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LPS stimulation-induced regulation of LECT2 expression via TLR4 in hepatocytes

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Leukocyte cell-derived chemotaxin 2 (LECT2), a secreted protein, is implicated in various physiological and pathological processes. As a hepatokine, LECT2 is predominantly synthesized and secreted by hepatocytes, with elevated levels being associated with multiple human inflammatory diseases. Although LECT2 plays a critical role in liver and systemic inflammation, the intracellular signaling mechanisms governing its expression under inflammatory conditions remain unclear. This study demonstrates that lipopolysaccharide (LPS) directly induces LECT2 expression in AML12 mouse hepatocytes. Use of a TLR4-specific inhibitor confirmed that LPS-induced LECT2 expression is mediated via its canonical receptor, TLR4. Furthermore, the p38 MAPK pathway was identified as a key mediator of this response, as evidenced by pharmacological modulation with a p38-specific inhibitor and agonist. Promoter analysis of the Lect2 gene revealed the presence of a putative AP−1-like binding site, suggesting transcriptional regulation by AP−1. Overexpression of c-Fos and c-Jun, along with ChIP-qPCR analysis, confirmed that AP−1 directly binds to Lect2 promoter, and regulates its transcription in response to LPS. Together, these findings reveal a novel TLR4/p38 MAPK/AP−1 signaling axis that, during inflammation, regulates LECT2 expression in hepatocytes, providing new insights into the molecular mechanisms underlying liver inflammation and LECT2-mediated pathophysiology. [BMB Reports 2025; 58(6): 250-256]

INTRODUCTION

Leukocyte cell-derived chemotaxin 2 (LECT2), a secreted protein predominantly synthesized by hepatocytes (1, 2), is involved in diverse physiological and pathological processes (3). Initially identified as a chemotactic factor promoting leukocyte migration, LECT2 has since been recognized as a multifunctional protein with significant roles in immunity, inflammation, and metabolic regulation.

LECT2 functions as a critical component of the innate immune system across various vertebrate species, displaying both antimicrobial properties and immunomodulatory effects. In bacterial infections, LECT2 enhances macrophage function through CD209a receptor phosphorylation (4), as demonstrated in a murine sepsis model, where LECT2 orchestrates innate immune responses by mediating macrophage activation (5). This immune modulation extends to other species; in chickens, *Salmonella enteritidis* infection induces LECT2 expression in heterophils (6), while in lampreys, LECT2 exhibits antibacterial effects against *Escherichia coli* by activating lymphocytes, following lipopolysaccharide (LPS) exposure (7).

Beyond its protective antimicrobial functions, LECT2 also contributes to inflammatory pathologies. In the Lipopolysaccharide/D-galactosamine (LPS/D−GalN)-induced acute liver injury model, *Lect2* knockout mice exhibited significant attenuation of hepatic damage with decreased IFN-γ production in hepatic NK and NKT cells, suggesting that in this context, LECT2 acts as a pathological mediator (8). Similarly, in non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NAFLD/NASH), LECT2 exacerbates disease progression by promoting macrophage infiltration into the liver, while shifting macrophage polarization toward the M2 phenotype (9).

Despite the established importance of LECT2 in immune function and inflammation, characterization of the molecular mechanisms regulating its expression remains inadequate. Research on chicken LECT2 (chLECT2) has provided initial insights, demonstrating that Polyinosinic:polycytidylic acid (Poly I:C), a TLR3 agonist, induces chLECT2 transcription in DF−1 cells through NF-κB and Activator Protein-1 (AP−1) transcription factors (10). However, whether similar regulatory mechanisms operate in mammalian hepatocytes, particularly in response to bacterial components, remains unknown.

The liver, the largest gland in the human body, comprises mainly parenchymal hepatocytes ([70-85]% of liver volume) (11). As a critical barrier against blood-borne pathogens, the liver is constantly exposed to microbial products, such as LPS, via the portal circulation. Hepatocytes play a key role in innate immunity by constitutively producing immune mediators and

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expressing Toll-like receptors (TLRs), including TLR4, which mediate recognition of bacterial components (12). LPS, a major component of the outer membrane of Gram-negative bacteria, activates TLR4, and induces robust inflammatory signaling (13). Understanding the molecular mechanisms of these hepatocyte responses to bacterial components is essential to comprehend liver inflammatory conditions and the regulation of liver-secreted immune modulators.

Although LECT2 has been implicated in liver and systemic inflammation, the intracellular signaling pathways regulating its expression under inflammatory conditions remain largely undefined. This study investigated the effect of LPS on LECT2 expression in murine hepatocytes. Our results demonstrate that LPS induces LECT2 expression via the TLR4/p38 MAPK signaling pathway. Moreover, we provide evidence that the AP−1 transcription factor contributes to the transcriptional regulation of *Lect2* gene, revealing a molecular mechanism by which inflammatory stimuli upregulate LECT2 production in hepatocytes.

RESULTS

Lect2 expression in mouse tissues and its induction by LPS in hepatocytes

First, to determine the tissue-specific expression of *Lect2*, real-time RT−PCR analysis of various mouse tissues was performed. As expected, *Lect2* mRNA in the liver was highly expressed, whereas in other organs, no detectable expression was observed (Fig. 1A), confirming that in mice, the liver is the primary source of *Lect2*.

Next, to investigate the pro-inflammatory regulation of *Lect2*, AML12 mouse hepatocytes were treated with different concentrations of LPS. Both (0.5 and 1) μg/ml LPS significantly increased *Lect2* mRNA expression and LECT2 protein levels (Fig. 1B, C). Given that LECT2 is a secreted protein, its concentration in the cell culture supernatant was also measured. Consistent with intracellular LECT2 upregulation, LPS treatment led to increased LECT2 secretion (Fig. 1D). Time-course analyses using Western blot and RT-qPCR revealed that LPS stimulation induced a time-dependent increase in both LECT2 protein and *Lect2* mRNA levels (Fig. 1E, F), indicating sustained transcriptional and translational activation. Taken together, these results demonstrate that LPS directly induces both the expression and secretion of LECT2 in hepatocytes, highlighting a liver-intrinsic mechanism of LECT2 regulation during inflammatory conditions.

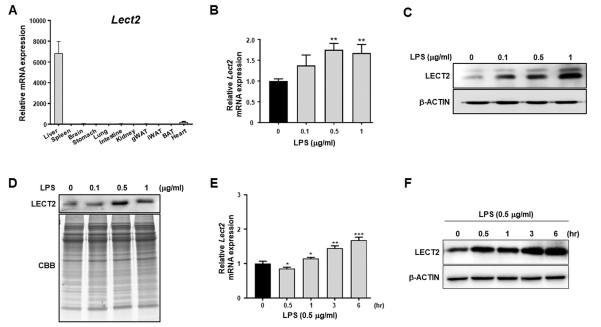

Fig. 1. *Lect2* expression in mouse tissues and its induction by LPS in hepatocytes. (A) The mRNA expression of *Lect2* in different tissues of adult mice was detected by RT-qPCR. After normalizing to β-actin, the relative expression level of *Lect2* in different tissues was compared with that in the iWAT. (B) AML12 cells were treated with different concentrations of LPS for 24 h, and the expression of mouse *Lect2* mRNA was measured by quantitative real-time polymerase chain reaction (qRT-PCR). (C) LPS-induced LECT2 protein level of hepatocytes. (D) LPS-induced protein level of secretory LECT2 of hepatocytes. AML12 cells were treated with the designated concentration of LPS (0, 0.1, 0.5, and 1 μg/ml) for 6 h. Cell lysates were extracted and examined by western blotting with anti-LECT2 antibodies. An anti β-actin antibody was used as an internal control. The blots were prepared in duplicate for each independent western blot for the LECT2 protein. The down blot is Commasie Brilliant Blue (CBB) staining. (E, F) *Lect2* mRNA and protein expression levels of AML12 cells treated with LPS (0.5 μg/ml) were analyzed in a time-dependent manner (0, 15, 30, 60 min, 3 h, 2 h, and 24 h). Values of experiments are represented as mean ± SD (n = 3, independent experiments). *P < 0.05; **P < 0.01 or ***P < 0.001 compared to control as determined by one-way ANOVA.

LPS-induced LECT2 expression in hepatocytes is mediated by the TLR4 pathway

Toll-like receptors TLR4 and TLR2 are the primary pattern recognition receptors responsible for sensing bacterial components, including lipopolysaccharide (LPS) from Gram-negative bacteria (14). Among them, TLR4 plays a more critical role in mediating LPS-induced signaling in hepatocytes, compared to TLR2 (15). To evaluate the involvement of TLR4 in LPS-induced LECT2 expression, AML12 hepatocytes were treated for 6 h with either vehicle control, LPS (1 μg/ml), TAK−242 (a selective TLR4 inhibitor, 3 μM), or LPS plus TAK−242. As expected, LPS treatment significantly increased LECT2 expression, while TAK−242 alone had no effect. Notably, pre-treatment with TAK−242 completely abrogated the LPS-induced upregulation of LECT2 (Fig. 2A, B). These findings indicate that LPS-induced LECT2 expression in hepatocytes is mediated specifically through

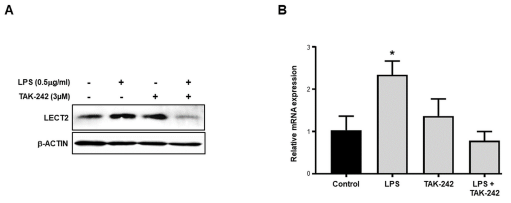

Fig. 2. LPS induces the expression of LECT2 by hepatocytes via the TLR4 pathway. (A) Expression of LECT2 protein level in AML12 cells treated with LPS (1 μg/ml) in the presence or absence of the TLR4 inhibitor TAK-242, (3 μM) for 6 h. (B) Expression of *Lect2* mRNA in AML12 cells treated with LPS (1 μg/ml) in the presence or absence of the TLR4 inhibitor TAK-242 (3 μM) for 24 h. Values of experiments are represented as mean ± SD (n = 3, independent experiments). *P < 0.05 compared to control as determined by one-way ANOVA.

the TLR4 signaling pathway, confirming that under inflammatory conditions, hepatocyte-intrinsic TLR4 activation is required for LECT2 induction.

p38 MAPK plays a central role in TLR4-mediated LECT2 expression in mouse hepatocytes

To elucidate the downstream signaling mechanisms by which LPS induces LECT2 expression, a pharmacological approach was employed, targeting key pathways activated downstream of TLR4. Prior to LPS exposure, AML12 hepatocytes were pre-treated with selective inhibitors. Notably, inhibition of p38 MAPK with SB203580 (3 μM) resulted in a significant reduction in LPS-induced LECT2 protein expression (Fig. 3A). In contrast, inhibition of JNK (SP600125, 3 μM), PI3K (LY294002, 3 μM), or NF-κB (Bay 11−7085, 3 μM) showed no significant effect on LECT2 expression, suggesting a specific role for the p38 MAPK pathway in regulating LPS-induced LECT2 production. To ensure that the suppression of LECT2 was specifically due to interference with LPS signaling, and not a direct effect of SB203580, AML12 cells were treated with SB203580 alone, LPS alone, or their combination. Consistent with previous findings, SB203580 alone did not affect basal LECT2 expression, but when co-administered, effectively inhibited LPS-induced LECT2 upregulation (Fig. 3B). To further confirm that p38 MAPK activation is downstream of TLR4, p38 phosphorylation following LPS stimulation was assessed. LPS-induced phosphorylation of p38 was markedly reduced by pre-treatment with the TLR4 inhibitor TAK−242, indicating that p38 activation is TLR4-dependent (Fig. 3C, D).

In addition, to validate the role of p38 MAPK in LECT2 regulation, AML12 cells were treated with anisomycin (5 μM), a known agonist of p38 MAPK. Anisomycin treatment for 6 h induced dose-dependent increases in both LECT2 protein and *Lect2* mRNA expression (Fig. 3E, F), further supporting the functional role of this pathway. Collectively, these findings indicate that LPS-induced LECT2 expression in hepatocytes is mediated through the TLR4/p38 MAPK signaling axis, establishing p38 as a key molecular regulator of LECT2 production under inflammatory conditions.

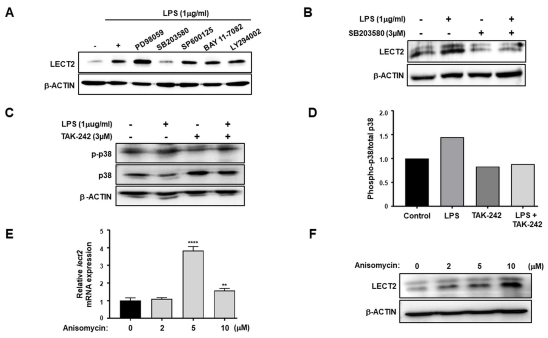

Fig. 3. LPS induces expression of LECT2 in hepatocytes by TLR4-mediated p38 MAPK activation. (A) Expression of LECT2 protein in AML12 cells treated with PD98059 (a MEK inhibitor 3 μM), SB203580 (p38 inhibitor, 3 μM), SP600125 (JNK inhibitor, 3 μM), Bay11-7082 (NF-κB inhibitor 3 μM) and LY294002 (PI3K inhibitor 3 μM) for 1 h, and then treated with or without LPS (1 μg/ml) for 6 h. (B) LPS activates p38, and LPS-activated p38 was reduced by p38 inhibitor in hepatocytes. AML12 cells were treated with LPS (1 μg/ml) for the designated times. AML12 cells were incubated for 6 h with LPS (1 μg/ml) alone or SB203580 (3 μM) in the absence or presence of LPS and the protein levels of LECT2 were measured using Western blot. (C, D) Expression of p-p38 protein level in AML12 cells treated with LPS (1 μg/ml) in the presence or absence of the TLR4 inhibitor TAK-242 (3 μM) for 6 h. Western blot analysis showing differential expression of p-p38 and p38 and their quantification normalized against p38. (E, F) The expression levels of LECT2 protein and mRNA levels in AML12 cells treated with various concentrations of anisomycin, p38 activator for 6 h. The expression level of LECT2 protein and mouse *Lect2* mRNA was measured by western blot with anti-LECT2 antibody and quantitative real-time polymerase chain reaction (qRT-PCR). Values of experiments are represented as mean ± SD (n = 3, independent experiments). **P < 0.01 or ****P < 0.0001 compared to control as determined by one-way ANOVA.

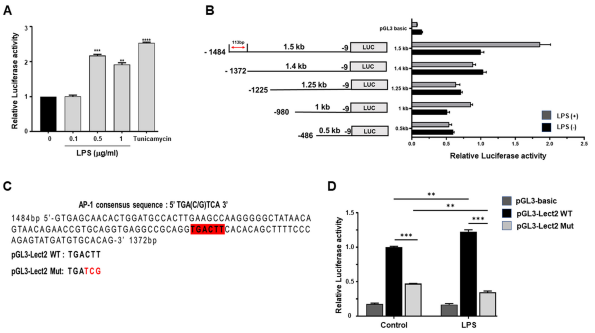

Fig. 4. LPS and TLR4 signaling pathway regulates *Lect2* gene promoter. (A) Effect of LPS molecules on *Lect2* promoter activity. AML12 cells were co-transfected with a *Lect2* promoter luciferase reporter (1.5 kb-Luc) and treated with different concentrations of LPS for 6 h and Tunicamycin. (B) Promoter identification by luciferase assay. Luciferase assay on promoter sequence with 5′ deletions to identify the promoter activity. The left panels show the scheme of the constructs. The right panels show the quantification of luciferase emission with the indicated constructs normalized to an empty vector. The plots represent the mean ± SD of three independent experiments. AML12 were transfected with different *Lect2* promoter constructs subjected to progressive deletions (full-length −1484 construct, and −1372, −1225 −980, and −486 deletion), and the relative luciferase activity (RLU) determined in cells stimulated for 6 h with LPS (0.5 μg/ml) or in unstimulated resting control cells. Luciferase activity was normalized for total protein content. (C) Schematic representation of the location of the putative AP-1 site (−1484 bp/−1372 bp). (D) AP-1 binding site identification of *Lect2* promoter by luciferase assay. AML12 cells were transiently transfected with the wild-type *Lect2* promoter, WT pGL3-Lect2, or with an AP-1-binding site-specific mutated promoter, pGL3-Lect2 Mut with or without LPS (0.5 μg/ml) for 6 h. The firefly luciferase activity of each sample was normalized for total protein content. Values of experiments are represented as mean ± SD (n = 3, independent experiments). **P < 0.01, ***P < 0.001, or ****P < 0.0001 compared to control as determined by one-way ANOVA.

Identification of promoter regulatory elements required for LPS-induced Lect2 gene activation

To investigate the promoter region responsible for LPS-induced *Lect2* gene transcription, a luciferase reporter plasmid (pGL3−Lect2) containing the 5′-flanking region of the mouse *Lect2* gene (−1,484 to −9) was generated. AML12 hepatocytes were co-transfected with this construct, and exposed to various concentrations of LPS. Upon stimulation with (0.5 and 1) μg/ml of LPS, luciferase activity increased approximately 2-fold, indicating that the cloned promoter fragment was responsive to inflammatory stimuli (Fig. 4A). Tunicamycin, previously reported to induce LECT2 expression via ER stress, was used as a positive control (16). To define the critical regulatory region responsible for this LPS response, four serial 5′-deletion constructs of the *Lect2* promoter were created, and their luciferase activity following LPS stimulation assessed. Interestingly, none of the truncated constructs retained LPS responsiveness, suggesting that the LPS-responsive element resides within the −1,484 to −1,372 region of the promoter (Fig. 4B). *In silico* analysis of this region identified a putative AP−1 binding site located between positions −1,457 and −1,451 (Fig. 4C). To assess the functional significance of this motif, a site-directed mutant of the AP−1 site (pGL3−Lect2 Mut) was generated, and its activity compared to the wild-type reporter construct. Under basal conditions (without LPS), the AP−1 mutant exhibited ~50% lower luciferase activity compared to the wild-type, indicating a role for AP−1 in maintaining basal *Lect2* promoter activity. Upon LPS stimulation, the wild-type construct showed a robust increase in luciferase activity, whereas the AP−1 mutant failed to respond (Fig. 4D). These findings demonstrate that a cis-acting AP−1-like element located in the −1,484/−1,372 region of the *Lect2* promoter is necessary for LPS-induced transcriptional activation, implicating AP−1 as a key transcription factor mediating the inflammatory regulation of LECT2 expression in hepatocytes.

AP−1 directly regulates Lect2 promoter activity in hepatocytes

AP−1 is a heterodimeric transcription factor composed of members of the Jun family (e.g., c-Jun, JunD) and Fos family (e.g., c-Fos), often in combination with ATF family proteins (e.g., ATF2). Upon activation of MAPK signaling pathways, c-Jun and c-Fos are synthesized and dimerize to form the active AP−1 complex, which regulates inflammatory gene expression (17). To assess the functional role of the putative AP−1 binding site in the Lect2 promoter, AML12 hepatocytes were co-transfected with the pGL3−Lect2 reporter construct and either c-Jun or c-Fos overexpression plasmids, individually, or in combination. Co-expression of c-Jun and c-Fos significantly enhanced *Lect2* promoter activity, resulting in a ~2-fold increase in luciferase expression. In contrast, transfection with either c-Jun or c-Fos alone failed to stimulate promoter activity, suggesting that functional *Lect2* gene activation requires heterodimeric formation of the AP−1 complex (Fig. 5A). Consistent with this observation, Western blot analysis revealed increased LECT2 protein levels in cells co-transfected with both c-Jun and c-Fos, while transfection with either factor alone had no significant effect on LECT2 expression (Fig. 5B). These results confirm that the heterodimeric AP−1 complex is necessary and sufficient to promote *Lect2* gene expression in hepatocytes.

To determine whether AP−1 directly binds to the endogenous *Lect2* promoter, chromatin immunoprecipitation was performed, followed by qPCR (ChIP-qPCR), using an anti-c-Fos antibody. Fig. 5C shows that the 113 bp *Lect2* promoter region (−1,484 to −1,372) containing the AP−1 binding site was enriched in the c-Fos immunoprecipitated chromatin, confirming that AP−1 directly interacts with the *Lect2* promoter *in vivo*. Taken together, these findings demonstrate that the heterodimeric AP−1 transcription factor (c-Jun/c-Fos) specifically binds and activates the *Lect2* promoter in response to inflammatory stimuli in mouse hepatocytes, establishing AP−1 as a key transcriptional regulator of LPS-induced Lect2 expression.

DISCUSSION

This study demonstrates that lipopolysaccharide (LPS), a widely used experimental endotoxin model to stimulate the innate immune response, induces LECT2 expression in mouse hepatocytes rapidly, and independently of immune cells. These findings are consistent with previous reports in other vertebrate models. For example, in Arctic lamprey (*Lampetra japonica*),

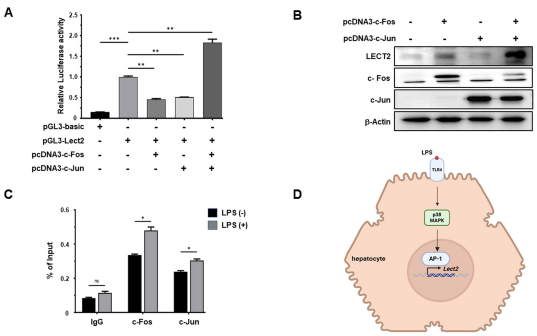

Fig. 5. AP-1 directly regulates *Lect2* promoter activity in hepatocytes. (A) Regulation of *Lect2* promoter activity by activator protein-1 (AP-1). AML12 cells were co-transfected with the wild-type *Lect2* promoter pGL3-Lect2, either alone or with c- Jun, c-Fos or both c-Jun and c-Fos overexpression plasmids. The firefly luciferase activity of each sample was normalized for total protein content. (B) LECT2 protein expression levels in AML12 cell transfected for 6 h with c-Fos, c-Jun or with c-Fos and c-Jun expression plasmid together. (C) Binding of AP-1 to the *Lect2* promoter. Chromatin immunoprecipitation assay using an anti-c-Jun and anti-c-Fos antibody and the primers harboring the AP-1-binding site in LPS-treated (for 1 h) AML12 cells. (D) Proposed mechanism of LPS-induced LECT2 expression in hepatocytes. Values of experiments are represented as mean ± SD (n = 3, independent experiments). *P < 0.05; **P < 0.01 or ***P < 0.001 compared to control as determined by one-way ANOVA.

LPS treatment upregulates *Lect2* mRNA expression in the heart and intestine (7), while in grass carp, Gram-negative bacterial exposure significantly increases *Lect2* mRNA levels in systemic tissues, particularly the head, kidney, and liver (18). Similarly, in zebrafish embryos and larvae, LPS challenge has been shown to enhance LECT2 expression (19).

Our results indicate that the primary receptor responsible for mediating LPS-induced LECT2 expression is hepatic TLR4. Given the rapid response time observed, it is likely that LPS acts directly on hepatocyte TLR4, rather than indirectly through immune cell intermediates. Upon LPS binding, TLR4 initiates two major signaling cascades: one that is MyD88-dependent, activating multiple downstream pathways including the IKK−NF-κB axis and three key mitogen-activated protein kinase (MAPK) pathways: ERK1/2, JNK, and p38 MAPK (20, 21). Importantly, these pathways exhibit significant crosstalk, with TAK1 serving as a common upstream kinase for both NF-κB and JNK signaling. This interconnection allows for complex regulation of inflammatory responses, with NF-κB often modulating JNK-mediated effects on cell survival and apoptosis (22). Recent evidence indicates that inhibition of the TLR4/JNK/NF-κB signaling axis attenuates M1 macrophage polarization in LPS-stimulated conditions (23). Our findings strongly implicate the p38 MAPK pathway in LPS-induced *Lect2* gene transcription. Inhibition of p38 MAPK with a pharmacological inhibitor significantly reduced LECT2 expression following LPS treatment, while activation of p38 MAPK markedly enhanced LECT2 levels, confirming its regulatory role in this context.

We further investigated the transcriptional mechanisms underlying this regulation. Activator protein-1 (AP−1) is a well-known transcription factor complex involved in inflammation, cellular differentiation, and stress responses (24). Upon MAPK activation, transcription of Fos and Jun family genes is induced, promoting formation of the AP−1 complex, which binds to TPA response elements (TREs) within target gene promoters (25). Our study demonstrates that AP−1 is involved in the transcriptional activation of *Lect2* in response to LPS, as supported by promoter analysis, c-Fos and c-Jun overexpression, and ChIP-qPCR data. Interestingly, NF-κB inhibition using Bay 11−7085 showed no significant effect on LPS-induced LECT2 expression, suggesting that NF-κB is non-essential for LECT2 regulation in AML12 cells. This finding contrasts with prior work in the HepG2 cell line, where NF-κB was implicated in LPS-induced LECT2 expression (26). These discrepancies highlight cell-type specific differences in LPS signaling, with normal murine hepatocytes (AML12) relying more on the p38 MAPK/AP−1 axis, while malignant hepatocyte models may involve distinct transcriptional regulators, such as NF-κB. Also, previous studies have shown that endoplasmic reticulum (ER) stress enhances LECT2 expression via ATF4 binding to the Lect2 promoter (16). Given that ATF4 belongs to the AP−1 family, further investigation is warranted to determine whether ATF4 cooperates with c-Fos/c-Jun in LPS-induced Lect2 transcription.

In summary, our data establish that the TLR4/p38 MAPK/AP−1 signaling pathway is a key regulatory axis for LPS-induced LECT2 expression in hepatocytes. These findings elucidate a novel molecular mechanism of LECT2 regulation under inflammatory conditions, while also suggesting that targeting p38 MAPK or AP−1 may offer therapeutic potential for conditions characterized by LECT2 overproduction, such as LECT2-associated amyloidosis.

MATERIALS AND METHODS

Materials

LPS (*Escherichia coli* 026:B6, L2654) and tunicamycin (T7765) from Sigma Aldrich, St Louis, MO, USA. Resatorvid (TAK-242), Adezmapimod (SB 203580), PD98059, LY294002, SP600125, BAY 11-7082, and Anisomycin from TargetMol, Boston, USA.

Cell culture and culture condition

AML12 cells were cultured in a DMEM/F12 containing 10% FBS, 1% insulin-transferrin-selenium (ITS), 40 ng/ml dexamethasone, 1% penicillin, and 1% streptomycin, following ATCC instructions. For long-term culture, cells were subcultured every 3 days.

Plasmid constructs

For overexpression experiments, the following plasmid DNA constructs were used: pcDNA3-FLAG-Fos WT (#8966 Addgene) and pcDNA3.1-Jun (#187902 Addgene).

Western blot analysis

Cell lysate extractions and tissues were prepared using radioimmunoprecipitation assay (RIPA) buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150mM NaCl; 50mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0), as described previously (27, 28). Antibodies against LECT2, Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, and β-Actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Protein precipitation using trichloroacetic acid

Culture media containing proteins secreted from AML12 cells were mixed with an equal volume of 20% trichloroacetic acid (TCA; Sigma-Aldrich) and incubated for 30 min on ice. TCA-mixed samples were centrifuged at 12,000 rpm for 10 min, and supernatants were removed. Pellets were washed using prechilled acetone approximately 4-5 times and boiled with 1× SDS-PAGE sample buffer for 10 min. Samples were analyzed by Western blot for LECT2. Coomassie Brilliant Blue staining was performed with SUN-gel staining solution (LPS solution, Seoul, South Korea) as a loading control.

RNA isolation, cDNA synthesis, and real-time Polymerase Chain Reaction (PCR) analysis

Total RNA was extracted from cell lines used and collected

liver tissues using easy-BLUE™ (iNtRON Biotechnology) according to the manufacturer's instructions, as described previously (29). cDNA (1 μg) was synthesized from RNA using RT Master Mix (Takara Bio). The following primers were used for real-time PCR amplification: LECT2: F, 5′-GGACGTGTGACA GCTATGGC-3′ and R, 5′-TCCCAG TGA ATGGTGCATACA-3′, β-Actin: F, 5′-GGCTGTATTCCCCTCCATCG-3′ and R, 5′-CCA GTTGGTAACAATGCCATGT-3′.

Luciferase constructs and cloning

For the murine *Lect2* promoter, a reporter construct was made by cloning a ~1.5 kb fragment into the pGL3 basic vector containing firefly luciferase. The mouse hepcidin 1.5 kb promoter was constructed by amplifying the PCR product using the forward primer 5′-CTGTGCACATCATACTCTGG-3′ (SacI) and the reverse primer 5′-TGCTTCTTGTTTCTTCCTCT-3′ (XhoI). The product was digested with SacI and XhoI restriction enzymes and inserted into the pGL3 basic vector at the corresponding sites. All cloned fragments were originally amplified from genomic AML12 DNA. The *Lect2* promoter sequence was confirmed by automatic sequencing. Site-directed mutagenesis to inactivate the AP-1 binding site was carried out within the pGL3-*Lect2* construct containing putative AP-1 site between (-1451 and -1457) upstream of the *Lect2* gene using the EZchange™ Site-directed Mutagenesis kit (Enzynomics, South Korea) following the manufacturer's instructions and confirmed by sequencing, as described previously (30). The *Lect2* promoter constructs (2 μg) were transiently transfected into AML12 cells in 6-well plates using the Lipofectamine 2000 according to the manufacturer's protocol.

Chromatin immunoprecipitation-qPCR (ChIP-qPCR)

For ChIP-qPCR experiments, AML12 cells were plated on 10 cm culture dishes. The plating density was controlled such that the culture would reach ~80-90% confluency at the time of collection. 12 h post-plating, LPS (0.5 μg/ml) was added to the cell culture for 1h. Cells were crosslinked in 1% formaldehyde solution for 10 min at room temperature and then added into 1 ml 1 × glycine buffer for 5 min. ChIP assay was performed using the Enzymatic Chromatin IP Kit (Cell Signaling Technology, 9003, Danvers, MA, USA), as described previously (31). Using the micrococcal nuclease in the kit, the nucleoprotein complexes were digested to yield DNA fragments ranging from 200 to 500 bp. The following antibodies were used: anti-c-Fos (#2250; Cell Signaling Technology, USA), anti-c-Jun (#2250; Cell Signaling Technology, USA). For immunoprecipitation reactions, samples were incubated for 12 h at 4°C with rotation. The immunoprecipitate was eluted and reverse-cross-linked, after which the DNA fragments were purified. The DNA products were quantified by qPCR (SimpleChIP® Universal qPCR Master Mix, Cell Signaling), and the primers used for amplifying the promoter of *Lect2* were forward: 5′-AAGCTGTG TGAAGTCACCTG-3′ and reverse: 5′-GACTCATGGCTGAAG ACACC-3′.

Statistical analysis

All quantified data were statistically analyzed using GraphPad Prism. For bar graphs, statistical significance was determined by a two-tailed unpaired Student's *t-test* for comparisons between two groups or a one-way ANOVA test for comparisons among multiple groups. Statistical differences with $P < 0.05$ were considered statistically significant.

ACKNOWLEDGEMENTS

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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