primary antibody at 1:100 in blocking solution for 1 h, and rhodamine-conjugated goat anti-rabbit secondary antibody (Pierce, Rockford, IL, USA) at 1:100 for 1 h. The cells were visualized on an Olympus microscope (Olympus, Melville, NY, USA) using a digital imaging system (Molecular Dynamics, Sunnyvale, CA, USA).

10. Electrophoretic mobility shift assay (EMSA)

Activated HSCs were stimulated with LPS (100 ng/ml) for 1 h or with IL-1β for 15min. Nuclear extracts were prepared as described.[28] Eight micrograms of protein was incubated with a radiolabeled probe containing the NF-κB consensus site (top: 5'-GCAGAGGGGACTTTCCGAGA-3';bottom: 5'-GTCTCGGAAA GTCCCCTCTG-3'), separated by electrophoresis and analyzed by autoradiography as described previously.[14] For supershift analysis and competition assays, nuclear extracts were preincubated with antibodies to p65, p50, c-Rel or CBF-1 (Santa Cruz) or 100-fold excess of unlabeled oligonucleotide.

11. RNase protection assay

Total RNA was isolated from human activated HSCs culturing in DMEM containing 0.5% FCS after treatment of LPS (10 ng/ml) or IL-1β (5 ng/ml) for 18 h with or without pretreatment of Polymyxin B (10 IU/ml) for 2 h by the TRIzol method (Invitrogen), according to the manufacturer's instructions. RNase protection assays were performed as described.[32] Chemokines mRNA levels were

determined by a using human cytokine multiprobe template set (hCK-5; PharMingen, San Diego, CA, USA). Thirty μg of total RNA was hybridized with 10^5 cpm of riboprobe. Twenty μg of yeast tRNA was hybridized as a negative control. The protected riboprobes were visualized by autoradiography and quantitated by phosphoimager analysis (Molecular Dynamics, Sunnyvale, CA, USA).

12. IL-8 and MCP-1 enzyme-linked immunosorbent assay

IL-8 and MCP-1 secretion into culture media from activated human HSCs were measured by ELISA according to the manufacturer's instruction (R & D Systems).

13. Detection of surface ICAM-1 and VCAM-1 expression by flow cytometry

Cultured HSCs were resuspended in PBS containing 2mM EDTA and then incubated with 10% normal goat serum in PBS for 10 min on ice to block Fc receptors. HSCs were washed twice and incubated with the mouse anti-human ICAM-1 Ab, mouse anti-human VCAM-1 Ab (Santa Cruz Biotechnology), or isotype

mouse anti-human $IgG_{1\alpha}$ Ab for 30 min on ice. Cells were washed twice and incubated with FITC-conjugated goat anti-mouse Ab (Pierce Biotechnology) for 30 min on ice. Cells were again washed twice and analysed using a FACScan™ (Becton Dickinson, San Jose, CA, USA) and Summit™ software (Cytomation, Inc., Fort Collins, CO, USA).

14. Statsitics

Data were analyzed using Mann-Whitney U-test. Data were considered to be statistically significant with $p = <0.05$.

III. Results

1. Detection of LPS Receptors in Human HSCs

The expression of the LPS receptor molecules, CD14, TLR4, and MD2 in human HSCs was first assessed by RT-PCR. In quiescent HSCs, low levels of mRNA encoding TLR4 were detected, whereas mRNA for CD14 and MD2 was not detected. mRNAs for CD14, TLR4 and MD2 were up-regulated in both *in*

vitro culture-activated HSCs (from normal portion of human liver) and *in vivo* activated HSCs (from HCV-induced cirrhotic liver) (Figure 1A). Activation of HSCs *in vivo* was confirmed by DNA microarray analysis. Compared to quiescent HSCs *in vivo* activated HSCs showed upregulation of α1(I) collagen and smooth muscle α-actin, that are known markers of HSC activation[14] (data not shown). In the presence of PMA, THP-1 cells differentiate toward a more monocyte-like phenotype and coordinately up-regulate the expressions of mRNAs for CD14, TLR4, and MD2,[33] serving as a positive control. Human CD14 and TLR4 proteins were expressed in both activated HSCs and THP-1 cells, as assessed by Western blotting (Figure 1B). LPS stimulation for 6 h did not affect the expression of CD14 and TLR4 in culture-activated HSCs or THP-1 cells.

2. LPS activates NF-κB in culture-activated human HSCs

We examined the LPS signal transduction pathway leading to NF-κB activation in culture-activated human HSCs. IκB kinase (IKK) activity was detectable at 5 min and peaked at 60 min after

LPS stimulation (Figure 2A). TNF-α (10 ng/ml) and IL-1β (5 ng/ml) were used as positive controls. Figure 2B (*upper panel*) shows decreased IκBα steady-state levels at 60-120 min and

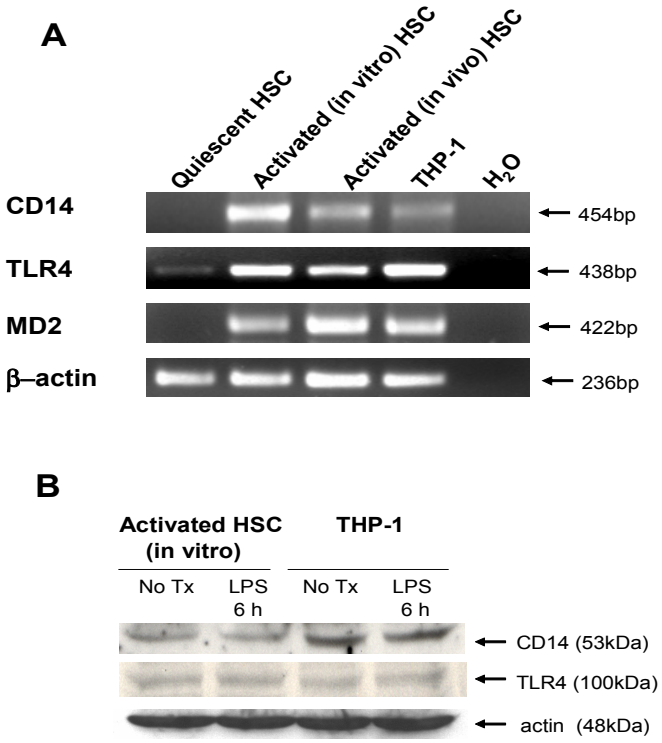

Figure 1. Expression of LPS receptors in human HSCs. (A) CD14, TLR4, and MD2 mRNA expression was assessed by RT-PCR. Total RNA was obtained from quiescent human HSCs, *in vitro* culture-activated human HSCs (from normal portion of human liver), *in vivo* activated human HSCs (from HCV-induced cirrhotic liver), or differentiated THP-1 cells. Data represent HSCs isolated from three normal portion of human livers and two human cirrhotic livers. THP-1 cells were differentiated by treatment with 10 ng/ml of PMA for 18 h, and were used as a positive control. (B) CD14 and TLR4 protein expression was assessed by Western blotting in culture-activated HSCs and PMA-treated THP-1 cells with or without LPS (100 ng/ml) for 6 h. Actin expression was measured to assess equal protein loading. (No Tx), No treatment.

resynthesis at 240 min following LPS stimulation. A proteosome inhibitor, MG-132, was used to assess IκBα phosphorylation, since phosphorylated IκBα is ubiquinated and rapidly degraded by a proteosome complex.[28] After preincubation with MG-132 (10μM) for 30 min, phosphorylated IκBα was detected from 60 min following LPS stimulation (Figure 2B, *middle panel*). Western blotting of α-tubulin was shown as a control for equal loading of samples (Figure 2B, *low panel*). These data demonstrated that LPS activated IKK that correlated with increased endogenous IκBα phosphorylation and degradation in activated human HSCs.

An NF-κB-driven luciferase reporter assay showed that LPS induced NF-κB transcriptional activity in a time- (Figure 3A) and dose- dependent (Figure 3B) manner in activated HSCs. Polymyxin B is a cationic cyclic polypeptide antibiotic that binds the lipid A moiety of LPS, inactivating its biological function.[34] Preincubation of Polymyxin B (10 IU/ml) for 30 min before stimulation completely inhibited LPS-induced NF-κB activation, but did not inhibit IL-1β-induced NF-κB activation in HSCs (Figure 3C).

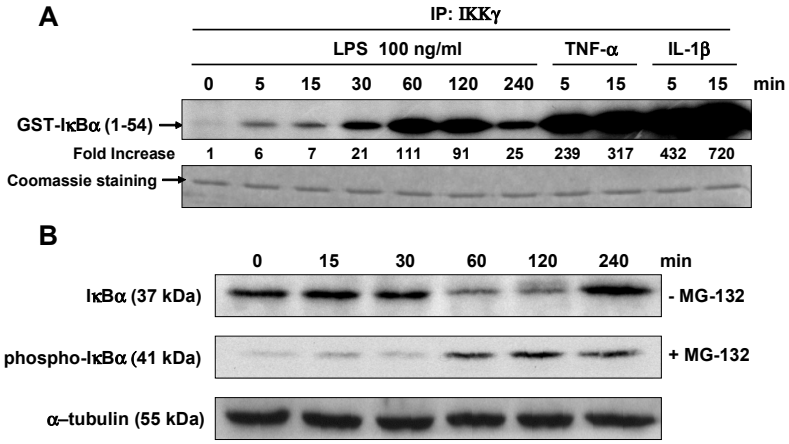

Figure 2. LPS stimulates IKK activity, IκBα phosphorylation and IκBα degradation. (A) Time course of activation of IKK in response to LPS in activated human HSCs. Cells were cultured in DMEM containing 10% FBS. HSCs were stimulated with LPS (100 ng/ml) for the indicated times. TNF-α (10 ng/ml) and IL-1β (5 ng/ml) were used as positive controls. An *in vitro* kinase assay for IKK was performed using GST-IκBα (amino acid 1-54) as a substrate. Coomassie staining was used to demonstrate equal protein loading. (B) HSCs were stimulated with LPS (100 ng/ml) in the presence or absence of MG-132 (10 ng/ml). A proteosome inhibitor, MG-132, was used to assess IκBα phosphorylation. IκBα steady-state level and IκBα phosphorylation were assessed by Western blotting. A representative of three independent experiments is shown.

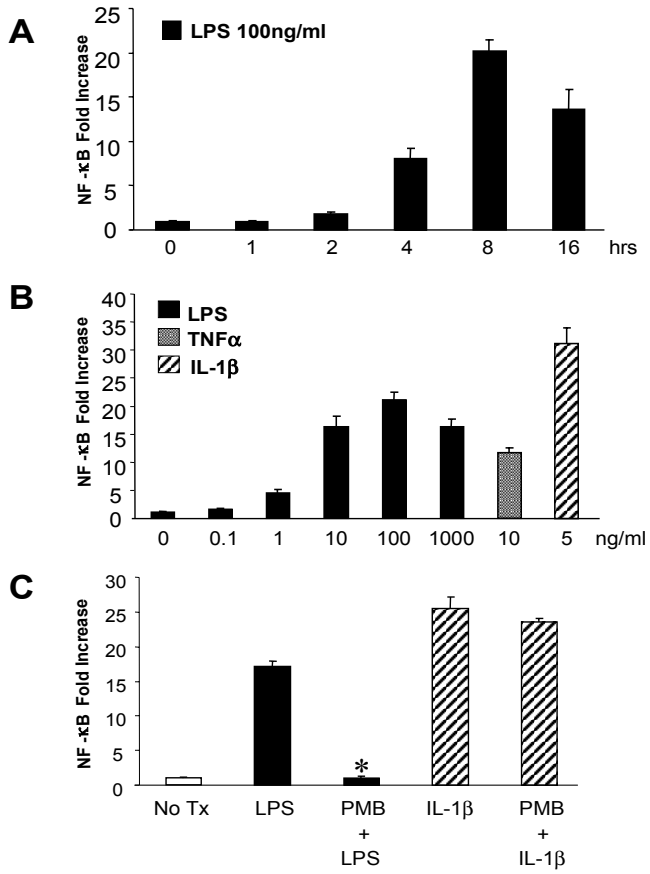

Figure 3. LPS-induced NF-κB transcriptional activity is inhibited by Polymyxin B. Activated human HSCs were infected with the Ad5NF-κBLuc (MOI 500) for 12 h in DMEM containing 0.5% FCS. At 20 h post-infection, HSCs were stimulated with (A) LPS (100 ng/ml) for the indicated times (0-16 h), or (B) with the indicated concentrations of LPS, TNF-α, and IL-1β for 8 h or (C) with LPS (100 ng/ml), or IL-1β (5 ng/ml) for 8 h with or without pretreatment of Polymyxin B (10 IU/ml) for 30 min. Cells were lysed, and NF-κB-mediated luciferase activity was quantified. Data represent the mean ± SD of 3 independent experiments and are expressed as fold-increase over unstimulated cells. All measurements of luciferase activity were normalized to the protein concentration. *: P<0.01, when compared with HSCs which were treated with LPS. (PMB), Polymyxin B.

3. LPS-induced NF-κB activation is serum-dependent and is mediated by TLR4

Lipid A is the LPS component that mediates its biological effects.[35] However, contamination of LPS with highly bioactive "endotoxin proteins" can also activate NF-κB through TLR2.[36] To rule out this possibility, we stimulated HSCs with synthetic lipid A, which is free of contaminating proteins. In the presence of serum, lipid A activated NF-κB in HSCs in a dose-dependent manner (Figure 4A). These results demonstrate that LPS-induced NF-κB activation in HSCs is dependent on the presence of serum, a source of LBP that is required for LPS to act through TLR4.[5,15] Polymyxin B eliminated lipid A-induced NF-κB activation in human HSCs, confirming this response was due to LPS but not to contaminants (Figure 4B).

To examine whether LPS acts directly through TLR4, we stimulated HSCs with lipid A after preincubation with anti-TLR4 blocking Ab (HTA 125). Preincubation of HTA 125 for 30 min significantly reduced lipid A-induced NF-κB activation in HSCs, whereas isotype antibody (mouse $IgG_{2\alpha}$) had no effect (Figure 4B). The C3H/HeJ mouse strain is homozygous for a mutant *Lps* allele

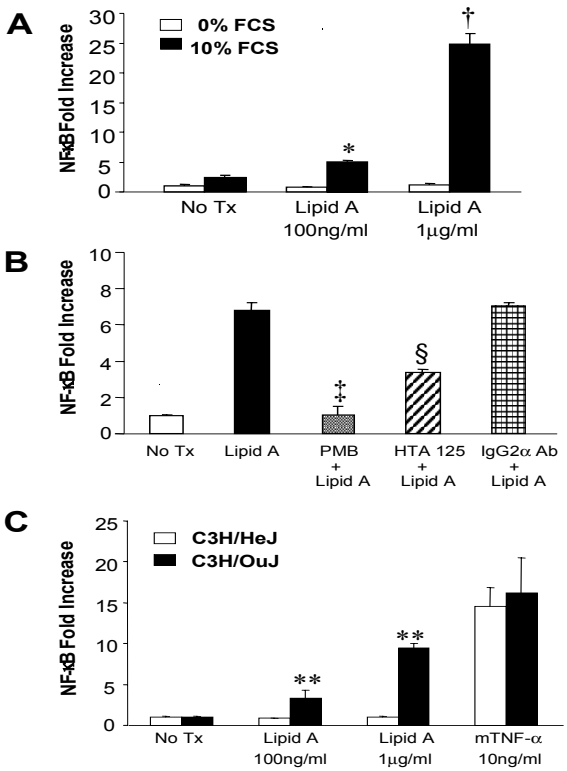

Figure 4. Lipid A-induced NF-κB activity depends on the presence of serum and is blocked by anti-TLR4 blocking Ab (HTA 125) or Polymyxin B. Activated human HSCs were infected with the Ad5NF-κBLuc (MOI 500) for 12 h. At 20 h post-infection, HSCs were treated with (A) lipid A for 8 h with 10% FCS or without serum, or (B) with 1μg/ml of lipid A with 10% FCS for 8 h with or without preincubation of Polymyxin B (10 IU/ml), HTA 125 (20μg/ml), or IgG_2 Ab (20 μg/ml) for 30 min. Activated HSCs from C3H/HeJ or C3H/OuJ mice were infected with the Ad5NF-κBLuc (MOI 500) for 12 h. At 20 h post-infection, HSCs were treated with lipid A or mouse TNF-α (10 ng/ml) for 8 h with 10% FCS (C). Luciferase assays were performed as described in "Materials and Methods". Data represent the mean ± SD of 3 independent experiments and are expressed as fold increase over unstimulated cells. All measurements of luciferase activity were normalized to the protein concentration. *, †: $p<0.01$ when compared with HSCs stimulated with Lipid A without serum; ‡, §: $p<0.01$ when compared with HSCs stimulated with Lipid A only; **:$p<0.01$when compared to HSCs from C3H/HeJ mice. (PMB), Polymyxin B, (mTNF-α), mouse TNF-α.

(*Lps*$^{d/d}$) that confers hyporesponsiveness to LPS. In contrast to C3H/HeJ mice, substrain C3H/OuJ (*Lps*^{n} homozygotes) that diverged from the same stock as C3H/HeJ mice, exhibits vigorous responses to LPS. We compared lipid A-induced NF-κB activation in activated HSCs derived from TLR4-sufficient C3H/OuJ mice and TLR4-deficient C3H/HeJ mice. Lipid A induced NF-κB activation in HSCs from C3H/OuJ mice, but failed to induce NF-κB activation in HSCs from C3H/HeJ mice (Figure 4C). These results provide further evidence that TLR4 mediates LPS-induced NF-κB activation in HSCs.

Untreated HSCs cultured with 10% FCS showed a cytoplasmic staining of p65 as demonstrated by immunofluorescent p65 staining (Figure 5A), whereas LPS treatment induced p65 nuclear translocation in HSCs (Figure 5B). However, no p65 translocation was found in HSCs cultured without serum following LPS stimulation (Figure 5C). IL-1β was used as a positive control (Figure 5D). Polymyxin B blocked LPS-induced, but not IL-1β-induced p65 nuclear translocation (Figure 5E-F). Preincubtion of HTA 125 for 30 min inhibited LPS-induced p65

nuclear translocation (Figure 5G), but preincubation of isotype Ab (mouse $IgG_{2\alpha}$) did not (Figure 5H). These results demonstrate that LPS-induced NF-κB activation is serum dependent and TLR4 is the major receptor for LPS-induced NF-κB activation.

4. LPS increases NF-κB DNA binding activity in activated human HSCs

In activated HSCs, at least three NF-κB DNA binding complexes have been observed: complex 1 (p65:p65 homodimer), 2 (p65:p50 heterodimer), and 3 (novel component) based on their relative electrophoretic mobilities in response TNF-α or IL-1β.[37] LPS or IL-1β stimulation increased NF-κB DNA binding activity in activated HSC, as demonstrated by EMSA (Figure 6). Supershift EMSA experiments were performed to identify constituent proteins of the NF-κB DNA binding activities. We also did a supershift assay with antibody against recombination signal binding protein Jκ (RBP-Jκ) also designated CBF1, which is a transcriptional factor that binds κB binding site of IL-6 promotor resulting in transcriptional repression.[38] The supershift complexes were obtained with anti-p65 and anti-p50; by contrast, no

supershifts were obtained with anti-c-Rel, or anti-CBF1 (Figure 6).

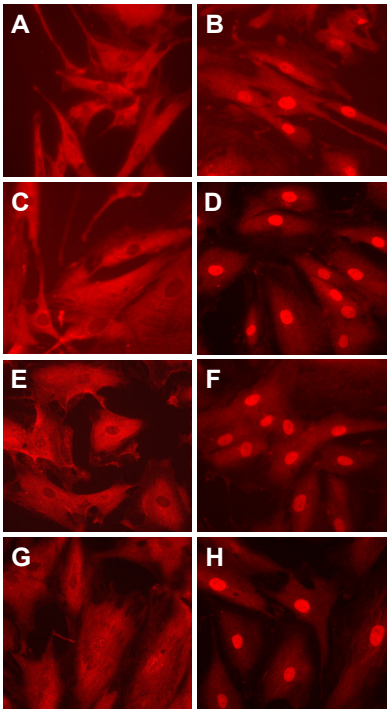

Figure 5. LPS-induced p65 nuclear translocation is blocked by HTA 125 or Polymyxin B. Immunofluorescent staining was used to assess the p65 nuclear translocation in HSCs. Cells were cultured in DMEM with 10% FBS (A, B, D-H) or in DMEM without serum (C). After 1 h of stimulation with LPS (10 ng/ml) or 15 min stimulation with IL-1β (5 ng/ml), cells were fixed and then stained with anti-p65 primary antibody, and then a rhodamine-conjugated secondary antibody. Cells were treated as follows: (A) DMEM, (B, C) LPS (10 ng/ml), (D) IL-1β (5 ng/ml), (E) LPS (10 ng/ml) after preincubation of polymyxin B (10 IU/ml) for 30 min, (F) IL-1β (5 ng/ml) after preincubation of polymyxin B (10 IU/ml) for 30 min, (G) LPS (10 ng/ml) after preincubation of HTA 125 (20 μg/ml) for 30 min, (H) LPS (10 ng/ml) after preincubation of $IgG_{2\alpha}$ Ab (20 μg/ml) for 30 min.

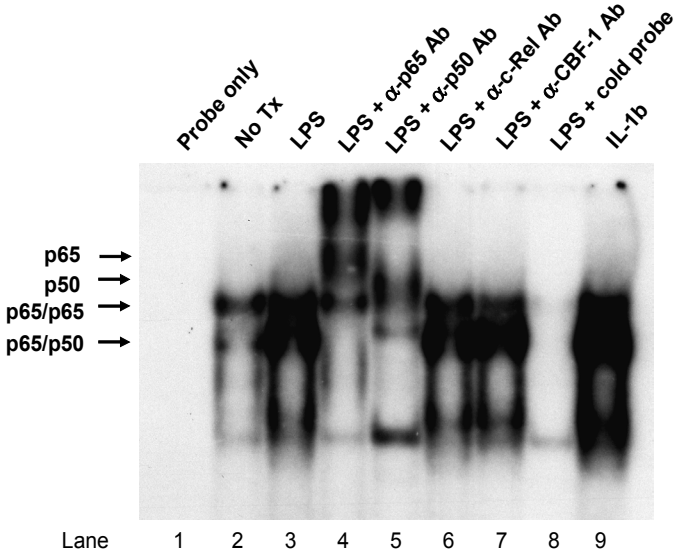

Figure 6. LPS stimulates NF-κB DNA-binding activity in activated human HSC. Culture-activated HSCs were incubated with DMEM alone (*Lane 2*), LPS (100 ng/ml) for 1 h (*Lane 3*) or IL-1β (5 ng/ml) for 15 min (*Lane 9*). Nuclear extracts (8μg) were assayed for NF-κB binding activity by EMSA using a radiolabeled consensus NF-κB site as a probe. *Lane 1*, probe without nuclear extract. *Lane 4-7*, Nuclear extracts from LPS-treated HSCs were incubated with p65, p50, c-Rel or RBP-Jκ antibodies, respectively, and then incubated with radiolabeled probe. Antibody supershift, produced by binding of the p65 and p50 antibody are identified by arrow. *Lane 8* is the same as *lane 3* with the addition of 100-fold molar excess cold oligonucleotide as competitor.

5. LPS activates JNK in activated human HSCs

The JNK/AP-1 pathway is an important regulator of inflammation and host defense response. LPS increased JNK activity, which peaked 9-fold at 60 min after stimulation (Figure 7A). TNF-α and IL-1β increased JNK activity 14- and 19-fold at 15 min after stimulation, respectively. LPS induced phosphorylation of c-Jun, a JNK substrate, a maximum at 60 min after stimulation (Figure 7B), correlating with the time course of JNK activation.

6. LPS induces the production of inflammatory chemokines including IL-8 and MCP-1 in activated HSCs

As shown by RNase protection assay (Figure 8A), LPS induced mRNA expression of IL-8 (10-fold) and MCP-1 (2.4-fold) in HSCs. Preincubation of Polymyxin B for 30 min before stimulation completely inhibited LPS-induced, but not IL-1β-induced increase in IL-8 and MCP-1 mRNA levels (Fig. 8A). Both LPS and IL-1β stimulated IL-8 and MCP-1 secretion. Polymyxin B blocked LPS-induced, but not IL-1β-induced chemokine secretion (Figure 8B).

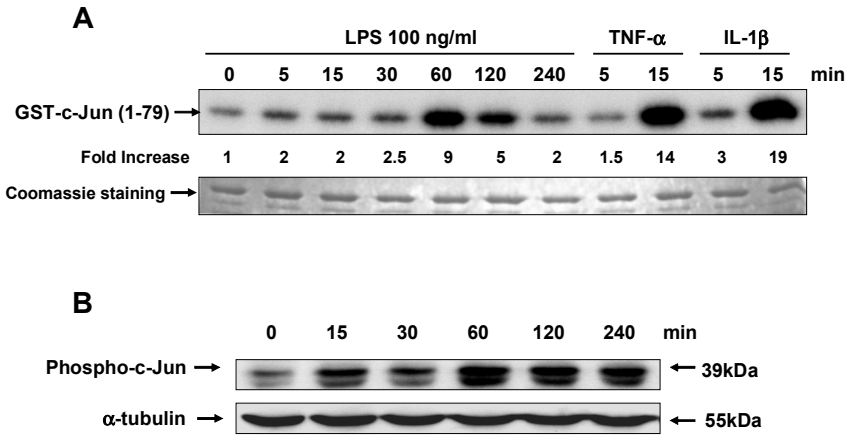

Figure 7. LPS activates JNK in activated human HSCs. HSCs cultured in DMEM containing 10% FCS were stimulated with LPS (100 ng/ml), TNF-α (10 ng/ml), and IL-1β (5 ng/ml) for the indicated times. (A) An *in vitro* kinase assays for JNK was performed using GST-c-Jun as a substrate. Coomassie staining was used to demonstrate equal protein loading. (B) HSCs cultured in DMEM containing 10% FCS were stimulated with LPS (100 ng/ml) for the indicated times. Western blotting was performed using Ab specific for phosphorylated c-Jun as described in "Materials and Methods". A representative of three independent experiments is shown.

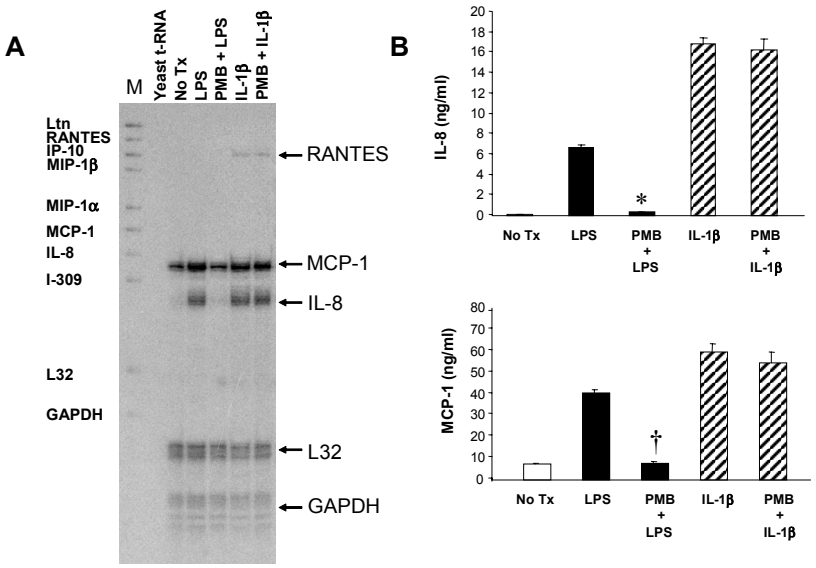

Figure 8. LPS induces the secretion of IL-8 and MCP-1 in activated human HSCs. (A) RNase protection assay was performed to assess mRNA expression of chemokines in HSC. Total RNA was isolated from HSCs culturing in DMEM containing 0.5% FCS after stimulation with LPS (10 ng/ml) or IL-1β (5 ng/ml) for 18 h with or without pretreatment of Polymyxin B (10 IU/ml) for 30 min. Chemokine mRNA levels were determined by a using human cytokine multiprobe template set (hCK-5; PharMingen, San Diego, CA). Thirty μg of total RNA was hybridized with 10^5 cpm of riboprobe. Twenty μg of yeast tRNA was hybridized as a negative control. Lane M contains the radiolabeled markers. Migration of the protected bands is indicated. (B) Secreted IL-8 and MCP-1 were quantified by ELISA. HSCs culturing 0.5% FCS containing DMEM were stimulated with LPS (10 ng/ml) or IL-1β (5 ng/ml) for 24 h with or without pretreatment of Polymyxin B (10 IU/ml) for 30 min. Data represent the mean ± SD of 2 experiments in triplicates. *: $p<0.01$, when compared with HSCs stimulated with LPS; †: $p<0.01$, when compared with HSCs stimulated with LPS. (PMB), Polymyxin B.

7. LPS-induced IL-8 production depends on NF-κB and JNK activation in HSCs

The IκB super-repressor is expressed in Ad5IκB infected HSCs (Figure 9A). This IκB super-repressor (Ad5IκB) completely inhibited LPS-induced NF-κB activation in activated HSCs (Figure 9A). ELISA showed that LPS-induced IL-8 secretion was nearly completely blocked by Ad5IκB (Figure 9B). Activated HSCs were preincubated with DMSO vehicle or 20 μM of SP600125, a selective JNK inhibitor[39] for 2 h and then subsequently stimulated with LPS for 1 h. c-Jun phosphorylation was induced in control and DMSO-treated HSCs by LPS stimulation, but inhibited by SP600125 (Figure 9C). IL-8 secretion was induced in control and DMSO treated HSCs by LPS stimulation, but inhibited about 70% by SP600125 (Figure 9D).

8. LPS upregulates the cell surface expression of adhesion molecules including ICAM-1 and VCAM-1 in activated HSCs

To assess the expression of the adhesion molecules ICAM-1 and VCAM-1, flow cytometry analysis was performed. ICAM-1

and VCAM-1 proteins were expressed on the activated HSCs (Figure 10A-B). LPS significantly upregulated the cell surface expression of ICAM-1 and VCAM-1, an effect that was blocked by polymyxin B (Figure 10A-B).

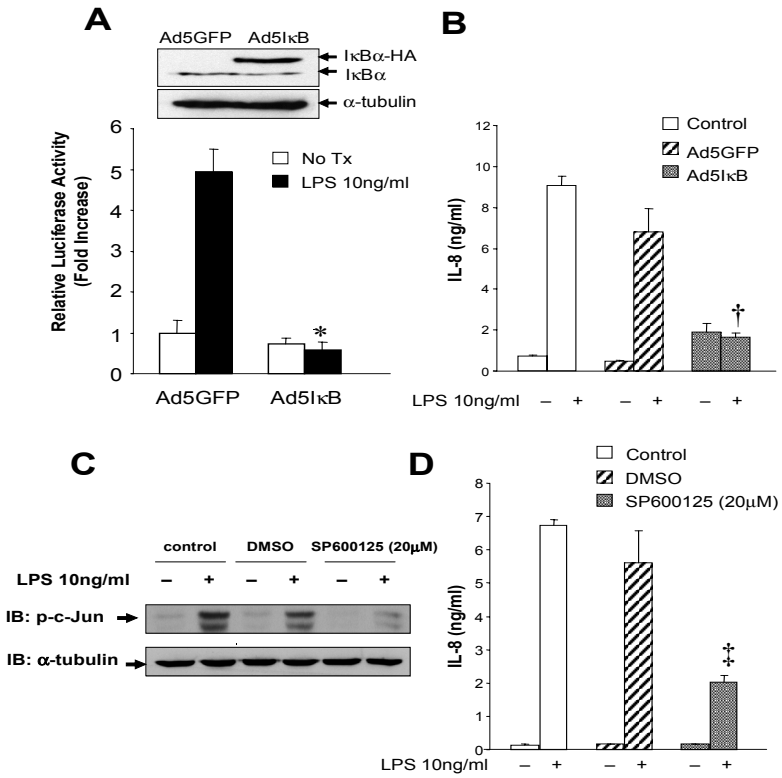

Figure 9. LPS-induced IL-8 secretion is dependent on NF-κB and JNK activity in activated human HSCs. (A) HSCs were co-infected with Ad5NF-κBLuc virus (MOI 500) together with Ad5IκB or Ad5GFP virus (MOI 1000) for 20 h. Infection of Ad5IκB was confirmed by Western blotting of IκBα expression. The hemagglutinin (HA)-tagged IκBα has higher molecular weight than the endogenous IκBα (upper panel). At 20 h post-infection, cells were stimulated with LPS (10 ng/ml) and 10% FCS for 8 h. Cells were lysed, and NF-κB-mediated luciferase activity in each sample was quantified (low panel). (B) HSCs were either left uninfected (control) or infected with Ad5IκB or Ad5GFP virus at a MOI of 1000 particles/cell for 12 h. Cells were stimulated with LPS (10 ng/ml) with 0.5% FCS for 24 h. Secreted IL-8 was quantified by ELISA. (C) HSCs were either left untreated as control, or preincubated with DMSO or SP600125 (20 μM) for 2 h and then stimulated with LPS (10 ng/ml) with 10% FCS for 1 h. Whole cell extracts (20 μg) were assessed for phospho-c-Jun expression using Western Blotting. (D) Human HSCs were either left untreated as control, or preincubated with DMSO or SP600125 (20 μM) for 2 h and then stimulated with LPS (10 ng/ml) with 0.5% FCS for 24 h. Secreted IL-8 was quantified by ELISA from culture supernatant of HSCs. The values in all data represent the mean ± S.D. from two experiments in triplicates. *: P<0.01 when compared with HSCs which were infected with Ad5GFP and stimulated with LPS; †: P<0.01 when compared with HSCs which were infected with Ad5GFP and stimulated with LPS; ‡: P<0.01 when compared with HSCs which were preincubated with DMSO and stimulated with LPS.

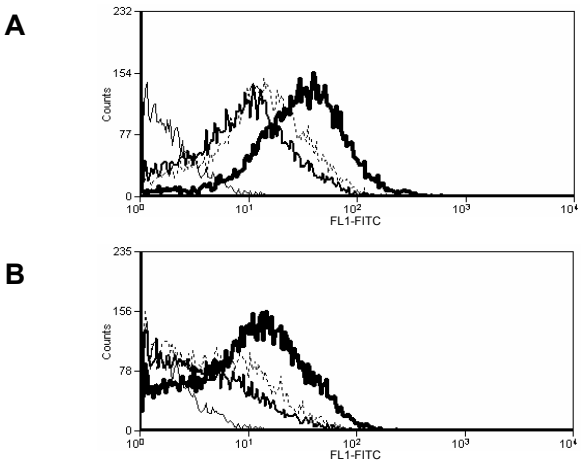

Figure 10. LPS-induced upregulation of ICAM-1 and VCAM-1 is inhibited by Polymyxin B. HSCs were stimulated for 24 h with 10 ng/ml LPS, stained for ICAM-1 (*A*) or VCAM-1 (*B*), and analysed by flow cytometry. *Thin solid line*, Isotype-matched control Ab; *Thick solid line,* ICAM-1 or VCAM-1 expression on untreated HSCs; *Thickest solid line,* ICAM-1 or VCAM-1 expression on LPS-stimulated HSCs; *dotted line*, ICAM-1 or VCAM-1 expression on LPS-stimulated HSCs pretreated with polymyxin B (10 IU/ml) 30 min before each stimulation. Figure is representative of four independent experiments. (FL), fluorescence.

IV. Discussion

There is increasing evidence for a role of portal bacteremia in hepatic inflammation in particular in alcoholic liver disease. The critical cell in the inflammatory process is the Kupffer cell which can be directly activated by bacterial products such as LPS and produce a wide variety of cytokines and reactive oxygen species.[5,7] However, the mechanism by which this inflammatory response can progress to fibrosis is largely unknown. One thesis is that the products of Kupffer cells including TGF-β and reactive oxygen species activate HSCs and therefore there is an indirect effect. Alternatively, the bacterial products can directly stimulate the HSCs or the two pathways can be synergistic. The underlying hypothesis of the present study was that there is a direct role for bacterial products on HSC signal transduction. This is largely been validated in our present studies in which an intact TLR4 pathway was demonstrated in activated human HSCs.

Our study demonstrated that three critical components of LPS signaling receptor complex CD14, TLR4, and MD2 are expressed

in both *in vitro*- and *in vivo*- activated human HSCs. Meanwhile, in quiescent HSCs, low levels of mRNA for TLR4 were detected, but mRNA for CD14 and MD2 was not detected. These results suggest that activation of HSCs is associated with up-regulation of LPS receptors. Activation of HSC results in the acquisition of myofibroblast-like properties, including cell proliferation, pro-inflammatory properties and increased collagen synthesis.[40] This activation process is associated with a dramatic up-regulation of specific cell surface receptors, including receptors for PDGF, TGF-β1, and vasoactive substances.[41] Therefore, the finding that LPS receptors are up-regulated in activated HSCs suggests that receptors mediating the inflammatory actions of HSCs are co-ordinantly up-regulated.

To assess LPS signaling in human HSCs, we investigated the activation of NF-κB and JNK. NF-κB is a critical transcriptional activator involved in the rapid induction of a number of cytokines involved in the inflammatory response.[42] We demonstrated that LPS directly induced NF-κB activation in a time- and dose-dependent manner in activated human HSCs. NF-κB binding activity was increased in activated human HSC after LPS

stimulation, as demonstrated by EMSA. Supershift EMSA experiments demonstrated that p65:p50 and p65:p65 NF-κB dimers are major NF-κB DNA binding complexes induced by LPS in activated HSCs. These findings are consistent with previous reports showing p65 is major NF-κB component in response to TNF-α or IL-1β in activated HSC.[14,28] Activation of LPS-responsive cells, such as monocytes and macrophages, occurs after LPS interacts with circulating LBP.[15] Analysis of LBP-deficient mice showed that LBP was essential for the rapid induction of an inflammatory response by low concentrations of LPS or Gram-negative bacteria.[43] As expected, LPS-induced NF-κB activation in activated human HSCs was dependent on the presence of serum, a source of LBP.

Preincubation with anti-TLR4 blocking Ab (HTA125) before lipid A stimulation significantly decreased NF-κB activation. Immunofluorescent staining also showed that TLR4 bloking Ab (HTA125) inhibited LPS-induced p65 nuclear translocation in HSCs. These results demonstrate that LPS-induced signaling in human HSCs requires TLR4. C3H/HeJ mice are characterized by hyporesponsiveness to LPS.[22,44] C3H/HeJ mice have a missense

mutation within the coding region of the *Tlr4* gene, resulting in a nonconservative substitution of a highly conserved proline by histidine at codon 712.[22] Lipid A failed to induce NF-κB activation in HSCs derived from C3H/HeJ mice, but not from its corresponding control mice, confirming LPS-induced proinflammatory pathway is mediated by TLR4 in HSCs.

Activated JNKs play an essential role in the activation of transcriptional factors, such as c-Jun, ATF-2, and Elk-1.[45] In macrophages, activated JNKs mediate the expression of cytokines such as TNF-α, IL-1, and IL-6,[46] chemokines such as RANTES,[47] all of which often lead to inflammatory responses. We demonstrated that LPS is an effective inducer of JNK activation in activated human HSCs. The kinetics of LPS-induced NF-κB and JNK activation in human HSCs were delayed (maximal 60 min) compared with TNF-α or IL-1β (maximal 15 min), suggesting that the assemblage/recruitment of signaling proteins is slower in endotoxin than in cytokine-stimulated cells.

Chemokines such as MCP-1 and IL-8 may perpetuate liver fibrosis due to their proinflammatory properties. MCP-1 expression is elevated in patients with chronic viral hepatitis and

in experimental liver injury models.[8,9] In cultured HSC, proinflammatory cytokines including TNF-α, IL-1β and IFN-γ potently stimulate MCP-1 expression.[8,12] Activated HSCs promote hepatic inflammation by production of potent neutrophil chemoattractant such as cytokine-induced neutrophil chemoattractant (CINC), the rat homolog to human IL-8.[48] Similarly, serum and hepatic levels of IL-8 correlate with severity of chronic viral hepatitis and liver cirrhosis.[10] LPS induces the expression of MCP-1[12] and MIP-2[13] in activated HSCs in rat. Our study demonstrated that purified LPS induced both MCP-1 and IL-8 mRNA and protein expression in activated human HSCs. HSCs also contribute to hepatic inflammation by upregulating expression on their cell surface of leukocytes adhesion molecules including ICAM-1,[49] and VCAM-1[50] in response to TNF-α or INF-γ. In this report, we demonstrated that LPS upregulated the expression of ICAM-1 and VCAM-1 in HSCs. Thus, LPS may participate in hepatic inflammation by aiding with neutrophil transmigration out of the hepatic sinusoid and into the liver parenchyma during LPS-induced liver injury.

The expression of chemokines (MCP-1, IL-8) and adhesion

molecules (ICAM-1, VCAM-1) genes are regulated by NF-κB.[42] In HSCs, NF-κB is upregulated upon cell activation, and is further induced by cytokines such as TNF-α, IL-1β resulting in the induction of inflammatory chemokines and cell adhesion molecules.[14] We showed that LPS-induced IL-8 secretion was blocked by Ad5IκB, which provides an exogenous nondegradable IκB, blocking NF-κB activation. In HSC, JNK is upregulated upon cell activation, is required for activation of HSC, stimulates HSC proliferation, and decreases collagen α1(I) expression.[39] However, the role of JNK in the inflammatory process in HSCs is unclear. We demonstrated that LPS-induced IL-8 production is partially inhibited by selective inhibition of JNK activation. This study provides a rationale for novel therapies for LPS-induced hepatic inflammation by targeting NF-κB or JNK.

Interestingly, the expression of chemokines such as MCP-1 and MIP-2 induced by LPS was reported as being dependent on the activation state of HSCs in rats. Only fully activated HSCs responded directly to LPS with the release of chemokines in rat.[12,13] This observation may be related to a changes in the expression of LPS receptors between activated and quiescent

HSCs. On the other hand, it may be due to an unidentified postreceptor defect of the NF-κB signaling pathway in quiescent HSC like TNF-α or IL-1β- induced response.[14] Further studies are needed to clarify the exact role of LPS in quiescent HSCs.

In conclusion, our study characterized LPS-induced proinflammatory signaling in activated human HSCs. LPS directly acts through TLR4 and then activates NF-κB and JNK to induce proinflammatory chemokines and adhesion molecules in activated human HSCs. Therefore, HSCs in addition to Kupffer cells may be a target for LPS-induced liver injury, and provide a direct link between inflammatory and fibrotic liver injury.

V. Conclusions

Bacterial LPS stimulates Kupffer cells and participates in the pathogenesis of alcohol-induced liver injury. However, it is unknown whether LPS directly affects HSCs, the main fibrogenic cell type in the injured liver. This study characterizes LPS-induced signal transduction and pro-inflammatory gene

expression in activated human HSCs.

The results as below were obtained:

1. Culture-activated HSCs and HSCs isolated from patients with hepatitis C virus-induced cirrhosis express LPS-associated signaling molecules including CD14, TLR4, and MD2.

2. Stimulation of culture-activated HSCs with LPS results in a rapid and marked activation of NF-κB, as assessed by *in vitro* kinase assays for IκB kinase (IKK), IκBα steady state levels, p65 nuclear translocation, NF-κB-dependent luciferase reporter gene assays and electrophoretic mobility shift assays. Lipid A induces NF-κB activation in a similar manner.

3. Both LPS- and lipid A-induced NF-κB activation is blocked by preincubation with either anti-TLR4 blocking antibody (HTA125) or Polymyxin B.

4. Lipid A induces NF-κB activation in HSCs from TLR4-sufficient (C3H/OuJ) mice but not from TLR4-deficient (C3H/HeJ) mice.

5. LPS also activates c-Jun N-terminal Kinase (JNK), as assessed by *in vitro* kinase assays.

6. LPS upregulates IL-8 and MCP-1 gene expression and secretion. LPS-induced IL-8 secretion is completely inhibited by the I B super-repressor (Ad5I B) and partially inhibited by a specific JNK inhibitor, SP600125.

7. LPS also upregulates cell surface expression of ICAM-1 and VCAM-1.

In conclusion, human activated HSCs utilize components of TLR4 signal transduction cascade to stimulate NF- B and JNK and upregulate chemokines and adhesion molecules. Thus HSCs are a potential mediator of LPS-induced liver injury.

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국문요약

Bacterial lipopolysaccharide 가 사람 간성상세포에서 Toll-like receptor 4 를 매개로 유발하는 염증반응 및 세포내 신호전달과정

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백 용 한

그람음성 세균 내독소는 간내에서 Kupffer 세포를 자극하여 염증성 사이토카인을 분비시키고 활성산소를 유리시키는 등의 작용으로 알코올성 간질환의 중요한 병인으로 간주되고 있다. 간성상세포는 간내 섬유화 및 염증반응에 중심적인 역할을 하는 세포이다. 그러나 현재까지 세균 내독소가 간성상세포에 직접적으로 작용하는지, 작용한다면 어떤 영향을 나타내는지 알려져 있지 않다. 본 연구에서는 세균 내독소가 간성상세포에 직접 작용하여 일으키는 염증성 반응과 이에 관계하는 신호전달체계를 규명하고자 하였다.

1. 배양에 의해 활성화되거나, C형 간염에 의한 간경변증으로 생체내에서 활성화된 사람 간성상세포에서 세균 내독소의 수용체인 TLR4, CD14, MD2 가 발현되었다.
2. 내독소로 사람 간성상세포를 자극했을 때 내독소의 용량과 자극시간에 비례하여 NF-κB가 현저히 활성화되었다.
3. 내독소와 lipid A에 의한 NF-κB 활성화는 TLR4 차단항체와 내독소길항제인 Polymyxin B에 의해 억제되었다.
4. 간성상세포에서 Lipid A에 의한 NF-κB 활성화는 TLR4 유전자

변이로 기능이 상실된 C3H/HeJ 마우스에서는 관찰되지 않았으며 TLR4 기능이 정상인 C3H/OuJ 마우스에서만 관찰되었다.
5. 사람 간성상세포에서 내독소는 c-Jun N-termianl Kinase (JNK)를 활성화 시켰다.
6. 사람 간성상세포에서 내독소는 IL-8과 MCP-1의 발현을 현저히 증가시켰으며 이러한 효과는 NF-κB 억제물질인 IκB super-repressor (Ad5IκB)에 의해 완전히 억제되었고 선택적 JNK 억제제인 SP600125에 의해서 부분적으로 억제되었다.
7. 사람 간성상세포에서 내독소는 세포표면에 발현된 ICAM-1과 VCAM-1의 발현을 유의하게 증가시켰다.

결론적으로 활성화된 사람 간성상세포는 그람음성 세균 내독소에 반응하여 내독소 수용체인 TLR4 관련 신호전달체계를 이용하여 NF-κB 및 JNK가 활성화되고 뚜렷한 염증성 반응을 나타내었다. 따라서 간성상세포는 내독소 유발성 간손상에서 중요한 역할을 하는 것으로 생각된다.

핵심되는 말 : 간성상세포, 간섬유화, 간내 염증반응, toll-like receptor, 세균 내독소