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REVIEW ARTICLE

Mechanisms of NLRP3 inflammasome-mediated hepatic stellate cell activation: Therapeutic potential for liver fibrosis

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Abstract The liver injury leads to an inflammatory response, which causes the activation of hepatic stellate cells (HSCs) that further secrete ECM proteins and play an important role in liver fibrosis. Moreover, the inflammatory response is a driving force for fibrogenesis, which is triggered by many types of injuries. Exaggerated inflammatory immune responses are mediated by cytoplasmic protein complexes known as inflammasomes, which are involved in many chronic liver diseases. Inflammasomes are pattern recognition receptors (PRRs) that can sense any microbial motifs known as pathogen-associated molecular patterns (PAMPs), and host- or environmental-derived stress signals known as damage-associated molecular patterns (DAMPs). The inflammasomes cause caspase-mediated proteolytic cleavage of pro-IL-1β and pro-IL-18 into active IL-1β and IL-18. In this review, we provide a comprehensive summary of the important roles of NLRP3 inflammasome in the pathogenesis of liver fibrosis with an emphasis on several direct and indirect pathways responsible for the NLRP3 inflammasome-mediated HSCs activation and fibrogenesis. In addition, we discuss the general pharmacological and genetics strategies for the inhibition of NLRP3 inflammasome activation and its downstream signaling with examples of emerging pharmacotherapeutics, targeting the NLRP3 inflammasome signaling as well as a possible way to develop effective and safer NLRP3 inflammasome inhibitors.

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Introduction

Chronic tissue injuries lead to fibrogenesis, a process of forming scarring tissues, that results in multiple organs failure, which accounts for one-third of total deaths globally.[1] Fibrosis is a reversible deposition of collagen and other fibrotic extracellular matrix components, thereby gradual loss of proper functioning of an organ.[2] Besides, persistent fibrosis results in irreversible complications like cirrhosis and liver failure,[3] which is responsible for more than one million deaths worldwide annually.[4] According to a recent WHO report, cirrhosis is the 9th topmost cause of death in lower-middle-income countries in 2016.[5] The most common insults to the liver include chronic alcohol abuse, non-alcoholic fatty liver disease (NAFLD), and hepatitis C virus (HCV) infection.[3] The liver injury leads to an inflammatory response, which causes the activation of hepatic stellate cells that further secrete ECM proteins and play an important role in liver fibrosis.[6] In general, there are four phases of the fibrotic responses: i) initiation, which is driven by primary injury to the organ, ii) activation of effector cells, iii) involves the expansion of extracellular matrix, and iv) involves dynamic deposition extracellular matrix with impaired resorption.[7,8] Moreover, the persistent inflammatory response acts as the driving force for the entire fibrotic process and triggers many types of injuries.[9] Exaggerated inflammatory immune responses generated through several stimuli are mediated by cytoplasmic protein complexes known as inflammasomes, which are involved in the majority of acute and chronic liver diseases.[10]

Inflammasomes are pattern recognition receptors (PRRs) that can sense any microbial motifs known as pathogen-associated molecular patterns (PAMPs), and host- or environmental-derived stress signals known as damage-associated molecular patterns (DAMPs). The inflammasomes are oligomerized and cause caspase-mediated proteolytic cleavage of pro-IL-1β and pro-IL-18 into active IL-1β and IL-18, which are important mediators for the progression of liver fibrosis.[11] There are various inflammasomes, such as NLRP1/3/6, NLRC4, AIM2, and NAIP have been identified.[12] Nevertheless, NLRP3 inflammasome is the most characterized and widely reported in liver fibrosis and other acute/chronic liver diseases. Furthermore, hepatocyte injury/death by apoptosis, necrosis, pyroptosis, or activation of hepatic stellate cells (HSCs) induced by different stimuli, such as the release of DAMPs and PAMPs, that trigger NF-κB-mediated upregulation of NLRP3 inflammasome components, which further facilitated fibrogenesis and exaggerated fibrosis.[13]

Inflammasomes and their family

Inflammasomes are multimeric protein complexes present in the cytoplasm of both parenchymal and non-parenchymal cells that mediate the inflammatory process.[14] Under the homeostatic condition, NLRP3 inflammasome presents in its inactive form and activated by many signals, and plays a crucial role in promoting an inflammatory cell death known as pyroptosis.[15] Further, inflammasomes are cytosolic complexes usually made up of a sensor protein, an adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1.[10] These are activated through a variety of stimuli like PAMPs and DAMPs and the activated inflammasome assembly causes caspase-1 activation, which subsequently leads to catalysis of pro-IL-1β and pro-IL-18 to their active forms.[12,16] Inflammasomes are classified based on their sensor proteins, which are responsible for the initiation of inflammasome formation and these sensors are either of the NOD-like receptor (NLR) family such as NLRP1, NLRP2, NLRP3, NLRP6, NLRP7, and NLRC4, or the HIN-200 family member, like AIM2.[12] The NLR family is a first sensor protein discovered to form inflammasomes, which comprised of 22 genes in humans and 33 genes in mice.[17]

NLRP3 inflammasome and mechanisms of activation

NLRP3 inflammasome is a large multiprotein complex (>700 kDa), consists of NLR, NLRP3 which is bridged to pro-caspase-1 by a homotypic interaction via ASC.[18] NLRP3 is a trifold protein consisting of an amino-terminal pyrin domain (PYD), a middle NACHT domain (domain present in NAIP, CIITA, HET- E, and TP1), and a carboxy-terminal leucine-rich repeat domain (LRR domain). The PYD is responsible for its interaction with ASC, while the NACHT domain is responsible for ATPase activity, which is essential for its functioning. LRR domain has an auto-inhibitory function by folding back onto the NACHT domain. The ASC also has two domains, one is an amino-terminal PYD linked to the NLRP3, and the other is the carboxy-terminal caspase recruitment domain (CARD). The last part is the pro-caspase-1, which also has an amino-terminal CARD, a central large catalytic domain (p20), and a carboxy-terminal small catalytic subunit domain (p10).[19]

Stimulation of NLRP3 by various PAMPs and DAMPs occurs through the NATCH domain and results in its oligomerization with NimA related kinase 7 (NEK7). Oligomerized NLRP3-NEK7 causes the recruitment of ASC via interaction of their PYD—PYD domains and further condensation of multiple ASC filaments into a single macromolecular structure known as ASC speck. This assembled ASC causes recruitment and activation of pro-caspase-1 through their CARD—CARD interactions and finally enable its self-cleavage and activation to caspase-1.[19–21]

Activation of NLRP3 is a two-step process that involves an initial priming step and a latter step of post-translational modifications (PTMs) of the NLRP3 (Fig. 1). Priming involves the transcriptional upregulation of the inflammasome components like NLRP3, caspase-1, and pro-IL-1β. Moreover, NF-κB regulates activation of NLRP3 via recognition of PAMPs and DAMPs through PRRs such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-containing protein 2 (NOD2) or cytokines such as TNF and IL-1β.[22] In addition, the expression of NLRP3 is regulated by NF-κB at the transcriptional level.[22] The priming step initiates the last step of the inflammasome activation, which is the PTMs of NLRP3 like ubiquitylation, phosphorylation, and sumoylation. These PTMs result in the stabilization of NLRP3 into an inactive but competent form in the cytoplasm.[19,23]

NLRP3 activation involves a set of multiple and non-exclusive upstream signals like K^+ efflux, Cl^- efflux, Ca^{2+} flux, lysosomal disruption, and mitochondrial ROS[19] (Fig. 1). K^+ efflux acts as one of the main upstream signals for NLRP3 activation, which is demonstrated by nigericin and ATP-mediated activation of P2X purinoceptor 7 (P2X7) resulting in the maturation of IL-1β via K^+ efflux associated mechanism in peritoneal macrophages.[19,24] Furthermore, Ca^{2+} flux is a critical signal that activates the NLRP3 inflammasome, which is established by the calcium-sensing receptor (CASR)-mediated NLRP3 activation via phospholipase C-mediated calcium release from the endoplasmic reticulum.[25] Moreover, Cl^- efflux is involved in NLRP3 activation, which is shown in LPS-induced macrophages, where chloride (Cl^-) intracellular channel proteins CLIC1 and CLIC4 participated in priming and activation of NLRP3 inflammasome.[26]

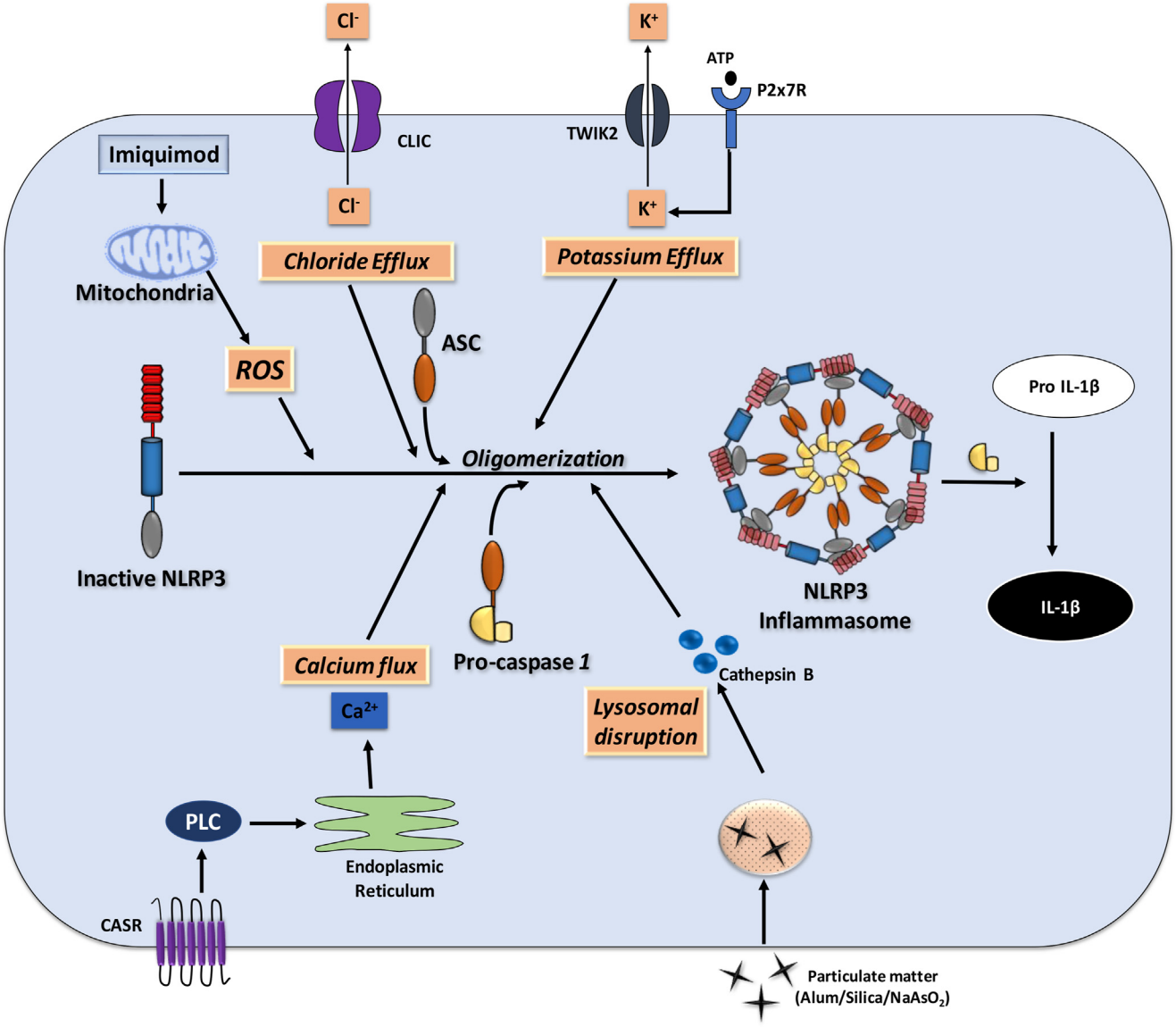

Figure 1 Mechanisms of NLRP3 inflammasome activation. NLRP3 is usually auto-repressed under physiological conditions but gets activated by undergoing oligomerization with ASC and pro-caspase-1 and releases active caspase-1, which is responsible for the conversion of pro-IL-1β (inactive) to IL-1β (active). The activation of NLRP3 inflammasome occurred mainly via 5 major upstream signals. 1) Potassium efflux: activation of the purinergic receptor via ATP causes potassium efflux that results in activation of NLRP3 complex; 2) calcium flux: activation of the calcium-sensing receptor causes phospholipase C mediated release of calcium ions from endoplasmic reticulum that results in NLRP3 oligomerization; 3) chloride efflux: efflux of chloride ions via chloride intracellular channel proteins CLIC1 and CLIC4 also acts as a signal for NLRP3 oligomerization; 4) lysosomal disruption: rupture of lysosomes due to large particulate materials also results in cathepsin B-mediated NLRP3 activation; and 5) reactive oxygen species (ROS): various ligands like imiquimod can induce mitochondrial stress resulting in the production of ROS that further cause the activation of NLRP3 inflammasome. ASC: adaptor protein apoptosis speck-like protein with a CARD domain; ATP: adenosine triphosphate; CASR: calcium-sensing receptor; P2x7R: P2x purinoceptor 7; PLC: phospholipase C; TWIK2: two-pore domain weak inwardly rectifying K^+ channel 2.

Lysosomal disruption is also one of the proposed upstream signals for NLRP3 activation. Large particulate maters, such as silica and aluminum salt crystals activated the NLRP3 inflammasome via lysosomal damage and rupture.[27] This is evidenced by the fact that cathepsin B inhibitor impairs NLRP3 inflammasome activation in human cells.[27,28] Similarly, another study showed that long-term exposure of arsenic ($NaAsO_2$) resulted in lysosomal disruption and a subsequent release of cathepsin-B that results in NLRP3 activation in the HSCs.[29] Mitochondrial reactive oxygen species (ROS) are generated as byproducts during oxidative phosphorylation but excess production of ROS during stress conditions, in turn, activates the NLRP3 inflammasome.[30] This mechanism has been reported with administration of imiquimod, a Toll-like receptor-7 (TLR7) ligand that resulted in the release of mitochondrial ROS via blockade of quinone oxidoreductases NQO2 and mitochondrial complex I activated the NLRP3 inflammasome[31] (Fig. 1).

NLRP3 inflammasome and liver fibrosis

NLRP3 inflammasome mediated an inflammatory response, which plays a critical role in fibrogenesis and a plethora of evidence support the central role of NLRP3 in the progression of liver fibrosis[32–35] (Fig. 2). The expression analyses of inflammasomes in experimental models of fibrosis showed that NLRP1, NLRP3, and AIM2 inflammasomes are significantly expressed in the Kupffer cells and moderately expressed in HSCs, and activation of these inflammasomes led to caspase-1 mediated maturation of inflammatory cytokines like IL-1β and IL-18.[36]

Role of NLRP3-mediated IL-1β in liver fibrosis

NLRP3 inflammasome causes the maturation of IL-1β through caspase-1; thus, it directly linked to inflammation and progression of liver fibrosis. IL-1β is one of the main inflammatory mediators and responsible for the proliferation and activation of HSCs and liver fibrosis, which is delineated by a study carried out in ATP-binding cassette transporter b4 knockout (*Abcb4*$^{-/-}$) mice and as well as in murine HSCs.[37] Further, IL-1β also increases the levels of fibrotic markers, like collagen-α1(I), TGF-β1, and TIMP-1.[38,39] This demonstrates that IL-1β is an important mediator for the progression of liver fibrosis. Moreover, another study carried out in obese diabetic mice fed with methionine/choline-deficient (MCD) diet developed NAFLD through activation of NLRP3 which further resulted in hepatic inflammation and fibrosis, whereas, treatment with a selective NLRP3 inhibitor, MCC950, resulted in normalized hepatic caspase-1 and IL-1β expression and reduced the ALT/AST ratio, hepatic inflammation, and fibrosis.[34] Besides, an *in vitro* study showed that MCC950 obliterated IL-1β release from macrophages and Kupffer cells by blocking cholesterol crystal-mediated NLRP3 activation in myeloid cells.[34]

Recently, we have reported the protective role of glibenclamide (GLB) against thioacetamide (TAA)-induced hepatic damage associated with NLRP3 inflammasomes signaling. Our findings demonstrated that GLB substantially reduced the hepatic damage and fibrosis through down regulation of NLRP3-mediated fibrogenesis as evidenced by a significant reduction in the expression of α-SMA, TGF-β1, NLRP3, ASC, caspase-1, and IL-1β.[40] Further, sitagliptin, an oral hypoglycemic agent, alleviated TAA-induced centrilobular necrosis in the liver via reduction of NLRP3 inflammasome activation along with decreased expression of TNF-α, IL-1β, and NF-κB in mice.[41] NLRP3 mediated inflammatory process leading to hepatic fibrosis has been demonstrated in choline-deficient amino acid-defined (CDAA) diet-fed mice, which is further reduced by andrographolide (a diterpenoid) administration.[32] Andrographolide treatment also resulted a reduction of macrophage infiltration in the liver and decreased mRNA level of the inflammasome, pro-inflammatory, and pro-fibrotic genes.[32] Interestingly, in a recent study with chlorogenic acid (CGA), a polyphenolic compound found in coffee, fruits, and vegetables, shown to protect against CCl_4-induced hepatic damage by blocking NLRP3 activation as evident by reduced expression of NLRP3, pro-caspase-1, caspase-1, pro-IL-1β and IL-1β.[42] Further, CGA has been proved to efficiently attenuates the liver fibrosis in LX-2 cells and CCl_4-induced liver fibrosis in rat by blocking TGF-β1/Smad 7 signaling.[43] Together, activation of NLRP3 inflammasome results in the activation of IL-1β signaling and HSCs, which contributed to the development and progression of liver fibrosis in a wide range of experimental models (Fig. 2).

Direct pathways for NLRP3 inflammasome-mediated HSCs activation

The direct pathway includes the activation of NLRP3 inflammasomes directly via various PAMPs, DAMPs, or cell surface receptors, which leads to the activation of HSCs and leads to liver fibrosis (Fig. 3).

Evidence from transgenic mouse models

An *in vitro* study carried out in primary mouse HSCs by monosodium urate (MSU) crystals administration to activate the inflammasomes and the HSCs isolated from wild-type mice showed increased mRNA expression of TGF-β and collagen 1 and also stained positive for α-smooth muscle actin (α-SMA); but these effects are abolished in HSCs isolated from *NLRP3*$^{-/-}$ and *ASC*$^{-/-}$ mice. Further, *NLRP3*$^{-/-}$ and *ASC*$^{-/-}$ mice showed a lower susceptibility to CCl_4 or TAA induced HSCs activation and fibrosis as compared to wild-type mice having functional inflammasome machinery.[44] Similarly, Wree et al showed the significance of NLRP3-initiated pyroptotic cell death by using *NLRP3* knock-in mice expressing mutant (D301N) NLRP3. The mutant mice showed NLRP3 activation that resulted in excessive hepatic inflammation along with HSC activation and collagen deposition in the liver, characterized by many markers of HSCs activation and fibrosis.[45] Additional evidence using *NLRP3* knock-in and knock-out mice showed that NLRP3 inflammasome activated the HSCs leading to the development of hepatic fibrosis.[46] Similarly, exposure of palmitic acid in human and rodent HSC cell lines or primary HSCs leads to induce fibrosis and significantly high expression of NLRP3 inflammasomes indicating its key role in HSC activation.[47]

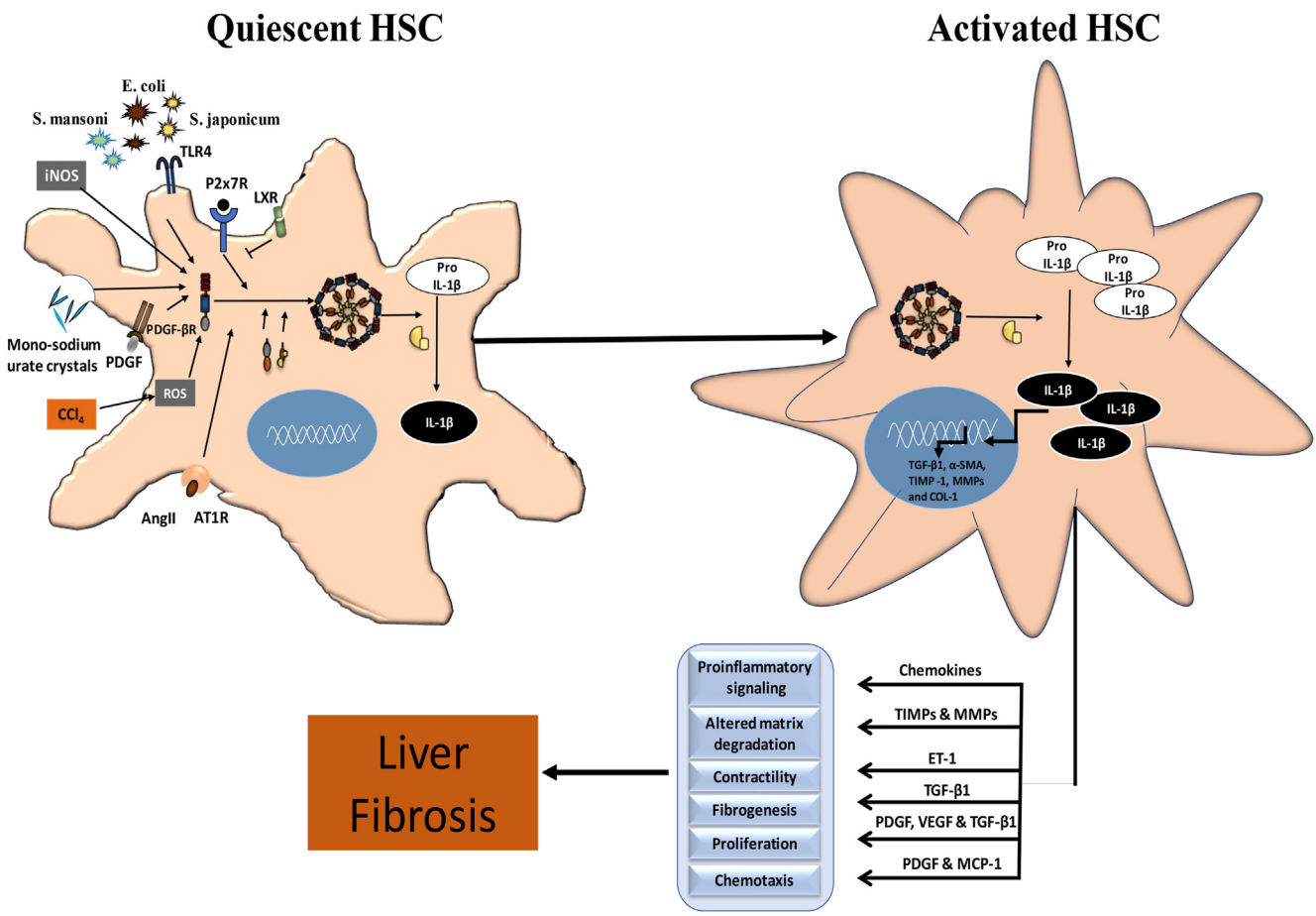

Figure 2 NLRP3 inflammasome-mediated HSC activation and liver fibrogenesis. NLRP3 inflammasome is present in the cytoplasm of HSCs and is activated by various agents like PAMPs (*S. mansoni*, *E. coli*, *S. japonicum*); DAMPs (toxic agent-associated damage to cells), or through receptors like purinergic receptors (P2x7R), angiotensin II receptors (AT_1R) and growth factor receptors (PDGF-βR). The activation of the NLRP3 inflammasome, in turn, activated the HSC (myofibroblast). Activated HSC further secretes various factors that result in an aggravated inflammatory response, chemotaxis of immune cells, altered matrix degradation, contractility and proliferation of myofibroblasts, and fibrogenesis. AngII: angiotensin II; AT_1R: angiotensin II type 1 receptor; COL-1: collagen type 1; CCl_4: carbon tetrachloride; ET-1: endothelin 1; iNOS: inducible nitric oxide synthase; LXR: liver X receptor; MCP-1: monocyte chemoattractant protein 1; MMP: matrix metalloproteinases; P2x7R: P2x purinoceptor 7; PDGF: platelet-derived growth factor; PDGF-βR: platelet-derived growth factor-β receptors; ROS: reactive oxygen species; α-SMA: α-smooth muscle actin; TIMP: tissue inhibitors of matrix metalloproteinases; TLR4: toll-like receptor 4; TGF-β1: transforming growth factor β1; VEGF: vascular endothelial growth factor.

Loss of NLRP3 function in knockout mice (*NLRP3*$^{-/-}$) and *NLRP3* gain-of-function in tamoxifen-inducible knock-in mice studies highlighted that *NLRP3* knock-in mice showed significant fibrosis, while *NLRP3*$^{-/-}$ mice are protected against CDAA diet-induced fibrosis.[48] Further, a study conducted using *NLRP3*^{A350V} knock-in mice showed severe liver inflammation and fibrosis characterized by neutrophil infiltration, increased expression of chemokines like CXCL1 and CXCL2, activation of macrophages, and high levels of TNF and IL-17.[49] In addition, *TNF* and *IL-17* knock-out mice showed a significant reduction in inflammation and fibrosis compared to NLRP3 mutants, whereas *TNF* knock-out mice showed a more prominent effect.[49] This proves that the NLRP3 mediates hepatic injury and fibrosis through TNF and IL-17 activation. Furthermore, aldosterone administration in wild-type mice development liver fibrosis in 4 weeks along with an increase expression of NLRP3, IL-1β, α-SMA and type I collagen, whereas *NLRP3*$^{-/-}$ knockout mice showed protection against aldosterone-induced liver fibrosis via down regulation of NLRP3 inflammasome and reduction of HSC activation.[50]

Evidence from parasitic infection models of liver fibrosis

Bacterial, parasitic, and viral infections in the liver commonly result in persistent liver inflammation that is further induced the fibrosis.[51] The NLRP3 inflammasome acts as a mediator of an infection-mediated inflammatory process leading to fibrosis, which is demonstrated by the contribution of NLRP3 inflammasome signaling in Schistosomiasis induced liver fibrosis (SSLF).[52] The BALB/c mice infected with *Schistosoma japonicum* showed a significant

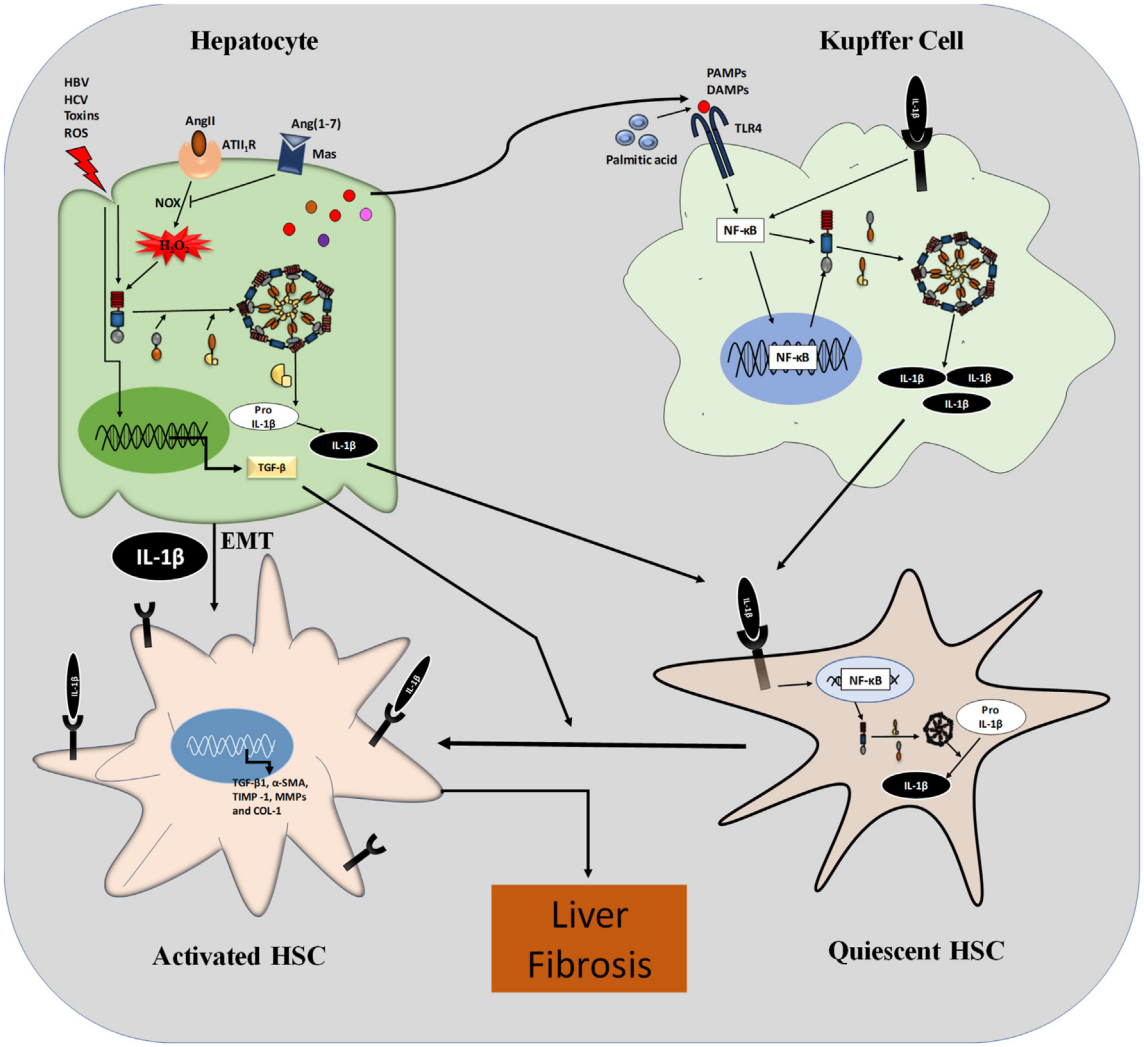

Figure 3 Activation of NLRP3 inflammasome in the hepatocyte and Kupffer cells leading to HSCs activation and liver fibrogenesis. Damage to the hepatocytes due to various DAMPs or PAMPs results in the activation of the NLRP3 inflammasomes. Also, angiotensin II (AngII) acts on the angiotensin-II type 1 receptor ($ATII_1R$) present on the surface of hepatocytes and results in the NADPH oxidase derived H_2O_2 mediated NLRP3 inflammasome activation. The activated NLRP3 inflammasome causes the activation and release of IL-1β, which subsequently activates HSCs. Additionally, IL-1β and NOX-derived H_2O_2-activated NLRP3 inflammasome cause the epithelial—mesenchymal transition (EMT), i.e., the conversion of hepatocytes to myofibroblasts. Damaged hepatocytes also release TGF-β, which acts as a pro-fibrogenic cytokine and converts quiescent HSCs to activated HSCs. Besides, damaged hepatocytes also release DAMPs that are recognized by toll-like receptors (TLR) present on Kupffer cells and activate the NLRP3 inflammasomes. TLR4 receptors on Kupffer cells are responsible for the recognition of various PAMPs and DAMPs and activate the NF-κB-mediated expression of the NLRP3, ASC, and pro-caspase 1, thereby the activation of NLRP3 inflammasome. NLRP3-mediated the release of IL-1β from Kupffer cells binds to its receptors present on the HSCs, which resulted in NF-κB-mediated NLRP3 activation, and ultimately activation of the HSCs (myofibroblast phenotype). These activated HSCs further release various pro-inflammatory mediators, pro-fibrogenic factors resulting in liver fibrosis. AngII: angiotensin II; Ang (1—7): angiotensin (1—7); $ATII_1R$: angiotensin-II type 1 receptor; CCl_4: carbon tetrachloride; CDCA: chenodeoxycholic acid; EMT: epithelial—mesenchymal transition; H_2O_2: hydrogen peroxide; HBV: hepatitis B virus; HCV: hepatitis C virus; LPS: lipopolysaccharides; Mas: Mas receptor; NOX: NADPH oxidase; ROS: reactive oxygen species; TGF-β: transforming growth factor-β; TLR4: Toll-like receptor 4.

increase in the expression of NLRP3 and NF-κB in liver tissue, Kupffer cells, and HSCs, which is decreased with administration of MCC950, a selective NLRP3 inhibitor.[52] Additionally, a similar study highlighted the protective role of Taurine in the *Schistosoma japonicum*-induced liver fibrosis by suppressing the TXNIP/NLRP3 inflammasome pathway and downstream molecule IL-1β.[53] Another study carried out in mice infected with *Schistosoma japonicum* for 6 weeks or soluble egg antigen (SEA) used for *in vitro* stimulation of HSCs resulted NLRP3 inflammasomes activation, which clearly demonstrated that NLRP3 inflammasome activated HSCs and initiated hepatic fibrosis.[54] Furthermore, *E. coli* transfection in HSC-T6 cells, a rat hepatic stellate cell line, resulted in the activation of

NLRP3 inflammasome and HSCs, which further secreted pro-fibrogenic factors, like TGF-β1 and IL-1β.[33] Moreover, *Schistosomiasis mansoni* infection led to mitochondrial damage and resulted an increase ROS production and activation of NLRP3 and AIM2 inflammasomes along with an increase NF-κB expression.[55] Thus, the NLRP3 inflammasome is a key player in infection-associated inflammation, activation of HSCs, and the progression of liver fibrosis.

Evidence of HSCs activation via different cell surface receptors

Purinergic receptor P2x7 (P2x7R) is one of the frequently responsible modulators of hepatic inflammation and fibrosis and its role in the activation of HSCs has been reported using the LX-2 cells (human HSCs) model. Pre-treatment with A438079, a selective antagonist of P2x7R, resulted in a reduce mRNA expression of NLRP3, α-SMA, and type I collagen as well as collagen deposition.[56] Another study reported that administration of 25-OCH_3-PPD (25-methoxyldammarane-3β,12β,20-triol), a ginsenoside isolated from *Panax ginseng*, attenuated TAA-induced liver fibrosis in mice through blocking the P2x7R-mediated NLRP3 inflammasome activation.[57] In addition, 25-OCH_3-PPD reduced the expression α-SMA via regulation of LXRs and P2x7R-NLRP3 in an *in vitro* study.[57]

Activation of NLRP3 is also mediated via inducible nitric oxide synthase (NOS2), which demonstrated in CCl_4 or bile duct ligation models of hepatic fibrosis using pharmacological and genetic inhibition approaches. Administration of MRI-1867, a hybrid cannabinoid-1 receptor/NOS2 (CB_1R/iNOS) inhibitor reduced NLRP3 expression in *CNR1*$^{-/-}$ mice, but not in *NOS2*$^{-/-}$ mice demonstrating that iNOS is an essential pathway in the regulation of NLRP3 signaling.[58] Intriguingly, tetramethylpyrazine (TMP), a compound obtained from Ligusticum chuanxiong Hort, significantly protected the liver against CCl_4-caused injury and fibrogenesis by suppressing levels of inflammatory cytokines, including TNF-α, NLRP3, NF-κB and IL-1β as well as improved liver histological architecture.[59] Moreover, TMP also inhibited inflammatory cytokine expression in HSCs associated with disrupting platelet-derived growth factor-b receptor (PDGF-βR)/NLRP3/caspase 1 pathway in the *in vitro* experiment.[59]

NF-κB signaling regulates various genes involved in inflammation; it is chronically active in many inflammatory diseases, such as inflammatory bowel disease, arthritis, sepsis, gastritis, asthma, atherosclerosis.[60,61] Dong et al reported that TLR4/NF-κB signal pathway was involved in NLRP3 inflammasome activation in palmitic acid-exposed HSCs and high-fat diet-induced NASH.[62] Zhao et al reported that tenofovir alafenamide fumarate/tenofovir disoproxil fumarate prevented progression and promoted reversion of liver fibrosis through assembling TGFβ1/Smad 3 and NF-κB/NLRP3 inflammasome signaling pathways.[63] Further, NF-κB signaling is a necessary prerequisite for the activation of the NLRP3 inflammasome in primary hepatocytes.[64] Similarly, hemistepsin A (HsA) administration significantly decreased ALT and AST activities in a CCl_4-induced liver fibrosis model. Furthermore, HsA, isolated from *Hemistepta lyrata* (Bunge) attenuated CCl_4-mediated collagen deposits and profibrogenic genes expression in hepatic tissue through inhibition of NF-κB/Akt-dependent signaling.[65]

Yes-associated protein (YAP), a major downstream effector of the Hippo signaling pathway, acts as a transcriptional regulator by activating the transcription of genes involved in cell proliferation and suppressing apoptotic genes, thereby YAP signaling pathway allows the cellular control of organ size and tumor suppression.[66] In addition, YAP is regulated by mechanical signals such as ECM rigidity, strain, shear stress, or adhesive area that are dependent on cytoskeletal integrity.[67] Moreover, Hippo/YAP1 signaling has been extensively studied in the liver and other organs over the recent years, and components of upstream Hippo signaling and the downstream effectors YAP and TAZ are involved in a multitude of cellular functions including cell proliferation, survival, development, differentiation, metabolism, and cross-talk with the immune system.[68] Wang et al reported that vitamin D receptor agonist calcipotriol suppressed the NLRP3 inflammasome activation by activating YEP1 to alleviate liver injury and retard fibrogenesis in cholestasis.[69] Jin et al reported that silencing YAP enhanced the tetramethylpyrazine induction of activated HSC senescence.[70] In addition, Li et al reported that treatment with anti-inflammatory agent magnesium isoglycyrrhizinate inhibited hippo/YAP signaling pathway,[71] whereas, the overexpression of Hippo/YAP signaling effector YAP completely diminished magnesium isoglycyrrhizinate-induced anti-inflammatory and anti-fibrotic effects. Alsamman et al findings showed that ceramide as a critical regulator of YAP/TAZ signaling and HSC activation and highlight ceramidase inhibition as a therapeutic target for the treatment of fibrosis.[72] Furthermore, activation of YAP suppressed ECM synthesis, and hepatic damage, HSC activation and fibrosis in a mouse model of liver ischemia-reperfusion injury.[73]

The Hedgehog signaling pathway is a crucial regulator of animal development and is present in all bilaterians, and the malfunction of this pathway is associated with many diseases such as fibrosis, COPD, and cancer.[74,75] Li et al reported that disruption of hedgehog/Gli 1 signaling in myeloid-specific *Foxo 1* knockout mice deteriorated liver function, diminished Snail, and augmented RIPK3 and NEK7/NLRP3 in ischemia/reperfusion stressed livers, which indicated hedgehog/Gli1 function is the key in oxidative stress-induced liver inflammation and necroptosis.[76] Sheng et al reported that the cluster of differentiation 47-signal regulatory protein alpha (CD47-SIRPα) signaling activates the hedgehog/smoothened (SMO)/GLI family zinc finger 1 (Gli1) pathway, which controls NEK7/NLRP3 activity through a direct interaction between Gli1 and Notch 1 intracellular domain and these pathways could be a potential therapeutic target in mesenchymal stem cell-mediated immunotherapy of sterile inflammatory liver injury.[77] Moreover, a naturally occurring flavonoid procyanidin B2 has been reported to inhibit the sonic hedgehog signaling pathway and showed protective potential against cold stimulation-induced liver injury.[78] In addition, the Hedgehog-YAP signaling pathway also regulates glutaminolysis to control activation of HSCs in acute and chronic liver injury in patients and mouse liver.[76] Thus, these reports indicated that Hedgehog/YAP and Hippo/YAP

pathways play an important role in HSC activation and liver fibrogenesis in varieties of stimuli.

Indirect pathways for IL-1β-mediated HSCs activation

The indirect pathway includes the activation of HSCs via IL-1β released from NLRP3 inflammasomes present in either hepatocytes or Kupffer cells, and by various DAMPs. The IL-1β released from Kupffer cells or hepatocytes results in NF-κB mediated activation of NLRP3 inflammasome that subsequently activates the HSCs leading to liver fibrosis.[79] The indirect pathway in Kupffer cells involved the activation of inflammasomes by various PAMPs and DAMPs resulted release of IL-1β and IL-18, which leads to activation of HSCs and fibrosis.[79] An *in vitro* study using Kupffer cells proved that palmitic acid administration increased the expression of NLRP3 inflammasomes when provided with TLR2 ligand.[80] These findings have also been confirmed using *TLR2*$^{-/-}$ mice fed a CDAA diet for 22 weeks showed significant protection against the development of NASH and liver fibrosis compared to WT mice.[80] This demonstrated that palmitic acid constitutively works with TLR2 to activate NLRP3 inflammasomes.[80] Another example of the indirect activation of NLRP3 inflammasomes is shown by angiotensin II (AngII) infusion induced hepatocyte epithelial—mesenchymal transition (EMT) leads to liver fibrosis in rat via NOX-derived H_2O_2-activated NLRP3 inflammasome/IL-1β/Smad pathway, which is blocked by administration of angiotensin-(1—7) [Ang-(1—7)] in BDL model of fibrosis.[81] Similarly, another study reported that AngII-induced collagen synthesis via induction of NLRP3 inflammasomes through NADPH oxidase (NOX)-dependent oxidative stress and hepatic fibrosis, which is inhibited by Ang-(1—7) via reducing the NOX-dependent oxidative stress and NLRP3 activation as well as increasing GSH and Nrf 2 levels in BDL model.[82] At the high physiological level, chenodeoxycholic acid (CDCA), a major primary bile acid, acts as an endogenous death signal and activated the NLRP3 inflammasome in macrophages, thereby facilitated the hepatic inflammation and fibrosis.[83] Thus, NLRP3 activation leads to an increased expression and/or secretion of IL-1β, which promoted HSCs activation and fibrosis (Fig. 2, 3).

Alcohol directly or indirectly disrupts the intestinal barrier, which increases the gut permeability, and resulted to increase leakage of gut microbiota and its products, mainly LPS.[84] The LPS subsequently translocated into the liver from the GI tract through the portal vein and serves as a DAMP to induce pro-IL-1β gene expression by binding to the TLR4 receptor on Kupffer cells.[85,86] The swelling and rupture of hepatocytes initiated by NAPQI, a toxic metabolite of acetaminophen, led to the release of DNA fragments into the extracellular space,[87] resulting in a massive DNA deposition within the necrotic areas, which promotes the secretion of IL-1β and IL-18 through TLR9/NF-κB signaling pathways, consequently leading to neutrophil infiltration to the DNA-rich area, further exacerbating the liver injury. Further, myeloid-specific NLRP3 inflammasome activation leads to hepatocyte proptosis and severe liver inflammation and fibrosis, which is another mechanism of indirect NLRP3-mediated liver damage.[45] ER stress is a significant trigger of NAFLD progression, which follows hepatic lipid accumulation, and therapies designed to reduce ER stress and proven to be beneficial in the treatment of NAFLD and liver fibrosis.[88—90] Therefore, the above described indirect mechanisms lead to activate NLRP3 inflammasome and IL-1β release, thereby promoting the liver injury and/or HSC activation.

Inhibition of NLRP3 inflammasome activation and its downstream signaling

NLRP3 inflammasomes are the core component mediating the inflammatory response leading to inflammation that further leads to fibrosis, and its inhibition could serve as an important approach to lessen liver fibrosis. Few drugs have been approved to inhibit NLRP3-dependent cytokines but are associated with side-effects due to lack of specificity. Furthermore, several new small molecules and old drugs (repurposing) that directly and indirectly affect the NLRP3 inflammasome activity has been reported in many inflammatory conditions including inflammatory bowel disease.[91,92] However, so far, no drug has been approved which can directly inhibit NLRP3 inflammasome. Several strategies to inhibit the activation of NLRP3 inflammasome and its downstream signaling are discussed in this section.

Inhibition of NLRP3 transcription and PTMs

Transcription of NLRP3 inflammasome can be inhibited by blocking the TLR-mediated increase in NLRP3 expression in HSCs, Kupffer cells, and hepatocytes. But this is not an ideal approach due to its lack of specificity and can result a blockade of non-NLRP3 inflammasomes and off-target effects. TLRs act as the pattern recognition receptors (PRRs) for various PAMPs and their signaling can be blocked by inhibition of IL-1 receptor-associated kinase 4 (IRAK4). IRAK4 is an essential kinase for TLR-mediated activation of NF-κB and its inhibitors are already under development.[93] However, these inhibitors are incapable to completely block NLRP3 transcription, as there are multiple pathways involved in priming of NLRP3 inflammasomes.[94]

PTMs are essential for the regulation of NLRP3 activation and controlling the PTMs and can regulate the activation of NLRP3. However, there is still very limited knowledge of the PTMs, and the specificity of their targets is still poorly understood. Deubiquitylation is an important PTM involved in NLRP3 activation and several deubiquitinase inhibitors are being developed for treating cancers which can be tested for NLRP3 inhibitory potential in various liver fibrotic conditions.[95] Moreover, TLR priming induced the phosphorylation (Ser 198) of NLRP3 via JUN N-terminal kinase 1 (JNK1) and promoted NLRP3 self-association and activation.[91,96] Nevertheless, phosphorylation can suppress NLRP3 activation as well.[97] In addition, the suppression of phosphorylation of NLRP3 by bile acids and prostaglandin E2 via protein kinase A (PKA)-mediated mechanism attenuated its ATPase activity and activation.[98,99] On other hand, sumoylation of NLRP3 at multiple sites by the protein E3 SUMO protein ligase MUL1 confines its activation, whereas

desumoylation of NLRP3 by sentrin-specific protease 6 (SENP6) and SENP7, promotes inflammasome activation.[100] Therefore, these PTMs can control and determine the NLRP3 activity and its biological function.

Epigenetically, chromatin structure and transcription of various genes is regulated by the overall acetylation levels on the histone proteins. In general, histone acetylation promotes the transcription of genes and deacetylation of histones results in transcriptional repression.[101,102] The balance between the acetylated and deacetylated states of histones is controlled by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs).[103] Recently it has been reported that NLRP3 acetylation level is modified by sirtuin 2 (SIRT2), a cytosolic deacetylase in macrophages, and acetylation of NLRP3 facilitated the assembly and activation of the NLRP3 inflammasome, whereas deacetylation inactivated the NLRP3 and thereby reversed the aging-associated inflammation and insulin resistance.[104,105] Similarly, zinc-dependent HDACs also regulated the NLRP3 acetylation and pharmacological inhibition of HDACs attenuated inflammation and protects dopaminergic neurons in Parkinson's disease[106] as well as reduced oxidative stress and NF-κB signaling and exerts neuroprotection in Alzheimer's disease.[107] In addition, HDACs inhibitors reported as anti-fibrotic and anti-diabetic molecules via reducing the steatosis, insulin resistance, beta-cell death and fibrogenesis in several experimental models.[108–113] Thus, these studies highlighted that PTMs can control and determine the overall effect on NLRP3 activity, and further studies are needed to elucidate these mechanisms in detail.

Inhibition of IL-1β

IL-1β is the most important downstream molecule in NLRP3 inflammasome-mediated pro-inflammatory pathway. Blockade of IL-1β has been the most successful strategy for the treatment of NLRP3 inflammasome-driven disorders like cryopyrin-associated periodic syndromes (CAPS), deficiency of IL-1 receptor antagonist (DIRA), and rheumatoid arthritis.[114] Inhibitors of IL-1 receptors blocked IL-1β signaling; three monoclonal antibodies have already been approved by the FDA.[114] Anakinra was the first drug approved to treat CAPS[114] and rheumatoid arthritis in humans[115]; followed by canakinumab and rilonacept. The efficacy of these drugs can be tested in liver fibrosis; however, these drugs fail to inhibit NLRP3-mediated pyroptosis and actions of other pro-inflammatory cytokines such as IL-18 and HMGB1 as well as are associated with different off-target effects.

Inhibition of ASC

ASC acts as the adaptor molecule connecting NLRP3 with caspase-1; thus, inhibition of ASC can prevent further downstream signaling of NLRP3. However, ASC is also essential for the functioning of many other non-NLRP3 inflammasomes and its inhibition can dysregulate some other critical physiological processes.[116] There is very limited knowledge available about the oligomerization of ASC and its contribution to the overall role in NLRP3 activity, which makes it more difficult to consider as a drug target.[94] IC-100 is a monoclonal antibody that uniquely inhibits the adaptor ASC component of multiple inflammasomes. It inhibits ASC by blocking inflammasome formation, activation, and initiation of the inflammatory response as well as disrupting their structure and function, thereby preventing durability of the inflammatory response (https://www.zyversa.com/inflammasomes/asc-inhibitor).[117] The pre-clinical data of IC 100 has shown therapeutic efficacy in many inflammatory diseases such as stroke, brain injury, multiple sclerosis and acute lung injury.[117,118] Diacerein and its analogs also inhibited the expression of ASC, NLRP3, and/or formation of NLRP3 inflammasome complex.[119] Notably, a randomized placebo-controlled trial concluded that a 2-year treatment with diacerein significantly reduced liver fibrosis in diabetic patients with NAFLD.[120] Besides, a four-week treatment of diacerein attenuated left ventricular fibrosis and improved cardiac function by reducing the inflammatory response after myocardial infarction in rats via inhibiting caspase-3 activity, and NF-κB transcription.[121] Furthermore, quercetin interfered with ASC oligomerization and prevents IL-1β, thereby inhibits inflammasome activation in mouse model of vasculitis.[122] More studies are required to delineate the exact mechanisms of ASC oligomerization and its role in NLRP3 inflammasomes-mediated liver fibrosis for possible exploration as a new therapeutic target.

Inhibition of caspase-1

Caspase-1 has been extensively targeted since its discovery owing to its direct involvement in the activation and release of cytokines IL-1β and IL-18. Two small molecule reversible pharmacological inhibitors of caspase-1 have also been developed, namely VX740 (pralnacasan) and VX765.[123,124] These small molecule inhibitors are prodrugs in nature and converted to an active form by cleavage through plasma esterases.[123,124] Both compounds are evaluated up to phase II clinical trials for epilepsy and psoriasis, but trails are discontinued due to hepatotoxicity.[125] These hepatoxic effects might be due to the critical functional role of caspase-1 in the other inflammasomes under physiological conditions. Mechanistic studies will provide more insights into the isoform-specific roles of caspase-1 inhibitors in the activation of various inflammasomes and their associated downstream signaling. Another approach for the repurpose of caspase-1 inhibitors, VX740 and VX765, is the utilization of nanotechnology-based strategies and to implement the novel drug delivery methods for successful delivering these drugs in the targeted cells or pathological tissues to minimize the hepatic and other adverse effects.

Inhibition of NLRP3 activation

Numerous compounds reported to inhibit NLRP3 inflammasome, but their mechanisms of inhibition are not well characterized. The most studied and well-characterized direct NLRP3 inhibitors are summarized in Table 1. Moreover, it is very challenging to synthesize a direct antagonist of NLRP3, because of its complex and multimeric structure as well as the lack the crystal-structures of active forms of

Table 1 Summary of the experimental studies described the inhibitory potential of various compounds on NLRP3 inflammasome activation in several pathological conditions, and the potential anti-fibrotic activities.

Inhibitors	Models used for NLRP3 activity	Mechanisms of NLRP3 inhibition	Potential anti-fibrotic activity reported
MCC950	Mouse BMMs primed with LPS	Directly attached to the Walker B motif within the NLRP3 NACHT domain and blocked ATP hydrolysis, thus inhibited activation of NLRP3.[128,129]	Attenuated hepatic fibrosis in murine models.[34,130] Reduced Schistosoma japonicum-infected associated liver fibrosis in mice via suppression NF-κB signaling.[52] Reduced BDL-induced cholestatic liver injury and disease progression in mice.[131]
	BMDMs PD model	Inhibited ASC oligomerization.[126,132]	
3,4-Methylenedioxy-β-nitrostyrene (MNS)	BMDMs	Inhibited the NLRP3 ATPase activity by cysteine modification.[129,133]	β-Nitrostyrene and its analogues attenuated LPS-mediated acute lung injury via the inhibition of neutrophil-platelet interactions.[134] No anti-fibrotic activity reported
CY-09	BMDMs	Binds directly to the ATP-binding motif of NLRP3 NACHT domain, thus inhibiting the NLRP3 ATPase activity.[129,135]	Reduced hepatic steatosis and NAFLD in mice.[136]
β-Hydroxybutyrate (BHB)	BMDMs	Inhibited K^+ efflux, thereby decreased ASC oligomerization and reduced IL-1β/18 secretions.[129,137]	Reduced hepatic lipid accumulation and fibrosis, and alcohol and age associated liver damage.[138–140]
Glibenclamide or Glyburide	BMDMs	Inhibited ATP sensitive K^+ channels resulting in indirect inhibition of NLRP3 activation.[129,141]	Reduced IL-1β, TGF-β1, and fibrosis in human hepatic stellate cell line infected with *B. abortus*,[142] as well as ameliorated hepatic fibrosis via downregulation of NLRP3-mediated fibrogenesis.[40] Attenuated bladder fibrosis by inhibition of NLRP3 and IL-1β.[143]
Parthenolide	BMDMs	Inactivation of caspase-1 by direct alkylation of cysteine residues, thus the inhibition the NLRP3 ATPase activity.[144,145]	Exhibited pro-apoptotic effects in HSCs and reduced TAA-induced hepatic fibrosis in rats.[146] Ameliorated peritoneal fibrosis by downregulating the TGF-β/Smad signaling.[147] Alleviated bleomycin-induced pulmonary fibrosis by suppression NF-κB/Snail signaling.[148]
OLT1177	J774 cells	Inhibited the NLRP3 ATPase activity.[129,149]	No anti-fibrotic activity reported
Tranilast	BMDMs	Directly binds to the NATCH domain of NLRP3 and blocks NLRP3 oligomerization.[91]	Attenuated NASH in rats by inhibiting TGF-β1,[148] and reduced the profibrogenic mediators and improved hepatic functions in *S. mansoni*-infected mice.[150] Attenuated pulmonary fibrosis by suppressing TGFβ-mediated ECM production.[151] Ameliorated the diabetic kidney fibrosis[152] and unilateral ureteral obstruction-induced fibrosis in rats.[153]
Oridonin	BMDMs	Inhibited NLRP3 activation by binding to the cysteine 279 of NATCH domain and blocking the interaction between NLRP3 and NEK7.[91]	Reduced CCl_4-induced liver fibrosis in mice,[154] and inhibited human HSCs proliferation and fibrogenesis by downregulating TGF-β1-mediated ECM *in vitro*[155] Inhibited bleomycin-induced lung fibrosis by suppressing TGFβ/Smad pathway.[156] Ameliorated cardiac fibrosis and remodeling in mice.[157]

(*continued on next page*)

Table 1 (*continued*)

Inhibitors	Models used for NLRP3 activity	Mechanisms of NLRP3 inhibition	Potential anti-fibrotic activity reported
Fc11a-2	DSS-induced colitis in mice	Inhibited cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 and indirectly suppressed the activation of NLRP3.[129,158]	No anti-fibrotic activity reported

BMMs: bone marrow derived macrophages; DSS: dextran sulfate sodium.

NLRP3 inflammasomes.[126] Recently, Sharif and co-worker reported a cryo-electron microscopy structure of inactive human NLRP3 in complex with NEK7, which potential facilitated the designing and development of more specific NLRP3 inhibitors.[127]

Conclusions and future directions

Chronic liver diseases like fibrosis, cirrhosis, hepatitis, and liver cancer are the growing health concerns.[159] Inflammation plays a central role in the progression of liver fibrosis, which can be induced by either host- or pathogen-derived molecules. Inflammasomes are the core molecules regulating the inflammatory process via caspase-1-mediated release of inflammatory cytokines, like IL-1β and IL-18. Expression of NLRP3 inflammasome has been reported in most hepatic cells, like HSCs, Kupffer cells, and hepatocytes. HSCs are the primary cells responsible for liver fibrosis, and several direct and/or indirect compelling pieces of evidence reported the upregulation of NLRP3 expression in the activated HSCs through DAMPs or PAMPs (Fig. 3). Inhibition of NLRP3 can be explored by several pharmacological approaches, novel drug delivery methods, and genetic strategies; however, the targeted inhibition of downstream molecules of NLRP3 inflammasome-mediated pathways is a challenging and nascent research area. The NLRP3 inflammasome has been implicated in liver fibrosis, and the use of specific NLRP3 inhibitors has been reported to attenuate liver fibrosis in *in vivo* and *in vitro* studies.

The present research to employ the NLRP3 inhibitors is entirely engrossed in NLRP3-driven disorders like CAPS, DIRA, and RA for the therapeutic approach. Further, specific NLRP3 inhibitors are proven as a successful approach for the treatment of liver fibrosis. However, these small molecules like canakinumab, anakinra, and rilonacept only inhibit IL-1β and are relatively less efficacious with a poor safety profile. Moreover, MCC950 is the most studied and efficacious NLRP3 inhibitor evaluated in the MCD diet- and *S. japonicum* infection-induced liver fibrosis mouse models. MCC950 has failed in phase I clinical trials due to hepatotoxicity.[160,161] But further mechanistic studies and chemical structure optimization of MCC-950 can reduce its toxicity. Also, characterization of the crystal structure of the active forms of NLRP3 and molecular mechanisms of its activation will be useful for therapeutic targeting of NLRP3 inflammasomes. Therefore, targeted NLRP3 inhibition can be a promising therapeutic approach for the treatment of liver fibrosis.

Author contributions

Conceptualization - HVC, DKD, SK, and GBJ; writing, review, and editing - HVC, DKD, SK, and GBJ; supervision - GBJ. All the authors read and approved the review.

Conflict of interests

Authors declare no conflict of interests.

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