Noncanonical Wnt5a/JNK Signaling Contributes to the Development of D-Gal/LPS-Induced Acute Liver Failure

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Original Article

Abstract—Acute liver failure (ALF) is a deadly clinical disorder with few effective treatments and unclear pathogenesis. In our previous study, we demonstrated that aberrant Wnt5a expression was involved in acute-on-chronic liver failure. However, the role of Wnt5a in ALF is unknown. We investigated the expression of Wnt5a and its downstream c-Jun N-terminal kinase (JNK) signaling in a mouse model of ALF established by coinjection of D-galactosamine (D-Gal) and lipopolysaccharide (LPS) in C57BL/6 mice. We also investigated the role of Box5, a Wnt5a antagonist, in vivo. Moreover, the effect of Wnt5a/JNK signaling on downstream inflammatory cytokine expression, phagocytosis, and migration in THP-1 macrophages was studied in vitro. Aberrant Wnt5a expression and JNK activation were detected in D-Gal/LPS-induced ALF mice. Box5 pretreatment reversed JNK activation and eventually decreased the mortality rate of D-Gal/LPS-treated mice, with reduced hepatic necrosis and apoptosis, serum ALT and AST levels, and liver inflammatory cytokine expression, although the latter was not significant. We further demonstrated that recombinant Wnt5a (rWnt5a)-induced tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) mRNA expression and increased THP-1 macrophage phagocytosis in a JNK-dependent manner, which could be restored by Box5. In addition, rWnt5a-induced migration of THP-1 macrophages was also reversed by Box5. Our findings suggested that Wnt5a/JNK signaling plays an important role in the development of ALF and that Box5 could have particular hepatoprotective effects in ALF.

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INTRODUCTION

Acute liver failure (ALF) is a serious life-threatening syndrome characterized by abrupt hepatocyte necrosis, which results in altered coagulation and mentation [1]. Due to the rapid progression to multiorgan failure and devastating complications, ALF is a disorder with high mortality and resource costs. Although the pathogenesis of ALF is complicated and remains unclear, it is generally

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believed that ALF develops with direct cellular damage caused by viruses, drugs, and other uncommon sources, as well as, more importantly, immune-mediated inflammatory injury [2, 3]. Furthermore, many studies have indicated that the dysfunction of monocytes and macrophages along with their induced inflammatory cytokines play significant roles in the initiation and progression of ALF [4, 5]. ALF induced by D-galactosamine (D-Gal) and lipopolysaccharide (LPS) has been widely used as an animal model to elucidate pathogenesis and evaluate the efficiency of hepatoprotective agents [6, 7]. LPS, part of the outer membrane of gram-negative bacteria, can greatly stimulate the secretion of proinflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) [8]. D-Gal, a specific hepatotoxic agent, can greatly increase hepatocellular death by enhancing their sensitivity to inflammatory injury [9].

Wnt proteins are a large family of secreted glycoproteins that participate in multiple cellular processes, such as cell proliferation, differentiation, migration, polarization, and apoptosis [10, 11]. Recently, an increasing number of studies have begun to focus on the involvement of noncanonical Wnt proteins, particularly Wnt5a, in inflammation [11–13]. Wnt5a expression was upregulated in the sera of patients with sepsis, and it induced the expression of proinflammatory cytokines in macrophages in response to microbial stimulation [14–16]. Moreover, suppression of Wnt5a signaling impaired the ability of macrophages to clear bacterial infection both in vitro and in vivo [17]. As a noncanonical Wnt protein, Wnt5a can activate c-Jun N-terminal kinase (JNK) signaling in inflammatory processes [12, 18]. JNK is a stress-activated kinase in the mitogen-activated protein kinase (MAPK) family. Activated JNK was demonstrated to mediate the hepatotoxic effects in ALF [19, 20]. Regulation of JNK signaling has shed new light on the treatment of ALF [21].

In our previous study, we suggested that Wnt5a expression in sera and peripheral blood mononuclear cells (PBMCs) of patients with acute-on-chronic hepatitis B liver failure (ACHBLF) was significantly increased, and the higher the level of Wnt5a was, the worse the prognosis [22]. However, the role of Wnt5a, especially Wnt5a/JNK signaling, in ALF has not been studied. In this study, we aimed to investigate the role of Wnt5a/JNK signaling and its antagonist Box5 in ALF using a D-Gal/LPS-induced ALF model and THP-1 cells.

**MATERIALS AND METHODS**

**Animals**

Male C57BL/6 mice (6–8 weeks old, weighing 18–25 g) were purchased from Pengyue Laboratory Animal Breeding Co. Ltd. (Jinan, China). All mice were housed under controlled conditions with a standard temperature and humidity, 12 h of light–dark circulation, and free access to food and water. LPS was purchased from Solarbio, D-Gal was purchased from Aladdin, and they were dissolved in sterile normal saline (NS). First, mice were randomly divided into a control group (n = 10) and a D-Gal/LPS group (n = 10). Mice in the D-Gal/LPS group received a combination of D-Gal (500 mg/kg) with LPS (10 µg/kg) intraperitoneally, while mice in the control group received NS only. Five hours later, the mice were sacrificed to obtain liver tissues and blood samples. Second, to investigate the hepatoprotective effect of Box5, sixty additional mice were randomly divided into NS (n = 30) and Box5 groups (n = 30). The mice were injected with Box5 (1 mg/kg; Box5 group) or NS (NS group) intraperitoneally 1 h before D-Gal/LPS administration. In detail, twenty mice in each group were monitored for 72 h after D-Gal/LPS administration for the survival analysis, and ten mice in each group were sacrificed 5 h after D-Gal/LPS administration for other experiments. All animal experiments were approved by the Ethical Committee of Qilu Hospital (Qingdao), Shandong University.

**Cell Culture**

The human monocyte cell line THP-1 was purchased from the Chinese Academy of Medical Sciences and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). THP-1 cells were induced to differentiate into macrophages by incubation with 100-nM phorbol 12-myristate 13-acetate (PMA, Selleckchem) for 24 h. To investigate the effect of Wnt5a on THP-1 macrophages, different concentrations (50, 100, 500 ng/ml) of recombinant Wnt5a (rWnt5a, R&D Systems) were added to the medium of THP-1 macrophages with different exposure periods (1, 3, 6 h).
Histology Staining, Immunohistochemistry and Double Labeling Immunofluorescence Staining

Fresh liver tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4-µm thickness. Deparaffinized sections stained with hematoxylin and eosin (H&E) were evaluated under a light microscope for histological examination. Additionally, the sections were incubated with primary antibody against Wnt5a (1:100; Bioss), followed by incubation with horseradish peroxidase (HRP)-labeled secondary antibody for immunohistochemistry staining. Target proteins were stained with diaminobenzideine (DAB) and evaluated under a light microscope. For double labeling immunofluorescence staining, sections were first incubated with primary antibody against F4/80 (1:3000; Servicebio) overnight and incubated with fluorochrome-conjugated secondary antibodies the next day. Subsequently, sections were incubated with the second primary antibody against Wnt5a (1:200; Bioss) overnight and stained with fluorochrome-conjugated secondary antibodies on the third day. Nuclear staining was conducted using 4′, 6-diamidino-2-phenylindole (DAPI). Fluorescence images were captured under a fluorescence microscope.

Analysis of Apoptosis

Apoptosis was analyzed using a One Step TdT-mediated dUTP Nick-End Labeling (TUNEL) Apoptosis Assay Kit (Beyotime) according to the manufacturer’s suggestions. Sections were deparaffinized and treated with 20-µg/ml proteinase K for 30 min at 37 °C, and then washed three times. Subsequently, these sections were incubated in the dark with TUNEL for 1 h at 37 °C. Finally, fluorescence-labeled images were visualized under a fluorescence microscope.

Liver Function Assays

Blood samples were taken from the hearts of the mice and centrifuged at 3000 rpm for 10 min to separate serum. Liver function markers alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by microplate assay using the corresponding determination kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer’s instructions.

RNA Extraction and qPCR

Total RNA was extracted from liver tissues and cells using TRIzol and was reverse transcribed into cDNA by using RevertAid First Strand cDNA Synthesis Kits (Thermo Fisher Scientific). qPCR was performed using Blaze Taq™ SYBR® Green qPCR Mix 2.0 (GeneCopoeia). Target gene expression was normalized with reference to GAPDH. Primer sequences for qPCR amplification are shown in Table 1.

Western Blot Analysis

To extract total proteins, liver tissues and cells were homogenized in a cold RIPA buffer with phenyl methane sulfonyl fluoride (PMSF) protease inhibitor and phosphatase inhibitor. Protein concentrations were determined by the Bicinchoninic Acid (BCA) Protein Table 1 Primers for real-time PCR

| Primers | Sequences |
|---------|-----------|
| Human wnt5a | 5'-GTC TTGAGCTTGGG-3' 5'-AGCTGTGCTATCAGGA-3' |
| Human IL-6 | 5'-AGCCACTACCTTGTGAGTAC-3' 5'-GCCCTTGGCTGTCATGAC-3' |
| Human IL-1β | 5'-GTGGGATGAGGATCAGTTTC-3' 5'-TAGTGGTGGTCGAGATTCTGA-3' |
| Human TNF-α | 5'-CTGCTGCATTGAGATGT-3' 5'-GATGACTGACTGCTGGG-3' |
| Human IL-10 | 5'-ATGCTTCTAGATCCTGGA-3' 5'-AAATCGATGACGCCGCTA-3' |
| Human GAPDH | 5'-GACCCGCTAAAGCGGAGA-3' 5'-TGTTGAAGACGCCGATGGA-3' |
| Mice wnt5a | 5'-GAATCCCAATTGCAACCTCACC-3' 5'-GCTCTCTGGTACATTTGCCC-3' |
| Mice IL-6 | 5'-TTCATCCGAGCTGCTCTCT-3' 5'-CAGAATTGGCACTCACAAC-3' |
| Mice IL-1β | 5'-GAATGCCCACCTTTTGACAGTG-3' 5'-TGGATGCTTCATCAGGACAG-3' |
| Mice TNF-α | 5'-TCTTCTCATTGCTGTTGG-3' 5'-CACTGTTGTGGCTAGAC-3' |
| Mice IL-10 | 5'-CTTACTGACTGGCATGAGGA-3' 5'-GCAGCTGCTTGGCATGAC-3' |
| Mice GAPDH | 5'-CCATGTCATGCTGGTGAACC-3' 5'-GCCATGAGGCGAGGATGTT-3' |

IL-6 interleukin-6, IL-1β interleukin-1β, TNF-α tumor necrosis factor-α, IL-10 interleukin-10
Assay Kit (Sparkjade). Equal protein samples were separated by SDS–PAGE, transferred onto PVDF membranes, and incubated with primary antibodies against Wnt5a (1:250; R&D Systems), JNK (1:1000; Cell Signaling Technology), phospho-JNK (p-JNK; 1:1000; Cell Signaling Technology), and β-actin (1:1000; Cell Signaling Technology). After staining with HRP-conjugated secondary antibody, protein bands were visualized using the ECL Super Kit (Sparkjade).

Migration Assay

Cell migration was evaluated using 24-well transwells with 8.0-μm pore polycarbonate membrane inserts. Approximately 1 × 10^5 THP-1 cells were suspended in 100-μl serum-free RPMI 1640 medium containing PMA and rWnt5a at different concentrations (50, 100, 500 ng/ml). Cells were seeded in the upper chamber, and 600-μl medium containing 20% FBS was added to the lower chamber. After incubation for 24 h, the cells that migrated to the underside of the upper chamber were fixed in paraformaldehyde and stained with crystal violet. Cell images were visualized under an inverted microscope.

Phagocytosis Assay

A neutral red uptake assay was used to measure the phagocytic ability of THP-1 macrophages. After induction by PMA for 24 h, the cells were cultured with rWnt5a for 1 h, 3 h, and 6 h at various concentrations (50, 100, 500 ng/ml). Then, 100-μl natural red (0.1%, Solarbio) was added to the cells and incubated at 37 °C. Two hours later, the cells were washed three times and lysed overnight at 4 °C with a 200-μl mixture of anhydrous ethanol and acetic acid (volume ratio of 1:1). Finally, the optical density was read at 550 nm, and the value represented the phagocytic ability of the THP-1 macrophages.

Statistical Analysis

Data are expressed as the means ± standard error of mean (SEM). Differences were determined by two-tailed Student’s *t* test or one-way analysis of variance. Statistical analysis was performed and presented using GraphPad Prism 5.0 software. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Hepatic Damage Caused by D-Gal/LPS Administration

With the use of H&E and TUNEL staining, we found that hepatocellular necrosis and apoptosis occurred more frequently in the mice in the D-Gal/LPS group than in those in the control group (Fig. 1a and b). Serum ALT and AST, typical markers of liver damage, were significantly elevated in D-Gal/LPS-treated mice compared to those in the control group (*P* < 0.05; Fig. 1c). Additionally, the mRNA levels of TNF-α, IL-6, IL-1β, and interleukin-10 (IL-10) in the liver tissues of mice in the D-Gal/LPS group were increased compared to those in the control group (*P* < 0.05 for all; Fig. 1d).

Aberrant Wnt5a Expression and JNK Activation in Liver Tissues Induced by D-Gal/LPS

To investigate the expression of Wnt5a in liver tissues, we first detected Wnt5a protein levels by immunohistochemistry staining. As shown in Fig. 2a, a remarkable upregulation of Wnt5a expression was observed in the D-Gal/LPS group compared to the control group, which was further confirmed by quantitative analysis of immunohistochemistry staining (*P* < 0.05; Fig. 2b). Additionally, we observed that liver Wnt5a was primarily expressed in the necrotic area (Fig. 2a), where there were many infiltrating macrophages, but not on viable hepatocytes. To determine the correlation between Wnt5a expression and macrophages, we explored the colocalization of Wnt5a and F4/80 (a pan-macrophage marker) in the liver sections of mice in the control and D-Gal/LPS groups using double labeling immunofluorescence staining. The results in Fig. 2c indicated that Wnt5a was primarily coexpressed with F4/80.

Interestingly, two distinct protein bands of Wnt5a were detected when we further validated our findings by western blot. Notably, the intensities of the upper band (∼58 kDa) were increased in mice in the D-Gal/LPS group (*P* < 0.05), whereas the expression of the lower band (∼43 kDa) was not significantly different compared with those in the control group (*P* > 0.05; Fig. 2d). However, to make matters more interesting, the mRNA expression of Wnt5a tended to be significantly decreased in mice in the D-Gal/LPS group compared with that in the control group (*P* < 0.05; Fig. 2e). To investigate JNK activation in
D-Gal/LPS-treated mice, we performed western blotting with antibodies against p-JNK (activated JNK) and JNK (total JNK). As shown in Fig. 2f, mice in the D-Gal/LPS group showed higher p-JNK/JNK ratios than those in the control group (P < 0.05).

Hepatoprotective Effect of Box5 by Inhibiting Wnt5a/JNK Signaling

To gain insight into Wnt5a signaling in ALF, we evaluated the effect of Wnt5a inhibition with its antagonist Box5. As shown in Fig. 3a, Box5 efficiently alleviated hepatocyte necrosis and hepatic hemorrhage and restored the liver structure. In addition, the number of TUNEL-positive cells (Fig. 3b) and liver damage markers ALT and AST (P < 0.05; P < 0.05; Fig. 3c) were also reduced by Box5. We further analyzed the survival rates of mice in the Box5 group and NS group. As indicated in Fig. 3d, the survival rate of the mice in the Box5 group was significantly lower than that in the NS group (P < 0.05). However, although the mRNA expression of inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IL-10 tended to decrease in mice pretreated with Box5 compared with that in mice given NS, the difference was not significant (P > 0.05 for all; Fig. 3e). To better understand JNK signaling, we further detected whether Box5-pretreated mice show reduced JNK activation. The results suggested that the liver p-JNK/JNK ratio in mice in the Box5 group was reduced compared with that in the NS group (P < 0.05; Fig. 3f).

Mediation of Downstream Gene Expression, Migration, and Phagocytosis of THP-1 Macrophages by rWnt5a

To evaluate the effects of exogenous Wnt5a in vitro, we incubated THP-1 macrophages with rWnt5a at different concentrations or durations. Over a 6-h period, the mRNA expression of TNF-α and IL-6 was distinctly increased at 1 h in THP-1 macrophages treated with 50-ng/ml rWnt5a (P < 0.05; P < 0.05; Fig. 4a). However, no significant difference was detected in the mRNA expression of IL-1β or IL-10 (P > 0.05; P > 0.05; Fig. 4a). Subsequently, we investigated whether cellular functions such as migration and phagocytosis were affected
by rWnt5a. As shown in Fig. 4b and c, we found that the migration of THP-1 macrophages was remarkably improved by incubation with rWnt5a for 24 h, especially after incubation with 50 or 100-ng/ml rWnt5a (P < 0.05). Moreover, the phagocytosis (A550 nm) of THP-1 macrophages was also significantly enhanced when they were exposed to 100-ng/ml rWnt5a for 1 h (P < 0.05; Fig. 4d). However, no significant differences in phagocytosis were detected among cells incubated with 50 or 500-ng/ml rWnt5a for a 6-h duration (P > 0.05 for all; Fig. 4d).

Finally, we detected JNK signaling in THP-1 macrophages treated with rWnt5a. In 1 h, activated JNK rapidly reached a maximum in THP-1 macrophages induced by 50-ng/ml rWnt5a (P < 0.05; Fig. 4e), and higher JNK activation was observed in THP-1 macrophages treated with 50 or 100-ng/ml rWnt5a for an hour compared to cells treated with 500-ng/ml rWnt5a or untreated cells (P < 0.05 for all; Fig. 4f).

Most Effects Exerted by rWnt5a on THP-1 Macrophages Were Reversed by Box5 and SP600125

To better understand Wnt5a/JNK signaling in vitro, we investigated whether inflammatory cytokine expression, phagocytosis and migration of THP-1 macrophages induced by rWnt5a could be reversed by Box5 and a specific JNK inhibitor, SP600125. The data suggested that rWnt5a-induced JNK activation was blocked by 200-µM Box5 (P < 0.05; Fig. 5a) and 40-nM SP600125 (P < 0.05; Fig. 5b). In contrast to the robust upregulation induced by rWnt5a, Box5 and SP600125 remarkably abrogated the increased mRNA expression of IL-6 and TNF-α (P < 0.05; P < 0.05; Fig. 5c and d). Moreover, Wnt5a-induced THP-1 macrophage phagocytosis was also reversed by Box5 (P < 0.05; Fig. 5e) and SP600125 (P < 0.05; Fig. 5f). Finally, as shown in Fig. 5g and i, the
improved migration of THP-1 macrophages induced by rWnt5a was also reversed after coincubation with Box5 ($P < 0.05$). However, SP600125 had no effect on rWnt5a-induced THP-1 macrophage migration ($P < 0.05$; Fig. 5h and j).

**DISCUSSION**

In our study, aberrant Wnt5a expression and JNK activation were detected in a D-Gal/LPS-induced ALF mouse model. In vivo, pretreatment with Box5, a Wnt5a antagonist, restored JNK activation and attenuated D-Gal/LPS-induced liver failure, as indicated by changes in liver pathology and ALT/AST levels, in addition to decreasing the mortality rate. In vitro, we demonstrated that downstream inflammatory cytokine expression and phagocytosis by THP-1 macrophages induced by rWnt5a were dependent on JNK activation, which could be reversed by Box5. In addition, rWnt5a-induced THP-1 macrophage migration was also reversed by Box5.

ALF is a deadly clinical disorder characterized by overwhelming hepatocyte death and rapid deterioration of normal liver function. Central to the pathogenesis of ALF is dysfunction at inflammation and immune responses to various causes, which in turn exacerbates hepatocellular necrosis and apoptosis [23, 24]. Resembling human ALF, coinjection of D-Gal and LPS into mice in our study resulted in large necrotic foci in the liver, increased hepatocellular apoptosis, loss of liver function and elevated expression of inflammatory cytokines.

Since it was first reported that cytokines such as IL-6, interleukin-8 (IL-8), and interleukin-15 (IL-15) were upregulated by Wnt5a in rheumatoid arthritis synovial fibroblasts [25], there has been a huge interest in Wnt5a signaling in the inflammatory process. A subsequent study indicated that Wnt5a promotes interleukin-12 (IL-12) synthesis and enhances human mononuclear cell inflammation induced by microbial stimulation [15]. Consistent with another study indicating that Wnt5a was upregulated in the sera of patients with sepsis [14], our previous study showed that serum Wnt5a...
was increased in patients with ACHBLF compared with that in patients with chronic hepatitis B and healthy controls [22]. In our present study, immunohistochemistry suggested that liver Wnt5a protein expression was significantly elevated in mice in the D-Gal/LPS group compared to that in the control group. However, two forms of Wnt5a with distinct molecular weights were detected in liver tissues, when we further characterized our results by western blot. We observed an increase in the larger molecular weight band of Wnt5a (∼58 kDa) in mice administrated D-Gal/LPS. Similar findings of two or more Wnt5a isoforms in one tissue have been observed in murine lung tissue [26] and neurons [27], although the Wnt5a forms in those studies had different molecular weights than ours. Wnt proteins rely heavily on posttranslational modifications, such as glycosylation and palmitoylation, for their secretion and function, and they can also agglomerate into multimeric or oligomerized complexes [28, 29]. Perhaps this partly explains our results, which undoubtedly need further in-depth study to confirm. To gain more insight into Wnt5a in ALF, we also evaluated Wnt5a transcription by qPCR. Interestingly, Wnt5a mRNA expression in liver tissues of mice in the D-Gal/LPS group was decreased compared with that in the control group, which was the opposite of its protein expression pattern. This contradictory expression of Wnt5a transcription and translation in the liver tissue of D-Gal/LPS mice

Fig. 4 Recombinant Wnt5a (rWnt5a) induced downstream regulation, migration and phagocytosis of THP-1 macrophages. a Relative mRNA levels of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and interleukin-10 (IL-10) in THP-1 macrophages treated with 50 ng/ml rWnt5a for 1 h, 3 h, and 6 h. b Transwell migration assay for THP-1 macrophages treated with different concentrations of rWnt5a (50, 100, 500 ng/ml) for 24 h (magnification, ×200). c Quantification analysis of migratory cells in five fields counted using ImageJ. d Neutral red uptake assay for THP-1 macrophages exposed to different concentrations of rWnt5a (50, 100, 500 ng/ml) for 1 h, 3 h, and 6 h using western blotting. e Assessment of JNK activation in THP-1 macrophages treated with rWnt5a at 50, 100, 500 ng/ml using western blotting. Data are shown as the means ± standard error of mean (SEM). *P < 0.05.
was similar to that observed in hepatocellular carcinoma and paracarcinoma liver tissues [30]. Thus, we speculated that in addition to posttranslational modifications, which require further exploration in the future, other mechanisms involved in the regulation of Wnt5a protein expression remain unclear. Nevertheless, these data suggested that Wnt5a signaling was involved in the development of ALF.

To determine the potential role of Wnt5a in ALF, we evaluated the effect of Wnt5a inhibition by its antagonist Box5 on the development of ALF. Box5 has been described to be a competitive inhibitor of Wnt5a through binding to its receptor to inhibit the biological activity of Wnt5a signaling [31]. Our results in the present study clearly showed that Wnt5a inhibition by Box5 alleviated pathologic severity, ameliorated liver function, and decreased the mortality rate of ALF mice. In addition, Box5 pretreatment also reduced the stimulation of inflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IL-10, induced by D-Gal/LPS, although the effect was not significant. These results were consistent with those suggested by Li et al. in diabetic nephropathy [32]. Taken together, these results indicated that Wnt5a inhibition by Box5 could be a potential therapeutic strategy for ALF.

Noncanonical Wnt5a signaling comprises two main pathways: the Wnt5a/Ca2+ pathway and the Wnt5a/JNK or planar cell polarity (PCP) pathway [33]. JNK signaling has been widely demonstrated to be involved in inflammation [32, 34, 35]. Activated JNK participated in the stimulation of many inflammatory cytokines by LPS [35, 36]. Phosphorylation of JNK mediated hepatotoxicity in acetaminophen-induced ALF [20]. Moreover, by attenuating JNK-mediated mitochondrial translocation, D-Gal/LPS-induced ALF could be ameliorated [37]. JNK inhibition with two different JNK inhibitors in vivo markedly reduced hepatic necrosis and apoptosis in paracetamol-induced ALF [38]. In this regard, we focused on JNK signaling as the downstream pathway of Wnt5a in our present research. As expected, activated JNK was detected in the liver tissues of mice in the D-Gal/LPS group, and this was restored by Box5 pre-treatment. In summary, the results showed that Wnt5a/JNK signaling plays an important role in the presence and progression of ALF.
Basal Wnt5a expression was observed in PBMCs, PBMC-derived macrophages, alveolar macrophages, microglia, and macrophage cell lines [12]. We found that Wnt5a was overexpressed on liver macrophages but not hepatocytes. Monocyte and macrophage dysfunction is central to ALF development [4]. It is widely known that liver macrophage recognition and phagocytosis of pathogens or debris and the subsequent stimulation of inflammatory cytokine expression are necessary for the initiation and propagation of ALF; furthermore, monocytes are recruited to differentiate into macrophages to expand the macrophage pool and promote tissue destruction. Considering the above aspects, we explored the role of Wnt5a/JNK signaling in the activation of THP-1 macrophages in vitro; these cells are originally a monocyte cell line, but they were induced to differentiate into macrophages by PMA in our study. The results indicated that rWnt5a could induce increased mRNA expression of TNF-α and IL-6 and enhance THP-1 macrophage phagocytosis and migration. With the exception of migration, the regulation of these cellular events was dependent on JNK signaling, as the JNK inhibitor SP600125 completely abolished these effects. Box5 not only restored the above modulations of THP-1 macrophages but also blocked the activation of JNK induced by rWnt5a. In summary, our findings support that JNK signaling participates in the regulation of macrophages and that Wnt5a is a regulator of JNK signaling. Furthermore, we show that Wnt5a/JNK signaling participates in the development of ALF, probably by regulating the activation of macrophages.

Currently, the mainstay of ALF management is supportive care and liver transplantation [39]. Unfortunately, few disease-specific or general interventions are available to improve outcomes in supportive systems [40]. Although liver transplantation is the determined cure for ALF, this treatment is challenged by the availability of suitable organs, its high cost and the possibility of rejection after transplantation. Hence, exploring other strategies to prevent liver failure and increase overall survival has been of interest. Targeting molecules that are involved in and determine the prognosis may contribute to the treatment of ALF. In our present study, we demonstrated that Wnt5a signaling plays an important role in the development of D-Gal/LPS-induced ALF mice and that its antagonist Box5 could improve the prognosis of these ALF mice. It is not only helpful to reveal the pathogenesis of ALF but also provides new ideas for reducing the incidence and mortality of ALF.

There are some limitations in our present study. First, in our present study, we initially suggested that aberrant Wnt5a signaling was detected in D-Gal/LPS-induced ALF mice and that Box5, a Wnt5a antagonist, specifically attenuated D-Gal/LPS-induced liver failure. Although the results are convincing since all the experiments have been repeated at least three times, the role of Wnt5a signaling in ALF should be validated and explored more in a larger number of mice in our future studies. Second, the D-Gal/LPS-induced ALF model in our present study cannot completely mimic the full development and progression of human liver failure. However, no single animal model has been established that can accurately reproduce all facets of human ALF up to date. Considering that the ALF model induced by D-Gal and LPS is useful to study inflammatory liver injury and test potential hepatoprotective agents [6, 7], we regard this model as the optimal model for our study. Third, the mechanism underlying the contradictory expression of Wnt5a mRNA and protein remains unclear. Since our study focused more on the function of the Wnt5a protein, more research concerning the regulation of transcription and translation is needed in the future. Fourth, the Wnt5a-knockout mouse model would be considered the most accurate experimental tool to investigate its function. Unfortunately, homozygous Wnt5a-knockout mice exhibit perinatal lethality due to developmental defects [41], and Wnt5a-siRNA knockdown in mice may not block liver Wnt5a protein expression, as suggested by our above results. Therefore, we selected Box5, a Wnt5a antagonist, to inhibit the Wnt5a protein in mice. If more insight into Wnt5a is further determined, a conditional Wnt5a-knockout mouse model may be the best option in future studies.

In conclusion, our findings provide strong evidence that aberrant Wnt5a/JNK signaling mediated massive hepatocellular necrosis and apoptosis, increased serum ALT and AST, and elevated inflammatory cytokine expression in D-Gal/LPS-induced ALF mice. Box5, a Wnt5a antagonist, efficiently abolished these effects and eventually improved the outcomes of D-Gal/LPS-induced ALF mice. Moreover, Wnt5a, expressed primarily on liver macrophages, was demonstrated to induce the activation of THP-1 macrophages in a JNK-dependent manner, which was also reversed by Box5. Overall, our results supported that Wnt5a/JNK signaling is involved in the development of ALF, partly via the regulation of macrophages, and Box5 may be a potential effective agent for the treatment of ALF.
AUTHOR CONTRIBUTION

Xiang-Fen Ji designed the study, carried out the experiments, and wrote the first draft of the manuscript. Fei Sun performed some experiments. Jing-Wei Wang analyzed some data. Yu-Chen Fan analyzed some data and was involved in editing the manuscript. Kai Wang revised the manuscript critically for important intellectual content.

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DATA AVAILABILITY

All data generated or analyzed during the study are included in this published article.

Declarations

Ethics Approval All the animal experiments were approved by the Ethical Committee of Qilu Hospital (Qingdao), Shandong University.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

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