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TLR4 promotes liver inflammation by activating the JNK pathway

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Abstract. – OBJECTIVE: Drug-induced liver injury has become a serious public health problem that cannot be ignored. Although the mechanism of acetaminophen (APAP)-induced liver injury has been investigated for several decades, there are still many deficiencies. However, only a deeper study of its mechanism can provide more effective measures of prevention and treatment for APAP-induced liver injury. The aim of this study was to investigate whether toll-like receptor 4 (TLR4) participates in and regulates APAP-induced liver injury, which may provide a new direction for the prevention and treatment of clinical drug-induced hepatitis.

MATERIALS AND METHODS: WT mice were treated with APAP (300 mg/kg) or equivalent PBS. The livers of mice were taken at 1 h, 3 h, 6 h and 12 h after treatment. Real-time fluorescence quantitative polymerase chain reaction (RT-qPCR) was used to detect TLR4 mRNA expression level in the liver. After TLR4 involving in APAP-induced liver injury was confirmed, we investigated the relationship between TLR4 expression and hepatic inflammation. WT and TLR4-/- mice received APAP (3000 mg/kg) intraperitoneal injection after 16 h of fasting; serum was collected after 8 h and 24 h, and serum alanine aminotransferase (ALT) and reduced glutathione (GSH) activity were measured. Rat liver tissue was observed for histological changes by hematoxylin and eosin (H&E) staining. RT-qPCR and enzyme-linked immunosorbent assay (ELISA) assay were performed to analyze proinflammatory cytokines expression (such as TNF-α, IL-1β, MCP-1, IL-6). After isolating mononuclear cells (MNCs) in the liver of mice, flow cytometry was used to detect cell activation level and infiltration of macrophages and neutrophils. Western blotting was used to analyze the activation of phosphorylated JNK and p38 signaling pathways in livers of WT and TLR4-/- mice. In addition, after stimulated with APAP, the silence of TLR4 in RAW264.7 cells could activate phosphorylated JNK and p38 signaling pathways.

RESULTS: After APAP stimulation, WT mice exhibited more severe liver injury than TLR4-/- mice, with higher ALT levels, lower GSH levels, and more necrotic or apoptotic cells. TLR4-/- mice have lower levels of inflammatory cytokines including MCP-1 and IL-6; at the same time, the number of infiltrating macrophages and neutrophils in liver tissue of TLR4-/- mice was significantly lower than that of WT mice. The activation of JNK signaling pathway was strikingly enhanced in WT mice treated with APAP, but no significant difference was observed in the activation of JNK phosphorylation in TLR4-/- mice after the same dose of APAP stimulation. Similarly, in RAW264.7 cells, the activation of phosphorylated JNK and p38 was remarkably inhibited by TLR4-siRNA, but was activated in the control group, which was consistent *in vivo*.

CONCLUSIONS: APAP-treated TLR4-/- mice showed milder liver injury compared to WT mice. It was confirmed that TLR4 could activate the JNK signaling pathway to induce the secretion of inflammatory factors and the infiltration of macrophages to promote APAP-induced liver injury. This finding might provide a new prevention and treatment idea for clinical drug-induced hepatitis.

Key Words:
APAP, Drug-induced liver injury, TLR4.

Introduction

Drug-induced liver injury (DILI) is a drugs' common side effect and one of the leading causes of acute liver failure and liver transplantation in Western countries[1]. It is also the main reason leading to a failure of many new drugs development and has been alerted by the Drug Supervision Department after its launch and withdrawn

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from the market[2,3]. Acetaminophen (APAP) is an acetanilide antipyretic analgesic drug. Because of its safe and commonly used dose, it is an over-the-counter drug in most countries, including China. It is the number one antipyretic analgesic drug on the international pharmaceutical market. In China, it has been used as an adjunct to acute and chronic hepatitis for several decades[4], but taking excess APAP is one of the leading causes of DILI globally[5]. In addition, due to individual differences lead to different severity of symptoms, it is difficult to clarify its pathogenesis[6]. After a small amount of APAP enters the liver, only a small amount of biologically active N-acetyl-to-benzenequinoneimine (NAPQI) is produced, and NAPQI can be detoxified in conjunction with reduced glutathione (GSH). However, after taking too much APAP, a large amount of NAPQI will be generated in the liver to excessively consume GSH[7]. Glutathione peroxidase, as the main inactivating enzyme of peroxide, has its function inhibited in the absence of GSH, and its inactivation ability is weakened, leading to the accumulation of peroxide in the body[8]. Some investigations have suggested that the macrophage and its subsequent cascade of inflammatory responses play an important role in APAP-induced liver damage[9], and its role in promoting the development and progression of DILI requires further research. Toll-like receptor (TLR) is a family of receptors that play a crucial role in inflammation and immune responses. In particular, TLR4 plays a role in inducing inflammation and antiviral cytokines[10,11]. Researches have suggested that the pro-inflammatory response induced by TLR4 is regulated by myeloid differentiation of MyD88 or Toll/Integrin-1 receptors, including adaptor protein (TRIF)-dependent mechanisms that activate key transcription factors of pro-inflammatory cytokines[12]. It has also been reported [13,14] that TLR4 may directly activate basic inflammatory pathways such as Jun N-terminal kinase (JNK) and nuclear factor-kappa B (NF-κB) to maintain chronic inflammatory conditions. In this study, we suggested that TLR4 may play a vital role in the promotion of macrophage infiltration and secretion of inflammatory cytokines through activating the JNK signaling pathway, ultimately aggravating liver damage. In this study, we first observed the involvement of TLR4 in the development of DILI by detecting TLR4 nucleic acid and protein expression. Afterwards, we verified its induction of liver inflammatory response by biochemical and pathological analysis, and found that it indeed participated in APAP-induced hepatic inflammation by promoting macrophage infiltration and pro-inflammatory cytokine secretion. Furthermore, we proved its key role in inducing JNK signaling pathway activation both *in vitro* and *in vivo* experiments. Therefore, our results suggest that TLR4 plays a crucial role in APAP-induced liver injury and may be a potential therapeutic target for drug-induced hepatitis.

Materials and Methods

Animal

The experimental mice used in this study were all raised in the Specific-Pathogen-Free (SPF) Experimental Animal Center, and were free to feed and drink in single cages. The TLR4$^{-/-}$ mice used in this study were from the Jackson Laboratory in the United States. We used 8-9 week WT and TLR4$^{-/-}$ mice for experiments with 6-8 mice in each group. This study was approved by the Animal Ethics Committee of Third People's Hospital of Shenzhen City Animal Center.

Animal Model Establishment

8-week-old male WT and TLR4$^{-/-}$ mice were injected intraperitoneally with 300 mg/kg APAP to induce acute liver injury. Specimens were collected after 8 hours and 24 hours.

Biochemical and Pathological Analysis of Liver Injury in Mice

Serum from WT and TLR4$^{-/-}$ mice treated with APAP 8 h and 24 h was sent to the laboratory for detection of alanine aminotransferase (ALT) levels. GSH levels in liver tissue were measured according to the GSH assay kit instructions (Abcam, Cambridge, MA, USA). The liver tissue was sent to Pathology Department for hematoxylin and eosin (H&E) staining, and the damage around the central vein was observed under a microscope.

Real-Time Fluorescence Quantitative Polymerase Chain Reaction (RT-qPCR) Experiments

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse cells and total RNA was extracted, then the RNA was reverse transcribed into complementary Deoxyribose Nucleic Acid (cDNA) and gene expression levels were detected by qPCR. The experiment was repeated three times. Primer sequences are shown in the Table I.

Table I. RT-qPCR primer pairs.

	Primer pairs (5′-3′)
GADPH	Forward: TGAAGGTCGGAGTCAACGGATT
	Reverse: CCTGGAAGATGGTGATGGGATT
IL-1β	Forward: TGGACCTTCCAGGATGAGGACA
	Reverse: GTTCATCTCGGAGCCTGTAGTG
IL-6	Forward: TACCACTTCACAAGTCGGAGGC
	Reverse: CTGCAAGTGCATCATCGTTGTTC
MCP-1	Forward: GCTACAAGAGGATCACCAGCAG
	Reverse: GTCTGGACCCATTCCTTCTTGG
TNF-α	Forward: GGTGCCTATGTCTCAGCCTCTT
	Reverse: GCCATAGAACTGATGAGAGGGAG

Western Blot

After the liver was collected, the total protein was extracted and electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE). The protein was transferred to the membrane at 80 V for 60 min, which was then blocked at room temperature for 2 h and incubated successively with primary antibodies (JNK, p38, and β-actin were 1:1000 dilution) and secondary antibody (horseradish peroxidase (HRP) labeled IgG antibody 1:10000 dilution).

Extraction of Liver Mononuclear Cells (MNCs)

The digestive solution containing collagenase was poured into the liver, which was grounded and filtered on a sieve to prepare a single cell suspension. After separation by centrifugation, the separation liquid was resuspended, the red blood cell lysate was lysed and precipitated. Finally, it was washed again with a buffer containing 0.5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and resuspended in 100 μL of buffer containing 2% fetal bovine serum (FBS), which were the liver mononuclear cell (MNC) suspensions.

Flow Cytometry

In the resuspended MNCs cell suspension, Fc receptor blocker was added first to anti-CD16/32, incubated for 10 min to block non-specific binding, and then flow antibody was added. PE-Cyanine7 labeled anti-mouse CD11b, APC Labeled anti-mouse F4/80, fluorescein isothiocyanate (FITC) labeled anti-mouse Ly6C, PE labeled anti-mouse Ly6G, APC Vio770 labeled anti-mouse CD45, all antibodies, were purchased from eBioscience (San Diego, CA, USA). 1 ml of 2% (fetal bovine serum) FBS was used to wash the cells, which were centrifuged at 400 g for 5 min and resuspended with 400 μl of 2% FBS.

Determination of Serum Cytokine Levels

Mouse serum was collected 8 hours and 24 hours after APAP treatment following the protocol of enzyme-linked immunosorbent assay (ELISA) kit (Bio Legend, San Diego, CA, USA). The microplate reader was used to measure the optical density (OD) of each well at A562 nm wavelength and A450 nm wavelength. A standard curve of cytokine concentration and absorbance values were plotted in Excel.

In Vitro

Control (siCO) and siTLR4 were purchased from Santa Cruz Biotech. (Santa Cruz, CA, USA). RAW264.7 cells were transfected with siCO or siTLR4 (50 nM) using siRNA transfection reagent (TransIT-TKO, Mirus, Madison, WI, USA). After 24 hours of transfection, cells were harvested at 5 min, 20 min, 60 min, 4 h, and 8 h after 10 mM APAP treatment. After protein extraction, Western blot was used to detect the JNK signaling pathway activation.

Statistical Analysis

All experiments were repeated at least three times. All figures in the chart and text were expressed as mean ± standard error. *t*-test was used to compare the differences between two groups. Multiple-group comparisons were performed using one-way ANOVA followed by Post-Hoc Test (Least Significant Difference) and Bonferroni was used to detect significance between groups. All statistical analyses were performed using Graphpad Prism (v6.0, La Jolla, CA, USA). $p<0.05$ was considered statistically significant.

Results

TLR4 Gene Participates in the Pathological Process of APAP-Induced Drug-Induced Liver Injury

To investigate whether TLR4 gene participates in the pathogenesis of APAP-induced drug-induced liver injury, we randomly divided WT male mice of the same age and same weight into two groups and injected intraperitoneally with equal volumes of APAP and PBS. The mice were sacrificed by carbon dioxide asphyxiation at 1 h, 3 h, 6 h and 12 h after injection and the liver tissue of the mice was collected. We used qPCR to detect the expression level of TLR4 mRNA (Figure 1). It could be observed that as time increased, TLR4 mRNA was significantly increased after APAP injection compared to the PBS group and could

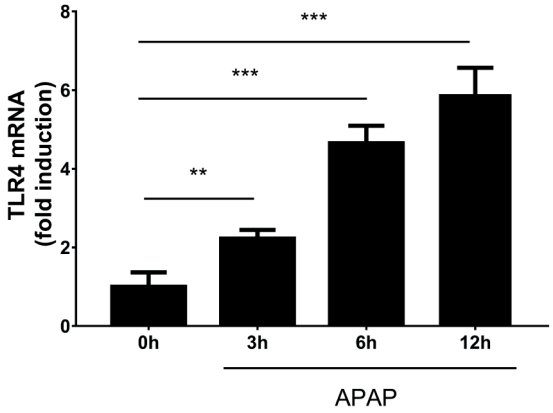

Figure 1. TLR4 gene is involved in the pathological process of APAP-induced drug-induced liver injury. 1 hour, 3 hours, 6 hours, and 12 hours after administration of PBS or APAP injection, ***A,*** TLR4 mRNA expression level in liver of mice was detected by qPCR at each time point; ***B,*** The expression of TLR4 protein in liver of mice at each time point was detected by Western blot (6-8 mice per group). ** $p<0.01$.

last for 12 hours. These results suggested that TLR4 is involved in the pathogenesis of APAP-induced liver injury.

TLR4 Knockdown Reduces APAP-induced Liver Injury

To analyze the role of the TLR4 gene in APAP-mediated drug-induced liver injury, we injected APAP intraperitoneally into male WT mice and TLR4$^{-/-}$ mice. After 8 h and 24 h, blood and liver tissues were collected. After blood centrifugation, serum ALT levels were determined. The results showed that, compared with WT mice, the ALT levels in TLR4$^{-/-}$ mice were significantly lower (Figure 2A). Histological analysis of liver tissue sections revealed that TLR4$^{-/-}$ mice were less sensitive to APAP-induced hepatic injury and histiocytes necrotic cells were significantly reduced than the WT group (Figure 2B). After 8 and 24 hours of APAP injection, liver glutathione levels were significantly increased in TLR4$^{-/-}$ mice (Figure 2C). The above data indicated that defects in the TLR4 gene attenuate liver damage after overabundance APAP treatment.

Defects in TLR4 Gene Can Aggravate Liver Inflammation

After clarifying that the TLR4 gene positively regulates APAP-induced liver injury, we further explored its mechanism. We examined the ex-

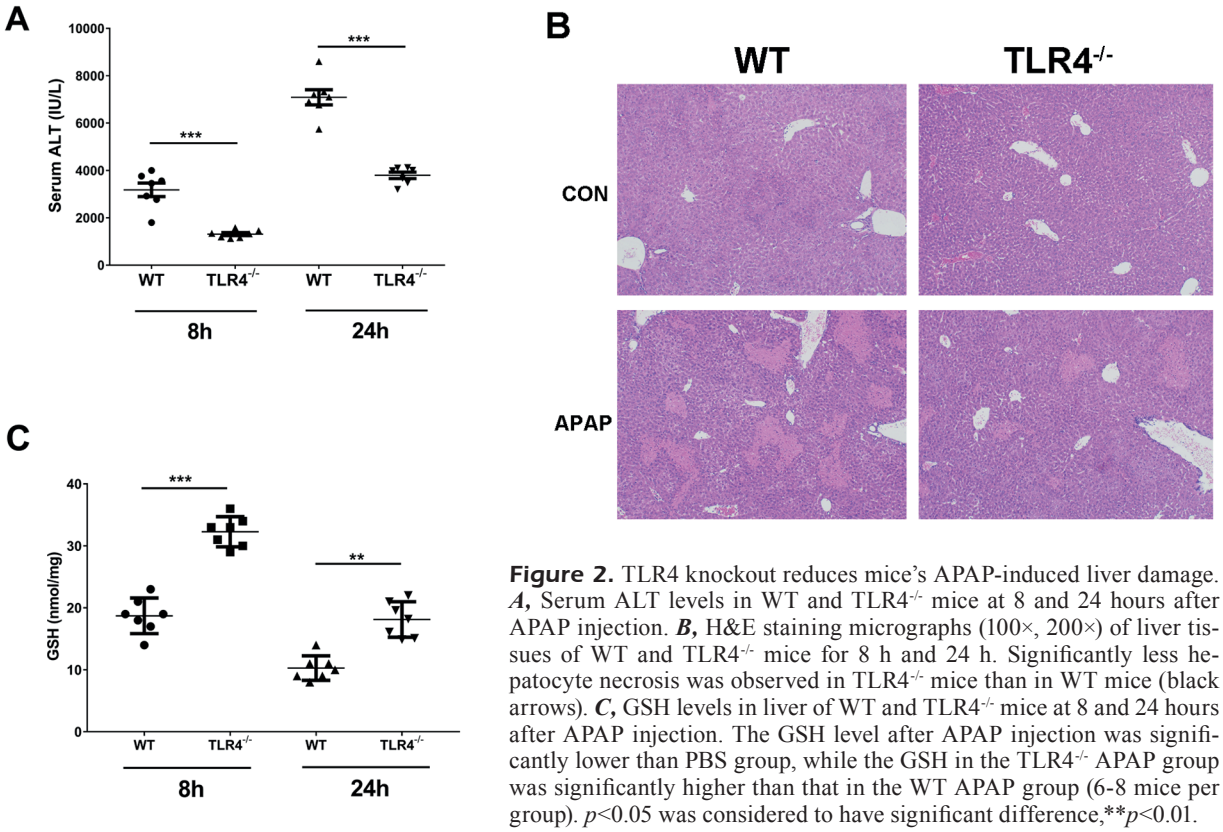

Figure 2. TLR4 knockout reduces mice's APAP-induced liver damage. ***A,*** Serum ALT levels in WT and TLR4$^{-/-}$ mice at 8 and 24 hours after APAP injection. ***B,*** H&E staining micrographs (100×, 200×) of liver tissues of WT and TLR4$^{-/-}$ mice for 8 h and 24 h. Significantly less hepatocyte necrosis was observed in TLR4$^{-/-}$ mice than in WT mice (black arrows). ***C,*** GSH levels in liver of WT and TLR4$^{-/-}$ mice at 8 and 24 hours after APAP injection. The GSH level after APAP injection was significantly lower than PBS group, while the GSH in the TLR4$^{-/-}$ APAP group was significantly higher than that in the WT APAP group (6-8 mice per group). $p<0.05$ was considered to have significant difference,** $p<0.01$.

pression levels of inflammatory cytokines in the WT and TLR4$^{-/-}$ mice liver after APAP injection 24 hours. The levels of IL-6 and MCP-1 mRNA in the liver of APAP-treated TLR4$^{-/-}$ mice were significantly lower, whereas the expression levels of IL-1β and TNF-α were not difference between WT and TLR4$^{-/-}$ mice (Figure 3A). We further detected the expression levels of these inflammatory cytokines in mouse serum and found that consistently with the qPCR results, the levels of IL-6 and MCP-1 in TLR4$^{-/-}$ mice were significantly lower than WT mice (Figure 3B). These data indicated that the TLR4 gene can aggravate APAP-induced liver injury by promoting the secretion of inflammatory factors.

TLR4 Regulates the Secretion of Proinflammatory Cytokines by Regulating the Infiltration of Macrophages

Researches have shown that the TLR4 gene is involved in the inflammatory response of adipose tissue and macrophages, so we wanted to verify whether TLR4 also regulates liver damage and inflammatory response through macrophages in APAP-induced liver injury. We investigated macrophages infiltrate in liver tissue. After 24 hours of APAP administration, liver tissues were taken and liver cells were grouped by flow cytometry. The total number of liver infiltrating macrophages ($CD11b^+Ly6C^+$) and neutrophils ($CD11b^+Ly6G^+$) was significantly higher in TLR4$^{-/-}$ mice than in WT mice (Figure 4), detecting that macrophages promoted the secretion of inflammatory factors, which is the main cause of severe liver damage in TLR4$^{-/-}$ mice.

TLR4 Activates Subsequent Cascade Inflammation by Activating JNK Signaling Pathway

After intraperitoneal injection of APAP in WT mice and TLR4$^{-/-}$ mice for 8 hours, the activation of JNK and p38 signaling pathways was found in

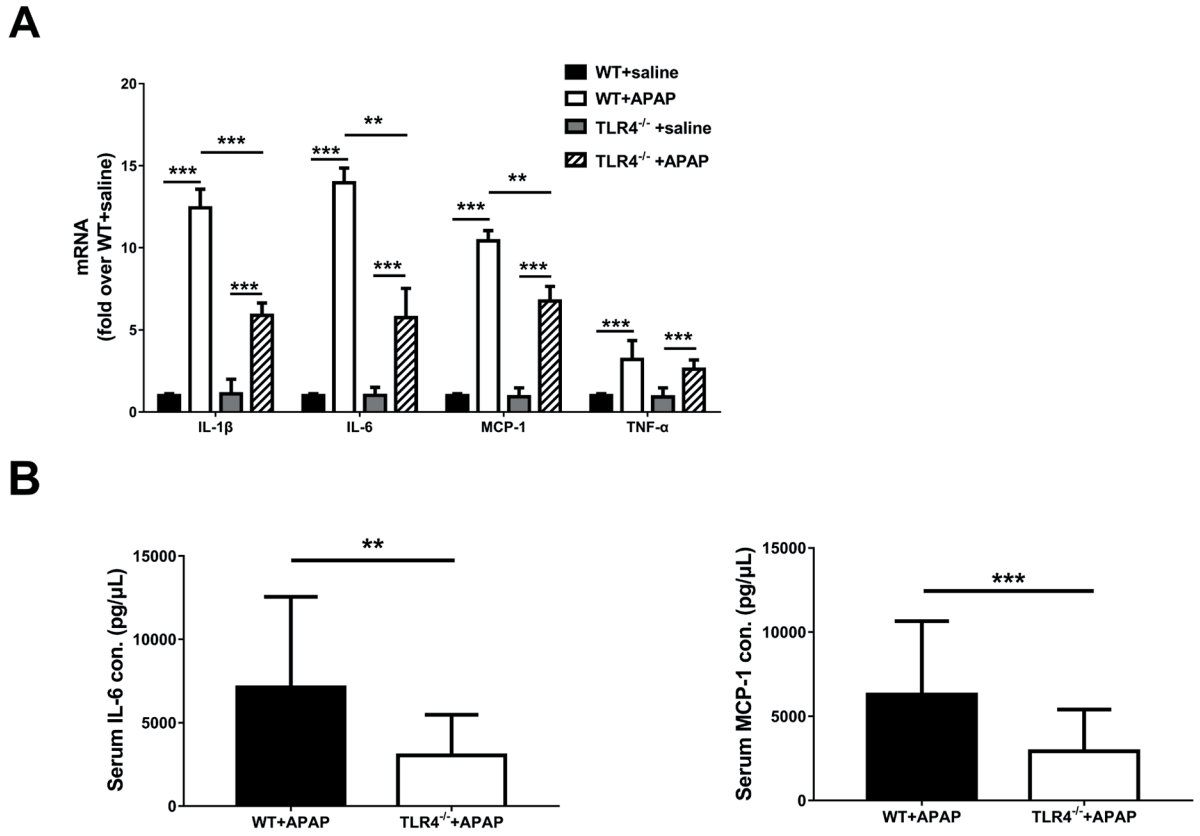

Figure 3. Defects in TLR4 gene reduce liver inflammation. ***A,*** mRNA levels of various inflammatory cytokines in the liver of WT and TLR4$^{-/-}$ mice were measured by qPCR after 24 hours of APAP treatment. ***B,*** Serum IL-6 and MCP-1 expression levels were measured by ELISA. It can be observed that, in WT and TLR4$^{-/-}$ mice, the expression levels of inflammatory cytokines in tissues and serum of treated groups are much higher than that of control groups, while the expression levels of IL-6 and MCP-1 in TLR4$^{-/-}$ treated groups are much lower than WT group. 6-8 mice in each group, each experiment was repeated 3 times. *p<0.05; **p<0.01.

liver tissue. It was showed that WT mice significantly activated JNK and p38 after APAP, there was no difference in activation between APAP and PBS groups in TLR4$^{-/-}$ mice (Figure 5A).

In vivo studies displayed that TLR4 induced liver inflammation through binding to JNK, and we conducted further *in vitro* experiments. As shown in Figure 5B, in the siTLR4 group of RAW264.7 cells, the JNK and p38 signaling pathways were not detected at 5 minutes, 20 minutes, 60 minutes, 4 hours, and 8 hours after APAP stimulation, but were significantly activated in the control group. These data indicated that TLR4 activates the JNK signaling pathway both *in vivo* and *in vitro* to exert a role in promoting macrophage activation and secretion of inflammatory factors, thereby aggravating APAP-induced liver injury.

Discussion

In this study, we demonstrated that administration of APAP activated JNK signaling pathways in liver tissues and cells, induced macrophage infiltration and secretion of proinflammatory cytokines, thus mediated liver damage. The pathogenesis of DILI induced by APAP has three main steps. Firstly, the role of drugs or their metabolites: I, direct injury causes hepatocyte stress response (intrinsic pathway); II, activation of immune response (extrinsic pathway) is also a crucial step; III, direct damage to mitochondrial function. Secondly, MPT. Thirdly, MPT eventually lead to liver cell apoptosis and necrosis[15]. Previous investigations[16,17] have reported that TLR4 is involved in the pathogenesis of multiple liver diseases, especially in the induction of inflammatory responses. Our findings showed that knockout of TLR4 significantly decreased APAP-induced liver injury in mice, with lower ALT levels and less infiltration of necrotic cells around the portal vein. In view of the mechanism of liver tissue injury, we first considered the possibility of an inflammatory response. Combining with the previous literature on mechanisms of TLR-induced inflammatory responses[18-20], we have found that the expression of IL-6 and MCP-1 was essential in APAP-induced liver injury that involves TLR4. After confirming that TLR4 is directly involved in APAP-induced liver injury and inflammation, we speculated that macrophages play a major role in promoting inflammatory secretion[21-24], and found that macrophages and neutrophils infiltrating in the liver tissue are important in the secretion of inflammatory factors. In the process of APAP-induced inflammatory response leading to liver injury, its upstream mechanism still has not been clearly elucidated. We investigated the activation of signal pathways in mice after APAP stimulation, surprisingly discovering the activation of phosphorylated JNK. It was observed that TLR4 induces activation of macrophages by secreting pro-inflammatory cytokines through the activation of JNK i*n vitro* and *in vivo*, which finally aggravates APAP-induced liver injury. It is of great significance to clarify the pathogenesis of DILI for prevention and target treatment of DILI and. However, the current study suggests that the mechanism of DILI is complex and involves various factors such as drug metabolism, mitochondrial function impairment, immune response, signal transduction, genetics and environment. Therefore, the occurrence of DILI is the result of the combined effects of multiple

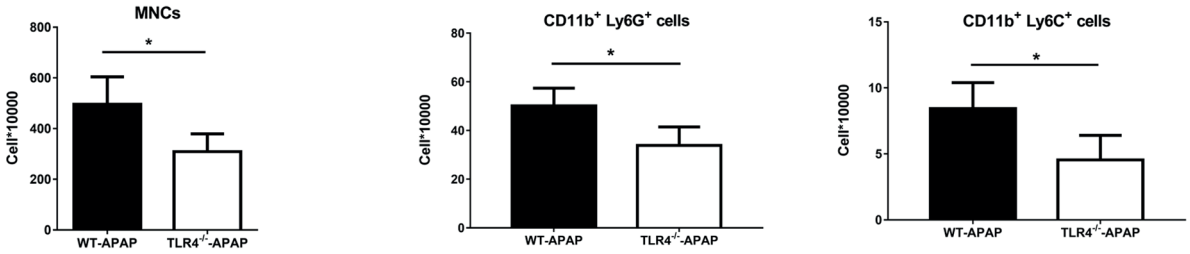

Figure 4. TLR4 gene regulates the secretion of hepatic proinflammatory cytokines by regulating macrophage infiltration. After 24 hours of APAP treatment, liver MNCs were isolated. After staining with the antibody of the flow cytometry, it was observed that infiltration of Ly6C^{+}CD11b^{+} macrophages and Ly6G^{+}CD11b^{+} neutrophils was significantly reduced in TLR4$^{-/-}$ mice liver compared to WT mice (6-8 mice per group). Each set of experiments was repeated 3 times. *p<0.05; **p<0.01; ***p<0.001.

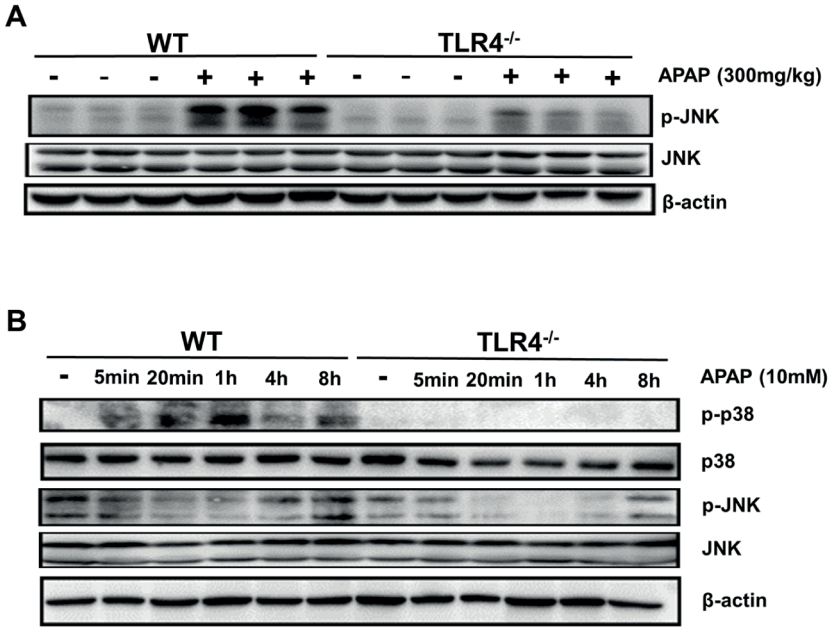

Figure 5. TLR4 activates subsequent cascade inflammation by activating the JNK signaling pathway. ***A,*** Liver tissue was harvested 8 hours after APAP injection and Western blot was used to detect phosphorylation of JNK and p38. It was observed that phosphorylation of JNK and p38 in liver tissue of WT mice were significantly increased after APAP stimulation, whereas activation of JNK and p38 phosphorylation was significantly reduced in TLR4$^{-/-}$ mice APAP group. ***B,*** Transfection and culture of RAW264.7 cells, 5 min, 20 min, 1 h, 4 h and 8 h after APAP stimulation, JNK and p38 phosphorylation were detected by Western blot. It was observed that phosphorylated JNK and P38 activation occurred in the RAW264.7 siCO group after APAP stimulation 5 minutes, whereas phosphorylation of JNK and P38 did not activate in the siTLR4 group (6-8 mice per group). Each set of experiments was repeated 3 times. *p<0.05; **p<0.01.

factors. Through the above studies, TLR4 and JNK play important roles in APAP-induced hepatitis. Our results proved that TLR4 may be a possible therapeutic intervention target for acute hepatitis and liver failure.

Conclusions

TLR4 promoted the infiltration of macrophages in liver tissue by activating the JNK signaling pathway *in vivo* and *in vitro*, thereby inducing the secretion of inflammatory factors and regulating the liver's damage response to APAP.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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