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Gasdermin D plays a key role as a pyroptosis executor of non-alcoholic steatohepatitis in humans and mice

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**Abbreviations:** NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; WT, wild type; MCD, methionine-and-choline-deficient; HFD, high fat diet; NAS, NAFLD activity score; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GGT, gamma glutamyl transpeptidase; CHO, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; AUROC, area under the receiver operating characteristic; TBARS, thiobarbituric acid reactive substances; NF-κB, nuclear factor-κB; SREBP-1c, sterol regulatory element binding protein isoform 1c; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase isoform-1; Cyp, cytochrome P450; ACO, acyl-CoA oxidase; LCAD, long-chain acyl-CoA dehydrogenase; LXR, liver X-activated receptor; PPAR, peroxisome proliferator-activated receptor; AAV, Adeno-associated virus; ALD, alcoholic liver disease; GSDMD, gasdermin D; GSDMD-N, cleaved gasdermin-N domain; GSDMD-FL, GSDMD full-length; TGF-β1, transforming growth factor beta 1; α-SMA, alpha-smooth muscle actin; IL-1β, interleukin (IL)-1 beta; TNF-α, tumor necrosis factor alpha; MCP-1, monocyte chemoattractant protein-1; EV, empty vector.

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Abstract

Background & Aims: Gasdermin D (GSDMD)-executed programmed necrosis is involved in inflammation and controls interleukin (IL)-1β release. However, the role of GSDMD in non-alcoholic steatohepatitis (NASH) remains unclear. We investigated the role of GSDMD in the pathogenesis of steatohepatitis.

Methods: Human liver tissues of non-alcoholic fatty liver disease (NAFLD) patients and control subjects were obtained to evaluate GSDMD expression. GSDMD knockout (GSDMD<sup>−/−</sup>) mice, obese db/db mice and their wild-type (WT) littermates were fed with methionine-and-choline-deficient (MCD) or control diet to induce steatohepatitis. The GSDMD<sup>−/−</sup> and WT mice were also used in a high fat diet (HFD)-induced NAFLD model. In addition, Alb-Cre mice were administered an adeno-associated virus (AAV) vector that expressed the gasdermin-N domain (AAV9-FLEX-GSDMD-N) and were fed with either MCD
or control diet for 10 days.

**Results:** GSDMD and its pyroptosis-inducing fragment GSDMD-N were upregulated in liver tissues of human NAFLD/NASH. Importantly, hepatic GSDMD-N protein levels were significantly higher in human NASH and correlated with the NAFLD activity score (NAS) and fibrosis. GSDMD-N remained a potential biomarker for the diagnosis of NASH. MCD-fed \textit{GSDMD}^{−/−} mice exhibit decreased severity of steatosis and inflammation compared with WT littermates. GSDMD was associated with the secretion of pro-inflammatory cytokines (IL-1β, TNF-α, and MCP-1) and persistent activation of the NF-κB signaling pathway. \textit{GSDMD}^{−/−} mice showed lower steatosis mainly by reduced expression of the lipogenic gene SREBP-1c and upregulated expression of lipolytic genes, including PPARα, ACO, LCAD, Cyp4a10 and Cyp4a14. \textit{Alb-Cre} mice administered with AAV9-FLEX-GSDMD-N showed significantly aggravated steatohepatitis when fed with MCD diet.

**Conclusion:** GSDMD plays a key role as a pyroptosis executor in the pathogenesis of steatohepatitis of NASH by controlling cytokine secretion, NF-κB activation, and lipogenesis.

**Lay summary**

NAFLD has become one of the most feared chronic liver diseases since it is the most rapid growing indication for adult liver transplantation and a major cause of hepatocellular carcinoma. However, the mechanisms involved in the transformation of simple steatosis to steatohepatitis remains unclear. Here we
show that GSDMD-driven pyroptosis is prominent in patients with non-alcoholic steatohepatitis (NASH), and GSDMD-N remains a potential biomarker for the diagnosis of NASH. GSDMD is involved in the pathogenesis of NASH mainly by regulating lipogenesis, the inflammatory response, and the NF-κB signaling pathway amplification cascade, which opens up new sight for the treatment of NASH in humans.

Introduction

Non-alcoholic fatty liver disease (NAFLD) represents a multi-step liver biological disorder with increased risk of cirrhosis and tumorigenesis\textsuperscript{1, 2}. Extensive basic and translational research have vividly mimicked the key aspects of steatohepatitis, and have offered the opportunity to take a reductionist approach to assess the genes or dietary factors involved in the pathogenesis of NAFLD\textsuperscript{3, 4}. Toxic lipid accumulation in the liver acts as the primary insult which initiates and propagates damage leading to hepatocyte injury and resultant inflammation\textsuperscript{5, 6}. It is important to note that inflammation in the liver is believed to be the compelling feature that transforms simple steatosis to steatohepatitis, which perpetuates hepatocellular injury and subsequent cell death, and promotes liver fibrosis\textsuperscript{7-9}. However, the molecular basis behind the inflammatory response leading to steatohepatitis is still largely unknown.

Inflammatory caspases, including caspase-1, murine caspase-11, and human caspase-4/5, play important roles as mediators of inflammation\textsuperscript{10-12}. 
Accumulating evidence has suggested that excessive activation of inflammatory caspases is implicated in the pathogenesis of hepatitis C virus infection, alcoholic liver disease (ALD), as well as non-alcoholic steatohepatitis (NASH). Pyroptosis is the dominant response following the activation of inflammatory caspases, leading to pore formation in the plasma membrane, cell swelling, and massive release of the pro-inflammatory cellular contents. Gasdermin D (GSDMD), a generic substrate for inflammatory caspases, has been shown to play a specific role in inflammatory caspase-mediated pyroptosis and also acts as a downstream effector of multiple inflammasomes. GSDMD exerts its pyroptosis executor function by releasing the cleaved gasdermin-N domain (GSDMD-N) that bears intrinsic pyroptosis-inducing activity and controls interleukin (IL)-1β release. As evidenced by the crucial role of pyroptosis in immunity and disease, excessive uncontrolled pyroptosis may be detrimental to the host. Yet, despite these important functions, the potential effects of GSDMD and the mechanism of its action in steatohepatitis are still unknown. Moreover, no evidence is currently available on whether GSDMD-driven pyroptosis may represent a new avenue for therapeutic intervention of steatohepatitis. In this study, we investigated the significance of GSDMD in human and experimentally-induced NAFLD, and in particular, in steatohepatitis, and elucidated the pivotal role of GSDMD in steatohepatitis pathogenesis through its involvement in mediating the NF-κB signaling pathway amplification cascade and lipogenesis. More importantly,
we explored the clinical impact of GSDMD-N in NASH patients, and demonstrated that GSDMD-N is positively correlated with the NAFLD activity score (NAS) and fibrosis, which opens up new insight into the potential treatment of NASH in humans.

**Materials and methods**

**Human samples**

Human liver tissue samples of NAFLD/NASH were selected from bariatric surgery patients with no history of liver disease of other etiology from the Xijing Hospital, the Fourth Military Medical University. Histological assessments were determined by two pathologists in a double-blind manner. Samples from control subjects with normal liver histology were obtained from laparoscopic surgery patients with other diseases, and had normal levels of transaminases with no evidence of chronic liver disease, diabetes, or hypertension. NAFLD patients were classified into two histological groups according to the NAS. Patients with NAS of 0 to 2 were considered as not having a diagnosis of NASH (NAFLD group), while patients with scores of ≥5 were diagnosed with NASH (NASH group). Fibrosis was staged using a 4-point scale. All subjects had given written informed consent and all the procedures were performed according to the Clinical Research Ethics Committee of the Fourth Military Medical University, which meets the ethical guidelines of the 1975 Declaration of Helsinki.

**Mice models**
All animal studies were approved by the Institutional Animal Care and Use Committee at the Fourth Military Medical University, which are in accordance with NIH guidelines. Mice were housed in a pathogen-free animal facility at 22 ± 2 °C under controlled 12 hours light/dark cycle. Mice were given regular chow or special custom diets when indicated, and had access to autoclaved water ad libitum.

The GSDMD−/− mouse was a generous gift from Professor Feng Shao (National Institute of Biological Sciences, Beijing, China). Eight-to-ten week old male GSDMD−/− mice (C57BL/6 strain) and male wild type (WT) littermate controls were fed randomly either with methionine-and-choline-deficient (MCD) or control diet (Medicine Biomedical, China) for 4 weeks to trigger steatohepatitis, or for 8 weeks to establish fibrosing steatohepatitis. Age matched obese db/db mice (C57BL/6 strain) were fed with MCD or control diets for 10 days to induce steatohepatitis.

In a separate set of experiments, GSDMD−/− mice and WT controls were fed with a high fat diet (HFD) or control diet (Research diets, USA) for 4 or 11 weeks to induce steatosis. Long-term (36 weeks) HFD feeding was also used in WT mice to induce steatohepatitis. The HFD provides 60% kcal from fat, 20% kcal from proteins, and 20% kcal from carbohydrates.

In addition, eight-week old male Alb-Cre mice (C57BL/6 strain) were administered an adeno-associated virus (AAV9)-FLEX-GSDMD-N vector (containing 1-279 residues of the N-terminal cleavage fragments with an
N-terminal Flag tag) or AAV9-FLEX-control (containing a Flag tag) through the
tail vein at a dose of $10^{12}$ vg per 200 µL per mouse, respectively\textsuperscript{26}. The mice
were subjected to MCD or control diets for 10 days to induce steatohepatitis.

For the adoptive transfer study, macrophages were obtained from the
peritoneal cavities of MCD-fed WT mice four days after intraperitoneal injection
of 3% Brewer thioglycollate medium (Sigma-Aldrich, USA). Briefly, the mouse
peritoneal cavity was lavaged with 1 mL PBS. The peritoneal fluid was
dispensed and cultured with DMEM/F12 medium supplemented with 10% FBS.
After 2 hours of incubation, non-adherent cells were removed by gentle
washing with warm PBS. MCD-fed \textit{GSDMD}^{-/-} mice were intraperitoneally
injected with purified macrophages ($4.5 \times 10^6$ cell/mouse) 48 hours before
harvest\textsuperscript{27}.

\textbf{Cell culture and treatments}

The immortalized mouse hepatocyte cell line AML-12 was purchased from
ATCC (CRL 2254), and no evidence of mycoplasma contamination was
detected by using PCR-based assay test. AML-12 cells were cultured in
DMEM/F12 medium and transfected with a GV362 vector carrying a
\textit{GSDMD-N} expression cassette or control vector. Experiments were performed
on cells rendered quiescent by 24 hours of incubation in serum-free medium.
Then the cells were cultured in MCD medium or control medium with or without
anti-tumor necrosis factor-alpha (TNF-\textit{\alpha}) (1:100, Abcam) or with anti-IL-1\textit{\beta}
(1:100, Abcam) neutralizing antibodies.
Results

Characteristics of the patient population

The main clinicopathological parameters of the patients evaluated in this study are described in Supplementary Table 1. The histological characteristics of the patients are shown in Supplementary Table 2. There were no statistically differences in age or gender between the three histological groups. Serum alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), and high-density lipoprotein cholesterol (HDL-C) levels did not differ statistically among the groups; whereas, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (CHO), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) levels were higher in the NASH group.

GSDMD and its pyroptosis-inducing fragment GSDMD-N are upregulated in liver tissues of NASH patients

To investigate the involvement of GSDMD in NAFLD/NASH, we first evaluated the protein expression of full-length GSDMD (GSDMD-FL) and its pyroptosis-inducing fragment GSDMD-N in liver biopsies from 14 NASH patients, 15 NAFLD patients, and 16 control subjects. Both hepatic GSDMD-FL and GSDMD-N protein levels were upregulated in NAFLD/NASH patients compared to normal controls as determined by Western blotting (Fig. 1A and 1B and Supplementary Fig. 1A). In particular, GSDMD-N protein levels were higher in NASH patients compared to NAFLD patients (median [25th
(Q25), 75th (Q75) percentiles: 1.99 [1.27, 2.90] vs. 1.11 [0.83, 1.23]; p<0.05) (Fig. 1B), suggesting that hepatic GSDMD-N production is prominent in patients with NASH.

**GSDMD-N is associated with lobular inflammation and represents a potential biomarker for the diagnosis of NASH**

We next evaluated the clinical impact of GSDMD-FL and GSDMD-N expression. GSDMD-N was positively correlated with lobular inflammation (rho: 0.554, p<0.01) and ballooning (rho: 0.390, p<0.05), which are two major histological characteristics of NASH (Table 1). In addition, GSDMD-N was also correlated with NAS (rho: 0.532, p<0.01) and fibrosis (rho: 0.477, p<0.01) (Table 1).

To further evaluate the potential utility of GSDMD as a biomarker for the diagnosis of NAFLD/NASH, the area under the receiver-operating characteristic (AUROC) curve was calculated. Both GSDMD-FL and GSDMD-N exhibited high potential cutoff values to distinguish patients with NAFLD or NASH from control subjects (Fig. 1C and 1D). In particular, GSDMD-N showed a high overall accuracy in discriminating NASH from NAFLD patients (AUROC: 0.83 [0.67–0.98], p<0.01) (Fig. 1D). Taken together, GSDMD-N could act as a biomarker for the diagnosis NASH in humans.

**GSDMD increased the severity of MCD-induced steatohepatitis**

To address the involvement of GSDMD in the murine model of steatohepatitis, we first evaluated GSDMD-N expression in MCD-fed db/db mice (C57BL/6...
strain). Similar to human liver tissues, GSDMD-N protein expression was elevated in steatohepatitis induced by MCD (Supplementary Fig. 1B). To demonstrate the role of GSDMD in the development of steatohepatitis, 

\( \text{GSDMD}^{-/-} \) and WT mice were fed with a control diet or a MCD diet for 4 weeks to trigger steatohepatitis. In contrast to the WT mice, the knockout of GSDMD expression mitigated the serum levels of ALT (\( p<0.05 \)) and hepatic lipid accumulation (\( p<0.05 \)) (Fig. 2A). Consistently, hematoxylin and eosin (H&E) staining and Oil-red O staining showed a significant reduction of hepatic steatosis and necroinflammation in \( \text{GSDMD}^{-/-} \) mice compared with WT controls (Fig. 2B). Moreover, the histopathological analysis also revealed reduced lipid accumulation (1.67 ± 0.52 vs. 2.67 ± 0.52, \( p<0.01 \)) and inflammatory cell infiltration (0.83 ± 0.75 vs. 1.67 ± 0.52, \( p<0.05 \)) compared with the MCD-fed WT mice (Supplementary Table 3), suggesting that GSDMD promotes the development of steatohepatitis.

**GSDMD is required for hepatic nutritional fibrosis**

It is well documented that fibrosis is a salient feature of steatohepatitis\(^{28,29}\). To investigate whether GSDMD is involved in hepatic fibrosis, \( \text{GSDMD}^{-/-} \) and WT mice were fed with control diet or MCD diets for 8 weeks to induce nutritional fibrosis\(^6,22\). Hepatic transforming growth factor \( \beta 1 \) (TGF-\( \beta 1 \)) and \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) mRNA levels were significantly higher in WT mice as determined by real-time PCR (\( p<0.05 \)) (Fig. 2C and 2D). Consistently, hepatic hydroxyproline content was decreased in \( \text{GSDMD}^{-/-} \) mice (\( p<0.05 \)) (Fig. 2E).
The development of severe intraparenchymal pericellular fibrosis was observed in WT mice compared with $GSDMD^{+/+}$ mice as indicated by Sirius Red staining (Fig. 2F). Immunohistochemistry staining also revealed improved $\alpha$-SMA expression in MCD fed $GSDMD^{-/-}$ mice (Fig. 2F), suggesting that GSDMD is required for the development of hepatic nutritional fibrosis in MCD-induced steatohepatitis.

**GSDMD promoted hepatic cytokine secretion, macrophage infiltration, and persistent activation of the NF-\(\kappa\)B signaling pathway**

Inflammation plays a pivotal role in NASH development. Specifically, GSDMD is involved in inflammatory caspase-mediated innate immune defenses and also controls mature IL-1\(\beta\) release without affecting its maturation\(^{11}\). To investigate whether the less severe inflammation observed in the steatohepatitis of $GSDMD^{-/-}$ mice could be a result of changes in cytokine expression known to be modulated by GSDMD, hepatic levels of specific cytokines were evaluated. Consistent with the improved liver histology, knockout of GSDMD significantly reduced hepatic production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-\(\alpha\)), monocyte chemoattractant protein-1 (MCP-1), and IL-1\(\beta\) compared to WT controls (Fig. 3A).

As the NF-\(\kappa\)B signaling pathway plays a pivotal role in the pathogenesis of steatohepatitis, and the cytokines IL-1\(\beta\), TNF-\(\alpha\), and MCP-1 are associated with the activation of NF-\(\kappa\)B in steatohepatitis\(^{30-32}\), the effects of GSDMD on
NF-κB activation was therefore investigated. An NF-κB p65 nuclear DNA-binding activity assay showed markedly enhanced activity in WT mice compared to GSDMD$^{-/-}$ mice fed with MCD diet (Fig. 3B). Consistently, p65 was extensively activated in WT mice compared to GSDMD$^{-/-}$ mice, as shown by the increased phosphorylation of p65 by Western blotting (Fig. 3C).

It is known that IL-1β and MCP-1 are mainly produced by activated macrophages$^{11,22}$. We investigated whether GSDMD could regulate macrophage infiltration in steatohepatitis. Hepatic mRNA levels of the macrophage-specific antigen F4/80 were significantly decreased in GSDMD$^{-/-}$ mice fed with MCD diet compared with WT controls (Fig. 3D). In keeping with these results, a large amount of macrophages can be observed in MCD-fed WT mice compared with GSDMD$^{-/-}$ mice as determined by F4/80 immunohistochemistry staining (Fig. 3E). We also analyzed CD4$^{+}$T cell and CD8$^{+}$T cell infiltration, and found that there were no significant differences of CD4$^{+}$T and CD8$^{+}$T cell infiltration in WT and GSDMD$^{-/-}$ mice (Supplementary Fig. 2). These data suggested that GSDMD is involved in hepatic inflammatory recruitment in MCD-induced steatohepatitis mainly by regulating the infiltration of macrophages.

To further address whether NF-κB activation was associated with GSDMD-regulated macrophage infiltration, adoptive transfer of macrophages was performed using a murine model. Adoptive transfer of WT macrophages into GSDMD$^{-/-}$ mice lead to a significant exacerbation of p65 phosphorylation...
as determined by Western blotting (Fig. 3F), suggesting that the damaging effect of GSDMD on steatohepatitis pathogenesis at least partly depended on hepatic macrophage infiltration.

**GSDMD induced steatohepatitis by mediating the expression of lipogenic and lipolytic genes**

To seek an explanation for the reduced steatosis that developed in *GSDMD*−/− mice, we assessed the expression of genes known to be involved in hepatic fatty acid regulation. Compared with MCD-fed WT mice, *GSDMD*−/− mice fed a MCD diet showed increased mRNA expression of key lipolytic genes, including peroxisome proliferator-activated receptor α (PPARα) and its downstream target molecules acyl-CoA oxidase (ACO), long-chain acyl-CoA dehydrogenase (LCAD), and the cytochrome P450 (Cyp) family of enzymes Cyp4a10 and Cyp4a14. In addition, MCD-fed *GSDMD*−/− mice also showed lower mRNA expression of sterol regulatory element binding protein isoform 1c (SREBP-1c). However, other lipogenic genes, such as fatty acid synthase (FAS), stearoyl-CoA desaturase isoform-1 (SCD-1), liver X-activated receptor α (LXRα), and liver X-activated receptor β (LXRβ) were not significantly different between the MCD-fed WT and *GSDMD*−/− mice (Table 2). These findings indicated that GSDMD influences hepatic lipogenesis mainly by directly or indirectly (such as via IL-1β or MCP-1) regulating the expression of lipolytic genes.

**Pyroptosis-inducing activity of GSDMD-N domain caused extensive**
steatohepatitis in cultured hepatocytes

GSDMD exerts its pyroptotic function through the GSDMD-N domain. To further address the functional significance of GSDMD-mediated pyroptosis in steatohepatitis, we overexpressed the GSDMD-N domain in AML-12 hepatocytes cultured in control or in MCD medium (Fig. 4A). There was an increase in the medium levels of ALT, hepatic TG content, and hepatic lipid peroxide activity as measured by the thiobarbituric acid reactive substance (TBARS) assay, after MCD treatment (Fig. 4B-4D). In addition, NF-κB activation was intensified in AML-12 hepatocytes overexpressing GSDMD-N and cultured with MCD medium (Fig. 4E). Importantly, we found a mild increase in medium levels of ALT, hepatic lipid peroxide, and NF-κB activation under normal culture conditions after GSDMD-N transfection, suggesting that GSDMD-N is responsible for the hepatocyte injury observed in steatohepatitis.

To further address whether the activation of NF-κB was dependent on GSDMD-N-induced cytokine cascades, AML-12 hepatocytes cultured in MCD medium (transfected with GSDMD-N or control vector) were exposed to anti-IL-1β Ab and/or anti-TNF-α blocking Ab (Supplementary Fig. 3). There was a significantly decreased expression of phosphorylated p65 in AML-12 cells after the blockade of IL-1β, especially in hepatocytes overexpressing GSDMD-N. Moreover, reduced phosphorylation of p65 was also observed after anti-TNF-α Ab treatment (Fig. 4F), suggesting that the activation of NF-κB by GSDMD was at least partially dependent on GSDMD-N induced
cytokine cascades.

**GSDMD-N-driven pyroptosis increased the severity of MCD-induced steatohepatitis in mice**

To further confirm the effects of GSDMD-N in steatohepatitis, an additional MCD-induced steatohepatitis model was used. *Alb-Cre* mice were administered vector AAV9-FLEX-GSDMD-N or the control vector AAV9-FLEX-control (Fig. 5A). H&E staining showed significantly increased hepatic steatosis and necroinflammation in mice administered AAV9-FLEX-GSDMD-N (Fig. 5B). Furthermore, histological analysis showed significantly increased levels of liver steatosis and inflammation in mice administered AAV9-FLEX-GSDMD-N (Fig. 5C). Consistently, mice administered AAV9-FLEX-GSDMD-N showed aggravated liver injury as evidenced by increased serum levels of ALT and hepatic TG content, accompanied with intensified nuclear DNA-binding activity of NF-κB p65 and SREBP-1c (Fig. 5D and 5E). Importantly, mice administered AAV9-FLEX-GSDMD-N showed mild liver injury, nuclear DNA-binding activity of NF-κB p65 and SREBP-1c and IL-1β production even under normal circumstances.

To investigate whether GSDMD-N-driven pyroptosis could amplifies other cytokine or chemokine production in NASH, hepatic levels of cytokines and chemokines were evaluated by a cytokine profiling assay (Supplementary Table 4). Upregulation of GSDMD-N promoted pro-inflammatory chemokine and cytokine production, including CCL2, CCL5, CXCL9, CXCL10 and TNF.
These data suggested that GSDMD-N-driven pyroptosis is a key regulator of inflammation in steatohepatitis.

**GSDMD is implicated in the pathogenesis of HFD-induced NAFLD**

Since the MCD diet in vivo is used as a model of steatohepatitis. To further precisely evaluate the role of GSDMD in NAFLD, the HFD-induced model was used. There was no significantly differences in liver TG content between GSDMD\(^{-/-}\) mice and WT mice fed with HFD for 4 weeks, while GSDMD\(^{-/-}\) mice exhibited less hepatic TG content compared to WT controls fed with a HFD for 11 weeks (Fig. 6A and 6B). H&E staining revealed that GSDMD\(^{-/-}\) mice developed less steatosis when fed with a HFD for 11 weeks (Fig. 6C). Given the significant role of GSDMD-N in inflammation, we further evaluated GSDMD-N expression in long-term (36 weeks) HFD feeding steatohepatitis in WT mice. In this additional model, GSDMD-N protein expression was significantly elevated (Supplementary Fig. 1C). Taken together, these data suggested that GSDMD was involved in the pathogenesis of NAFLD/NASH.

**Discussion**

The most important finding from this study is that GSDMD-induced pyroptosis was involved in human and murine steatohepatitis. GSDMD\(^{-/-}\) mice fed a MCD diet showed significantly reduced steatohepatitis compared with WT control mice fed with the same diet. Consistently, a noticeable improvement in liver inflammation, as well as a reduction in serum ALT levels and hepatic TG content, was observed in MCD-fed GSDMD\(^{-/-}\) mice. In addition, liver fibrosis
was strongly attenuated in $GSDMD^{−/−}$ mice after 8 weeks of MCD induction. These data suggest that GSDMD plays an important role in the process of steatohepatitis.

The molecular mechanisms by which GSDMD exerts its broad range of functions in steatohepatitis were subsequently investigated. Inflammation is a remarkable feature in steatohepatitis. GSDMD has been reported to execute programmed necrosis in inflammation and to control pro-inflammatory cytokine IL-1$\beta$ release$^{11}$. IL-1$\beta$ has long been proven to be an important pro-inflammatory cytokine that drives the pathogenesis of liver inflammation, steatosis, injury, and fibrosis, and often amplifies the effects of other cytokines$^{8, 33-35}$. Evidence from previous studies has indicated that IL-1$\beta$ sensitizes primary mouse hepatocytes to TNF-$\alpha$-induced cytotoxicity and could induce a significant production of TNF-$\alpha$ and MCP-1, suggesting that these cytokines are functionally related$^{36, 37}$. Therefore, we evaluated the effect of GSDMD on the secretion of these cytokines in steatohepatitis and found that $GSDMD^{−/−}$ mice showed a significant reduction in the hepatic production of IL-1$\beta$, TNF-$\alpha$, and MCP-1 (Fig. 3A). TNF-$\alpha$ is a key pro-inflammatory factor involved in human NASH and diet-induced murine steatohepatitis by activating inflammatory cells and inducing insulin resistance$^{38, 39}$. MCP-1 is a crucial pro-inflammatory factor in steatohepatitis by inducing inflammation and stimulating lipogenesis$^{7, 22}$. NF-$\kappa$B has been reported to be a critical signaling pathway involved in inflammatory recruitment and liver injury in steatohepatitis.
In addition, NF-κB acts as a key upstream regulator of IL-1β, TNF-α, and MCP-1 expression, which in turn can activate NF-κB; thus, the effect of GSDMD on NF-κB signaling was investigated. We demonstrated that the phosphorylation of p65 was significantly lower in MCD-fed *GSDMD*⁻/⁻ mice as evidenced by decreased NF-κB p65 nuclear DNA-binding activity and weakened phosphorylation of p65 by Western blotting (Fig. 3C). These data suggested that GSDMD-promoted steatohepatitis is at least in part related to the regulation of pro-inflammatory cytokine secretion and NF-κB signaling pathway activation.

It is well recognized that MCP-1 stimulates macrophage recruitment. Macrophages are the most important innate immune cells involved in pyroptosis and are crucial to the damage process implicated in steatohepatitis. Our study revealed that GSDMD deficiency reduced hepatic macrophage infiltration (Fig. 3D and 3E). Moreover, the phosphorylation of p65 was partially enhanced by transferring WT macrophages to MCD-fed *GSDMD*⁻/⁻ mice. Overall, these results indicated that macrophages are an important cell population responsible for the pathogenic effects of GSDMD in steatohepatitis.

The underlying mechanism leading to excessive liver TG accumulation in steatohepatitis can be attributed to an enhanced uptake and synthesis of fatty acids and inhibition of fatty acid oxidation. Our data showed that knockout of GSDMD expression significantly reduced hepatic TG content and steatosis.
(Fig. 2A). This reduction was associated with reduced activity of the lipogenic gene SREBP-1c and upregulated expression of lipolytic genes, including PPARα and its downstream target molecules ACO, LCAD, and the Cyp family of enzymes Cyp4a10 and Cyp4a14 (Table 2). SREBP-1c is a critical transcription factor for the regulation of target genes involved in lipid synthesis and storage of TGs\(^{42,43}\). The reduced steatosis observed in GSDMD\(^{-/-}\) mice fed with a MCD diet may be a result of SREBP-1c downregulation, and thus a decrease in TG content. Moreover, the impairment of fatty acid oxidation systems represents another important mechanism in the accumulation of liver TGs. ACO and LCAD, which are regulated by PPARα, are the key enzymes involved in fatty acid oxidation systems in the liver\(^{44}\). We found that PPARα, and its downstream targets ACO, LCAD, Cyp4a10, and Cyp4a14 were significantly upregulated in GSDMD\(^{-/-}\) mice fed with MCD diet compared with WT mice, suggesting that GSDMD could influence hepatic lipogenesis by regulating the lipolytic genes expression.

The induction of GSDMD in human NASH and in murine steatohepatitis, and the mitigated lipid accumulation and IL-1β secretion of dietary steatohepatitis in GSDMD\(^{-/-}\) mice led us to hypothesize that GSDMD-driven pyroptosis, rather than cytokine secretion itself, is probably the key determinant of dietary steatohepatitis. Thus, we further addressed the functional significance of pyroptosis in hepatocytes by overexpressing GSDMD-N, which is the functional fragment responsible for pyroptosis\(^{11}\). 
AML-12 hepatocytes transfected with GSDMD-N vector in MCD cultured medium showed greater liver injury as evidenced by elevated medium ALT and hepatic lipoperoxide levels, accompanied by increased NF-κB activation, and steatosis (Fig. 4B-4E). The severe steatohepatitis was also observed in MCD-fed Alb-Cre mice administered with AAV9-FLEX-GSDMD-N, indicating that the aggravated steatohepatitis was mainly caused by GSDMD-N-driven pyroptosis (Fig. 5B-5E). More importantly, we identified that overexpression of the GSDMD-N domain could spontaneously induce mild liver injury even without MCD treatment, indicating that GSDMD-N-dominant pyroptosis is an indispensable mechanism involved in the pathogenesis of steatohepatitis. It is well known that Lipopolysaccharide (LPS) or inflammatory caspases (caspase-1, murine caspase-11, and human caspase-4/5) are required for the cleavage of the GSDMD-N domain. As elevated levels of LPS and inflammatory caspases are often observed in NASH patients and in murine steatohepatitis, this provides a prerequisite for the cleavage of the GSDMD-N domain and subsequent pyroptosis\textsuperscript{25}. In the present study, we also confirmed that the expression of these inflammatory caspases is elevated in human NASH samples (caspase-1/4/5) and in murine steatohepatitis models (caspase-1) (Supplementary Fig. 1D-1F). Taken together, our data demonstrate that GSDMD-driven pyroptosis plays a pivotal role in the pathogenesis of steatohepatitis (Fig. 6E). These findings open up new insight into the treatment of steatohepatitis in humans.
Conflict of interest statement: The authors have no conflicts of interest to disclose.

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Author contributions: JL and KCW designed the experiments, supervised the study and revised the paper; FS commented on the study and provided the material support. BX, MZJ, YC and WJW completed the main experiments, wrote the first draft of the paper, prepared the figures and analyzed the data; DC and XWL helped with the main experiments; ZZ and DZ helped with the preparation of human samples; DMF and YZN helped with the experimental design and paper writing; All authors have reviewed the final version of the manuscript and approve it for publication.

References


Figures and Figure legends

**Fig 1. Upregulation of GSDMD-N in liver tissues represents a potential biomarker for the diagnosis of human NASH.** (A) GSDMD-FL protein levels were increased in liver tissues of human NAFLD/NASH compared with normal controls as determined by Western blotting. (B) Pyroptosis-inducing fragment GSDMD-N protein levels were upregulated in human NAFLD/NASH tissues, particular NASH tissues compared with normal liver tissue as indicated by Western blotting. (C and D) Receiver-operating characteristic (AUROC) curves of GSDMD-FL and GSDMD-N in diagnosing NAFLD in all subjects, NASH in NAFLD patients and NASH in all subjects. Data are expressed as the Median (25th, 75th percentiles), Kruskal-Wallis test were used.

**Fig 2. GSDMD increased the severity of MCD-induced steatohepatitis.** (A) Serum ALT and liver TG content in WT and *GSDMD*−/− mice fed with a control diet or a MCD diet. (B) Representative H&E and Oil-red O stained sections of WT and *GSDMD*−/− mice. (C) Hepatic mRNA levels of TGF-β1 and (D) α-SMA in WT and *GSDMD*−/− mice fed with a control diet or a MCD diet. (E) Hepatic collagen area and hydroxyproline content in WT and *GSDMD*−/− mice. (F) Representative Sirius Red and α-SMA stained sections of WT and *GSDMD*−/− mice. Data are mean ± SD (n = 6/group). *p <0.05, **p <0.01, ***p <0.001 vs. same genotype mice fed with a control diet. Scale bars, 20µm.

**Fig 3. GSDMD promoted hepatic cytokine secretion, macrophage infiltration, and persistent activation of the NF-κB signaling pathway.** (A)
Hepatic IL-1β, TNF-α and MCP-1 secretion; (B) NF-κB p65 nuclear DNA-binding activity assay; (C) Hepatic protein expression of NF-κB p65; (D) Hepatic mRNA levels of F4/80; (E) Representative immunohistochemistry stained sections of F4/80 in WT and GSDMD−/− mice fed with a control diet or a MCD diet. (F) Hepatic protein expression of NF-κB p65 in GSDMD−/− mice adoptive transferred with WT macrophages. Data are mean ± SD (n = 6/group).

* p <0.05, ** p <0.01, *** p <0.001 vs. same genotype mice fed with a control diet.

Scale bars, 20µm.

Fig 4. Pyroptosis-inducing activity of GSDMD-N domain caused extensive steatohepatitis in cultured hepatocytes. (A) Representative scheme of the structure of the GV362 vector. (B) Medium ALT; (C) Hepatic TG content; (D) Hepatic peroxide; (E) NF-κB p65 nuclear DNA-binding activity assay in AML-12 hepatocytes transfected with GSDMD-N or control vector cultured in control or in MCD medium. Data are mean ± SD from three independent experiments. ** p <0.01, *** p <0.001 vs. AML-12 cells transfected with the same vector in control medium, # p <0.05 AML-12 cells transfected with GSDMD-N vector vs. control vector cultured in control medium. (F) Protein expression of NF-κB was determined by Western blotting. Data are mean ± SD from three independent experiments. ** p <0.01, *** p <0.001 AML-12 cells cultured in MCD medium without blockade vs. AML-12 cells transfected with the same vector cultured in control medium. # p <0.05, ## p <0.01, ### p <0.001 AML-12 cells treated with anti-IL-1β Ab and/or anti-TNF-α Ab.
Ab vs. AML-12 cells transfected with the same vector cultured in MCD medium.

**Fig 5. GSDMD-N-driven pyroptosis increased the severity of MCD-induced steatohepatitis in mice.** (A) Representative scheme of the structure of the AAV9-FLEX-GSDMD-N adeno-associated virus; (B) Representative H&E stained sections of *Alb-Cre* mice administered vector AAV9-FLEX-GSDMD-N or the control vector AAV9-FLEX-control; (C) Histological analysis of liver sections; (D) Serum ALT and hepatic TG content; (E) Nuclear DNA-binding activity of NF-kB p65 and SREBP-1c. Data are mean ± SD (n = 6/group). *p < 0.05, **p < 0.01, ***p < 0.001 mice fed with a MCD diet vs. mice administered with the same AAV vector fed with a control diet, #p < 0.05 mice administered with AAV9-FLEX-GSDMD-N vs. AAV9-FLEX-control treated with a MCD diet. Scale bars, 20µm.

**Fig 6. GSDMD is implicated in the pathogenesis of HFD-induced NAFLD.** (A and B) Hepatic TG content in WT and *GSDMD*−/− mice fed with a control diet or a HFD for 4 weeks or for 11 weeks. (C) Representative H&E stained sections of WT and *GSDMD*−/− mice fed with a control diet or a HFD for 11 weeks. Data are mean ± SD (n = 6/group). *p < 0.05, **p < 0.01, ***p < 0.001 vs. same genotype mice fed with a control diet. Scale bars, 20µm. (D) Schematic diagram for the mechanisms of GSDMD in the promotion of dietary steatohepatitis. GSDMD plays a key role as a pyroptosis executor in the pathogenesis of steatohepatitis of NASH by promoting pro-inflammatory
cytokines secretion, exacerbating NF-κB activation, thus directly or indirectly facilitating liver fibrosis and lipogenesis.
Figure 4

A. Diagram of Gasdermin-N (1-279AA) and Mouse GSDMD N-terminus.

B. Bar graph showing Medium ALT (U/L) with p < 0.01.

C. Bar graph showing Medium triglyceride (nmol/L) with p < 0.05.

D. Bar graph showing Hepatic TBARS assay (ng/mg protein) with p < 0.01.

E. Bar graph showing NF-κB p65 binding activity (OD 450 nm) with p < 0.01.

F. Bar graph showing Relative p-p65 protein levels with * and **.

Legend:
- Control diet
- MCD diet
- GSDMD-N
- MCD medium
- IL-1β blockade
- TNF-α blockade
- Flag-GSDMD-N
- NF-κB p65
- p-NF-κB p65
- β-actin
Figure 5

A

AAV9

CMV bGlobin-FLEX-MCS-EGFP-WPRE-hGH polyA

B

Control

GSDMD-N

Control

GSDMD-N

Control diet

MCD diet

C

Histological scores of liver sections

<table>
<thead>
<tr>
<th>Scores</th>
<th>AAV9-FLEX-control</th>
<th>AAV9-FLEX-GSDMD-N</th>
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<tr>
<td></td>
<td>Control</td>
<td>MCD</td>
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<tr>
<td>Steatosis</td>
<td>0.00 ± 0.00</td>
<td>1.00 ± 0.63*</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.17 ± 0.41</td>
<td>1.00 ± 0.63*</td>
</tr>
</tbody>
</table>

D

Serum ALT (U/L)

Control GSDMD-N

p<0.05

E

Hepatic triglyceride (µg/mg protein)

Control GSDMD-N

p<0.05

NF-κB p65 binding activity (OD 450 nm)

Control GSDMD-N

p<0.001

SREBP-1c binding activity (OD 450 nm)

Control GSDMD-N

p<0.05
Figure 6

A. Control diet vs HFD (4 weeks)
B. Control diet vs HFD (11 weeks)

C. H&E staining of WT vs GSDMD

D. Schematic diagram of hepatic stellate cells and lipogenesis pathways.
## Tables

### Table 1. Correlations with GSDMD-FL and GSDMD-N in NAFLD/NASH patients.

<table>
<thead>
<tr>
<th>Factors</th>
<th>GSDMD-FL</th>
<th></th>
<th>GSDMD-N</th>
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<tr>
<td></td>
<td>rho</td>
<td>p value</td>
<td>rho</td>
<td>p value</td>
</tr>
<tr>
<td>Age (years)</td>
<td>-0.091</td>
<td>0.652</td>
<td>0.088</td>
<td>0.649</td>
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<tr>
<td>BMI (kg/m²)</td>
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<td>0.418</td>
<td>0.205</td>
<td>0.285</td>
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<tr>
<td>ALT (IU/L)</td>
<td>0.053</td>
<td>0.783</td>
<td>0.514</td>
<td>0.004</td>
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<tr>
<td>AST (IU/L)</td>
<td>0.214</td>
<td>0.264</td>
<td>0.609</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CHO (mmol/L)</td>
<td>0.292</td>
<td>0.124</td>
<td>0.095</td>
<td>0.624</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>0.221</td>
<td>0.249</td>
<td>0.188</td>
<td>0.328</td>
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<tr>
<td>NAS</td>
<td>0.228</td>
<td>0.234</td>
<td>0.532</td>
<td>0.003</td>
</tr>
<tr>
<td>Steatosis</td>
<td>0.051</td>
<td>0.794</td>
<td>0.554</td>
<td>0.002</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>0.293</td>
<td>0.123</td>
<td>0.554</td>
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<tr>
<td>Ballooning</td>
<td>0.202</td>
<td>0.293</td>
<td>0.390</td>
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<tr>
<td>Fibrosis</td>
<td>-0.059</td>
<td>0.762</td>
<td>0.477</td>
<td>0.009</td>
</tr>
</tbody>
</table>

**NOTE.** Spearman's correlation coefficient was used to estimate the association of GSDMD-FL and GSDMD-N levels and several factors of interest.

†p value corresponds to Ho: rho = 0.
Table 2. Hepatic mRNA expression of lipogenic and lipolytic genes in WT and GSDMD\(^{-/-}\) mice fed with a MCD or a control diet for 4 weeks.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Wild-type mice</th>
<th>GSDMD(^{-/-}) mice</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>MCD</td>
</tr>
<tr>
<td>Lipogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LXRα</td>
<td>0.98 ± 0.16</td>
<td>1.67 ± 0.40**</td>
</tr>
<tr>
<td>LXRβ</td>
<td>0.97 ± 0.27</td>
<td>1.43 ± 0.14*</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.18 ± 0.28</td>
<td>2.42 ± 1.24*</td>
</tr>
<tr>
<td>FAS</td>
<td>1.06 ± 0.22</td>
<td>1.50 ± 0.13*</td>
</tr>
<tr>
<td>SCD-1</td>
<td>0.62 ± 0.35</td>
<td>0.03 ± 0.13**</td>
</tr>
<tr>
<td>Lipolytic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPARα</td>
<td>1.37 ± 0.34</td>
<td>0.58 ± 0.17***</td>
</tr>
<tr>
<td>ACO</td>
<td>1.03 ± 0.19</td>
<td>0.24 ± 0.09***</td>
</tr>
<tr>
<td>LCAD</td>
<td>0.81 ± 0.09</td>
<td>0.24 ± 0.10***</td>
</tr>
<tr>
<td>Cyp4a10</td>
<td>0.77 ± 0.27</td>
<td>1.18 ± 0.57*</td>
</tr>
<tr>
<td>Cyp4a14</td>
<td>0.80 ± 0.15</td>
<td>3.71 ± 2.49**</td>
</tr>
</tbody>
</table>

NOTE. Specific mRNA expression values were normalized to the expression of β-actin. Data are expressed as the mean ± SD (n=6/group). *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) (MCD vs. control diet in same genotype mice. #\(p<0.05\), ##\(p<0.01\), ###\(p<0.001\) (GSDMD\(^{-/-}\) mice vs. WT mice fed with a MCD diet).
Highlights

1. Hepatic N-terminal cleavage fragments of GSDMD (GSDMD-N) production is prominent in patients with non-alcoholic steatohepatitis (NASH), and is associated with lobular inflammation and hepatic ballooning.

2. GSDMD-N is a potential biomarker for the diagnosis of NASH.

3. GSDMD is involved in steatohepatitis by mediating macrophage infiltration, the NF-κB signaling pathway amplification cascade, and lipogenesis.