Cell death mechanisms in human chronic liver diseases: a far cry from clinical applicability

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Summary

The liver is constantly exposed to a host of injurious stimuli. This results in hepatocellular death mainly by apoptosis and necrosis, but also due to autophagy, necroptosis, pyroptosis and in some cases by an intricately balanced combination thereof. Overwhelming and continuous cell death in the liver leads to inflammation, fibrosis, cirrhosis, and eventually hepatocellular carcinoma. While data from various disease models may suggest a specific (predominant) cell death mode for different etiologies, the clinical reality is not as clear cut. Reliable and non-invasive cell death markers are not available in general practice and assessment of cell death mode to absolute certainty from liver biopsies does not seem feasible, yet. Various etiologies probably induce different predominant cell death modes within the liver. Although, the death modes involved may change during disease progression. Moreover, current methods applicable in patients are limited to surrogate markers for apoptosis (M30), and possibly for pyroptosis (IL-1 family) and necro(pto)sis (HMGB1). While markers for some death modes are not available at all (autophagy), others may not be specific for a cell death mode or might not always definitely indicate dying cells. Physicians need to take care in asserting the presence of cell death. Still the serum-derived markers are valuable tools to assess severity of chronic liver diseases. This review gives a short overview of known hepatocellular cell death modes in various etiologies of chronic liver disease. Also the limitations of current knowledge in human settings and utilization of surrogate markers for disease assessment are summarized.

Introduction

All living cells eventually die. In our current knowledge about cell death, two general types are observed and have been defined by the Nomenclature Committee on Cell Death (NCCD): accidental cell death (ACD) due to physicochemical stress and regulated cell death (RCD), involving distinct pathways and activation of the cellular machinery leading to specific forms of cell demise. Historically morphological alterations of cells have been employed to discern different types of cell death. Enlarged and swollen cells, disruption of membrane integrity, size increase of organelles and distended albeit intact nucleus, which undergoes karyolysis in later stages, have been ascribed to necrosis, the currently only entity of ACD. Cell shrinkage, accompanied with membrane blebbing, and nuclear fragmentation have been associated to apoptosis, which was the first type of RCD described. By now it is clear that mere morphological characterization of cell death cannot distinguish all possible variants of cell demise. Biochemical measurement of regulators, effectors and their specific activity in a given setting are imperative to characterize cell death. Moreover, recent findings show that on the one hand RCD modes can be altered by blocking of regulative components leading to a switch of cell death mode, on the other hand for most types of RCD regulators and effectors are kept in check by counter-regulatory mechanisms (a schematic overview of current interaction between more common cell death modes is given in Figure 1). Thus, even activation of caspase-3, deemed a definite sign for ongoing apoptosis, might not lead to cell demise in all settings. For the many (seemingly) different RCD modes observed by now, only few have been studied extensively in clinical settings of liver diseases. Surrogate markers, detectable in serum have been ascribed to specific cell death modes, though in some cases that may not be sufficient to even assure cellular demise at all. This overview aims to summarize current knowledge about occurrence and contribution of known cell death modalities in various liver diseases. Also evidence will be critically assessed, if data is sufficient to state that a specific cell death mode may be predominant in various settings of liver injury.

Basic concepts of different cell death modalities

Apoptosis

Apoptosis is the most prominent mode of RCD and according to many studies the most important mechanism of liver cell demise [1–3]. Though, apoptosis does not only occur after injury or in disease in mature tissues. Apoptosis is also a crucial process during morphogenesis and development of tissues [4,5]. Morphologic hallmarks of apoptosis include the presence of rounded cells, chromatin condensation, nuclear fragmentation, plasma membrane blebbing, and formation of apoptotic bodies containing organelles and cytoplasm [6,7]. Biochemical indicators of apoptosis are mitochondrial outer membrane permeabilization (MOMP), phosphatidylserine exposure, and the activation of caspases, a family of cysteine-dependent specific proteases [8]. It is important to highlight that morphological classification of cell death should be considered with caution. Particular morphological patterns of cell demise may be observed during biochemically different cell death modes [8,9]. Therefore, biochemical classification of cell death is preferable to morphological assessment, although single biochemical readouts are not sufficient to define a type of cell demise [8,9]. Apoptosis is sub-categorized into extrinsic apoptosis and intrinsic apoptosis [8].

Extrinsic apoptosis refers to apoptosis induced by the activation of death cell receptors, by interaction of extra-cellular stress signals [10–13] (Figure 2). Known death ligands and receptors are: i) FAS/CD95 ligand, which is bound by the receptor FAS/CD95; ii) TNF-α and TNF-related apoptosis inducing ligand (TRAIL), which are bound by TNF-α-receptor 1 (TNFR1), and TRAIL 1-2 receptors, respectively. Death receptors are transmembrane receptors and ligand binding outside the cell leads to conformational change and/or dimerization of the intracellular cell death domain (DD) [14]. This

conserved sequence enables not only dimerization or multimerization of receptors but also recruitment of different proteins. Depending on the involved receptor among recruited factors are receptor-interacting protein kinase 1 (RIP1), Fas associated protein with a DD (FADD), c-FLIP (FADD-like IL-beta converting enzyme (FLICE) inhibitor protein), cellular inhibitors of apoptotic proteins (cIAPs), and procaspase-8 [15,16]. TNFR1-like receptors also require recruitment of TNFR associated DD (TRADD). The generated multiprotein complex is called the death inducing signal complex (DISC), and regulates the activation of initiator caspases 8 and 10 [17]. c-FLIP and cIAPs act as pro-survival factors by inhibiting caspases [18–20]. Once caspase 8 and 10 are activated, they cleave the effector caspases 3, 6 and 7, which finally lead to cell demise. Extrinsic apoptosis can be suppressed by the use of pancaspase inhibitors, for example Z-VAD-fmk [21].

In parenchymal liver cells, generally considered type II cells, extrinsic cell death is induced by MOMP. Cleavage of BH3-interacting domain death agonist (BID) by caspase 8 generates truncated (t-)BID. t-BID interacts with BAX and BAK (pro-apoptotic BCL-2 protein family members; see also table 1) to form mitochondrion-permeabilizing pores [22–25], leading to release of cytochrome c, SMAC-DIABLO, endonuclease G (ENDOG), and apoptosis-inducing factor (AIF) [26,27]. In hepatocytes, the release of cytochrome c promotes formation of the apoptosome and subsequent activation of the effector caspases 3, 6 and 7. Although hepatocytes generally require MOMP to amplify extrinsic apoptosis signaling they are also able to undergo apoptosis in a mitochondrion-independent manner, given a sufficiently strong extrinsic signal [28]. An overview of different extrinsic apoptosis mechanisms is given in Figure 2.

Intrinsic apoptosis can be triggered by a wide variety of intracellular stress stimuli including but not limited to DNA damage, oxidative stress, cytosolic Ca^{++} overload, and ER (endoplasmic reticulum) stress. Intrinsic apoptosis is regulated by BCL-2 family members [29,30]. All described stressors converge in a mitochondria-dependent control mechanism [31], where pro-apoptotic and anti-apoptotic signals are integrated, leading to MOMP, when pro-apoptotic signals predominate [31–33]. As described above, MOMP results in mitochondrial dysfunction due to a block of ATP generation, loss of mitochondrial trans-membrane potential, generation of reactive oxygen species (ROS), and the release of mitochondrial inter-membrane space proteins into the cytosol with subsequent assembly of the apoptosome [31,34].

There is also crosstalk between c-Jun N-terminal kinase (JNK) signaling and apoptosis. In response to TNFR1 signaling or to ROS JNK plays a key role in death signaling cascades triggered by TNFa and other toxins [35], including free fatty acids-induced hepatocyte lipoapoptosis [36]. Sustained ER stress is also able to trigger a mitochondrial pathway of apoptosis, which involves the stress sensor inositol-requiring enzyme 1, apoptosis signal-regulating kinase 1, and downstream activation of JNK [37]. Finally it has been shown that JNK can phosphorylate specific Bcl-2 proteins and induce MOMP, seemingly independent of classic cell death signals for apoptosis [38,39] Though, the initiation of cell death due to JNK can also occur in a necrotic type, possibly with mutual activation of RIP3 (see below) [40,41], complicating interpretation of JNK activation in cell death.

Necrotic cell death

In contrast to apoptosis (as process of tissue homeostasis and remodeling) necrosis implies a pathological condition, in particular for acute and massive hepatic injury

[1,42,43]. Morphological changes observed in necrosis include formation of vacuoles, karyorrhexis (which also occurs during apoptosis), and karyolysis [8]. Historically, necrosis was seen as unregulated process, where cells undergo lysis due to severe physicochemical stress [44]. While this type of ACD can occur, current findings have identified mechanisms regulating the process of necrosis [44–46]. Three possible types of regulated necrosis have been identified, yet, which seem to converge on mitochondrial damage and loss of inner membrane integrity of mitochondria:

- Mitochondrial permeability transition pore (MPTP) related regulated necrosis;
- Necroptosis, induced by extracellular signals during inhibition of caspases;
- PARP-dependent regulated necrosis.

Current knowledge on necrosis and regulative molecular mechanisms have been excellently summarized by Karch and Molkentin [44]. In the following only the core concepts of regulated necrosis and necroptosis are outlined.

Regulated necrosis

Necrosis is characterized by loss of mitochondrial integrity, loss of ATP, and a stop in the ATP-dependent ion pumps leading to swelling and cell rupture. The uncontrolled release of intracellular components, e.g. high-mobility-group-protein B1 (HMGB1) and HDGF, leads to an inflammatory response by the immune system [42,47]. Mitochondrial permeability transition pore (MPTP), regulated by cyclophilin D, induces loss of phosphorylative oxidation, generation of ROS, and depletion of ATP [48]. Assembly of the MPTP occurs under conditions of calcium overload and increased ROS within mitochondria, as is the case in ischemia/reperfusion injury [49]. Opening of the MPTP results in ATP consumption rather than production by mitochondria [49]. ATP

depletion is a key biochemical event and cause of necrosis [31,50]. Depleted ATP-reserves block execution of apoptosis, which could occur since MPTP formation subsequently leads to MOMP and release of factors causing apoptosome assembly [51]. It was observed that Bax and Bak are necessary for MPTP-mediated necrosis and that mitochondria from mice deficient in Bax and Bak or cyclophilin D are resistant to MPTP [51–53].

Upon mitochondrial damage AIF and ENDOG are released and translocate into the nucleus, where DNA fragmentation is induced [54–56]. Release of mitochondria-derived endonucleases seems to occur as consequence of apoptotic as well as regulated necrotic cell demise [57–59]. A prominent example for DNA fragmentation by endonucleasis is acetaminophen toxicity [60]. Results from murine models demonstrate RIPK1-dependent but RIPK3-independent, non-necroptotic cell death [61–63]. Though, these findings have not been confirmed in the clinical situation, yet.

Necroptosis

In contrast to accidental cell death, programmed necrosis or necroptosis represents a programmed cell death biochemically separated from apoptosis [45,46,64]. DNA damage or stimulation of the pro-apoptotic cell death receptors TNFR1, TNFR2, TRAIL1-2, and FAS can induce necroptosis in different type of cells under low ATP concentrations, or when inhibitors of caspases are present [65]. Signaling via TNF-α is considered most important for necroptosis [66]. When caspase 8 is inhibited (e.g. by the use of caspase inhibitor drugs or genetic knockdown) binding of TNF-α to TNFR1 is able to induce necroptosis [67–69]. Conversely, caspase 8 is a main inhibitor of necroptosis by cleavage of receptor interacting kinase-1 (RIPK1), a crucial signal

transducer for necroptosis. RIPK1 and RIPK3 regulate necroptosis and determine the necrotic response to TNF-α [67,68] by formation of the necrosome (including FADD, cFLIP, and caspase 8). Within the necrosome (or riptosome) RIPK3 phosphorylates the mixed linage kinase like protein (MLKL), which integrates into membranes. This leads to release of intracellular components into extracellular space and subsequent inflammatory responses and membrane rupture [70,71]. The anti-apoptotic cIAPs can also counter necroptosis by polyubiquitinilation of RIPK1, resulting in the activation of the transcription factor NF-κB (pro-survival signal) [72]. Another downstream effector of RIPK1/RIPK3 may be poly(ADP-ribose) polymerase 1 (PARP1) (involved in DNA repair, and transcription regulation), reducing ATP [73], Though, PARP1 might also exert a RIPK1/RIPK3 independent necrosis program [74] or might serve as a switch between apoptosis and necrosis [75]. It is still a matter of debate if necroptosis plays a role in pathophysiologic settings, as liver RIPK-3 expression is low [76], except for NASH [40].

Pyroptosis

Pyroptosis is a recently discovered pathway of programmed cell death downstream of inflammasome activation. Despite its dependence on caspase activation (in this case caspase-1), pyroptosis is morphogically similar to necrosis, as it leads to membrane rupture or pore formation. Inflammasomes are multiprotein complexes which may vary in composition. So-called canonical inflammasomes either contain (often multiple copies) Apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain) (ASC) or one protein of the nucleotide-binding oligomerization domain-like receptor (NLR) family. The most prominent members of the NLR family

are NLRP1, NLRP3, and NLRC4. The inflammasome complex itself is activated during bacterial infections via PAMPs (pathogen associated molecular patterns, i.e. bacterial products and compounds), DAMPs (danger associated molecular patterns: usually intracellular localized substances as ATP or HMGB1, nucleic acids, and some lipoproteins), or ligation of TNF-α to the TNFR1. Specificity of stimulus and induced type of inflammasome has been reviewed comprehenseively by de Vasconcelos et al. [77]. Inflammasome activation leads to self-cleavage of caspase 1, which is the main effector caspase in pyroptosis. In mice also caspase-11 and in human caspases-4 and -5 can be recruited by inflammasomes. Upon caspase-1 activation the proinflammatory cytokines pro-IL-1β and pro-IL-18 are cleaved and released. IL-1 and IL18 in addition to the release of intracellular components during pyroptosis confer a strong inflammatory stimulus in the affected tissue. Among the many substrates cleaved by caspase 1 is gasdermin, which seems to be essential for cell death execution as pyroptosis [78,79]. Of note, inflammasome activation can also induce apoptosis in parallel to pyroptosis [47,80–82].

Autophagy

Autophagy, or macroautophagy, is an intracellular process maintaining cellular homeostasis and energy production by degradation of cytosolic components. Autophagy also removes unnecessary or dysfunctional cellular components as well as long-lived proteins from the cell [83,84]. Morphologic characteristics of autophagy include vast cytoplasmic vacuolization due to formation of double-membrane vesicles called autophagosomes. Autophagosomes contain cytosolic organelles and proteins and are fused with lysosomes resulting in the formation of autolysosomes. The autolysosome content is then degraded by acidic hydrolases. Products of this process are released into

the cytoplasm for recycling or energy generation. Autophagy is particularly important for liver cell homeostasis. In the absence of injury hepatocytes are in a quiescent state and may be susceptible to accumulation of missfolded and dysfunctional proteins [83,85]. The basal level of autophagy in the liver is increased under different situations such as starvation or hypoxia to promote cell survival [83,86]. The importance of autophagy in the liver becomes obvious in patients with alpha-1-antitrypsin (α1AT) deficiency. These patients have an altered autophagy process and improperly folded α1AT accumulates within hepatocytes inducing damage [87]. Autophagy is generally considered a protective process for the liver [88,89], and its inhibition has been linked to cellular stress and apoptosis and necrosis [90]. In contrast, activation of autophagy related mediators may also block apoptosis execution and rescue cells from death. Conversely caspase-mediated cleavage of Beclin 1, an important autophagy related mediator, inhibits its capacity to facilitate autophagy [91]. In settings of caspase inhibition, cell death can occur due to autophagy [92,93]. This may be mediated by regulators of autophagy as Beclin-1 and ATG5 [91,94] or indirect *via* generation of toxic ROS concentrations by autophagy [95]. The relevance of autophagic cell death or autosis for clinical settings is unclear, yet, as these findings derive from *in vitro* experiments. Monitoring autophagy is a very difficult task, and available methods are unable to distinguish high autophagy rates from cases in which the last step of autophagy is blocked [96]. According to the NCCD autophagy is present in a particular model system when markers of autophagy, such as LC3/*Atg8*, are used in parallel to blockers of autophagy flux [9]. Unambiguous identification of autophagy in human samples requires detection of multiple steps of this process, i.e. formation of autophogosomes visualized by electron microscopy, which can be challenging. Thus, a deeper understanding of autophagic processes in actual human pathobiology is needed,

before autophagy related genes or proteins might serve for diagnostic or even therapeutic purposes.

Crosstalk between apoptosis, necrosis, and autophagy

As has already been mentioned the biochemical identity of a cell death mode might not be as clear cut, as often thought. Many stimuli can induce regulated demise of a cell in various forms, for example endoplasmic reticulum (ER) stress can trigger both apoptosis and autophagy [97]. This is due to interrelated and sometimes redundant mechanisms. The result, which mode of cell death is finally executed, depends on the state of the cell and its energy reserves, presence of infectious agents (i.e. HBV blocks execution of apoptosis), and many other factors. For example programmed necroptosis is one example of the crosstalk between apoptosis and necrosis [46]. Important cell death hubs connecting different regulated cell death modes are ATP (energy balance), p53, and BCL-family proteins. Since Apoptosis is an ATP-dependent process, substantial depletion of ATP leads to a switch from apoptotic to necrotic cell demise [98]. Factors affecting mitochondrial ATP production, such as PARP1 are considered important molecular regulators of the interface between apoptosis and necrosis [73]. The mitochondrial effector protein AIF controls the caspase-independent apoptotic cell death [56]. However, AIF has also been implicated in necroptotic cell demise after DNA damage together with PARP1 [99] (see below).

The p53 response to injury (e.g. DNA damage or hypoxia) results in the stimulation of the apoptotic machinery either indirectly, via FAS/CD95, BAX, PUMA or BID, or directly via MOMP [100–103]. p53 controls not only the intrinsic and extrinsic apoptosis pathways but has also a role in necrosis [104], and seems to interact with

cyclophilin D, a key regulator of the MPTP. p53 can also induce autophagy through the inhibition of the mammalian target of rapamycin (mTOR) [105]. BCL-2 family proteins regulate the integrity of mitochondria [25,106], and a crucial step in intrinsic apoptosis is MOMP due to BCL-2 family proteins. Within the BCL-2-family proteins three major groups are known, comprising anti-apoptotic proteins, proteins able to form pores in the outer membrane of mitochondria, and BH3 only proteins which act either anti- or pro-apoptotic (see table 1 for an overview of BCL-2 proteins). However, members of the BCL-2 family seem to be also involved in MPMT formation during necroptosis [38,51–53].

In summary, the complex regulation of the various types of cell death (which have not been described to completeness in the above paragraphs) makes it difficult to identify clear-cut cases *in vivo*. In an organism cell death of multiple forms may occur at the same time or subsequently due to tissue injury. As the situation regarding stimuli, energy supply, and previous damage of each individual cell may influence outcome of cell death, even a single damaging process or cell death signal might not lead to the same results in all cells. In addition, it should be noted that many of the general mechanisms described here were the results of studies performed in cell lines, murine embryonal fibroblasts, or very specific genetic-(multi-)knockout models. Many of these results still await confirmation in primary hepatocytes, relevant *in vivo* models and in most cases in human tissue samples. It is well known, that pre-clinical findings often cannot be transferred to the situation in the patient. This makes clinical application of cell death on the one hand as diagnostic tool and on the other hand as therapeutic target a highly challenging task. In the following we describe current knowledge in chronic liver disease on the above described cell death types, although in some cases evidence may be sparse.

Cell death mechanisms in various chronic liver diseases

NAFLD/NASH

In the course of the obesity pandemic, non-alcoholic fatty liver disease (NAFLD) has increased to similarly epidemic proportions all over the world [107,108]. NAFLD progresses to non-alcoholic steato-hepatitis (NASH) in about 20% and is characterized by the presence of inflammation with different degrees of fibrosis [109]. Although the driving force for disease progression is still under investigation, apoptosis of hepatocytes is supposed to be a key step for development from simple steatosis (NAFL) towards NASH [110–112]. Lipotoxicity is one of the major suspects as cause for hepatocellular damage, caused by free fatty acid accumulation [113,114]. Hepatocellular apoptosis has been found in human NASH and expression of death ligands and death receptors is increased in liver tissue of NASH patients [110,115]. In addition effector genes of apoptosis (PUMA, BIM) have been observed elevated in NASH [116,117]. Concurrently caspase 8 activation has been identified in NASH patients [118], suggesting an extrinsic activation of apoptosis pathways. Cytokeratin 18 (CK-18) cleavage by caspases generating a neo-epitope [119] detectable in sera of NAFL and NASH patients [120] will be discussed below. Apart from apoptosis related genes and factors expression of RIPK3 seems to be increased in livers from patients with NASH [40,121]. Higher amounts of RIPK3 and MLKL in liver tissue of NASH patients were observed compared to controls and still slightly higher as in steatosis [122]; although mere raised expression of these proteins does not necessarily imply ongoing necroptosis. After all serum concentrations of HMGB1 were similar in NAFL and NASH. Positive staining in immunohistochemistry also has to be interpreted with care, because of possible unspecific staining of biliary cells [40]. In addition, other

functions for RIP3K in hepatocarcinogenesis and cholestasis have been reported [123], in particular the role of RIP kinases in inflammatory signaling should be considered [124]. Caspase 8 null mice under an methionine-choline-deficient (MCD) diet, showed over-expression of RIPK3 and severe hepatocyte damage [40], also suggesting necroptosis. Though, the MCD model mimics histological features of human NASH, in particular the inflammatory component, but does not alter metabolic parameters or induce obesity [125,126]. Unfortunately many experimental works on NASH still use the MCD model, that might be relevant for the progressive inflammatory changes observed in NASH, but lacks any relevance to the underlying human clinical situation of NAFLD with obesity, adipocyte hypertrophy, and (hepatic) insulin resistance. Nevertheless, if the findings from human liver tissue can be confirmed, it might be worth to explore, if a shift from caspase-8-dependent apoptosis to necroptosis occurs during progression from NAFL to NASH. Cellular components inhibiting caspase-8 would thus be an interesting target to counter NAFLD progression [127].

A hallmark of NASH is the inflammatory component and activation of resident macrophages (Kupffer cells) and infiltrating monocytes/macrophages [128–130]. Apoptosis has long been considered a non-inflammatory process, in contrast to necrosis. However, by now there is evidence supporting that apoptosis, through TNFR1 and FAS signaling, can induce inflammation i.e. by production of chemokines and pro-inflammatory cytokines [131]. Especially Kuppfer cells react to this by release of TNF-α, or FAS-L, and TRAIL [129,132], which again act as death ligands on hepatocytes. Thus a vicious circle is generated, with continued and mutually reinforcing apoptosis and inflammation leading to fibrosis [133].

Another process possibly affecting progress of NAFLD could be autophagy, mainly by modulating the storage of lipids within the hepatocytes, although the mechanisms are

not fully elucidated [134,135]. Autophagy promotes resistance of hepatocytes to injury by FFA and oxidative stress and defective autophagy is linked to ER stress, which again promotes insulin resistance. Dysfunctional autophagy in obesity or NAFLD may lead to activation of mTOR and promotion of insulin-resistance [136–138]. This theory is corroborated by elevated ER stress and p62 expression in NASH, indicating impaired autophagic flux [139]. While in hepatocytes autophagy has a protective role, autophagy seems to promote hepatic stellate cell activation [140]. It is noteworthy that monitoring autophagy is very complex and conclusions from single or even a few autophagy related factors should be taken with caution. The gold standard for detection of altered autophagy, measuring autophagic flux, cannot be assessed in human samples currently.

For other cell death modes in NAFLD, clinical data are not available or conflicting. Pyroptosis or at least NLRP3 as important inflammasome component seems to be essential for NASH-induced fibrogenesis in an murine MCD model [141], with the above described limitations of this model. Moreover, NLRP3 expression was correlated to release of cell death markers (M65) and liver injury in human NAFLD [142].

Cell death in NAFLD seems to be predominantly apoptotic, although an increase of necroptosis during development from NAFL to NASH could be possible. Though, this hypothesis should be addressed in thoroughly conducted clinical studies. Thus, research efforts should be focused on the identification of factors that could establish which patient will progress from steatosis to an inflammatory/fibrogenic state beyond the well-studied ALT and CK18 [143,144]. Since no therapeutic options are currently available for NASH prophylactic measures may be the best option. Keeping up healthy life style choices regarding food, activity, and sufficiently intense exercise is the most promising preventive program against NAFLD.

Alcoholic liver disease

The incidence of alcoholic liver disease (ALD) is increasing steadily worldwide. Alcohol-induced liver injury includes fatty liver, fibrosis and alcoholic hepatitis [145]. Alcoholic hepatitis is a necro-inflammatory process that may progress to fibrosis and cirrhosis [146]. It was demonstrated in ALD that both apoptosis and necrosis participate in the pathophysiology of the hepatocyte injury [110,147,148]. Recent findings suggest also a role for necroptosis and pyroptosis as mechanisms of cell demise in the liver [2,149]. Alcohol is metabolized by the cytosolic alcohol dehydrogenase (ADH) and by the microsomal ethanol oxidation system (mainly located in zone 3, where the expression of CYP2E1 is high) into acetaldehyde, which induces hepatocyte apoptosis [150,151]. Excessive acute and chronic alcohol consumption leads to generation of ROS and subsequently increases oxidative stress in the liver [152], which in part is triggered by Kupffer cells [153]. Generation of ROS and depletion of the antioxidant glutathione lead to mitochondrial damage and release of cytochrome c, which in turn activates caspases [154]. It has been demonstrated that alcohol also induces mitochondrial dysfunction, ER stress, altered proteasome function and other mechanisms of cell damage [155]. DAMPS are released after necrotic cell death, and stimulate activation of macrophages and neutrophils, as well as fibrogenesis [35]. Further liver damage in ALD arises by increased bacterial endotoxin levels (LPS) and PAMPS, which induce Kupffer cells via TLR4 to produce TNF-α, IL-6, and ROS leading to hepatocellular death by apoptosis [156,157].

Currently no data on autophagy in human alcoholic liver disease is available. Results from animal models suggest, that autophagy could be beneficial in alcoholic liver damage by removal of damaged mitochondria and excess lipid droplets [89]. However,

ethanol seems to decrease autophagy possible via an AMPK dependant mechanism [158,159].

In ALD cell death seems to be the consequence of both necrosis and intrinsic apoptosis, due to ER-stress- or ROS-induced mitochondrial injury. Additional contributions of other cell death modes cannot be excluded, though clinical data on these is scarce. Autophagy may play a rather protective role in ALD. It remains to be elucidated if the necrotic type of cell death observed mainly in ALD could actually represent the regulated necroptotic type.

Strategies to reduce liver damage in ALD are restricted to acute hepatitis and implicate the use of anti-TNF molecules, corticosteroids, pentoxiphylline, and N-acetyl cysteine (NAC); however, other strategies are focused in the use of prebiotics to modify the gut microorganisms, pancaspase inhibitors, IL-1 receptor antagonists, and antioxidants (Reviewed in [160]).

Cholestatic diseases

Cholestasis or obstruction of bile flow can occur due to genetic, obstructive, inflammatory, or toxic disorders. Accumulation of toxic bile salts within the liver leads to hepatocyte apoptosis, biliary proliferation, and apoptosis of biliary epithelial cells [161]. Bile acid concentrations within the hepatocytes are tightly regulated by farnesoid X receptor (FXR) [162,163]. It is possible to experimentally mimic cholestasis by bile duct ligation (BDL) in mice, which induces apoptosis of hepatocytes [164]. During experimental cholestasis, toxic and mainly hydrophobic bile acids accumulate within hepatocytes [165] resulting in extrinsic activation of death receptors in addition to death ligand dependent extrinsic apoptosis [166–170]. Deregulated biogenesis of

mitochondria by bile acids additionally leads to intrinsically induced apoptosis [171]. This elevated hepatocellular apoptosis in bile obstruction has been linked to fibrogenesis via engulfment of apoptotic bodies by Kupffer cells and hepatic stellate cells [133,172–174]. These findings were supported by increased expression of FAS-L in infiltrating mononuclear cells and FAS in biliary epithelial cells of PBC patients [175]. While many models suggest apoptosis by bile acids as major cell demise mechanism, necrosis can also be triggered in cholestatic liver diseases [21,176]. *In vivo* apoptosis and necrosis may coexist, or secondary necrosis can occur following dysfunctional apoptotic cell death [21,164,177]. Strikingly the few larger studies in human tissue and primary human cells indicate a predominantly necrotic, or possibly as we now know necroptotic, cell demise [178,179]. Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, exerts cytoprotective effects on hepatocytes and biliary epithelial cells / cholangiocytes specifically in PBC. Though, UDCA is less effective in other biliary diseases and may even aggravate the situation [180–184]. This is an important example how differing mechanisms, in this case apoptotic cell death in mice vs. necro(pto)tic cell death in humans may limit information from animal and *in vitro* models. While UDCA is a promising and important therapeutic agent, it's use is restricted to specific biliary diseases in human, despite broad applicability in murine models.

Chronic viral hepatitis

Chronic viral hepatitis B and C generate a persistent inflammatory process and continuous stimulation of the immune system within the liver, resulting in hepatocyte death mainly by apoptosis [185–187]. HCV replication within the hepatocytes can lead to apoptosis and may stimulate the production of diverse cytokines and chemokines

(e.g. TNF-a, IFN-g, IL-12, IL-10, IP-10, and RANTES), which again can induce apoptosis [188–190]. Diverse HCV proteins such as core and NS3 can induce apoptosis of hepatocytes via TRAIL, TNF-α or FAS [191,192]. These death ligands are also produced by effector cells, mainly T and NK T cells to eliminate infected hepatocytes [185]. However, HCV virus can also induce apoptosis of activated T cells facilitating immune evasion [193]. Persistent apoptosis of infected hepatocytes lead to disease progression and fibrogenesis [194]. Chronic HBV infection is also associated with apoptosis and livers from HBV infected patients showed increased levels of death ligands [195,196]. HBx protein has demonstrated a dual role on hepatocytes. HBx can induce apoptosis of hepatocytes or may exert a pro-survival effect through the repression of lethal 7 protein (let-7), which acts as a repressor of STAT3 [197]. Generally in chronic viral liver diseases apoptosis is the predominant cell death mode. Therefore, some therapeutic approaches against HBV and HCV infection aim to reduce or eliminate the amount of apoptotic hepatocytes to prevent inflammation and stimulation of fibrogenesis. In a setting of HBV-induced acute liver failure, anti-viral therapy has been shown to effectively reduce viral load and surrogate markers of cell death and to improve patient survival [198]. Autophagy is one process that may facilitate pathogen removal by host cells. However, during HBV as well as HCV infection autophagy is induced [199–202], since both pathogens seem to utilize the autophagy machinery for replication [200,203]. While blocking of apoptosis might be beneficial for disease progression (i.e. fibrogenesis) and inhibition of autophagy might reduce HCV replication, the arrival of new and potent direct acting anti-viral drugs have curbed interest in these approaches.

Hepatocellular carcinoma

As described above, cell death is not only an important feature of chronic liver diseases but also a central mechanism for disease progression in most etiologies. Thus, inhibition of apoptosis or other cell demise modes is essential including to prevent hepatocarcinogenesis [204–206]. In contrast, when HCC is established, strategies aimed at increasing cancer cell death are required. Persistent hepatocyte cell death is linked to hepatocarcinogenesis [206–210]. In line with this, patients with chronic hepatitis B or C with elevated ALT have higher risk to develop HCC in comparison with patients with normal ALT [211–213]. Patients with HCC actually exhibit elevated serum concentrations of M30 and expression of pro-apoptotic ligands is increased in tumor surrounding tissue. Expression of anti-apoptotic regulators is increased in parallel, suggesting opposing signals at play in the tumor vicinity [214]. Indeed, hepatocellular carcinoma cells themselves are resistant to apoptosis induction, making approaches to selectively enhance sensitivity to TRAIL or Fas agonists promising.

The role of autophagy in cancer is complex and depends largely on context. In general, in HCC reduced autophagy has been observed [215,216], which was correlated with poor prognosis [215,217]. However, it was demonstrated that autophagy has a pro-tumoral effect protecting HCC cells from damage and promoting their invasion capacity [218,219]. Due to the complexity of autophagy and the contrasting observations in HCC, pharmacological modulation of autophagy in HCC is still in preclinical stages.

In summary, strategies aimed at minimizing hepatocyte damage could have a positive impact in prevention of HCC growth [220]. When HCC is established, selective induction of cell death of HCC cells could represent an urgently needed therapeutic alternative, i.e. by gene transfer [221–224]. Other strategies include the inhibition of XIAP [225] or the use of histone-deacetylase inhibitors (NCT00943449256). Current therapeutical options, apart from liver resection or transplantation, as selective internal

radiotherapy (SIRT), transcatheter arterial chemoembolization (TACE), or radiofrequency ablation (RFA) indeed also lead to death of tumor cells. Though, this probably occurs mostly via unregulated necrotic cell damage.

Cholangiocarcinoma

Cholangiocarcinoma (CCA) is an epithelial cell malignancy of the biliary tree displaying markers of cholangiocyte differentiation. It is the second most common primary hepatic cancer and its incidence in Western Countries is increasing. CCA is characterized by a dismal overall survival because of limited therapeutic options. CCA tumour development and progression appears to be maintained by potent survival signals. Co-activation of survival associated networks results in a blockage of tumour cell death. One of the key cytokines generated in inflammation is IL-6, which is elevated in the serum of patients with biliary tract cancer [161] and exhibits enhanced expression in the tumor stroma of patients with CCA. IL-6 inhibits cell death by activating the transcription factor signal transducer and activator of transcription 3 (STAT3), which in turn can upregulate survival factors such as myeloid cell leukemia sequence 1 (Mcl-1) and Bcl-xL. Suppressor of cytokine signaling 3 (SOCS3), an endogenous feedback inhibitor of IL-6, is epigenetically silenced via methylation of its promoter in CCA [226]. Treatment with demethylating agents restored IL-6 induction of SOCS3. For CCA demethylating agents might inhibit the procarcinogenic effects of IL-6 and thus pave the way for pro-apoptotic drugs [227]. Currently no cell death related therapy of CCA is available or in clinical testing, as information on cell death in CCA is scarce.

Markers of cell death

As already mentioned above, it is challenging to assess specific cell death modes in a clinical setting. First a biopsy would be required to retrieve liver cells, second a large set of parameters including mRNA and protein expression but also post-translational changes as phosphorylation, ubiquitination, or cleavage of specific proteins by caspases must be assessed. In fact it is probably impossible with current methods to generate an encompassing dataset describing the actual situation of cell demise in human liver tissue during disease. Even if this was possible, only a single time point would be available upon a liver biopsy. Since cell death is *per se* a highly dynamic process and changes of cell death mode may occur during disease course, a single measurement may not give sufficient information to interpret ongoing liver injury. Until methods arise, that may allow detection of *in situ* cell demise in humans, surrogate markers, associated to specific types of cell death are in use. These non-invasive markers may increase information on liver disease, in particular regarding severity of the injury. The most prominent marker set is M65 and M30, which can detect CK-18 either full length or a caspase-cleaved form, respectively. CK-18 is an epithelial cell marker and thus not specific for liver disease. Though, in established liver disease M65 and M30 have been shown to increase with severity of liver injury [43,120,190,228]. In diagnosed liver disease it is thus reasonable to assume that increased serum M65 indicates ongoing hepatocellular cell death, without further information on the mode of death. Elevated serum M30 indicates ongoing apoptosis of hepatocytes. Consequently, M30 or M65 have been employed to detect severity of NAFLD [120,229,230], ALD [228,231,232], or fibrosis / cirrhosis [190,233,234]. Both cell death markers in combination with other non-invasive factors could achieve high accuracy in prediction of fibrosis stage or could differentiate etiologies of liver disease [43,235]. To our knowledge caspase-1, activated during pyroptosis does not expose the neoepitope on CK-18, which is detected by M30,

making it indeed an apoptosis specific marker. Conversely, release of IL-1beta and IL-18 (and other IL-1 family members) seem to be specifically occurring in conditions of caspase-1 activation. Elevated levels of these have been detected in NASH and ASH [236–239]. If these truly indicate cell death due to pyroptosis or mere inflammasome activation without execution of cell death remains to be elucidated. There are also no data on caspase-4 or -5 activity in human chronic liver diseases, which may also contribute to pyroptosis and release of specific markers. A long known marker, gaining new attention is High-mobility group box 1 (HMGB1), which is supposed to be released only under severe cellular stress and membrane rupture, as well as by immune cells [240,241]. Although initially identified as unspecific released during necrosis, HMGB1 is now known to be released under many different conditions from a variety of cells [240,242]. Moreover, HMGB1 is subject to wide-ranging post-translational modification such as acetylation, methylation, and oxidation leading to different effects on target cells or completely impairing uptake. Modifications and functions of this complex molecule have been summarized well by other groups [240,242,243]. Current data suggests, that de- or un-acetylated HMGB1 with all three Cystein residues reduced or with a disulfide bond between Cys23 and Cys45 derives from necrosis or pyroptosis [242]. Unfortunately it is not clear, which types of HMGB1 were analyzed in the following clinical studies. Serum HMGB1 seems to correlate with severity of alcoholic liver injury as well as HCV-induced fibrosis [244,245]. Though, monocytes and macrophages are able to actively release HMGB1 [246,247], complicating interpretation related to cellular death in liver diseases with strong inflammatory background. Although caspase 3 has been identified as a major player in liver apoptotic cell demise no consistent results are available for plasma caspase 3 as a marker of liver cell death. It might be worth to explore a role as marker for types of liver injury inducing mostly

apoptotic cell death (i.e. NAFLD). To evaluate the full potential of these markers, a full panel for M30, M65, caspase 3, IL-1beta, IL-18, and HMGB1 (in the above described necrosis-specific conformation) in various etiologies of liver disease with differing severity and fibrosis stages as reference would be imperative. Unfortunately no such comprehensive study on known surrogate markers of cell death modes has been performed, yet.

Concluding remarks

Regulated cell death occurs in all chronic liver diseases and the type of predominant cell death may be specific for different etiologies. Current data suggest that cell demise in viral etiologies and NAFLD is mostly apoptotic but may switch to necroptosis during progression to NASH. ALD leads to a necrotic injury with intrinsic apoptosis, although no reliable information is available, if the necrotic type of injury could in fact be necroptotic or pyroptotic. For chronic liver diseases with biliary component many models suggest apoptosis induced by bile acids as predominant mode, though the clinical reality seems to indicate rather necro(pto)tic cell demise in this setting. Established tumours in the liver seem to develop resistance to apoptosis and approaches to increase susceptibility to this (or other) cell death modes could be feasible options.

Regarding the complicated mechanisms by which different cell death modes are interrelated and may be switched, results from in vitro and in vivo models have to be taken with care. Many findings from cell lines and murine models either still await confirmation in primary human cells or human tissue samples or do not correspond to the actual clinical situation in humans. Apart from this, it is extremely challenging to identify with certainty a specific cell death mode in actual human liver disease.

While surrogate markers for various cell death modes might help to assess severity of liver injury and monitor disease progression, therapeutic options utilizing cell death are far from common clinical usage. In part this may be due to a switch of cell death mode rather than complete abolishment of cell demise in a real life setting of human disease, when inhibitors i.e. of apoptosis are applied. A similar situation is given for autophagy. Although some already approved drugs may affect/promote autophagy (e.g. rapamycin or metformin) the lack of selectivity, and the many gaps in the molecular and cellular knowledge of the role of autophagy in the liver limit its application in the clinic.

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Figure Legends:

Figure 1: Summary of known interactions of major cell death pathways. Many factors and stimuli can lead to cellular demise. Necrosis occurs only as accidental cell death, due to severe cellular damage. Regulated cell death modes occur after ligation of specific extracellular signals to receptors or intracellular stimuli. Apoptosis is probably the main outcome, when regulated cell death is activated. Necroptosis and Pyroptosis are only executed, when apoptosis is inhibited, i.e. by inhibition of executioner caspases. Autophagy is usually a process to recycle resources or degrade damaged proteins or organelles within cells. Autophagy can either promote cell survival by blocking apoptosis or lead to cell death, depending on extent of damage and status of the cell. Remains of dying cells, either as apoptotic bodies or as debris, can elicit an immune response, which may facilitate release of pro-inflammatory or pro-apoptotic signals by immune cells. MOMP: mitochondrial outer membrane permeabilization.

Figure 2: Extrinsic activation of apoptosis. The extrinsic pathway of apoptosis can be initiated by two general mechanisms. The first mechanism involves binding of a death ligand (FASL, TRAIL) to a corresponding death receptor. The transmembrane death receptors form multimers *via* the cytoplasmic tails and recruit multiple proteins to the death-inducing signaling complex (DISC). The DISC contains pro-caspase-8 (or -10) which is activated to caspase-8 (or 10), when proapoptotic stimuli predominate survival signals. Caspase-8 proteolytically cleaves effector caspases (i.e. caspase-3) in type I cells, leading to execution of apoptosis. In type II cells pro-apoptotic signaling requires amplification by mitochondrial outer membrane permeabilization (MOMP). Caspase cleavage of BID to tBID leads to pore formation in the mitochondrial outer membrane and MOMP. The second mechanism initiating extrinsic apoptosis involves dependence

receptors (DCC or UNC5B). These receptors are activated in the absence of their ligand (netrin-1). Active receptors can directly convert pro-caspases to the active executioner caspases (i.e. caspase 9) or lead to MOMP. tBID, truncated BID. Figure adapted from [9].

Tables

Table 1: Bcl-2* family proteins are important regulators of apoptosis and mitochondrial outer membrane permeabilization (MOMP), which can act either pro- or anti-apoptotic.

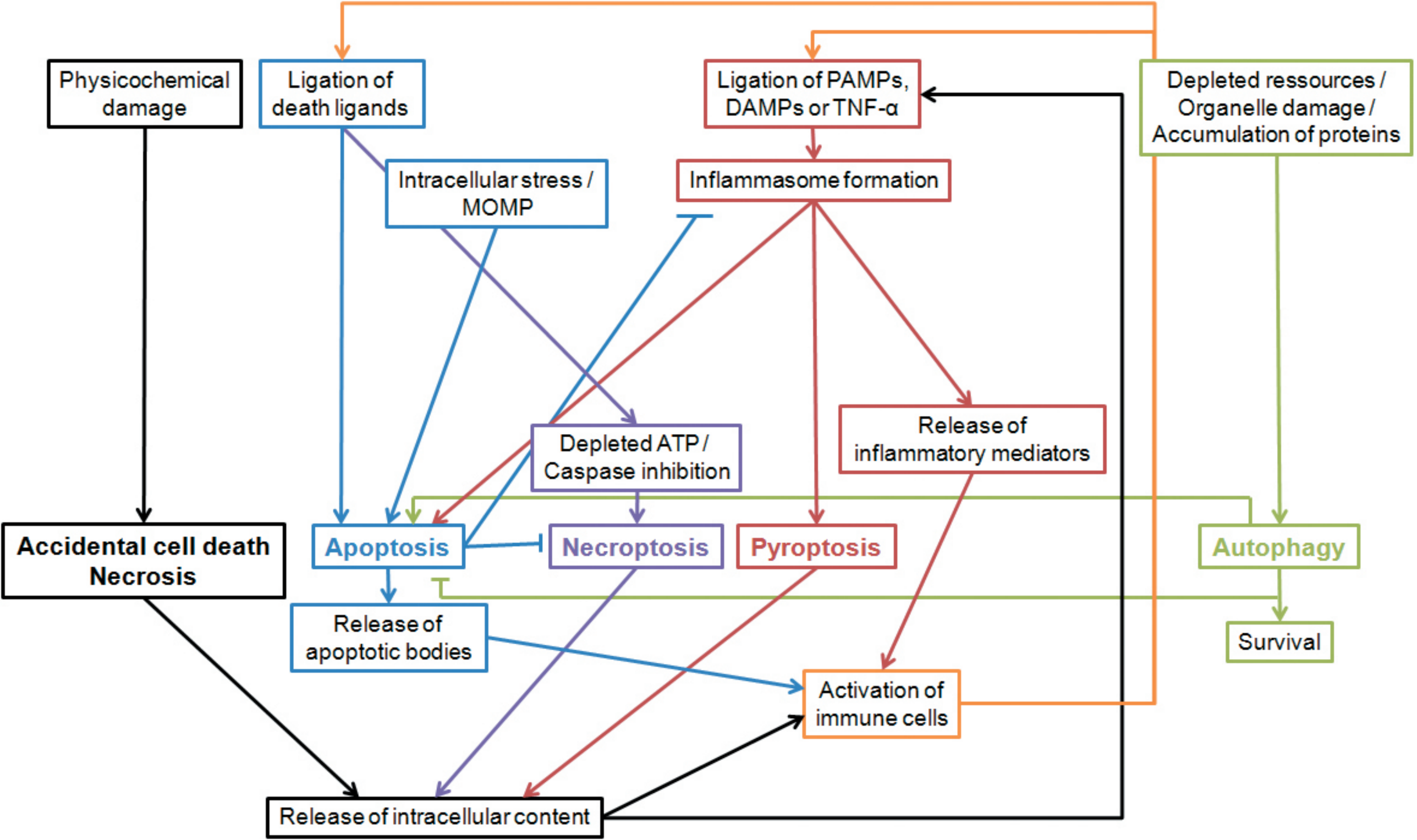
Pro-survival members	**Pro-apoptotic members of the Bax/Bak family** (pore formation in mitochondrial outer membrane)	**BH3 domain-only members** (pro- or anti-apoptotic depending on context and dimerization)
Bcl-xL	Bak	Bim
Bcl-2	Bax	Bid
Mcl-1	Bcl-xS	PUMA
Bcl-w	Bok/Mtd	Bad
A1/Bfl1		Bik
Nr13		Nix
Boo/Diva		Noxa
		Bnip3
		Hrk

*: B-cell lymphoma 2

Table 2: Overview of cell death modes in chronic liver disease and possible implications.

Disease	Primary cell death mechanism	Secondary cell death mechanism	Relevant markers of cell death	Clinical significance
Non-alcoholic fatty liver disease / Non-alcoholic steatohepatitis	apoptosis	necroptosis, autophagy	M30 M65	Indicator of disease severity Indicator of disease severity
Alcoholic liver disease	Necrosis / apoptosis	necroptosis	HMGB1 M65 M30	Disease marker / indicator of disease severity
Cholestatic diseases	In models and in PBC: apoptosis In human disease: probably necrosis	Necrosis / necroptosis	M65 M30	Disease severity / progression
Chronic viral hepatitis	apoptosis	Necrosis / autophagy	M65 M30 HMGB1 sTRAIL sTNFR1-2	Disease progression Disease progression

HMGB1: High-mobility-group-protein B1; M30: caspase-cleaved neoepitope of cytokeratin 18 (CK18), surrogate marker for apoptosis; M65: full lenght CK18, surrogate marker for overall cell death; sTRAIL: soluble TNF-related apoptosis inducing ligand; sTNFR1-2: soluble Tumor-Necrosis-Factor Receptor 1/2.


Physicochemical damage
Ligation of death ligands
Intracellular stress / MOMP
Ligation of PAMPs, DAMPs or TNF-α
Inflammasome formation
Depleted ressources / Organelle damage / Accumulation of proteins
Depleted ATP / Caspase inhibition
Release of inflammatory mediators
Accidental cell death Necrosis
Apoptosis
Necroptosis
Pyroptosis
Autophagy
Release of apoptotic bodies
Survival
Activation of immune cells
Release of intracellular content

Death ligands

Death receptors

DISC

pro-caspase 8

pro-survival

caspase 8

BID

tBID

MOMP

Netrin

Dependence receptors

pro-caspase 9

MOMP

caspase 9

Effector caspases

Apoptosis