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The Receptor Interacting Protein Kinases in the Liver

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Abstract

The Receptor Interacting serine/threonine Kinase1 and 3 (RIPK1, RIPK3) are regulators of cell death and survival. RIPK1 kinase activity is required for necroptosis and apoptosis, while its scaffolding function is necessary for survival. Although both proteins can mediate apoptosis, RIPK1 and RIPK3 are most well-known for their role in the execution of necroptosis via the pseudokinase mixed lineage domain like (MLKL). Necroptosis is a caspase-independent regulated cell death program which was first described in cultured cells with unknown physiologic relevance in the liver. Many recent reports have suggested that RIPK1 and/or RIPK3 participate in liver disease pathogenesis and cell death. Notably, both proteins have been shown to mediate inflammation independent of cell death. Whether necroptosis occurs in hepatocytes, and how it is executed in the presence of an intact caspase machinery is controversial. In spite of this controversy, it is evident that RIPK1 and RIPK3 participate in many experimental liver disease models. Therefore, in addition to cell death signaling, their necroptosis independent role warrants further examination.

Keywords

RIPK1; RIPK3; cell death; necroptosis; apoptosis

The Receptor Interacting Protein Kinase Family

The Receptor Interacting serine/threonine Protein Kinase (RIPK) family is comprised of seven members, all of which share a homologous kinase domain with varying intermediary and C-terminal domains [Figure1A.]. These unique non-kinase domains differentiate the proteins and largely dictate their function[1]. RIPK1, the originally identified member of the family, harbors a C-terminal death domain (DD) which engages death receptors (DR) such as tumor necrosis factor (TNF), TNF related apoptosis inducing ligand (TRAIL), CD95 (FAS) directly, or indirectly via adaptors such as TNF-receptor-associated death domain (TRADD) and Fas-associated protein with death domain (FADD). In between RIPK1's N-terminal kinase domain and its DD there is an intermediary domain with a RIP homotypic

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interaction motif (RHIM) that enables its interaction with other proteins with similar domains such as RIPK3, TIR-domain-containing adapter-inducing interferon-β (TRIF), and DNA-dependent activator of IRFs/Z-DNA binding protein 1 (DAI). This RHIM interaction is important for Toll-Like Receptor-3 and 4 (TLR3 and TLR4) engagement of RIPK1 via the adaptor protein TRIF[2]. RIPK1's kinase activity is generally thought to be necessary for killing by these stimuli[3–5]. RIPK2 (a.k.a CARDIAK/RICK) has a caspase activation and recruitment (CARD) domain on its C terminus. RIPK2 plays an essential role in modulation of innate and adaptive immune responses and NOD1/NOD2 mediated NFκB activation[6]. RIPK3 shares the family's N-terminal kinase domain and on its C-terminus has a RHIM domain which enables its interaction with RIPK1[7]. This RIPK1/RIPK3 interaction serves to activate RIPK3 as the initiation signal for the formation of the necrosome (discussed below). RIPK4 (DIK/PKK) and RIPK5 (SgK288) are characterized by ankyrin repeats in their C-terminus[1]. RIPK6 and RIPK7, more commonly known as Leucine-Rich Repeat Kinase 1 and 2 (LRRK1 and 2), share the serine/threonine kinase of the RIPKs, however they contain leucine rich repeat motifs, GTPase domains, as well as, Ras of complex proteins/C-terminal of Roc (Roc/COR) domains[1]. Interestingly variants of RIPK7/LRRK2 have been associated with familial and sporadic Parkinson's disease and Crohn's disease[8,9]. In general, the functions of RIPK4-7 are poorly understood. For the purpose of this review we will focus on the most studied RIP kinases in the liver: RIPK1 and RIPK3.

RIPK1, was first described as a FAS receptor interacting protein and as a mediator of cell death[10]. The structure of RIPK1 and whether it is in a kinase active conformation or not, dictates its function. When ubiquitinated, RIPK1 is in closed conformation and forms a platform that facilitates congregation of multiprotein complexes resulting in NFκB and MAPK activation[11,12]. In its kinase-active form, RIPK1 recruits RIPK3 to its RHIM domain initiating cell death pathways. RIPK1 is constitutively expressed in most tissues. However, stress signals, death receptor ligands, or T cell activation can induce the protein[1,10]. RIPK3 was described subsequent to RIPK1 in 1999 as a novel protein with an N-terminal kinase domain that was closely homologous with RIPK1's[7]. RIPK3 was shown to contain the RHIM domain enabling its binding to RIPK1, and RIPK3 over-expression was shown to induce apoptosis[7]. RIPK3 tissue distribution is not uniform and the protein is difficult to detect in hepatocytes under basal conditions[7,13]. In fact, the presence of RIPK3 in the liver and whether it is induced and in which models is a matter of intense debate[14]. Interestingly, unlike RIPK1−/− mice which die within three days of birth[11], RIPK3−/− mice showed no phenotype[15]. However, Kinase-Dead RIPK1 Knock-in mice are viable and fertile, while some Kinase-Dead RIPK3 mice (D161N mutant) die at embryonic day 11.5[5] [Figure1B.]. Both proteins can mediate apoptosis, through DRs. When caspase-8 is inhibited or absent, TNF can result in an alternative cell death sub-routine termed necroptosis, which is regulated in execution, and necrotic in morphology[16]. Since the discovery that RIPK1 and RIPK3 mediate the TNF death signal to activate the necroptosis effector mixed lineage domain like (MLKL), these kinases have been largely studied in the context of necroptosis[17]. These signaling events can occur downstream of various DRs (TNF, FAS, TRAIL, TLR, etc) with some variations. For simplicity we will focus on the extensively studied TNF signaling pathway[18–21].

RIPKs and Apoptosis

TNF is a potent inducer of cytokines and inflammation. TNF activation can also result in the activation of caspases, DNA fragmentation, chromatin condensation, plasma membrane blebbing, cell shrinkage, and ultimately formation of apoptotic bodies[22]. Binding of TNF to TNFR results in the formation of an intracellular signaling protein complex called Complex-I [Figure2.][23,24]. RIPK1 and TRADD are recruited to the receptor and bind via their respective DDs. TRADD recruits TNF receptor associated factor-2 (TRAF2) and the cellular inhibitor of apoptosis (cIAPs). The cIAPs ubiquitinate RIPK1 which leads to the recruitment of linear ubiquitin chain assembly complex (LUBAC, composed of HOIL, HOIP, Sharpin), transforming growth factorβ (TGFβ)-activated-kinase-1 (TAK1), TAB1/2 and the IKK complex (IKK1/ 2 and NEMO)[23]. Phosphorylation of the IKK complex by TAK1 leads to phosphorylation and proteasomal degradation of IkBα which normally sequesters NFκB and this results in NFκB liberation, nuclear translocation, and the transcription of anti-apoptotic NFκB target genes such as the caspase 8 inhibitor cellular FLICE inhibitory protein (cFLIP), and the de-ubiquitinases cylandromatosis (CYLD) and TNF Tumor necrosis factor alpha-induced protein 3 (TNFAIP3 or A20)[25]. RIPK1 can also mediate TNF-induced activation of the mitogen-activated protein kinases (MAPKs), such as ERK, p38, and JNK[12,26]. Therefore, the end result of Complex-I activation is inflammation and survival. In contrast transition of this multi-protein cluster from Complex-I to Complex-II results in cell death. RIPK1's post-translational modifications control cell fate by determining whether signaling pathways stream through Complex-I or Complex-II. When TNF induced transcription is compromised and RIPK1 is de-ubiquitinated by enzymes such as CLYD, it can move from Complex-I to the pro-death Complex-II [Figure2.]. Recently inhibitory phosphorylation of RIPK1 by the TAK1/P38 activated MAPKAP kinase-2 (MK2) has been described as a key regulator of RIPK1 function[27–30]. Inhibitory phosphorylation of RIPK1 (on S321 and S336) by MK2 suppresses the cytotoxic potential of RIPK1 and prevents TNF induced cell death[28–30]. In addition, RIPK1 can be inhibited by caspase8/cFLIP mediated cleavage and IKK mediated phosphorylation preventing its incorporation into Complex-II[31]. How RIPK1 phosphorylation by MK2 prevents Complex-II formation remains unclear, however since the phosphorylated residues are very close to the capsase8/cFLIP cleavage target it has been suggested that this modification can result in a conformational change exposing the cleavage site[32]. There are multiple variations and compositions of Complex-II. Complex IIa which is comprised of TRADD, FADD, RIPK1 and caspase 8 results in RIPK1 independent apoptosis via capsase-8 [Figure2.][24]. In Complex-IIb RIPK1 associates with FADD and caspase-8 resulting in apoptosis which is RIPK1 kinase dependent[24]. A complex similar to Complex-IIa can form when cIAPs are depleted (independent of TNF signaling) called the Ripoptosome consisting of RIPK1, RIPK3, FADD, Caspase-8 and cFLIP[33–35]. Complex-II and the Ripoptosome trigger apoptosis by activating caspase 8 to form homodimers which requires the kinase activity of RIPK1[24,34,35]. In type I cells such as lymphocytes caspase-8 activates the executioner caspases (3 and 7) resulting in apoptosis, in type II cells such as hepatocytes, apoptosis requires mitochondrial participation through caspase-8 mediated cleavage of Bid resulting in mitochondrial outer membrane pore opening (MOMP) and cytochrome C release. This results in the formation of the apoptosome activating caspase 9, which in turn activates the effector caspases (caspase 3/7) to induce apoptosis. This has been

reviewed in detail[22]. In addition to DR signaling and TLRs, anticancer drugs and DNA damage can activate RIPK1 dependent apoptosis[36]. RIPK3 can also result in apoptosis, unlike the RIPK3−/− mice that have no phenotype, kinase-dead RIPK3 knock-ins (RIPK3D161N) die at E11.5[5]. RIPK3 kinase inhibitors cause a conformational change in RIPK3 resulting in RIPK1 recruitment, caspase-8 activation and apoptosis[37]. Interestingly not all kinase dead RIPK3 mice develop spontaneous apoptosis, for example the K51A mutant is viable and fertile indicating that the lack of kinase activity is not the impetus for apoptosis induction. Rather certain mutations can cause conformational change in the protein resulting in RIPK1 recruitment and ultimately activation of the Ripoptosome[38]. An intriguing open question especially in liver disease models is whether the RIPK1 inhibitor, necrostatin-1 (nec-1), can similarly affect RIPK1 platform function by affecting protein conformation.

RIPKs and Necroptosis

Despite being a critical member of the DR induced Complex-I and participating in apoptosis, RIPK1's claim to fame is due to its link with the more recently described cell death sub-routine, necroptosis. Necroptosis occurs in certain cell types when TNF binds to TNFR in the presence of a pan-caspase inhibitor (+/− an IAP antagonist)[36]. It was first described in cells stimulated with TNF or FAS in the presence of a pan caspase inhibitor, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (ZVAD-fmk) requiring the kinase activity of RIPK1[18,39,40]. RIPK1 kinase inhibitors, necrostatins (Nec), were later shown to block this form of morphologically necrotic and regulated cell death, confirming the role of the protein in necroptosis execution[41,42]. When caspase-8 is absent or inhibited, RIPK1 binds to RIPK3 via the RHIM domain, forming a complex called the necrosome which also includes FADD (+/− TRADD) [Figure2.]. RIPK3 then oligomerizes via its RHIM domain and autophosphorylates leading to activation of the pseudokinase, MLKL[43]. RIPK3 is necessary for necroptosis execution while RIPK1 is most likely dispensable, as recruitment of RIPK3 by other RHIM-containing proteins, such as TRIF and DAI, can promote RIPK3 activation[44,45]. This has been reported with certain viruses such as herpes simplex and murine cytomegalovirus where RHIM containing proteins can directly engage RIPK3 independent of RIPK1[46]. MLKL, the only known effector of necroptosis, is a pseudokinase with an N-terminal effector domain which is kept inactive by MLKL's C-terminal pseudokinase domain[43,47–49]. It is hypothesized that RIPK3 and MLKL exist as pre-assembled non-active complexes and that auto-phosphorylation of RIPK3 results in an open conformational shift exposing the N-terminal four-helical bundle domain and releasing MLKL, leading to oligomerization and translocation to plasma membrane and cell lysis by membrane rupture[47–53].RIPK3 is the only known activator of MLKL and therefore it is believed that cells that do not express RIPK3 do not undergo necroptosis[36]. One intriguing aspect of the relationship between RIPK1 and RIPK3 lies in the observation that in addition to collaboration with RIPK3 to promote cell death, RIPK1 can inhibit RIPK3 under certain conditions. RIPK1 specific knockout in intestinal epithelium or keratinocytes resulted in spontaneous RIPK3 mediated cell death and inflammation[54,55]. The precise mechanism by which this occurs remains unknown.

RIPKs and Inflammation

While RIPKs were initially described as mediators of cell death and survival, emerging evidence indicates a necroptosis-independent role for the RIPKs in inflammation [Figure3.][56]. The most obvious evidence for this lies in the relationship between RIPK1 and NFκB, the main transcription factor for the expression of inflammatory genes. Ubiquitinated RIPK1's scaffolding function mediates NFκB activation downstream of TNF. Although RIPK1 seems to be dispensable for NFκB activation, some studies have suggested decreased expression of NFκB responsive genes without RIPK1[57–59]. RIPK1 and RIPK3 can participate in inflammatory pathways such as TLR signaling, cytokine production, NOD-like receptor protein 3 (NLRP3) and inflammasome activation independent of MLKL and their role as mediators of necroptosis[60–62]. The effect of RIPK1 and RIPK3 in inflammation was initially assumed to be due to the inflammatory nature of necroptotic cell death from MLKL mediated plasma membrane rupture and release of intracellular immunogenic molecules. However there is accumulating evidence that the RIPKs directly influence inflammation. An example is in the TNF alpha induced protein 3 (Tnfaip3)/A20 global knockout mouse. A20−/− mice have systemic inflammation resulting in cell death. Deletion of RIPK3 or a catalytic inactive RIPK1 significantly delay mortality in these mice while MLKL deletion does not[63]. Liver leukocytes isolated from RIPK3−/− mice and stimulated by α-GalCer showed significantly reduced production of cytokines (TNF, IL-4 and IFNγ) compared to WT cells suggesting a role for RIPK3 in NKT cytokine production[64]. Macrophages lacking cIAPs secret larger quantities of pro-inflammatory TNF and IL-6 which required RIPK1 and RIPK3, but not MLKL[65].

Inflammasome dependent cell death or pyroptosis, is characterized by the assembly of a multi-protein complex (NLRP3-ASC-Caspase-1) resulting in activation of caspase-1 and release of the pro-inflammatory cytokines IL-1β and IL-18[56]. RIPK3 can activate caspase-1 and promote IL-1β cleavage thereby contributing to inflammatory cytokine production[61,62,66,67]. RIPK3 has also been shown to activate the NLRP3 inflammasome pathway in macrophages downstream of TLR3 in response to RNA virus infection[68]. RIPK1 has been associated with the alternative NLRP3 pathway in human monocytes stimulated by LPS downstream of TLR4 which requires FADD and caspase-8[69]. Interestingly in a mouse model of pyroptosis where NLRP3 was hyperactivated and mutant mice suffered shortened survival, poor growth, and severe liver inflammation; RIPK1 and RIPK3 did not contribute to disease[70]. Detailed discussion of pyroptosis and inflammasome mediated cell death and inflammation is beyond the scope of this topic and have been recently reviewed extensively[56,71,72]. The RIPKs role in the inflammasome and in necroptosis may not be mutually exclusive and there may be cross talk between the two pathways[56]. Therefore, a growing body of evidence suggests that the RIPKs are multi-functional proteins with prominent roles independent of their actions as cell death mediators [Figure3.]. These non-necroptotic properties should be taken into account when interpreting experimental findings[14].

RIPKs in the liver

Cell death is an important driver of liver disease and both apoptosis and necrosis occur in the liver with apoptosis being the more common death pathway[22,73–75]. RIPK1 and RIPK3 have been studied in various liver injury models, mainly in the context of determining the contribution of necroptosis [Figure4.]. Hepatocytes robustly express DRs and many disease states such as NASH, viral hepatitis, alcoholic hepatitis, cholestatic liver disease and auto-immune liver disease have been shown to result in hepatocyte apoptosis[76,77]. One significant argument against the occurrence of necroptosis in the liver is that under numerous apoptosis-inducing experimental settings pharmacologic or genetic prevention of apoptosis in the liver has been demonstrated to rescue without a switch to necroptosis[14]. A second important point is that it is not clear that hepatocytes contain all the necessary machinery for necroptosis. RIPK1 and MLKL are clearly expressed in the liver, however RIPK3 expression under basal conditions has been controversial[7,13,14,78–80]. RIPK3 induction has been suggested in some models and human diseases[81–85]. While RIPK3 is difficult to detect in freshly isolated hepatocyte it is robustly expressed in the non-parenchymal cell fraction, making it possible that necroptosis occurs in certain cell types in the liver[78]. A RIPK3 independent model for necroptosis in hepatocytes has been suggested and since MLKL is clearly present in the liver, it is hypothesized that other MLKL activators exist[79,80].

RIPKs in APAP toxicity and hepatocyte Death

RIPK1 was the first family member studied in liver disease due to the availability of nec-1, the RIPK1 kinase inhibitor which was shown to protect against necrotic cell death in multiple organ systems[41,42]. Using nec-1 multiple investigator demonstrated a survival benefit against acetaminophen (APAP) induced liver injury in mice and in hepatocytes *in vitro*[86–90]. Unlike the consistent and reproducible results with nec-1, RIPK3's role in APAP and whether necroptosis occurred in the liver became the source of controversy[14,78]. Since the cell death mode in APAP is largely necrotic, the abrogation of injury with nec-1 was initially interpreted as inhibition of necroptosis. However nec-1 is neither specific to RIPK1, nor necroptosis[38]. Nec-1 was discovered to be identical to the indoleamine 2,3-dioxygenase (IDO) inhibitor methyl-thiohydantoin-tryptophan (MTH-Trp)[91]. IDO is a potent immuno-modulator and is the rate-limiting step of tryptophan catabolism and it has been shown to regulate pathways that lead to pro and anti-inflammatory cytokine production. Therefore additional evidence was needed regarding the role of RIPK1 in APAP induced liver injury[91]. Using an antisense oligonucleotide knockdown approach, we observed that RIPK1 knockdown in liver protected significantly from APAP induced cell death and provided a survival benefit to mice. In keeping with the controversial reports on this matter, RIPK1's role in APAP toxicity was soon challenged[92]. The off target effects of nec-1 combined with fact that necroptosis requires the participation of RIPK3 and MLKL led us to explore APAP toxicity in RIPK3 and MLKL knockout mice to settle the question of APAP-induced necroptosis in the liver[78]. Previously it was reported that RIPK3−/− mice were protected against APAP at early (6hours) but not late (24hrs) time points[88]. We were unable to detect any difference in liver injury from RIPK3−/− mice compared with WT animals at either time[78]. However, knockdown of RIPK1 in RIPK3−/− mice afforded a significant protection, suggesting that RIPK1 was functioning independent in RIPK3 and necroptosis to protect

against APAP toxicity[78]. To confirm this, we used MLKL−/− mice and were unable to detect any attenuation of APAP-induced cell death in MLKL−/− mice[78]. These results were later independently confirmed by others[79]. Therefore, the mere participation and even induction of the RIPKs in any disease model does not indicate that the cell death subroutine is necroptosis. In fact, many of the studies in different models and organ systems showing protection using nec-1, RIPK1 kinase dead, and RIPK3−/− mice were not reproducible in MLKL−/− mice, pointing to the multifactorial and non-necroptotic functions of these proteins[63].

RIPKs in Immune Mediated Liver Injury Models

Compounds that activate the immune system such as Concanavalin A (ConA) and α-galactosylceramide (α-GalCer) are widely used substitute models for human auto-immune hepatitis. Unlike autoimmune hepatitis which is mainly adaptive immune mediated, these compounds generate an innate immune response. ConA, a plant lectin, activates CD4+ T cells, NKT cells, KC and macrophages to induce an immune-mediated hepatitis[93]. The mode of cell death in ConA has been a source of debate but the preponderance of the evidence suggest it is a necrotic subroutine (no protection by caspase inhibition) although some apoptosis may occur in the early phases of injury[94–96]. Unlike APAP which is not dependent on external DR signaling, ConA hepatitis is mediated through cytokines and DR. IFN-γ, IL-4, IL-6, FAS and TRAIL have all been reported as mediators of ConA hepatitis and cell death, but the major cytokine contributing to toxicity is known to be TNF[96]. The necrotic mode of cell death combined with the dependency on DR signaling has led multiple investigators to study necroptosis in ConA induced liver injury. Pre-treatment of nec-1 was suggested by some to markedly protect against ConA[97–99]. RIPK1 kinase inhibition has been proposed to result in decreased Poly ADP-ribose polymerase-1 (PARP-1) activation, and decreased IL-33 suggesting that RIPK1 is an upstream mediator of these signaling events in ConA[97,98]. However, *in vivo* RIPK1 knockdown in the liver (with antisense) resulted in exacerbated injury and increased mortality from ConA which was almost completely prevented by zVAD-fmk[57]. Using RIPK1 kinase dead knock-in mice (Ripk1-K45A) and nec-1s, Filliol and colleague have reported that RIPK1 kinase activity drives liver injury induced by ConA, as shown by reduced levels of serum transaminases and improved histology[100]. Interestingly, RIPK1 liver specific knockout RIPK1-LPCKO resulted in more pronounced injury and higher ALT. In agreement with the aforementioned study, the cell death mode in these RIPK1 deficient hepatocytes was caspase-dependent apoptosis[100]. Furthermore, RIPK1 deletion in parenchymal cells resulted in a more robust inflammatory response. This enhanced sensitivity was TNF mediated as treatment with etanercept, which neutralizes TNF, protected against ConA[100]. These studies elucidate the complexity with the RIPK1 protein as inhibiting its kinase and platform activities resulted in alternative outcomes. In contrast to the studies reporting benefit with RIPK1 kinase inhibition(genetically or using nec-1), others have observed a worsening of injury and increased mortality (up to 50%) with the more specific nec-1s[87]. Interestingly these same investigators reported that despite aggravation of liver injury with nec-1s, a modest attenuation was observed in RIPK3 global knockouts[87]. Kang and colleagues have also reported that ConA injury is attenuated in RIPK3−/− mice[64]. However, the contribution of

RIPK3 to ConA has been challenged by two independent reports indicating no difference in outcomes of liver injury in RIPK3−/− compared to WT mice[79,101]. Interestingly, Gunther and colleagues were unable to detect any RIPK3 in hepatocytes and failed to detect an induction or increase in transcripts following ConA treatment[79]. However, despite no role for RIPK3, they reported less liver injury in both MLKL−/− mice and in nec-1s pre-treated mice with ConA suggesting an alternative MLKL activation pathway in hepatocytes[79]. Further exploration of this alternative, non-canonical necroptotic pathway to MLKL activation is warranted.

The glycolipid α-galactosylceramide (α-GalCer) which is a specific and potent activator for invariant natural killer T cells (NKT) has also been used to study immune-mediated liver injury[57]. This model resembles the ConA model, but is relatively less severe and restricted to NKT cells, innate immune cells uniquely abundant in the liver. Using both α-GalCer and ConA models, Kang et al have reported attenuation in RIPK3 −\− mice with less cytokine (TNF, IL-4) and IFNγ expression. RIPK3 in bone marrow (BM) cells was essential as ConA-induced liver injury was significantly attenuated in the WT animals that were transplanted with RIPK3−/− BM. This protective effect was suggested to be due to the effect of RIPK3 on cytokine production from NKT cells deficient in RIPK3 and not due to the prevention of hepatocyte necroptosis[64]. Contrary to the findings by Kang et al, we found no difference in liver injury between global RIPK3−/−, MLKL−/− mice and strain matched controls treated with α-GalCer[57]. However, knockdown of RIPK1 markedly aggravated α-GalCer-induced hepatitis and resulted in 90% post treatment mortality as early as 6 hours post α-GalCer[57]. Accompanying the exacerbated liver injury, RIPK1 knockdown significantly increased serum levels of proinflammatory cytokines and chemokines (TNF and IFNγ) 6hrs post injection correlating with increased inflammatory cell infiltration[57]. The liver cell death was accompanied by caspase 3 activation and inhibited by TNF neutralization and pan-caspase inhibitors without a switch to necroptosis[57]. This increased sensitivity to TNF was not seen in RIPK1 kinase dead D138N mice or with nec-1 and seems to be due to the scaffolding functions of RIPK1[57]. Increased sensitivity of hepatocytes lacking RIPK1 to TNF mediated apoptosis has been reported using lipopolysaccharide (LPS) and unmethylated CpG oligodeoxynucleotide (CpG-DNA) to induce liver injury as well[57,102]. In RIPK1 knockdown livers low dose LPS resulted in massive hepatocyte apoptosis[57]. This injury is thought be mediated by macrophage-produced TNF, as macrophage depletion using clodronate or the use of a TNF neutralizing antibody can rescue from liver injury[57,102]. While certain aspects of these studies, namely the effect of RIPK3 deletion and RIPK1 inhibitors in immune-mediated hepatitis has been controversial, a common theme that becomes evident is that deletion of RIPK1 aggravates immune-mediated liver injury by sensitizing hepatocytes to TNF mediated apoptosis. The discrepancies in many of these reports could be minimized by using standard dosing regimens and appropriate controls (especially when DMSO is the vehicle). Additionally, interpretation of effects in animal models where littermate controls are not used should be done with caution[14].

RIPKs in Fatty Liver Disease and Alcoholic Liver Disease

Alcoholic and non-alcoholic steatohepatitis (ASH and NASH) are major public health problems and leading causes of liver related mortality worldwide and in the U.S.[103,104]. These distinct disease entities share many common features such as steatosis, Mallory bodies, inflammation, parenchymal fibrosis, and hepatocyte injury leading to cell death. In both entities a major driver of disease progression is cell death with apoptosis as the main subroutine[75,105]. This is demonstrated by the fact that caspase 8 liver specific KO (casp8-LKO) or caspase inhibition greatly protects against steatohepatitis without a switch to necroptosis in NASH[106–108]. Studies examining the role of the RIPK proteins in NASH have yielded contradictory results. This may be partly due to the various models employed to induce NASH in rodents. Studies using diets deficient in methionine and choline (MCD) were commonly used to induce steatohepatitis in mice. However, MCD has fallen out of favor as mice on these diets, lose weight and do not exhibit the metabolic syndrome phenotype despite developing steatohepatitis[109]. Using the MCD model, an increase in RIPK3 protein as early as two weeks has been reported[84]. In contrast to the earlier studies showing protection in casp8-LKO mice, using the MCD diet Gautheron and colleagues found higher ALTs and worse fibrosis with deletion of caspase-8 which was somewhat attenuated by RIPK3 knockout[84] and accompanied by increased pMLKL staining[110]. Increased RIPK3 expression has also been reported using immuno-staining of human NASH and NAFLD livers[84,85]. Afonso and colleagues reported increased RIPK3 and pMLKL expression with High Fat Choline Deficient (HFCD) feeding and attenuation of liver injury, inflammatory cytokines, and fibrosis with RIPK3 deletion[85]. Interestingly they reported increased RIPK3 protein not only in biopsy samples from NASH patients but also in liver biopsies from patients with other chronic liver diseases such as PBC, ASH, Hepatitis B and C (HBV, HCV)[85], raising questions about the specificity of RIPK3 staining or perhaps suggesting that induction of RIPK3 is a common feature of chronic liver disease regardless of underlying etiology[14]. In contrast to reports of amelioration of injury in RIPK3−/− using the MCD diet, using the Western type High Fat Diet (HFD) mice lacking RIPK3 exhibited significantly worse steatosis, fibrosis, and inflammation compared to WT mice[82]. The WT mice in this study expressed no basal RIPK3 or pMLKL, but after 12 weeks on the HFD both proteins increased in the fatty liver by immunohistochemistry (IHC)[82]. However, despite the high expression of pMLKL these mice had very little injury and cell death[82]. Further complicating matters was the observation that RIPK3−/− mice exhibit glucose intolerance at baseline on a chow diet[82]. The role of RIPK3 in alcoholic liver injury using the Lieber-DeCarli model of alcohol feeding has been explored[83,111]. In contrast to the HFD study, RIPK3−/− mice on a Lieber-DeCarli alcohol diet had a modest attenuation of injury compared to WT controls[83]. The authors also reported increased RIPK3 protein immunostaining in human liver biopsy samples with alcoholic liver disease[83]. Another group recently studied the mode of cell death in Lieber-DeCarli model using caspase8-LPCKO mice and reported increased apoptosis with caspase-8-LPCKO with no significant RIPK1, RIPK3 and MLKL induction[111]. The Gao-binge model of alcohol feeding has also been reported to result in increased RIPK3 protein[81]. The authors found lower transaminase elevations and decreased steatosis from global RIPK3 deletion, but no difference in inflammation and hepatitis. They could not detect a transcriptional change in RIPK3, and

attributed the increased protein levels of RIPK3 to impaired hepatic proteasome function[81]. The contribution of necroptosis to cell death in NASH is arguable and apoptosis seems to be the predominant cell death pathway[75]. Studies using hepatocyte specific MLKL knockout and littermate controls are needed to settle the matter.

However, given recent reports of insulin resistance in RIPK3−/− mice[82], it is compelling to imagine a necroptosis independent contribution of RIPK3 to NASH pathogenesis.

RIPKs in HCC

Hepatocellular carcinoma (HCC), the most common primary liver tumor which often occurs in the context of chronic inflammation. Cell death is both a consequence and a cause of liver inflammation and therefore a strong link exists between hepatocyte death and HCC development. The role of the RIPKs has been explored in a few models of HCC[59,112,113]. In the human hepatoma cell line HepG2 cells, deletion of RIPK1 or cFLIP sensitized to TRAIL mediated apoptosis[114]. Surprisingly, low RIPK1 expression which is seen in some human HCCs has been associated with a worse prognosis and decreased survival[112,115]. Conditional deletion of the mitogen activated protein 3 (MAP3)-kinase, TAK1, in liver parenchymal cells (TAK1-LPCKO) results in hepatic inflammation, cholestasis, fibrosis which leads to HCC[116]. These TAK1-LPCKO mice exhibited defective NF-kB activation, increased spontaneous apoptosis and increased RIPK3 protein expression. TAK1-caspase8-LPCKO resulted in less injury and less caspase 3 cleavage without a switch to necroptosis[116]. The combination of global RIPK3 knockout with TAK1-LPCKO resulted in less necrosis, less cholestasis and decreased ALT. At 38 weeks TAK1-caspase8-LPCKO did not exhibit any tumors while TAK1-LPCKO-RIPK3−/− mice exhibited a massive tumor burden suggesting a role for RIPK3 in modulating hepatocyte apoptosis[116]. TAK1LPCKO-RIPK3−/− mice had increased JNK activation and the addition of the JNK inhibitor significantly decreased hepatocytes proliferation (Ki67 and cyclin D1 levels)[116]. NEMO liver parenchymal cell knockout (NEMO-LPCKO) results in hepatitis, hepatocyte apoptosis and HCC, suggesting a tumor suppressor function of NF-kB signaling in the liver[117]. The development of hepatitis and HCC in NEMOLPC-KO mice is triggered by FADD- and caspase-8-dependent apoptosis. Surprisingly NFKB-LPCKO mice, which lack all subunits in the liver parenchymal cells, have a much milder phenotype compared to NEMO-LPCKO suggesting that NEMO has NF-kB-independent pro-survival functions in hepatocytes[113]. The role of RIPK1 in mediating cell death from the NEMO-LPKO mice has been explored using a genetic kinase dead RIPK1 knock-in mouse (D138N)[113]. The addition of the kinase dead RIPK1 to the NEMO-LPCKO strongly inhibited hepatocyte death, prevented liver damage and HCC development. In contrast, the global deletion of RIPK3−/− in the NEMO-LPCKO did not prevent these outcomes, suggesting that RIPK3/MLKL-driven necroptosis does not play an important role in this model[113]. Further supporting this was the fact that the dying hepatocytes stained positively for cleaved caspase 3, and ablation of caspase-8 or FADD inhibited cell death indicating RIPK1 kinase dependent apoptosis as the cell death subroutine. The expression of kinase inactive RIPK1, fully restored the expression of cFLIP and partly normalized the expression of TRAF2 and cIAP1 proteins in the NEMO-deficient livers. However, RIPK1 ablation in LPC using RIPK1 floxed mice could not prevent the death of NEMO-deficient hepatocytes showing that kinase independent platform properties

of RIPK1 are required to prevent hepatocyte apoptosis (this was prevented with the additional deletion of TRADD)[113]. FADD and caspase-8 co-immunoprecipitated with RIPK1 suggesting that NEMO prevents formation of a RIPK1/FADD/Casp8 complex and that in the absence of NEMO RIPK1 mediates hepatocyte apoptosis via its kinase activity through this complex. In a recent report genetic deletion of RIPK1 in parenchymal cells (RIPK1-LPCKO) had no spontaneous phenotype. However when exposed to LPS/TNF these mice exhibited enhanced apoptosis due to the proteasomal degradation of TRAF2 leading to caspase dependent cell death[112]. The mode of cell death was caspase-8 dependent apoptosis as the additional knockout of caspase 8 in these mice completely rescued the phenotype[112]. Unlike the RIPK1-LPCKO, inhibiting the kinase activity of RIPK1 genetically or pharmacologically did not sensitize to TNF mediated cell death suggesting the platform function of RIPK1 is instrumental in cell survival. Interestingly like RIPK1-LPCKO, the TRAF2-LPCKO did not have a spontaneous phenotype but the double KO of RIPK1/TRAF2 resulted in hepatitis, impaired NF-kB activation and caspase 8 dependent apoptosis and HCC[112]. In this study human liver HCC samples higher expression of RIPK1 and TRAF2 was associated with a significant survival benefit and low expression of these proteins predicted a worse prognosis[112]. In contrast, another study from China has reported increased RIPK1 expression in HCC samples as a poor prognostic indicator after resection[118]. Patients expressing more RIPK1 by immunostaining of tumor were more likely to have advanced stage, intra-hepatic metastasis, and portal vein invasion. Interestingly, patients with increased RIPK1 in HCC were more likely to be HBV infected[118].

Van and colleagues have used the diethylnitrosamine (DEN) induced HCC model in RIPK1-LPCKO mice and have also observed increase in apoptosis with RIPK1 deletion and reduction in tumor size[59]. Interestingly while ablation of either RIPK1 or RelA in liver parenchymal cells did not cause spontaneous liver pathology, mice with combined deficiency of RIPK1 and RelA in LPCs showed increased hepatitis, increased hepatocyte apoptosis (cleaved caspase-3) and developed spontaneous chronic liver disease and cancer[59]. Lack of RIPK1 kinase activity did not inhibit DEN-induced liver tumor formation, confirming that kinase-independent functions of RIPK1 promote carcinogenesis in a TNFR1-dependent manner[59]. An interesting finding here was that unlike previous studies, RIPK1-LPCKO resulted in impaired NF-κB signaling and reduction in the TNF-induced expression of A20, baculoviral IAP repeat-containing 3 (Birc3), nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor-alpha (Nfkbia), and Tnf[59]. It has now been shown in multiple models that knockdown and knockout of RIPK1 *in vivo* sensitizes hepatocytes to TNF mediated apoptotic cell death[57,59,100,112]. RIPK1 appears an attractive therapeutic target for HCC treatment if targeting cancer cells for RIPK1 ablation or knockdown and sparing healthy hepatocytes becomes possible.

RIPKs in other liver diseases

The contribution of the RIPKs to cholestatic and viral liver disease is less extensively characterized. RIPK1 but not RIPK3 deletion completely rescues the growth retardation, ductopenia and cholestatic phenotype of liver parenchymal cell specific deletion of the IKKα/β subunits (IKKα/β-LPCKO)[115]. Despite ductopenia and ongoing injury, no HCC developed in the IKKα/β-LPKO livers suggesting a failure of cholangiocyte proliferation.

Staining for Ki-67 revealed increased proliferation in the combined IKKα/β-LPKO/RIPK1 KO compared to IKKα/β-LPKO[115]. The authors proposed that RIPK1 is the direct phosphorylation target of the IKK subunits and functions as an anti-proliferative protein in cholangiocyte homeostasis[115]. The mode of cell death in this model was proposed to be a combination of apoptosis and necrosis without a significant contribution from necroptosis as RIPK3−/− did not affect the phenotype[115]. Afonso and colleagues have examined the effect of RIPK3 deletion in a bile duct ligation (BDL) model of liver injury[119]. No difference was detected after three days however, two weeks post BDL, RIPK3−/− mice displayed less liver injury compared to strain matched controls[119]. RIPK3 deficiency attenuated inflammatory cell infiltration but did not affect BDL induced fibrosis[119]. How necroptosis is activated in this model with intact caspase-8 is unclear. High levels of RIPK3 and MLKL expression in the liver of PBC patients (hepatocytes and cholangiocytes) using immunostaining was also reported[119]. Cell death in viral hepatitis is due to a cytotoxic CD8+ T cell immune response[120]. Viral hepatitis is associated with increased expression of DR ligands such as soluble FAS, TRAIL and TNF resulting in apoptotic hepatocyte death[121–125]. There is not much data on the RIPKs in viral hepatitis. In an *in vitro* HCV culture system it was reported that the addition of nec-1 to a pancaspase inhibitor abated HCV related cell death and resulted in improved survival compared to caspase inhibition alone[126].

Concluding Remarks

RIPK1 and RIPK3 are the two most studied proteins in the RIPK family. Both proteins are multi-functional and can mediate apoptosis, necroptosis or activate inflammatory pathways independent of cell death. It is well established that RIPK1 kinase activity is necessary for necroptosis, but how RIPK1 is activated and what target protein it phosphorylates remain unclear[38]. RIPK1 kinase inhibition does not only block necroptosis; it also interferes with TNF induced apoptosis in certain contexts[24,35]. Therefore, it is important to consider that the effect of necrostatins on hepatocytes and in animal models can be due to interference with apoptosis or necroptosis[38]. Nec-1 has also been known to have off target effects and studies using nec-1 should be interpreted with caution[91]. Necroptosis is thought to have evolved as an alternative cell death pathway when apoptosis is not possible. Recently the positive effects of catalytic inactive RIPK1 kinase dead and RIPK3 ablation in different pathologies was challenged by Newton et al. as MLKL loss was not as effective as RIPK3 knockout or catalytic inactive RIPK1 in these models, re-iterating the MLKL independent roles of the RIPKs[63]. In addition to its kinase activity, ubiquitinated RIPK1 can form a platform and participate in the activation of signaling cascades by approximating multiprotein complexes. For example, RIPK1 serves to facilitate NFκB and MAPK activation. This platform function of RIPK1 is important for the integrity of hepatocyte function as hepatocyte RIPK1KO or knockdown sensitizes to TNF mediated apoptosis (without affecting NFκB activation in most reports)[57–59,100,112]. It is therefore not surprising that in many animal models of liver disease, RIPK1 kinase inhibition genetically or pharmacologically had a different effect than RIPK1 ablation in liver parenchymal cells. Furthermore, the effect of RIPK1 in the immune cells and non-parenchymal liver cells in these injury models has not been fully established[14].

RIPK3's role in the liver has been quite controversial. Although RIPK3 is most known as the kinase activating MLKL for necroptosis execution, RIPK3 can also mediate apoptosis.

Mutant kinase inactive RIPK3D161N protein and RIPK3 small molecule inhibitors promote apoptosis, due to the induction of a conformational change in RIPK3 resulting in RIPK1 mediated caspase-8 recruitment[5,37]. Whether necroptosis occurs in liver disease and in hepatocytes in particular is controversial[14,106]. It is generally accepted that cells that do not express RIPK3 do not undergo necroptosis[36]. Therein lies one of the controversies regarding the RIPKs and necroptosis in liver disease, as RIPK3 is not expressed in hepatocytes under basal conditions[7,13,78,79]. However, increased expression of RIPK3 in both animal models of acute and chronic liver injury and human liver diseases has been suggested by some investigators, but not all[81–85,87,119]. In one model, due to a lack of increase in RIPK3 mRNA, impaired proteasome function was implicated as the cause of increased RIPK3[81]. Much of the work on the RIPKs was done by immunostaining of liver tissue and most investigators have interpreted increased RIPK3 staining as the “induction of necroptosis” bringing up the issues of specificity of these stains on injured liver, and the quality of the reagents used[14]. In addition, most genetic knockout studies use complex embryonic multiprotein knockout models which could result in unforeseen compensatory biologic consequences and complicate interpretation of data and extrapolation to human physiology. Since these critiques are universal limitations of experimental models multiple scientific approaches and more consistent findings by independent laboratories are necessary to draw firm conclusions regarding RIPK3 and necroptosis in the liver. We have been unable to detect RIPK3 in diseased human livers (unpublished data). In mice we were only able to detect RIPK3 in the NPC fraction of the liver including immune cells and endothelial cells and others have confirmed this data[78,79]. However, MLKL is robustly expressed. A few key points and open questions remain that will guide future research in the field. Why is MLKL present in hepatocytes in the absence of RIPK3 and what other protein(s) can activate it? What are the non-necroptosis functions of RIPK1 and RIPK3 in the liver? What is the role of the RIPKs in the non-parenchymal liver cells? Given the heterogeneity of the liver and its diverse cell population, a more detailed examination of the effect of the RIPKs in the liver in a cell specific manner should be undertaken. It is evident more than ever that non-necroptotic functions of the RIPKs in the liver (including apoptosis) may contribute to some of the findings in liver disease models. It is also important to consider that the cell death functions and non-cell death/ inflammatory functions of the RIPKs may not be mutually exclusive. Finally, the most pressing clinical issue remains, which is to understand if necroptosis occurs with an intact apoptotic machinery and in the presence of caspase-8 in the physiologic setting or if activation of necroptosis is a potential complication of the use of caspase inhibitors therapeutically in human liver disease. There is still much to be learned about the functions of the RIPKs and future studies will shed some light on the complex biology of these multifunctional proteins.

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Abbreviations

ALT	Alanine Aminotransferase
APAP	Acetaminophen
α-GalCer	α-galactosylceramide
ASC	Apoptosis-associated Speck-like protein containing a Carboxy-terminal CARD
ASH	Alcoholic Steatohepatitis
Birc3	Baculoviral IAP repeat-containing 3
cIAP	cellular Inhibitor of Apoptosis
cFLIP	cellular FLICE inhibitory protein
ConA	Concanavalin A
CYLD	cylandromatosis
DAI	DNA-dependent Activator of IRFs/Z-DNA binding protein 1
DD	Death Domain
DR	Death Receptor
DEN	Diethylnitrosamine
ERK	Extracellular signal-regulated kinase
FADD	Fas-Associated protein with Death Domain
DMSO	Dimethyl Sulfoxide
IHC	Immunohistochemistry
IDO	Indoleamine 2,3-Dioxygenase
KO	Knockout
KC	Kupffer cell
LRRK1 and 2	Leucine-Rich Repeat Kinase 1 and 2
LPCKO	Liver Parenchymal Cell Knockout
LUBAC	linear ubiquitin chain assembly complex
MCD	Metheionine Choline Deficient Diet
MTH-Trp	methyl-thiohydantoin-tryptophan

MLKL	Mixed lineage kinase domain-like
MOMP	mitochondrial outer membrane pore opening
MKK2	MAP kinase-activated protein kinase 2
NAPQI	N-Acetyl-p-benzoquinone imine
NF-kB	nuclear factor kB
NEMO	NF-kappa-B Essential Modulator
Nfkbia	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
NAFLD	non alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NK	natural killer cell
NLRP3	NOD-like receptor protein 3
NPC	non-parenchymal cells
Roc/COR	Ras of complex proteins/C-terminal of Roc
RIPK1	Receptor Interacting serine/threonine Protein Kinase 1
RIPK3	Receptor Interacting serine/threonine Protein Kinase 3
RHIM	RIP Homology Interaction Motif
TNF	Tumor Necrosis Factor
Tnfaip3	TNF alpha induced protein 3
TGFβ	transforming growth factorβ
TAK1	-activated-kinase-1
TAB2	TAK1 Binding protein-2
TAB3	TAK1 Binding protein-3
TLR3 and TLR4	Toll-Like Receptor-3 and 4
TRAIL	TNF Related Apoptosis Inducing Ligand
TRADD	TNF Receptor Associated Death Domain
TRAF	TNF Receptor Associated Factor
TRIF	TIR-domain-containing adapter Inducing Interferon-β
WT	wild type

ZVAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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Main Concepts and Learning Points

- RIPK1 and RIPK3 participate in both apoptotic and necroptotic cell death and this is largely context dependent
- RIPK1 and RIPK3 have cell death independent functions
- RIPK1 knockdown and knockout sensitizes hepatocytes to apoptotic cell death in TNF/DR mediated cell death models, indicating its scaffolding function mediates hepatocyte survival
- RIPK3 is undetectable in hepatocytes under basal conditions; however, it may be induced in certain diseases
- Whether necroptosis occurs in hepatocytes is unclear
- The contribution of RIPK1 and RIPK3 in various liver disease models has been examined and is discussed below

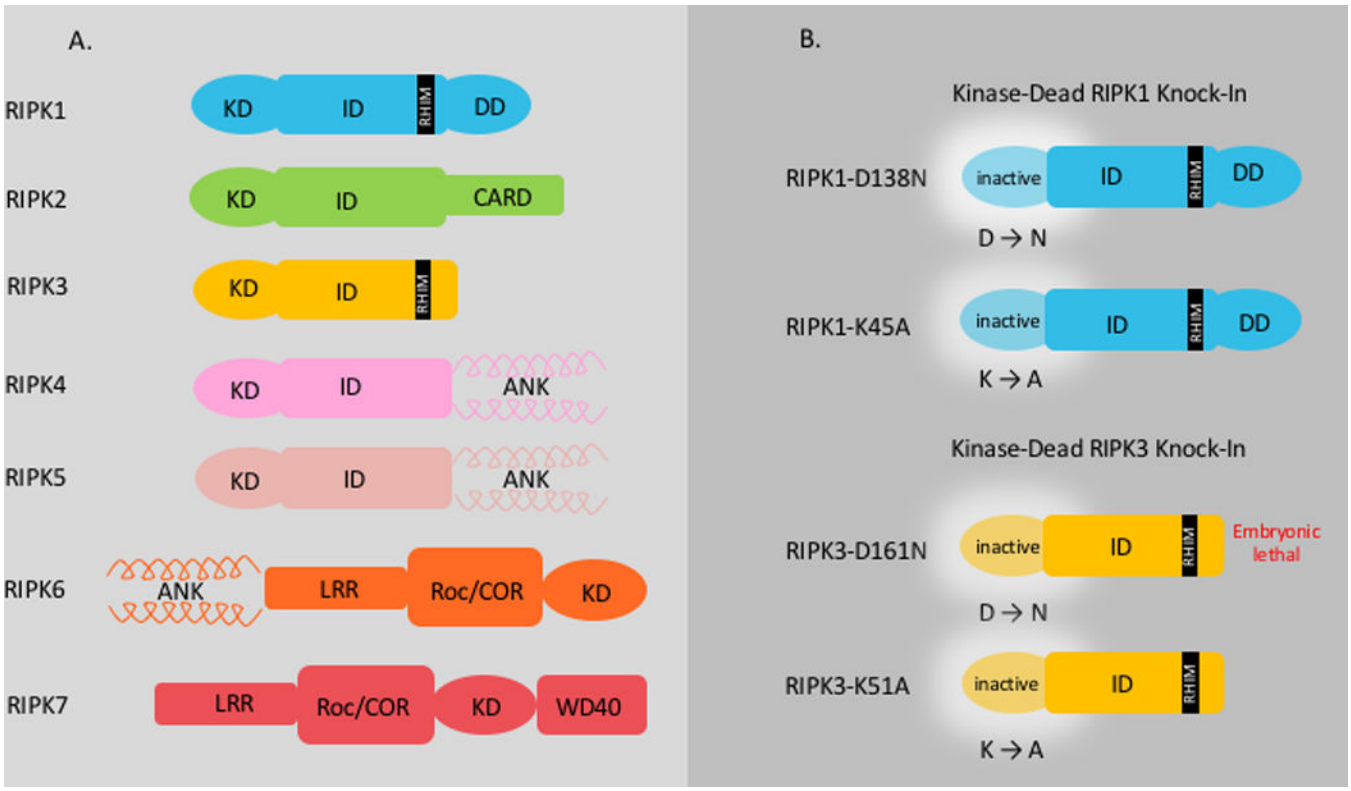

Figure 1.

Panel A: The Receptor Interacting Kinase family is comprised of seven diverse members with a homologous Kinase Domain (KD). Each protein contains additional specific domains which are unique to the protein and dictate its function.

Panel B: The mutated Kinase-Dead Knock-In RIPK1 and RIPK3 mice that have been generated to study cell death are depicted. **RIPK1-D138N:** In order to render the kinase domain inactive, the catalytically important HKD (Asp-Phe-Gly) motif is altered. The conserved aspartate (D) at position 138 is mutated to asparagine (N) resulting in a "kinase-dead" protein. The mice are viable and fertile. **RIPK1-K45A**: A point mutation in the catalytic lysine (K) to alanine (A) in exon 3 of the RIPK1 gene eliminates all kinase activity resulting in a "kinase-dead" protein. These mice are viable and fertile. **RIPK3 D161N:** The aspartate (D) at position 161 in the catalytically important DFG (Asp-Phe-Gly) motif of the kinase domain of RIPK3 is mutated to an asparagine (N), rendering the protein catalytically inactive or "kinase-dead". The mice generated with this mutation are not viable and die at E11.5. **RIPK3-K51A:** The lysine (K) at position 51 on the ATP-binding pocket is mutated to an alanine (A) resulting in "kinase-dead" RIPK3. These mice are viable and fertile. Abbreviations: ANK: Ankyrin Repeats, CARD: caspase activation and recruitment, KD: Kinase Domain, ID: Intermediary Domain, RHIM: RIP Homeotypic Interaction Motif, LRR: Leucine Rich Repeats, RIPK1: Receptor Interacting Kinase, roc/COR: Ras of complex proteins/C-terminal of Roc, WD40: WD40 Domain.

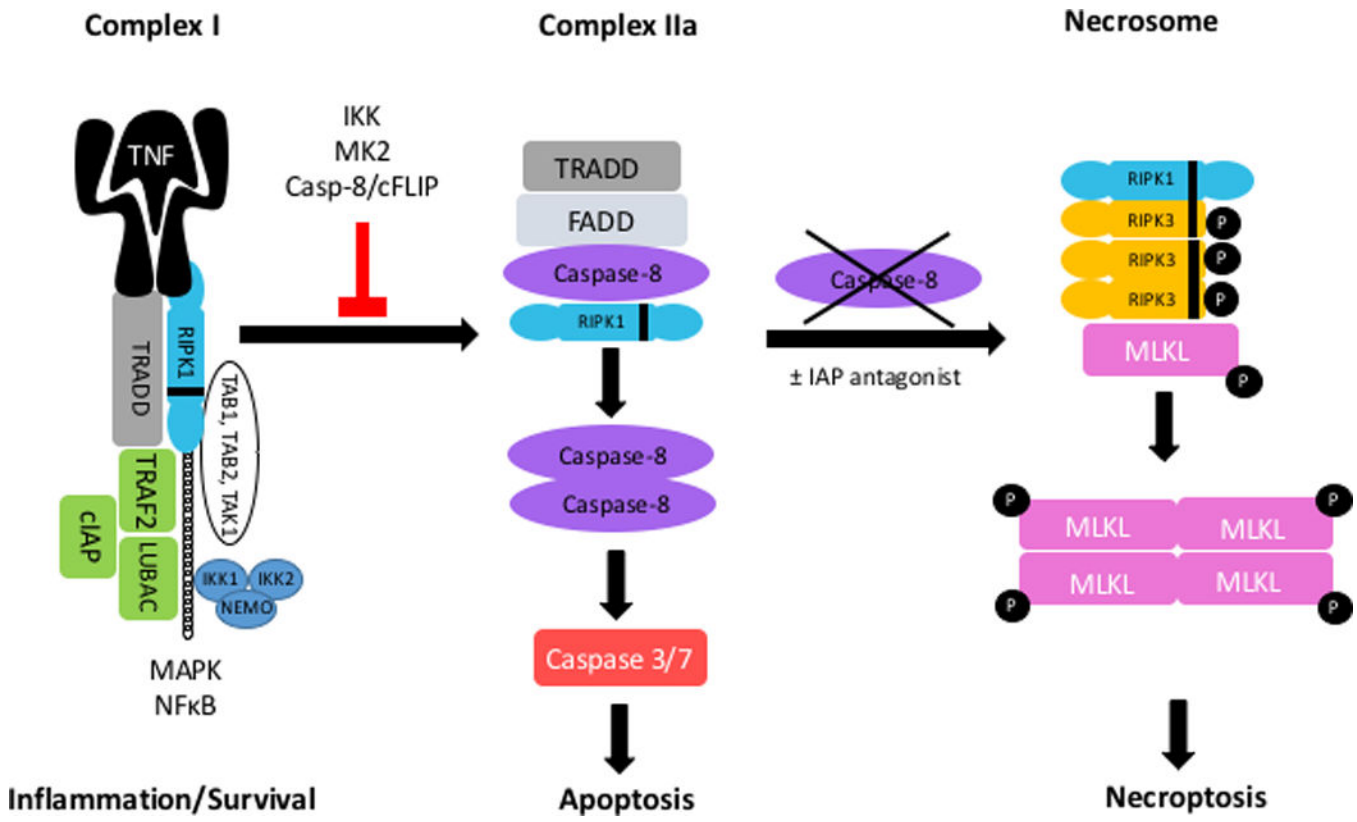

Figure 2.

TNF binding to its receptor results in the formation of Complex-1 which includes TRADD, RIPK1, TRAF2, IAP1, IAP2 and LUBAC. Ubiquitination of RIPK1 results in the formation of a platform and subsequent recruitment of IKK1, IKK2, NEMO and TAB/TAK-1 complexes. This results in the activation of NF-κB and MAPK cascade, promoting stress response, inflammation and survival. When RIPK1 is not ubiquitinated, Complex-II forms made up of TRADD, FADD and caspase-8 and RIPK1 (IIa) or RIPK1/RIPK3, FADD, Caspase 8 and FLIPL (IIb, not shown) which leads to caspase-8-mediated activation of caspase 3/7 and apoptosis. In certain cells when caspase-8 is inhibited RIPK1, RIPK3, and MLKL form the necrosome, resulting in MLKL phosphorylation leading to its oligomerization and translocation to cell membrane to induce necroptosis.

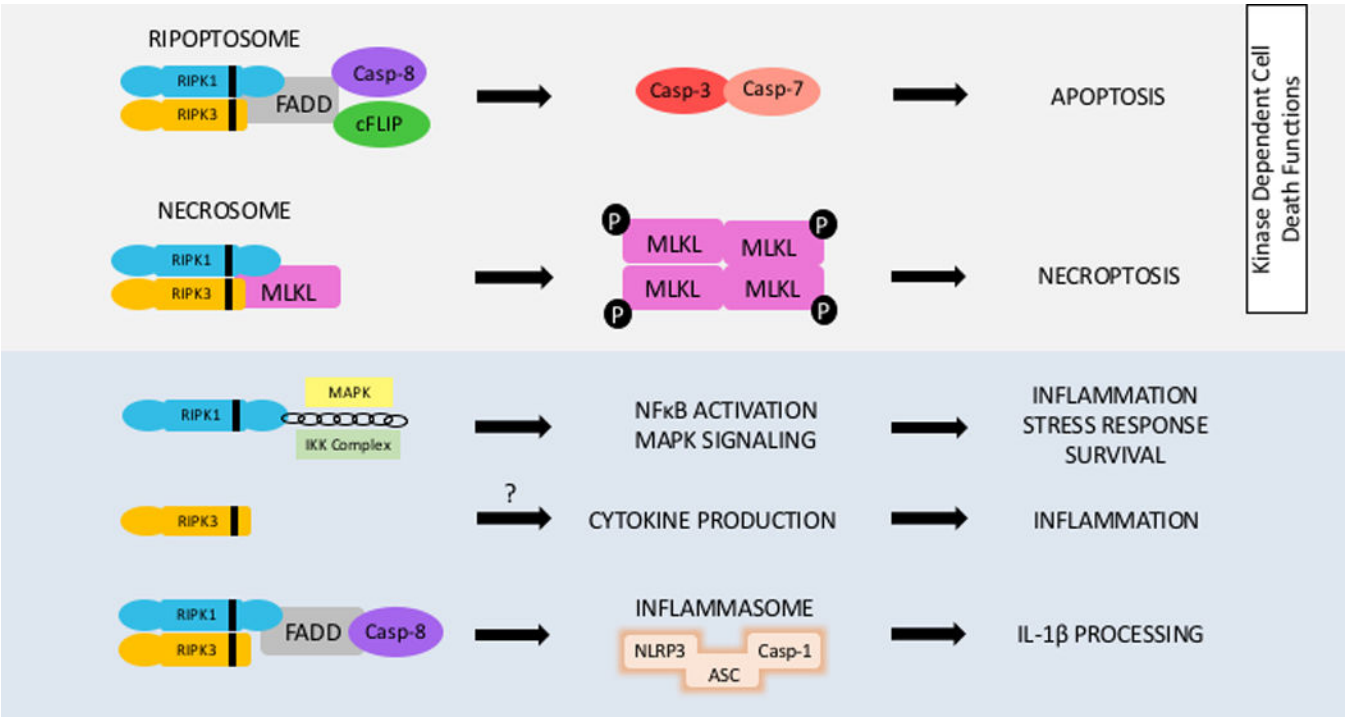

Figure 3.

Although the RIPKs are most known in the context of necroptosis they are multifunctional proteins. The kinase activity of RIPK1 mediates apoptosis and necroptosis while its scaffolding activity enables NFκB activation, inflammation and cell survival. RIPK3's necroptosis independent roles include activation of cytokine secretion in certain models and inflammasome activation via caspase-8. Both RIPK1 and RIPK3 have been implicated in activation the NLRP3 inflammasome pathway.

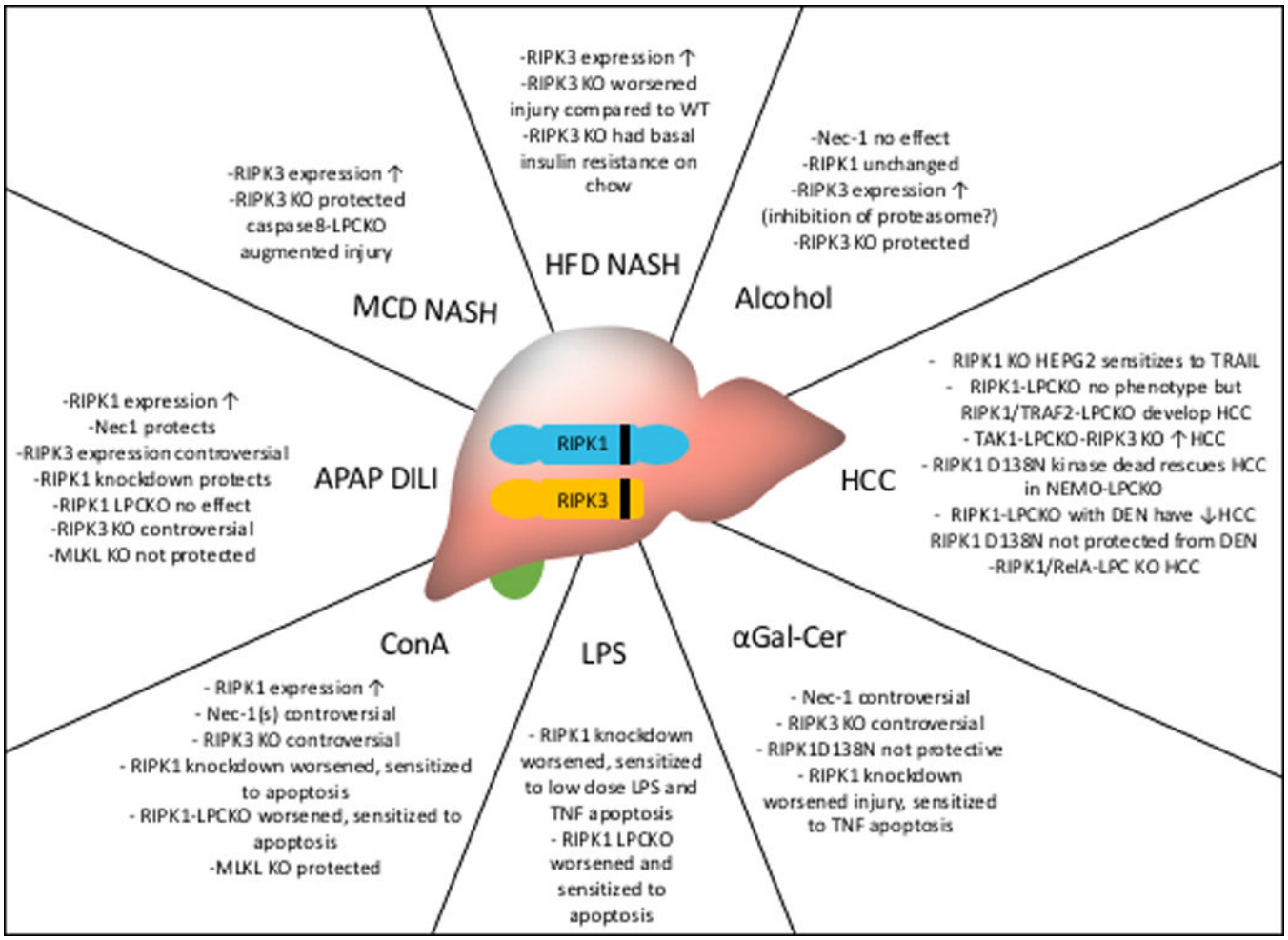

Figure 4.

RIPK1 and RIPK3 have been studied in various models of liver disease, generating significant controversy.