

Inflammasomes in non-alcoholic fatty liver disease

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1. ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a leading liver disorder in the world. Inflammation is one of the most important pathological events during the development of NAFLD and also represents the hallmark between simple steatosis and non-alcoholic steatohepatitis (NASH). Inflammasomes are novel protein complex platforms assembled in response to pattern-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Currently, there are several identified inflammasomes, including nod-like receptor protein (NLRP)-1, 2, 3, 6, 10, 12, NLRP4 and absent in melanoma 2 (AIM2) inflammasomes. In the liver, inflammasomes are primarily expressed in immune cells. However, increasing evidence suggests that their expressions in other types of cells in the liver are also present. In general, inflammasomes are up-regulated in various liver diseases. In NAFLD, it is reported that the levels of inflammasome components (e.g. NLRPs, caspase-1, IL-1 β and IL-18) are elevated. Silence of these components attenuates hepatic injury. Collectively, the main purposes of this review are to examine the recent progress of hepatic inflammasome research and to discuss possible directions of therapeutic strategy and development against NAFLD.

2. INTRODUCTION

The term non-alcoholic fatty liver disease (NAFLD) actually include a spectrum of abnormal liver conditions which are not attributed to the abuse of

alcohol, from the development of simple steatosis (fat accumulation in the liver without inflammation) to non-alcoholic steatohepatitis (NASH; with hepatic steatosis, inflammation and fibrosis). In contrast, chronic excessive use of alcohol (over 12 ounces of 4 - 5% beer, or 6 ounces of 8 - 10% wine, or 2 ounces of 45% hard liquor/whiskey everyday) can lead to alcoholic fatty liver disease (AFLD), alcoholic steatohepatitis (ASH), alcoholic cirrhosis and cancer (1). It should be noted that, although necessary, excessive alcohol use is not sufficient to cause AFLD, since only 1 in 5 heavy drinkers develops alcoholic hepatitis, and 1 in 4 develops cirrhosis (2). The prevalence of NAFLD in adults is 10-30% and its prevalence is increasing in Western countries as well as in China because of the epidemic rise in obesity and diabetes (3-5). It is estimated that 20% of NASH cases will slowly progress to cirrhosis and even liver cancer (6).

Pathogenic mechanisms related to the initiation and progressions of NAFLD remain not fully characterized. Despite the fact that the causative factors for AFLD and NAFLD are somehow different, both diseases share the same natural history (i.e. from simple steatosis to hepatitis to cirrhosis). In addition, the clinical symptoms of AFLD and NAFLD are quite similar (7). The liver and the gastrointestinal tract are the main sites conducting alcohol metabolism. After alcohol consumption, alcohol dehydrogenase and acetaldehyde dehydrogenase induce the reduction of nicotinamide adenine dinucleotide (NAD) to NADH (reduced form of NAD), which then cause fatty

liver through the inhibition of gluconeogenesis and fatty acid oxidation. Cytochrome P450 2E1, another metabolic pathway of alcohol, will be induced to produce free radicals to damage the liver, leading to the occurrence of inflammation, chemoattraction, necrosis and apoptosis (8). For NAFLD, to date, the most recognized disease model is the “multiple-parallel hit model” in which the dysregulated lipid metabolism and insulin resistance are considered as the first hit to the liver. Then the following hits include fibrosis, necrosis, apoptosis, oxidative stress and inflammation which will lead to the progression from steatosis to NASH (9). Among these pathological events, the role of inflammation in the pathogenesis of NAFLD received massive attention during the past years because it is the central event that links the upstream insulin resistance/steatosis and downstream cell injury, which is related to the progression of cirrhosis (10).

In clinical practice, NAFLD can be retarded or reversed to normal hepatic conditions when appropriate treatments are implemented. To date, there are two major categories of NAFLD therapies: (1) lifestyle interventions (such as weight reduction, dietary modification, and physical exercise) and (2) pharmaceutical therapies. Weight reduction and dietary modification are the most recognized strategies for the prevention and control of NAFLD (11).

3. INFLAMMATION IN NAFLD

The main difference between simple steatosis and NASH is the development of chronic inflammation. Recent reviews consider simple steatosis to be a benign and non-progressive condition, while NASH may reflect different disease entities (12). Patients with steatohepatitis may show none or low level of steatosis, suggesting that inflammation could occur first (13). In addition, treatment with anti-tumor necrosis factor (TNF) antibody and anti-diabetic drug metformin improved hepatic steatosis in *ob/ob* mice (14). Applications of antioxidants are also shown to attenuate hepatic inflammation in alcohol-induced liver diseases (15). Very interestingly, loss of Kupffer cells, the local macrophage of the liver, leads to steatotic development probably due to decreased level of interleukin-10 (IL-10) (16).

Inflammation during the pathogenesis of NASH is also positively related with disrupted lipid metabolism, oxidative stress, and apoptosis. It is well known that increased lipid accumulation leads to lipid peroxidation and inflammation. For example, in the liver, oxidized low-density lipoprotein (LDL) can bind to scavenger receptors on the surface of Kupffer cells (e.g. CD36) to promote inflammatory responses (17). Other studies also found that TNF- α was capable of inhibiting hepatic cholesterol elimination and activating its synthesis, contributing to the decrease of high-density lipoprotein (HDL)-cholesterol and the increase of LDL-cholesterol (18, 19). Oxidative

stress is also a direct inducer and downstream target of hepatic inflammation during the progression of NASH. Increased oxidative stress in NASH patients results in pro-oxidative and pro-inflammatory environments. For example, hepatic oxidative stress activates transcriptional factor nuclear factor-kappa B (NF- κ B), which in turn increases the secretion of pro-inflammatory cytokines and chemokines, resulting in elevated apoptosis, neutrophil chemotaxis, and hepatic stellate cell activation (20). Our studies also demonstrated that, when appropriate antioxidant/hepato-protective agents were applied, the increased expression of inducible nitric oxide synthase (iNOS), which is the key link between oxidative stress and inflammation, in NASH rodents were significantly inhibited (21, 22).

The Toll-like receptor (TLR)-mediated pathways, including c-Jun NH2-terminal kinase (JNK), is another key event in the progression of NASH. TLR4 is the receptor in response to lipopolysaccharide (LPS) and other kinds of endotoxins. The interaction between TLR4 and its adaptor protein myeloid differentiation factor 88 (MyD88) triggers a downstream signaling cascade, leading to activation of the NF- κ B pathway (23). In a murine model, genetic deletion of TLR4 shows significantly attenuated hepatic injury induced by NASH through the actions of Kupffer cells (24). Moreover, in leptin deficient *ob/ob* mice, it is suggested that probiotic treatment prevented histological changes and insulin resistance associated with NASH (14). Since high-fat diet feeding induces the decrease of bifidobacteria, which can reduce intestinal endotoxin levels and improve mucosal barrier functions (25), a link between modulation of gut bacteria, endotoxemia, and inflammation in NASH should be considered in the therapy of NAFLD.

4. INFLAMMASOMES

4.1. Discovery

In 2002, Martinon *et al.* reported a caspase-activating complex that they called the inflammasome (comprises caspase-1, caspase-5, Pycard/Asc, and NALP1, a Pyrin domain-containing protein sharing structural homology with NODs) in a cell-free system (26). They found that immune-depletion of Pycard can abrogate pro-inflammatory caspase activation and pro-IL-1 β processing after the challenge of LPS. This is the first time that the term inflammasome was introduced. However, the first member of the inflammasome family – Nod-like receptor protein (NLRP3) was discovered in 2001. Hoffman *et al.* identified a gene with four distinct mutations that segregated with the disorder familial cold auto-inflammatory syndrome (FCAS) and Muckle-Wells syndrome (MWS). This gene, called CIAS1 (referred to NLRP3 later), encodes a protein with a pyrin domain, a nucleotide-binding site (NBS, NACHT subfamily) domain and a leucine-rich repeat (LRR) motif region (27). After that, other members of the inflammasome family have been identified one after another.

4.2. Classification

To date, a number of inflammasomes have been discovered and characterized, including NLRP1, 2, 3, 6, 10, 12, NLRC4 and AIM2. Although differing in ligand recognition, downstream pathway, and biological functions, the activation of caspase-1 is the core function of almost all of the inflammasomes.

The NLRP1 inflammasome is the first functionally described member of the inflammasome family. It consists of NACHT, PYD (pyrin domain) and LRR domains. NLRP1 can be activated by anthrax toxin or chemotherapy (28, 29). Importantly, in the presence of ASC, its activity can be further enhanced (30). Unlike other inflammasomes, NLRP1 inflammasome is able to be located in the nucleus (31).

The NLRP2 inflammasome was discovered in 2012. Minkiewicz *et al.* reported that human astrocytes express an inflammasome consisting of NLRP2, ASC, and caspase-1. Upon the activation by ATP, NLRP2 is activated to promote the processing of inflammatory caspase-1 and IL-1 β (32). However, the expression pattern of NLRP2 inflammasome in other organs as well as its specific functions in various diseases needs further investigations.

Unlike other inflammasomes and many innate receptors, the NLRP3 inflammasome is a proteolytic caspase-1-activating platform in which caspase-1 does not involve in apoptosis too much. Instead, it is capable of cleaving the pro-forms of IL-1 β and IL-18 in the cytoplasm, to release these two potent pro-inflammatory cytokines from the cell. Activated caspase-1 also has the ability to induce the secretion of IL-1 α and high mobility group box 1 (HMGB1), as well as to initiate a lytic form of cell death named pyroptosis (33). The NLRP3 inflammasome responds to a spectrum of stimuli, including various types of molecules, bacteria, and viruses (34). Therefore, it becomes a novel therapeutic target for related diseases, including metabolic disorders and pathogen infections (35).

The NLRP6 inflammasome plays vital roles in maintaining intestinal homeostasis and balance of intestinal microbiota through regulation of IL-18 (36). Mice with NLRP6 deficiency are more susceptible to intestinal inflammation and chemically induced colitis, suggesting its functions in mucosal self-renewal and proliferation (37). A new report found that the NLRP6 inflammasome regulates goblet cell mucus secretion through induction of autophagy. Alteration of its expression damages mucus layer, leading to susceptibility to infection (38).

NLRP10 is the only NLR lacking the putative ligand-binding leucine-rich-repeat domain. Thus, it is considered as the negative regulator of other inflammasomes (39). Moreover, NLRP10 cannot function

through an inflammasome to regulate caspase-1. Instead, deficiency of NLRP10 in mice shows a profound defect in helper T-cell-driven immune responses to a spectrum of stimuli (e.g. LPS) (40).

NLRP12 is found to be associated with ASC and then to form an active IL-1 β -maturing inflammasome (40). It is also involved in the pathogenesis of periodic fever syndromes (PFS) (41) and the host defense against *Yersinia pestis* (42). A new study identified that NLRP12/NLRP3-dependent activation of caspase-1 might be a central event in mediating the hypersensitivity to secondary bacterial infection in malaria (43). Alongside the roles of NLRP1 and NLRP3, NLRP12 participates in the protection from acute colitis through negative regulation of non-canonical NF- κ B signaling. That is, Nlrp12 $^{-/-}$ mice exhibit more severe colitis upon dextran sodium sulphate (DSS) administration than its wild-type littermates (44).

One of the major functions of the NLRC4 inflammasome is to activate caspase-1 to fight against the infection of Gram-negative bacteria, such as *Salmonella*, *Legionella*, and *Shigella*. For example, the NLRC4 inflammasome is activated upon the cytosolic delivery of flagellin or the bacterial rod protein PrgJ through the type III secretion system (T3SS) in the infected macrophage by *Salmonella* (45). Very recently, the phosphorylation of NLRC4 at Ser533 is found to be vital for its activation with the help of kinase Pkc δ (46) or not (47).

AIM2 is a cytosolic inflammasome sensing double-strand DNA (dsDNA). It can be activated by virus, bacterial, and mammalian host DNA to trigger the release of caspase-1 (48, 49). In autoimmune diseases, the AIM2 inflammasome recognizes the mammalian DNA which acts as a contributory factor to the disease pathogenesis (50). In the central nervous system (CNS), the AIM2 inflammasome is triggered by dsDNA in cells harboring intracellular bacterial and then to secrete pro-inflammatory cytokines (51).

4.3. Signaling pathway of the NLRP3 inflammasome

Although a variety of molecular structures such as pattern-associated molecular pattern (PAMP)- and damage-associated molecular pattern (DAMP)-containing molecules are capable of activating NLRP3 inflammasome, at current stage, it is considered that NLRP3 is not likely to recognize these motifs except for cellular homeostasis (e.g. cell stress). One of these examples is endoplasmic reticulum (ER) stress. Several ER stressors can induce the assembly of the NLRP3 inflammasome and the secretion of IL-1 β and IL-18, probably independent of known unfolded protein response (UPR) initiators (PERK, IRE1 α , and ATF6) (52). Our study and other recent studies also identified the key role

of thioredoxin-interacting protein (TXNIP) in ER stress-induced activation of the NLRP3 inflammasome (53-55), although another recent study using TXNIP-knockout macrophages found that TXNIP cannot directly activate NLRP3 (56).

Intracellular Ca^{2+} can also promote the NLRP3 inflammasome activation. For instance, one study suggests that this process is through the release of mitochondrial reactive oxygen species (mROS) and mitochondrial DNA (mtDNA) (57). G protein-coupled Ca^{2+} -sensing receptors, CASR and GPRC6A, are able to trigger the NLRP3 activation, via a PLC-IP3-IP3R cascade (58). Moreover, recent studies found that Ca^{2+} -permeable channels, such as transient potential melastatin-like 2 (TRPM2), TRPV2 and TRPM7, are required for the activation of NLRP3 on the basis of Ca^{2+} influx (59, 60).

Intrinsic and extrinsic apoptotic pathways are inducers of the NLRP3 inflammasome. Recent studies revealed that the inhibitors of apoptosis (IAPs) regulate inflammasomes in both positive and negative manners. Deletion of the gene encoding cIAP2 impairs the activation of NLRP3 (61). Very interestingly, deletion of all three IAPs (XIAP, cIAP1, and cIAP2) leads to NLRP3-caspase-1-dependent and caspase-8-dependent IL-1 β activation (62). As a key component of the extrinsic apoptotic pathway, caspase-8 can trigger the cleavage of both IL-1 β and IL-18 by engaging cell surface receptors, such as FAS (63), TLRs (64), and C-type lectin receptor dectin-1 (65). It activates IL-1 β through two distinct pathways: active caspase-8 directly process IL-1 β in response to signals outside the cells, while deficiency or insufficient activation of caspase-8 leads to NLRP3 activation through RIP3 signaling (66).

In recent literatures, two important regulators of inflammasomes were identified and characterized namely guanylate-binding protein 5 (GBP5) and double-stranded RNA-dependent protein kinase (PKR, also known as EIF2AK2) (67, 68). GBP5 is a member of the GBP family which promotes the activation of the NLRP3 inflammasome by ATP, nigericin, and bacteria. It binds to the PYRIN domain of NLRP3 and forms a tetrameric structure to modulate the NLRP3-ASC oligomerization. Upon induction of agonist, PKR is autophosphorylated. When PKR is inactivated by genetic deletion or pharmacological inhibition, the responses of inflammasome to double-stranded RNA, ATP, monosodium urate, adjuvant aluminium, rotenone, live *Escherichia coli*, anthrax lethal toxin, DNA transfection and *Salmonella typhimurium* infection are significantly impaired. However, He *et al.*, reported that cells isolated from *Pkr*-deletion mouse strains, PKR shows no obvious effect on NLRP3 activation (69). Reason for contradictory observations regarding PKR on NLRP3 remains unclear.

4.4. Inflammasomes in the liver

Hepatocytes are the most abundant epithelial cells in the liver. In an adult human, there are approximately 10^{11} hepatocytes in total, which represent around 60% of all cells and around 80% mass of the liver. The liver is also comprised of immune cells, such as Kupffer cells, neutrophil leukocytes, dendritic cells, T/B cells, and NK/NKT cells. Innate immune cells are the major source of inflammasome production in the liver. There is increasing evidence that inflammasomes are functionally active in non-immune cells, including hepatocytes (70), stellate cells (71), endothelial cells (72), and myofibroblasts (73). Kupffer cells express most kinds of inflammasomes except NLRP1 (31).

HBV core antigen is able to induce the secretion of IL-18 from human peripheral blood mononuclear cells (PBMCs) and from HBeAg negative patients, suggesting the possible role of inflammasome activation in chronic HBV infection (74, 75). Du *et al.* showed that in HBV associated glomerulonephritis (HBV-GN) patients, the expression of AIM2 in renal biopsy specimen is significantly increased to regulate the expression of caspase-1, IL-1 β , and IL-18 (76).

In chronic HCV infection patients, the serum and hepatic levels of IL-1 β are increased. When anti-HCV therapy is applied, serum expression of IL-1 β and caspase-1 is reduced (77). In HCV-infected human hepatoma cells, the HCV is sensed by NLRP3 protein to recruit ASC for the assembly of inflammasome and the secretion of IL-1 β (78). Negash *et al.* also confirmed that the increased level of NLRP3 inflammasome and IL-1 β from Kupffer cells might be a component of HCV immunopathology in HCV-infected patients (79).

Acetaminophen (APAP)-induced hepatotoxicity is still the leading cause of drug-induced liver injury in the U.S. (80). In this case, DAMPs are released from damaged hepatic cells to initiate inflammatory responses through TLRs and NLRs/inflammasomes (e.g. ATP and MSU) (81). Controversial results have been reported on the role of inflammasomes in APAP-induced hepatotoxicity. A majority of studies found that IL-1 β is up-regulated at both transcriptional and translational levels in APAP-induced toxicity in animal models (82-84). But a small clinical study in children and adolescents pointed out that APAP overdose did not induce the elevation of serum IL-1 β level (85). Blocking antibodies against IL-1 α , IL-1 β , and IL-1R lead to amelioration of APAP-induced liver injury (83). Deficiency of IL-18 also reproduces similar results (72). However, another study found that IL-1R knockout mice, or mice lacking of inflammasome components (NLRP3, ASC, and caspase-1) showed no protection against APAP-induced liver injury (86). These contradictory results may be attributed to the diversity of IL-1 and IL-18 roles in the pathogenesis of liver diseases and the

observation timing, animal age, and gender discrepancy in the experimental design (87).

In the clinics, the main features of ischemia-reperfusion (I/R) injury include cellular necrosis, release of DAMPs, inflammation, oxidative stress, and disruption of liver sinusoidal endothelial cells (88). In the presence of antioxidant N-acetylcysteine, caspase-1 is inhibited and the liver I/R injury is attenuated (89). Consistent with this result, silence of NLRP3 ameliorates hepatic I/R injury via inhibition of caspase-1 and NF- κ B activity (90). Application of IL-1R antagonist or IL-18 neutralizing antibody also significantly ameliorates hepatic I/R injury in animal models, probably through TLR4 and TLR9 (91-93).

In both clinical patients and animals with alcoholic fatty liver disease (AFLD), the serum level of IL-1 β is significantly increased when compared with normal subjects (94). It is also found that IL-1 β signaling is required for the development of alcohol-induced liver steatosis and IL-1R antagonist ameliorates inflammasome-dependent alcoholic steatohepatitis (95). Our recent study also found that the NLRP3 inflammasome components (NLRP3, ASC, IL-1 β , and caspase-1) are up-regulated in a cell-based alcoholic liver injury model (55).

4.5. Inflammasomes in NAFLD

Up-regulation of both IL-1 β mRNA and protein is observed in diet-induced NASH mice models, methionine-choline-deficient (MCD) diet, high-fat diet (HFD) and choline-deficient amino acid-defined (CDAA) diet (96-99). Our HFD-induced NASH rat model shows similar results (100). When IL-1 α , IL-1 β , or IL-1R is deleted, the hepatic injury induced by NASH is attenuated (101, 102). In line with this result, mice deficient of IL-1R antagonist showed severe hepatic fat accumulation and fibrosis after an atherogenic diet feeding, when compared with their wild type littermates (103). Knockout of NLRP3 or addition with pan-caspase inhibitor (VX-166) also exhibits improved hepatic function after the diet induction of NAFLD (95, 104). In human NAFLD and type 2 diabetes patients, increased mRNA expression of the NLRP3 inflammasome components is observed and weight loss management decreases such expression (101, 105).

During NASH, molecular triggers of inflammasomes include DNA, saturated fatty acids and LPS. Csak *et al.* reported that saturated, but not unsaturated, fatty acids induce the activation of caspase-1 and the release of IL-1 β upon stimulation of LPS. They also found that fatty acid-treated hepatocyte is capable of inducing inflammasome activation in hepatic mononuclear cells (105). Another study further confirmed that saturated fatty acid palmitate, but not unsaturated oleate, induces the activation of the NLRP3-ASC inflammasome, leading to caspase-1, IL-1 β and IL-18 production. Autophagy and

increased mROS were also involved in this process (99). Besides that, Kupffer cells are important bridge linking TLR signal transduction and inflammasome activation. Depletion of Kupffer cells markedly decreases the expression of both hepatic and serum level of IL-1 β (96). In addition, Kupffer cells promote hepatic steatosis via IL-1 β -dependent suppression of peroxisome proliferator-activated receptor alpha (PPAR α) activity after chronic HFD feeding (97).

The molecular mechanism of transition from steatosis to NASH is partly explained by a recent study showing that altered interactions between the gut microbiota and the host, produced by defective NLRP3 and NLRP6 inflammasome sensing, may govern the rate of progression of NASH (106). This highlights the importance of maintaining the homeostasis of the gut-liver axis under pathological conditions. Other studies, however, found that inflammasome activity contributes to liver fibrosis and transition from simple steatosis to NASH. By using both knockout mice and knock-in mice expressing constitutive active NLRP3, they found that activation of NLRP3 is required for hepatocyte pyroptosis, inflammation and fibrosis developments in NASH (107, 108). Our recent study also found that bee's honey is able to attenuate hepatic injury induced by NASH through targeting the TXNIP-NLRP3 pathway (109).

5. CONCLUSION

In conclusion, inflammasomes are novel protein complexes that modulate a spectrum of exogenous and endogenous danger signals to secrete IL-1 β and IL-18. In recent years, research data suggested that activation of inflammasomes contribute to the pathogenesis of type 2 diabetes and obesity (110-113). As a contributor and a direct consequence of metabolic syndrome, the role of inflammasome complexes in the initiation and progression of NAFLD has been extensively studied. However, several key questions remain largely unknown and warrant further investigations:

- (1) The role of inflammasomes other than NLRP3 and NLRP6 in the pathogenesis of NASH should be clearly defined;
- (2) The importance of the gut-liver axis in liver diseases receives much attention. However, detailed mechanisms that influence the microbiota homeostasis and hepatic inflammation needs further detailed study;
- (3) The inter-organs crosstalk during obesity and insulin resistance development needs to be defined. The liver may directly or indirectly "communicate with" adipose tissue, intestine, pancreas, muscles, and the entire circulation system to affect the metabolic status of the body;
- (4) Gene polymorphism studies on suitable patient cohorts may help to determine the link between the prevalence of metabolic syndrome and genetics.

Also, it should shed light on the early-stage prediction of metabolic diseases.

- (5) Identification of selective pharmacological drugs that inhibit inflammasome pathways should provide solid proof of the significance of inflammasomes in the development of metabolic inflammation. For example, glyburide is an anti-diabetic drug directly targeting the NLRP3 inflammasome (111). Development of such drugs may significantly accelerate the clinical therapeutic development against metabolic syndrome and other chronic inflammatory liver diseases.

Additional research information addressing the above questions may not only offer a better understanding of the inflammasome pathways in the liver diseases, particularly in NAFLD/NASH, but it will also hopefully lead to novel and specific therapeutic strategy against obesity-related conditions in the entire body.

6. ACKNOWLEDGEMENT

Supported by the National Natural Science Foundation of China (Grant No. 31300813) and Small Project Fund, The University of Hong Kong.

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Key Words: Non-alcoholic Fatty Liver; Inflammasome, Metabolic Disorders, Review

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