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Role of pyroptotic cell death in the pathogenesis of NASH

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Abstract: Nonalcoholic fatty liver disease (NAFLD) represents a huge threat to public health of the whole world. Around 25% of NAFLD patients will progress to nonalcoholic steatohepatitis (NASH), which has been predicted to be the main reason for liver transplantation in the United States in 2020. Extensive effort has been devoted to investigating the underlying molecular mechanisms of NASH pathogenesis and developing new promising treatments. Recent studies have demonstrated that pyroptosis, an inflammatory programmed cell death mediated by inflammasome and gasdermin-D (GSDMD), is involved in the development and progression of NASH. This review aims to summarize the recent findings regarding the role of pyroptosis and related molecules in the pathogenesis of NASH.

Introduction

Due to the change of lifestyle, the incidence of non-alcoholic fatty liver disease (NAFLD) has increased rapidly in recent decades worldwide. According to the statistics by the World Health Organization, there are around 25-30 % of population suffering from NAFLD in western countries, and this number is also rising in Asian countries in recent years (Estes *et al.*, 2018; Fan *et al.*, 2017; Sheth, 1997; Younossi *et al.*, 2016). NAFLD is usually asymptomatic, but occurs with other metabolic syndromes frequently, such as obesity, insulin resistance, hyperlipidemia, high blood pressure, and sometimes with complications such as cardiovascular diseases (Di Bonito *et al.*, 2019; Rinella, 2015; Shi *et al.*, 2019). The pathological progression spectrum of NAFLD is simple steatosis, nonalcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular cancer (HCC) (Anstee *et al.*, 2019; Calzadilla Bertot and Adams, 2016; Pierantonelli and Svegliati-Baroni, 2019). Approximately 10-20 % of NAFLD patients will progress to NASH, a more severe subtype of chronic liver disease and the aggressive form of NAFLD (Rinella, 2015; Younossi *et al.*, 2016). It is unclear why some NAFLD patients progress to NASH, while the majority of them can remain in the benign stage of simple steatosis. It is predicted that the prevalence of NASH will become the leading cause of hepatocellular carcinoma or liver transplantation in the USA by 2020 (Goldberg *et al.*, 2017). However, the diagnostic testing methods and clinical treatment for NASH are currently very limited, and so far, there is still no approved pharmacotherapy for NASH (Sumida and Yoneda, 2018).

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The mechanisms underlying the pathogenesis of NASH have received extensive studies, and a "two-hit" theory has been broadly accepted for many years (Day and James, 1998; Fang *et al.*, 2018). According to this traditional 'two-hit' theory, the development of NASH is caused by two hits as hepatic fat accumulation (hepatic steatosis) acts as the first hit to sensitize the hepatocytes, for later attack by the second hit to cause the progression into steatohepatitis. The second hit originates from various factors including oxidative stress, intestinal microbiota, mitochondrial dysfunction, inflammatory cytokines, and/or endoplasmic reticulum (ER) stress, (Day and James, 1998). However, recently growing evidence suggests that hepatic steatosis may not be limited to the 'first hit' and itself without another hit may also cause the liver injury, and this evidence has challenged the 'two-hit' theory. Then a modified 'multiple hits' model has been proposed suggesting that multiple pathogenic factors act in parallel to drive the development of NASH and the pathogenic drivers in different NASH patients may be heterogeneous and different (Fang *et al.*, 2018; Takaki *et al.*, 2013; Tilg and Moschen, 2010).

In defining the pathogenic drivers for NASH development, it is now accepted that NASH is caused by liver lipotoxicity (Noureddin and Sanyal, 2018; Svegliati-Baroni *et al.*, 2019). Excessive accumulation of lipid molecules including free fatty acids, free cholesterol, and diacylglycerols, etc., in liver results in lipotoxic burden on hepatocytes and leads to the occurrence of (ER) stress, mitochondrial dysfunction, oxidative stress, and finally cell death in hepatocytes (Marra and Svegliati-Baroni, 2018; Noureddin and Sanyal, 2018; Ogawa *et al.*, 2018). Injured hepatocytes then recruit and activate immune cells, resulting in chronic inflammation in the liver. One of the key questions that remain to be clarified is how toxic lipid molecules induce

cell death in hepatocytes and which cell death model(s) is (are) involved. Previous studies suggest that lipotoxicity kills hepatocytes by apoptosis, which is a programmed and noninflammatory mode of cell death (Kanda *et al.*, 2018). In the recent decade, knowledge regarding different forms of cell death, like necroptosis, pyroptosis and autophagy, and the molecular mechanisms regulating these modes of cell death have grown very fast. Accumulating evidence suggests that the mode of cell death in hepatocytes during NASH pathogenesis is not limited to apoptosis, but also includes necroptosis as well as pyroptosis, two forms of proinflammatory cell death (Beier and Banales, 2018; Dara *et al.*, 2016; Schwabe and Luedde, 2018). In this review, we will focus on pyroptosis and summarize the recent advances about the involvement of pyroptosis in NASH pathogenesis.

Pyroptosis

Pyroptosis was originally identified as an inflammatory cell death involved in the innate immune response against pathogen infection (Cookson and Brennan, 2001). It has been regarded as caspase-1-mediated lytic cell death induced by different inflammasome complexes for a long time. Inflammasomes are multimeric protein complexes that typically comprise a sensor protein, an adaptor protein and the zymogen procaspase-1 (Lu and Wu, 2015; Martinon *et al.*, 2002; Sharma and Kanneganti, 2016; Vanaja *et al.*, 2015). Various pathogen-associated or danger-associated molecular patterns (PAMPs or DAMPs) are recognized by specific inflammasome sensors, leading to the oligolization of corresponding inflammasomes (Malik and Kanneganti, 2017; Vanaja *et al.*, 2015). Then procaspase-1 is cleaved and activated through proximity-induced self-cleavage, and the active caspase-1 next cleave pro-interleukins 1β and 18 (pro-IL-1β and pro-IL-18) into mature cytokines (Malik and Kanneganti, 2017; Vanaja *et al.*, 2015). IL-1β is a potent inducer of inflammation, vasodilation and immune cell extravasation, and also has roles in shaping adaptive immune responses (Bent *et al.*, 2018; Joosten *et al.*, 2013). IL-18 promotes the production of interferon (IFN)-γ by T_H1 cells, NK cells, and cytotoxic T cells, enhances the development of T_H2 cells, and promotes local inflammation (Bent *et al.*, 2018; Dinarello *et al.*, 2013; Van De Veerdonk *et al.*, 2011). In addition to the maturation of pro-inflammatory cytokines IL-1β and IL-18, inflammasome-induced caspase-1 activation also results in pro-inflammatory cell death, with the features of cell membrane lysis and release of intracellular contents, which are distinguished from canonical apoptosis. This form of cell death was referred to as 'pyroptosis' by Cookson *et al.* (2001).

Although the form of pyroptotic cell death has been recognized for a long time, its regulation and relevant mechanisms have been clarified until recent years. First, apart from caspase-1, another caspase, caspase-11 in mice and caspase-4/5 in humans have also been found to mediate the occurrence of pyroptosis induced by cytosolic LPS or Gram-negative bacterial infections (Ng and Monack, 2013; Yang *et al.*, 2015), suggesting that pyroptosis may not be limited to macrophages and play more prevalent roles. Subsequently, two independent studies identified the key executioner molecule of pyroptosis, gasdermin D (GSDMD), which is the substrate of inflammatory caspase-1 and caspase-11/4/5 (Kayagaki *et al.*, 2015; Shi *et al.*, 2015). GSDMD contains two conserved domains, the N-terminal gasdermin-N domain, and the C-terminal gasdermin-C domain, which are bound by a middle linker region. GSDMD is kept in an autoinhibitory state by its gasdermin-C domain binding to its gasdermin-N domain (Shi *et al.*, 2017). Active caspase-1 and caspase-11/4/5 cleave GSDMD at D276 in the linker region and gasdermin-N domain is thereby released and oligomerized to bind to phosphoinositide or cardiolipin in cell membrane, leading to the formation of membrane pores with an inner diameter of 10–15 nm (Huang *et al.*, 2015; Kayagaki *et al.*, 2015; Shi *et al.*, 2015). The formation of membrane pores by the oligomerized gasdermin-N terminal disrupts the cellular osmotic potential and causes the swelling and lysis of the cells, leading to the release of the pro-inflammatory cytokines such as IL-1β and IL-18 and other cell content. The discovery of GSDMD and the establishment of GSDMD knockout animals marked the milestone of pyroptosis research and incited the enthusiasm of studying pyroptosis in different physiological and pathological processes, including cardiovascular diseases, cancer, and liver diseases like NASH.

Pyroptosis in NASH

Regarding the pathogenesis of NASH, it was first found that pyroptosis inducer, NOD-like receptor protein 3 (NLRP3) inflammasome, was involved in the development of NAFLD and NASH. NLRP3 inflammasome, as the most extensively studied inflammasome, consists of the cytoplasmic inflammasome sensor NLRP3, adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the effector procaspase-1. NLRP3 inflammasome can be activated by diverse stimuli, including different pathogen-associated molecular patterns (PAMPs) like bacterial toxins and viruses, and danger-associated molecular patterns (DAMPs) like uric acid, fatty acid and ATP (Swanson *et al.*, 2019), etc. NLRP3 inflammasome activation results in the cleavage and activation of caspase-1, which further cleaves pro-IL-1β, pro-IL-18 as well as GSDMD, leading to the occurrence of pyroptosis and the release of the mature cytokines IL-1β and IL-18. Growing evidence suggests that NLRP3 inflammasome plays important roles in different inflammation-relevant diseases as microbial infection, atherosclerosis, Alzheimer's disease, Type 2 diabetes, as well as NASH (Swanson *et al.*, 2019). Several studies reported that the expression of NLRP3 and other relevant molecules are all significantly increased in both NASH mouse models and NASH patients (Csak *et al.*, 2011; Matsuzaka *et al.*, 2012; Wree *et al.*, 2014). Wree *et al.* (2014) found that NLRP3 overexpression aggravated the hepatic inflammation and fibrosis in the choline-deficient amino acid-defined (CDAA)-induced NAFLD model, whereas NLRP3 genetic deficiency significantly attenuated the symptoms of hepatomegaly, liver injury and fibrosis in the same mouse model (Wree *et al.*, 2014). Mridha *et al.* (2017) obtained similar results using NLRP3 selective inhibitor MCC950, which reduced liver fibrosis in MCD (methionine and choline-deficient) diet-fed mice. These studies suggested that NLRP3 inflammasome plays an important role in the

pathogenesis of NAFLD, and might serve as a potential target for the treatment of NAFLD and NASH. In addition to the role of inflammasome sensor NLRP3, the inflammasome effector, caspase-1, has also been found to contribute to the inflammation and fibrogenesis in MCD-induced NASH model (Dixon *et al.*, 2012). Dixon *et al.* (2013) reported that a high-fat diet (HFD) significantly increased the expression of pre-caspase-1, IL-1β and genes involved in lipogenesis in the liver of NASH model mice. Moreover, lack of caspase-1 reduced the levels of triglyceride (TG), plasma cholesterol and free fatty acids in mouse plasma, and alleviated the liver inflammation (Dixon *et al.*, 2013). Regarding the inflammasome activation product, IL-1β, its role in the pathogenesis of NAFLD and NASH is consistent with NLRP3 and caspase-1. Overexpression of IL-1β induced hepatocyte inflammation and restricted the expansion of fat cells, thereby resulted in a large amount of adipose tissue ectopic and interfered with liver fat metabolism (Nov *et al.*, 2013). Kamari *et al.* (2011) found that both the protein level and the mRNA level of IL-1β were elevated in various dietary-induced NASH models. Similarly, IL-1β-deficient mice had significantly decreased liver inflammation and liver fibrosis than wild type mice.

With the above findings that the components of the inflammasome pathway, including NLRP3, caspase-1, and IL-1β, all play roles in the pathogenesis of NAFLD or NASH, it is reasonable to speculate that pyroptosis, as another important outcome induced by inflammasome activation, may also contribute to the progression of NASH. The identification of GSDMD as the critical executor of pyroptosis and the establishment of GSDMD knockout mice enable the assessment of pyroptosis in the pathogenesis of NASH. Xu *et al.* (2018) recently published in the *Journal of Hepatology* and reported that GSDMD plays a crucial role in the development and progression of steatohepatitis. Their results demonstrated that *Gsdmd*$^{-/-}$ mice exhibited attenuated liver injury, hepatic lipid accumulation, necroinflammation as well as hepatic fibrosis in MCD diet-induced steatohepatitis compared to wild type mice. In addition, *Gsdmd*$^{-/-}$ mice also developed less steatosis in the HFD-induced NAFLD model, indicating a pivotal role of GSDMD in different murine models of NAFLD and NASH. Moreover, overexpression of the gasdermin-N domain (GSDMD-N), which bears intrinsic pyroptosis-inducing activity by injecting AAV9-FLEX-GSDMD-N in *Alb-Cre* mice not only increased the severity of MCD-induced steatohepatitis but also spontaneously caused liver injury even in control diet-fed mice, suggesting that GSDMD-N-induced pyroptosis is a crucial contributor for the pathogenesis of steatohepatitis. Importantly, Xu *et al.* (2018) also showed that the protein expression of GSDMD and its pyroptosis-inducing fragment GSDMD-N are both upregulated in liver tissues of NAFLD/NASH patients. Furthermore, the level of GSDMD-N is positively correlated with the NAFLD activity score (NAS) and fibrosis in patients with NASH, suggesting that GSDMD is also involved in human steatohepatitis and GSDMD-N might act as a potential biomarker for NASH diagnosis (Xu *et al.*, 2018).

Conclusions

As outlined in this review, the members that participate in regulating pyroptosis, including NLRP3, caspase-1, IL-1β as well as GSDMD, have been shown to contribute to the pathogenesis of NAFLD and NASH. Hence, pyroptosis might play a significant role in steatohepatitis, and pyroptosis signaling molecules may be potential pharmaceutical targets for the treatment of NASH (Wree *et al.*, 2016). However, despite these exciting findings about the role of pyroptosis in NASH pathogenesis, many aspects during the process still remain elusive and require further research, such as which factors induce the activation of inflammasome and lead to the occurrence of pyroptosis during the progression of steatosis, how GSDMD causes liver damage and fibrosis and what the underlying mechanism is, whether there is negative regulator of pyroptosis, etc. Future efforts should be devoted to answering these questions, so as to provide a better theoretical basis for clinical treatment development for NASH.

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Conflicts of Interest

The authors declare that there is no conflicts of interest regarding the publication of this article.

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