Cell Death in Hepatocellular Carcinoma

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The progression of liver tumors is highly influenced by the interactions between cancer cells and the surrounding environment, and, consequently, can determine whether the primary tumor regresses, metastasizes, or establishes micrometastases. In the context of liver cancer, cell death is a double-edged sword. On one hand, cell death promotes inflammation, fibrosis, and angiogenesis, which are tightly orchestrated by a variety of resident and infiltrating host cells. On the other hand, targeting cell death in advanced hepatocellular carcinoma could represent an attractive therapeutic approach for limiting tumor growth.

programmed cell death hepatocellular carcinoma

1. Introduction

The liver is an essential organ that exerts important and critical functions (glucose storage, lipid and cholesterol homeostasis, detoxification and processing of xenobiotics, endocrine regulation, blood volume regulation, and immune surveillance). Portal vein supplies around 80% of the blood from the gut to the liver, draining into the hepatic lobules through the hepatic sinusoids [1]. A highly organized liver zonation creates oxygen and metabolic gradients or zones with different and specialized hepatocytes functions. This particular microarchitecture is configured by liver sinusoids, discontinuous and specialized capillaries lined by a fenestrated monolayer of liver sinusoidal endothelial cells (LSECs). The basal side of LSECs interacts with hepatocytes and hepatic stellate cells (HSCs) in the space of Disse, while their luminal side interacts with liver resident immune cells, including Kupffer cells (KCs), hepatic natural killer (NK) cells, and NKT cells and also tissue-resident lymphocytes (T and B cells) [2] [3]. KCs are the largest population of tissue-resident macrophages and play an important role in maintaining immune tolerance due to their phagocytic and antigen presentation activity [4]. Likewise, an immunosuppressive environment, an elevated expression of immune checkpoint molecules and an incomplete activation of CD4+ and CD8+ T cells are crucial for an efficient immune tolerance [5]. Intrahepatic innate immune responses are beneficial during acute hepatitis, as the enhance both inflammation and tissue healing. However, sustained immune activation in chronic liver diseases (CLD) may represent the basis for chronic inflammation. The resulting cirrhotic microenvironment promotes the initiation and progression of hepatocellular carcinoma (HCC), despite the underlying molecular mechanisms of the different etiologies [6].

2. Cell Death and Inflammation: A Road to HCC

HCC is the most prevalent primary liver cancer and the third most common cause of cancer death worldwide, with a 5-year survival of 18% [7]. HCC is closely associated with chronic inflammation and fibrosis, representing the

common end-stage of CLD from excessive alcohol intake, viral hepatitis, and non-alcoholic fatty liver disease (NAFLD) [8]. In this regard, hepatocellular death is a common trigger and sensitive parameter of liver disease progression, and it is well known that specific cell death responses promote liver disease progression through different mechanisms [9]. It has been stated that the relative contribution of cell death to hepatocarcinogenesis depends on the underlying disease. For instance, HBV infection and NAFLD may induce HCC development in the absence of chronic liver injury and/or fibrosis, suggesting alternative cell-death-independent mechanisms that promote carcinogenesis [10]. Nevertheless, it is important to highlight that 80% of HCC occurs in the context of a fibrotic or cirrhotic liver, with significant levels of hepatocellular cell death and inflammation [11].

In liver homeostasis, a balance between the loss and replacement of hepatocytes occurs in a highly regulated manner. Liver regeneration is closely associated with its capacity for xenobiotic and toxin-detoxifying functions after excessive exposure to insults such as food-derived toxins or infections by viruses, bacteria, and/or parasites [12][13]. Therefore, cell death is a crucial feature in chronic inflammatory diseases [14].

Although different modes of cell death have been described, apoptosis and necrosis have traditionally been the most studied and characterized. Apoptosis has conventionally been associated with highly controlled, host-induced cell death in scenarios of injury, representing a key mechanism to prevent malignant transformation. On the contrary, necrosis is still largely considered an “immunogenic” form of cell death. Nevertheless, a growing number of recent studies have shown that specific and distinct programmed cell-death processes, distinct from apoptosis and necrosis, might be involved in the modulation of compensatory proliferation and immune cell activation in CLD [15][16][17].

3. Apoptosis

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Morphological hallmarks of apoptosis involve DNA fragmentation, plasma membrane blebbing, and cell shrinkage, which lead to cell fragmentation into organelle-containing apoptotic bodies, triggered by activated aspartate-specific proteases, known as caspases [9][18]. Apoptosis is classically considered non-inflammatory or low inflammatory, with a minimal release of damage-associated molecular patterns (DAMPs) due to the rapid removal of apoptotic bodies [19].

Receptor-mediated apoptotic pathways are typically promoted by key regulatory molecules such as the tumor necrosis factor (TNF) family of death receptor ligands (e.g., Fas ligand [FasL]) and TNF-related, apoptosis-inducing ligand (TRAIL), all of which are highly expressed in hepatocytes [20]. Importantly, both mechanisms might be linked, due to the cytochrome c release-mediated activation of caspase-3, the ultimate executioner of apoptosis. The binding of TNF to TNF receptor 1 (TNFR1) induces the formation of complex I, which is involved in nuclear factor-ƙB (NF-ƙB)-dependent expression of pro-survival genes such as the apoptosis regulator B cell lymphoma 2 (Bcl 2) family or the encoding cellular FLICE-like inhibitory protein (c-FLIP), among others (**Figure 1**A). In fact, TNF-mediated cell death only occurs if NF-ƙB-dependent antiapoptotic signals are suppressed. In that scenario, the lack of inhibition by c-FLIP results in a switch from survival responses towards cell death by the formation of

complex IIa or complex IIb, both involved in caspase-8 activation and apoptosis [13]. Complex IIa is formed upon dissociation of the adaptor protein TNFRSF1A-associated via death domain (TRADD) from complex I and its association with FAS-associated death domain protein (FADD), whereas complex IIb or ripoptosome is composed by the receptor-interacting protein kinase 1 (RIPK1), FADD, and caspase-8 (**Figure 1**B).

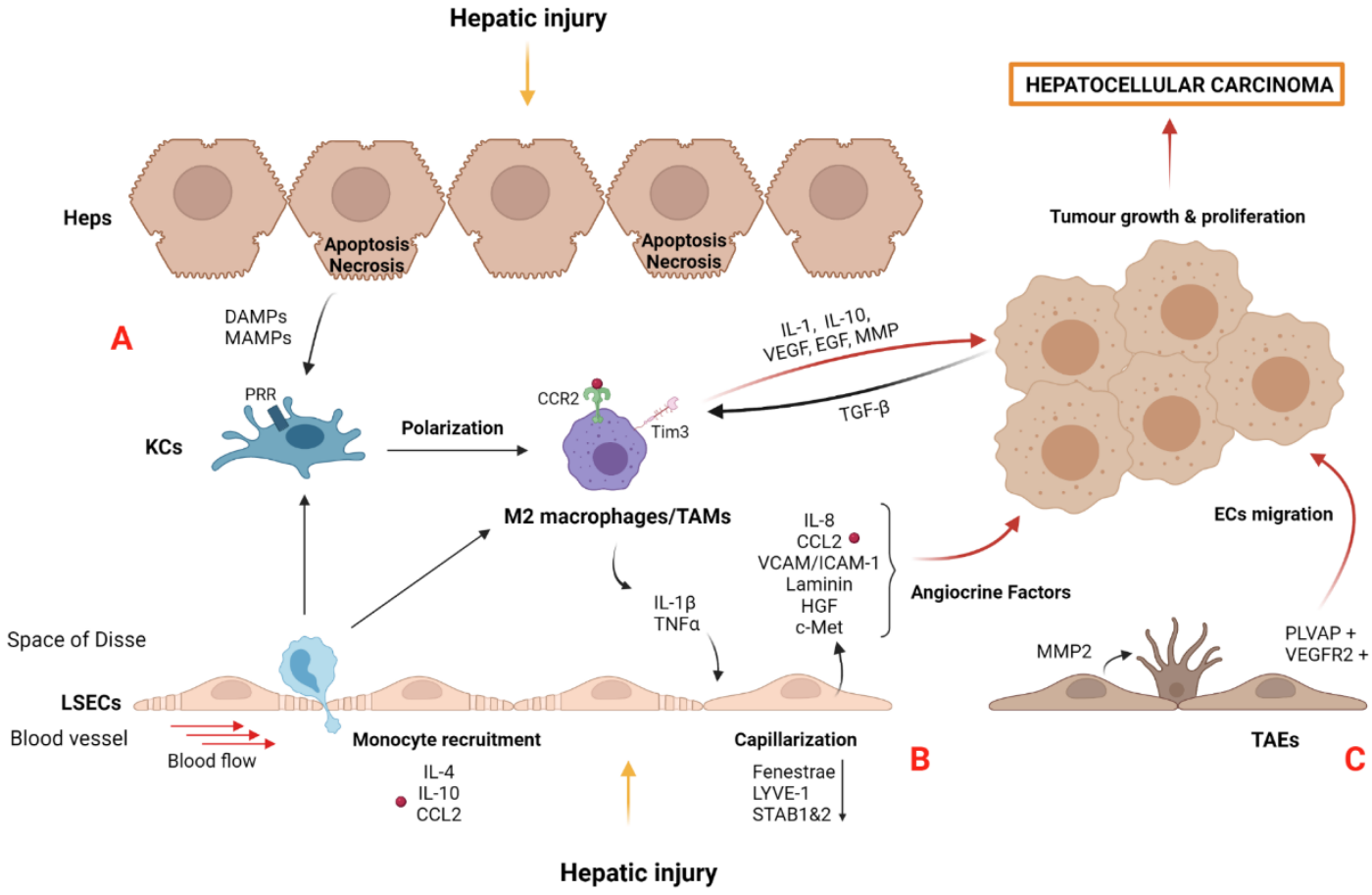

Figure 1. Programmed cell death pathways initiated by TNF and FAS: (**A**) The TNF–TNFR1 cascade simultaneously activates proapoptotic and antiapoptotic signals, and diverse signaling complexes are formed. Complex I, composed by the adaptor protein TRADD, the receptor RIPK1, and the E3 ligase TRAF2, enables the recruitment of the inhibitor NF-ƙB kinase complex, comprising IKKα/β and NEMO. Subsequently, the inhibitor of NF-ƙB (IƙBα) is ubiquitinated and NF-ƙB translocates to the nucleus, promoting the expression of survival and antiapoptotic genes such as Bcl2 and c-FLIP. (**B**) In the FAS/TRAIL-mediated apoptotic pathway, binding of FASL/TRAIL to their cognate receptors results in FAS clustering and binding to FADD. TRADD dissociates from complex I and associates with FADD to form complex IIa, which triggers caspase-8 activation and, consequently, apoptosis. Complex IIb, or ripoptosome, is highly dependent on RIPK1 activity, and represents an alternative death complex that induces apoptosis through direct activation of caspase-8. (**C**) In contrast, caspase-8 can be inhibited under certain physiological or pharmacological conditions, promoting the shift from the ripoptosome-induced apoptosis to necroptosis, a form of programmed necrosis via complex IIc or necrosome. RIPK3 mediates the phosphorylation of MLKL, resulting in its conformational change and oligomerization. This allows MLKL to bind to plasma membrane lipids and form a pore that leads to cell lysis. Release of cellular components (cytokines, chemokines and DAMPs) perpetuates liver inflammation and fibrogenesis in a pro-tumoral microenvironment, thus

promoting HCC development. Abbreviations: Bcl2, B-cell lymphoma 2; Casp-3, caspase-3; c-FLIP, cellular FLICE-like inhibitory protein; DAMPs, damage-associated molecular patterns; FAS, FS-7-associated surface antigen; FADD, FAS-associated death domain protein; FASR, FAS receptor; FASL, FAS ligand; MLKL, mixed lineage kinase domain-like protein; NF-ƙB, nuclear factor kappa-light-chain-enhancer of activated B cells; RIPK1, serine/threonine-protein kinase 1; TNF, tumor necrosis factor; TRADD, TNFRSF1A-associated via death domain; TRAF2, receptor-associated factor; TRAIL, Targeting TNF-related apoptosis-inducing ligand. Created with BioRender.com.

Mechanistically, diverse molecules arise as key determinants in apoptotic cell death such as caspase-8 and receptor interacting protein kinase 1 (RIPK1). Recent studies have demonstrated the dual role of caspase-8 in HCC development, triggering apoptosis and promoting cell proliferation by sensing DNA damage in a nonapoptotic function. HCC patients with lower levels of caspase-8 exhibited better overall survival compared with those with high caspase-8 expression. In addition, a significant correlation between caspase-8 and high levels of Ki67 expression was also observed in HCC patients [21]. Importantly, caspase-8 can serve two distinct roles in response to TRAIL receptor engagement. As well as promoting apoptosis through its protease activity, caspase-8 acts as a scaffold for the assembly of a caspase-8-FADD-RIPK1 “FADDosome” complex, leading to NF-ƙB-dependent inflammation, which can be uncoupled from apoptosis and does not require caspase proteolytic activity [22]. This TRAIL-dependent cytokine and chemokine production was observed in different carcinogenic cell types, and an accumulation of tumor-supportive immune cells in the cancer microenvironment has been described in an endogenous TRAIL/TRAIL-R-mediated chemokine (C-C motif) ligand 2 (CCL2) secretion [23].

Regarding the role of RIPK1 in liver cancer, several studies have recently emerged with contradictory results that need to be clarified. On one hand, specific RIPK1 gene deletion in hepatocytes induced the TNF-dependent proteasomal degradation of TNF receptor-associated factor 2 (TRAF2). This mechanism resulted not only in a caspase-8 hyperactivation, but also in an impaired NF-kB activation, promoting the spontaneous development of HCC [24]. On the other hand, another study demonstrated that NEMO, an important signaling adaptor protein in the NF-kB activation pathway, was shown to prevent hepatocarcinogenesis and steatohepatitis by inhibiting RIPK1 kinase activity-dependent apoptosis of hepatocytes [25]. Interestingly, new roles of RIPK1, promoting inflammation, liver fibrosis, and HCC, as well as cell death, have recently been described. In particular, RIPK1 activation is able to promote the transcription of key proinflammatory chemokines such as CCL2, favoring the C-C chemokine receptor type 2 (CCR2)+ macrophage infiltration as well as regulating HSC activation [26]. Similarly, the pharmacological inhibition of RIPK1 reduces necroinflammatory and fibrotic NASH features in HFD-fed mice [27].

Further studies are needed to elucidate the RIPK1 molecular mechanism regulating liver injury. The use of promising RIPK inhibitors, such as necrostatin [28][29], is discussed in the therapeutic approach section.

4. Necrosis

Traditionally, necrosis has been considered as a dysregulated, accidental, and inflammatory cell death type that culminates in ATP depletion due to mitochondrial dysfunction, loss of membrane integrity, and cell swelling [12][30].

Mitochondrial dysfunction during necrosis occurs due to a prolonged opening of the mitochondrial permeability transition (MPT) pore, which leads to a loss of oxidative phosphorylation and ATP depletion. Necrotic cell death is a significant feature that has been modulated by using JNK and MPT inhibitors in diverse liver injury scenarios [31]. In a mouse model of acetaminophen-induced liver injury, the JNK inhibitor SP600125 completely prevented necrosis when administered after acetaminophen dosing [32]. Similarly, the MPT inhibitor cyclosporine A demonstrated a protective effect in preventing the permeabilization of the mitochondrial membrane and, consequently, reduced hepatic necrotic areas [33].

In the necrosis setting, the release of cellular constituents and DAMPs, such as interleukin (IL)-33 or high-mobility group protein B1 (HMGB1), into the extracellular environment, a process called “oncosis”, elicits a significant proinflammatory response that can also trigger damage to neighboring cells [30]. The non-histone chromosomal protein HMGB1 is considered the prototypical DAMP, released passively by injured or necrotic cells. HMGB1 acts as a proinflammatory cytokine by stimulating necrosis-induced inflammation via toll-like receptor (TLR) 4 activation and the release of cytokines such as IL-8 [34]. Previous studies associated HMGB1 with the prognosis of HCC, suggesting that an overexpression of DAMPs could be a novel, effective, and supplementary biomarker for HCC [35]. Recent data from mouse models have shown that HMGB1 activates ductular reactions via cell-extrinsic mechanisms and RAGE receptor, and promotes hepatocyte transformation [36]. In addition, HMGB1 signaling induces HCC development indirectly through IL-6/Stat3-miR-21-mediated metalloproteinases (MMP) activity [37]. Nevertheless, exactly how DAMPs induce hepatocarcinogenesis remains uncertain, and more studies are needed to unravel this question.

5. Necroptosis

Necroptosis is the best-studied form of regulated or programmed necrosis, sharing some features with necrosis and apoptosis. Unlike apoptosis, cells undergoing necroptosis show disruptions to their cell membrane, which is a key characteristic of necrosis [38]. Similar to apoptosis, necroptosis uses the same upstream TNF molecular mechanism, resulting in a backup pathway to ensure cell death in situations of apoptosis inhibition [39]. It is well known that activated caspase-8 acts as a crucial executioner of apoptosis, but its suppression might shift the balance to necroptosis [40]. In this scenario, RIPK1, RIPK3, and the mixed lineage kinase domain-like protein MLKL make up the complex IIc, or necrosome, which perforates membrane structures and induces subsequent cell lysis. As a result, necroptosis triggers organelle and cellular swelling and the release of a variety of DAMPs into the extracellular space, eliciting a robust immune response by recruiting different immune cells to heal the damaged tissue [41][42] (**Figure 1**C).

Several studies have demonstrated the role of RIPK3, activating innate immunity via pattern recognition receptors (PRR) such as NLRPL3 inflammasome activation [43][44]. Apart from the contribution of necroptosis to the inflammatory pro-carcinogenic environment, a direct role of this cell death pathway in HCC has also been studied. Remarkably, the repression of necroptosis was observed in all common human hepatoma cell lines, such as Huh-7, HepG2, and Hep3B, due to a methylation-dependent loss of RIPK3 expression, suggesting that evading this cell death mechanism could be important for the malignant transformation of tumor cells. Therefore, the restoration of

RIPK3 expression sensitized cells to chemotherapy, indicating that epigenetic modifications might be an interesting approach to increase chemosensitivity in certain types of HCC [45].

6. Pyroptosis and the Inflammasome

6.1. Pyroptosis

Pyroptosis is a highly proinflammatory cell death mode, predominantly dependent on caspase-1 and caspase 4/5/11 [15][46]. Distinct microbes and host factors activate the pyroptotic cascade, inducing the release of intracellular substances comprising IL-1β, IL-18, IL-33, HMGB-1, and heat shock protein (HSP) through caspase-dependent pore formation, swelling, and rupture of the cell.

Pyroptosis might be triggered by canonical and noncanonical signaling pathways depending on the activated caspase involved. In the canonical pathway, DAMPs and pathogen-associated molecular patterns (PAMPs) are detected by intracellular sensors known as inflammasomes. Inflammasomes are danger-sensing, multimeric protein complexes composed by three elements: (i) A sensor molecule belonging to the NOD-like receptor family such as NLRP3, NLRP1, NLRP4, NLRP6, and NLRP9; (ii) The PYHIN family member AIM2, an adaptor protein with CARD or PYD domains with oligomerization function; (iii) The effector molecule pro-caspase-1 [47]. The most extensively studied and well-characterized inflammasome in liver disease is the NLRP3 complex, which, in response to low-threshold signals, is able to fine-tune the inflammatory response. As shown in **Figure 2**, the activation of NLRP3 has recently been described as a two-step process. A priming signal (signal 1), resulting from the binding of PAMPs to its cognate TLR, is required to upregulate the transcription of NLRP3, caspase-1, pro-IL-1β and pro-IL-18 genes. Subsequently, a second activation signal (signal 2), provided by DAMPs and/or PAMPs, triggers inflammasome assembly and oligomerization in the cytosol. There, these complexes activate the serine protease caspase-1, which, in turn, induces the maturation of IL-1β and IL-18 and the cleavage of gasdermin D (GSDMD), a pore forming protein that has been recently identified as the ultimate executor of pyroptosis by creating pores in the cell membrane [47] (**Figure 2**A).

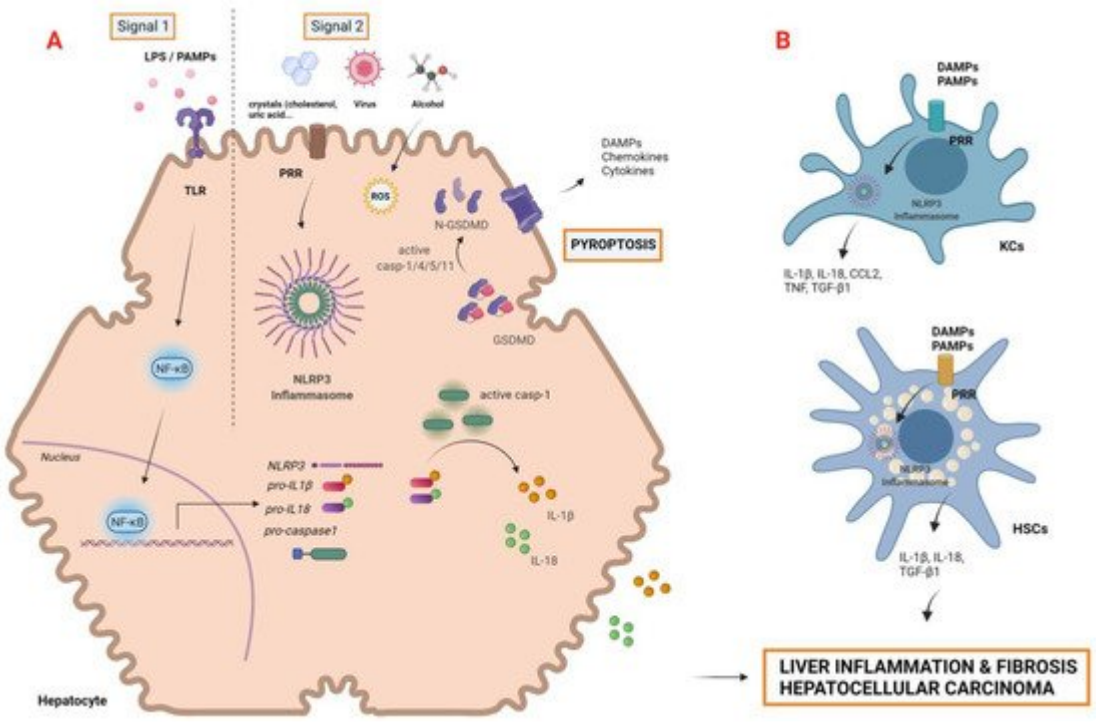

Figure 2. Activation of the NLRP3 inflammasome and canonical pyroptosis in liver cells. Various stimuli of liver damage might cause hepatocyte cell death and gut dysbiosis, leading to high exposure to DAMPs and PAMPs that activate liver inflammasomes: (**A**) In hepatocytes, NLRP3 inflammasome activation requires two steps: the priming signal (SIGNAL 1) is initiated by PAMPs such as LPS, which bind to their corresponding TLR and upregulate the expression of pro-IL-1β, pro-IL-18, pro-caspase-1, and NLRP3 genes via NF-ƙB signaling activation. The second signal (SIGNAL 2) is triggered by PAMPs and/or DAMPs and activates NLRP3, which, in turn, activates pro-caspase-1. Activated casp-1 cleaves IL-1β and IL-18 precursors into mature and proinflammatory forms that are secreted to extracellular space. Casp-1 and, alternatively, caspase-4/5/11 might also cleave protein GSDMD. The N-terminal fragment of GSDMD (N-GSDMD) forms pores in the plasma membrane, inducing pyroptosis by cell swelling and osmotic lysis. (**B**) Inflammasome activation and pyroptosis also occurs in nonparenchymal cells. DAMPSs and gut-derived PAMPs activate Kupffer cells via PRRs, triggering the production of IL-1β and, subsequently, CCL2 and TNF. NLRP3 activation in HSC also induces the expression of the profibrogenic molecule TGF-β. Together, these events result in liver inflammation and fibrosis, perpetuating liver damage and hepatocellular carcinoma. Abbreviations: CCL2, C-C motif chemokine ligand 2; DAMPS, damage-associated molecular patterns; GSDMD, gasdermin D; HSCs, hepatic stellate cells; KCs, Kupffer cells; LPS, liposaccharide; NF-ƙc, nuclear factor kappa-light-chain-enhancer of activated B cells NLRP3, NLR family pyrin domain containing 3; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; TLR, toll-like receptor; TNF, tumor-nuclear factor; TGF-β, transforming growth factor beta. Created with BioRender.com.

In noncanonical pyroptosis, lipopolysaccharide (LPS), derived from gram-negative bacteria, binds the caspase recruitment domain (CARD) of pro-caspases-4, 5, and 11, promoting the oligomerization and subsequent cleavage of GSDMD. Importantly, in that pathway, the pore in the plasma membrane is formed, but not the cleavage and maturation of proinflammatory cytokines IL-1β and IL-18. However, recent studies have shown that caspase-11

may activate the NLPR3-dependent caspase-1 inflammasome as well, and hence indirectly stimulate the release of intracellular cytokines [48][49].

6.2. Inflammasomes in Liver Diseases and HCC

The importance of inflammasomes in liver disease has increased in the last decade, due to its contribution to hepatocyte damage, immune cell activation, and the amplification of liver inflammation [17][50]. Furthermore, several inflammasome receptors (NLRP1, NLRP3, AIM2) are expressed in KCs, LSECs, periportal myofibroblasts and HSCs, participating directly or indirectly in the promotion of liver fibrosis (**Figure 2**B). One study reported an upregulation of the profibrogenic cytokine transforming growth factor (TGF-β1) in HSCs through the stimulation of inflammasome by uric acid crystals [51]. Similarly, KCs' inflammasomes can be activated in response to gut-derived PAMPs and hepatocyte-derived DAMPs, producing IL-1β and IL-18, which, in turn, contribute to HSC activation [52].

Increased hepatic levels of caspase-1 and NLRP3 were found in alcoholic liver disease (ALD) patients, and high levels of IL-1β were detected in the serum of severe forms of alcoholic hepatitis [53][54]. Additionally, several studies have shown NLRP3 inflammasome activation in hepatocytes and KCs in the progression of NAFLD [55][56]. Accordingly, the gene expression of pro-IL-1β correlated with the profibrogenic collagen-1 gene in patients with liver steatosis, suggesting an association between NLRP3 inflammasome and the progression from NAFLD to NASH [57].

Although several studies have reported the importance of the NLRP3 inflammasome as a regulator for tumor control, its role in human cancers remains controversial. On one hand, NLRP3 inflammasome activation operates as a pro-tumorigenic factor, promoting proliferation, survival, metastasis, angiogenesis, and immunosuppression [50][58]. For instance, in breast cancer, IL-1β production promotes the infiltration of myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), providing an inflammatory microenvironment and favoring tumor progression [59]. Although pro-carcinogenic effects have also been described in gastric and prostate cancers, the direct role of NLRP3 inflammasome in liver cancer remains elusive and poorly described. On the other hand, several studies have reported evidence of the protective anti-tumorigenic effects of NLRP3 inflammasome by directly activating pyroptotic cell death or secreting death-inducing cytokines [60]. In colorectal liver metastases, the activation of NLRP3 inflammasome in KCs triggers IL-18 production, stimulating the maturation of NK cells and priming the FasL-mediated apoptosis of tumor cells [61][62]. Concordantly, the expression of all NLRP3 inflammasome components was either completely lost or significantly downregulated in human HCC, showing a significant correlation with advanced stages and poor pathological differentiation [63]. In addition, the reconstitution of NLRP3 inflammasome through the E2/ERβ/MAPK estrogen pathway seems to reverse the malignant phenotype of HCC, reinforcing its tumor-suppressive effect [64].

Given the complex nature of the NLRP3 inflammasome and its seemingly contradictory functions in carcinogenesis, future research needs to address important aspects, such as the driving factors in tumor activation and cross-talk pathways. Targeting the NLRP3 inflammasome, alone or in combination with chemotherapy, may be a promising potential therapeutic approach in cancers.

7. Autophagy

Autophagy is an intracellular lysosomal pathway that plays a pivotal role in homeostasis maintenance in diverse physiological processes. Considered as a “self-eating” process, capable of resisting metabolic stress by recycling cellular components, autophagy has a controversial dual function in the pathophysiology of liver cancer [65]. The up- and downregulation of this catabolic mechanism was described in HCC, suggesting that autophagy may act as both a tumor promotor and suppressor during malignancy [66]. Furthermore, studies based on the inhibition of autophagy demonstrated that basal autophagy plays a suppressive role in the initial dysplastic stage by maintaining genomic stability, removing damaged mitochondria (mitophagy) and preventing malignant transformation with accumulated mutations [67]. However, once the tumor is established, in a proliferative stage, autophagy would promote tumorigenesis, supporting cell growth [68]. p62, a ubiquitin-binding autophagy receptor, is accumulated in premalignant liver diseases and most HCCs. Its expression is needed and sufficient for activation of the transcription factor NRF2 and mTORC1, the induction of c-Myc, and the protection of HCC-initiating cells from oxidative-stress-induced death [69]. Importantly, autophagy sustains an oxidative metabolism, which is required for tumor development. In a liver damage scenario, active autophagy promotes reactive oxygen species (ROS) generation, resulting in an upregulated oxidative stress that may induce cell death, followed by compensatory cell proliferation [70]. Interestingly, the impact of autophagy-mediated metabolic reprogramming on therapeutic resistance is largely unclear, especially in liver cancer [71][72][73][74]. Sorafenib, an oral multi-kinase inhibitor that is widely used in advanced-stage HCC, results in a significant prolonged survival rate in liver cancer patients [71]. Nevertheless, a considerable number of HCC patients are refractory to sorafenib treatment due to, at least, the potential induction of autophagy through different mechanisms, such as the activation of the AMPK/mTOR signaling pathway or FOXO-3 mRNA methylation and stabilization [72][73][74]. In that sense, strategies focused on autophagy modulation, combined with sorafenib, have been recently described as new therapeutic options with which to overcome drug resistance in HCC [75][76][77].

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