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Chi3l1 regulates APAP-induced liver injury by promoting macrophage infiltration

Y. WANG[1], M. ZHONG[2], W. WANG[3], Y.-H. LI[1]

[1]Department of General Surgery, China-Japan Union Hospital of Jilin University, Changchun, China
[2]Department of Respiratory, China-Japan Union Hospital of Jilin University, Changchun, China
[3]Department of Breast Surgery, Jilin Province Tumor Hospital, Changchun, China

Abstract. – OBJECTIVE: This study aims to investigate the role of Chi3l1 in Acetaminophen (APAP)-induced liver injury.

MATERIALS AND METHODS: *In vivo* model of liver injury was established in mice administrated with APAP (250 mg/kg) or equivalent phosphate-buffered saline (PBS). Mouse liver tissues were collected at 1 h, 3 h, 6 h, 12 h, and 24 h after treatment, respectively. ALT levels and apoptosis were evaluated. Additionally, we established APAP-induced acute liver injury model in wild-type (WT) mice and Chi deficient (Chi3l1$^{-/-}$) mice. Pathological of liver tissue were observed by hem y-lin and eosin (HE) staining. Mononuclear (MNCs) were isolated from mouse liver, and amounts of infiltrating macrophages and ne trophils were then counted b ytometry Serum levels of cytokines cted by enzyme-linked immunoso assa ELISA). Bone marrow-derived n phage MDMs) were extracted from ea se.

RESULTS: After A P n, Chi3l1$^{-/-}$ mice showed more re liv y than that of WT mice, whi was mani as higher ALT levels a re necrotic ptotic cells. Compa T mice, Chi$^{-/-}$ mice expressed h er lev inflammatory cytokines (M P-1 and IL- crophage-associated m ules (CD68 a 86), as well as the ounts of infiltrati macrophages and rophil addition, higher expression inflam tory cytokines were found in BM xt d fro T mice treated with those l sates ved from Chi3l1$^{-/-}$ mice thos on ted cells. APAP-treated $^{-/-}$ mic d more severe liver injury tha t of W e.

C LUSIONS: Our study confirmed that Chi e liver function from APAP-in- d injury inhibiting the secretion of inflam- ory factors and macrophage infiltration.

rds:
Drug-induced liver injury, Chi3l1.

uction

duced liver (DILI), as a com- ide effect of drugs, is one of the leading ses of acute patic failure and liver trans- ntation in W rn countries[1]. DILI is the r reason for failure of many new drugs d ment, w is also being concerned by the department[2,3]. Acetaminophen (APAP) is an acetanilide antipyretic analgesic, which is one of the most popular non-prescrip- in the pharmaceutical market due to safe and reliable dose. In China, APAP has been used as an adjunct to acute and chronic hepatitis for several decades[4]. Overdose of APAP is one of the leading causes of DILI in the world[5], accounting for more than 50% of reported DILI. Severities of symptoms caused by APAP are varied because of individual differences, which makes it difficult to determine its pathogenesis[6]. After a small amount of APAP enters the liver, it produces biologically active N-acetyl-to-benzenequinoneimine (NAPQI), which further exerts its detoxification function by combining with reduced glutathione (GSH). However, overdose of APAP will cause NAPQI accumulation in the liver and excessive consumption of GSH[7]. Glutathione peroxidase is the main inactivating enzyme of peroxides. GSH deficiency significantly inhibits glutathione peroxidase, thereby leading to accumulation of peroxides in the body[8]. It has been reported that macrophages and their related inflammatory responses exert an important role in APAP-induced liver damage, while their roles in promoting the occurrence and progression of DILI require further explorations[9]. Chi3l1 (chitinase 3 like 1) is a chitinase-like soluble secretory protein without any enzymatic activity[10]. It can be produced by a variety of cells, including neutro-

Corresponding Author: Yuhui Li, BM; e-mail: 43790964@qq.com

phils, macrophages, chondrocytes, synoviocytes, smooth muscle cells, endothelial cells and tumor cells[10,11]. Accumulating evidences have suggested that elevated level of Chi3l1 is associated with poor prognosis of liver diseases, such as liver fibrosis, non-alcoholic fatty liver, alcoholic liver disease and hepatocellular carcinoma[12,13]. We speculated that Chi3l1 exerts a crucial role in promoting macrophage infiltration and secretion of inflammatory factors, thereby aggravating liver damage. In this study, we first confirmed that Chi3l1 participated in the development of APAP-induced DILI by detecting the mRNA expression of Chi3l1. Second, we verified that APAP could induce hepatocyte apoptosis, macrophage infiltration and inflammatory response. Therefore, our results suggested that Chi3l1 participated in APAP-induced liver injury and might be served as a potential therapeutic target for drug-induced hepatitis.

Materials and Methods

Animal Rearing

Chi3l1$^{-/-}$ mice used in this study were ...rted from the Jackson Laboratory in the U... States. 8-week old wild-type (WT) and Chi... mice were fed in our SPF (specific ...thogen-free Experimental Animal Center ... mice per group. All mice were suppli... with f... ccesses to food and drinking wat... d kept ... single cage. This study was a...d by ... Ethics Committee o... ...ty Animal Center.

Animal Mode... ...lishment

WT and C...l$^{-/-}$ ... ...ere intraperitoneally injected wi... 290 mg/kg ... to induce acute liver inj... Mice tissues ... ollected after admini... ...ion fo... 8 h and 24 h, respectively.

Biochemical and Pathological Analysis

Serum samples collected fr... WT ... Chi3l1$^{-/-}$ mice treated with APAP ... 8 h and 24 h were used for detecting acti... of ALT and AST. GSH level in liver tissue ... easured according to the instructi... of GS... ...y ki... (Jiancheng, Nanjing, C...). Liver tis... observed under a mic... ope af... hemato... and eosin (HE) stai...

Real-Time Flu... sce... Quantitativ... ...lymera... ...hai... Reaction ...PCR)

Total R... ...xtracted from ...ver tissues by TRIzol ...hod (... ...gen, Carlsbad, CA, USA) and reversely transcr... complementary Deoxy... Nucleic Acid ...NA) as previously d... ...bed. RNA concentration was detected us... spectrometer ...nd those samples with A260/ ...80 ratio of ... ...0 were selected for the fol... ...ng qRT-PCR ...action. QRT-PCR was then p... ...ed bas... ...n the instructions of SYBR Prem... ...M (TaKaRa, Otsu, Shiga, Japan). The experiment was repeated 3 times in ...rallel. QRT-PCR reaction parameters were as ... ...naturalization at 95°C for 60 s, exten... ...on a... ...5°C for 30 s and annealing at 60°C for 40 s, for a total of 40 cycles. Primer sequences were shown in Table I.

Extraction of Liver Mononuclear Cells (MNCs)

Liver tissue of mouse was completely digested with collagenase and then ground to prepare for the single cell suspension. After centrifugation at a gradient density, the red blood cell lysate was used to lyse the pellet. The lysate was washed with the buffer containing 0.5% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and finally the cells were resuspended using 100 µL of 2% fetal bovine serum (FBS) containing buffer.

Table I. q... ...prime...

	...ward	Reverse
mC... ...DH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
m...	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
...-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
...TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
...-β	TCCTTCACGGAGAGACACCT	GGCTGGGAACCATTAGTC
C...	GGGGGATCCATGGGCAATCCTTAT	TCGGGTGACCTTGCTTAGACGTGCAGG

Flow Cytometry

Fc receptor blocker and anti-CD16/3 antibody were added to the MNCs cell suspension and incubated for 10 min to block non-specific binding sites. Subsequently, the corresponding flow cytometry (Partec AG, Arlesheim, Switzerland) antibody was incubated in dark for 15 min. Next, 1 mL of 2% FBS was used to wash the cells. After centrifugation at 400 g for 5 min, 400 µL of 2% FBS were added for resuspension. The antibodies used in this assay were as follows: 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-tag anti-mouse CD45. All antibodies were purchased from eBioscience (San Diego, CA, USA).

Determination of Serum Cytokine Levels

After APAP treatment in mice for 8 h and 24 h, serum samples were collected and subjected to experimental procedures according to the enzyme-linked immunosorbent assay (ELISA) kit (Bio Legend, San Diego, CA, USA). The optical density (OD) values of each well at the wavelength of 562 nm and 450 nm were measured with a microplate reader (Bio-Rad, Hercules, CA, USA). Excel was used to draw a standard curve of cytokine concentration and absorbance value.

Cell Extraction and Culture

After APAP injection, WT mice the muscle tissue was [illegible] and the femur and tibia tissue were dissected and soaked in sterile phosphate-buffered saline (PBS). Bone marrow cells were flushed and cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) medium (HyClone, South Logan, UT, USA) containing 10 ng/mL M-CSF (macrophage colony-stimulating factor). After culturing for 6 days, cell lysate derived from Chi3l1$^{-/-}$ mouse (1:2) was added to BMDMs extracted from WT mice and incubated for 17 h. Expression levels of inflammatory factors in BMDMs extracted from mice were examined. Cells in the control group did not receive any treatment. The liver tissue of Chi3l1$^{-/-}$ mice received APAP treatment was homogenized in PBS. After frozen and thawed in liquid nitrogen, liver tissue was incubated to a 37°C water bath. The liver lysate was then washed with a 70-µm sieve and the supernatant was harvested.

Statistical Analysis

All experiments were repeated at least three times. Data were expressed as mean ± SD (Standard Deviation). The independent-sample *t*-test was used to compare the intergroup differences. Multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). Bonferroni was performed to detect significance between groups. GraphPad Prism (6.0) (La Jolla, CA, USA) was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

Chi3l1 Was Involved in the Pathological Process of APAP-Induced DILI

To investigate whether Chi3l1 participates in the pathogenesis of APAP-related drug-induced liver injury, we randomly assigned WT mice into two groups. Mice received intraperitoneal injections of APAP or PBS, respectively. After injection for 1 h, 3 h, 6 h, 12 h, and 24 h, respectively, mice were sacrificed by carbon dioxide asphyxiation for collecting liver tissues. We detected mRNA level of Chi3l1 by qRT-PCR (Figure 1). The results showed that compared with the PBS group, mRNA level of Chi3l1 remarkably increased after injection of APAP in a time-dependent manner, which lasted for 24 h. These results suggested that Chi3l1 might be involved in the pathogenesis of APAP-induced liver injury.

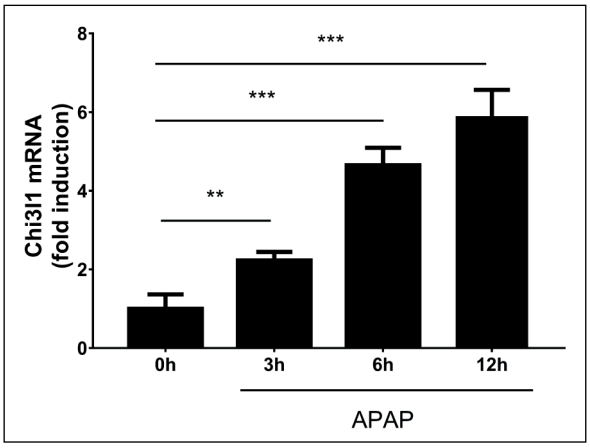

Figure 1. Chi3l1 was involved in the pathological process of APAP-mediated drug-induced liver injury. The mRNA expression of Chi3l1 in the liver of mice was detected by qRT-PCR after PBS or APAP injection for 1 h, 3 h, 6 h, 12 h and 24 h (**$p<0.01$).

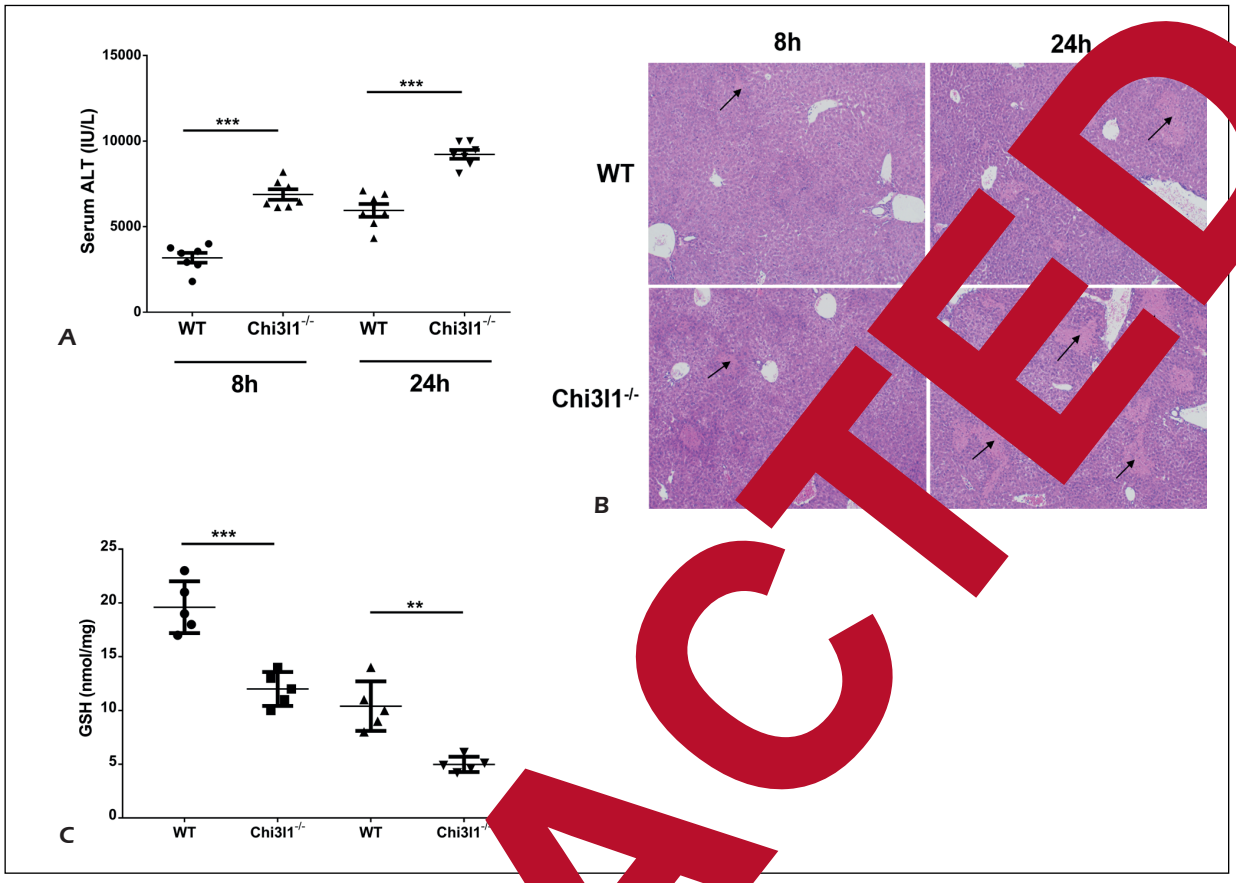

Figure 2. Chi3l1 knockout exacerbated APAP-induce[d] [l]iver injury in mice. **A**, Serum ALT levels in WT and Chi3l1$^{-/-}$ mice at 8 and 24 h after APAP injection. **B**, HE staining micrographs (magnification 100×, 200×) of liver tissues of WT and Chi3l1$^{-/-}$ mice at 8 h and 24 h; significant hepatic necrosis was observed in Chi3l1$^{-/-}$ mice (black arrows). **C**, GSH levels in livers of WT and Chi3l1$^{-/-}$ mice 24 h after APAP injection. GSH levels were significantly lower in APAP group than those of the PBS group, and GSH levels in the Chi3l1$^{-/-}$ group were significantly lower than that of the WT group (**p<0.01).

Knockout of Chi3l1 Exacerbated APAP-Induced Liver Injury in Mice

To study the role of Chi3l1 in APAP-mediated drug-induced liver injury, we intraperitoneally injected APAP into WT and Chi3l1$^{-/-}$ mice. Mice blood and liver tissues were collected after injection for 8 h and 24 h, respectively. Serum level of ALT was measured after centrifugation and the results showed that the ALT levels in Chi3l1$^{-/-}$ mice were remarkably higher than those of WT mice (Figure 2A). Pathological results of tissue sections revealed that Chi3l1$^{-/-}$ mice were more sensitive to APAP-induced liver injury since they exhibited extensive histiocytic necrosis (Figure 2B). GSH levels in the liver tissues of Chi3l1$^{-/-}$ mice were also markedly higher than those of WT mice (Figure 2C). The above data indicated that knockout of Chi3l1 exacerbated liver damage in mice after APAP treatment.

Knockout of Chi3l1 Aggravated the Inflammatory Response in Liver

After clarifying that Chi3l1 negatively regulated APAP-induced liver injury, we further explored its mechanisms by examining expressions of inflammatory cytokines in the liver after APAP injection. The mRNA levels of IL-6 and MCP-1 in liver tissues of APAP-treated Chi3l1$^{-/-}$ mice were remarkably higher than those of WT mice. However, no significant differences in mRNA levels of IL-1β, TNF-α, and IFN-γ were found between Chi3l1$^{-/-}$ mice and WT mice (Figure 3A). Protein levels of IL-6 and MCP-1 in CHI3L1$^{-/-}$ mice were also remarkably higher than those of WT mice (Figure 3B). All these results demonstrated that the Chi3l1 exacerbated APAP-induced liver injury by promoting the secretion of inflammatory factors.

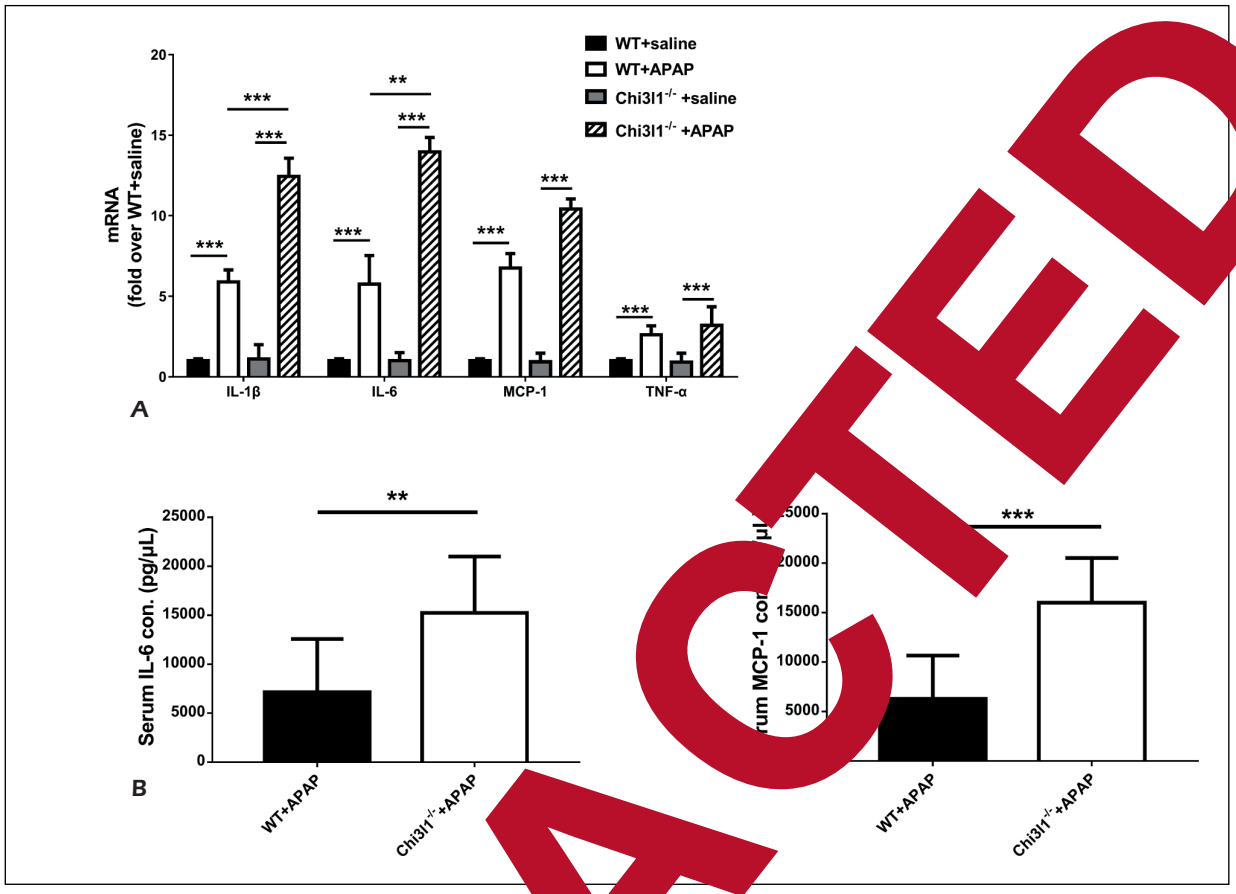

Figure 3. Knockout of Chi3l1 aggravated the inflamm onse in liver. **A**, The mRNA levels of various inflammatory cytokines in the liver of WT and Chi3l1$^{-/-}$ mice were meas y qRT-PCR after 24 h of APAP treatment. **B**, Serum IL-6 and MCP-1 levels were measured by ELIS ression lev inflammatory cytokines in tissues and serum of mice treated with APAP were much higher than trol group. xpression levels of IL-6 and MCP-1 in Chi3l1$^{-/-}$ group were much higher than those in the W up (*p< **p<0.01).

Chi3l1 Regulat he Apopt of Hepatocyt Secretion of Pro-Infla tokines Via Macr hage Infil n

It is r ted that Chi3l volved in the inflam ry re ction of adip se tissues and macr ages. I e, we investigated whether Ch ould gulate er damage and inflami ponses ough macrophages in APAP-in liver ry. Our results demonstrated that t ophage marker CD68 and pro- ammato macrophage marker CD86 wer markably higher in liver tissues of C han those of WT mice (Figure In addition, we examined the macrophage tration in liver tissues. The data showed that a PAP treatment for 24 h, the total amounts of trating macrophages (CD11b^{+}Ly6C^{+}) and neutrophils (CD11b^{+}Ly6G^{+}) in the liver of Chi3l1$^{-/-}$ mice were higher than those of WT mice (Figure 4B). Subsequently, we further observed the role of inflammatory macrophages in liver tissues of APAP-induced inflammation *in vitro*. BMDMs in WT and Chi3l1$^{-/-}$ mice were extracted, respectively. Apoptosis results elucidated that positive staining of Annexin V and 7-AAD in macrophages extracted from Chi3l1$^{-/-}$ mice was much more pronounced than those of WT mice (Figure 4C). Besides, levels of IL-6 and MCP-1 in the BMDMs incubated with lysates extracted from Chi3l1$^{-/-}$ mice were higher than those of the untreated group (Figure 4D). All above data confirmed that increased secretion of inflammatory factors by macrophages was the major cause of severe liver damage in Chi3l1$^{-/-}$ mice.

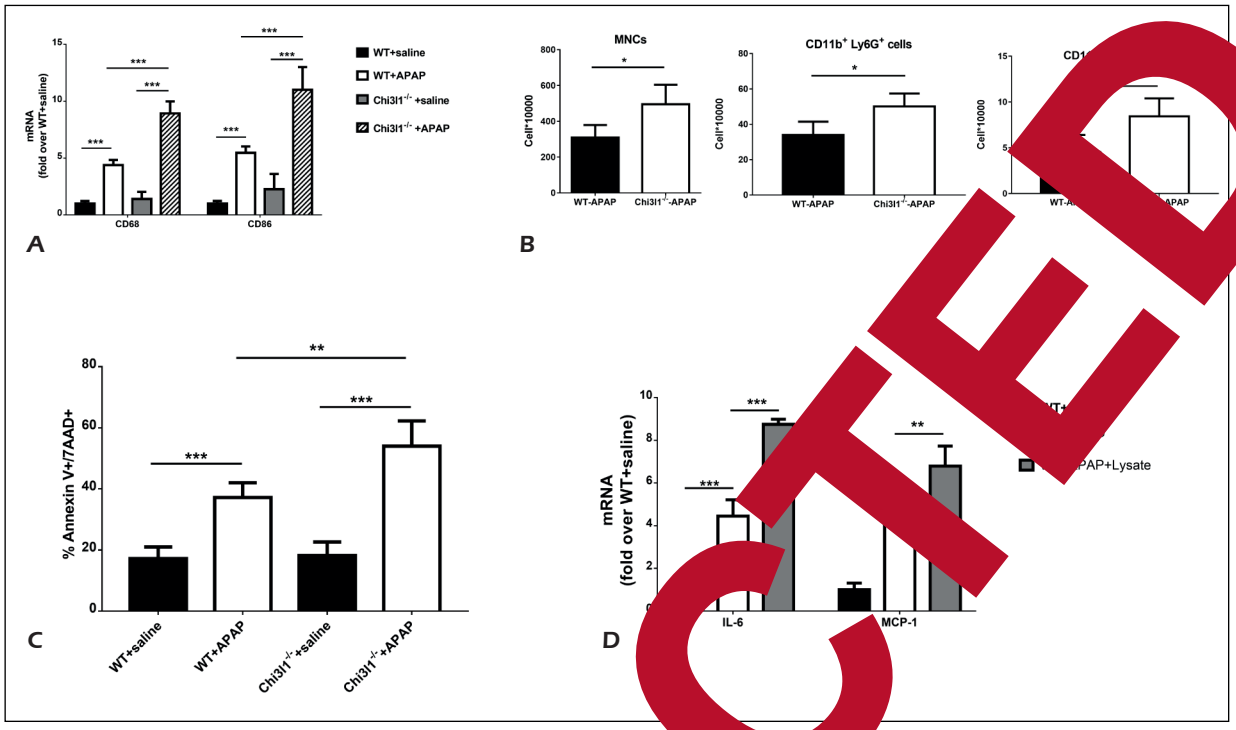

Figure 4. Chi3l1 regulated the apoptosis of hepatocytes and the sec… ammatory cytokines via macrophage infiltration. **A**, After 24 h of APAP treatment, mac… hage-related marker… and CD86 mRNA levels in livers of WT and Chi3l1$^{-/-}$ mice were measured by qRT-PCR. **B**… f APAP treatment, liver MNCs were isolated. Significantly increased Ly6C^{+}CD11b^{+} macrophages and Ly6G^{+}… lls were observed in the liver of Chi3l1$^{-/-}$ mice compared to WT mice. **C**, After 24 h of APAP treatmen… ice, Bl… xtracted from WT and Chi3l1$^{-/-}$ mouse. Positive macrophage was detected by Annexin V and 7-AAD… Aft… ubati… of liver lysates of Chi3l1$^{-/-}$ mice, expressions of inflammatory factors in BMDMs of wild-type mice wa… (*p<0.05, **p<0.01, ***p<0.001).

Discussi…

In this study, we d… trate… treatment induced t… se… Chi3l1 in mice, which in tur… ediated … damage by regulating macr… ge activatio… ltration and secretion … and MCP-1. …AP-induced DILI i… des … major steps. Firstly, the drug its… or its meta… directly damage the hepa… te stress respo… ntrinsic pathway) o… ivate the immune re…onse (extrinsic path… APA… o directly leads to mitochondri… funo… Seco… y, mitochondrial permeabi… tion p… MPT) opens. Thirdly, MPT eve… lea… apoptosis and necrosis … cell… us studies[4,12,15,16] have reported that Ch… is involved in the pathogen… of various liver diseases, especially in i… s induced by inflammation and … ls. Our results revealed that IL-6 and MCP-… vels in liver tissues and serum samples of … mice were remarkably higher than those of w… mice. The role of TNF-α in APAP-induced liver injury is controversial. Studies have reported that TNF-α receptor knockout could inhibit APAP hepatotoxicity in mice[17]. However, some researchers believed that APAP-induced damage is similar in TNF-α knockout mice and WT mice. In our study, no significant difference in mRNA level of TNF-α was observed between WT and Chi3l1$^{-/-}$ mice. After initially confirming the involvement of Chi3l1 in APAP-mediated DILI, we hypothesized that Chi3l1 could directly induce the secretion of these inflammatory factors. A great number of studies have shown that infiltrating macrophages and neutrophils participate in inducing acute liver inflammation in the mouse hepatitis model[18-21]. Therefore, we also considered the role of macrophage infiltration in regulating hepatic inflammatory cytokine secretion by Chi3l1. We found that both the infiltration of CD11b^{+}Ly6C^{+} macrophages and CD11b^{+}Ly6G^{+} neutrophils in the liver of Chi3l1$^{-/-}$ mice were upregulated, further indicating the key role of Chi3l1 in the macrophage infiltration of liver. The source of

macrophages in liver inflammation has been well studied. Whether this process is a result induced by liver intrinsic macrophages or bone marrow-derived macrophages deserves further investigation. We extracted bone marrow-derived macrophages to observe the direct effect of hepatic lysates on secretion of inflammatory factors by myeloid-derived macrophages. Our data confirmed that Chi3l1 was involved in the activation of myeloid macrophages and their subsequent cascade of inflammatory responses. It is of great significance to clarify the pathogenesis of DILI to improve the preventive and therapeutic efficacy of DILI. Current researches have suggested that various aspects are involved in DILI pathogenesis, such as drug metabolism, mitochondrial function impairment, immune response, signal transduction, genetics and environmental factors. Therefore, DILI is a result of the combined effects of multiple factors. Chi3l1 is greatly involved in the macrophage-mediated hepatic inflammation response in the APAP-mediated drug-induced hepatitis model. Our results demonstrated that the Chi3l1 might be a potential therapeutic target for acute hepatiti[illegible] liver failure.

Conclusions

APAP-treated Chi3l1$^{-/-}$ mic[illegible] more severe liver injury than that [illegible] Chi3l1 protects the liver functio[illegible]m AP[illegible]nduced injury by inhibiting the [illegible]tion [illegible]tory factors and mac[illegible]ha[illegible]ion, which provides a new dir[illegible]n for t[illegible]vention and treatment of clini[illegible]rug-induced [illegible]tis.

Conflict of Interests

The autho[illegible]are no competing [illegible]s.

Refe[illegible]ces

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