

Necroptotic Cell Death in Liver Transplantation and Underlying Diseases: Mechanisms and Clinical Perspective

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Cell death is a natural process for the turnover of aged cells, but it can also arise as a result of pathological conditions. Cell death is recognized as a key feature in both acute and chronic hepatobiliary diseases caused by drug, alcohol, and fat uptake; by viral infection; or after surgical intervention. In the case of chronic disease, cell death can lead to (chronic) secondary inflammation, cirrhosis, and the progression to liver cancer. In liver transplantation, graft preservation and ischemia/reperfusion injury are associated with acute cell death. In both cases, so-called programmed cell death modalities are involved. Several distinct types of programmed cell death have been described of which apoptosis and necroptosis are the most well known. Parenchymal liver cells, including hepatocytes and cholangiocytes, are susceptible to both apoptosis and necroptosis, which are triggered by distinct signal transduction pathways. Apoptosis is dependent on a proteolytic cascade of caspase enzymes, whereas necroptosis induction is caspase-independent. Moreover, different from the “silent” apoptotic cell death, necroptosis can cause a secondary inflammatory cascade, so-called necroinflammation, triggered by the release of various damage-associated molecular patterns (DAMPs). These DAMPs activate the innate immune system, leading to both local and systemic inflammatory responses, which can even cause remote organ failure. Therapeutic targeting of necroptosis by pharmacological inhibitors, such as necrostatin-1, shows variable effects in different disease models.

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In this review, we will discuss the mechanisms of necroptosis, and we will focus on liver transplantation and liver diseases, such as acute liver failure, fatty liver diseases, cholestatic liver diseases, chronic viral hepatitis, and primary liver cancer. Furthermore, we will review the clinical relevance of necroptotic cell death and its therapeutic potential by targeting cell death in liver diseases.

Abbreviations: AIH, autoimmune hepatitis; APAP, acetaminophen; ATP, adenosine triphosphate; CCA, cholangiocarcinoma; cIAP, cellular inhibitor of apoptosis protein; ConA, concanavalin A; CYLD, cylindromatosis; CYP2E1, cytochrome P450 2E1; DAMP, damage-associated molecular pattern; DC, dendritic cell; Drp1, dynamin-related protein 1; FADD, Fas-associated protein with death domain; FLIP, FLICE-inhibitory protein; GalN, D-galactosamine; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HFD, high-fat diet; HMGB1, high-mobility group box 1; IDO, indoleamine 2,3-dioxygenase; IFN γ , interferon γ ; IKK- α , inhibitor of nuclear factor kappa B kinase subunit beta; IKK- β , inhibitor of nuclear factor kappa B kinase subunit beta; IL, interleukin; IP, intraperitoneally; IRI, ischemia/reperfusion injury;

Cell death is a fundamental process that is essential in embryonic and (neo)natal development and homeostasis in all organs, including the liver. Cell death is a means of removing aged and damaged cells that otherwise might play a role in organ dysfunction and cancer development. For instance, if transformed hepatocytes with genetic aberrations become resistant to cell death, this may lead to cancer initiation and tumorigenesis.⁽¹⁾ In response to the overwhelming cellular stress, hepatocytes can die through active suicide, termed “apoptosis.” Another type of cell death, termed “necrosis,” is a more passive killing of cells. Apoptosis is characterized by a cascade of specific intracellular events leading to so-called programmed cell death, whereas necrosis occurs as a consequence of extracellular events leading to physical damage and nonregulated (nonprogrammed) cell death.⁽²⁾ In addition to apoptosis and necrosis, a new form of cell death that shared both properties of apoptosis and necrosis was identified approximately a decade ago. This form of programmed necrosis has been termed “necroptosis.” The molecular

events involved in necrosis, programmed apoptotic, and necroptotic cell death are summarized in Fig. 1.

Necroptosis is characterized as an active and well-regulated form of necrosis that is morphologically and biochemically distinct from apoptosis. A special feature of necroptosis is the loss of integrity of the plasma membrane and subsequent release of damage-associated molecular patterns (DAMPs), triggering inflammation and exacerbating tissue damage, ie, so-called necroinflammation.⁽³⁾ Necroptosis is one of the main contributors to necroinflammation. Inflammation and the subsequent immunological response play an important role in many liver diseases as well as in ischemia/reperfusion injury (IRI) or rejection after liver transplantation. Necroptosis is increasingly considered to play a role in the pathophysiology

of these processes, and insight into necroptosis offers promising therapeutic intervention methods to treat liver diseases by targeting necroptosis. For instance, in hepatocellular carcinoma (HCC) or biliary cancer, resistance to apoptosis severely hampers the efficacy of chemotherapy (due to anticancer drug resistance). In this field, the pharmacological switch of cell death toward necroptosis may lead to therapeutic applications in drug-resistant cancers. This review focuses on necroptosis and summarizes not only related regulatory mechanisms but also the clinical relevance and bench-to-bedside translational potential in liver diseases and liver transplantation.

Mechanisms of Necroptosis

Necroptosis can be initiated by a range of factors, such as IRI, release of reactive oxygen species (ROS), antineoplastic events, and calcium overload.⁽⁴⁾ Furthermore, intracellular factors, such as tumor necrosis factor α (TNF- α), Fas ligand, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), interferon γ (IFN γ), double-stranded RNA, and adenosine triphosphate (ATP) depletion, are also known to be involved in inducing necroptosis.⁽⁵⁾ The binding of TNF- α to tumor necrosis factor receptor 1 (TNFR1) is one of the most extensively studied signaling pathways promoting not only necroptosis but also apoptosis and activation of the nuclear factor kappa B (NF- κ B) pathway (Fig. 1A, left side).⁽⁶⁾ Generally, TNF- α can trigger the formation of prosurvival- and proinflammatory-related complexes and caspase-dependent apoptosis.⁽⁷⁾ Specifically, TNFR1 recruits TNFR1-associated death domain (TRADD) protein, tumor necrosis factor receptor-associated factor 2 (TRAF2), receptor-interacting serine/threonine-protein kinase (RIPK) 1, cellular inhibitor of apoptosis protein (cIAP) 1/2, and the linear ubiquitin chain assembly complex (LUBAC) to form the complex I, which contributes to the activation of the NF- κ B signaling pathway.⁽⁸⁾ After dissociation from TNFR1, complex I will be transformed into complex IIa (comprising TRADD, Fas-associated protein with death domain [FADD], FLICE-inhibitory protein [FLIP], and procaspase 8), leading to activation of caspase 8 and rendering RIPK1-independent apoptosis.⁽⁹⁾ Conversely, the formation of complex IIb, consisting of RIPK1, RIPK3, FADD, FLIPs, and caspase 8, can be promoted by knockdown of the nuclear factor kappa B

IV, intravenously; JNK, c-Jun N-terminal kinase; KC, Kupffer cell; LO2, human fetal hepatocyte cell line; LPS, lipopolysaccharide; LUBAC, linear ubiquitin chain assembly complex; MCD, methionine-choline-deficient; miRNA, microRNA; MLKL, mixed-lineage kinase domain-like; mRNA, messenger RNA; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Nec, necrostatin; NEMO, nuclear factor kappa B essential modulator; NET, neutrophil; NF- κ B, nuclear factor kappa B; NKT, natural killer T cell; NLRP3, nucleotide-binding oligomerization domain-like receptor protein 3; NPC, nonparenchymal cell; PARP-1, poly(adenosine diphosphate ribose) polymerase; PGAM5, phosphoglycerate mutase 5; PLC, parenchymal liver cell; PMH, primary mouse hepatocyte; pMLKL, pseudokinase mixed-lineage kinase domain-like; po, per os; RIPK, receptor-interacting serine/threonine-protein kinase; ROA, retro-orbital administration; ROS, reactive oxygen species; TAB1, transforming growth factor β -activated kinase 1 binding protein-1; TAK1, transforming growth factor β -activated kinase 1; TLR, toll-like receptor; TNF- α , tumor necrosis factor α ; TNFR1, tumor necrosis factor receptor 1; TRADD, tumor necrosis factor 1-associated death domain; TRAF2, tumor necrosis factor receptor-associated factor 2; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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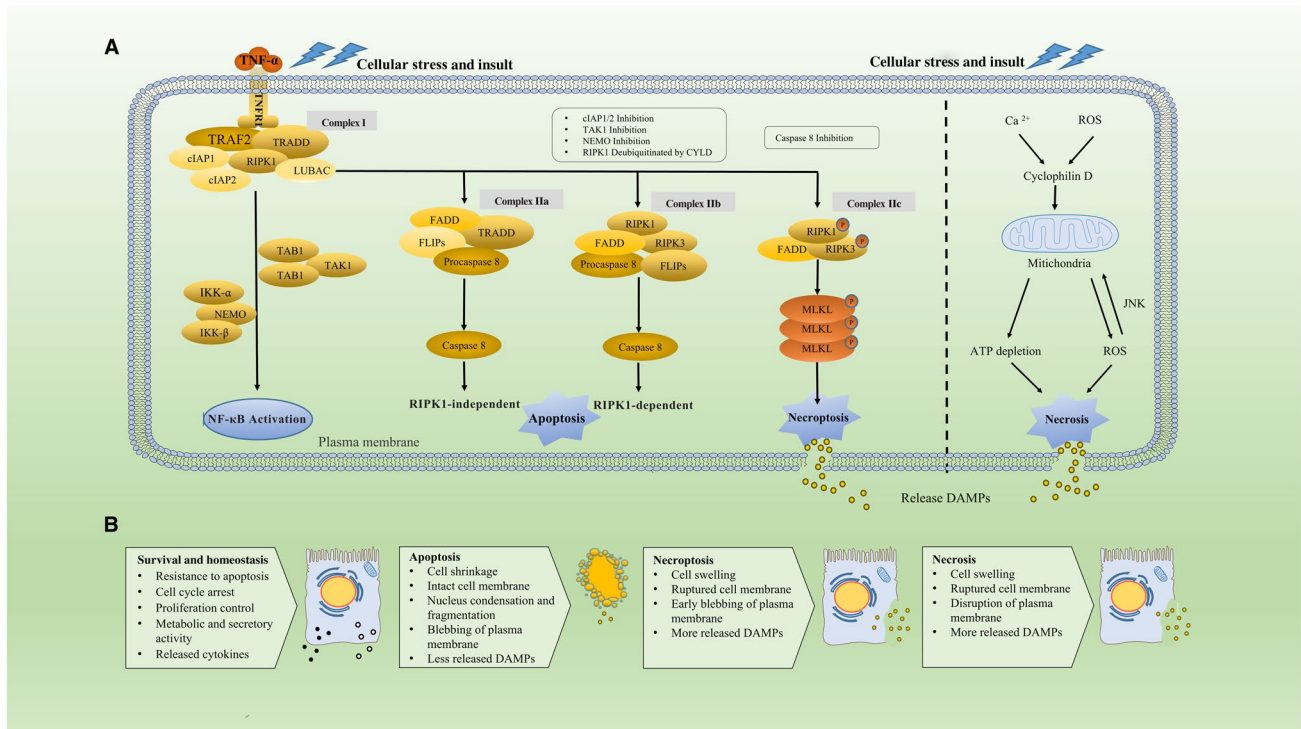


FIG. 1. Distinct molecular and morphologic features of apoptotic, necroptotic, and necrotic cell death. (A) Molecular pathways of cell death in PLCs. The binding of TNF- α and TNFR1 recruits TRADD, TRAF2, RIPK1, cIAP1/2, and LUBAC and forms the complex I leading to the activation of the NF- κ B signaling and a prosurvival pathway. Following the dissociation from TNFR1, complex I is transformed into complex IIa, which includes TRADD, FADD, FLIPs, and procaspase 8, and contributes to the activation of caspase 8 and subsequent RIPK1-independent apoptosis. Hyperactivation of cylindromatosis (CYLD) deubiquitinates RIPK1 and thus destabilizes complex I and promotes the formation of complex IIb, which is involved in RIPK1-dependent apoptosis. Complex IIb consists of RIPK1, RIPK3, FADD, FLIPs, and caspase 8, and it can be promoted by inhibition of NEMO, cIAPs, or TAK1. Nevertheless, once caspase 8 is inhibited, RIPK3 is activated to interact with RIPK1 and binds to MLKL, forming the complex IIc (necrosome) by which necroptosis is promoted. RIPK3 phosphorylates MLKL in the complex IIc and thereby triggers oligomerization of MLKL, driving the permeabilization step. Nonprogrammed cell death by necrosis is characterized by mitochondrial impairment with resulting ATP depletion and triggering of the ROS-JNK loop. After the cell membrane ruptures in necrotic or necroptotic cells, intracellular DAMPs are released and act as activators and amplifiers of necroinflammation. Conversely, release of a lower amount of DAMPs from apoptotic cells leads to much milder necroinflammation. (B) Summary of hallmark events and characteristics of cell survival and cell death by apoptosis, necroptosis, or necrosis.

essential modulator (NEMO), blockage of cIAPs, or transforming growth factor β -activated kinase 1 (TAK1).⁽¹⁰⁾ Hyperactivation of cylindromatosis deubiquitinates RIPK1 and, thus, destabilizes complex I, promoting the formation of complex IIb, which is involved in RIPK1-dependent apoptosis.⁽¹¹⁾ However, when caspase 8 is down-regulated or inhibited, RIPK3 is activated to interact with RIPK1, which binds to mixed lineage kinase domain-like (MLKL). Together, this forms the cytosolic complex IIc (necrosome) by which necroptosis is initiated (Fig. 1A, right side).⁽¹²⁾ Necrostatin (Nec)-1 is a well-investigated inhibitor of necroptosis by targeting the catalytic and allosteric functions of

RIPK1,⁽¹³⁾ and with that, preventing the formation of the necrosome.

From the above, it is clear that RIPK1 and RIPK3 do not play an exclusive role in the modulation of cell death. Moreover, RIPK1 is the switch node between the prosurvival pathway and apoptosis and/or necroptosis.⁽¹⁴⁾ As the ultimate execution step, RIPK3 phosphorylates MLKL in the complex IIc and thereby triggers oligomerization of MLKL, which is indispensable for its translocation to the plasma membrane.⁽¹⁵⁾ These oligomers can destabilize the plasma membrane through a pore-forming complex or by incapacitating Ca²⁺ or Na⁺ channels indirectly.⁽¹⁶⁾ The late permeabilization step, characterized by an increase

of intracellular osmotic pressure and the opening of membrane pores, represents one of the hallmarks of necroptotic cell death.⁽¹⁷⁾

Necroinflammation is a form of sterile inflammation triggered by DAMPs that are released from necrotic cells through the ruptured membrane. Necroptosis is one of the most important initiators. DAMPs act as activators and amplifiers of the inflammation response⁽¹⁸⁾ and can be categorized into 2 groups:

1. Molecules with no inflammatory activity in normal cells but which, upon release, exhibit immune activity (ie, heat shock proteins and extracellular ATP).
2. Alarmins that exhibit specific cytokines initiating an inflammatory response once released (ie, interleukin [IL] 1 α and IL33).

DAMPs are recognized by a series of receptors called “pattern recognition receptors,” such as toll-like receptors (TLRs) and nucleotide oligomerization domain-like receptors, which activate the innate immunity and thereby evoke the release of cytokines that, in turn, induce more necrosis and trigger an inflammatory cascade reaction.⁽¹⁹⁾ This vicious inflammatory circle is strongly associated with the development of chronic liver disease and liver fibrosis⁽³⁾ and is involved in acute liver injury, graft injury, and rejection after liver transplantation.⁽²⁰⁾

Necroptosis in Liver Diseases

DRUG-INDUCED LIVER INJURY

Drug-induced liver injury is mostly caused by acetaminophen (APAP) toxicity.⁽²¹⁾ It has been reported that necrosis, independent of caspase and the TNF receptor, is mostly involved in APAP-induced liver injury⁽²²⁾ and that the role of apoptosis is limited as was demonstrated by the insensitivity to caspase inhibitors (Table 1).⁽³²⁾ In a murine model, both genetic blockage and chemical inhibition of RIPK1 could ameliorate APAP-induced liver injury.⁽²⁷⁾ In contrast, Li et al.⁽³¹⁾ reported that neither Nec-1 nor RIPK1 silencing was able to protect human hepatocytes from cell death, suggesting a differential role of RIPK1 in mice or humans undergoing APAP-induced liver injury. Likewise, Ramachandran et al.⁽²⁵⁾

demonstrated that RIPK3 is an early mediator of drug-induced liver injury. Knockout of RIPK3 protected mice from APAP toxicity but only during a very short time frame (no more than 24 hours *in vivo* and 48 hours *in vitro*). One potential explanation for this is that the abrogation of RIPK3 cannot alleviate the secondary injury that occurs after APAP overdose.⁽³³⁾ Deutsch et al.⁽²⁸⁾ also reported increased expression of RIPK3 in liver tissue of patients with hepatic failure caused by APAP overdose. Silencing or chemical inhibition of RIPK3 protects human hepatocytes from drug-induced liver injury.⁽³¹⁾ However, a study by Dara et al.⁽²³⁾ shows that knockout of RIPK3 did not alleviate liver injury and the activation of RIPK3 is not observed in the murine APAP-induced liver injury model. Furthermore, they also reported that the MLKL messenger RNA (mRNA) level is elevated quickly after APAP treatment and knockout of MLKL cannot rescue mice from APAP-induced liver injury,⁽²³⁾ demonstrating that the up-regulation of MLKL is not the mediator but the consequence of APAP toxicity. Therefore, the involvement of necroptosis in APAP-induced liver injury is still questionable. The role of RIPK1 independent of RIPK3 and MLKL in such injury needs to be clarified. Moreover, further investigation should focus not only on animal models but should also include clinical human samples because there might be species-specific differences within these processes.

IMMUNE-MEDIATED LIVER INJURY

Immune-mediated liver injury plays a critical role in liver diseases through innate and adaptive immune responses. Specifically, autoimmune hepatitis (AIH) is a severe necroinflammatory liver disease that progressively contributes to liver failure and mortality.⁽³⁴⁾ Administration of the lectin concanavalin A (ConA), a T cell mitogen, to mice is the most used model to study immune-mediated liver injury. Typically, in this model, early stage apoptosis is followed by massive necrosis at later stages. The kinase activity of RIPK1 is elevated when stimulated with ConA and cell death is caspase-independent.⁽³⁵⁾ However, knockout of RIPK1 in parenchymal liver cells (PLCs) could not rescue liver damage because of exacerbated TNF- α -mediated and caspase-dependent apoptosis (Table 2).⁽²⁸⁾ Actually, under steady-state conditions, RIPK1 also functions

TABLE 1. Necroptosis in APAP-Induced Liver Injury

Researchers	Subject (Mice)	APAP Treatment	Findings
Dara et al. ⁽²³⁾	Male C57BL/6n	300 mg/kg IP	<ul style="list-style-type: none"> RIPK1 knockout protects mice from APAP toxicity, but no protection is found in knockout of RIPK3 or MLKL mice. RIPK1, but not RIPK3, level in cytoplasm increases after APAP treatment in PMH. High expression of RIPK3 in NPC but low expression in PMH. Protection of Nec-1 in vitro and in vivo. JNK acts downstream of RIPK-dependent necrotic signaling.
An et al. ⁽²⁴⁾	Male C57BL/6	300 mg/kg IP	<ul style="list-style-type: none"> APAP triggers hepatic caspase-independent and RIPK-dependent necrosis. RIPK1 and RIPK3 increase after APAP treatment, but RIPK3 increases earlier than RIPK1. JNK acts downstream of RIPK-dependent necrotic signaling. Both Nec-1 and JNK inhibitor protect mice from lethal APAP intoxication. Nec-1 can decrease RIPK1 and RIPK3 expression after APAP treatment, but JNK inhibitor cannot. RIPK3 is absent in liver lysates from untreated mice.
Ramachandran et al. ⁽²⁵⁾	Male C57Bl/6J	200 mg/kg IP	<ul style="list-style-type: none"> RIPK3 increases early after APAP treatment. RIPK3 inhibition reduces cellular necrosis, accompanied with reduced mitochondrial oxidant stress, JNK activation, and Drp1 translocation. Protective effect of RIPK3 knockout is lost at 24 hours in vivo and 48 hours in vitro. Protective effect of Nec-1 is lost at 48 hours in vitro. Protection of RIPK3 knockout is not caused by inhibition of protein adduct formation.
Takemoto et al. ⁽²⁶⁾	Male C57BL/6	800 mg/kg IP	<ul style="list-style-type: none"> RIPK1 and RIPK3 increase after APAP treatment and are colocalized with CYP2E1. Nec-1 protects against APAP-induced hepatic injury in vivo and in vitro by inhibiting ROS production and suppresses mitochondrial dysfunction.
Zhang et al. ⁽²⁷⁾	Male C57Bl/6J	300 mg/kg IP	<ul style="list-style-type: none"> Dabrafenib protects mice and human hepatocytes from APAP hepatotoxicity by inhibiting RIPK3. RIPK3 silencing partially reversed the APAP-induced loss of the cell viability of QSG-7701 cells and HL-7702, 2 kinds of human hepatocyte cell lines. Nec-1 inhibition or RIPK1 silencing did not reduce APAP-induced cell death in human hepatocyte cells.
Deutsch et al. ⁽²⁸⁾	Male C57BL/6	500 mg/kg IP	<ul style="list-style-type: none"> Blockade of RIPK1 or RIPK3 ameliorates APAP toxicity. RIPK1 and RIPK3 are absent in normal hepatocytes but extensively expressed in the liver from APAP-treated mice. Elevated expression of RIP3 occurs in the liver of patients with hepatic failure from severe APAP toxicity, but expression was absent in the normal human liver. Nec-1s was similarly protective against APAP injury. NLRP3^{-/-} mice are protected from APAP injury. Blockade of RIPK1 and RIPK3 diminishes inflammasome activation, immune cell infiltration, and sterile inflammation after APAP administration.
Yan et al. ⁽²⁹⁾	Male C57BL/6	300 mg/kg IP	<ul style="list-style-type: none"> RIPK3 and MLKL mRNA increase at 2 hours after APAP treatment. Knockout of RIPK3 cannot alleviate APAP toxicity. A pan caspase inhibitor (Z-VAD-FMK), but not Nec-1, inhibits TNFα/APAP-induced cytotoxicity on human fetal hepatocyte line (LO2) cells a kind of normal hepatic cell line.
Lee et al. ⁽³⁰⁾	C3H/He	400 mg/kg po	<ul style="list-style-type: none"> No change of RIPK1 level is found after APAP treatment compared with control mice. RIPK3 is not expressed in the livers of normal control mice but increases after APAP treatment.
Li et al. ⁽³¹⁾	Male C57Bl/6J	300 mg/kg IP	<ul style="list-style-type: none"> Dabrafenib targets RIPK3 and disrupts the interaction between RIPK3 and MLKL and exhibits an inhibitor of necroptosis. Dabrafenib prevents APAP-induced necrosis in normal human hepatocytes.

as a scaffold protecting hepatocytes from apoptosis through NF- κ B activation or NF- κ B-independent pathways.⁽⁴⁰⁾ This means that RIPK1 plays a dual role in the ConA-induced liver injury. Deutsch et al.⁽²⁸⁾ reported the elevated expression of RIPK3 in liver tissue of AIH patients, but genetic silence of RIPK3 in a murine model could not relieve ConA hepatitis.

Conversely, Kang et al.⁽³⁸⁾ demonstrated that genetic silencing of RIPK3 reduced the ConA-induced elevation of serum aminotransferase concentrations as well as inflammatory markers such as IFN γ and TNF protein. This discrepancy may arise from using different gene modification strategies in these studies. Actually, as reported by Kang et al.,⁽³⁸⁾ the expression of RIPK3

TABLE 2. Necroptosis in ConA-Induced Liver Injury

Researchers	Subject (Mice)	Con A Treatment	Findings
Liedtke et al. ⁽³⁶⁾	Unclear	25 mg/kg IV	<ul style="list-style-type: none"> Caspase 8 deletion protects against Fas- and LPS-mediated liver injury but enhances nonapoptotic liver injury. High RIPK1 is expressed upon ConA treatment. FADD-RIPK1-RIPK3 complex is promoted upon ConA treatment in caspase 8–deleted mice. Caspase 8 deletion was protective when ConA was administered together with GalN, which induces apoptosis in addition to necrosis. JNK signaling is also associated with necrosis induction in these animals. Deletion of both caspase 8 and NEMO protects against steatosis and hepatocarcinogenesis but triggers massive liver necrosis, cholestasis, and biliary lesions.
Jouan-Lanhouet et al. ⁽³⁷⁾	Female C57Bl/6	20 mg/kg ROA	<ul style="list-style-type: none"> PARP-1 is activated in ConA-induced hepatitis. ConA-induced hepatitis is inhibited by Nec-1 or PJ-34 (a pharmacological inhibitor of PARP-1) pretreatment.
Kang et al. ⁽³⁸⁾	C57Bl/6	20 mg/kg IP	<ul style="list-style-type: none"> Both deletion of RIPK3 and pharmacological inhibition of Drp1 protect mice from NKT-mediated induction of acute liver damage. PGAM5 is a key mediator of RIPK3-mediated activation of NKT cells but does not play a role in necroptosis. RIPK3 deficiency reduces transaminase levels, inflammatory cell infiltrates, and apoptotic cells in ConA-treated mice. Mice lacking TNFR1 are resistant to ConA-induced liver injury and inflammation. RIPK1 does not play a role in RIPK3-dependent activation of cytokine production.
Deutsch et al. ⁽²⁸⁾	Male C57BL/6	20 mg/kg IV	<ul style="list-style-type: none"> RIPK1 and RIPK3 expression is elevated in mice with ConA hepatitis. RIPK3 deletion can only protect against early injury of ConA hepatitis. RIPK1 deletion markedly exacerbates ConA hepatitis, resulting in increased apoptotic cell death in the liver but can also reduce intrahepatic inflammatory infiltrate. Expression of RIPK3 is elevated in the liver of patients with hepatic failure from AIH. Exacerbation of hepatocyte injury is found in ConA plus Nec-1–treated mice and can be protected by caspase 8 blockage.
Filliol et al. ⁽³⁵⁾	Male C57BL/6	20 mg/kg IV	<ul style="list-style-type: none"> ConA treatment in mice can induce TRAIL-mediated caspase-independent cell death of hepatocytes and be partially prevented by co-treatment of Nec-1. RIPK1 kinase activity drives hepatocyte necroptosis following ConA injection but also serves as a scaffold protecting hepatocytes from massive apoptosis in the same model. Blockage of RIPK1 in mice triggers TNF-α–promoted apoptosis and can be protected by caspase inhibitor.
Le Cann et al. ⁽³⁹⁾	C57Bl/6	12 mg/kg IV	<ul style="list-style-type: none"> Both Sibiriline and Nec-1s can significantly decrease liver damage by reducing the size of perivascular and parenchymal zones of necrosis in ConA hepatitis.
Filliol et al. ⁽⁴⁰⁾	Alfp-Cre transgenic mice	12 mg/kg IP	<ul style="list-style-type: none"> RIPK1 deletion sensitizes mice to Fas-induced liver injury due to increased hepatocyte apoptosis. Hepatolysis is observed in RIPK1-deleted mice upon being treated with ConA.
He et al. ⁽⁴¹⁾	Unclear	25 mg/kg IV	<ul style="list-style-type: none"> Hepatic PGAM5 mRNA levels were elevated in patients suffering from AIH. ConA-induced liver inflammation was associated with elevated levels of PGAM5 protein in liver tissues. PGAM5 deletion protects mice from ConA-induced hepatocellular necrosis and liver injury downstream of inflammatory cell infiltration and activation. T cells activated by ConA produce high levels of cytokines, including IFNγ, TNF-α, and IL2. PGAM5 deficiency protects mice from ConA-induced liver injury downstream of inflammatory cell infiltration and activation.

in natural killer T cells (NKTs) is much higher compared with hepatocytes. RIPK3 is involved in the function of NKTs, a crucial step in ConA hepatitis, through activation of RIPK3–phosphoglycerate mutase 5 (PGAM5)–dynamin-related protein 1 (Drp1)/nuclear factor of activated T cell signaling. This is in accordance with results of He et al.⁽⁴¹⁾ that showed that PGAM5–Drp1 axis–mediated mitochondrial fission

drives the hepatic necrosis in ConA hepatitis. In addition, pseudokinase mixed-lineage kinase domain-like (pMLKL) has been reported to be elevated in human AIH biopsies, and in murine models, it has been proven that ConA hepatitis is driven by an MLKL–dependent pathway that occurs independent of RIPK3.⁽⁴²⁾ Taken together, the actual pathway of programmed necrosis during ConA hepatitis is still not clear. Although the

involvement of necroptotic mediators, such as RIPK1, RIPK3, and MLKL, has been reported, they are more likely to exhibit independent roles in diverse signaling pathways that drive programmed necrosis. Moreover, as demonstrated by Liedtke et al.,⁽³⁶⁾ simultaneous blockage of RIPK1 and caspase 8 during ConA hepatitis completely inhibited liver injury by suppressing necrosis as well as apoptosis and might be a potential therapy for immune-mediated liver injury.

FATTY LIVER DISEASES

Nonalcoholic fatty liver disease (NAFLD) is a common pathological condition associated with obesity, diabetes, and metabolic syndrome, resulting in fat accumulation in the liver without the history of alcohol abuse. NAFLD can develop into nonalcoholic steatohepatitis (NASH), where fat accumulation triggers inflammation (hepatitis).⁽⁴³⁾ NAFLD/NASH is currently the most rapidly expanding indication for liver transplantation in developed countries. Progressive steatohepatitis is associated with extensive apoptosis in hepatocytes induced by free fatty acids.⁽⁴⁴⁾ Several novel agents inhibiting apoptosis have been applied in clinical trials, but no significant protective effect was observed so far.^(45,46) In addition, inhibition of apoptosis by blocking caspase 8 in a murine model for alcohol-induced liver injury could not mitigate hepatic cell death, which might imply a possible switch from apoptosis to necroptosis.⁽⁴⁷⁾ This is in line with results of Gautheron et al.⁽⁴⁸⁾ that showed that deletion of caspase 8 renders mice more susceptible to methionine-choline-deficient (MCD) diet-induced liver steatosis, including extensively increased RIPK3 expression and subsequent massive liver injury and fibrosis. In concordance with these mouse models, significant elevation of RIPK3 in liver biopsies from NASH and NAFLD patients has also been reported.^(48,49) Circulating RIPK3, pMLKL, and necrosis markers were also found to be increased in the serum of NAFLD patients.⁽⁴⁹⁾ Deficiency of RIPK3 attenuates murine MCD-induced liver injury, steatosis, inflammation, fibrosis, and oxidative stress.⁽⁴⁹⁾ However, in mice that were fed a high-fat diet (HFD) to induce NAFLD, the absence of RIPK3 exacerbated liver injury with increased inflammation and hepatocyte apoptosis as well as early fibrotic responses.⁽⁵⁰⁾ The discrepancy between these findings may arise from the difference between the MCD and HFD models. Generally, mice treated with HFD exhibit glucose intolerance and

insulin resistance similar to NAFLD patients, whereas MCD-fed mice do not show these features and might be a more appropriate model for NASH.⁽⁵¹⁾ This also reveals the potential different role of necroptosis in NASH and NAFLD patients. In further studies on NASH/NAFLD as well as other disease models, the differences between the various types of cell death and also a careful evaluation of using the right model should obviously be taken into consideration.

CHOLESTATIC DISEASES

Cholestatic diseases are defined by a reduction in bile flow caused by impaired secretion by hepatocytes or cessation in bile flow through intrahepatic or extrahepatic bile ducts. Cholestasis often occurs as a result of chronic liver and biliary diseases, such as hepatitis, primary sclerosing cholangitis, primary biliary cirrhosis, and graft-versus-host disease.⁽⁵²⁾ Accumulation of toxic bile acids can induce apoptosis of both hepatocytes and cholangiocytes and is strongly associated with hepatocarcinogenesis as was shown in a rodent model.⁽⁵³⁾ Unfortunately, potent caspase inhibitors could only moderately alleviate liver injury after bile duct ligation, which implies that apoptosis is not the only type of cell death in cholestasis. As also described for NAFLD/NASH, a switch to necroptosis may occur in cholestatic diseases.⁽⁵⁴⁾ Afonso et al.⁽⁵⁵⁾ reported high expression levels of RIPK3 and MLKL in primary biliary cirrhosis patients, demonstrating the occurrence of necroptosis in this disease. They also found that RIPK3 deficiency could inhibit necroinflammation that occurred during bile duct ligation. Actually, in the case of cholestasis, caspase 8-dependent apoptosis was found to drive compensatory proliferation in hepatocytes and nonparenchymal cells (NPCs) through c-Jun N-terminal kinase (JNK) activation. However, when the necroptotic pathway is triggered, activation of RIPK3 inhibits caspase 8-dependent activation of JNK in both PLCs and non-PLCs. This, in turn, limits the immune response and compensatory proliferation of PLCs. Subsequently, the development of jaundice and cholestasis is promoted, and inflammatory hepatocarcinogenesis is impeded.⁽⁵⁶⁾ Another study also showed that inactivated phosphorylation of RIPK1 in PLCs inhibits the compensatory proliferation of both hepatocytes and intrahepatic biliary cells, which promotes cholestasis.⁽⁵⁷⁾ This indicates the possible independent role of RIPK1 and RIPK3

in cholestatic diseases, which needs to be clarified in further studies.

VIRAL HEPATITIS

Chronic viral hepatitis (hepatitis B virus [HBV] and hepatitis C virus [HCV]) is a major cause of chronic liver disease worldwide. HBV/HCV-related liver injury constitutes a major risk factor for cirrhosis and HCC.⁽⁵⁸⁾ Necroinflammation in viral hepatitis is strongly associated with liver fibrosis progression and hepatocarcinogenesis.^(58,59) However, the mechanism of both necroptosis and necroinflammation in chronic viral hepatitis remains unclear due to a limited number of studies. Afonso et al.⁽⁴⁹⁾ demonstrated high expression of RIPK3 in liver tissue from HBV and HCV patients by immunostaining, which indicates the potential involvement of necroptosis in chronic viral hepatitis. HCV has been proven to influence the death receptor-mediated pathway and the apoptotic pathway. Consequently, caspase inhibitors, such as IDN-6556 (Emricasan, a caspase inhibitor), were reported to be used as a potential therapy,⁽⁶⁰⁾ but again, the possible necroptotic switch should also be taken into account here. Lim et al.⁽⁶¹⁾ found that the HCV-induced cell death of human hepatoma cells could be rescued not only using a pancaspase inhibitor but also by Nec-1. However, the researchers did not provide evidence for the type of cell death involved. In addition, HBV-related hepatoma cell lines have been described to express high levels of apoptosis inhibitors, which may explain the resistance of HCC-HBV to apoptosis induction therapy.⁽⁶²⁾ The HBV X protein-induced microRNA (miRNA) 21 could suppresses cell apoptosis in HCC by targeting IL12.⁽⁶³⁾ In addition, HBV core protein could also inhibit Fas-mediated apoptosis in HCC by regulating membrane-bound Fas/Fas ligand and soluble Fas expression.⁽⁶⁴⁾ Taken together, this implies that targeting necroptosis might be further explored as a potentially interesting therapy for HBV-related HCC-HBV.

LIVER CANCER

HCC and cholangiocarcinoma (CCA) are the most common primary malignancies in the liver. They differ markedly in their morphology, metastatic potential, and responses to therapy. It has been suggested that both forms may arise from liver progenitor cells and have potentially overlapping pathways of oncogenesis.⁽¹⁾ The regulatory molecules and tissue

context that commit transformed hepatic cells toward HCC or CCA are still largely unknown, but a recent mouse study showed that hepatocytes with aberrantly activated oncogenes give rise to CCA when embedded in a necroptosis-dominated hepatic microenvironment.⁽⁶⁵⁾ DAMPs released by necroptotic hepatocytes can activate immune cells to secrete various specific cytokines to form a robust inflammatory environment, determining the outgrowth of CCA from transformed hepatocytes. In contrast, hepatocytes that harbor the same oncogenic driver will give rise to HCC if it is not adjacent to necroptotic hepatocytes. Notably, different from hepatocytes, transformed cholangiocytes can only develop into CCA and not HCC.⁽⁶⁶⁾ This finding may explain why CCA exhibits resistance to apoptosis, which is possibly due to higher endogenous expression of myeloid cell leukemia 1.⁽⁶¹⁾ Further work is warranted to unravel the extracellular and intracellular mechanisms and interactions between cytokines and transformed hepatocytes in detail, which, in turn, will be helpful to find new targeted therapies against HCC and CCA.

Necroptosis in Liver Transplantation

Liver transplantation is widely accepted as the only effective intervention for patients with end-stage liver disease. During liver transplantation, IRI is inevitable, although ischemia-free transplantations are feasible as described recently.⁽⁶⁷⁾ IRI is a detrimental process that not only damages the liver graft but is also associated with distant organ damage, such as acute kidney injury after liver transplantation.⁽⁶⁸⁾ Apoptosis and necrosis are the 2 most important types of cell death related to IRI. With strict morphological criteria, Gujral et al.⁽⁶⁹⁾ demonstrated that only a small subset of sinusoidal endothelial cells and hepatocytes underwent apoptosis after warm ischemia followed by reperfusion. Necrosis appears to be the dominant type of cell death in IRI, especially during reperfusion injury, accounting for more than 90% of total cell death and, consequently, caspase inhibitors cannot prevent IRI-related cell death. A schematic overview of necroptosis in liver transplantation is shown in Fig. 2. Haga et al.⁽⁷⁰⁾ demonstrated that hypoxia/reoxygenation leads to an increased expression of necroptosis mediators in murine liver cell lines, which subsequently could be

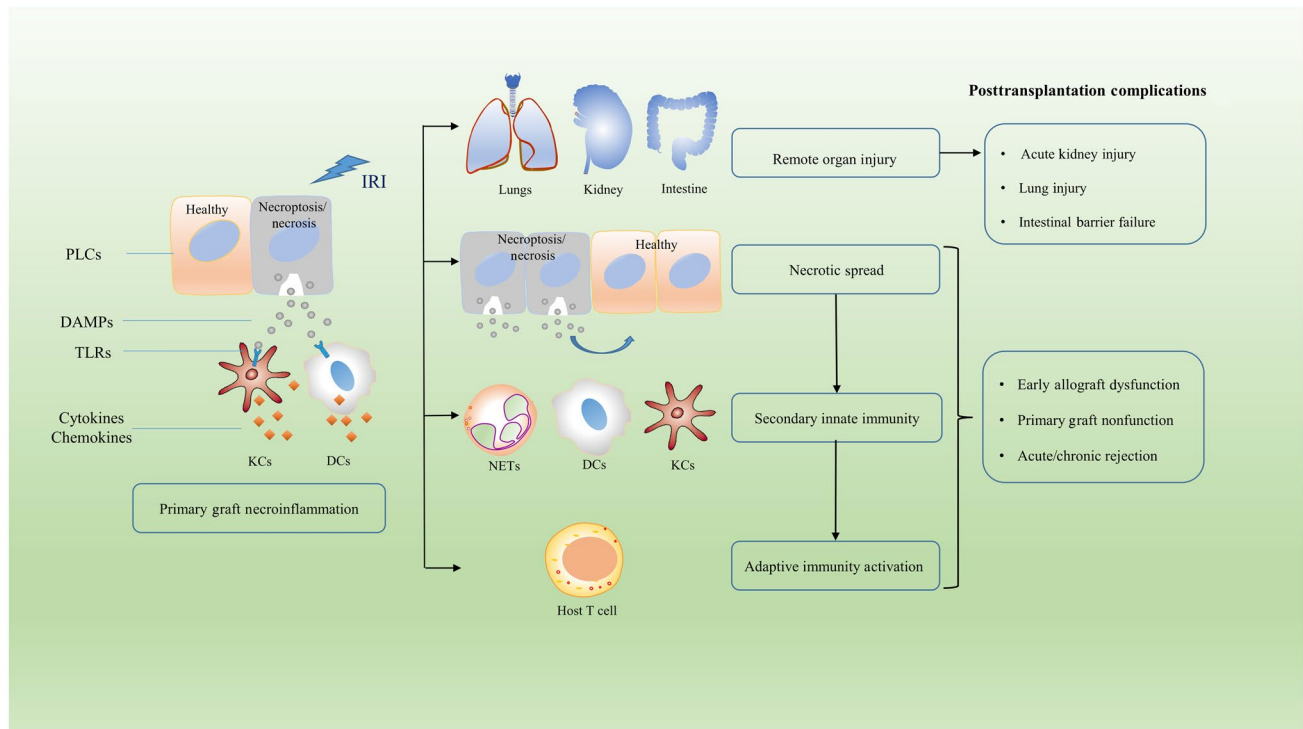


FIG. 2. Schematic overview of necrosis and necroinflammation during liver transplantation. During ischemia and reperfusion injury, both necroptosis and necrosis of PLCs can occur. Rupture of the cell membrane facilitates the release of intracellular DAMPs and subsequent inflammatory responses. TLRs on both KCs and DCs are activated that promote the production and release of cytokines and chemokines. This will trigger migration of innate immune cells to the liver graft but also give rise to necrotic spread by further induction of necroptosis in surrounding cells. This necrotic spread could cause early allograft dysfunction or total graft failure causing primary nonfunction. Furthermore, this necrotic spread and necroinflammation can lead to remote organ injury outside the graft. Robust innate immunity can also activate host T cells and evoke adaptive immune response that is associated with acute and chronic rejection after transplantation.

inhibited by Nec-1. Several studies also interpret the role of necroptosis in IRI using a mouse hepatic IRI model. Hong et al.⁽⁷¹⁾ reported that RIPK1 and RIPK3 expression is dramatically increased and is accompanied by the formation of the RIPK1/RIPK3 complex after IRI. Treatment with Nec-1 not only mitigated necroptosis but also decreased the serum levels of TNF- α and IL6. Nevertheless, 2 other studies done using similar animal models reveal the converse conclusion that necroptosis is not involved in the IRI process,^(72,73) demonstrating controversial findings resulting in unclear and contradicting conclusions in this field. Also, there is no protective effect observed after administration of Nec-1 or cyclosporine A, which is an inhibitor of mitochondrial permeability transition. This indicates that neither necroptosis nor mitochondrial permeability transition contributes to necrosis in hepatic IRI. This contradictory conclusion may arise from variations

during the establishment of the model and needs more careful analysis. Administration of Nec-1 before ischemia occurs or before reperfusion is done might also explain this discrepancy in results because the injury type and signaling are differential in various stages during transplantation surgery.⁽⁶⁹⁾ Furthermore, Liss et al.⁽⁷⁴⁾ found that hepatic IRI in the setting of steatosis led to increased expression of RIPK1, RIPK3, and MLKL, which might indicate the potential involvement of necroptosis in IRI in steatotic liver grafts.

Necroinflammation resulting from IRI has proven to determine the fate of the liver graft and the outcome of patients undergoing liver transplantation (Fig. 2). Necroptosis is one of the critical triggers of necroinflammation, though its role in hepatic IRI is still unclear. Following hepatic IRI, DAMPs are released from necrotic cells and exacerbate inflammation and liver injury.⁽⁷⁵⁾ Activation of TLRs by DAMPs

also recruits innate immune cells to the graft and ultimately contributes to graft rejection.⁽⁷⁶⁾ High-mobility group box 1 (HMGB1), a nuclear protein regulating transcription, is one of the most-investigated DAMPs, which binds to TLR4 and induces the generation of proinflammatory cytokines and subsequent graft liver damage.⁽⁷⁷⁾ Increased plasma levels of HMGB1 are a marker of hepatocellular injury in recipients.⁽⁷⁸⁾ Down-regulation of nuclear HMGB1 by small interfering RNA in a mouse IRI model protected the liver from IRI and alleviated inflammation response.⁽⁷⁹⁾ Moreover, a clinical trial demonstrated that a TLR4 single-nucleotide polymorphism, leading to diminished binding with HMGB1, reduced the risk of graft loss after liver transplantation.⁽⁸⁰⁾ In general, necroinflammation is a promising new therapeutic target for liver transplantation. Furthermore, the booming development of ex vivo normothermic machine perfusion should also be addressed because this can provide an appropriate model for the investigation on necroptosis and necroinflammation in human liver grafts.

Pharmacological Strategies Targeting Necroptosis

Although apoptosis inhibitors have been used in a clinical trial against apoptosis-mediated liver injury, the protective effect of these inhibitors remains questionable and might be dependent on whether or not the switch from apoptosis to necroptosis is made.⁽⁸¹⁾ Therefore, the clinical application of necroptosis inhibitors should also be considered. Nec-1 is the first and extensively used compound identified as an inhibitor of necroptosis acting on the kinase activity of RIPK1.⁽⁸²⁾ Because of its striking specificity for necroptosis, it is also widely interpreted as indicating proof of necroptosis in various conditions. However, even though Nec-1 was described more than a decade ago, it has only been used in preclinical trials to treat amyotrophic lateral sclerosis.⁽⁸³⁾ Low potency and the short half-life of Nec-1 in vivo restrains its clinical application. Moreover, Nec-1 is not exclusively inhibiting for necroptosis because it also acts on the indoleamine 2,3-dioxygenase (IDO) enzyme that regulates the innate and adaptive immune system.⁽⁸⁴⁾ Instead, the development of Nec-1s, which is more stable, has great potential in vivo. Nec-1s lacks IDO inhibitory activity and possesses a longer half-life in vivo than Nec-1, which makes Nec-1s more promising for

bench-to-bed translation.⁽⁸⁵⁾ Administration of radio immunoprecipitation assay 56, another RIPK1 inhibitor, is also a potential therapy against necroptosis that has been validated in murine systemic inflammatory response syndrome.⁽⁸⁶⁾

Considering the crosstalk of RIPK1 in apoptosis, necroptosis, and NF- κ B pathways, inhibition of RIPK3 kinase activity seems to be a more specific and therefore more potential therapeutic option targeting necroptosis. RIPK3 inhibitors can also be applied in some RIPK1-independent pathologic conditions, such as viral infection and pancreatitis.⁽⁸⁷⁾ Compounds including low concentrations of GSK'840, GSK'843, GSK'872, and GW392 have been verified in vivo and in vitro to inhibit necroptosis.^(82,88) However, RIPK3 inhibitors used in high concentrations might induce apoptosis by activating caspase 8.⁽⁸⁹⁾ Furthermore, necrosulfonamide, which targets MLKL, is found to inhibit necroptosis in vitro. More studies are needed to investigate the efficiency and safety of necrosulfonamide in vivo.

Different from the above inhibitors, some drugs that are already used in the clinic also inhibit necroptosis activity. Their safety in patients has already been validated. For instance, dabrafenib is a US Food and Drug Administration-approved drug for metastatic melanoma treatment,⁽⁹⁰⁾ and Li et al.⁽³¹⁾ demonstrated that dabrafenib could inhibit RIPK3 and alleviate APAP-induced liver injury in vitro and in vivo. Sorafenib is a clinically used drug to treat HCC and acute myeloid leukemia,⁽⁹¹⁾ and it can inhibit both RIPK1 and RIPK3 kinase activity and thus protects against TNF-induced systemic inflammatory response syndrome and kidney IRI.⁽⁹²⁾ Likewise, ponatinib and pazopanib, 2 anticancer agents, were also found to be inhibitors of necroptosis in human cells.⁽⁹³⁾ Phenytoin, a clinically used anti-convulsant drug, can also block necroptosis in systemic inflammatory response syndrome and was shown to be efficient in a kidney IRI model. In addition, melatonin is synthesized endogenously by the pineal gland and functions as an indirect antioxidant. It has been demonstrated to attenuate carbon tetrachloride-induced liver injury and to prevent fibrosis by inhibiting necroptosis-associated inflammatory signaling.⁽⁹⁴⁾

Conclusion

In this review, we strived to illuminate the role of necroptosis in acute and chronic liver diseases as well as in liver transplantation. Although our primary view was

on necroptosis, the crosstalk between apoptosis and necroptosis should not be ignored. In most cases, these 2 types of cell death are intermixed in the pathogenesis of liver diseases, although the role of necroptosis in some liver diseases is still unconfirmed. The switch between apoptosis and necroptosis should be noted when using either caspase inhibitors or necroptosis inhibitors. For instance, caspase inhibitors can prevent apoptosis but may induce necroptosis in turn, which gives rise to detrimental effects, ranging from less effectiveness to massive secondary inflammation response. Insight into necroptosis also offers a convincing explanation for the limited clinical benefits of caspase inhibitors. Actually, necroptosis mediators, such as RIPK1 and RIPK3, can also be involved in other signaling pathways, mitochondrial dysfunction, and apoptosis. This highlights the difficulty to target cell death in pathological situations. Further studies should be focused on the investigation of the potential crosstalk between various cell-survival and cell-death signaling pathways in liver diseases. It is also notable that RIPK1, RIPK3, and MLKL could function independently in some liver diseases, such as acute liver injury and cholestatic diseases, through a nonnecroptosis pathway. What would be important to help the research field forward is consensus on the precise definition of necroptosis and its morphology and signaling hallmarks. In addition, the results of some recent studies using experimental mouse models exhibit controversial results, which could be caused by the differences in genetic background of the mouse strains and the different experimental conditions used in the studies. For instance, in studies focusing on fatty liver disease, diverse treatment may very well lead to inconsistent results when choosing either MCD or HFD models. Related studies involving humans are still restricted to clinical liver biopsies. It is also a promising direction to use in vitro experimental models derived from patients, such as organoids,^(95,96) which represent individual or patient-specific pathological features. In addition, as a severe consequence of necroptosis, necroinflammation plays a crucial role in both acute and chronic liver diseases. Several of these DAMPs could serve as potential biomarkers for the evaluation of liver injury, for instance, during normothermic graft preservation by machine perfusion for liver transplantation. In summary, necroptosis is a promising therapeutic target for treatment of liver diseases and during graft preservation and should be further explored both to deepen our understanding of how liver cells die and

clarify the clinical perspective for translation of the knowledge in medical practice.

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