Cell death and inflammation – a dangerous but vital liaison

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Abstract

The immune system has developed multiple ways to fight infection. Yet, it is constantly tasked with overcoming newly developing pathogenic mechanisms of resistance to host immunity. In most mammals, the stimulation of both, innate and adaptive immune receptors can result in gene activation and cell death induction by apoptosis and necroptosis. RIPK1 and RIPK3 are key mediators of necroptosis; however, new findings support their role in the regulation of cell death-independent proinflammatory signaling. Here, we discuss the biological functions of RIPK1 and RIPK3 and how they regulate cell death and inflammation as well as the interplay between them. Finally, we discuss recent advances in our knowledge of linear ubiquitination which, alongside RIPK3 and Caspase-8, exerts regulatory functions on RIPK1-mediated inflammation. Together, this review examines the complex interplay between RIPK1, RIPK3 and LUBAC which is important to regulate cell death and inflammatory signaling.

Host defense against tissue damage

Tissue damage, for instance as a consequence of breakdown of cellular membranes, is dangerous, not the least because it can cause infection; the same is obviously true the other way around. Consequently, many organisms can sense both, damage and pathogens, a feat achieved via so–called **damage- and pathogen-associated molecular patterns (DAMPs and PAMPs)** (see glossary), respectively. Molecular patterns associated with different forms of damage, or various pathogens, are recognized by a family of receptors referred to as pattern-recognition receptors (PRRs). PRRs are expressed by both, immune and non-immune cells. The mammalian PRR family consists of Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs) and DNA sensors such as DNA-induced activator of IFN (DAI, also known as ZBP1) and Cyclic GMP-AMP synthase (cGAS). Triggering of PRRs results in the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) signaling, and subsequent induction of inflammatory cytokines and chemokines including, amongst others, tumor necrosis factor (TNF) and interleukin-1β (IL-1β). Engagement of certain PRRs also results in the activation of interferon regulatory factor (IRF) 3 and IRF7 and subsequent induction of type I interferons (IFNs)[1]. These cytokines play crucial roles in triggering the mammalian innate immune response by binding to their respective receptors, including TNF receptor (TNFR) superfamily

(SF) members. In addition to their ability to further induce inflammatory mediators via MAPK and NF-κB activation, some TNFR-SF members, are known as death receptors (DRs). This includes TNFR1, CD95 (Fas/APO-1), death receptor 3 (DR3) and two different receptors for the TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-R1 (DR4) and TRAIL-R2 (DR5). These receptors have a cytoplasmic death domain (DD) that allows them to transduce a regulated pro-death signal resulting in **apoptotic or necroptotic cell death** (see glossary)[2, 3].

Apoptosis and necroptosis are tightly regulated processes that are executed by specialized proteins, such as Caspase-8, RIPK1, RIPK3 and MLKL[2, 3]. The discovery of these factors has revolutionized the way we understand cell death and disease. We are, however, only starting to understand how these molecules are regulated and, importantly, the interplay between them, including how they regulate each other. Recent breakthrough investigation has not only made tremendous steps in understanding the roles of these molecules in cell death regulation but has also uncovered that they bear important non-death functions. These cell death-independent functions are related to the regulation of inflammatory signaling, the battle against pathogen and the regulation of key developmental processes such as hematopoiesis. This review will explore the most recent findings on these topics and discuss future avenues for investigation.

Signal transduction by death receptors

The different signaling outputs downstream of DRs strongly rely on the many components of the signaling complexes that are formed upon DR engagement by their cognate ligands. For example, ligation of TNFR1 by TNF results in the formation of a membrane-bound signaling complex referred to as the TNFR1 signaling complex (TNFR1-SC) or complex I of TNFR1 signaling (**Figure 1**). Formation of the TNFR1-SC is initiated by the TNF-induced recruitment of the adaptor proteins TNFR1-Associated Via Death Domain (TRADD), the Receptor Interacting Protein Kinase 1 (RIPK1), TNF Receptor Associated Factor 2 (TRAF2 and/or TRAF5) and the E3 ubiquitin ligases cellular Inhibitor of Apoptosis (cIAP) proteins 1 and 2 (cIAP1/2)[4]. TNFR1-SC-recruited cIAP1/2 ubiquitinate various components of the TNFR1-SC, including RIPK1 and cIAP1, enabling the recruitment of another E3 ubiquitin ligase, the Linear UBiquitin chain Assembly Complex (LUBAC), which exclusively generates linear, also referred to as methionine 1 (M1)-linked, ubiquitin chains on substrates, including RIPK1, TRADD and TNFR1[5, 6]. This highly ubiquitinated protein complex enables efficient recruitment and retention of the kinase complexes comprised of the Transforming growth factor-β-activated kinase 1 and TAK1-binding protein 2 and 3 (TAK1/TAB2/3) and the Inhibitor of nuclear factor κ-B kinase subunits α, β and NF-κB essential modulator (IKKα/IKKβ/NEMO), with NEMO itself also being modified by M1-linked ubiquitin[7]. Recently, the non-canonical IKKs, TANK-Binding

Kinase 1 (TBK1) and IKKε were identified to form part of the TNFR1-SC to which they are recruited by NEMO, via the adaptor proteins TRAF family member-associated NF-κB activator (TANK) and Nucleosome assembly protein 1 (NAP1), in a linear-ubiquitin-dependent manner in cancer cell lines and primary mouse embryonic fibroblasts (MEFs)[8]. Activation of TAK1/TAB2/3 and IKKα/IKKβ/NEMO results in subsequent gene induction through activation of NF-κB and MAPK signaling (**Figure 1**).

Under certain circumstances, the stimulation of TNFR1 by TNF can also induce cell death in mammalian cells. In these cases, a cytoplasmic complex, called complex-II, is formed. This cell death-inducing complex originates from the TNFR1-SC and contains RIPK1 and/or TRADD to which the DD-containing adaptor protein Fas-associated DD (FADD) is recruited[9, 10] (**Figure 1**). FADD brings caspase-8 to the complex which, upon recruitment-mediated activation, initiates apoptosis induction. However, complex-II-associated RIPK1 can also recruit and activate RIPK3 which triggers necroptosis execution[11-13]. Both, apoptosis and necroptosis are tightly regulated by the cellular FLICE-like inhibitory protein (cFLIP)[14]. All isoforms of cFLIP can form heterodimers with caspase-8 in complex II. The heterodimer between the long isoform of cFLIP ($cFLIP_L$) and Caspase-8 can cleave RIPK1 and RIPK3 and prevent necroptosis[15, 16] but it is not clear whether this enzymatic activity is responsible for this functional effect.

In addition to ubiquitin ligases, various deubiquitinases (DUBs) are important in regulating the signals triggered by TNF, including CYLD (cylindromatosis) and TNFα -induced protein 3 (A20) which are recruited to the TNFR1-SC where they regulate the gene-activatory and cell death-inducing output of TNF signaling (reviewed in [17, 18]). CYLD-mediated cleavage of polyubiquitin chains has been shown to function as a negative regulator of NF-κB [19, 20]. However, the role of A20 in TNF-signaling is independent of its DUB activity since it was shown that A20 binds and protects M1 chains from cleavage [21]. Thereby, A20 likely interferes with recruitment of IKKα/β/NEMO kinase complex by binding to linear chains generated in, for instance, RIPK1 and TNFR1 [21]. A20 and CYLD, however, have opposing roles on the regulation of cell death induction by TNF. Whereas A20 prevents cell death by protecting linear ubiquitin chains from degradation, CYLD cleaves them and sensitizes cells to TNF-induced death[21, 22]. Induction of cell death by CYLD is thwarted by caspase-8/$cFLIP_L$-mediated cleavage[23]. Furthermore, the DUB known as OTU deubiquitinase with LINear linkage specificity (OTULIN) regulates TNF signaling outcomes by modulating LUBAC function[21, 24, 25] (**Figure 1**). This demonstrates the high level regulation of cell death and inflammation downstream of DRs, ubiquitination and deubiquitination events being prominent regulatory checkpoints.

Cell death can be induced independently of death receptors by binding of intracellular bacterial, viral or host danger signals to NLRs (different inflammatory cell death pathways are reviewed in [26]). This results in the assembly of a multi-protein complex called inflammasome. The best characterized inflammasome complex contains NLRP3 and activates caspase-1, thereby inducing maturation IL-1β and IL-18 and cleavage of gasdermin D. Gasdermin D then drives cell death by pyroptosis by forming pores in the membrane which is accompanied by the release of inflammatory mature IL-1β and IL-18[27]. Recent work has also revealed a role for caspase-8 in processing and activation of Gasdermin D[28-30].

RIPK1 – the molecular switch between life and death

It is now established that, whereas the **scaffolding function** (see glossary) of RIPK1 is protective, the kinase activity of RIPK1 is pro-death. Indeed, RIPK1 deficiency induces postnatal lethality in mice which is prevented by combined loss of RIPK3 and caspase-8, but not by their individual loss[31, 32]. By contrast, mice that express a kinase-dead version of RIPK1 (*Ripk1*^{K45A} *or Ripk1*^{D138N}) are viable and resistant to necroptosis-dependent LPS-induced hypothermia, TNF-induced shock or Vaccinia Virus infection [33, 34]. This indicates that the kinase activity of RIPK1 is crucial to mediate necroptosis in mice in a variety of necroptosis-inducing insults. Therefore, RIPK1 is a double-edged sword that can prevent, but also allow cell death, at least in mouse models (reviewed in[35]).

Posttranslational modifications on RIPK1 play a critical role in this functional switch by preventing RIPK1 from recruiting and activating RIPK3 at cell death complexes. For instance, RIPK1 phosphorylation at specific sites when present in the TNFR1-SC prevents its auto-phosphorylation (e.g. S166), recruitment to complex-II and cell death in human and mouse cells[36, 37] (**Figure 1**). Likewise, ubiquitination of RIPK1 can play a key role in the regulation of cell death and inflammation (reviewed in [35, 38]).

An acceptor site for K63 linkages on RIPK1 is K377, which was proposed to be crucial for optimal NF-κB activation and prevention of cell death since human and mouse cells bearing a K377R mutation in RIPK1 display attenuated induction of NF-κB and MAPK pathways and are sensitized to TNF-, poly-IC- and LPS-induced cell death *in vitro*[38]. Yet, the relevance of this ubiquitination site *in vivo* remains to be determined. Using MEFs derived from mice bearing a cIAP1 mutation in the ubiquitin associated (UBA) domain (*Ciap2*$^{-/-}$*Ciap1*^{UBAmut}), another report showed that the UBA domain of cIAP1, is required to repress RIPK1 auto-activation. This is achieved by cIAP1-UBA-mediated ubiquitination of several Ub-acceptor lysines on RIPK1 since *Ciap2*$^{-/-}$*Ciap1*^{UBAmut} MEFs displayed fewer ubiquitinated lysines, which promoted its autoactivation[39]. In addition, cIAP1-generated K48-linkages on RIPK1 target it for proteasomal degradation, consequently limiting its stability[39]. Therefore, in the absence of

a functional UBA domain, an increased amount of RIPK1 molecules with fewer ubiquitinated lysines induces exacerbated TNF-mediated cell death.

In the last two years, several kinases that prevent RIPK1 auto-phosphorylation have been identified (reviewed in[40]). The canonical kinases IKKα and IKKβ phosphorylate RIPK1 at several sites in the TNFR1-SC, thereby preventing its transition to complex II as demonstrated using MEFs deficient for each individual kinase or by IKKα/β inhibition[41]. The non-canonical IKKs TBK1 and IKKε also phosphorylate RIPK1 and this activity is required to prevent TNF-induced RIPK1 kinase activity-dependent cell death *in vitro* and *in vivo*[8, 42]. This was evidenced by the fact that MEFs lacking TBK1 were sensitized to TNF-induced cell death which was prevented by treatment with a RIPK1 kinase inhibitor. In addition, whereas TBK1 inhibition sensitized mice to TNF-induced shock, expression of kinase-dead instead of wildtype RIPK1 prevented embryonic lethality of *Tbk1*$^{-/-}$ mice[8, 42] (Table 1). Although not recruited to the TNFR1-SC, the p38 MAPK target, MAP kinase-activated protein kinase 2 (MK2) phosphorylates RIPK1 at S321 and S336 and might constitute a secondary checkpoint in the control of RIPK1-mediated cell death, given that the mere phosphorylation of RIPK1 by MK2 is not strictly required to prevent TNF-induced cell death but only upon depletion of cIAP1/2 or TAK1, which was shown in primary murine cells either treated with pharmacological inhibitors or in genetic models of cells lacking RIPK1 or MK2[43-45] (**Figure 1**).

The kinases that control RIPK1 auto-phosphorylation cannot substitute for each other, i.e. when only one of these distinct cell death checkpoints fails to operate properly, TNF induces cell death[8, 41-45] (**Figure 3a**). It is tempting to speculate that TNF signaling may serve the purpose of a 'litmus test' which might probe for proper immune signaling, checking whether TNF is capable of inducing the activation of MAPKs, the NF-κB-activating kinases IKKα and IKKβ , and the kinases TBK1 and IKKε required for□□□□□□□□ion of IFN-γ downstream of several immune receptors, albeit not TNFR1[1, 8]. If TNF signaling were to fail this litmus test in a given cell, TNF would induce its death and, thereby, the release of a multitude of DAMPs[8, 41-45]. In doing so, TNF, previously referred to as the 'fire alarm of the immune system'[46], would chime the big ben of fire alarms.

RIPK3 – killing me… not so softly

Necroptosis is induced as a result of the formation of a hetero-amyloid complex between RIPK1 and RIPK3 that associates via their respective RIPK homotypic interaction motif (RHIM), called the necrosome[11, 13, 47]. The kinase activities of both RIPK1 and RIPK3 are required for canonical necroptosis induction **(Figure 1)**[33, 34, 48]. RIPK3, in turn, phosphorylates and, thereby, activates the pseudokinase mixed lineage kinase domain-like (MLKL)[49, 50]. However, certain PRRs, such as TLR3 and TLR4, can drive RIPK3 activation via the adapter

protein TIR-domain-containing adaptor-inducing IFN-β (TRIF) and the DNA-induced activator of IFN (DAI, also known as ZBP1)[51, 52], two additonal RHIM-containing proteins **(Figure 1)**.

Spontaneous necroptosis is observed in mutant mice lacking the RHIM domain of RIPK1, resulting in embryonic lethality **(Table 1)**[37, 53]. This aberrant necroptosis is mediated by DAI and, to a lesser extent, also by TRIF. These findings were genetically demonstrated by loss of DAI in mice, which rescued *Ripk1*$^{\Delta RHIM}$ mice from lethality[37]. Furthermore, deficiency in RIPK3 or DAI allowed *Ripk1*$^{-/-}$Casp-8$^{-/-}$ mice to be born, demonstrating that RIPK1 could serve an essential survival function by interfering with aberrant RIPK3/DAI-induced lethal necroptosis[31, 32, 37].

The mechanism of activation of DAI/RIPK3-mediated necroptosis under physiological conditions remains to be determined. DAI has long been thought of as a specific sensor of dsDNA, yet, endogenous RNA was recently found capable of activating DAI. This was demonstrated using MEFs infected with murine cytomegalovirus (MCMV). In this context, cell death induction upon infection required RNA synthesis but not viral DNA replication and DAI was found to directly bind to RNA via its Z-binding domain (ZBD)[54]. Supporting the notion of DAI as an RNA sensor, another report showed that DAI-deficient mice are susceptible to lethal infection by Influenza A virus (IAV) [55]. These studies indicate that RIPK1 may prevent non-canonical necroptosis induced by endogenous RNA, challenging the role of DAI as an exclusive DNA sensor[56].

In essence, the more we learn about necroptosis execution, especially about the role of RIPK3 in this process, the more we come to realize that its regulation is more complex than originally anticipated. There are several open questions concerning the interactions of different RHIM-domain-containing proteins and how they regulate necroptosis (see “outstanding questions”). Although it now appears that RHIM-dependent oligomerization of RIPK3 is a common execution step in necroptosis induction, further and robust investigation is required to understand the nature of RIPK3 activation.

MLKL – the key to membrane opening

Once RIPK3 has been activated it undergoes a conformational change that induces its autophosphorylation, enabling the phosphorylation and activation of MLKL[57-60]. Activation of MLKL results in its recruitment to the plasma membrane where it can induce the formation of pores and the release of cellular content, including ions, leading to deleterious osmotic pressure ultimately resulting in disruption of membrane integrity. The mechanism of MLKL execution of necroptosis is still debated but it is believed that the pseudokinase interacts with phosphatidylinositol phosphates (PIPs) in the membrane and oligomerizes therein as

demonstrated by crosslinking and oligomerization assays as well as lipid/protein coprecipitations under necroptotic conditions[58, 61]. Moreover, a recent report described the MLKL channel as an octamer that is formed by pre-assembled tetramers; these MLKL tetramers were thought to form within the necrosome and to be released before or during translocation to the plasma membrane[62].

Activation of MLKL, however does not represent a point-of-no-return in the execution of necroptosis[63-65]. It has been shown that the endosomal sorting complexes required for transport (**ESCRT) machinery** (see glossary), which accumulates at MLKL-damaged sites in the membrane, can facilitate membrane repair and, consequently, survival. Presumably, the ESCRT machinery, by localizing to the site of damage in the plasma membrane, enables the release of such damaged membrane domains and, with them, accumulated MLKL, in the form of membrane-surrounded bubbles[63-65]. Indeed, silencing of ESCRT components sensitized cells to necroptosis following activation of RIPK3 or MLKL by expression of the dimerizers RIPK3-2Fv or hMLKL$^{1–181}$-2Fv[56-58]. These studies suggest that, if the MLKL-activating stimulus is sufficiently powerful to allow the pore-forming activity of activated MLKL to surpass the repair activity of the ESCRT pathway, the cell might undergo necroptosis[63-65]. It appears there is still much to be discovered regarding MLKL regulation and the mechanisms of necroptosis execution and it will be exciting to learn about posttranslational modifications of MLKL (e.g. ubiquitination), as these may play a role in regulating the outcomes of necroptosis.

RIPK3 – now going apoptotic

Other than a key inducer of necroptosis, RIPK3 can also regulate apoptosis. A role of RIPK3 in the regulation of apoptosis is prominent during viral infection. For instance, mice constitutively lacking RIPK3 or both MLKL and FADD, but not MLKL alone, are more susceptible to Influenza A virus (IAV) infection than their wild-type counterparts. This reveals an important role for RIPK3-mediated apoptosis in antiviral immunity[66].

Under sterile conditions, RIPK3 can also induce apoptosis. A few years back, two individual studies reported that different mutations leading to the abrogation of RIPK3 kinase activity harbored opposing phenotypes in mice. Whereas *Ripk3*$^{K51A/K51A}$ mice were born and resistant to necroptosis stimuli, *Ripk3*$^{D161N/D161N}$ mice were embryonically lethal due to aberrant RIPK1 and caspase-8 dependent apoptosis since this lethality was prevented by concomitant deletion of RIPK1 and caspase-8 (**Table 1**)[33, 48]. In addition, high concentrations of two specific RIPK3 inhibitors, GSK'843 and GSK'872, allosterically activated RIPK3, triggering the formation of a FADD/RIPK1/Caspase-8 platform that ultimately killed murine cell lines and MEFs via apoptosis[48]. These findings indicated that conformational changes caused by the RIPK3 D161N mutation enabled RIPK3 to induce apoptosis (**Figure 2a and b**).

A recent report extended on these findings and provided important insight into the function of the kinase domain of RIPK3[62]. *Ripk1*$^{-/-}$ mice, which normally survive embryogenesis, die at late gestation during embryogenesis when crossed to *Ripk3*$^{+/D161N}$ mice (*Ripk1*$^{-/-}$*Ripk3*$^{+/D161N}$ mice) (**Table 1**)[67]. This finding, together with the observation that heterozygosity of *Ripk3* does not induce embryonic lethality in RIPK1-null mice[31, 32], indicates that the D161 mutation might determine a conformational change that, in the absence of RIPK1, causes the activation of the wild-type copy of RIPK3, resulting in exacerbated necroptosis (**Figure 2a**). Although this remains to be directly shown, this study provides compelling evidence that whilst the D161N mutation of RIPK3 abrogates its kinase activity, it also locks RIPK3 in a configuration that favors homodimerization via the kinase domain and allosteric activation of wild-type RIPK3. Consistent with this model, a mutation of the homodimerization interface of RIPK3, R69H, abolishes the allosteric activation otherwise afforded by the D161N mutation, demonstrating the importance of the scaffolding function of RIPK3 in cell death regulation[67].

Lastly, MEFs lacking RIPK3 and treated with Smac mimetics or TAK1 inhibitors were protected from TNF-induced apoptosis. However, cells expressing kinase-dead (K50A) or RHIM-deficient RIPK3 were only partially protected. This means that in conditions of cIAP1/2 or TAK1 depletion, which induce RIPK1-dependent cell death, RIPK3 contributes to caspase-8 activation independently of its kinase activity or RHIM domain[68]. This again implies a scaffolding role for RIPK3 in apoptosis regulation (**Figure 2c**). Together, these studies support the notion that the kinase domain of RIPK3 also harbors scaffolding functions and that, together, these two functions may regulate apoptosis and necroptosis. Yet, future studies are needed to provide further mechanistic insight on these distinct roles of RIPK3.

Nonetheless, despite the decisive roles for RIPK1, RIPK3 and their respective kinase activities in the induction of cell death, they also exert cell-death-independent functions.

The life of RIPs – it's not all about death

It is well established that, at least in certain cell types, RIPK1 is involved in NF-κB-induced gene activation and production of various proinflammatory cytokines. In addition, RIPK3 has been shown to be involved in inflammation also independently of cell death as discussed in this section.

In a recently reported mouse model expressing a catalytically-inactive mutant of RIPK3 containing a four-amino-acid deletion (*Ripk3*$^{\Delta 20\text{-}23}$ mice), which phenotypically resembles a kinase-dead mutant that does not affect homodimerization (see discussion above), FADD-deficient mice were rescued from embryonic lethality (**Table 1**)[69]. This demonstrated that catalytically-inactive RIPK3 could interfere with aberrant necroptosis triggered when FADD is

absent[69]. However, unlike *Fadd*$^{-/-}$*Ripk3*$^{-/-}$ mice, which are viable, *Fadd*$^{-/-}$*Ripk3*$^{\Delta 20\text{-}23}$ mice succumb at birth due to increased production of inflammatory cytokines, such as TNF, IL-1β and IL-6, in the intestine[69]. Whether the inflammation in *Fadd*$^{-/-}$*Ripk3*$^{\Delta 20\text{-}23}$ mice is due to a function of FADD that requires the kinase activity of RIPK3 or due to a gain-of-function of the RIPK3 mutation (unleashed in the absence of FADD) remains to be determined. Nevertheless, this result shows that the kinase activity of RIPK3 can regulate proinflammatory signaling independently of necroptosis.

Both RIPK1 and RIPK3 have also been shown to promote TRIF-requiring but MyD88-independent cytokine production (TNF, CCL3, CCL4, GM-CSF and IL-6) by LPS upon caspase-8 inhibition *in vitro*, since bone marrow derived macrophages (BMDMs) deficient for RIPK1 or RIPK3 were unable to promote cytokine release in response to LPS plus zVAD, a pan caspase inhibitor[70]. Of note, cytokine release was not affected in MLKL-deficient BMDMs, indicating that RIPK1 and RIPK3 can enable macrophages to mount an inflammatory response independently from their roles in MLKL-dependent necroptosis[70]. Along these lines, LPS/zVAD-induced type I IFN production in BMDMs by the TLR4 pathway requires the kinase activities of both, RIPK1 and RIPK3[71]. In addition, enhanced IFN-β synthesis was observed after challenge with attenuated mutant strains of *Yersinia pseudotuberculosis* and *Klebsiella pneumoniae* upon caspase inhibition. This was again strictly dependent on the kinase activities of RIPK1 and RIPK3[71]. *In-vivo* injection of non-cytotoxic doses of LPS in mice also led to a RIPK1- and RIPK3-dependent acute inflammatory response and type I IFN production[70, 71]. However, in this *in-vivo* situation, caspase inhibition was not required, since acute inflammation was observed upon LPS treatment alone. This might be due to a limited expression of caspase-8 by BMDMs *per se* in response to LPS, although this remains to be assessed[71]. In contrast, in another study, loss of RIPK3 or of the kinase activity of RIPK1 had no impact on the response of mice to LPS-induced sepsis[72]. Although this differential response could be due to the varying doses of LPS used in the different studies, further investigation is required to determine the exact roles of RIPK1 and RIPK3 in LPS responses *in vivo*.

Similarly to how RIPK1 and RIPK3 mediate acute inflammation, they can also play important roles in fighting infection. For example, RIPK1 kinase activity was shown to be required to fight *Yersinia pseudotuberculosis* by promoting apoptosis and cytokine production by hematopoietic cells[73]. Studies involving RNA viral infection have also shown that RIPK3-deficient mice are more susceptible to infection by influenza A virus (IAV) than wildtype controls[66, 74-76]. The role of RIPK3 in this context might be due to both RIPK3-mediated apoptosis and necroptosis, as mentioned previously. However, the authors show RIPK3 interaction with the mitochondrial antiviral-signaling protein (MAVS), an adaptor in RLRs signaling platforms, and decreased IFN-β production. Yet, deficiency of RIPK3 in BMDMs had

no effect on necroptosis induction upon IAV infection in BMDMs[74]. This evidence suggests that RIPK3 might interfere with RIPK1–MAVS interaction, thereby limiting IFN-β production in BMDMs independently of necroptosis. The authors also showed that RIPK3-deficient BMDMs displayed impaired Protein Kinase R (PKR) signaling as detected by decreased phosphorylation of PKR and its downstream target phospho-eIF2α. This implies that RIPK3 can induce activation of the PKR pathway in BMDMs, stabilizing *Ifnb* (IFN-β) transcripts[74]. Therefore, although RIPK3 can dampen the production of IFN-β downstream of RLR signaling, it can also stabilize IFN-β mRNA, thereby providing an IFN-β response that appears to be sufficient to fight viral infection[74].

Another study reported that RIPK3 could restrict West Nile virus (WNV)-induced encephalitis in a cell death-independent manner. Specifically, whereas RIPK3-deficient mice exhibited enhanced mortality upon WNV infection compared to wild-type mice, MLKL- or MLKL/caspase-8-deficient mice were unaffected, implicating a cell death-independent role of RIPK3 in this process[77]. The authors further demonstrated that RIPK3-deficient cortical neurons displayed impaired neuronal chemokine and cytokine production, such as CCL2 and CXCL10, upon WNV infection, poly(I:C) or LPS[77]. This indicates that RIPK3 is crucial for several TLRs to induce the secretion of proinflammatory cytokines. Thus, this work implicated RIPK3 in the regulation of immune responses in the central nervous system (CNS) [77], but its exact functions warrant further investigation.

From another angle, RIPK3 has been proposed to be involved in the activation of inflammasomes[78]. Depletion of cIAP1/2 in BMDMs or BM derived dendritic cells (BMDCs) by Smac mimetics induced activation of caspase-1 and secretion of IL-1β. Moreover, deletion of RIPK3 interfered with this process after LPS treatment[79]. Another report additionally showed that caspase-1 and IL-1β activation in *Mlkl*$^{-/-}$ BMDM was similar to that in WT cells[80]. Together, these studies suggest that RIPK3 is required for inflammasome activation independently of necroptosis induction. Consistent with *in-vitro* experiments, IL-1β-dependent autoantibody-mediated arthritis is exacerbated in cIAP1/2-null mice and it is counteracted by deletion of RIPK3[79, 80]. Furthermore, deficiency in the DUB A20 triggers LPS- and poly(I:C)-induced IL-1β maturation in BMDMs which was prevented by treatment with the RIPK1 kinase inhibitor Necrostatin-1s or by constitutive loss of RIPK3[81] (**Table 1**). The authors show that ubiquitin linkages on pro-IL-1β are increased in the absence of A20 and that this ubiquitination was prevented in the absence of RIPK3, implying that A20-dependent IL-1β deubiquitination requires RIPK3. Together, these studies indicate that RIPK3 can promote NLRP3 inflammasome activation and IL-1β-mediated inflammatory responses. In addition, they suggest that the NLRP3 inflammasome can be controlled by ubiquitination events which, in turn, are tightly regulated by RIPK1 and RIPK3. Furthermore, RIPK1 and RIPK3 appear to be

required for RNA virus-induced activation of the NLRP3 inflammasome since RIPK3 deficiency or Necrostatin-1s treatment in BMDMs infected with vesicular stomatitis virus (VSV) interfered with inflammasome formation[75]. Although the RNA sensor that drives RIPK1/RIPK3-dependent NLRP3 activation is unknown, a proposed mechanism suggests that the mitochondrial protein GTPase Dynamin related protein 1 (DRP1) might be involved in this process since depletion of DRP1 from murine BMDMs also interfered with inflammasome assembly. In addition, RIPK3 deficiency and RIPK1 inhibition prevented DRP1 activation as well as generation of reactive oxygen species (ROS) from mitochondria in infected cells. Therefore the authors propose that RIPK1/RIPK3 might activate DRP1 which could in turn promote the release of ROS[75].

Collectively, these reports implicate RIPK1 and RIPK3 in innate immunity against pathogens, importantly in a manner that is not solely dependent on cell death regulation but also appears to involve cell death-independent proinflammatory signaling.

Control of RIPK1-mediated proinflammatory signaling

When examining the roles of RIP kinase proteins, reports have indicated that RIPK1 can play a pivotal role in **emergency hematopoiesis** (i.e. engraftment following BM transplantation, see glossary)[31]. Although fetal hematopoiesis in *Ripk1*$^{-/-}$ mice is normal and hematopoietic progenitors are unaltered at birth, fetal BM progenitors lacking RIPK1 have impaired capacity to self-renew and to repopulate the BM of lethally irradiated mice[47]. Loss of RIPK1 from the hematopoietic compartment results in cell death in the spleen, the thymus and the BM and a decrease in hematopoietic stem and progenitor cells (HSPC). These defects appear to be partly mediated by RIPK3-dependent necroptosis since dying cells that were negative for cleaved caspase-3 were observed in the RIPK1-deficient BM and spleen and full loss of RIPK3 prevented this[82]. However, hematopoietic precursors deficient in both RIPK1 and RIPK3 have been shown to lack long-term multi-lineage-repopulating activity; the mechanism for this has been attributed to TNF-induced apoptosis since *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ HSPCs remained sensitive to TNF-induced apoptosis *in vitro*[82]. These studies are relevant in that they indicate that RIPK1 is required for the integrity of major hematopoietic organs and emergency hematopoiesis in a process that depends on cell death, at least in mice.

Another study has shown that embryos which lack heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), one of the two essential LUBAC components, together with caspase-8 and RIPK3 (*Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Hoil-1*$^{-/-}$), display increased proinflammatory cytokine production and impaired fetal hematopoiesis, as demonstrated by a dramatic decrease in hematopoietic progenitors[83, 84] (**Table 1**). Of relevance, proinflammatory cytokines and hematopoietic defects were prevented upon removal of RIPK1. However, these defects were not observed in mice lacking

HOIL-1, caspase-8 and MLKL, which suggests that these defects are independent of cell death induction. Thus, the absence of caspase-8 and LUBAC function uncovers an essential function of RIPK3 in preventing RIPK1 from interfering with normal fetal hematopoiesis. How RIPK1 unleashes uncontrolled cytokine production and whether LUBAC and RIPK3 cooperate to counteract this activity warrants further investigation.

Nevertheless, based on currently available genetic models, some speculation can be made in this regard. For instance, since mice deficient in both caspase-8 and RIPK3 do not have impaired hematopoiesis that would cause embryonic lethality, LUBAC appears to be sufficient to keep RIPK1 in check to ensure proper hematopoiesis during embryogenesis. If this were true, it would indicate that LUBAC and RIPK3 might independently prevent aberrant RIPK1 signaling in the absence of caspase-8. However, deletion of caspase-8 alone can trigger enhanced inflammatory cytokine production in the absence of RIPK3 in mice, subsiding until birth, meaning that caspase-8 deficiency in itself can also promote deregulation of proinflammatory signaling[85]. Deletion of RIPK1 prevents this inflammatory output demonstrating that the inflammatory phenotype in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice is a result of aberrant RIPK1 signaling[85]. Since deregulated cytokine production in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice does not result in embryonic lethality but the one in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Hoil-1*$^{-/-}$ mice does, LUBAC alone may be able to restrict aberrant RIPK1-mediated signaling to a degree that is compatible with survival (**Figure 2b**). However, in order to maintain optimal regulation of RIPK1, it is likely that the three components, LUBAC, caspase-8 and RIPK3, act in concert rather than independently of each other.

Studies performed on mice with mutations in OTULIN confirm a role for linear ubiquitination, caspase-8 and RIPK3 in regulating RIPK1-mediated signaling during embryogenesis. Hypomorphic (W96R, D336E mutations or *Otulin*^{gumby} mice) or catalytically inactive OTULIN (C129A mutation) resembles LUBAC deficiency, as it results in embryonic lethality due to defective vascularization in mice[24, 25, 86]. Indeed, vascular defects in *Otulin*^{C129A} mice have been demonstrated genetically to be caused by exacerbated cell death as co-ablation of RIPK3 and caspase-8 normalized vascularization[25]. Although the vascular defects in *Otulin*^{gumby} mice were originally attributed to aberrant Wnt signaling, the role of cell death has not been investigated[86] (**Table 1**). At face value, the fact that OTULIN deficiency virtually phenocopies LUBAC deficiency in mice is counterintuitive, because OTULIN was originally thought of as a negative regulator of LUBAC[86]. However, it has been demonstrated that deficiency in OTULIN or in its activity can result in aberrantly increased linear ubiquitination of different LUBAC components prior to TNFR1-SC engagement and that this interferes with effective LUBAC recruitment to the TNFR1-SC in human cells[21]. These findings were later confirmed in murine cells[25]. Consequently, this creates a LUBAC hypomorph that cannot be recruited to signaling

complexes and act in them appropriately[24, 25]. Similar to LUBAC deficiency, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ *Otulin*^{C129A} mice succumb after birth due to aberrant production of type I IFNs[25]. Loss of one copy of RIPK1 normalized IFN production and rescued these mice from postnatal lethality, implicating RIPK1-mediated signaling as responsible for aberrant inflammation and lethality in these mice[25].

Collectively, these results show that RIPK3, caspase-8 and linear ubiquitination are important to block RIPK1-mediated cell-death-independent proinflammatory signaling during embryogenesis. It therefore appears that, similar to the regulation of RIPK1-mediated cell death, the non-cell-death aspects of RIPK1 signaling are placed under a stringent, triple control mechanism, namely one that relies on LUBAC, caspase-8 and RIPK3. However, unlike the triple control of RIPK1-mediated cell death, where a cell can die when any of the three kinases IKKα/β, TBK1/IKKε or MK2 fails[8, 41-45], in the case of the non-cell-death function of RIPK1 signaling, either one of the three control mechanisms is sufficient to prevent RIPK1 from exerting signaling that is lethal for the organism (**Figure 3a and b**).

Concluding remarks

During the past 10 years, significant steps towards the understanding of different programmed cell death pathways triggered by members of the TNFR-SF and PRRs have been made. Although several questions remain unanswered (see “outstanding questions”), this research has yielded the identification of key players of various programmed cell death pathways, the discovery of cell-death-driven inflammation and, based thereupon, the realization of new exciting perspectives to putatively target different forms of programmed cell death for the treatment of a variety of human diseases. At the same time, it is becoming increasingly clear that the very same players of these cell death pathways, including RIPK1, RIPK3, cIAP1/2, LUBAC and caspase-8, have important functions beyond their roles in cell death signaling. Current evidence suggests that these cell-death-independent functions are primarily related to the regulation of proinflammatory cytokine production. Moreover, these functions and outputs need to be carefully considered when seeking therapeutic intervention that perturbs one or more of these pathways; caution should be taken when attempting to inhibit any of these components with the aim of preventing pathological cell death and ensuing inflammation.

It is important to highlight that our increased understanding of the regulation of cell death and inflammation and of their interdependency in the form of the discovery of cell-death-driven inflammation, may prove crucial for the provision of effective treatments for patients suffering from auto-inflammatory disorders arising as a consequence of mutations in genes encoding, e.g. NEMO, A20, OTULIN, the different LUBAC components, TBK1, and RIPK1. Ultimately,

this line of research will also hopefully lead to the discovery of more effective therapeutic options for patients with other chronic inflammatory, autoimmune or neurodegenerative diseases. It will be exciting to follow future research that aims to further elucidate the mechanisms and biology of these complex signaling pathways.

Key Table, Table 1. Overview of mouse phenotypes bearing gene mutations in cell death regulators.

Genotype	Phenotype	Restored phenotype		Reference
	Spontaneous	Partial	Complete	
Casp8$^{-/-}$	Embr. lethal E10.5 Excessive cell death	*Tnfr1*$^{-/-}$ (E17.5)	*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$	32, 87-90
	Proinflammatory phenotype	*Ripk3*$^{-/-}$	*Ripk3*$^{-/-}$*Ripk1*$^{-/-}$	85
Fadd$^{-/-}$	Embr. lethal E10.5 Cardiovascular defects		*Ripk3*$^{-/-}$ *Ripk1*$^{-/-}$	32, 88, 91
Ripk1$^{-/-}$	Perinatally lethal Multiorgan cell death and inflammation	Myd88$^{-/-}$ *Tnfr1*$^{-/-}$ (~P12) *Trif1*$^{-/-}$ *Tnfr1*$^{-/-}$*Trif1*$^{-/-}$ (~P24) *Ripk3*$^{-/-}$ (~P4) *Mlkl*$^{-/-}$ (~P4) *Tradd*$^{-/-}$ (perinatal): intestinal inflammation *Dai(Zbp1)*$^{-/-}$ *Dai(Zbp1)*$^{-/-}$*Trif1*$^{-/-}$ (~P25)	*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ *Ripk3*$^{-/-}$*Tnfr1*$^{-/-}$ *Tradd*$^{-/-}$*Ripk3*$^{-/-}$ (runted adults) *Ripk3*$^{-/-}$*Dai(Zbp1)*$^{-/-}$ (runted adults) *Casp8*$^{-/-}$*Dai(Zbp1)*$^{-/-}$*Trif1*$^{-/-}$	31, 32, 37, 53, 92, 93
Ripk3$^{-/-}$	Viable			11, 12
Mlkl$^{-/-}$	Viable			49
Ripk1^{K45A}	Viable			94
Ripk1^{D138N}	Viable			33, 34
Ripk3^{K51A}	Viable			48
Ripk3^{D161N}	Embr. lethal E11.5 Excessive apoptosis	*Casp8*$^{+/-}$ (E15.5) *Ripk1*$^{+/-}$ (E18.5)	*Ripk1*$^{-/-}$*Casp8*$^{-/-}$	33
Ripk3$^{+/D161N}$ *Ripk1*$^{-/-}$	Embr. lethal E18.5 Excessive necroptosis			67
Ripk3$^{\Delta 20-23}$	Viable			69
Ripk3$^{\Delta 20-23}$ *Fadd*$^{-/-}$	Perinatal lethal Proinflammatory cytokine production			69

Ripk1$^{\Delta RHIM}$	Perinatally lethal Multiorgan cell death and inflammation		*Ripk3*$^{-/-}$ *Mlkl*$^{-/-}$ *Dai(Zbp1)*$^{-/-}$	37
Ikkα$^{-/-}$	Perinatal death Abnormal skin and limb development			95, 96
Ikkβ$^{-/-}$	Embr. lethal E14.5 Liver apoptosis			97, 98
Nemo$^{-/-}$	Embr. lethal E14.5 Liver apoptosis			99
Tbk1$^{-/-}$	Embr. lethal E14.5	*Ripk3*$^{-/-}$ (nd)	*Ripk1*^{D138N}	42, 100
cIAP1/2$^{-/-}$	Embr. lethal E10.5 Excessive cell death	*Ripk1*$^{-/-}$ (E14.5) *Ripk3*$^{-/-}$ (E14.5) *Tnfr1*$^{-/-}$ birth		101
Hoip$^{-/-}$	Embr. lethal E10.5 Excessive cell death	*Tnf*$^{-/-}$ (E15.5): Excessive cell death *Tnfr1*$^{-/-}$ (E17.5): ND *Ripk1*^{K45A}: Excessive cell death	*Mlkl*$^{-/-}$*Casp8*$^{-/-}$: runted mice	83, 102
Hoil-1$^{-/-}$	Embr. lethal E10.5 Excessive cell death	*Tnfr1*$^{-/-}$ (E16.5): ND *Ripk1*^{K45A}: Excessive cell death *Ripk3*$^{-/-}$*Casp8*$^{-/-}$: Proinflammatory cytokine production and haematoietic defects	*Mlkl*$^{-/-}$*Casp8*$^{-/-}$: runted mice *Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Ripk1*$^{-/-}$: runted mice	83
Cyld$^{-/-}$	Δexon 4: viable and fertile Δexons 1and 2: viable, sterile males			103, 104
Tnfaip3$^{-/-}$ (A20$^{-/-}$)	Multi-organ inflammation and premature death	*Asc*$^{-/-}$, *Nlrp3*$^{-/-}$, *Casp1*$^{-/-}$ and *Ripk3*$^{-/-}$ decreased levels of mature IL-1β in response to LPS, poly:(IC) and zymosan in BMDMs (survival nd). *Ripk3*$^{-/-}$ (25 weeks)	*MyD88*$^{-/-}$	72, 81, 105, 106

		Ripk1^{K45A} (25 weeks) *Mlkl*$^{-/-}$ (4 weeks)		
Otulin^{gumby}	Embr. lethal E10.5 Vascular defects, Aberrant Wnt signaling			86
Otulin^{C129A}	Embr. lethal E10.5 Excessive cell death	*Ripk1*^{D138N} (E14.5): nd *Tnfr1*$^{-/-}$ (E16.5): nd *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ (perinatal): Proinflammatory cytokine production *Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Ripk1*$^{-/-}$: nd	*Ripk3*$^{-/-}$*Casp8*$^{-/-}$*Ripk1*$^{+/-}$ Survived up to 3 months	25

Embr: embryonic; Δ: deletion/truncation; nd: non-determined

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1: Gene activation and cell death regulation by TNFR1-SC in mammals. Engagement of tumor necrosis factor (TNF) receptor 1 (TNFR1) by TNF induces formation of the TNFR1 signaling complex (TNFR1-SC) in which the kinase-containing functional units Transforming growth factor-β-activated kinase 1 (TAK1) and TAK1-binding protein 2 and 3 (TAK1/TAB2/3) and the Inhibitor of nuclear factor kappa-B kinase subunit alpha, beta and NF-kappa-B essential modulator (IKKα/IKKβ/NEMO), respectively induce activation of mitogen-activated protein kinases (MAPKs)- and nuclear factor κ-light-chain-enhancer of activated B cells (NF- □B)-mediated expression of survival genes and proinflammatory cytokines (left panel). Apart from this activity, these two kinase complexes act together with the kinases TANK Binding Kinase 1 (TBK1)/IKK□ to keep the kinase activity of Receptor interacting protein kinase 1 (RIPK1) in check. In addition, the MAP kinase-activated protein kinase 2 (MK2) regulates RIPK1 phosphorylation in the cytoplasm to prevent RIPK1 from going into cell death complexes. Ubiquitination events by cellular Inhibitor of Apoptosis (IAPs) and the Linear Ubiquitin chain assembly complex (LUBAC) as well as deubiquitination events by CYLD (cylindromatosis) and deubiquitinase with LINear linkage specificity (OTULIN) are important for the regulation of gene activation and cell death. The cell death complex or complex II can contain TNFRSF1A Associated Via Death Domain (TRADD), RIPK1, Fas-Associated DD (FADD), caspase-8/10 and RIPK3. In the canonical necroptosis pathway, when Caspase-8 activity is completely absent, RIPK1/RIPK3 signaling is unchecked. The kinase activities of RIPK1 and RIPK3 allow their stable oligomerization via their RIPK homotypic interaction motif (RHIM) domains and subsequent RIPK3 autophosphorylation and activation. Alternatively, other RHIM-containing proteins, such as DNA sensors such as DNA-induced activator of IFN (DAI, also known as ZBP1), can activate RIPK3 in the non-canonical necroptosis pathway, independently of RIPK1. Activated RIPK3 recruits mixed lineage kinase domain-like (MLKL) and induces its phosphorylation. MLKL then undergoes a conformational change leading to rupture of the plasma membrane and induction of necroptosis.

Figure 2: Role of RIPK3 in necroptosis and apoptosis regulation in mice. a) A point mutation in D161 renders the Receptor interacting protein kinase 3 (RIPK3) catalytic inactive and prevents necroptosis induction. This mutation induces a conformational change that activates RIPK1 to induce caspase-8/FADD-mediated apoptosis (left panel). Yet, if RIPK1 is lost, D161N mutant RIPK3 is able to activate a wild-type copy of RIPK3 to induce necroptosis. b) A point mutation in K51 also renders RIPK3 catalytic inactive but without affecting its scaffolding functions. Therefore, this mutation does not activate RIPK1-mediated apoptosis whilst blocking necroptosis. c) Upon loss of the cellular Inhibitor of Apoptosis 1 and 2 (cIAP1/2)

or the Transforming growth factor-β-activated kinase 1 (TAK1), the scaffolding function RIPK3 plays a role in RIPK1-mediated apoptosis.

Figure 3: Control on RIPK1-mediated proinflammatory signaling in mice. a) The three kinase complexes, Inhibitor of nuclear factor □-B kinase subunits α, β and NF-□B essential modulator (IKKα/IKKβ/NEMO), TANK-Binding Kinase 1 (TBK1) and IKKε and MAP kinase-activated protein kinase 2 (MK2) are essential to prevent RIPK1-autophosphorylation and the pro-death function of RIPK1 (left panel). When any of the three checkpoints fail, RIPK1 induces cell death (right panel). b) The Linear UBiquitina chain Assembly complex (LUBAC), caspase-8 (C8) and the Receptor Interacting protein 3 (RIPK3, R3) act in concert to regulate RIPK1-mediated proinflammatory signaling (left panel). In the absence of *Ripk3*, *Casp8* and the heme-oxidized IRP2 ubiquitin ligase 1 (*Hoil-1)*, RIPK1 induces embryonic lethality due to induction of proinflammatory cytokines and defective hematopoiesis (left panel). When, instead of *Ripk3*, mixed lineage kinase domain-like *(Mlkl)* is absent together with *Hoil-1* and *Casp8*, RIPK3 is able to keep RIPK1 in check to allow embryogenesis, yet it is not sufficient to block lethal inflammation during adulthood (middle panel). In the absence of *Ripk3* and *Casp8*, LUBAC is sufficient to prevent RIPK1-mediated embryonic lethality and to allow survival during adulthood. Yet, it is not sufficient to fully prevent RIPK1-mediated proinflammatory cytokines (right panel).

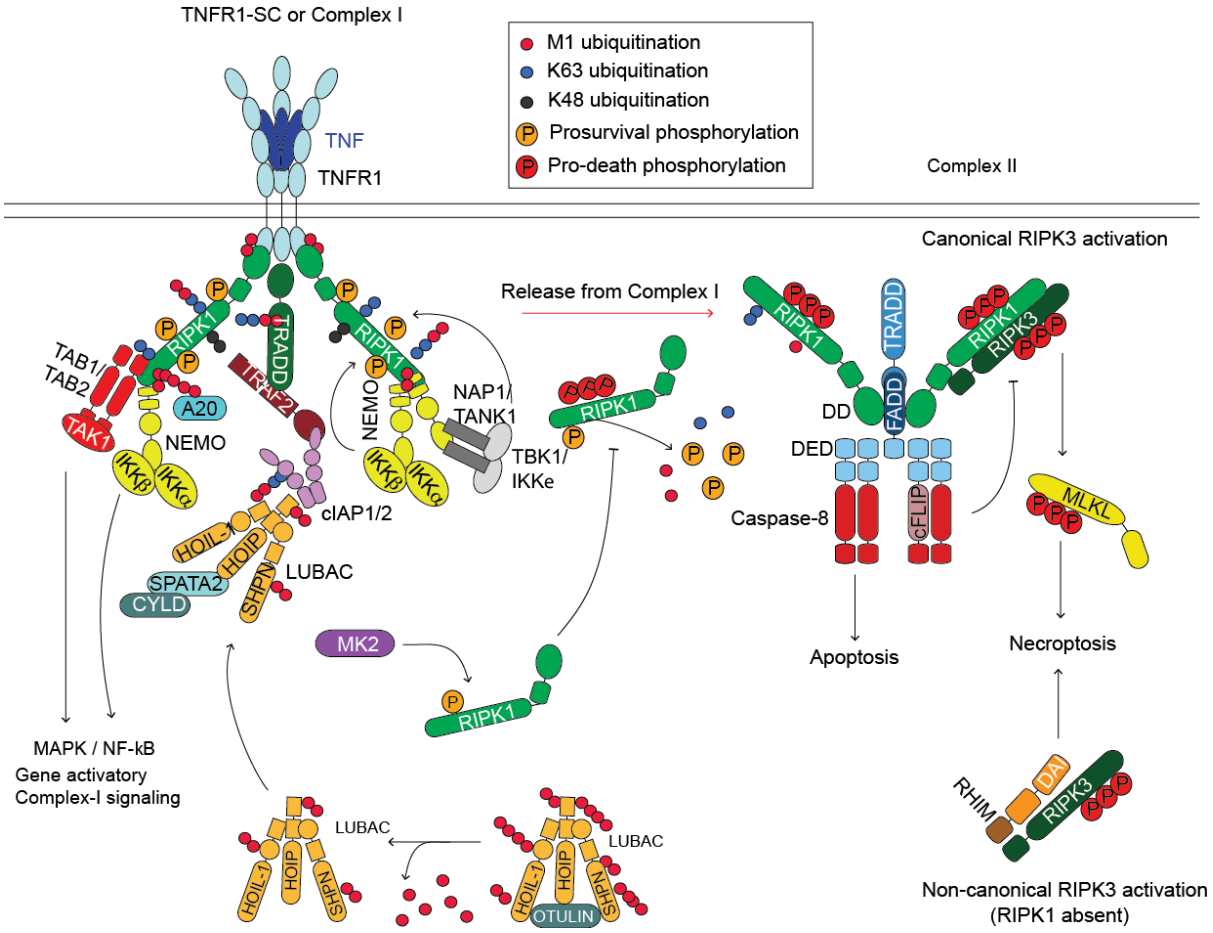

Figure 1

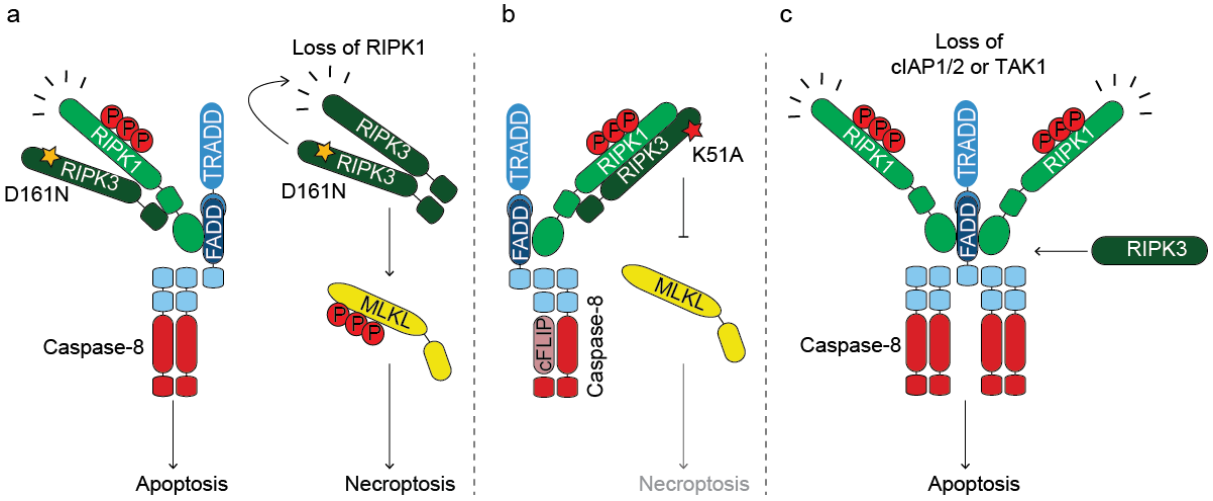

Figure 2

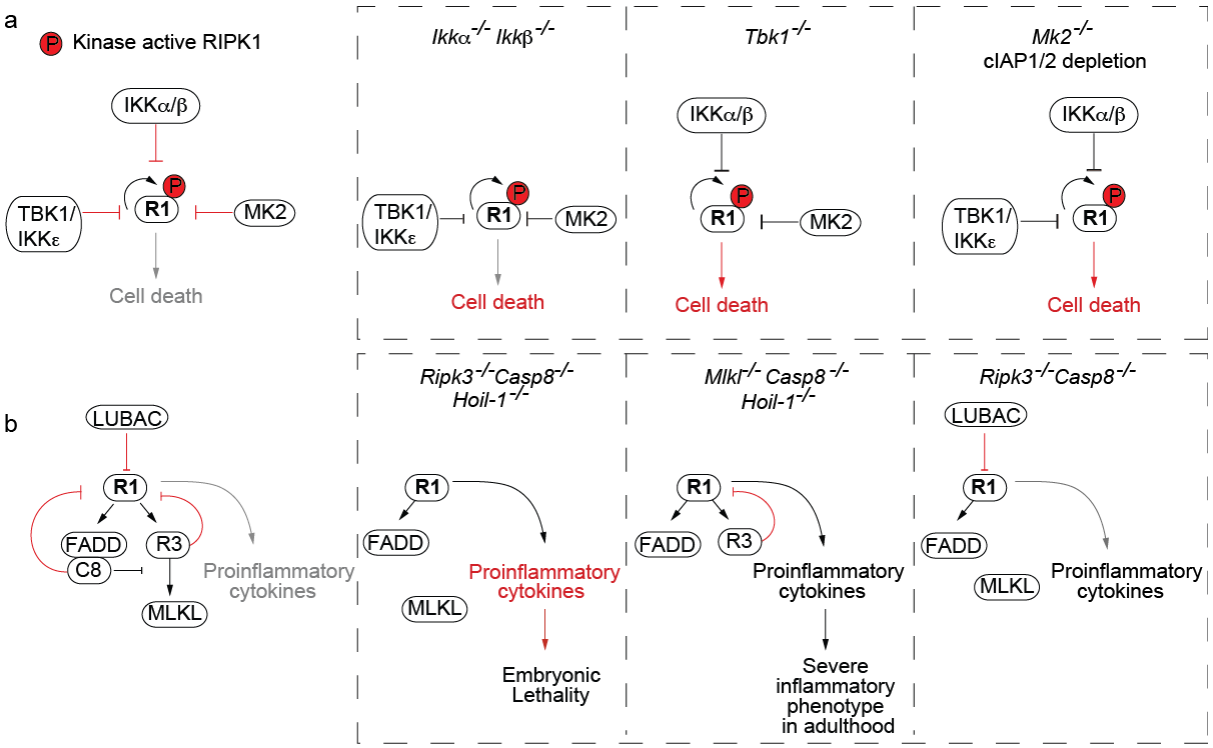
a
Kinase active RIPK1
IKKα/β
TBK1/
IKKε
R1
MK2
Cell death
Ikkα-/- Ikkβ-/-
Tbk1-/-
Mk2-/-
cIAP1/2 depletion
b
LUBAC
R1
FADD
R3
C8
MLKL
Proinflammatory
cytokines
Ripk3-/-Casp8-/-
Hoil-1-/-
Mlkl-/- Casp8-/-
Hoil-1-/-
Embryonic
Lethality
Severe
inflammatory
phenotype
in adulthood

Figure 3