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Lytic Cell Death in Metabolic Liver Disease

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Summary

Regulated cell death is intrinsically associated with inflammatory liver disease and is pivotal in governing outcomes of metabolic liver disease. Different types of cell death may coexist in the progression of metabolic liver disease to inflammation, fibrosis, and ultimately cirrhosis. In addition to apoptosis, lytic forms of hepatocellular death, such as necroptosis, pyroptosis and ferroptosis elicit strong inflammatory responses due to cell membrane permeabilization and release of cellular components, contributing to the recruitment of immune cells and activation of hepatic stellate cells. Controlling liver cell death, in turn, emerges with fundamental importance and offers novel opportunities for potential therapeutic intervention. This review summarizes the underlying mechanism of distinct lytic cell death modes and their commonalities, discusses its relevance to metabolic liver diseases of different aetiologies, and acknowledges the limitations of current knowledge in the field. We focus on the role of hepatocyte necroptosis, pyroptosis and ferroptosis in non-alcoholic fatty liver disease, alcohol-associated liver disease and other metabolic liver disorders, as well as potential of translation into human disease.

Keywords

Programmed cell death; Necroptosis; Pyroptosis; Ferroptosis; NAFLD; NASH; ASH; Hemochromatosis; Niemann-Pick disease; Gaucher's disease

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1. Introduction

Cells can be exposed to unrecoverable extra- or intra-cellular perturbations that disrupt cellular homeostasis and activate one of many signal transduction cascades, ultimately leading to cell death and liver injury. The regulated cell death (RCD) modalities are initiated and propagated by specific molecular mechanisms, with considerable interactivity. Moreover, each type of RCD is characterized by distinct morphological, biochemical, and molecular features with specific physiological consequences ranging from anti-inflammatory and tolerogenic to pro-inflammatory and immunogenic profiles.

Decades of research have revealed multiple forms of genetically encoded RCD pathways with increasingly recognized relevance in disease. Importantly, these novel RCD pathways can co-exist simultaneously in pathological contexts, and several share overlapping mechanisms that can act as a “backup” dying strategy to ensure organism homeostasis when a death-inducing cellular threshold is reached. In chronic liver diseases, viral, toxic, metabolic or autoimmune triggers cause hepatocellular death, followed by inflammation and compensatory proliferation, often closely linked to development of fibrosis, cirrhosis, and hepatocellular carcinoma [1]. Traditionally, apoptosis was considered as a highly regulated process, diametrically opposed to passive necrosis, itself considered as an accidental and uncontrolled form of cell death. Passive necrosis occurs when cells are irreparably damaged by external forces, resulting in rapid cytoplasmic and organelle swelling (oncosis), along with plasma membrane permeabilization and subsequent leakage of damage-associated molecular patterns (DAMPs) that trigger immune response. However, recent findings highlight multiple forms of regulated necrotic modalities, including necroptosis, pyroptosis and ferroptosis, that share key morphologic features with passive necrosis yet have well-defined and regulated causal mechanisms [2, 3]. Necroptosis is probably the best-understood form of regulated necrosis, since it shares multiple molecular components with the extrinsic apoptotic pathway and can act as a fail-safe mechanism to ensure cell death progression when apoptosis is abnormally inhibited, which may also be relevant in pathological conditions in the liver [4]. Nevertheless, other alternative forms of RCD, such as ferroptosis or pyroptosis may turn to be equally important as we gather experimental evidence for their relevance. Importantly, a better characterization of RCD in liver disease may lead to novel therapeutic opportunities. As the field continues to progress and novel signalling pathways that orchestrate RCD are still being characterized, this review summarizes current knowledge on the contribution of hepatocellular RCD modalities in metabolic liver disease. Experimental models and patient findings are addressed, as well as the pathophysiological relevance of each of the main types of RCD in hepatocytes, controversies and unsolved issues, and potential therapeutic applications. RCD of non-hepatocyte liver cells, immune cells and others will not be explored, although it may prove equally important in liver disease.

2. The Principles of Alternative Cell Death Modalities

2.1. Necroptosis

The first evidence of necroptosis was provided in 1996 by Ray *et al.*, who observed a lytic mode of cell death in pig kidney cells infected with cow pox virus, which was governed by

the expression of the viral cytokine response modifier A (CrmA), a caspase inhibitor [5]. In 2000, Holler *et al.* demonstrated that FAS, TRAIL and TNF receptors, the classical death receptors (DRs), initiated cell death by two alternative pathways, one relying on caspase-8 (*i.e.*, the classical extrinsic apoptotic pathway) and the other dependent on the receptor interacting protein kinase 1 (RIPK1) (*i.e.* the necroptosis) [6]. However, the term used to describe this novel mode of cell death appeared only in 2005, when Degterev *et al.* showed that necrostatin-1, a chemical compound that blocks the kinase activity of RIPK1, was able to inhibit cell death in TNF-treated cell lines [7]. Later, the two downstream core components of the necroptotic machinery, namely RIPK3 and mixed lineage kinase domain like pseudokinase (MLKL), have been identified in less than a decade [8–11]. Morphologically, necroptosis, which is primarily triggered following microbial infections and physicochemical stressors (*e.g.*, radiation or chemotherapy), exhibits the features of passive necrosis (*e.g.*, in response to extreme external factors) with increased cell volume, swelling of organelles, loss of membrane integrity, cellular collapse, leading to the release of DAMPs (*e.g.* high-mobility group box 1 [HMGB1]; interleukin [IL]-1 α , IL-33). Although no specific necroptotic DAMPs have been identified so far, their release during necroptosis provokes a strong inflammatory response that has been linked to the development of many diseases [12].

Necroptosis occurs typically upon DR activation (*e.g.*, TNFR1, CD95, TRAIL-R), and only if apoptotic signalling components are inactive, absent or inhibited (*e.g.*, caspase inhibition) (Figure 1). In this specific context, DR triggers the formation of a RIPK1/RIPK3 cell death platform, named the necrosome [13]. Inside, RIPK1 and RIPK3 adopt a hetero-amyloid structure through critical RIP homotypic interaction motif (RHIM) interactions [14]. Consequently, RIPK3 mediates the phosphorylation of MLKL, resulting in MLKL oligomerization and translocation to the plasma membrane [13]. Recent results suggest that casein kinase 1 (CK1) family proteins are necrosome components, which are required to directly phosphorylate serine 227 of human RIPK3 and induce necroptosis [15]. Finally, MLKL forms pore in the plasma membrane [16, 17], and increases permeability *via* the activation of A disintegrin and metalloproteinase (ADAM) proteases [18], Ca²⁺ influx by targeting the cation channel transient receptor potential melastatin related 7 (TRPM7) [19], and phosphatidylserine externalization [20]. Necroptosis activation requires MLKL phosphorylation, which is dependent on RIPK3 kinase activity, itself dependent on phosphorylation by RIPK1; hence, RIPK1 and/or 3 phosphorylation cannot be equated with necroptosis without MLKL phosphorylation. There are considerable crosstalk regulations between apoptosis and necroptosis in DR signalling pathways [21], and it seems that one cannot be achieved without inhibiting the other. On one hand, caspase-8 cleaves and inactivates RIPK1 and RIPK3, which has for consequence to suppress necroptosis [22, 23]. On the other hand, activity of RIPK3 determines whether cells die by necroptosis (*i.e.*, the necrosome formed) or apoptosis (*i.e.*, RIPK3 kinase activity inhibited) [24, 25].

The activation of necroptosis is not limited to DRs, and it can be triggered by multiple pathways, including toll-like receptors (*e.g.*, TLR3 and TLR4) [26], nucleic acid sensors (*e.g.*, Z-DNA-binding protein 1 [ZBP1, also known as DAI]) [27], retinoic acid inducible gene 1 protein (RIG1) [28], transmembrane protein 173 (TMEM173, also known as STING) [29], and adhesion receptors [30]. These signalling pathways are often independent of

RIPK1, but still have in common the phosphorylation of MLKL by RIPK3 and the MLKL-mediated pore formation in the plasma membrane [13]. Interestingly, the endosomal sorting complexes required for transport (ESCRT)-III complex, a membrane remodelling and scission machinery, limit MLKL-mediated necroptosis by promoting membrane repair [31]. As MLKL also regulates endosomal trafficking and extracellular vesicles generation [32], a delicate balance between membrane injury and repair exists and ultimately decides cell fate in necroptosis.

2.2. Pyroptosis

Pyroptosis was initially observed in 1992 by Zychlinsky *et al.*, who described a lytic form of cell death in *Shigella flexneri*-infected macrophages [33]. However, the term has emerged nearly a decade later, in 2001, when Cookson and Brennan demonstrated that *Salmonella-induced* macrophage death was dependent on caspase-1 [34]. Since this discovery, the number of pyroptotic caspases (as opposed to apoptotic caspases) has considerably increased, including caspase-1, caspase-11 and its human orthologs caspase-4 and 5 [35, 36], and even more surprisingly the apoptotic effector caspase-3 under specific circumstances [37, 38]. Pyroptosis, which occurs primarily upon infection with intracellular pathogens, is characterized by caspase-dependent pore formation in the plasma membrane, swelling, rupture of the cell, and release of pro-inflammatory IL-1 β and IL-18 [39]. In 2015, the pore-forming gasdermin D (GSDMD) was identified as the executioner of pyroptosis [40, 41]. Caspases-1 and -11 cleave GSDMD into a 31-kDa N-terminal GSDMD^{NT} fragment, which exhibits intrinsic pore-forming activity, and a 22-kDa C-terminal GSDMD^{CT} fragment that binds to GSDMD^{NT} to maintain the protein in an inhibitory state [40, 41]. The overexpression of GSDMD^{NT} alone results in induction of pyroptosis, whereas that of GSDMD^{CT} blocks GSDMD^{NT}-induced pyroptosis [41]. Interestingly, GSDMD belongs to a larger protein family, which consists of GSDMA, GSDMB, GSDMC, GSDMD, GSDME (also referred to as DFNA5 [deafness, autosomal dominant 5]), and DFNB59 [42]. Recently, GSDME was identified as an additional executioner of pyroptosis, able to switch caspase-3 mediated apoptosis induced by TNF or chemotherapy drugs to pyroptosis [38]. Although most of the gasdermins have been associated with the occurrence and development of various diseases, their precise function and molecular mechanism of activation remain largely unknown [43].

Pyroptosis is activated by canonical and non-canonical signalling pathways, which differ in the use of cytoplasmic multiprotein complexes named inflammasomes (Figure 2) [44, 45]. The canonical pathway begins with inflammasomes that recognize various exogenous and endogenous danger signals, including damage-associated molecular pattern molecules (DAMPs) and pathogen-associated molecular patterns (PAMPs). The canonical inflammasomes comprise a sensor protein belonging to the nucleotide-binding domain (NBD) and leucine-rich-repeat-(LRR)-containing (NLR) or the AIM2-like receptor (ALR) or pyrin family, an adaptor protein, apoptosis-associated speck-like protein containing a CARD (ASC), and an inactive zymogen, pro-caspase-1 [46]. When formed, canonical inflammasomes lead to the activation of caspase-1 that cleaves pro-IL-1 β and pro-IL-18 into their active forms. Then, IL-1 β and IL-18 are released extracellularly as a result of the pore formation in the plasma membrane by GSDMD^{NT}. On the other side, the non-canonical

pathway is dependent on caspase-11, which is able to cleave GSDMD independently of inflammasome priming. In this later case, GSDMD^{NT} signals back to canonical NLRP3 inflammasome, which in turn activates the caspase-1-dependent pathway [44–46].

2.3. Ferroptosis

Ferroptosis was originally observed in 2003 using erastin, a cell-permeable compound, which was only lethal to engineered human tumour cells expressing an oncogenic RAS mutation [47]. The term ferroptosis was coined in 2012 by the Stockwell lab to describe a non-apoptotic cell death caused by the accumulation of iron-dependent lipid peroxides, induced by erastin [48]. Ferroptosis, which principally exerts tumour-suppressor functions, is morphologically characterized by cell volume shrinkage and increased mitochondrial membrane density without typical apoptotic or necrotic manifestation [48]. Ferroptosis can be induced either in a canonical way by a direct or indirect inactivation of the glutathione peroxidase 4 (GPX4), the major protective mechanism of biological membranes against peroxidation damage, or in a non-canonical manner by increasing the labile iron pool [48]. The pathological relevance of ferroptosis has been evaluated using ferrostatin-1 (Fer-1), a lipid reactive oxygen species (ROS) scavenger that prevents ferroptosis induced by erastin [48–50].

In the canonical pathway, erastin interacts directly with the transporter solute carrier family 7 member 5 (SLC7A5), which disrupts the transport of amino acids into the cell by the system Xc⁻ (Figure 3) [48]. The Xc⁻ transporter system is composed of a regulatory subunit solute carrier family 3 member 2 (SLC3A2) and a catalytic subunit solute carrier family 7 member 11 (SLC7A11), which promotes the cellular uptake of cystine, the plasma precursor of cysteine, by exchange with glutamate [51]. In cells, cysteine is required for the synthesis of glutathione (GSH), a major redox regulatory system [52]. By indirectly blocking the Xc⁻ transporter, erastin inhibits GSH synthesis, which is used by GPX4 to eliminate the production of phospholipid hydroperoxides (PL-OOH), the major mediator of chain reactions in lipoxygenases [53]. System Xc⁻ inhibitors (*e.g.*, erastin, sulfasalazine, sorafenib and L-glutamate) are considered as class I ferroptosis inducers, whereas direct GPX4 inhibitors (*e.g.*, RAS-selective lethality protein 3 [RSL3], ML162 and altretamine) are referred to class II inducers [50].

The main ROS produced by cellular metabolism or accumulated upon cell transformation (*e.g.*, RAS-mediated) are the superoxide radical anion (O₂^{•-}) and hydrogen peroxide (H₂O₂). In the presence of free iron, these ROS are likely to be converted to hydroxyl radical (HO[•]), which is highly reactive to macromolecules (*e.g.*, PUFAs, polyunsaturated-fatty-acids). The reactions involving iron and leading to the hydroxyl or alkoxyl (RO[•]) radical are traditionally called Fenton reactions [54]. The oxidation of PUFAs, including arachidonic acid, by a catalytic pathway involving acyl-CoA synthetase long-chain family member 4 (ACSL4), lysophosphatidylcholine acyltransferase 3 (LPCAT3), and lipoxygenases (LOXs), is required for lipotoxicity in ferroptosis [55–57]. Compared with other proteins of its family, GPX4 is the only member able to reduce membrane phospholipid hydroperoxides highlighting its key role against lipid peroxidation, which is prominent in ferroptosis and contributes to plasma membrane permeabilization and consequently release of DAMPs, even

though the molecular mechanisms remain elusive [48]. Recently, the ferroptosis suppressor protein 1 (FSP1), previously known as apoptosis-inducing factor mitochondrial 2 (AIFM2), was identified as a key component of a non-mitochondrial CoQ₁₀ (ubiquinone) antioxidant system that acts in parallel to the GPX4 canonical pathway to halt the progression of lipid peroxides [58, 59]. In the non-canonical pathway, increased iron uptake by transferrin receptor (TFRC) and reduced iron export by ferroportin promote oxidative damage and ferroptosis [60, 61].

2.4. Commonalities

Unlike necrosis, which is accidental and uncontrolled, lytic RCD, including necroptosis, pyroptosis and ferroptosis, are genetically defined and employ complex machinery specifically designed to perforate the plasma membrane. This common phenomenon plays an evolutionarily conserved role in systemic immunity, combining the killing of cells with alerting the immune system through the release of DAMPs. Although these three forms of lytic RCD are at first glance dedicated to eliminate intracellular pathogens (*i.e.*, pyroptosis and necroptosis) or mutation-driven cancer cells (*i.e.*, ferroptosis), their chronic stimulation can lead to an excessive inflammation that may be detrimental and start damaging healthy cells, tissues, and organs [62]. In fact, these three RCD forms have already been involved in the development of many inflammatory diseases from different aetiologies, including liver diseases [63].

Accumulating evidence indicates that a chronic low-level of inflammation (also known as sterile-inflammation) is mediated by DAMPs [64]. As DAMPs are hallmarks of lytic RCD, and are recognized by pattern recognition receptors (PRRs), which are also used to induce lytic RCD (*e.g.*, TLRs and NLRs), DAMPs therefore appear to be the common denominators connecting lytic RCD pathways to each other [64]. A substantial number of DAMPs, including HMGB1, heat shock proteins (HSPs), ATP, and interleukins, have been differentially involved in pyroptosis, necroptosis or ferroptosis [65, 66]. However, further investigations are needed to identify which specific DAMPs might preferentially activate one lytic RCD or another.

Recent evidence suggests that caspase-8 may represent the molecular switch that controls apoptosis, necroptosis and pyroptosis, and prevents tissue damage [67, 68]. In addition to its enzymatic role in apoptosis and necroptosis, caspase-8 acts as a scaffolding protein that together with MLKL regulates NLRP3 inflammasome activation in macrophages [69]. These unexpected roles of caspase-8 involves the activation of the inflammasome and induction of pyroptosis under circumstances in which apoptosis and necroptosis are compromised, such as when the abundance of viral inhibitors may have driven the counteradaptation of pyroptosis as a host defence [70]. Questions that remain to be addressed include how the different modes of RCD influence disease progression and coordinate adaptive responses.

In the next section, we describe the potential roles of RCD in various metabolic liver diseases using an unexpurgated approach, in that the observations described as regulating RCD in these models are included regardless of whether they have been confirmed or even viewed skeptically.

3. Alternative Cell Death Modalities in Metabolic Liver Diseases

3.1. Non-alcoholic fatty liver disease and non-alcoholic steatohepatitis

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of chronic liver disease that ranges from simple steatosis to non-alcoholic steatohepatitis (NASH) and is strongly associated with obesity and the metabolic syndrome. NAFLD is estimated to affect up to one-third of the general population worldwide [71]. Although metabolic alterations caused by free fatty acid overload is considered a major cause for hepatocyte damage, the mechanisms driving disease progression from simple steatosis to NASH remain incompletely understood [72]. Indeed, lipotoxicity, oxidative stress, organelle dysfunction and inflammatory response exacerbate ballooning and increase hepatocyte cell death [73]. Chronic liver cell death may then trigger immune cell recruitment and hepatic stellate cell activation, hepatocyte turnover and clonal expansion, in a feed-forward loop that drives NAFLD progression. While historically much emphasis was placed on apoptosis and necrosis, more recently other types of RCD, such as necroptosis or pyroptosis, controlled by overlapping molecular machineries, and capable of acting as a backup of each other, have been implicated in NAFLD and NASH pathogenesis.

3.1.1. Necroptosis—Hepatocellular necroptosis has been described in human NASH and expression of RIPK3 and MLKL is increased in liver of biopsy-proven NASH and other chronic liver disease patients [74–77]. The systemic release of necrosome proteins RIPK1 and MLKL in patients displaying disease activity further supports the clinical importance of this pathway in NASH [78]. RIPK3 and MLKL expression was also increased in visceral adipose tissue from obese and type II diabetes patients, while RIPK3 expression correlated with MLKL and metabolic serum markers [79]. High levels of core components of necroptosis were detected in liver, adipose tissue and muscle in diabetic mice, whereas targeting diabetic mice with RIPK1 inhibitors or depletion of MLKL prevented insulin resistance and glucose intolerance, despite no effect on inflammation [80]. Indeed, both specific knockdown of RIPK1, RIPK3 or MLKL and the use of chemical inhibitors activated insulin-stimulated AKT signalling, key in glucose homeostasis. Further evidence from animal studies confirmed that RIPK3 increases in the liver of experimental models of NASH [74, 75, 81]. JNK activation was implicated in RIPK3-dependent liver damage and fibrosis in methionine-choline deficient (MCD) diet-fed mice, since inhibition of JNK diminished RIPK3 levels in the liver [75]. Similarly, in common bile duct ligation hepatic injury, necroptosis was induced and contributed to fibrosis [82], although the effects of necroptosis inhibition on different hepatic fibrosis models are yet to be consistently determined. RIPK3-MLKL-mediated necroptosis contributed also to ischemia reperfusion injury of steatotic livers [83]. *Mlkl*^{-/-} mice had decreased hepatic neutrophil infiltration and inflammation, while *Ripk3*^{-/-} or RIPK3 kinase-dead knock-in mice were protected against late ischemia reperfusion injury.

Interestingly, RIPK3 deficiency protected from liver injury, inflammation and fibrosis in MCD-fed mice [74, 75], but exacerbated liver steatosis and apoptosis, as well as adipose tissue inflammation, insulin resistance and glucose intolerance induced by high-fat diet [79, 81]. While, RIPK3 global knockout mice exhibit no phenotype, the mechanism for the

enhanced apoptosis in *Ripk3*^{-/-} mice on high fat diet is uncertain, and contradictory to that reported earlier in an alcohol model [84]. Nevertheless, *Ripk3*^{-/-} mice fed a choline-deficient high-fat diet as a mouse model of NASH developed insulin resistance as a result of increased compensatory apoptosis and inflammation in white adipose tissue [79]. A compartment protective function of RIPK3 in adipose tissue argues in favour of hepatocyte-specific RIPK3 inhibition in NASH. Moreover, if the increased hepatocellular apoptosis is indeed not directly determined by the absence of RIPK3 in hepatocytes, a nonparenchymal anti-inflammatory role of RIPK3 should be further dissected using conditional knockout models. Other functions of RIPK3 in hepatocarcinogenesis have been recognized [85], and its involvement in inflammatory signalling independent of necroptosis and mediation of cell death should not be neglected [86, 87]. RIPK3-deficient natural killer cells showed impaired immune responses to tumours and liver inflammation [88]. It is of interest that the strong induction of RIPK3 and MLKL in experimental models of NASH and in patients with chronic liver disease is required to sensitise to necroptosis. Epigenetic silencing of RIPK3, in contrast, has been associated with malignant transformation in hepatocellular carcinoma cells, while reestablishment of RIPK3 expression by promoter demethylation resensitized cells to chemotherapy-induced necroptosis [89], again implying that induction of RIPK3 is context-dependent.

Overall, RIP kinases are pleiotropic modulators of cell death and participate in the pathogenesis of many chronic diseases [90], suggesting that preventing the formation and/or activation of the necrosome might arrest disease progression in NASH. In a recent study, RIPA-56, a highly specific RIPK1 inhibitor, reduced hepatic inflammation and fibrosis in dietary obese mice, while reversing steatosis and dampening body weight gain [78]. Similarly, MLKL pharmacological inhibition decreased hepatic *de novo* fat synthesis and chemokine ligand expression in NASH [91]. Further studies in human NAFLD and NASH using robust hallmarks of necroptosis, as well as intervention studies in conditional knockouts and in models that better mimic the same degree of inflammation and fibrosis as human disease should provide additional insights on the therapeutic relevance of targeting necroptosis in the NAFLD context.

3.1.2. Pyroptosis—Lipotoxicity, innate immune response, cell death pathways, mitochondrial dysfunction and endoplasmic reticulum stress trigger chronic inflammation in the liver, potentially fuelling the transition from NAFL to NASH [92–94]. In this regard, activation of resident Kupffer cells and infiltrating macrophages is a remarkable feature of NASH pathogenesis [95]. While Kupffer cells are known to release TNF- α [96], further feeding a vicious cycle of inflammation and apoptosis leading to fibrosis, it is now apparent that inflammatory caspases, including caspase-1, murine caspase-11, and human caspase-4/5, play key important roles as mediators of inflammation [41]. This positions pyroptosis in non-alcoholic fatty liver development and progression to NASH.

Inflammasome activation triggers caspase activity and promotes inflammation and fibrosis in liver disease [97], while typical activators of inflammasomes such as fatty acids, DAMPs, and uric acid upregulate NLRP3 inflammasome components. Excessive activation of inflammatory caspases has been directly implicated in the pathogenesis of NAFLD in humans and mice, where pro-inflammatory cytokines released during pyroptosis are key

effector molecules [97–99]. Indeed, activation of IL-1 β signalling, downstream of inflammasomes, drives liver inflammation and fibrosis in experimental NASH [93] and amplifies the response of other cytokines [100]. Another generic substrate for inflammatory caspases, GSDMD, acts as a downstream effector of non-canonical inflammasome activation and plays a specific role in inflammatory caspase-mediated pyroptosis [43]. The GSDMD^{NT} domain with intrinsic pyroptosis-inducing activity was positively correlated with the NAFLD activity score and fibrosis in patients with NASH [99]. Further, *Gsdmd*^{-/-} mice fed an MCD diet showed significantly reduced steatohepatitis, noticeable improvement in liver inflammation, as well as reduced serum ALT levels and hepatic triglyceride content. In addition, liver fibrosis was strongly attenuated in *Gsdmd*^{-/-} mice after MCD feeding [99]. Interestingly, *Gsdmd*^{-/-} mice were protected from steatosis via downregulation of the lipogenic gene *Srebp1c* and induction of lipolytic genes, including *Ppara* and its downstream targets. Importantly, overexpression of the GSDMD^{NT} domain, functionally responsible for pyroptosis, spontaneously induced liver injury even without MCD treatment, indicating that GSDMD^{NT}-induced pyroptosis is a crucial mechanism involved in the pathogenesis of steatohepatitis.

PAMPs and DAMPs can directly induce pyroptotic cell death in hepatocytes or indirectly cause liver cell injury. Interestingly, compared to global NLRP3 knock-in mice, mice with myeloid-specific *Nlrp3* mutations lack detectable pyroptotic hepatocyte cell death and have less severe liver inflammation, HSC activation, and fibrosis [101]. Thus, in addition to immune cells, hepatocyte pyroptosis resulting from intrinsic inflammasome activation exacerbates inflammation and fibrosis in the liver, indicating that both immune cell- and liver-specific NLRP3 inflammasome activation are essential for liver injury [101, 102]. Hepatocyte-specific NLRP3 mutant animals are nevertheless needed for direct evidence of the crosstalk between hepatocytes and the other cell types in the onset and progression of liver injury in NAFLD and NASH.

3.1.3. Ferroptosis—Evidence for a potential role of ferroptosis in NAFLD and NASH is scarce. Nevertheless, secondary products of lipid peroxidation, such as malondialdehyde and 4-hydroxynonenal are utilized as oxidative stress markers in NASH patients [103], and Vitamin E, an antioxidant that suppresses lipid peroxidation, reduces serum ALT in NASH patients [104]. Moreover, iron accumulation from metabolic dysfunction, aggravates NASH, such as in liver siderosis observed in some NASH patients [105], and primary hemochromatosis exacerbates NASH [106], while iron removal ameliorates liver damage and serum ALT [107]. In a recent study, ferroptosis inhibitors, trolox and deferiprone, suppressed necrotic cell death, infiltration of inflammatory cells, and inflammatory cytokine expression in the onset of steatohepatitis in the choline-deficient, ethionine-supplemented (CDE) diet model [108], in which hepatic cell death is an early event in the context of steatosis [109]. Oxygenated PE, implicated in the ferroptosis pathway, was increased in the liver of CDE-fed mice, but normalized after trolox treatment. Consistently, the hepatic PC/PE ratio is decreased in NASH patients [110, 111]. Overall, evidence of ferroptosis in NAFLD and NASH deserves further exploitation in meaningful models of disease and in patients, particularly since no therapeutic options are currently available for NASH.

3.2. Alcohol-associated liver disease and alcoholic steatohepatitis

Alcohol-associated liver disease (ALD) is a major health problem worldwide and a significant source of liver injury, characterized by a wide spectrum of hepatic lesions from steatosis, steatohepatitis and fibrosis that may progress to cirrhosis and even hepatocellular carcinoma [112]. Cell injury, inflammation, oxidative stress, regeneration and bacterial translocation are key drivers of alcohol-induced liver injury [113]. ALD develops via a complex process involving parenchymal and non-parenchymal cells, as well as recruitment of other cell types to the liver in response to damage and inflammation. Importantly, cross-regulation by the effectors of different cell death pathways most likely influences liver inflammation in ALD. It is possible that apoptosis occurs in early steatosis ALD, followed by necroptosis in early ASH and pyroptosis in alcoholic hepatitis, in agreement with neutrophilic inflammation and endotoxemia and septicaemia, the most common cause of death from alcoholic hepatitis.

3.2.1. Necroptosis—The involvement of necroptosis has been suggested from studies where RIPK3 expression was induced in human ALD and in mice after ethanol exposure [84, 114]. RIPK3-mediated necroptosis and neutrophil-mediated liver inflammatory response were also highly correlated with poor prognosis in patients with end-stage alcoholic cirrhosis [115]. RIPK3 deficiency, in turn, effectively protected from ethanol-induced serum liver enzymes, steatosis and inflammation [84, 114]. Nevertheless, inhibition of RIP1 kinase activity blunted Gao-binge alcohol-induced hepatic inflammation but did not protect against chronic ethanol feeding-induced steatosis and liver injury, suggesting that alcohol-induced RIPK3-mediated necroptosis is independent of RIPK1 [114]. Other studies reported that ethanol-induced decrease of Nrf2 expression was abrogated by curcumin or gallic acid treatment, by suppressing RIPK3 and RIPK1 expression as well as DAMP release [116, 117]. Overall, RIPK1 involvement remains to be consistently demonstrated in ethanol-induced hepatic injury, RIPK3 depletion should be attempted in other relevant cell types, and MLKL targeting warrants further exploitation. While it appears clear that ethanol-induced necroptosis involves RIPK3, it is not yet known if MLKL-independent pathways are involved as reported in models of autoimmune arthritis [118]. Further, increased infiltration of inflammatory cells in the liver after alcohol exposure requires the inclusion of inflammatory cell-specific knockout mice in future studies to clarify the role of necroptosis in alcohol-induced liver pathogenesis.

3.2.2. Pyroptosis—Excessive and uncontrolled activation of inflammasomes has a damaging effect on the host, inducing uncontrolled inflammatory responses and the consequent cell death. Recent evidence shows that pyroptosis plays a prominent role in ALD pathogenesis. The NLRP3 inflammasome pathway is activated in hepatocytes in response to endotoxin challenge, a condition facilitated by alcohol intake [119]. Consistently, NLRP3 deficiency ameliorated alcohol-driven liver steatosis and injury [120], while caspase-4/11 was upregulated in liver tissue from patients with alcoholic hepatitis and in mice [121]. Pyroptosis triggered by gut-derived PAMPs and by metabolic-derived DAMPs (ATP and uric acid) resulted in release of inflammasome-dependent cytokines from immune cells in ALD patients and in cells exposed to ethanol [120, 122]. Caspase-1 was also activated in hepatocytes of patients with alcoholic hepatitis [123]. Interestingly, a recent study

demonstrated that alcohol elicits caspase-1-mediated pyroptosis through overexpression of thioredoxin-interacting protein (TXNIP), a member of the α -arrestin family, and this event is reversed by miR-148a [124]. MiR-148a levels, in turn, are deregulated in the liver of patients with alcoholic hepatitis or ethanol-fed animals. Consistently, hepatocyte-specific delivery of miR-148a alleviates alcoholic liver injury. These data together with the fact that serum ALT activities remained elevated after macrophage depletion supports the direct effect of alcohol on NLRP3 inflammasome activation in hepatocytes. Pyroptosis is thus fit for stimulating and effectively sustaining the inflammatory cycle in ALD, although the specific contribution of NLRP3 inflammasome in immune cells and hepatic stellate cells remains still debatable.

3.2.3. Ferroptosis—Iron overload and increased oxidative stress have been well documented in ALD [125]. Recent findings shed light on mechanisms underlying the alcohol-induced liver damage by describing that adipose-specific lipin-1 overexpression accelerated iron accumulation, caused lipid peroxidation, reduced GSH and GAPDH, and promoted ferroptotic liver damage in mice after ethanol administration [126]. Lipin-1 is a Mg^{2+} -dependent phosphatidic acid phosphohydrolase involved in the generation of diacylglycerol during synthesis of phospholipids and triglycerides. Ethanol-mediated inhibitory effects on adipose-specific lipin-1 expression were associated with experimental steatohepatitis in rodents. The hepatic ferroptotic signalling in the liver damage induced by adipose lipin-1 overexpression in ethanol-fed mice remains, however, to be elucidated.

3.3. Other metabolic liver disorders

3.3.1. Hemochromatosis—Studies on cell death in other metabolic liver disorders, including iron-overload diseases, and genetic disorders such as Niemann-Pick disease or Gaucher's disease are scarce. Iron is implicated in the pathogenesis of a number of human liver diseases. Hereditary haemochromatosis is the classical example of a liver disease caused by iron, but iron is an important contributor to the progression of other forms of chronic liver disease, including NAFLD. Iron-induced liver damage is likely potentiated by coexisting inflammation, which culminates with hepatocyte cell death and drives fibrogenesis [127]. Hereditary hemochromatosis is caused by mutations in genes whose protein products limit iron absorption. As a consequence, excess iron generates ROS thereby inducing cell death and global oxidative damage, ultimately leading to severe chronic complications, including hepatic cirrhosis. Nevertheless, the precise roles of iron and iron metabolism in cell death are largely unknown. A recent study investigated the role of iron homeostasis in SLC7A11-mediated ferroptosis and found that iron overload triggers ferroptosis both *in vitro* and *in vivo* [128]. Indeed, *Slc7a11* knockout was not sufficient to induce ferroptosis under basal conditions but facilitated iron overload-induced ferroptosis due to impaired cystine uptake and increased ROS production. Ferroptosis is thus a potential therapeutic target for treating iron overload-associated diseases, including hemochromatosis. Other lytic forms of cell death need to be explored and extended further to other metal-related liver disorders.

3.3.2. Niemann-Pick disease—Niemann-Pick disease, type C1 (NPC1) is an inborn error of metabolism that results in endolysosomal accumulation of unesterified cholesterol. Clinically, NPC1 manifests as cholestatic liver disease in the newborn or as a progressive

neurodegenerative condition characterized by cerebellar ataxia and cognitive decline. Neuroinflammation and necroptosis contribute to the NPC1 pathological cascade. Pharmacological and genetic inhibition of RIPK1 kinase activity increased lifespan in both *Npc1*^{-/-} mice treated with RIPK1 inhibitors, and *Npc1*^{-/-}/*Ripk1* kinase death double mutant mice [129]. Nevertheless, the increase in lifespan was modest, suggesting that the therapeutic potential of RIPK1 inhibition, as a monotherapy, is limited. Conversely, no increased survival was noticed in *Npc1*^{-/-}/*Ripk3* mice compared to *Npc1*^{-/-} mice. These data suggest that although necroptosis is occurring in NPC1, the effects of RIPK1 inhibition may be related to its RIPK3-independent role in neuroinflammation and cytokine production. Nevertheless, corroborating the role of alternative RCD sub-routines, cellular death of NPC1 fibroblasts was not mitigated by treatment with caspase inhibitors [129] or following neuronal overexpression of Bcl-2 [130]. More importantly, necroptosis inhibition and combination therapy with 2-hydroxypropyl- β -cyclodextrin has recently been suggested for NPC1 [131].

3.3.3. Gaucher's disease—Necroptosis appears to have a role in the pathological cascade of other lysosomal storage diseases. Gaucher's disease is an inherited metabolic disorder caused by mutations in the glucocerebrosidase gene (*GBA*), and the most common lysosomal storage disease. Neuronal cell death in mice with Gaucher's disease is caspase-independent and nonapoptotic. In striking contrast, RIPK3-mediated necroptosis has been implicated in pathology of Gaucher's disease [132]. In fact, levels of *Ripk1* and *Ripk3* were markedly upregulated in Gaucher's disease brains, both in mice and in patients, but were unaltered in brains obtained from mouse models of NPC1. This indicates that although brain inflammation and microglial activation are shared features of many lysosomal storage diseases, pathways of neuroinflammation are disease specific. Moreover, RIPK3 deficiency improved the clinical course of Gaucher's disease mice, with improved survival and motor coordination and beneficial effects on cerebral and hepatic injury. Ablation of *Ripk3* resulted in fewer CD68-positive Kupffer cells and decreased ALT activity, suggesting that hepatocyte injury was also attenuated. Development of necroptosis inhibitors with improved pharmacokinetics may pave the way for alternative therapeutic approaches for Gaucher's disease, for which innovative treatment is urgently required. The specific involvement of other RCD forms is yet to be determined.

4. Controversies and Unsolved Issues

4.1. Perspective of cell death in liver diseases

Cell death has been described as a clear and present danger for hepatocytes as they are directly exposed to portal blood from the intestines, participate in the xenobiotic metabolism of drugs and alcohol, play a central role in lipid, fatty acid, and bile acid metabolism, contact with prevalent hepatotropic viruses, and reside within a milieu of innate immune responding cells [133]. Yet elucidating the precise role for various modes of cell death in liver diseases has proven difficult, and unpredictable. For example, apoptosis as a driving force in liver injury and carcinogenesis has only recently been established despite intense study over the last two decades [134]. It will also take time and detailed analysis of relevant models to identify the potency and hierarchal relationship between apoptosis, necroptosis, pyroptosis

and ferroptosis in liver diseases. Also, perturbations with therapeutic intent targeting one mode of cell death may potentiate or even inhibit additional modes of liver injury further complicating this analysis. For example, caspase inhibitors employed to prevent apoptosis, may promote necroptosis by preventing caspase-8 proteolytic degradation of RIP1 kinase, or inhibit pyroptosis by blocking caspase-1 or -8. In this section of this review, we will acknowledge these controversies providing nuanced insight and a critical appraisal of the information described above.

4.2. Unresolved issues regarding necroptosis in liver diseases

The role of necroptosis in liver diseases remains to be firmly established. This uncertainty relates to current tools, models, and evolving information that permits a reinterpretation of prior studies. First, there is vigorous controversy regarding the expression of RIPK3 by hepatocytes, but under basal conditions expression appears to be quite limited [135, 136]. Whether they can be induced in various liver diseases remains unclear, as the antibodies employed for immunoblot analysis and immunohistochemistry are fraught with nonspecificity. We are not aware of any detailed proteomic studies which have been performed to address these questions, although the technology to examine phosphoproteins by this approach is widely available. Moreover, activating phosphorylation of RIPK3 and MLKL have been identified in the absence of cell death questioning the use of these phosphoproteins as biomarkers for necroptosis [135]. Second, RIPK3 deficiency does not phenocopy MLKL deficiency, and hence studies with RIPK3 knockout or kinase dead knock-in mice, are not sufficient to invoke necroptosis as a cause of cell death, as RIPK3 has diverse functions [137]. Moreover, inhibition of RIPK3 kinase often provokes apoptosis further confusing this approach in examining necroptosis in disease pertinent models [25]. The multifunctional aspect of RIPK3 biology in different cell types certainly needs to be taken into account when germ line vs. cell type specific knockout or kinase dead knock-in paradigms are employed. Third, caspase-8 limits necroptosis by proteolytically cleaving RIPK1 [138], and the genetic absence of caspase-8 in hepatocytes does not trigger widespread necroptosis [134]. Finally, genetic studies employing RIPK3 knockout mice suggest cholangiocyte, but not hepatocyte injury is ameliorated suggesting necroptosis mediates cholangiocyte but not hepatocyte injury [134]. The above observations will need to be reconciled with those presented above implicating necroptosis in liver disease (see future directions below).

4.3. Unresolved issues regarding pyroptosis in liver diseases

Pyroptosis requires caspase-dependent cleavage of GSDMD to plasma membrane pore-forming fragments [139]. Cleavage of GSDMD occurs via a canonical inflammasome caspase-1 dependent mechanism, a non-canonical inflammasome pathway involving caspases 4, 5 or 11 [139], or in an inflammasome-independent caspase-8 mediated pathway [140]. Although all cell types appear to be inflammasome competent, the best examples are in cells of the innate immune system, especially those interacting with intracellular organisms. Cell type specific GSDMD will be requisite to examine this form of cell death in hepatocytes. Interestingly, in shock models of liver injury, GSDMD germ line deficient mice display increased caspase-8 activation and apoptosis in the liver [141]. This is another

cautionary example where inhibiting one mode of cell death enhances a different mechanism of liver injury.

4.4. Unresolved issues regarding ferroptosis in liver diseases

Oxidative stress has long-been implicated in liver injury. Ferroptosis which is regulated by the availability of cytosolic free iron, cystine transport into the cell, GPX4 activity, and glutathione levels, leads to cell death by lipid peroxidation. A specific linchpin molecular executioner of ferroptosis has not been described, and hence genetic deletion of a final common pathway mediator is not yet feasible. In fact, propagation of lipid peroxidation is non-enzymatic and may prove difficult to target. In this regard, the role of ferroptosis in liver disease will be largely circumstantial, and we note that to date, antioxidant therapy has not been impactful in human liver diseases. Careful work will be necessary to dissect the ultimate role of ferroptosis in liver diseases.

5. Future directions and therapeutic implications

The contribution of different modes of cell death to various liver diseases will require carefully defined preclinical studies that address causality. These studies will need to focus on the terminal executioners of cell death, as upstream pathway mediators have multiple functions. For example, RIP kinases have diverse functions in addition to promoting necroptosis [90]. Such studies need to focus initially on hepatocyte cell death employing hepatocyte conditional knockout models of MLKL, GSDMD, and Bax plus Bak, to discern the interrelationships between necroptosis, pyroptosis and apoptosis. These studies will require development of genetic mouse models and hence will take time. Also, conditional knockouts in other cell types (*e.g.*, cholangiocytes, immune cells such as macrophages) will ultimately be necessary to complete this puzzle of cell death in liver injury. Finally, we note that cell death *per se* may be a biomarker for stressed, proinflammatory hepatocytes as recently suggested in NASH [142], and therefore, precluding cell death may not have the major impact on liver injury that we anticipate.

These various modes of cell death also represent unique therapeutic avenues for the treatment of cell death in human liver diseases. However, inhibiting one mode of cell death may potentiate other modes of cell death as noted above, negating a therapeutic benefit. Recently reported, caspase inhibition with Emricasan treatment did not improve clinical outcomes nor liver histology in patients with NASH fibrosis, resulting in more liver fibrosis and hepatocyte ballooning [143, 144]. Caspase inhibition may have directed cells to alternative, more inflammatory mechanisms of cell death, such as necroptosis. Indeed, Emricasan has previously been shown to shift cell death from apoptosis towards necroptosis in acute myeloid leukemia cells treated with birinapant, a second mitochondrial derived activator of caspases mimetic, which enhances cancer cell apoptosis [145]. MLKL inhibitors are being developed such as necrosulfonamide, which binds to the N-terminal 4 helical bundle of MLKL to prevent necroptosis [146, 147]. Indeed, a variety of targets and inhibitors are being investigated to block necroptosis, and we refer the reader to this literature [147]. Interestingly, necrosulfonamide also binds GSDMD and blocks pyroptosis

[148]. As these compounds and others become further developed as pharmacologic agents, we look forward to future human clinical trials with anticipation.

In summary, we now recognize several different forms of cell death, executed by different pathways and mediators. The mechanisms are being unraveled and their contribution to human liver diseases is being carefully examined. These insights provide further complexity to understanding liver injury, but these challenges also provide new and exciting therapeutic opportunities.

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Key points

- Regulated hepatocyte cell death drives metabolic liver disease progression to liver inflammation, fibrosis and cirrhosis.
- Accumulating evidence suggests that lytic cell death modalities such as hepatocyte necroptosis, pyroptosis and ferroptosis play pathophysiological roles in metabolic liver disease, while other functions in non-hepatocyte liver cells, immune cells and others have been suggested.
- Functional characterization of lytic cell death in appropriate models of metabolic liver disease and in patients might result in novel therapeutic strategies that target regulated hepatocyte cell death to prevent disease progression.

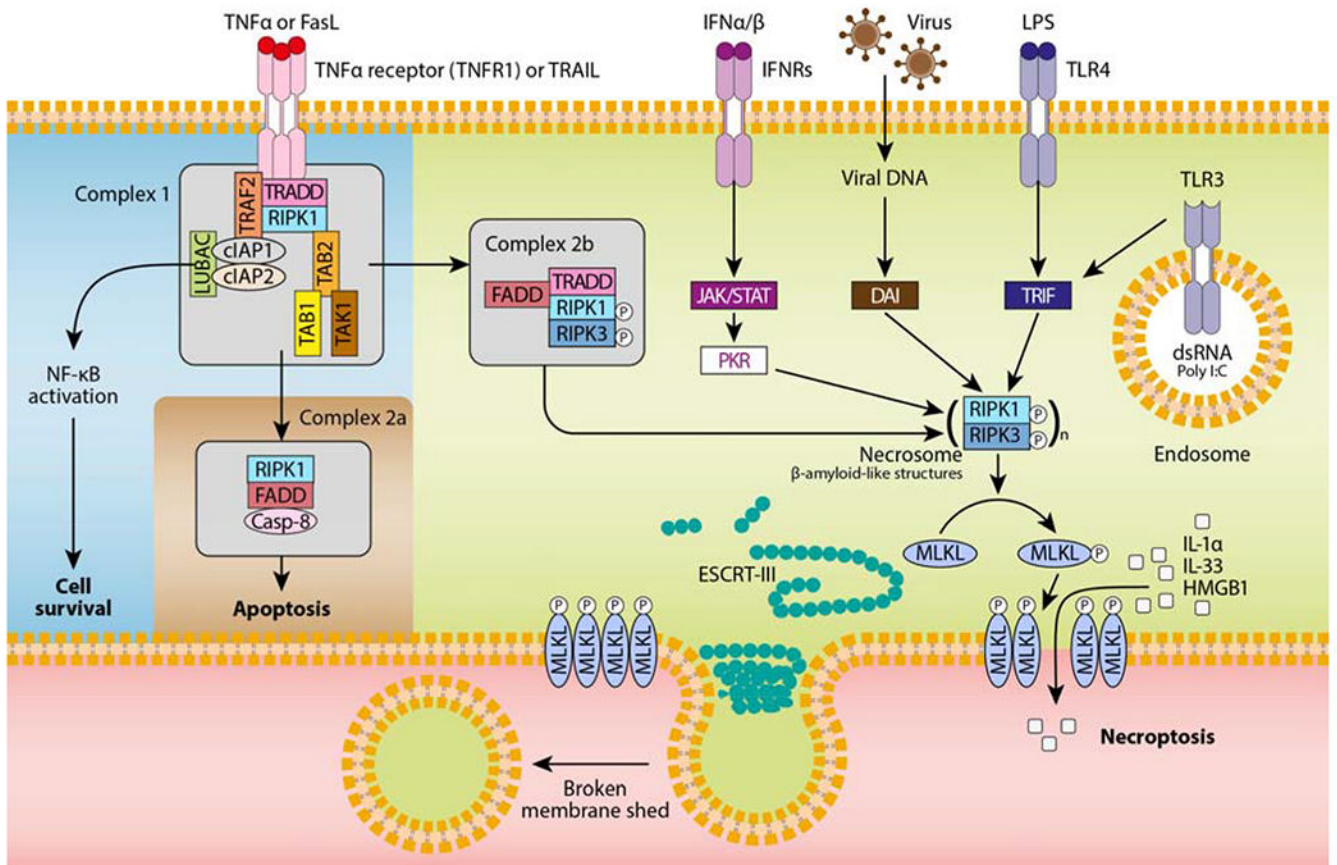


Figure 1. RIPK3- and MLKL-dependent necroptosis mediated by various stimuli.

TNF- α or FasL binding to their receptor induces the formation of a receptor proximal complex (complex 1), which consists of adaptor proteins (*e.g.*, TRADD, tumor necrosis factor receptor type 1-associated death domain; FADD, fas-associated protein with death domain; TAB, transforming growth factor beta-binding protein), ubiquitin ligases (*e.g.*, TRAF2, TNF receptor associated factor 2; LUBAC, linear ubiquitin chain assembly complex; cIAPs, cellular inhibitors of apoptosis), and kinases (*e.g.*, TAK, transforming growth factor beta-activated kinase 1; RIPK1, receptor interacting protein kinase 1). The ubiquitination of RIPK1 provides docking sites for the recruitment of key proteins leading to cell survival by nuclear factor κ B (NF- κ B)-dependent upregulation of pro-survival genes. Then, complex 1 internalization leads to the formation of complex 2a, which ultimately results in caspase-8-dependent apoptosis. Finally, when caspases or cIAPs are inhibited, RIPK1 associates with RIPK3 to form the necrosome (complex 2b), which in turn recruits the pseudokinase MLKL. RIPK3-mediated phosphorylation of MLKL results in MLKL translocation to the plasma membrane and pore formation. Other necroptotic stimuli such as IFN α/β , virus, pathogen-associated molecular patterns (*e.g.*, LPS, poly I:C) are also depicted. Although the nature of these signalling complexes is not completely characterized, the main mediators activating the necrosome are shown. To antagonize necroptosis, ESCRT-III components are recruited to localized sites of MLKL-directed membrane damage to shed broken membrane into blebs.

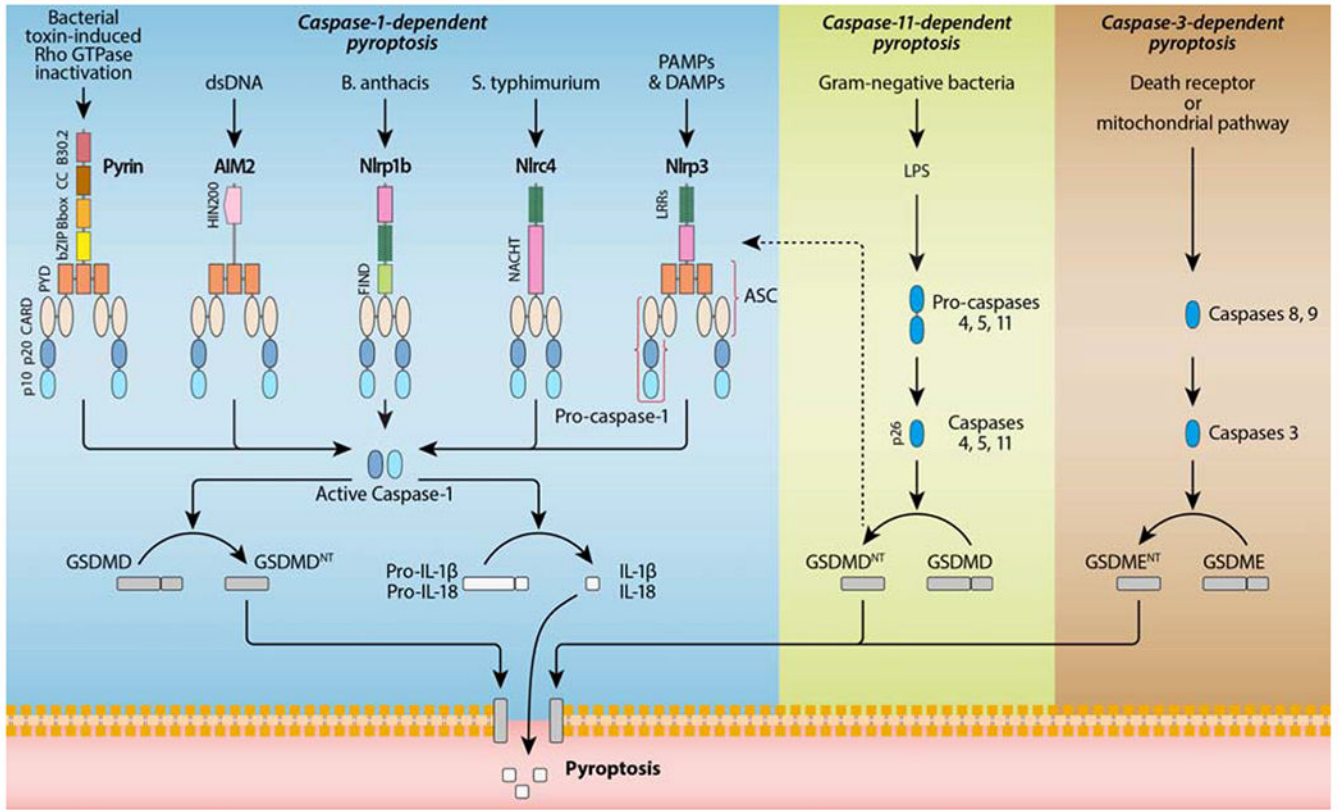


Figure 2. Caspase-1-dependent and -independent pyroptosis.

Caspase-1-dependent pyroptosis requires activation of the canonical inflammasomes, including NLRP1b, NLRP3, NLRC4, AIM2 and Pyrin. The NLRs are characterized by the combined presence of a nucleotide-binding and oligomerization domain (NACHT) and a variable number of LRRs. They also contain either a caspase recruitment domain (CARD) or pyrin domain (PYD) in their amino-terminus. The AIM2 protein is composed of a N-terminal PYD and a C-terminal DNA-binding HIN200 domain. The pyrin protein has a N-terminal PYD, a bZIP transcription factor domain, a B-box, a coiled-coil, and a C-terminal B30.2 domain. NLRP1b and NLRC4 recruit caspase-1 *via* their CARD domain, and the bipartite PYD-CARD adaptor protein ASC is required for assembly of the AIM2, NLRP3 and pyrin inflammasomes. The pyrin protein also binds directly to caspase-1 *via* its B30.2 domain. Caspase-1-independent pyroptosis requires the activation of caspase-11, which recognizes cytosolic LPS and cleaves GSDMD to initiate pyroptosis. In this pathway, GSDMD^{NT} also activates the NLRP3 inflammasome and thereby caspase-1-dependent maturation of IL-1 β and IL-18. Caspase-3-dependent pyroptosis requires the activation of Gasdermin E (GSDME). Caspase-3 can be activated by the mitochondrial and death receptor pathway. Active caspase-3 cleaves GSDME, to produce GSDME N-fragments (GSDME^{NT}), which then forms pores in the plasma membrane.

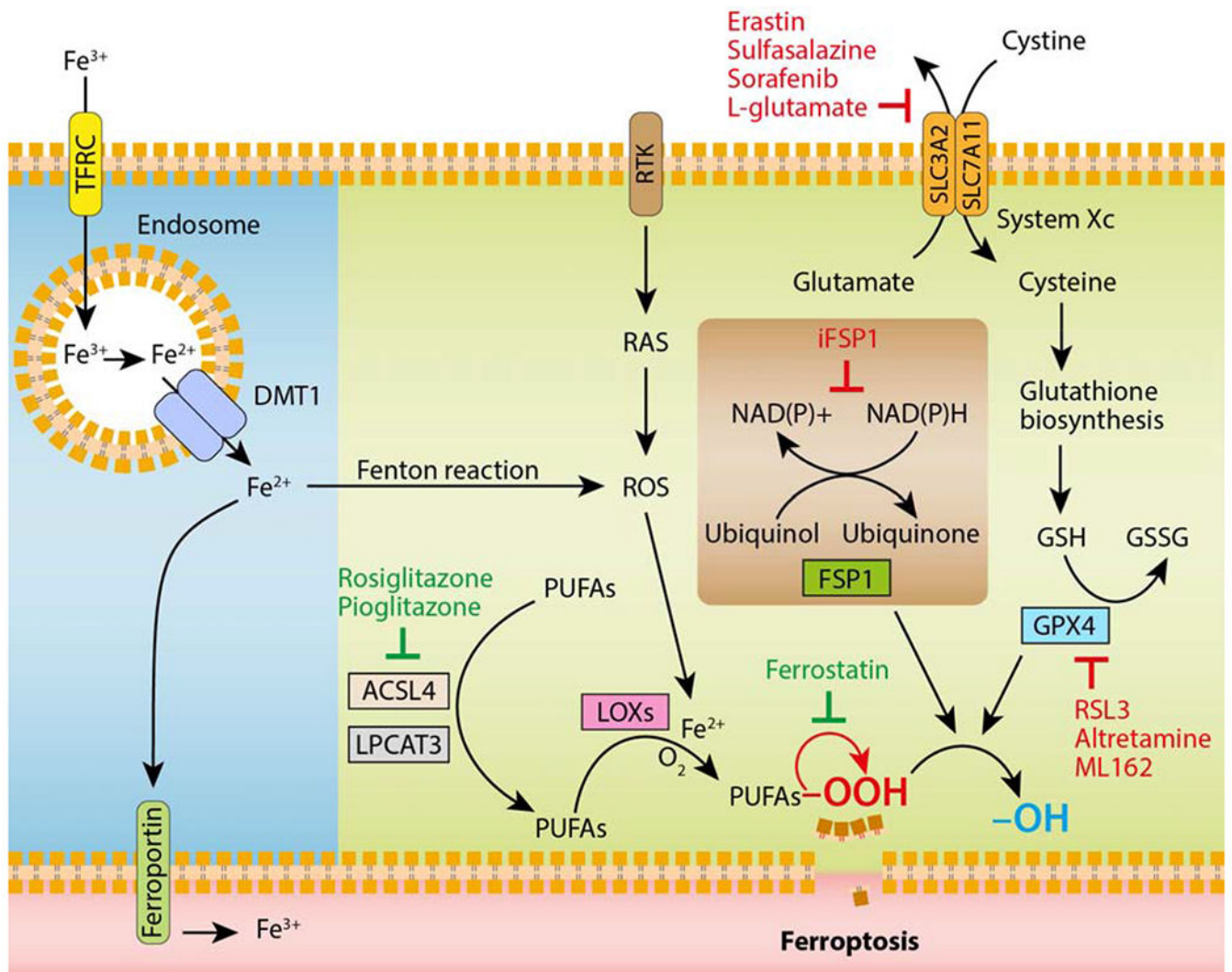


Figure 3. The core components controlling ferroptosis.

Glutathione (GSH) is a tripeptide essential for preventing damage caused by ROS, such as lipid peroxides. It is synthesized continuously from cysteine, glutamate and glycine. Cellular availability of cysteine is the limiting step for GSH synthesis. The Xc⁻ transporter, which consists of two subunits belonging to the family of solute transporters (SLC3A2 and SLC7A11), is responsible for the uptake of extracellular cystine, the precursor of cysteine. GSH provides electrons to the key regulator of ferroptosis, GPX4, which reduces lipid peroxides (-OOH) in plasma membranes to the corresponding alcohols (-OH). Recently, the enzymes ACSL4 and LPCAT3 that are directly involved in shaping the cellular lipid composition, have been shown to sensitize cells to ferroptosis. Oxidation of lipid bilayers during ferroptosis occurs both in an enzymatic (*i.e.*, LOXs, lipoxygenases) and non-enzymatic (*i.e.*, radical-mediated autoxidative; symbolized by the red arrow) manner. Alternatively, FSP1 can suppress ferroptosis by ubiquinol, the reduced form of ubiquinol, which traps lipid peroxy radicals. FSP1 catalyses the regeneration of ubiquinol using NAD(P)H. Cellular iron homeostasis, which is dependent on the coordination of iron uptake (*i.e.*, TFRC) and export (*i.e.*, ferroportin), directly regulates ferroptosis through Fenton

reactions and generation of ROS. Fe^{3+} is reduced to Fe^{2+} by metalloreductases in the endosome, and the divalent metal transporter 1 (DMT1) mediates the transport of Fe^{2+} from the endosome into a labile iron pool in the cytoplasm. Receptor tyrosine kinase (RTK) activates the oncogene RAS, which is known to induce oxidative stress through generation of ROS. Ferroptosis-inducing drugs are depicted in red, whereas ferroptosis inhibitors are shown in green.