# Serpina3c Deficiency Induced Necroptosis Promotes Non-Alcoholic Steatohepatitis Through β-Catenin/Foxo1/TLR4 Signaling

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# Abstract

**Background:** Hepatocyte death and liver inflammation have been recognized as central characteristics of nonalcoholic steatohepatitis (NASH); however, the underlying molecular mechanism remains elusive. The aim of this study is to determine the precise role of serpina3c in the progression of NASH.

**Methods:** Male *Apoe*$^{-/-}$/*serpina3c*$^{-/-}$ double knockout (DKO) and *Apoe*$^{-/-}$ mice were fed a high-fat diet (HFD) for 12 weeks to induce NASH. Several markers of steatosis and inflammation were evaluated. In vitro cell models induced by palmitic acid (PA) treatment were used to evaluate the beneficial effect of serpina3c on necroptosis and the underlying molecular mechanism.

**Results:** Compared with *Apoe*$^{-/-}$ mice, DKO mice exhibited a significantly exacerbated NASH phenotype that included hepatic steatosis, inflammation, fibrosis and liver damage, and increased hepatic triglyceride contents. We also indicated that the expression of the receptor-interacting protein 3 (RIP3) and phosphorylated mixed lineage kinase domain-like (MLKL) was increased in DKO mice. Our results found that serpina3c knockdown promoted necroptosis and lipid droplet formation under conditions of lipotoxicity in vitro. However, these phenomena were reversed by the overexpression of serpina3c. Mechanistically, downregulation of serpina3c expression promoted Foxo1 and β-catenin expression, and Foxo1 and β-catenin colocalized in the nucleus under conditions of lipotoxicity, consequently upregulating the expression of Toll-like receptor4 (TLR4). However, disruption of the Foxo1-β/catenin by Foxo1 and β-catenin inhibitors decreased TLR4 expression and ameliorated hepatic necroptosis in vitro.

**Conclusion:** Serpina3c plays a protective role against the progression of NASH by inhibiting necroptosis. Serpina3c, a Wnt/β-catenin inhibitor, inhibits necroptosis via β-catenin/Foxo1 by inhibiting TLR4 expression.


# Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease and comprises simple steatosis to more severe forms of disease, such as nonalcoholic steatohepatitis (NASH)[1]. It is now widely recognized that the mechanism underlying the pathogenesis of NASH is excessive lipid accumulation in hepatocytes that causes hepatocyte lipotoxicity and induces cell death. Hepatocyte death is accompanied by the massive recruitment of monocytes into the liver, and activation of macrophages leads to the release of many inflammatory factors and the occurrence of chronic inflammation[2]. Accumulating studies suggest that hepatic cell death plays critical roles in the promotion of liver fibrosis and progression of disease.

Multiple forms of cell death, including apoptosis, pyroptosis and necroptosis, are associated with steatohepatitis[3]. Receptor-interacting protein 3 (RIP3) is a molecular switch of cell necroptosis; the recruitment of RIP3 leads to the recruitment and phosphorylation of mixed lineage kinase domain-like protein (MLKL), eventually leading to perforated membrane structures that can eventually result in the rupture of cell membranes[4]. An early study showed that RIP3 expression is increased in the livers of

patients with chronic liver disease and that liver necroptosis is increased in high-fat diet-induced experimental models of NASH [5]. Necroptosis can be regulated by Toll-like receptor 4 (TLR4). TLR4 can bind to TRIF receptor-domain-containing adaptor inducing interferon-β (IFN-β)) and then with receptor-interacting protein kinase 3 (RIPK3) to induce necroptosis[6].

Serpina3c, a serine proteinase inhibitor (serpin), is mainly expressed in the liver and adipose tissue. Serpins, including serpina3k and serpina4, have been reported to be Wnt signaling inhibitors[7, 8]. Our previous research showed that serpina3c plays a protective role in metabolic diseases[9]. The absence of serpina3c expression promotes the onset of atherosclerosis and pancreatic dysfunction. In addition, Choi Y et al reported that serpina3c is a critical factor involved in adipogenesis, and inhibition of serpina3c may result in beneficial effects in the treatment of obesity[10]. The liver is the main metabolic organ of the human body. NASH is a kind of metabolic disease and the hepatic manifestation of metabolic syndrome (MetS). Given the role of serpina3c in metabolic syndrome, we focused our analysis on the pathogenic role of serpina3c in NASH.

In this study, we aimed to investigate the role of serpina3c in NASH using a high-fat, *Apoe*$^{-/-}$ mouse model, which is an appropriate model of NASH associated with MetS. *Apoe*$^{-/-}$ mice develop spontaneous hypercholesterolemia and atherosclerosis and are widely used as a mouse model to study fatty liver disease[11]. This study first indicated that the absence of serpina3c expression significantly increased HFD-induced liver damage, steatosis, fibrosis and macrophage infiltration and activation. Here, we also found that serpina3c negatively regulated hepatocyte necroptosis. Furthermore, we established the involvement of β-catenin/Foxo1/TLR4 signaling in serpina3c-induced necroptosis in hepatocytes.

# Materials And Methods

## Animals

Male *Apoe* $^{-/-}$mice, and *Apoe*$^{-/-}$*/serpina3c* $^{-/-}$(DKO) mice were used in this study. As our previous study[9], the generation of the serpina3c-knockout mice was commissioned by Beijing Biocytogen (Beijing Biocytogen Co., Ltd). Eight-week-old male DKO mice and their *Apoe*$^{-/-}$ littermates (n=10~12/group) were fed a HFD (10% fat, 2% cholesterol, and 0.5% sodium cholate) for 12 weeks. The mice were maintained at room temperature with a 12-hour light/dark cycle and given free access to food and water.

## Liver Function Examination

Levels of ALT, AST, and triglycerides, cholesterol was determined using the commerial kit (Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

## Liver histopathology

For histopathology, hepatic sections (8 μm) were used, and images were captured by bright-field microscopy (Olympus BX51 microscope). Three liver sections, spaced at 40 μm in tervals, were stained

per mouse, and three fields of view were captured per section, resulting in nine fields of view per mouse. Oil red O (ORO)-stained sections were assessed for steatosis. H&E-stained sections were assessed for lobular inflammation, and hepatocyte ballooning, the latter indicating hepatocyte injury, according to the scoring method developed by the Non-Alcoholic Steatohepatitis Clinical Research Network (NASH-CRN) [12], as previously described[13].Collagen analysis was performed by Sirius Red staining. For immunohistochemistry analysis, reagents included anti-α-SMC (diluted to 1:400; Sigma, USA), anti-mouse CD68 antibody (diluted at 1:400; Sigma, USA), and biotinylated anti-mouse IgG (PV9000, ZSGB-bio).

### Cell culture and treatment

AML12 hepatocytes (CRL-2254, ATCC) were cultured in DMEM/F12 with 10% FBS, supplemented with insulin, transferrin, selenium, dexamethasone and pen/strep. AML12 hepatocytes were exposed to 200 μM palmitic acid for up to 24 h to model lipotoxicity. (PA, P0500, Sigma) complexed to BSA or BSA alone as control for up to 24 h to model lipotoxicity. All lentiviruses were purchased from GenePharma (Shanghai, China): serpina3c-knockdown lentivirus LV-Mus serpina3c shRNA (LV-KD), serpina3c-overexpression lentivirus LV-Mus serpina3c (LV-OV) and control group (LV-NC). Lentivirus was transfected into AML12 cells according to the manufacturer's protocol. At 48 hours post-transfection, cells were harvested for Western blot analysis. Pre-treatment AML12 cells with a Wnt inhibitor IWR-1(10μM, HY-12238, MedchemExpress, China), Foxo1 antagonist AS1842856 (1μM, HY-100596, MedchemExpress, China) or necroptosis antagonist Necrosulfonamide (10μM, HY-100573, MedchemExpress, China) for 2 h before stimulation with PA.

### PI Staining

AML12 hepatocytes were treated with PA for 24 h and immobilized in 4% paraformaldehyde for 30 min and washed twice with PBS. After treatment with RNase A for 30 min at 37 ∘C. 10 mg/mL propidium iodide (PI, KeyGENBioTECH, China) was used to stain cell nuclei. After counterstaining with Hoechst 33342, we observed the cells using fluorescence microscopy.

### Immunofluorescence

AML12 cells were treated with 0.3% Triton (Triton X-100, Dilution with PBS) for 15 min, blocked with 5% BSA at 4°C and then incubated overnight with primary anti-Foxo1 and anti-β-catenin antibodies at 4°C. Subsequently, the specimens were incubated with the corresponding rhodamine- or FITC-conjugated secondary antibody at 37°C for 1 hour, and the nuclei were stained with DAPI for 5 minutes at room temperature. Digital images were acquired with an Olympus FV3000 confocal laser scanning microscope (Tokyo, Japan)

### Quantitative RT-PCR (qRT-PCR) Analysis

After being extracted using TRIzol reagent, total mRNA from the mouse liver and AML12 cells was collected and depurated using DNase. cDNAs were then obtained using a Transcript or First Strand cDNA synthesis kit (TaKaRa Bio, Japan). Quantitative real-time PCR was carried out on a StepOne Plus

RealTime PCR System (Thermo Fisher Scientific). The primers are as followed: Arg-1 forward (P1) CATATCTGCCAAAGACATCGTG, reverse (P2) GACATCAAAGCTCAGGTGAATC; ACC1 forward (P1) ACCTCAGTGGCGTCTCAGTATCC, reverse (P2) ACTGCCTGCCTGTCTCCATCC; ACOX forward (P1) CCAATGCTGGTATCGAAGAATG, reverse (P2) CGACTGAACCTGGTCATAGATT;

FASN forward (P1) TAAAGCATGACCTCGTGATGAA, reverse (P2) GAAGTTCAGTGAGGCGTAGTAG;

SREBF1 forward (P1) GCTACCGGTCTTCTATCAATGA, reverse (P2) CGCAAGACAGCAGATTTATTCA; IL-18 forward (P1) AGACCTGGAATCAGACAACTTT, reverse (P2) TCAGTCATATCCTCGAACACAG; MCP-1 forward (P1) TTCTGATCTCATTTGGTTCCGA, reverse (P2) TTCTGATCTCATTTGGTTCCGA; TGF-β forward (P1) CCAGATCCTGTCCAAACTAAGG, reverse (P2) CTCTTTAGCATAGTAGTCCGCT; CD36 forward (P1) CTTTGAAAGAACTCTTGTGGGG, reverse (P2) GTCTGTGCCATTAATCATGTCG; TNF-a forward (P1) ATGTCTCAGCCTCTTCTCATTC, reverse (P2) GCTTGTCACTCGAATTTTGAGA; IL-1β forward (P1) GCAGAGCACAAGCCTGTCTTCC, reverse (P2) ACCTGTCTTGGCCGAGGACTAAG; IL-6 forward (P1) AGTTGCCTTCTTGGGACTGATGTTG, reverse (P2) GGTATCCTCTGTGAAGTCTCCTCTCC. The quantified transcripts from the samples were normalized against β-actin gene expression. Majority of the results were calculated based on the ΔΔCt method; the results are presented as relative expression.

### Western Blotting

Protein was extracted from mouse liver and AML12 cells with different treatments using RIPA buffer. The protein concentration was quantified using a BCA assay (Beyotime). Total protein (20-30μg) was analyzed using 8%~10% SDS-PAGE electrophoresis. After blocking with 5% nonfat milk, the membranes were incubated with antibodies overnight at 4°C. The following antibodies were used in this study: anti-serpina3c (50375-RP01, Sino Biological, 1:1000), anti-β-actin (Proteintech, 1:1000), anti-JNK (9252, Cell Signaling Technology, 1:1000), anti-p-JNK(9255S, Cell Signaling Technology, 1:1000), anti-β-catenin (8480S, Cell Signaling Technology, 1:1000), anti-p-P65(3033S, Cell Signaling Technology, 1:1000), anti-P65(8242S, Cell Signaling Technology, 1:1000), anti-RIP3(95702S, Cell Signaling Technology, 1:1000), anti-p-MLKL(ab196436, abcom, 1:1000), anti-Foxo1(2880S, Cell Signaling Technology, 1:1000), anti-TLR4(ab13867, abcom, 1:1000). After three washes, the membranes were incubated with the secondary antibody at room temperature for 1 hour. The proteins on the blot were detected by a chemiluminescent detection system with LumiGLO and peroxide (Tanon). Densitometric analysis of the images was performed with ImageJ software (NIH, Littleton, CO, USA).

### Statistical Analysis

All data are expressed as the means ± SD from at least three independent experiments. Comparisons between groups were evaluated using a two-tailed Student's *t* test or one-way ANOVA with multiple comparisons. All statistical analyses were performed using SPSS, version 16.0. P values <0.05 were considered to be statistically significant.

## Results

## Serpina3c deficiency promoted liver injury and hepatic steatosis in HFD-fed *Apoe*$^{-/-}$ mice

In our previous study, the results showed that body weight, plasma total cholesterol levels, triglyceride levels, and plasma glucose levels were not significantly different in between HFD-fed *Apoe*$^{-/-}$ and DKO mice[14]. However, circulating levels of alanine aminotransferase (ALT) (Figure 1A) and aspartate aminotransferase (AST) (Figure 1B) were significantly increased in DKO mice compared with *Apoe*$^{-/-}$ control mice. The data indicate that serpina3c deficiency promoted liver injury. Among the markers of hyperlipidemia, liver triglycerides have been associated with NASH[15]. We found higher hepatic triglyceride levels in DKO mice than in *Apoe*$^{-/-}$control mice (Figure 1C), which supports a role of serpina3c in hepatic steatosis. Consistent with this conclusion, H&E staining and Oil Red O staining of liver sections revealed that the livers of DKO mice exhibited significant hepatocyte ballooning and steatosis (Figure 1D). The severity of NASH was assessed using the NASH Activity Score (NAS), an unweighted sum of scores for steatosis, lobular inflammation, and hepatocyte ballooning. Compared to *Apoe*$^{-/-}$ mice, DKO mice exhibited increased NAS scores (Figure 1E). To test the corresponding molecular mechanisms that accounted for the increased liver triglyceride content and lipid accumulation in DKO mice, we performed RT-PCR and analyzed the expression of liver lipid metabolism-associated genes. As expected, deficiency of serpina3c expression increased the liver expression of SREBP1c and CD36, although CD36 expression was not statistically significant. Serpina3c deficiency did not affect the liver expression of ACC1, FASN, or ACOX (Figure 1F). Therefore, these results illustrated that serpina3c played an essential role in liver injury and hepatic steatosis during NASH development.

## Serpina3c deficiency promoted hepatic inflammation response in HFD-fed *Apoe*$^{-/-}$ mice

Liver inflammation is one of the most important characteristics of NASH[16]. An increase in the inflammatory milieu in the liver is a hallmark of NASH. During the progression of liver injury, activated hepatic macrophages represent a major source of inflammatory mediators, including cytokines and chemokines, which are stimuli that sustain HSC activation and fibrogenesis. Thus, we performed immunohistochemistry for the macrophage marker CD68, and we observed that macrophage infiltration was significantly increased in DKO mice compared to *Apoe*$^{-/-}$ mice (Figure 2A). Given the effects of adhesion molecules on inflammatory cell infiltration, we assessed the expression of the adhesion molecule ICAM-1, and our results revealed significantly increased expression of the adhesion molecule ICAM-1 in DKO mice compared with *Apoe*$^{-/-}$ mice (Figure 2B). Moreover, consistent with macrophage infiltration in the liver of DKO mice, compared with *Apoe*$^{-/-}$ mice, serpina3c-knockout mice exhibited significantly increased hepatic production of proinflammatory cytokines, including TNF-a, monocyte chemoattractant protein-1 (MCP-1), and IL-18, and reduced expression of arginase-1 (Arg-1) and IL-1β (Figure 2C). As the JNK/NF-κB signaling pathway plays a pivotal role in the pathogenesis of steatohepatitis and the cytokines IL-18, TNF-α, and MCP-1 are associated with the activation of JNK/NF-κB signaling in steatohepatitis[17], the effects of serpina3c on NF-κB activation were investigated. JNK and p65 were extensively activated in DKO mice compared to *Apoe*$^{-/-}$ mice, as shown by the increased phosphorylation of JNK and p65 by western blotting (Figure 2D). These data suggested that serpina3c is

involved in the hepatic recruitment of inflammatory cells in HFD-induced NASH, mainly by the activation of JNK/NF-κB signaling and the infiltration of macrophages.

### Serpina3c deficiency promoted hepatic fibrosis in HFD-fed *Apoe*$^{-/-}$ mice

It is well documented that fibrosis is a salient feature of steatohepatitis. Transforming growth factor-beta1 (TGF-β1) is a cell factor that has been found to be involved in tissue fibrosis, and it plays an important role in NASH[18]. To investigate whether serpina3c is involved in hepatic fibrosis, hepatic mRNA levels of transforming growth factor-beta1 (TGF-β1) were measured by real-time PCR. The hepatic TGF-β1 mRNA levels were higher in DKO mice (Figure 3A). Collagen deposition was markedly increased in the liver sections of DKO mice compared with those of *Apoe*$^{-/-}$ mice, as shown by Sirius Red staining (Figure 3B). In addition, immunohistochemistry staining also revealed increased α-SMA expression in DKO mice (Figure 3C). These data suggested that serpina3c is required for the development of hepatic fibrosis.

### The effect of serpina3c on the necroptosis in vivo and in vitro

Multiple forms of cell death, including apoptosis, pyroptosis and necroptosis, are associated with NAFLD. The liver pathology results showed that hepatocyte death was appreciably increased in DKO mice. To further determine which cell death pathway is involved in hepatocellular death due to serpina3c deficiency, the protein expression pattern in the liver was analyzed by western blotting. Compared with *Apoe*$^{-/-}$ mice, DKO mice exhibited a dramatic increase in the expression of RIP3 and p-MLKL (Figure 4A), whereas the expression of cleaved-caspase 3, bcl-2 and GSDMD were unchanged between the two groups. The data indicated that necroptosis was the main mechanism of cell death in serpina3c-deficient cells. To further explore the effect of serpina3c on necroptosis in NAFLD, we treated AML12 cells with palmitic acid (PA) to establish an in vitro model of NAFLD after lentivirus transfection to overexpress and knock down serpina3c. First, AML12 cells were treated with PA (0, 100, 200, 300, 400 μM) for 24 h. We investigated the impact of PA on necroptosis in AML12 cells. As shown in Figure 4B, the hepatic TG content was markedly increased in AML12 cells treated with different concentrations of PA for 24 h in a concentration-dependent manner. Moreover, AML12 hepatocyte staining with propidium iodine (PI) and trypan blue showed dose-dependent increases in the size of the PI-positive and trypan blue-positive populations after treatment with PA (Figure 4C and 4D), indicating an increase in necroptosis. Next, we also examined the expression of the necrosome protein complex. The expression of RIP3 and p-MLKL was increased in a concentration-dependent manner (Figure 4E). As shown above, the hepatic TG content and necroptosis of AML-12 cells started to significantly increase in response to 200 μM PA; thus, PA at a concentration of 200 μM was used to treat the cells. AML12 cells were transfected with lentivirus to overexpress (LV-OV) or knockdown serpina3c (LV-KD), and negative control (LV-NC) lentivirus was used as a control. We found that LV-OV significantly increased the expression of serpina3c and LV-KD decreased the expression of serpina3c in AML12 cells (Figure 4F). We found that the protein expression of RIP3 and phosphorylated MLKL was strongly upregulated after the knockdown of serpina3c in AML12 cells treated with PA and obviously downregulated by the overexpression of serpina3c (Figure 4G). To

determine whether necroptosis could be attenuated via a necroptosis inhibitor, we treated AML12 cells with necrosulfonamide (Nec) for 2 h before stimulation with PA. After 24 h, we measured changes in necroptosis. The results showed that the protein expression of RIP3 and phosphorylated MLKL induced by serpina3c knockdown in AML12 cells was attenuated by the necroptosis inhibitor (Figure 4H). Knockdown of serpina3c resulted in a marked increase in the TG contents, as shown by Oil red O staining, and overexpression of serpina3c reduced the hepatic TG contents (Figure 4I). Moreover, necroptosis inhibitors also decreased the hepatic TG contents induced by serpina3c knockdown. Next, we found that knockdown of serpina3c increased the size of the PI-positive and trypan blue-positive populations, overexpression of serpina3c significantly inhibited necroptosis, and necroptosis inhibitors also reversed the necroptosis induced by serpina3c knockdown. (Figure 4J and 4K). Collectively, these various results thus showed that the specific role of serpina3c in PA-induced hepatocyte necroptosis is involved in the pathogenesis of NASH, which in turn can be rescued by Nec.

### The effect of serpina3c on β-catenin and Foxo1 expression and nuclear transfer

We next sought to dissect the molecular mechanisms underlying the regulation of necroptosis by serpina3c. Several studies have reported that serpin, as a Wnt inhibitor, downregulated the levels of β-catenin[19]. The Wnt/β-catenin pathway has been proven to play a key role in the development of necroptosis[20]. To investigate the potential effects of serpina3c on the regulation of β-catenin signaling, the total protein and nuclear protein levels of β-catenin were measured. The total protein levels of β-catenin were significantly increased in DKO mice versus *Apoe*$^{-/-}$ mice (Figure 5A). Moreover, the total protein and nuclear protein levels of β-catenin were significantly upregulated in serpina3c-knockdown cells, and these effects were reversed by serpina3c overexpression (Figure 5B and 5C). The regulation of forkhead box o1 (Foxo1) by β-catenin is particularly important in reducing liver necroptosis[21]. β-catenin binds to T-cell factor (TCF) to activate profibrotic genes and binds to Foxo1 to promote necroptosis under oxidative stress. Perhaps not surprisingly, as a result of the upregulation of β-catenin expression, the total protein levels of Foxo1 were increased and Foxo1 phosphorylation was decreased in DKO mice versus *Apoe*$^{-/-}$ mice in vivo and in LV-KD-transfected cells versus LV-NC-transfected cells in vitro; however, serpina3c overexpression reversed these effects in vitro (Figure 5A and 5B). Moreover, the nuclear protein levels of Foxo1 were significantly upregulated in serpina3c-knockdown cells, and this effect was reversed by serpina3c overexpression (Figure 5C). These data suggest that the absence of Serpina3c expression promotes Foxo1 and β-catenin expression in the nucleus. We then asked whether there is putative crosstalk between Foxo1 and β-catenin signaling in serpina3c-knockdown AML12 cells treated with PA. Immunofluorescence staining revealed increased nuclear Foxo1 and β-catenin expression in siRNA-serpina3c-transfected AML12 cells. Strikingly, both Foxo1 and β-catenin were colocalized in the nucleus (Figure 5D). However, the immunofluorescence staining of Foxo1 and β-catenin was decreased after AML12 cells were pretreated with recombinant serpina3c protein (Figure 5D). TLR4, a death receptor on the plasma membrane, is a downstream target gene of Foxo1. Studies have shown that increasing Foxo1 activity promotes innate TLR4-mediated inflammatory responses and tissue injury[22, 23]. We then analyzed the levels of TLR4 in vivo and in vitro. Our results showed increased TLR4 protein expression

when serpina3c expression was downregulated, but TLR4 protein expression was reduced by serpina3c overexpression in vitro (Figure 5A and 5B). Taken together, these results indicated that serpina3c, as a WNT inhibitor, prevented β-catenin activity and Foxo1/TLR4 expression.

### Serpina3c inhibits necroptosis via β-catenin/Foxo1/TLR4 signaling pathway

To further investigate the role of β-catenin/Foxo1 in the regulation of necroptosis by serpina3c in vitro, we treated AML12 cells with β-catenin and Foxo1 antagonists for 2 h before stimulation with PA. After 24 h, we observed changes in the activation of necroptosis. The results showed that compared with PA alone in the LV-KD group, inhibition of β-catenin with a β-catenin antagonist suppressed the protein expression of Foxo1, TLR4, RIP3 and p-MLKL in the LV-KD group (Figure 6A). This result suggested that serpina3c inhibited Foxo1/TLR4 expression through β-catenin inhibition. Then, we used a Foxo1 antagonist and found that PA alone in the LV-KD group, the Foxo1 antagonist suppressed protein expression of TLR4, RIP3 and p-MLKL in the LV-KD group (Figure 6B). Moreover, the number of the PI-positive and trypan blue-positive populations were significantly smaller in the LV-KD group treated with the β-catenin or Foxo1 antagonist than in the LV-KD group treated with PA alone (Figure 6C, 6D). Oil red O staining showed that the β-catenin or Foxo1 antagonist reversed the increased hepatic TG content induced by serpina3c downregulation (Figure 6E). Overall, serpina3c inhibited necroptosis via β-catenin/Foxo1/TLR4 signaling in vitro.

## Discussion

In this study, we first confirmed herein that serpina3c plays an important role in the progression of NASH. Most importantly, the results demonstrated that serpina3c deficiency significantly promoted NASH pathogenesis, including hepatic steatosis, inflammation and liver fibrosis, as well as noticeably exacerbated liver injury, which manifested as hepatocyte ballooning and increased serum ALT and AST levels. In addition, the results showed that serpina3c plays a protective role against necroptosis. The possible underlying molecular mechanism is that serpina3c, as a Wnt inhibitor, suppresses necroptosis via β-catenin/Foxo1/TLR4 signaling. Taken together, our data revealed the importance of serpina3c in NASH and the underlying mechanism, which sheds light on potential strategies for using serpin in the treatment of NAFLD.

NASH is the result of advanced NAFLD liver injury and contributes to liver-related morbidity and mortality. Many kinds of serpin, including serpina1 and serpina4, are mainly expressed in the livers of humans. The level of serpina1 was markedly reduced in patients with NASH, suggesting that serpina1 contributes to the pathogenesis of NASH[24]. Another study also demonstrated that the serpina4 gene expression levels were significantly downregulated in the livers from obese patients with NAFLD. These studies illustrate that serpin is associated with NAFLD, but currently, there is a lack of further research. In this study, our results showed that serpina3c knockout worsened steatosis, suggesting that serpina3c may play an important protective role in NAFLD. Our results also showed that the gene expression of SREBP-1c and CD36 was increased in DKO mice fed HFD, although CD36 gene expression was not statistically

significant. These data suggested that serpina3c deficiency led to the fatty acid disorder in liver. The contribution of necroptosis to the pathogenesis of NASH is the subject of great interest. It is now widely recognized that hepatic lipid accumulation and increased free fatty acid (FFA) levels cause lipotoxicity to hepatocytes and induce cell death[25]. In our study, hepatocyte nuclear marginalization and fragmentation were observed by HE staining in serpina3c-deficient cells. These histological changes indicate that under conditions of hepatic lipotoxicity, serpina3c deficiency causes hepatocellular death. Regarding the mechanism of cell death in NASH, our results showed that the expression of apoptotic marker proteins was unchanged. In addition to apoptosis, recent study has shown a worsening of ballooning and fibrosis in clinical trials of NASH using pan-caspase inhibitors, raising the possibility of a switch to a necroptotic form of cell death[26]. Necroptosis relies on the kinase cascade, which activation of RIPK3 promotes the formation of the “necrosome”, a complex containing RIPK1, RIPK3, and MLKL. Several studies have confirmed that canonical RIP3-MLKL signaling plays a critical role in the pathogenesis of NASH[27]. In this study, we showed that serpina3c knockout promoted the expression of RIPK3 and p-MLKL. In vitro, serpina3c inhibits PA-induced necroptosis, suggesting that serpina3c is able to inhibit necroptosis under conditions of lipotoxicity. However, our results also show that RIP3 and p-MLKL expression was not different in hepatocytes with reduced serpina3c expression without PA treatment. Thus, it is likely that necroptosis does not occur under physiological conditions, suggesting that Serpina3c deficiently increases the susceptibility of the liver to lipotoxicity and promotes cell necroptosis.

There is strong evidence that hepatocyte cell death drives inflammation and fibrosis in NASH[28]. The JNK/NF-κB signal is one of the important pathways associated with the inflammatory response. The activation of this pathway can lead to the expression of a large number of inflammatory cytokines and then induce a series of inflammatory responses. Multiple cytokines, including TNF-α, IL-6, and IL-8, are involved in the development of NASH[29]. Here, we proved that serpina3c deficiency greatly promoted activation of the JNK/NF-κB signaling pathway and increased the expression of the inflammatory cytokines IL-18, TNF-a, and MCP-1, which are downstream target genes of NF-κB. In addition, serpina3c deficiency contributes to the hepatic expression of the adhesion molecule ICAM-1, and indicate that serpina3c deficiency increased inflammatory cell infiltration in the liver, which is consistent with our previous reports and other reports about the anti-inflammatory effect of serpin in various metabolic diseases[30]. Fibrosis is an important prognostic marker in human NASH. Inflammatory cells infiltrating the damaged area can release various cytokines to activate cells that secrete a large amount of ECM. In this study, serpina3c deficiency presented profibrotic activity due to the expression of the master regulator of fibrosis initiation, TGF-β, and collagenous fiber deposition also increased. In summary, Serpina3c deficiency promotes the inflammatory response by activating the JNK/NF-κB signaling pathway, resulting in liver fibrosis.

The Wnt/β-catenin signaling pathway is involved in the regulation of various cellular actions, including apoptosis, necrosis and inflammation[31]. A study reported that serpina3k, which belongs to the same serpina branch as serpina3c, binds to low-density lipoprotein receptor-like protein 6 (LRP6) and blocks its Wnt ligand-induced dimerization with the Fz receptor, leading to the downstream inhibition of β-

catenin[32]. Activation of β-catenin signaling promotes endothelial cell programmed necrosis under conditions of hyperlipidemia[33]. In addition, activated β-catenin can enter the nucleus and bind to Foxo1 to activate Foxo1 and increase hepatocyte necroptosis[21]. Our data show that serpina3c knockdown activated β-catenin/Foxo1 and promoted necroptosis under conditions of lipotoxicity. β-catenin and Foxo1 inhibitors were used to further verify that serpina3c inhibited necroptosis through β-catenin/Foxo1. These observations corroborated the hypothesis that serpina3c ameliorates hepatic necroptosis via β-catenin/Foxo1 signaling. Furthermore, the present study reported that the Foxo1 pathway altered TLR4 expression, which plays an important role in necroptosis[34]. TLR4 is a downstream target gene of the transcription factor Foxo1 and contributes to the regulation of inflammation and necroptosis. Our study showed that the absence of serpina3c increased TLR4 expression by β-catenin/Foxo1. Taken together, our findings demonstrate that serpina3c, as a Wnt inhibitor that mediates β-catenin/Foxo1/TLR4 signaling, is a key regulator of hepatocyte necroptosis in NASH.

Remarkably, the present study highlights the importance of serpina3c as a key regulator of hepatocyte necroptosis in NASH. We demonstrated, for the first time, that in HFD-fed *Apoe*$^{-/-}$ mice, serpina3c deficiency exacerbates NASH histopathology. We also showed that serpina3c inhibits hepatocyte necroptosis, a key characteristic of NASH. Moreover, our study provided evidence that ceserpina3c, a Wnt inhibitor, prevents hepatocyte necroptosis via β-catenin/Foxo1/TLR4 signaling. The findings of the present study show that serpina3c may provide potential therapeutic strategies for NASH.

# Declarations

**Data availability** All authors have access to the entirety of the data underlying this manuscript. Access to the data can be granted at any time upon reasonable request.

**Animal research** the animal studies were approved by the Care of Experimental Animals Committee of Southeast University. All the manipulation of animals were performed in the Animal Center of Southeast University (No.: 20200326003).

**Consent to Participate** Not applicable.

**Consent to Publish** Not applicable.

**Plant Reproducibility** Not applicable.

**Clinical trials registeration** Not applicable.

**Author contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jing-jing Ji, Jia-qi Guo, Ya Wu, Ziwei Yang and Yu Jiang. The first draft of the manuscript was written by Ling-lin Qian and Yu-yu Yao and all authors commented on previous versions

of the manuscript. All authors read and approved the final manuscript. Gen-shan Ma revised the manuscript.

**Conflict of interest** the authors Ling-lin Qian, Jing-jing Ji, Yu Jiang, Jia-qi Guo, Ya Wu, Ziwei Yang, Gen-shan Ma and Yu-yu Yao declared that they have no conflict of interest.

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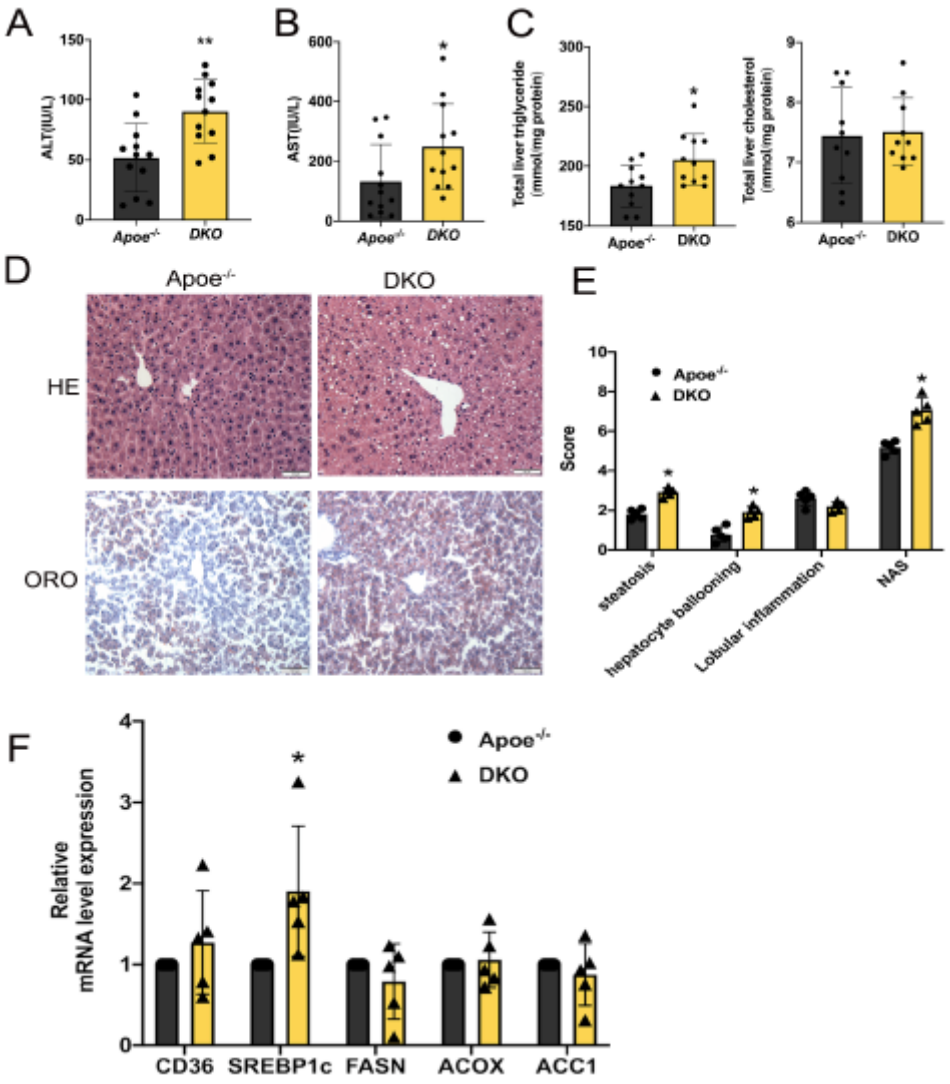
# References

1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and outcomes. Hepatology. 2016;64:73–84.
2. Ju C, Tacke F. Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies. Cell Mol Immunol. 2016;13:316–27.
3. Wree A, Holtmann TM, Inzaugarat ME, Feldstein AEJSiLD. Novel Drivers of the Inflammatory Response in Liver Injury and Fibrosis. Semin Liver Dis. 2019, 39.
4. Shojaie L, Iorga A, Dara L. Cell Death in Liver Diseases: A Review. Int J Mol Sci. 2020.
5. Afonso MB, Rodrigues PM, Carvalho T, Caridade M, Rodrigues CJCS. Necroptosis is a key pathogenic event in human and experimental murine models of non-alcoholic steatohepatitis. Clin Sci (Lond). 2015;129:721–39.
6. Kawai T, Akira SJI. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34:637–50.
7. Zheng X, Cui H, Yin Y, Zhang Y, Zong R, Bao X, Ma JX, Liu Z, Zhou Y. SERPINA3K Ameliorates the Corneal Oxidative Injury Induced by 4-Hydroxynonenal. Invest Ophthalmol Vis Sci. 2017;58:2874–83.
8. He Y, Han Y, Xing J, Zhai X, Wang S, Xin S, Zhang J. Kallistatin correlates with inflammation in abdominal aortic aneurysm and suppresses its formation in mice. Cardiovasc Diagn Ther. 2020;10:107–23.
9. Ji JJ, Qian LL, Zhu Y, Wu YP, Yao YYJCS. Serpina3c protects against high-fat diet-induced pancreatic dysfunction through the JNK-related pathway. Cell Signal. 2020:109745.
10. Choi Y, Choi H, Yoon BK, Lee H, Seok JW, Kim HJ, Kim JW: Serpina3c Regulates Adipogenesis by Modulating Insulin Growth Factor 1 and Integrin Signaling. *iScience* 2020, 23:100961.
11. Schierwagen R, Maybuchen L, Zimmer S, Hittatiya K, Back C, Klein S, Uschner FE, Reul W, Boor P, Nickenig G, et al. Seven weeks of Western diet in apolipoprotein-E-deficient mice induce metabolic syndrome and non-alcoholic steatohepatitis with liver fibrosis. Sci Rep. 2015;5:12931.
12. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.

13. Wilson RB, Chen YJ, Sutherland BG, Sawyez CG, Zhang R, Woolnough T, Hetherington AM, Peters KM, Patel K, Kennelly JP, et al. The marine compound and elongation factor 1A1 inhibitor, didemnin B, provides benefit in western diet-induced non-alcoholic fatty liver disease. Pharmacol Res. 2020;161:105208.
14. Qian LL, Ji JJ, Guo JQ, Wu YP, Ma GS, Yao YY. Protective role of serpina3c as a novel thrombin inhibitor against atherosclerosis in mice. Clin Sci. 2021;135:447–63.
15. Tomizawa M, Kawanabe Y, Shinozaki F, Sato S, Motoyoshi Y, Sugiyama T, Yamamoto S, Sueishi MJBR. Triglyceride is strongly associated with nonalcoholic fatty liver disease among markers of hyperlipidemia and diabetes. Biomed Rep. 2014.
16. Wang X, Zheng Z, Caviglia J, Corey K, Herfel T, ai BC, Masia R, Chung R, Lefkowitch J, Schwabe RJCM. Hepatocyte TAZ/WWTR1 Promotes Inflammation and Fibrosis in Nonalcoholic Steatohepatitis. Cell Metab. 2016:848–862.
17. PeeA AD, Leclercq I, Field J, George J, Jones B, Farrell GJG. NF-κB Activation, Rather Than TNF, Mediates Hepatic Inflammation in a Murine Dietary Model of Steatohepatitis. Gastroenterology. 2005;129:1663–74.
18. Dewidar B, Meyer C, Dooley S, Meindl-Beinker AN. TGF-beta in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019. *Cells*; 2019. p. 8.
19. Zhou T, Chen L, Huang CH, Lin Z, Zong R, Zhu C, Pan F, Ma JX, Liu ZG, Zhou Y. Serine Proteinase Inhibitor SERPINA3K Suppresses Corneal Neovascularization via Inhibiting Wnt Signaling and VEGF. *Invest Ophthalmol Vis Sci* 2014.
20. Zhang YZ, Wang L, Zhang JJ, Xiong XM, Zhang D, Tang XM, Luo XJ, Ma QL, Peng JJA. Vascular peroxide 1 promotes ox-LDL-induced programmed necrosis in endothelial cells through a mechanism involving β-catenin signaling. Atherosclerosis. 2018:128–138.
21. Li C, Sheng M, Lin Y, Xu D, Ke BJCD. Differentiation: Functional crosstalk between myeloid Foxo1–β-catenin axis and Hedgehog/Gli1 signaling in oxidative stress response. Cell Death Differ. 2020.
22. Fan WQ, Morinaga H, Kim JJ, Bae E, Spann NJ, Heinz S, Glass CK, Olefsky JM. FoxO1 regulates Tlr4 inflammatory pathway signalling in macrophages. Embo Journal. 2010;29:4223–36.
23. Kamo N, Ke B, Busuttil RW, Kupiec-Weglinski JW. PTEN-mediated akt/beta-Catenin/foxo1 signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury. Hepatology. 2013;57:289–98.
24. Zang S, Ma X, Zhuang Z, Liu J, Bian D, Xun Y, Zhang Q, Zhao F, Yang W, Liu J, Luo Y, Liu Y, Ye B, Ye D, Shi J.Increased ratio of neutrophil elastase to α1-antitrypsin is closely associated with liver inflammation in patients with nonalcoholic steatohepatitis, J Clinical and Experimental Pharmacology and Physiology. 2016, 43.
25. Noureddin M, Sanyal AJ. Pathogenesis of NASH: The Impact of Multiple Pathways. Curr Hepatol Rep. 2018;17:350–60.
26. Harrison SA, Goodman Z, Jabbar A, Vemulapalli R, Sanyal AJJJoH. A randomized, placebo-controlled trial of emricasan in patients with NASH and F1-F3 fibrosis. J Hepatol. 2019, 72.

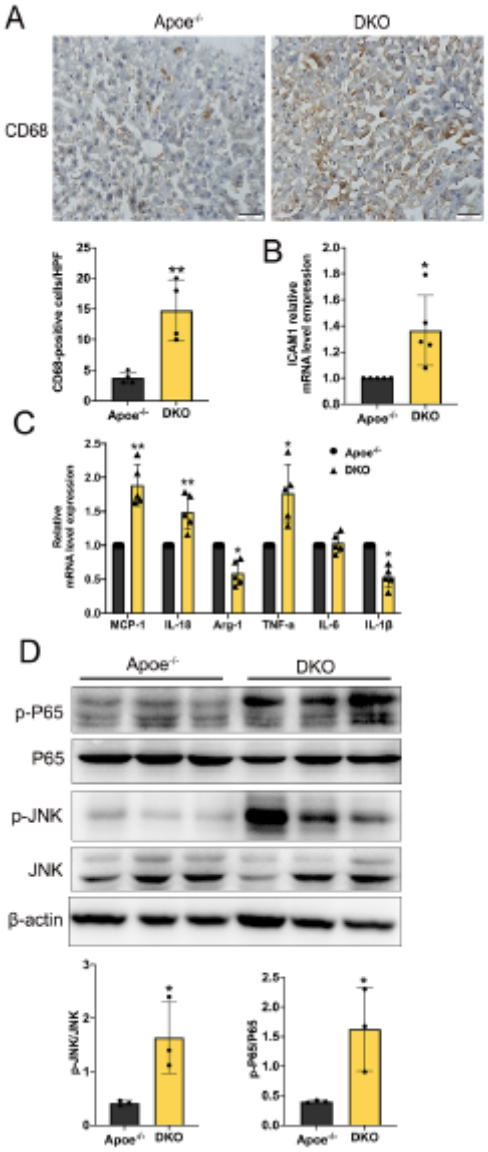
27. Wu XQ, Poulsen KL, Sanz-Garcia C, Huang E, McMullen MR, Roychowdhury S, Dasarathy S, Nagy LE. MLKL-dependent signaling regulates autophagic flux in a murine model of non-alcohol-associated fatty liver and steatohepatitis. J Hepatol. 2020;73:617–27.
28. Cazanave SC, Gores GJ. Mechanisms and clinical implications of hepatocyte lipoapoptosis. Clinical Lipidology. 2010;5:71–85.
29. Mendez-Sanchez N, Valencia-Rodriguez A, Coronel-Castillo C, Vera-Barajas A, Contreras-Carmona J, Ponciano-Rodriguez G, Zamora-Valdes D. The cellular pathways of liver fibrosis in non-alcoholic steatohepatitis. *Annals of Translational Medicine* 2020, 8.
30. Li B, Sheng Z, Liu C, Qian L, Wu Y, Wu Y, Ma G, Yao Y. Kallistatin Inhibits Atherosclerotic Inflammation by Regulating Macrophage Polarization. Hum Gene Ther. 2019;30:339–51.
31. Ming M, Wang S, Wu W, Senyuk V, Beau M, Nucifora G, Qian Z. Activation of Wnt/beta-catenin protein signaling induces mitochondria-mediated apoptosis in hematopoietic progenitor cells. J Biol Chem. 2012;287(27):22683–90.
32. Zhang B, Abreu JG, Zhou K, Chen Y, Hu Y, Zhou T, He X, Ma JX. Blocking the Wnt pathway, a unifying mechanism for an angiogenic inhibitor in the serine proteinase inhibitor family. Proc Natl Acad Sci U S A. 2010;107:6900–5.
33. Wilson RB, Chen YJ, Sutherland BG, Sawyez CG, Zhang R, Woolnough T, Hetherington AM, Peters KM, Patel K, Kennelly JP, et al: The marine compound and elongation factor 1A1 inhibitor, didemnin B, provides benefit in western diet-induced non-alcoholic fatty liver disease. *Pharmacological Research* 2020, 161.
34. Jorgensen I, Rayamajhi M, Miao EAJNRI. Programmed cell death as a defence against infection. Nat Rev Immunol. 2017;17:151–64.

# Figures



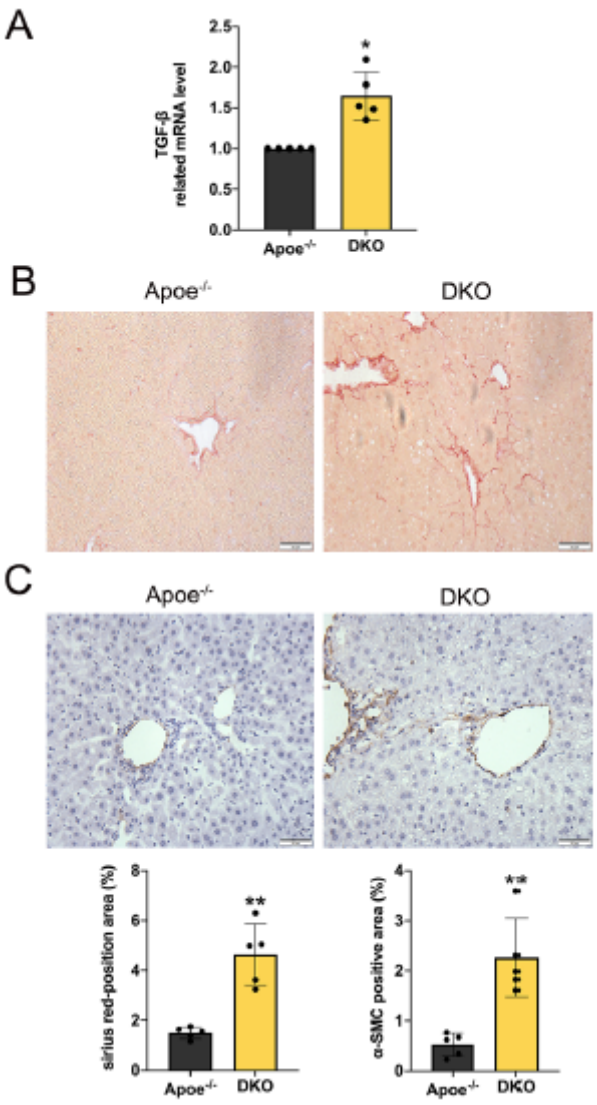

## Figure 1

Serpina3c deficiency promoted liver injury and hepatic steatosis in HFD-fed DKO mice. Male Apoe-/- mice and DKO mice were fed HFD for 12 weeks. A and B, ALT and AST levels in the plasma from Apoe-/- mice and DKO mice (n=12/group). C, Levels of triglyceride (TG) and cholesterol (TC) in the liver from Apoe-/- mice and DKO mice(n=12/group). D, Representative Haematoxylin and Eosin (HE) staining and Oil red O (ORO) staining of liver sections after HFD feeding for 12 weeks (n=5/group). Scale bars, 100μm. E, Scores for steatosis, hepatocyte ballooning, and lobular inflammation were determined in three H&E-stained hepatic sections per mouse, and were summed to generate NAFLD activity scores (NAS). F, Quantitative RT-PCR-assisted detection of CD36, SREBP1c, ACC, FASN, and ACOX in liver (n=5/group). All data represent the mean ± SD. *p < 0.05, vs. Apoe-/- mice group.



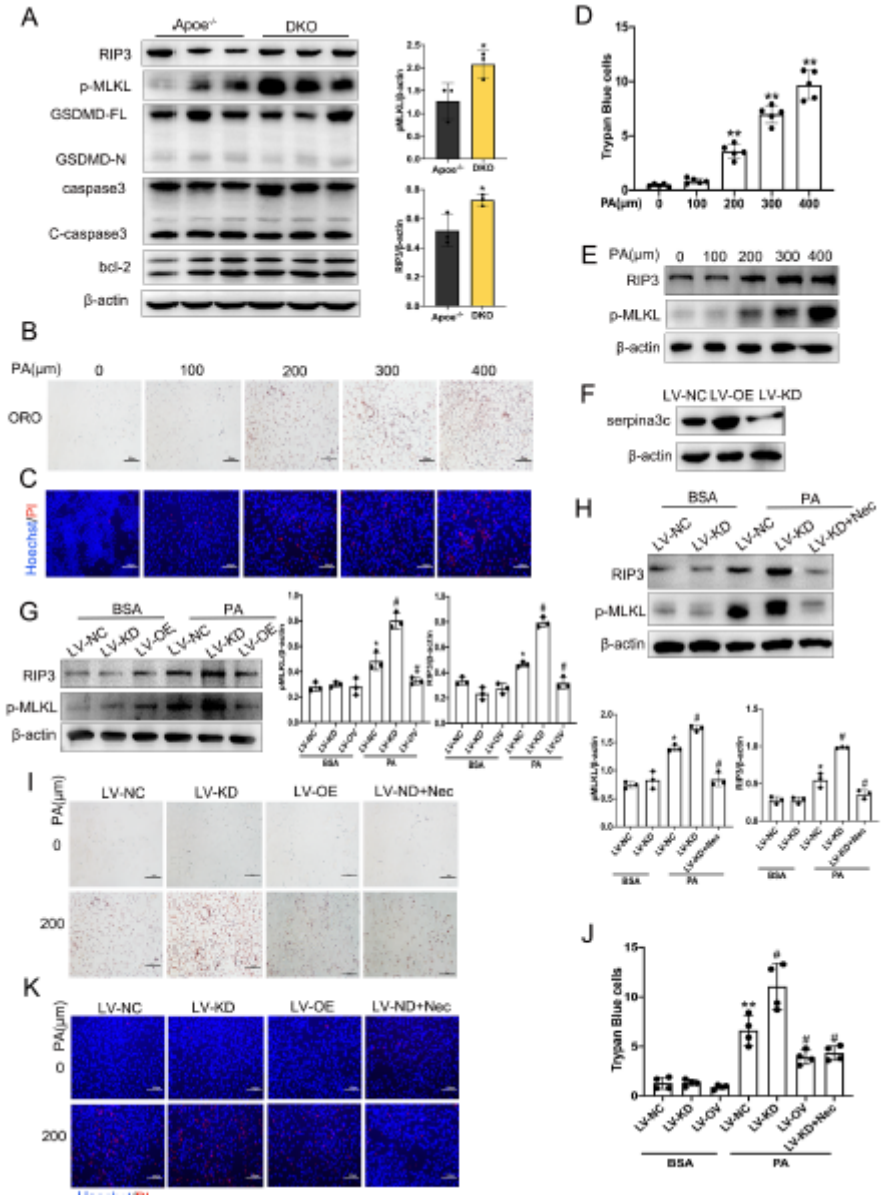

## Figure 2

Serpina3c deficiency promoted hepatic inflammation response in HFD-fed DKO mice A, Representative Immunostaining detected liver CD68 + macrophages in liver sections after HFD feeding for 12 weeks (n=5/group). Scale bars, 100μm. B, RT-PCR analyses of liver adhesion molecule ICAM-1 in liver from Apoe-/- mice and DKO mice (n=5/group). C, Quantitative RT-PCR-assisted detection of MCP-1, IL-18, Arg, TNF-α, IL-6 and IL-1β in liver (n=5/group). D, Immunoblot detection of liver p-JNK, JNK, p-P65 and P65 expression. Quantification of the blots normalized to the level of β-actin expression. (n=3/group). All data represent the mean ± SD. *p < 0.05, vs. Apoe-/- mice group.




## Figure 3

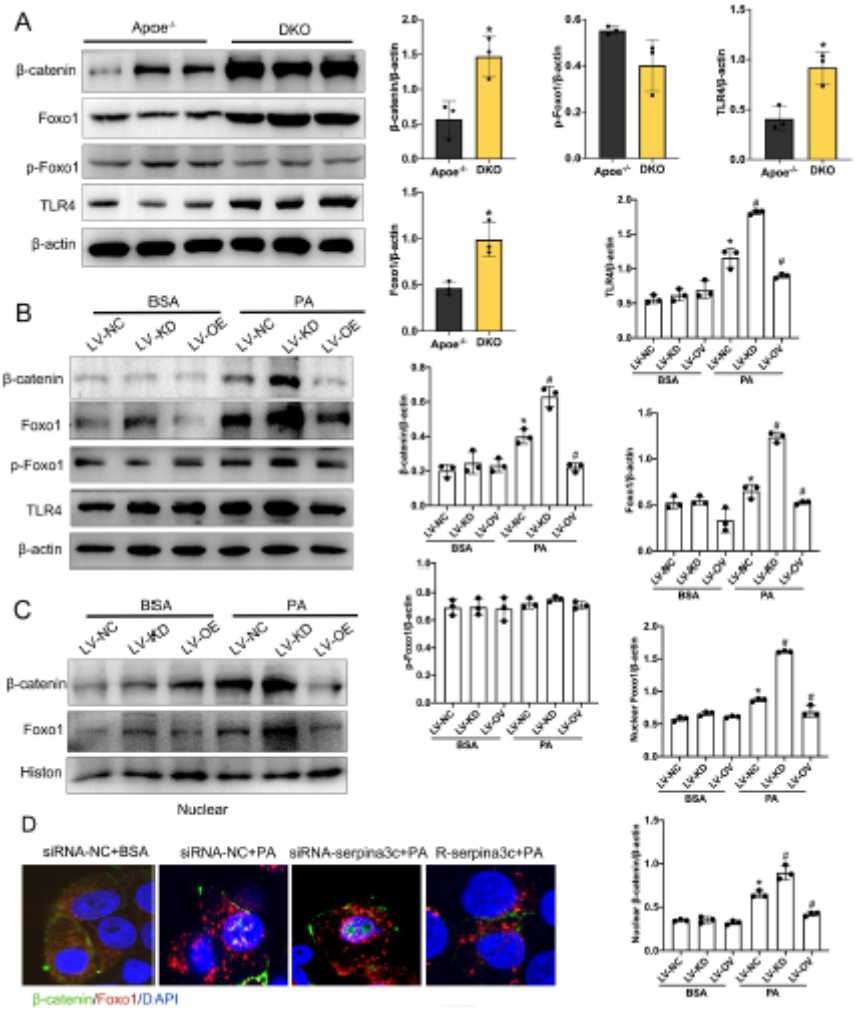
Serpina3c deficiency promoted hepatic fibrosis in HFD-fed DKO mice A, Quantitative RT-PCR-assisted detection of TGF-β in liver (n=5/group). B and C, Representative Sirius Red staining and α-SMA+ staining of liver sections after HFD feeding for 12 weeks (n=5/group). Scale bars, 100μm. All data represent the mean ± SD. *p < 0.05, vs. Apoe-/- mice group.




## Figure 4

The effect of serpina3c on the necroptosis in vivo and in vitro A, western blot assay analysis of liver RIP3, p-MLKL, cleaved-caspase 3, bcl-2 and GSDMD expression. Quantification of the blots normalized to the level of β-actin expression. (n=3/group). All data represent the mean ± SD. *p < 0.05, vs. Apoe-/- mice group. B, hepatocyte AML12 were treated with BSA or different concentrations PA for 24 h, followed by Oil-red O staining. Scale bar, 50μm. C, hepatocyte AML12 were treated with BSA or different concentrations PA for 24 h, followed by representative immunofluorescence staining of PI. Scale bar, 100μm. D, the number of cell necroptosis was tested by trypan blue after treatment with BSA or different concentrations PA for 24 h in AML12 cells. E, western blot assay analysis of RIP3, p-MLKL expression after treatment with BSA or different concentrations PA for 24 h in AML12 cells. Quantification of the blots normalized to the level of β-actin expression. F, Western blot analysis the expression of serpina3c in AML12 cell lysates transfection with control lentivirus (LV-NC), knockdown lentivirus (LV-KD) and overexpression lentivirus (LV-OV). G, Western blot analysis the expression of RIP3, p-MLKL in LV-NC, LV-

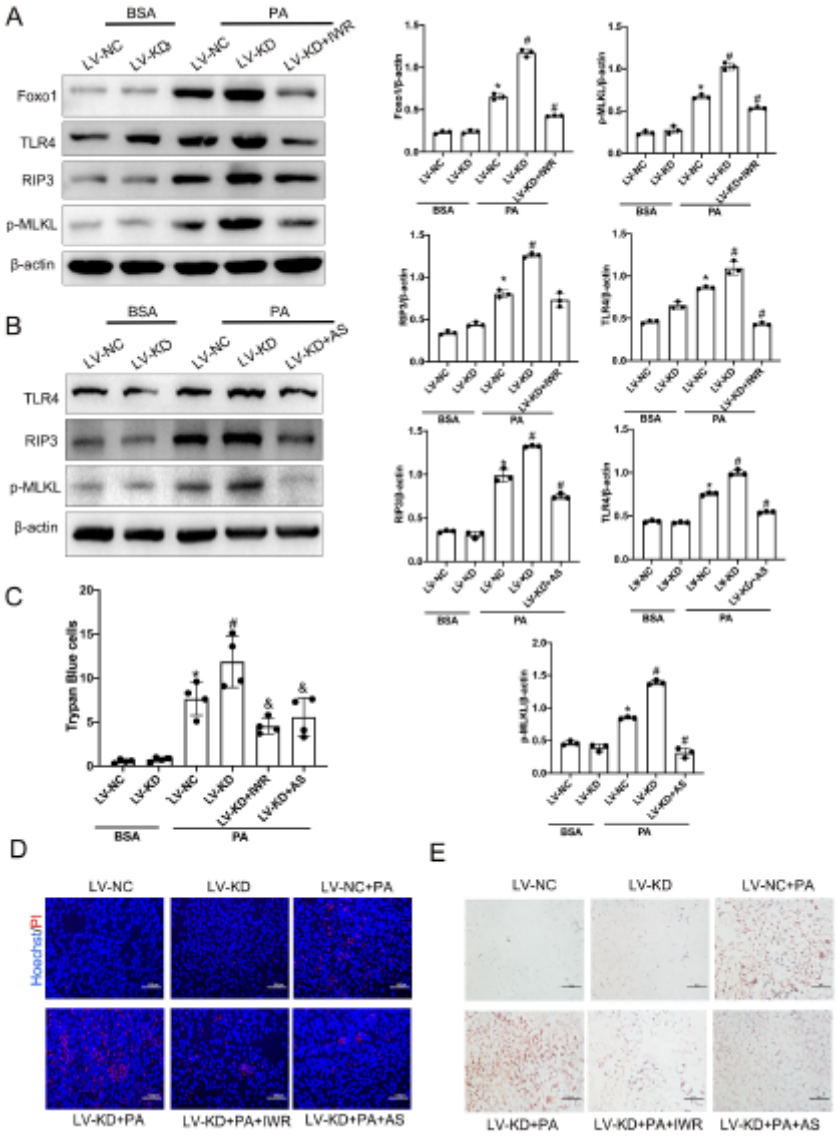
KD and LV-OV AML12 cells treatment with 200μM PA for 24 h. Quantification of the blots normalized to the level of β-actin expression. H, Immunoblot analysis of RIP3, p-MLKL after transfection with LV-NC and LV-KD with or without pretreatment with Necrosulfonamide (Nec) for 2 h before stimulation with PA. I, hepatocyte AML12 Oil-red O staining treatment with PA for 24 h. Scale bar, 50μm. J, the number of cell necroptosis was tested by trypan blue. K, Representative immunofluorescence staining of PI. Scale bar, 100μm. All data are expressed as mean ± SD from three independent experiment. *P<0.05, **P<0.01, vs. LV-NC. #P<0.05, ##P<0.01, vs. LV-NC+PA.




## Figure 5

The effect of serpina3c on β-catenin and Foxo1 expression and nuclear transfer A, western blot assay analysis of β-catenin and Foxo1 expression in liver. Quantification of the blots normalized to the level of β-actin expression. (n=3/group). All data represent the mean ± SD. *p < 0.05, vs. Apoe-/- mice group. B western blot assay analysis of total protein β-catenin, Foxo1 and TLR4 expression in PA-stimulated hepatocyte AML12. Quantification of the blots normalized to the level of β-actin expression. C, western blot assay analysis of total protein and nuclear β-catenin and Foxo1 expression in PA-stimulated hepatocyte AML12. Quantification of the blots normalized to the level of β-actin expression. D, Immunofluorescence staining for hepatocyte AML12 Foxo1 (red) and β-catenin (green) colocalization in

the nucleus after transfection knockdown serpina3c siRNA(siRNA-serpina3c) and control siRNA (siRNA-NC) in PA stimulation with or without recombinant serpina3c protein (R-serpina3c) pretreatment. Scale bars, 10 μm. All data are expressed as mean ± SD from three independent experiment. *P<0.05, **P<0.01, vs. LV-NC. #P<0.05, ##P<0.01, vs. LV-NC+PA.




## Figure 6

Serpina3c inhibits necroptosis via β-catenin/Foxo1/TLR4 signaling pathway AML12 hepatocytes were transfected with LV-NC and LV-KD, then pre-treated with or without IWR-1 or AS1842856 and challenged with PA for 24 h. A, western blot assay analysis of TLR4, Foxo1, p-MLKL and RIP3 expression. Quantification of the blots normalized to the level of β-actin expression. B, western blot assay analysis of TLR4, p-MLKL and RIP3 expression. Quantification of the blots normalized to the level of β-actin expression. C, the number of cell necroptosis was tested by trypan blue. D, Representative immunofluorescence staining of PI. Scale bar, 100μm. E, images of hepatocyte AML12 Oil-red O staining. Scale bar, 50μm. All data are expressed as mean ± SD from three independent experiment. *P<0.05, **P<0.01, vs. LV-NC. #P<0.05, ##P<0.01, vs. LV-NC+PA. &P<0.05, vs. LV-KD+PA.

# Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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