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CD31 induces inflammatory response by promoting hepatic inflammatory response and cell apoptosis

G.-Y. CHENG, Q. JIANG, A.-P. DENG, Y. WANG, J. LIU, Q. ZHOU, X.-H. ZHENG, Y.-Y. LI

Department of Pharmacy, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Gangying Cheng and Qian Jiang contributed equally to this work

Abstract. – OBJECTIVE: To investigate whether CD31 could regulate paracetamol-induced liver injury, thereby providing a new direction for the prevention and treatment of drug-induced hepatitis.

MATERIALS AND METHODS: Wild-type (WT) mice were treated with acetaminophen (APAP) (250 mg/kg) or isodose of phosphate-buffered saline (PBS). 1, 3, 6 and 12 h after the treatment, the messenger RNA (mRNA) and protein expression level of CD31 in the liver of mice were determined by Real-time reverse transcription polymerase chain reaction (RT-PCR) and Western blotting, respectively. Once CD31 was confirmed to be involved in APAP-induced liver injury, the acute liver injury model in WT mice and CD31 gene deficient (CD31-/-) mice induced by APAP was established. Serum samples were collected at 8 and 24 h after APAP injection (250 mg/kg), and the activity of serum alanine aminotransferase (ALT) was measured. The liver tissues of mice were isolated and analyzed by hematoxylin and eosin (HE) staining. Meanwhile, mononuclear cells (MNCs) were isolated from the liver tissues of mice. The number of infiltrating macrophages and neutrophils was detected by flow cytometry, and the activation level of these cells was analyzed. The expression levels of proinflammatory cytokines in liver tissues, such as TNF-α, IL-1β, keratinocyte chemoattractant (KC), MCP-1 and IL-6, were determined by RT-PCR. The expression levels of cytokines in serum were detected by enzyme-linked immunosorbent assay (ELISA). Moreover, the protein expression levels of JNK, Caspase-3, and cytochrome P450 2E1 (CYP2E1) in liver tissues were detected by Western blotting.

RESULTS: After APAP treatment, we found that WT mice were more sensitive to APAP-induced liver injury. The level of ALT in WT mice was significantly higher than that of CD31-/- mice, meanwhile, more necrotic or apoptotic cells were found in WT mice. Results also indicated that the expression levels of inflammatory cytokines, including KC, IL-1β, MCP-1 and IL-6, were significantly higher in WT mice. Meanwhile, the number of infiltrating macrophages and neutrophils in the liver tissues of WT mice were much more than that of CD31-/- mice.

CONCLUSIONS: APAP-treated CD31-/- mice exhibited less liver injury when compared with WT mice. We also confirmed that CD31 was greatly involved in APAP-induced inflammatory response by promoting hepatic inflammatory and cell apoptosis, which might provide a new strategy for the prevention and treatment of drug-induced hepatitis.

Key Words:
APAP, Drug-induced liver injury, CD31.

Introduction

Drug-induced liver injury (DILI) is a common side-effect of drugs, which is also one of the major causes of acute liver failure and liver transplantation in Western countries[1]. At the same time, DILI remains the main reason for the failure of many new drug research and development, as well as the warning from drug supervision department and the withdrawal of the market[2,3].

Acetaminophen (APAP) is an acetanil antipyretic analgesic. Due to its safe and reliable dosage, it is a frontline antipyretic analgesic in the international pharmaceutical market, which is also a non-prescription drug in most countries including China. However, over-administration of APAP has become one of the leading causes of

Corresponding Authors: Yuanyuan Li, MD; e-mail: frwb31@163.com
Xuehai Zheng, MD; e-mail: rnku11@163.com

DILI over the world[4], accounting for 50% of reported DILI. Meanwhile, the severity of relevant symptoms differs greatly in individuals. Therefore, the pathogenesis of DILI has not been fully elucidated[5].

Only a small amount of bioactive N-acetyl-to-benzenequinoneimine (NAPQI) is produced after small dose of APAP enters the liver. NAPQI can be combined with glutathione (GSH) for detoxification. However, a large amount of NAPQI produced by excessive APAP will accumulate in the liver, thereby over-consuming GSH[6]. Meanwhile, glutathione peroxidase (GSH-Px) is the main inactivation enzyme of peroxides. In the absence of GSH, the function of GSH-Px is significantly inhibited and its inactivation ability is weakened, eventually resulting in the accumulation of peroxides in human bodies[7]. So far, multiple metabolic enzymes related to the occurrence of DILI have been found. However, the role of drug metabolism in promoting the occurrence and progression of DILI still needs to be further studied.

CD31, a molecular with a weight of 130 kDa, is also known as platelet endothelial cell adhesion molecule-1 (PECAM-1/ CD31). It's known to all that CD31 belongs to the immunoglobulin superfamily, which plays an essential role in the clearance of senescent neutrophils[8]. Meanwhile, CD31 is present on the surface of platelets, neutrophils, monocytes, and certain types of T cells, as well as tight junctions between endothelial cells. The biological functions of CD31 may be involved in leukocyte migration, angiogenesis and integrin activation.

CD31 has been widely reported in the study of tumor invasion, metastasis and endothelial migration[9]. Previous studies have indicated that CD31 also has a regulatory role in inflammation in addition to its significant function in tumorigenesis. However, the specific pathogenesis mechanism and its interaction with ligands have not been clearly stated. Therefore, we suggested that CD31 was involved in the pathogenesis of APAP-induced liver injury.

In the present work, we first demonstrated that CD31 was involved in the development of APAP-induced DILI *via* detecting the mRNA and protein expression levels of CD31. Secondly, we detected the protein expression levels of JNK, Caspase-3, and cytochrome P450 2E1 (CYP2E1) in liver tissues, as well as the phosphorylation of CYP2E1, JNK and Caspase-3 *via* Western blotting. Next, we further confirmed whether CD31 participated in the direct damage of hepatocytes induced by APAP. Results also verified that CD31 was involved in inducing inflammatory cell infiltration, promoting the secretion of inflammatory cytokines and participating in APAP-induced hepatic inflammatory. Therefore, our study suggested that CD31 exerted an essential function in APAP-induced liver injury, which might be a potential therapeutic target for the treatment of drug-induced hepatitis.

Materials and Methods

Experimental Animals

All experimental mice used in this study were housed in a specific-pathogen-free (SPF) experimental animal center, with free access to standard feeding and drinking water. Meanwhile, each mouse was housed in a single cage. $CD31^{-/-}$ mice used in this study were imported from the Jackson Laboratory in the United States (West Grove, PA, USA). 8-9 week old wild-type (WT) and $CD31^{-/-}$ mice were selected for the following experiments, with 6-8 mice in each group. This study was approved by the Animal Ethics Committee of Huazhong University of Science and Technology Animal Center.

Establishment of Animal Model

Male WT and $CD31^{-/-}$ mice were intraperitoneally injected with 250 mg/kg APAP to induce acute liver injury. Serum samples were then collected at 8 h and 24 h after injection to detect the level of ALT.

Biochemical and Pathological Analysis of Liver Injury in Mice

After 8 h and 24 h of APAP treatment, the serum level of ALT in WT and $CD31^{-/-}$ mice was detected in the clinical laboratory. Collected liver tissues were stained with hematoxylin and eosin (HE) staining (Beyotime, Shanghai, China). Subsequently, the liver lesions were observed and evaluated under a light microscope.

Western Blotting

Total proteins extracted from liver tissues were lysed with cell lysis buffer and shaked on ice for 30 min, followed by centrifugation (14,000 g/min) at 4°C for 15 min. The concentration of extracted proteins was calculated by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). A total of 60 mg protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred

to polyvinylidene difluoride (PVDF) membranes. Next, the membranes were blocked with 5% milk at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies (JNK, Caspase-3 and β-actin, 1: 1000, Abcam, Cambridge, MA, USA) and corresponding secondary antibody (horseradish peroxidase (HRP)-labeled IgG, 1:10000) at room temperature for 1-2 h. Results were analyzed after color development and observation.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted total RNA was then reverse transcribed into cDNA by using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR reaction was performed using SYBR® Premix Ex TaqTM (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was repeated for three times. Primers used in this study were shown in Table I.

Extraction of Liver MNCs

After complete digestion with the solution containing collagenase, the liver tissues were ground and filtered through a cell strainer to prepare single cell suspension. Then, separating medium was re-suspended *via* gradient density centrifugation, followed by pyrolysis precipitation by erythrocyte lysates. Subsequently, the precipitation was washed again with 0.5% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) buffer, and re-suspended with 100 μL buffer containing 2% FBS. Finally, the suspension of MNCs was successfully prepared.

Flow Cytometry

In re-suspended MNCs cell suspension, Fc receptor antagonist and anti-CD16/32 were first added, followed by incubation for 10 min to block nonspecific binding. Afterwards, PE-Cyanine7-labeled anti-mouse CD11b, APC-labeled anti-mouse F4/80, FITC-labeled anti-mouse Ly6C, PE-labeled anti-mouse Ly6G and APC Vio770-labeled anti-mouse CD45 were obtained. All antibodies were purchased from eBioscience Company (San Diego, CA, USA). The mixture was cultured in the dark for 15 min, followed by additional washing with 1 mL 2% FBS and centrifugation at 400 g/min for 5 min. Finally, 400 μL 2% FBS was used for mixture re-suspension and subsequent flow cytometry detection.

Determination of Serum Cytokine Levels

Serum samples of mice were collected at 8 h and 24 h after APAP treatment. According to the instructions of the enzyme-linked immunosorbent assay (ELISA) kit (Bio Legend, San Diego, CA, USA), the optical density (OD) value of each well at 562 nm and 450 nm wavelength was measured by a microplate reader, respectively. The standard curve of cytokine concentration and absorbance values was performed, and the concentration of cytokines was finally determined.

Statistical Analysis

All experiments were repeated for at least three times. All experimental data were expressed as mean ± standard error. *t*-test was used to compare the differences between two groups. One-way ANOVA was used to analyze the differences among different groups, and Bonferroni test was used to test the significance of each group. Graphpad Prism (v6.0, La Jolla, CA, USA) was used for all statistical analysis. $p<0.05$ was considered statistically significant.

Results

CD31 was Involved in the Pathological Process of APAP-Induced DILI

To investigate whether CD31 was involved in the pathogenesis of APAP-induced DILI, WT

Table I. RT-qPCR primer pairs

Name	Forward	Reverse
mGAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
mIL-1α	ACGGCTGAGTTTCAGTGAGACC	CACTCTGGTAGGTGTAAGGTGC
mIL-1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
mIL-4	ATCATCGGCATTTTGAACGAGGTC	ACCTTGGAAGCCCTACAGACGA
mIL-6	TACCACTTCACAAGTCGGAGGC	CTGCAAGTGCATCATCGTTGTTC
mTNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG

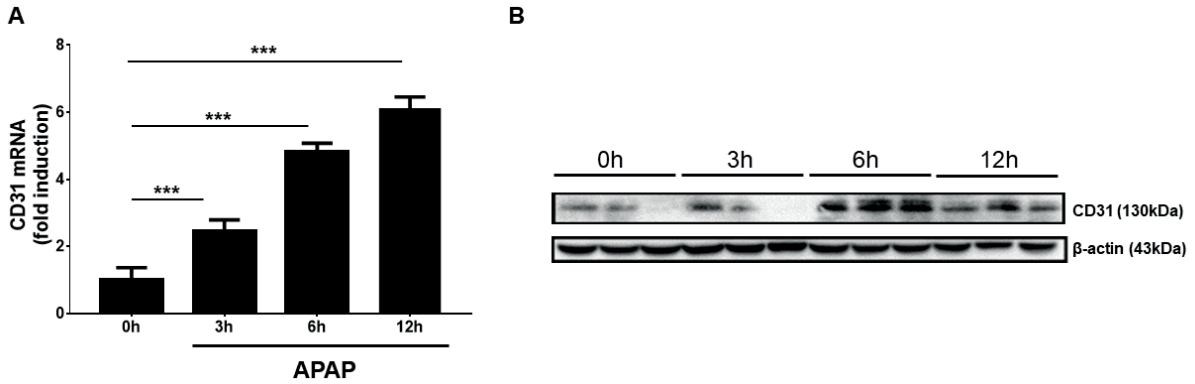

Figure 1. CD31 was involved in the pathological process of APAP-induced DILI. ***A,*** The mRNA level of CD31 in mouse liver was detected by RT-PCR at 1, 3, 6 and 12 h after PBS or APAP injection. Each group had 6-8 mice. ** $p<0.01$, *** $p<0.001$. ***B,*** The protein level of CD31 in mouse liver was detected by Western blotting at 1, 3, 6 and 12 h after PBS or APAP injection. Each group had 3 mice. The experiment was repeated for 3 times.

male mice with equal body weight were divided into two groups. Equal doses of APAP and PBS were injected to the corresponding group, respectively. 1, 3, 6 and 12 h after injection, mice were sacrificed by the carbon dioxide asphyxia method, and liver tissues were collected.

We detected the mRNA (Figure 1A) and protein expression levels (Figure 1B) of CD31 by qPCR and Western blotting, respectively. Results showed that as the time passed, the mRNA and protein levels of CD31 in the APAP group were remarkably increased after APAP injection when compared with those of the PBS group. Meanwhile, such trend lasted for almost 12 h. These results suggested that CD31 was involved in the pathogenesis of APAP-induced liver injury.

Knockout of CD31 Protected Mice from APAP-Induced Liver Injury

To investigate the function of CD31 in APAP-induced DILI, APAP was intraperitoneally injected into male WT and CD31$^{-/-}$ mice. After 8 and 24 h, blood samples and liver tissues were collected. The serum level of ALT was measu-

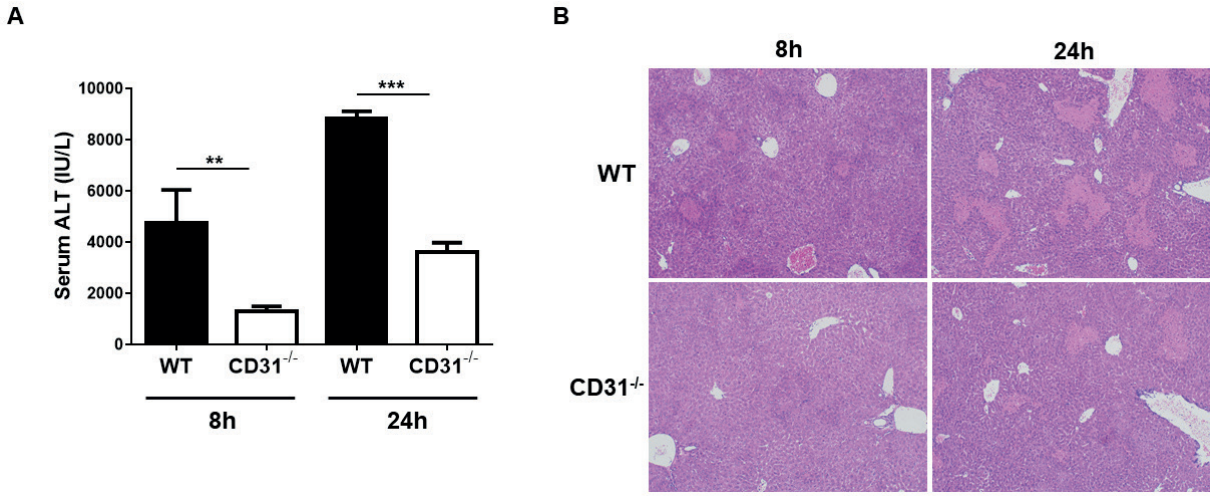

Figure 2. CD31 knockout protected mice from APAP-induced liver injury. ***A,*** Serum level of ALT in WT and CD31$^{-/-}$ mice at 8 and 24 h after APAP injection. Each group had 6-8 mice. ** $p<0.01$; *** $p<0.001$. ***B,*** HE staining images (100×) of liver tissues in WT and CD31$^{-/-}$ mice at 8 and 24 h. A large number of hepatocyte necrosis was observed in WT mice, whereas CD31$^{-/-}$ mice had less hepatic damage.

red, and the blood samples were centrifuged. Our data demonstrated that the level of ALT in CD31$^{-/-}$ mice were significantly lower than that of WT mice (Figure 2A). Histological analysis of liver tissues revealed that CD31$^{-/-}$ mice were less sensitive to APAP-induced liver injury (Figure 2B). After injection of APAP, WT mice exerted an significant wider range of necrotic cells in the liver, whereas CD31$^{-/-}$ mice had smaller necrotic area.

CD31 Participated in the Direct Damage of APAP to the Liver

Under normal circumstances, 85-95% APAP is metabolized to harmless substance in the liver, which can be eliminated from the body by UCP, glucuronosyltransferase and sulfotransferase. When APAP is overdosed, members of the cytochrome P450 enzyme family such as CYP1A2, CYP2E1 and CYP3A4, are involved in the metabolic process. Subsequently, these metabolic enzymes become the harmful metabolite of APAP, namely N-acetyl-p-benzoquinoneimine (NAPQI). Previous studies have demonstrated that the binding of GSH to NAPQI can reduce its toxicity *in vivo*. When GSH is depleted, NAPQI covalently binds to important macromolecules *in vivo* to form protein adducts, eventually leading to liver damage[10,11]. Other studies have shown that CYP2E1 knockout mice exert resistance to APAP-induced liver injury[12]. Therefore, we examined the protein expression of CYP2E1 and found that CYP2E1 was highly expressed in CD31$^{-/-}$ mice than that of WT mice, indicating less amount of NAPQI and lower toxicity in CD31$^{-/-}$ mice (Figure 3A). In addition, during the metabolism of APAP, the production of ROS induced by mitochondrial GSH clearance will continue to activate JNK phosphorylation[13]. Here, we detected the expression of JNK in both CD31$^{-/-}$ and WT mice, and found that the phosphorylation of JNK in the liver of WT and CD31$^{-/-}$ mice after APAP treatment was significantly higher than that of controls. Besides, the phosphorylation level of JNK in CD31$^{-/-}$ mice was significantly lower than that of WT mice (Figure 3B). Researches have demonstrated that the activation of Caspase-3 can lead to hepatocellular apoptosis. Therefore, we examined the expression level of Caspase-3 in the liver tissues of mice treated with APAP or PBS. Results indicated an obvious higher expression level of Caspase-3 in mice treated with APAP than controls. However, Caspase-3 activity in WT mice was remarkably higher than that of CD31$^{-/-}$ mice (Figure 3B). These results demonstrated that CD31 mediated the pathogenesis of drug-induced hepatitis through direct involvement of APAP in liver injury, whereas CD31 deficiency could alleviate APAP-induced liver injury.

Deletion of CD31 Alleviated the Inflammatory Response of Liver

We also examined the expression of inflammatory cytokines in the liver of WT and CD31$^{-/-}$ mice, respectively. Results indicated that the mRNA level of IL-1β in liver tissues of APAP-treated CD31$^{-/-}$ mice was remarkably lower than that of WT mice. However, there were no significant differences in the expression levels of IL-6, MCP-1 and TNF-α between WT and CD31$^{-/-}$ mice (Figure 4A). Meanwhile, the serum level of IL-1β in CD31$^{-/-}$ mice was significantly lower than that of WT mice after APAP treatment (Figure 4B). Previous studies have confirmed that CD31 participates in macrophage and adipose tissue inflammation. To investigate whether CD31 receptors were involved in APAP-induced infiltration of inflammatory cells in liver, hepatocytes were grouped via flow cytometry. Results found that, 24 h after APAP injection, the total number of hepatic infiltrating macrophages ($CD11b^+Ly6C^+$) and neutrophils ($CD11b^+Ly6G^+$) in CD31$^{-/-}$ mice was remarkably lower than those of WT mice (Figure 4C). These data indicated that the deletion of CD31 suppressed the infiltration of inflammatory cells in the liver of mice.

Discussion

In this study, we showed that CD31 deletion in APAP-induced hepatitis led to the reduction of hepatocyte necrosis, drug-metabolizing enzymes and phosphorylated expressions of JNK and Caspase-3. Moreover, the infiltration of inflammatory macrophages and neutrophils, as well as the secretion of IL-1β, were also significantly inhibited. As a surface marker of endothelial cells, CD31 plays an essential role in inflammation, stroke, immunity, angiogenesis and tumorigenesis[14-17]. Bone marrow transplantation has demonstrated that CD31 is upregulated in airway hyper responsiveness. Meanwhile, dendritic cells and macrophages are also involved in the induction of tracheal inflammation[18]. There are three major steps in the pathogenesis of APAP-induced DILI. The first is the reaction initiated by drugs or corresponding metabolites,

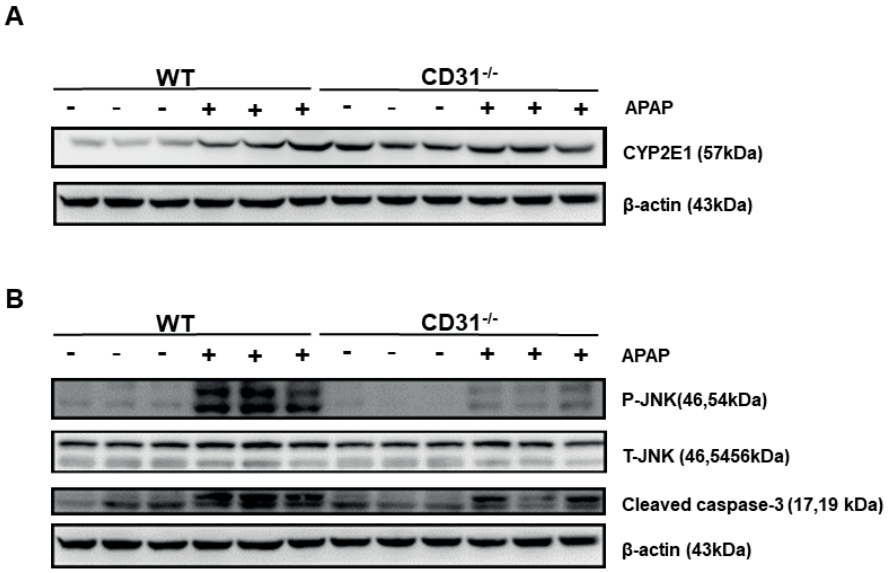

Figure 3. CD31 was involved in the direct damage of APAP to liver. ***A,*** After the mice were treated with APAP or an equal dose of PBS for 8 h, liver tissues of mice were collected and the expression of CYP2E1 was detected by Western blotting. The expression of CYP2E1 in the APAP group was significantly higher than that of the control group. Meanwhile, the expression of CYP231 in CD31$^{-/-}$ mice was higher than that of WT mice. ***B,*** The phosphorylation of JNK and the protein expression level of Caspase-3 in WT and CD31$^{-/-}$ mice at 8 h after the treatment of APAP or an equal dose of PBS were detected by Western blotting, respectively. Each group had 3 mice, and each experiment was repeated for 3 times.

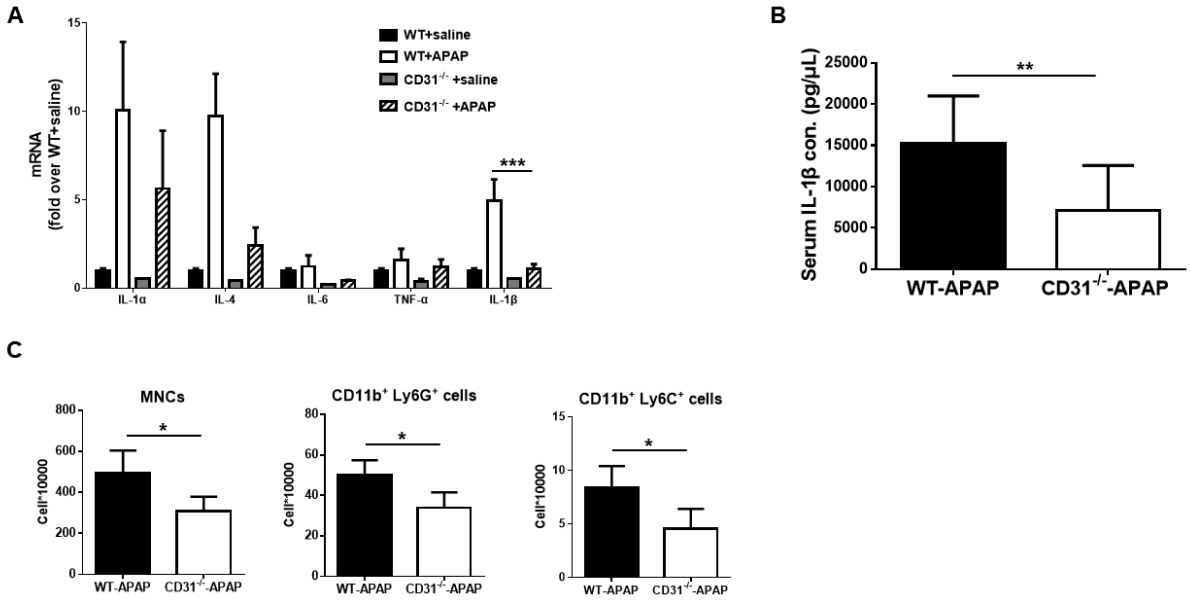

Figure 4. Depletion of CD31 alleviated the inflammatory response of liver. ***A,*** The mRNA levels of various inflammatory cytokines in the liver tissues of WT and CD31$^{-/-}$ mice were detected by RT-PCR 24 h after APAP treatment. The expression of inflammatory cytokines in WT and CD31$^{-/-}$ mice was much higher than that of the control group, while the expression of IL-1β in CD31-/- mice was much lower than that of WT mice. ***B,*** Measurement of serum IL-1β expression level. ***C,*** Liver MNCs were isolated at 24 h after APAP treatment. After staining with flow cytometry, the total number of hepatic infiltrating macrophages (CD11b^{+}Ly6C^{+}) and neutrophils (CD11b^{+}Ly6G^{+}) in CD31$^{-/-}$ mice was remarkably lower than that of WT mice. Each group had 6-8 mice, and each experiment was repeated for 3 times. * p<0.05; ** p<0.01; *** p<0.001.

including hepatocyte stress response induced by direct injury (internal pathway), the activation of immune response (external pathway) and direct damage to mitochondrial function. The second is mitochondrial permeability transition (MPT), and the last is apoptosis and necrosis of liver cells induced by MPT. Scholars have shown that the loss of mitochondrial membrane potential and membrane permeability is related to the mediation of JNK. However, the specific mechanism of this process still remains unclear[19]. Numerous studies[20-23] have shown that KC, infiltrating macrophages and neutrophils participate in inducing acute liver inflammation in mouse hepatitis models. In the present work, we found that the infiltration of $CD11b^{+}Ly6C^{+}$ macrophages and $CD11b^{+}Ly6G^{+}$ neutrophils in the liver of $CD31^{-/-}$ mice were significantly decreased, demonstrating the key role of CD31 in inducing liver inflammation. The level of IL-1β was also significantly reduced in APAP-treated $CD31^{-/-}$ mice when compared with that of WT mice. The role of TNF-α in APAP-induced liver injury, however, is controversial. It has been reported[24] that TNF-α receptor knockout mice can inhibit APAP hepatotoxicity. However, it has also been found that mice with knockout of TNF-α have a similar degree of damage as WT mice. In our study, there was no significant difference in the mRNA level of TNF-α between WT and $CD31^{-/-}$ mice. Previous researches have reported that TNF-α induces the activation of JNK by binding to TNF receptors[25]. In our investigations, the levels of APAP-stimulated JNK phosphorylation and cleaved Caspase-3 in the liver of $CD31^{-/-}$ mice were significantly lower than those of WT mice. Clarifying the pathogenesis of DILI is of great importance to prevent the occurrence of DILI and to search for novel therapeutic targets. However, current researches have suggested that the mechanism of DILI is complex, which involves many processes, such as drug metabolism, mitochondrial dysfunction, immune response, signal transduction, genetics and environment. Therefore, the occurrence of DILI is a combination of multiple factors. In summary, we suggest that CD31 plays a key role in liver inflammation response and direct cell injury in APAP-induced DILI model. Our results revealed that PP1, a CD31 inhibitor, might be an anti-inflammatory potential drug for the treatment of drug-induced hepatitis clinically. Moreover, CD31 gene could also serve as a potential therapeutic target for acute liver failure.

Conclusions

We found that CD31 regulates the direct damage of APAP by regulating CYP2E1 metabolism, JNK phosphorylation and Caspase-3 activation. Furthermore, CD31 can regulate hepatic inflammatory response by activating macrophages and secreting a series of pro-inflammatory cytokines.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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