Regulation of LECT2 expression via TLR4 in response to LPS stimulation in hepatocytes

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A Master's Thesis Submitted

to the Department of Medical Science
and the Committee on Graduate School
of Yonsei University in Partial Fulfillment of the
Requirements for the Degree of

Master of Medical Science

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June 2025

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ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude to my graduate advisor, Professor Kyung-Hee Chun, whose guidance, wisdom, and encouragement were essential to completing my master's project. My sincere thanks to my dissertation committee members, Professors Ho Geun Yoon and Jun-Young Seo, for their valuable insights and feedback that significantly shaped my research. I'm deeply grateful to Professors Jae-Woo Kim, Sang-Wook Park, and Jaewoo Choi from Yonsei University College of Medicine's Department of Biochemistry and Molecular Biology for creating such an inspiring academic environment. Special thanks to Dr. Hack Sun Choi, Dr. Myung Sup Kim, Dr. Jung-Hwan Baek, and Dr. Eunjin Koh for their thoughtful guidance throughout this journey. I particularly appreciate Sunyoung Park, M.Sc for her kindness and support, which truly made a difference in my work. I would also like to extend my sincere appreciation to the National Institute for International Education (NIIED) for awarding me the Global Korea Scholarship (GKS), which gave me the invaluable opportunity to study at Yonsei University. Finally, my deepest gratitude goes to my parents, whose unconditional love and unwavering support made this achievement possible. I couldn't have done this without you. I am also profoundly grateful to my two sisters and my brother for their constant encouragement and belief in me throughout this journey.

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Regulation of LECT2 expression via TLR4 in response to LPS stimulation in hepatocytes

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.

Key words : LECT2, LPS, TLR4, p38 MAPK, inflammation, AP-1, hepatocytes

1. 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 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.

2. MATERIALS AND METHODS

2.1. 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.

2.2. 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 sub-cultured every 3 days.

2.3. Plasmid constructs

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

2.4. 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 2mM EDTA, pH 8.0), as described previously [14,15]. Antibodies againstLECT2, Phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, and β-Actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

2.5. Protein precipitation using trichloroacetic acid

For analysis of secreted proteins, conditioned media from AML12 cells were collected and subjected to protein precipitation. Equal volumes of the media and 20% trichloroacetic acid (TCA; Sigma-Aldrich) were combined and incubated on ice for 30 minutes. Following incubation, samples were centrifuged at 12,000 rpm for 10 minutes, and the resulting

supernatants were discarded. The protein pellets were washed 4-5 times with pre-chilled acetone to remove residual TCA. The pellets were then resuspended in 1× SDS-PAGE sample buffer and heated at 100°C for 10 minutes to denature the proteins. Western blot analysis was performed to detect LECT2 protein expression. As a loading control, duplicate gels were stained with Coomassie Brilliant Blue using SUN-gel staining solution (LPS Solution, Seoul, South Korea).

2.6. 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 [16]. 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'-GGACGTGTGACAGCTATGGC-3' and R, 5'-TCCCAG TGA ATGGTGCATACA-3', β-Actin: F, 5'-GGCTGTATTCCCCTCCATCG-3' and R, 5'-CCA GTTGGTAACAATGCCATGT-3'.

2.7. 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 Lect2-Luc 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 [17]. 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.

2.8. 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 [18]. 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-crosslinked, 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'-AAGCTGTGTGAAGTCACCTG-3' and reverse: 5'-GACTCATGGCTGAAGACACC-3'.

2.9. 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.

3. RESULTS

3.1. *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 (Figure 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 (Figures 1B and 1C). 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 (Figure 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 (Figures 1E and 1F), 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.

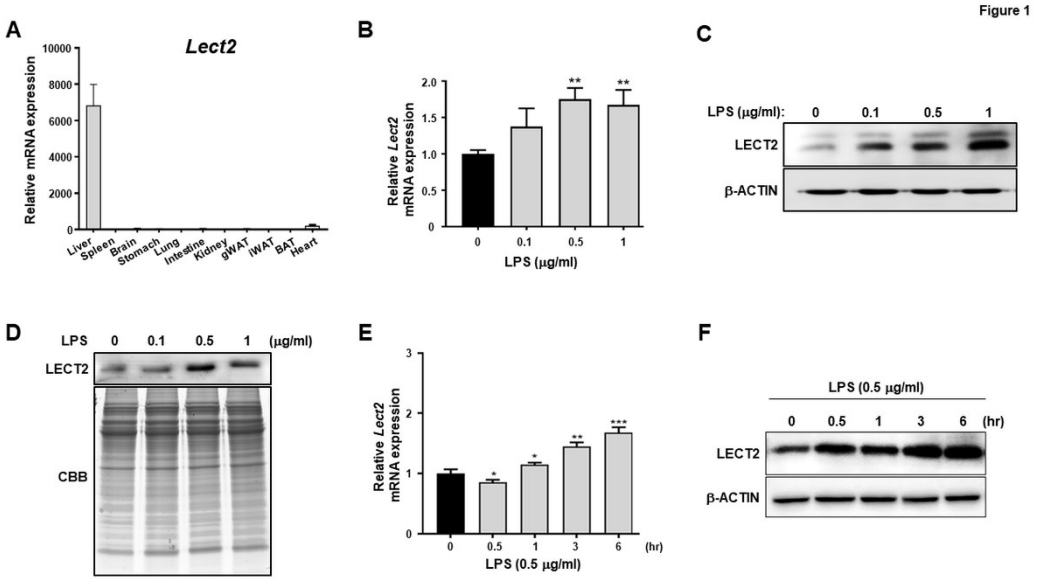

Figure 1. *Lect2* Expression in mouse tissues and its induction by LPS in hepatocytes. (A) The mRNA expression of *Lect2* in different tissues of adult mice was detected by RT-qPCR. After normalizing to β-actin, the relative expression level of *Lect2* in different tissues was compared with that in the iWAT. (B) AML12 cells were exposed to varying concentrations of LPS for a duration of 24 hours, and the levels of mouse Lect2 mRNA were quantified using 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 served as an internal control. Each independent western blot for the LECT2 protein was prepared in duplicate blots. The down blot is Commasie Brilliant Blue (CBB) staining. (E and F) *Lect2* mRNA and protein expression levels of AML12 cells treated with LPS (1 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.

3.2. 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 [19]. Among them, TLR4 plays a more critical role in mediating LPS-induced signaling in hepatocytes, compared to TLR2 [20]. 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 (Figures 2A and 2B). These findings indicate that LPS-induced LECT2 expression in hepatocytes is mediated specifically through the TLR4 signaling pathway, confirming that under inflammatory conditions, hepatocyte-intrinsic TLR4 activation is required for LECT2 induction.

Figure 2

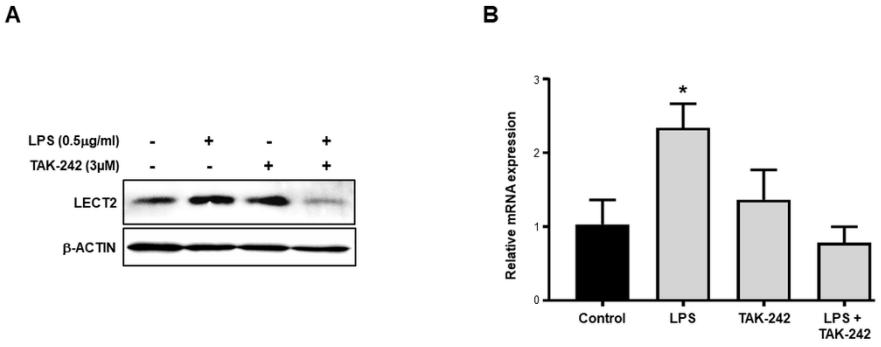

Figure 2. LPS induces expression of LECT2 by hepatocytes via the TLR4 pathway. (A) Expression of LECT2 protein level in AML12 cells treated with LPS (0.5 μg/ml) in the presence or absence of the TLR4 inhibitor TAK-242, (3 μM) for 6 h. (B) The expression of Lect2 mRNA in AML12 cells exposed to LPS (0.5 μg/ml) with or without 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.

3.3. 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 (Figure 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 (Figure 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 (Figures 3C and 3D).

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 (Figures 3E and 3F), 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.

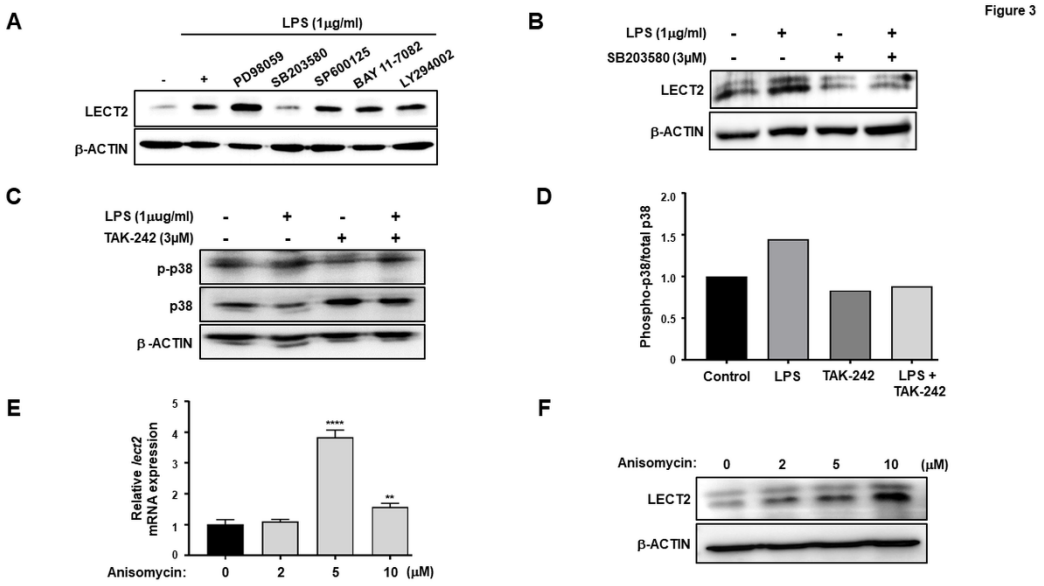

Figure 3. LPS induces expression of LECT2 in hepatocytes by TLR4-mediated p38 MAPK activation. (A) Expression of LECT2 protein in AML12 cells treated with PD98059 (a MEK inhibitor 3 μM), PD98059 (a MEK inhibitor 3 μM), SB203580 (p38 inhibitor, 3 μM), SP600125 (JNK inhibitor, 3 μM), Bay11-7082 (NF-κB inhibitor 3 μM) and LY294002 (PI3K inhibitor 3 μM) for 1 h, and then treated with or without LPS (0.5 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 and D) Expression of p-p38 protein level in AML12 cells treated with LPS (0.5 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 and 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.

3.4. 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 (Figure 4A). Tunicamycin, previously reported to induce LECT2 expression via ER stress, was used as a positive control [21]. 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 (Figure 4B). *In silico* analysis of this region identified a putative AP−1 binding site located between positions −1,457 and −1,451 (Figure 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 (Figure 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.

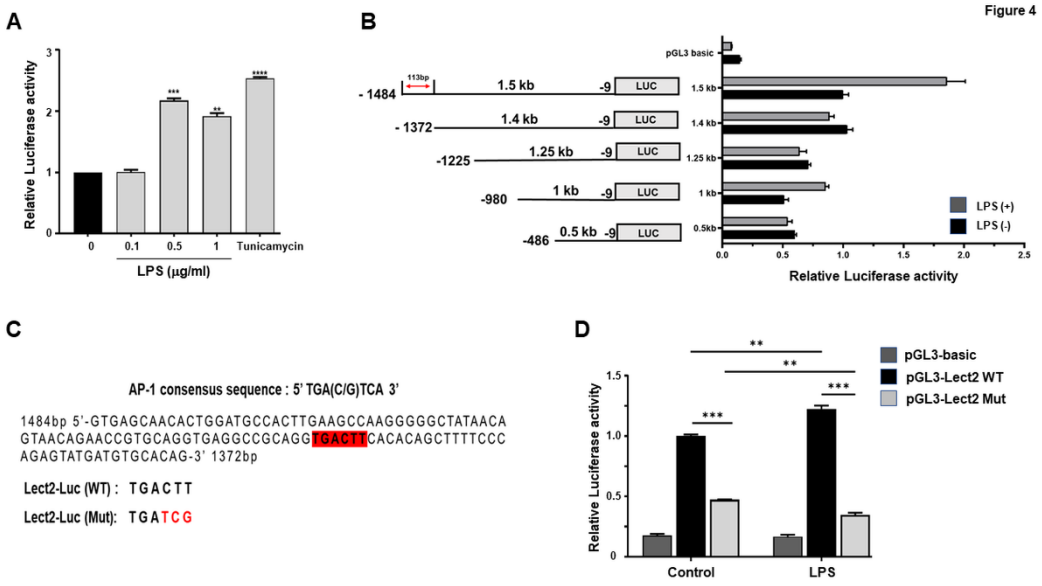

Figure 4. LPS and TLR signal pathway regulates *Lect2* gene promoter. (A) Effect of LPS molecules on *Lect2* promoter activity. AML12 cells were co-transfected with a *Lect2* promoter luciferase reporter (1.5kb-Luc) and treated with different concentrations of LPS for 6 h and Tunicamycin. (B) Promoter identification by luciferase assay. Identification of promoter activity through 5' deletion analysis using luciferase reporter assays. Schematic representations of the deletion constructs are shown in the left panels. Right panels display relative luciferase activity (mean ± standard deviation from three independent experiments) for each construct. 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 (-1484bp/-1372bp). (D) AP-1 binding site identification of *Lect2* promoter by luciferase assay. AML12 cells were transiently transfected with the wild-type *Lect2* promoter, WT Lect2-Luc, or with an AP-1-binding site-specific mutated promoter, *Lect2* (AP-1 MUT)-Luc 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.

3.5. 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 [22]. 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 (Figure 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 (Figure 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. Figure 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.

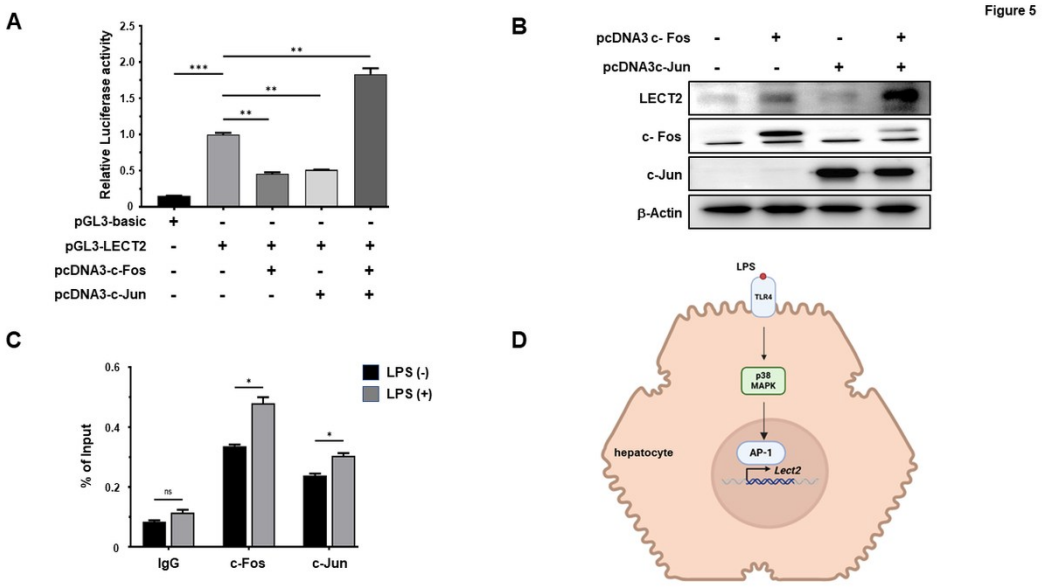

Figure 5. AP-1 directly regulates *Lect2* promoter activity in hepatocytes. (A) Regulation of *Lect2* promoter activity by activator protein-1 (AP-1). AML12 cells were co-transfected with the wild-type *Lect2* promoter, either alone or with c- Jun, c-Fos or both c-Jun and c-Fos overexpression plasmids. 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. A chromatin immunoprecipitation assay was conducted using antibodies against c-Jun and c-Fos, along with primers containing the AP-1-binding site, in AML12 cells treated with LPS for 1 hour. (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.

4. 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*), 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 [23]. Similarly, in zebrafish embryos and larvae, LPS challenge has been shown to enhance LECT2 expression [24].

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 [25,26].

p38 MAPK (MAPK14) is composed of four isoforms: p38α, p38β, p38γ, and p38δ, with p38α being the most prevalent member of the family. The p38α protein kinase plays a role in the transduction of various external signals that affect cellular functions such as inflammation, differentiation, proliferation, and apoptosis[27]. When the MAPK cascade is initiated by the engagement of external receptors, the upstream kinases undergo activation, subsequently leading to the activation of p38α. This process naturally involves phosphorylation at Thr180 and Tyr182 within the activation loop. Subsequently, p38α phosphorylates additional protein substrates including transcription factors[28]. p38α has been associated with numerous inflammatory conditions. In the context of skin injuries, the cell-specific removal of p38 indicates that p38α plays a dual role in modulating

inflammation [29]. Similarly, in a colitis model, the deletion of p38α specifically in myeloid cells resulted in reduced inflammation and a better disease state compared to wild-type mice[30]. In colorectal cancer, p38a signaling plays a dual role in cancer development. While p38a helps prevent inflammation-related epithelial damage and tumor formation, it also promotes the growth and survival of cancer cells[31]. In the liver, p38α inhibits cell proliferation by opposing the JNK–c-Jun signaling pathway[32]. Hepatocyte-specific deletion of p38α in mice reduces oxidative stress–induced NASH but leads to increased simple steatosis [33]. In HFD-induced NAFLD, pharmacological blockade of p38α alleviates hepatic lipid accumulation, decreases apoptosis, mitigates pro-fibrotic alterations, and reduces the expression of pro-inflammatory mediators[34]. Embryonic stem cells that lack p38 MAPK exhibit a reduced ability to generate IL-6 in response to IL-1 stimulation and demonstrate diminished activation of MAPK-activated protein kinase 2 (MAPKAPK2), which is a downstream target of p38α MAPK, under conditions of chemical stress[35]. The p38 signaling pathway enhances hepatic gluconeogenesis, as the activation of gluconeogenic genes by cAMP depends on p38 signaling.[36] Elevated levels of phosphorylated p38 in hepatocellular carcinoma (HCC) tissues have been identified as a marker of poor prognosis, associating with larger tumor size and the presence of satellite tumors [37].

Importantly, TLR4-dwonstream 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 [38]. Recent evidence indicates that inhibition of the TLR4/JNK/NF–κB signaling axis attenuates M1 macrophage polarization in LPS-stimulated conditions [39].

LPS derived from Gram-negative bacteria have been implicated in the pathogenesis of various metabolic diseases. Evidence indicates that LPS levels increase following ingestion of a high-fat meal, even among healthy individuals[40]. Similarly, in individuals with type 2 diabetes, a high-fat diet exacerbates metabolic dysfunction due to increased endotoxin exposure[41]. An obesogenic diet can increase circulating LPS levels, leading to metabolic

endotoxemia, a condition associated with metabolic inflammation and dysglycemia[42]. Moreover, alterations in the gut microbiota contribute to metabolic endotoxemia, inflammation, and related disorders, potentially by increasing intestinal permeability[43].

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 [44]. 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 [45]. 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 [46]. 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 [21]. 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.

5. CONCLUSION

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 and suggest that targeting p38 MAPK or AP-1 may offer therapeutic potential for diseases characterized by LECT2 overproduction, such as LECT2-associated amyloidosis. However, further validation through in vivo studies is required to confirm this mechanism.

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Abstract in Korean

간세포에서 LPS 자극에 대한 반응으로 TLR4를 통한 LECT2 발현 조절

백혈구 유래 케모탁신 2(Leukocyte cell-derived chemotaxin 2, LECT2)는 다양한 생리적 및 병리적 과정에 관여하는 분비 단백질로, 간호르몬(hepatokine)으로서 주로 간세포에 의해 합성 및 분비된다. LECT2 단백질의 높은 발현 수준은 여러 인간 염증성 질환들과 연관되어 있다. LECT2 단백질이 간 및 전신 염증에서 중요한 역할을 한다는 것은 알려져 있으나, 염증 조건 하에서 LECT2의 발현을 조절하는 세포내 신호전달 경로는 아직 명확히 밝혀지지 않았다. 본 연구에서는 lipopolysaccharide (LPS)가 AML12 마우스 간세포에서 LECT2 발현을 직접 유도할 수 있음을 제시하였다. TLR4 억제제를 사용한 실험을 통해, LPS에 의한 LECT2 발현이 해당 리간드의 수용체인 TLR4를 매개로 이루어짐을 확인하였다. 또한, p38 MAPK의 특이적 억제제 및 활성제를 이용하여 AML12 세포주에서 LPS에 의한 LECT2 조절 과정에 p38 MAPK가 관여함을 규명하였다. Lect2 프로모터 분석 결과, LPS에 의한 Lect2 전사 활성에는 AP-1 유사 서열 요소가 관여하는 것으로 나타났으며, c-Fos 및 c-Jun 과발현 플라스미드와 CHIP-qPCR 분석을 통해 AP-1 이형 이합체 전사인자의 역할이 확인되었다. 이상의 결과는 LPS에 의해 유도되는 LECT2 발현에 대한 분자적 기전을 제시한다

핵심되는 말: LECT2, LPS, TLR4, p38 MAPK, 염증, AP-1, 간세포