Review

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RIPK1 and RIPK3: critical regulators of inflammation and cell death

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RIPK1 and RIPK3 (receptor-interacting serine/threonine protein kinases 1/3) interact by virtue of their RIP homotypic interaction motifs to mediate a form of cell death called necroptosis, although mice lacking these kinases have very different phenotypes. RIPK1-deficient mice die soon after birth, whereas RIPK3-deficient mice are healthy. Necroptosis involves cell rupture and is triggered by tumor necrosis factor (TNF), Toll-like receptors (TLRs), or the T cell receptor (TCR) when pro-apoptotic caspase-8 is inhibited. Various mouse models of disease are ameliorated by RIPK3 deficiency, suggesting that necroptosis contributes to pathology. Genetic rescue experiments now reveal why RIPK3-deficient are viable but RIPK1-deficient mice are not. These and other experiments indicate unexpected complexity in the regulation of both apoptosis and necroptosis by RIPK1 and RIPK3.

Necroptosis under the spotlight

Cell death in response to different insults can be loosely categorized as either apoptosis or a form of necrosis. During apoptosis, members of the caspase family of intracellular cysteine proteases dismantle the cell into membrane-enveloped fragments called apoptotic bodies that are engulfed rapidly by neighboring healthy cells [1–3]. Apoptosis plays an important role in sculpting tissues during development, and efficient clearance of the cell corpse limits triggering of the innate immune system [4]. It is conceivable, however, that apoptosis in other contexts will be damaging and trigger inflammation. By contrast, cell rupture during necrosis is considered highly inflammatory because it releases intracellular components that activate innate immune cells. Necroptosis is a form of regulated necrosis that is executed by RIPK1 and/or RIPK3 when caspases are inhibited. Necroptosis has received much attention in recent years because its inhibition, either genetically or with small-molecule inhibitors, is reported to lessen disease severity in several mouse models (Table 1). Thus, while necroptosis appears to mediate host defense against some pathogens [5–8], its inhibition in certain contexts may have therapeutic potential. This review focuses on recent genetic and biochemical studies that reveal unexpected complexity in the regulation of cell death and inflammation by RIPK1 and RIPK3.

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Necroptosis signaling triggered by TNF receptor 1 (TNFR1) and TLRs

The proinflammatory cytokine TNF, in combination with a pan-caspase inhibitor such as zVAD.fmk, is sufficient to activate RIPK3-dependent necroptosis in some cell types, whereas robust killing of other cells requires TNF, caspase inhibition, and an antagonist of the inhibitor of apoptosis (IAP) proteins. Other death ligands, including FasL and Trail, as well as TLR agonists such as lipopolysaccharide (LPS) and poly(I:C) that activate signaling by TLR4 and TLR3 respectively, also stimulate RIPK3-dependent necroptosis in the presence of a pan-caspase inhibitor. Studies using the RIPK1 inhibitor necrostatin-1 or cells expressing catalytically inactive RIPK1 generally indicate that the kinase activity of RIPK1 is also required for killing by these stimuli [5,6,9–16].

TLR3 and TLR4 engage RIPK1 indirectly through the adaptor protein TRIF (TIR-domain-containing adapter-inducing interferon β) and this interaction relies on the RIP homotypic interaction motifs (RHIM) in RIPK1 and TRIF [17]. RIPK1 is recruited to TNFR1 through its death domain (DD), which can bind directly to the DD in TNFR1 or the DD-containing adaptor TRADD. This receptor-associated signaling complex, coined complex I [18], also contains the adaptor protein TRAF2 and the ubiquitin ligases cIAP1, cIAP2, and LUBAC (linear ubiquitin chain assembly complex composed of SHARPIN, HOIL-1, and HOIP). Complex I promotes the expression of proinflammatory genes by activating mitogen-activated protein kinase (MAPK) signaling pathways and the transcription factor NF-κB. Subsequent formation of a cytoplasmic complex II containing RIPK1, the DD-containing adaptor FADD, and caspase-8 drives cell death signaling. Auto-processing of caspase-8 initiates the apoptotic demise of the cell, whereas inhibition of caspase-8 activity causes cells expressing RIPK3 [5,11,19] and its pseudokinase substrate MLKL (mixed lineage kinase domain-like) [20–23] to die by necroptosis rather than apoptosis (Figure 1).

The precise events leading to the formation of complex II and activation of RIPK1 are unclear. Ubiquitylation of RIPK1 and possibly other components of complex I by cIAP1, cIAP2, and LUBAC [24–26] facilitates the recruitment of the TAK1 and IKK complexes, both of which appear to limit complex II assembly independently of their roles in NF-κB signaling [27–29]. This does not exclude, however, an important role for NF-κB target genes in modulating complex II assembly. For example, upregulation of *Tnfaip3*, encoding the deubiquitylating enzyme

Table 1. Disease models ameliorated in mice expressing catalytically inactive RIPK1 (*Ripk1*$^{kd/kd}$) or lacking RIPK3 (*Ripk3*$^{-/-}$)

Model	Tested in		Refs
	Ripk3$^{-/-}$	*Ripk1*$^{kd/kd}$	
Skin and multi-organ inflammation in *Sharpin* mutant mice		x	[16]
Systemic inflammation induced by TNF	x	x	[6,15,82]
Cerulein-induced pancreatitis	x		[11,19]
Atherosclerosis in *Ldlr* or *Apoe* mutant mice	x		[83]
dsRNA-induced retinal degeneration	x		[84]
rd10 model of retinitis pigmentosa	x		[85]
Kidney ischemia–reperfusion injury	x		[86]
Myocardial infarction	x		[87]
Steatohepatitis	x		[88]
Gaucher's disease	x		[89]
Ethanol-induced liver injury	x		[90]

A20, or *Cflar*, encoding the catalytically inactive homolog of caspase-8 called c-FLIP/CFLAR (cellular FLICE-inhibitory protein/CASP8 and FADD-like apoptosis regulator), can protect cells from TNF-induced death [30,31]. Inhibition of the kinase activity of TAK1 [27] was shown to enhance complex II assembly and cell death, but what TAK1 phosphorylates in its pro-survival role is unclear. The pro-survival function of the IKKγ/NEMO subunit is attributed to its binding to ubiquitylated RIPK1 and stabilizing complex I [28]. Consistent with the ubiquitylation status of RIPK1 influencing cell survival, compromised IAP or LUBAC activity, or deubiquitylation of RIPK1 by cylindromatosis (CYLD), favors complex II assembly [26,32–34]. Studies to date suggest that CYLD contributes to but is not essential for necroptosis [34–36], perhaps indicating overlapping roles for other deubiquitylating enzymes such as otulin [37]. Alternatively, deubiquitylation of RIPK1 promotes but is not essential for death signaling because what appears to be ubiquitylated RIPK1 has been observed in complex II [5,29,34]. More detailed

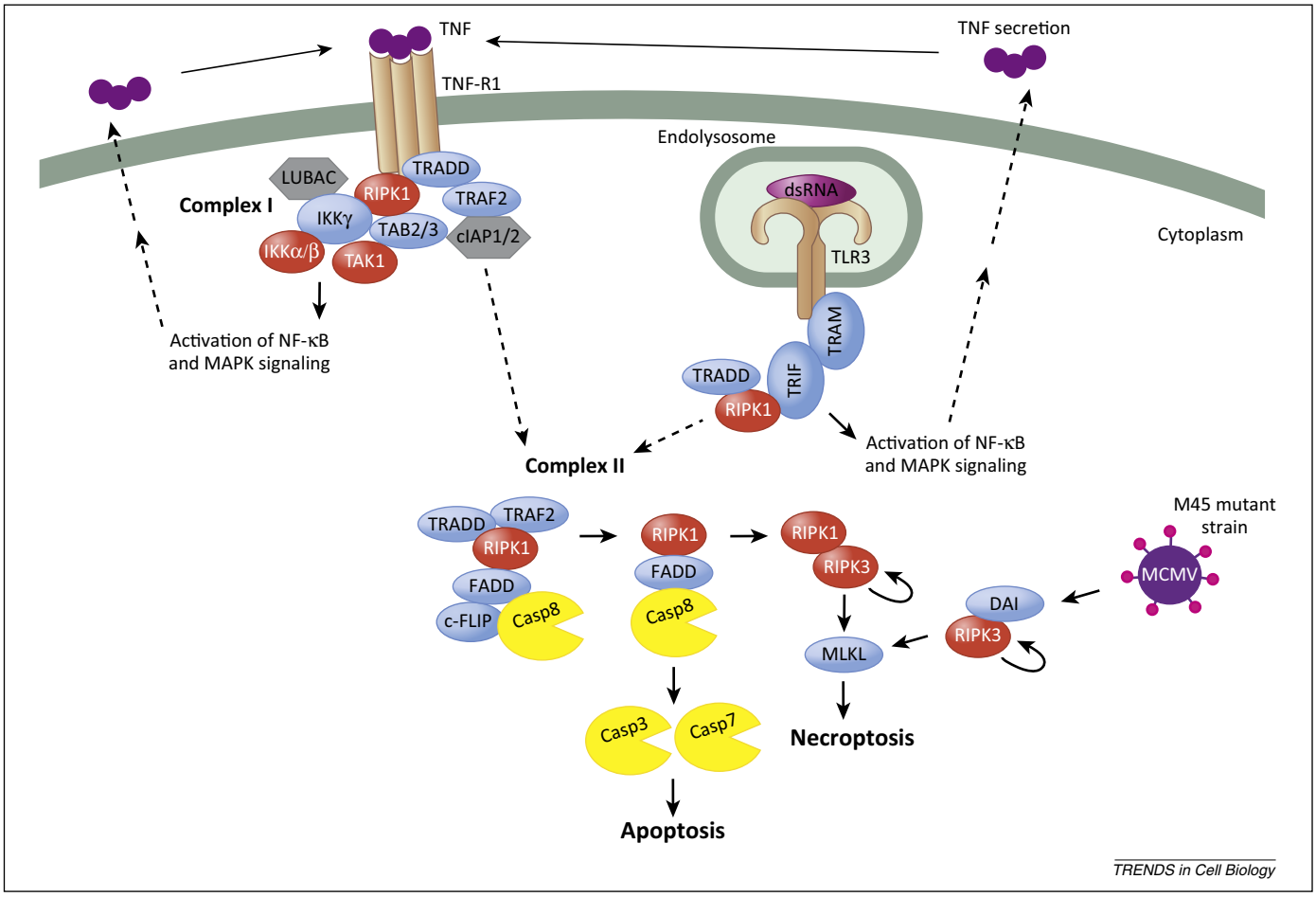

Figure 1. Necroptosis signaling by RIPK3 and MLKL. Activation of RIPK3 by TNFR1 and TLR3 requires catalytically-active RIPK1 and inhibition of c-FLIP/caspase-8 catalytic activity. RIPK3 autophosphorylation is required for the recruitment of the pseudokinase MLKL. Phosphorylation of MLKL by RIPK3 results in translocation of MLKL to membranes and cell rupture. M45 mutant MCMV triggers RIPK1-independent necroptosis. Kinases are red, ubiquitin ligases are grey, adaptor proteins are blue, and proteases are yellow. Abbreviations: c-FLIP, cellular FLICE inhibitory protein; CFLAR, CASP8 and FADD-like apoptosis regulator; MCMV, murine cytomegalovirus; MLKL, mixed lineage kinase domain-like; RIPK1/3, receptor-interacting serine/threonine protein kinase 1/3; TNFR1, tumor necrosis factor (TNF) receptor 1.

kinetic studies are needed that define the nature of the modifications to RIPK1 within the receptor-associated and cytoplasmic complexes.

Interestingly, necrostatin-1 blocks complex II formation and death induced by the combination of TNF, zVAD.fmk, and the translation inhibitor cycloheximide [5], or by TNF, zVAD.fmk, and an IAP antagonist [11]. These data imply that the kinase activity of RIPK1 is required for the interaction of RIPK1 with FADD and RIPK3. One possibility is that conformational changes induced by RIPK1 autophosphorylation expose the C-terminal RHIM and DD of RIPK1 for interactions. Consistent with the biochemical data and this model, the kinase activity of RIPK1 has been implicated in apoptosis induced by the combination of TNF and IAP antagonist, TNF and TAK1 inhibitor, or TNF and SHARPIN deficiency [6,16,38–40]. Inhibition of RIPK1 has also been shown to block caspase-8 activation and apoptosis in macrophages infected with the bacterial pathogen *Yersinia pestis* [41,42]. Note that the kinase activity of RIPK1 is dispensable for apoptosis induced by TNF and cycloheximide [6], which is in keeping with an earlier study that identified two distinct apoptosis signaling pathways downstream of TNFR1 [33]. Experiments involving RIPK1 inhibition *in vivo* should therefore be interpreted with caution because it cannot be assumed that it is necroptosis that is being inhibited.

Processing of caspase-8 zymogens within complex II into homodimers that mediate apoptosis is regulated by c-FLIP [31]. Incorporation of c-FLIP into complex II blocks apoptosis by interfering with caspase-8 cleavage [43]. Genetic studies in mice revealed that caspase-8 and c-FLIP also prevent necroptosis [44]. Specifically, most defects associated with caspase-8 or FADD deficiency in mice are rescued by eliminating RIPK3-dependent necroptosis [35,36,44–48], and defects associated with loss of c-FLIP are rescued only when both the FADD/caspase-8-dependent apoptosis and RIPK3-dependent necroptosis death programs are disabled [49]. Heterodimers of c-FLIP and caspase-8 have catalytic activity but exhibit subtle differences in substrate specificity compared to caspase-8 homodimers [50]. This catalytic activity is required for inhibition of necroptosis [44], but what the c-FLIP/caspase-8 heterodimer cleaves to prevent necroptosis remains elusive. Cleavage of CYLD by caspase-8 was proposed to suppress necroptosis, but it was inhibited by the viral serpin CrmA [51]. CrmA is a poor inhibitor of the c-FLIP/caspase-8 heterodimer [44], suggesting that CYLD was cleaved by caspase-8 homodimers during apoptosis rather than by c-FLIP/caspase-8 heterodimers. Caspase-8-dependent cleavage of RIPK1 and RIPK3 has also been described [52,53] and offers a simple mechanism for inhibition of necroptosis, but evidence for these kinases being cleaved by the c-FLIP/caspase-8 heterodimer specifically is lacking. If the caspase-8 cleavage site(s) in RIPK1 or RIPK3 are relevant then mutation of the key aspartate residue(s) in mice should yield embryonic lethality similar to caspase-8, c-FLIP, or FADD deficiency [54–56], the assumption being that the kinase activity and interactions of RIPK1 or RIPK3 are not perturbed by the point mutation(s).

When c-FLIP/caspase-8 catalytic activity is blocked, interaction of the RHIM domain in RIPK1 with the RIPK3 RHIM appears to trigger the recruitment of further RIPK3, causing intramolecular autophosphorylation of RIPK3 and subsequent recruitment of MLKL [20,57–59]. Phosphorylation of the C-terminal pseudokinase domain of MLKL by RIPK3 is proposed to induce conformational changes that expose the N-terminal domain and promote MLKL translocation to membranes [60–65]. Whether MLKL ruptures the plasma membrane on its own or by engaging other proteins is not clear.

Necroptosis that requires RIPK3 but not RIPK1

Not all necroptosis stimuli require RIPK1 to activate RIPK3. For example, infection with the M45-mutant strain of murine cytomegalovirus (MCMV) activates RIPK3 independently of RIPK1, but does require the host RHIM-containing protein DAI [8,66]. The RHIM-containing ICP6 protein of herpes simplex virus 1 is proposed to engage RIPK3 directly [7], although it remains to be seen if ICP6 can kill cells treated with necrostatin-1 or expressing catalytically inactive RIPK1. Further evidence for RIPK1-independent necroptosis was provided when RIPK3 or MLKL deficiency rescued some of the defects caused by RIPK1 deficiency in mice [14,67–69]. These studies, discussed in more detail below and summarized in Figure 2, reveal that although RIPK1 catalytic activity is needed for RIPK3 activation by some necroptosis stimuli, RIPK1 also serves as a brake on necroptosis signaling. Mice expressing catalytically inactive RIPK1 are viable, unlike mice lacking RIPK1, and this pro-survival function of RIPK1 is therefore independent of its kinase activity [6,14,15,70]. It is unclear what is engaging RIPK3 to promote necroptosis in the absence of RIPK1 *in vivo*. Signaling by interferons (IFNs) and TLRs is proposed to contribute to RIPK3 activation [68], but it has not been determined whether simultaneous elimination of these signaling pathways replicates the rescue offered by RIPK3 loss. It is also not clear how type I IFN signaling activates RIPK3. The kinase PKR was proposed to mediate RIPK3 activation by IFNs in fibroblasts [71], although a subsequent study found that necroptosis occurred normally in PKR-deficient macrophages [72].

Necroptosis in endothelial cells, keratinocytes, intestinal epithelial cells, and lymphocytes

Genetic studies in mice suggest that necroptosis is actively suppressed in many cell types both during development and in adult animals. For example, loss of FADD or caspase-8 causes embryonic lethality that is linked to RIPK1- and RIPK3-dependent loss of endothelial cells forming the vasculature of the developing yolk sac [44,45,73]. TNFR1 signaling has been implicated as a trigger of this necroptosis because TNFR1 deficiency delays the death of FADD- or caspase-8-deficient embryos by several days [68]. It cannot be the sole trigger, however, because mice lacking both caspase-8 and RIPK3 are viable [44,45] (Figure 2). Similarly, lethality and skin inflammation due to FADD deficiency in keratinocytes is rescued by RIPK3 loss, but is only delayed by loss of TNFR1 or CYLD catalytic activity [36].

FADD deletion in intestinal epithelial cells induces RIPK3-dependent Paneth cell loss, enteritis, and colitis but, interestingly, eliminating CYLD catalytic activity, the TLR signaling adaptor MyD88, or the gut microbiota only

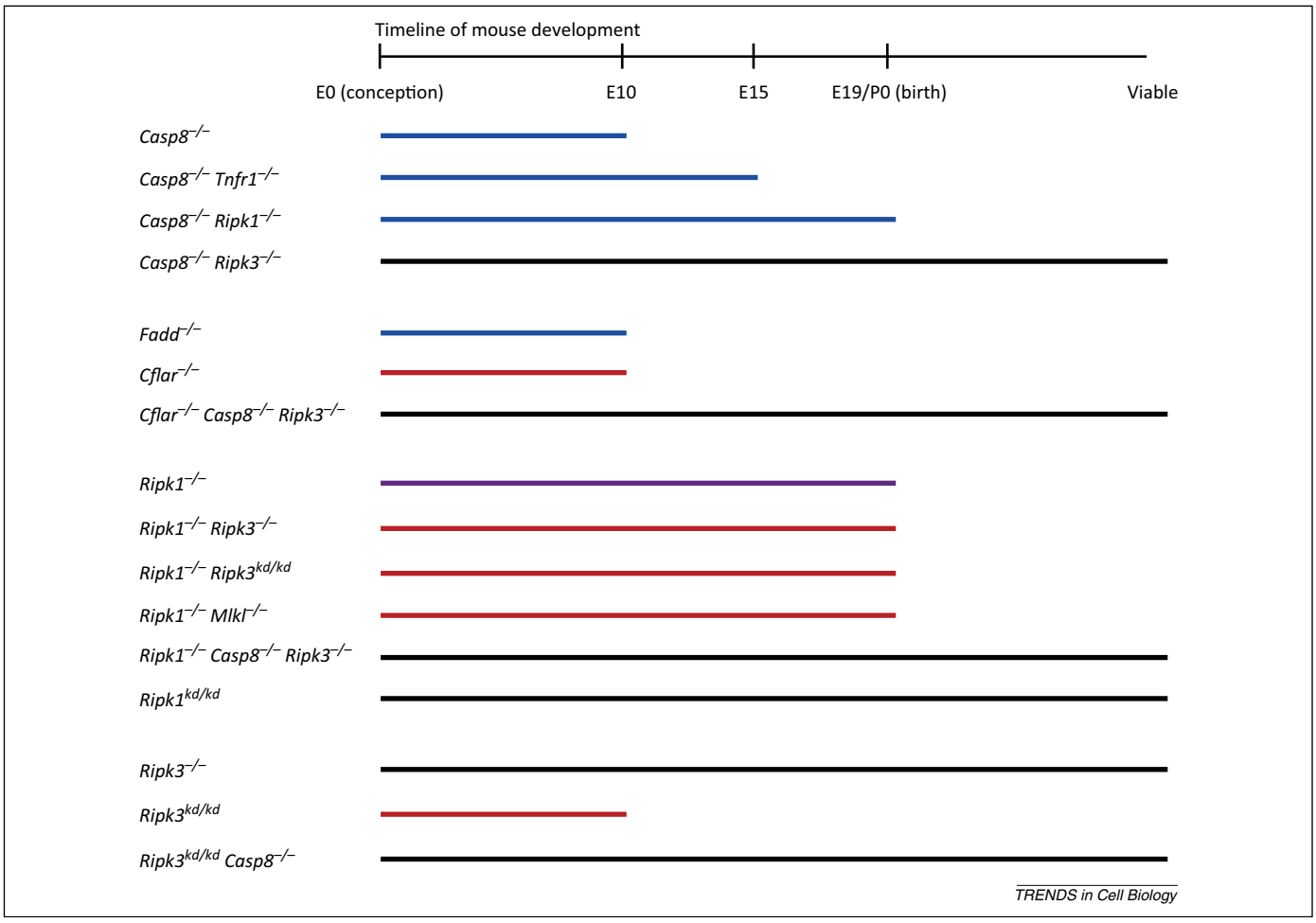

Figure 2. Time of death of mice mutant for genes controlling apoptosis or necroptosis. Mouse genotypes are indicated on the left and the length of the line on the right indicates how long each genotype survives. Blue lines indicate where death is due to inappropriate necroptosis, red lines indicate where death is due to inappropriate apoptosis, and purple lines indicate where death is due to apoptosis and necroptosis. Embryos are designated E0 when a vaginal plug is detected. P0 refers to newborn mice. Abbreviation: kd, D161N kinase dead mutant. *Cflar* encodes c-FLIP.

prevents colitis [35]. Thus, while TLR stimulation by bacteria in the gut and TNF production appear to drive necroptosis in the FADD-deficient colon, the triggers of the small intestinal phenotype remain unknown. Catalytically inactive RIPK1 failed to prevent intestinal inflammation caused by FADD deficiency [69], even in the colon where TNF was a driver of disease [35].

T lymphocytes are another cell type that undergo RIPK1- and RIPK3-dependent necroptosis when FADD or caspase-8 function is compromised, but death is induced only after stimulation of the TCR [44–47,73]. How signaling by the TCR engages the FADD/caspase-8 pro-survival function and/or RIPK1 and RIPK3 for necroptosis is unclear.

Suppression of apoptosis and necroptosis by RIPK1

Mice lacking RIPK1 die soon after birth with aberrant caspase-8-dependent apoptosis in the gut and systemic inflammation, the latter driven in large part by RIPK3/MLKL-dependent necroptosis [67]. Consequently, elimination of both RIPK3 and caspase-8 is required to obtain a viable RIPK1-deficient mouse [14,67,68]. Deletion of RIPK1 from only intestinal epithelial cells confirmed a cell intrinsic requirement for RIPK1 in preventing FADD/caspase-8-dependent apoptosis in the gut [69,74], whereas deletion of RIPK1 from only keratinocytes elicited RIPK3/MLKL-dependent skin inflammation [69]. Interestingly, apoptotic cells containing cleaved caspase-3 were detected in RIPK1-deficient skin [67,69]. It is unclear, however, if necroptosis was more prevalent than apoptosis because the field currently lacks an antibody that specifically labels mouse cells dying by necroptosis.

The kinase activity of RIPK1 is dispensable for its inhibition of FADD/caspase-8-dependent apoptosis and RIPK3/MLKL-dependent necroptosis *in vivo* because mice expressing catalytically inactive RIPK1 are viable [6,14,15]. Whether the RHIM and/or DD in RIPK1 are crucial to death inhibition remains to be determined. Disruption of these domains in mice might shed light on whether death inhibition is through direct interaction of RIPK1 with FADD and RIPK3, or by a more indirect mechanism.

Apoptosis induction by catalytically inactive RIPK3

An essential role for the kinase activity of RIPK3 in necroptosis was established when wild type but not catalytically inactive RIPK3 reconstituted necroptosis signaling in RIPK3-deficient cells [5,11]. What these experiments failed to predict, however, was that expression of catalytically

inactive RIPK3 D161N in mice would be lethal both during development and in the adult [15]. Developing embryos expressing inactive RIPK3 D161N exhibited RIPK1- and caspase-8-dependent apoptosis of endothelial cells in the yolk sac. Similarly, expression of RIPK3 D161N in adult mice caused apoptosis in several tissues including the intestine. Mice lacking RIPK3 are viable [75], which argues against RIPK3-dependent phosphorylation of another protein being crucial for inhibiting apoptosis. In addition, mice with a different catalytic residue in RIPK3 mutated do not exhibit embryonic lethality [76]. RIPK3 D161N may therefore adopt a conformation with a greater propensity to oligomerize and/or engage RIPK1 than does wild type RIPK3. Interestingly, small-molecule inhibitors of the kinase activity of RIPK3 were found to induce apoptosis in a similar manner to RIPK3 D161N [76].

Necroptosis-independent functions for RIPK1 and RIPK3

A common assumption is that RIPK1 and RIPK3 promote inflammation because necroptosis releases intracellular components that stimulate innate immune cells. It is possible, however, that RIPK1 and/or RIPK3 have additional functions beyond inducing cell death. Several recent reports focusing on macrophages or dendritic cells suggest that RIPK1 and/or RIPK3 regulate the production of proinflammatory cytokines in particular settings [39,77–81]. For example, macrophages lacking cIAP1, cIAP2, and XIAP secreted larger quantities of proinflammatory cytokines and chemokines, including TNF and IL-6, than did their wild type counterparts, and this required RIPK3 and the kinase activity of RIPK1, but not MLKL [39]. Lack of a role for MLKL argues that necroptosis probably was not involved, but can it be excluded that apoptosis mediated by RIPK1 and/or RIPK3 did not contribute to cytokine release? Elevated chemokine secretion was detected before caspase-3 activity, but it would be interesting to know if a pan-caspase inhibitor such as zVAD.fmk was as effective as necrostatin-1 at blocking cytokine production in MLKL-deficient macrophages treated with a pan-IAP antagonist. Intriguingly, RIPK1 inhibition and RIPK3 deficiency were not equivalent in suppressing cytokine production induced by IAP deficiency [39]. The authors obtained evidence that RIPK1 activity, but not RIPK3, increased the expression of TNF mRNA, but mechanistically it is unclear how this might occur.

Loss of IAPs, XIAP, or caspase-8 is reported to facilitate RIPK3-dependent secretion of IL-1β in response to LPS [77–79]. While cIAPs and caspase-8 are known to suppress the activation of RIPK3, it is not clear how XIAP regulates RIPK3. Whether RIPK3-dependent processing of IL-1β by caspase-1 and/or caspase-8 in these settings is a consequence of necroptosis remains controversial.

Concluding remarks

The role of RIPK3 and MLKL in mediating necroptosis is well established, but the contribution of necroptosis to human disease remains an unresolved question. The field, until recently, has been hampered by a lack of reagents to detect necroptosis in human tissues. A monoclonal antibody that recognizes phosphorylated human MLKL in tissue biopsies [60] may provide important insights because it should mark cells dying by necroptosis in the same way that an antibody to cleaved caspase-3 is used to identify cells dying by apoptosis. Sequence differences between human and mouse MLKL mean that an equivalent reagent is still needed to interrogate preclinical models of disease. Currently, one has to rely on the resistance of MLKL-deficient mice to invoke a role for necroptosis in disease models. The ability of RIPK3 and RIPK1 to trigger apoptosis in particular contexts means that a protective effect of RIPK3 deficiency or RIPK1 inhibition *in vivo* may not be due to inhibition of necroptosis. It is important to know if apoptosis and/or necroptosis is inhibited such that appropriate biomarkers can be developed to indicate when RIPK1 inhibition might be beneficial. A better understanding of how the kinase activity of RIPK1 is switched on might aid the development of reagents for detecting active RIPK1 specifically.

Discovery of the pro-survival role of RIPK1 unveiled unexpected complexity in the regulation of apoptosis and necroptosis. Whether this ability of RIPK1 to inhibit apoptosis and necroptosis might be exploited will require a better understanding of the kinase-independent scaffolding function of RIPK1. Another surprise was the pro-apoptotic behavior of RIPK3 inhibitors and the catalytically-inactive RIPK3 D161N mutant. Other ways of blocking necroptosis specifically have to be considered. Interfering with MLKL translocation to membranes may be an option [63], but addressing whether MLKL requires downstream effectors to induce necroptosis will be important because this might reveal additional opportunities for intervention. Such efforts are based on the assumption that MLKL deficiency will ameliorate inflammation and disease similarly to what has been observed with inhibition of RIPK1 and RIPK3 deficiency. As researchers continue to test MLKL-deficient mice in different disease models, it will be interesting to see how often this proves to be true.

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