Multitasking Kinase RIPK1 Regulates Cell Death and Inflammation

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Receptor-interacting serine threonine kinase 1 (RIPK1) is a widely expressed kinase that is essential for limiting inflammation in both mice and humans. Mice lacking RIPK1 die at birth from multiorgan inflammation and aberrant cell death, whereas humans lacking RIPK1 are immunodeficient and develop very early-onset inflammatory bowel disease. In contrast to complete loss of RIPK1, inhibiting the kinase activity of RIPK1 genetically or pharmacologically prevents cell death and inflammation in several mouse disease models. Indeed, small molecule inhibitors of RIPK1 are in phase I clinical trials for amyotrophic lateral sclerosis, and phase II clinical trials for psoriasis, rheumatoid arthritis, and ulcerative colitis. This review focuses on which signaling pathways use RIPK1, how activation of RIPK1 is regulated, and when activation of RIPK1 appears to be an important driver of inflammation.

Receptor-interacting serine threonine kinase 1 (RIPK1) was discovered more than two decades ago because of its ability to interact with the apoptosis-inducing death receptor Fas (Stanger et al. 1995). The carboxy-terminal death domain (DD) in RIPK1 (Fig. 1) binds to the intracellular DD of Fas or tumor necrosis factor receptor 1 (TNFR1) and to the DD in the adaptor proteins TRADD and FADD (Stanger et al. 1995; Hsu et al. 1996a; Chen et al. 2008; Ermolaeva et al. 2008; Pobezinskaya et al. 2008; Park et al. 2013). Another protein interaction motif in RIPK1, termed the RIP homotypic interaction motif (RHIM), mediates interactions with the RHIM-containing proteins RIPK3 (Sun et al. 2002), TRIF (also called TICAM-1) (Meylan et al. 2004; Kaiser and Offermann 2005), and ZBP1 (also called DAI) (Kaiser et al. 2008). Analyses of RIPK1-deficient cells indicate that RIPK1 is dispensable for Fas-induced apoptosis, but is needed in some cell types for optimal activation of nuclear factor (NF)-κB-dependent gene transcription by TNFR1 and TLR3 (Toll-like receptor 3) (Ting et al. 1996; Kelliher et al. 1998; Meylan et al. 2004; Cusson-Hermance et al. 2005; Vanlangenakker et al. 2011; Dannappel et al. 2014; Takahashi et al. 2014; Newton et al. 2016b; Van et al. 2017; Cuchet-Lourenço et al. 2018). The kinase activity of RIPK1, however, is dispensable for NF-κB signaling (Ting et al. 1996; Lee et al. 2004; Berger et al. 2014; Newton et al. 2014; Polykratis et al. 2014), instead being required for RIPK1 to engage the cell death machinery downstream from TNFR1.

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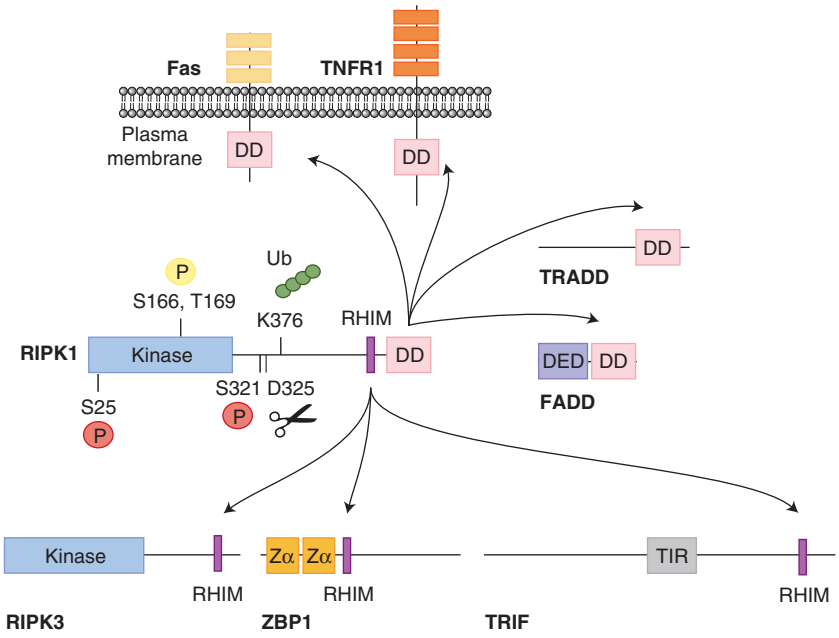

Figure 1. Domain organization and protein interactions of receptor-interacting serine threonine kinase 1 (RIPK1). RIPK1 has an amino-terminal kinase domain and carboxy-terminal RIP homotypic interaction motif (RHIM) and death domain (DD) motif. The RHIM and DD mediate interactions with other RHIM- or DD-containing proteins. Dimerization of RIPK1 via its DD promotes autophosphorylation of the kinase domain on Ser166. Thr169 in mouse RIPK1 is autophosphorylated as well, but this residue is not conserved in human RIPK1. Posttranslational modifications of RIPK1 that appear to limit activation of the kinase include ubiquitination (Ub) on mouse Lys376 (human Lys377), cleavage after mouse Asp325 (human Asp324) by caspase-8, phosphorylation on mouse Ser321 (human Ser320) by MK2, and phosphorylation on Ser25 by IκB kinase (IKK). DED, death effector domain; TIR, Toll/interleukin-1 receptor domain; Zα, Z-DNA-binding domain.

SIGNALING PATHWAYS THAT USE RIPK1

TNFR1 Signaling

RIPK1 is a component of what is termed TNFR1 complex I (Fig. 2; Micheau and Tschopp 2003). Ligated TNFR1 recruits TRADD and RIPK1, with TRADD serving as an adaptor for TRAF2 (Hsu et al. 1996b), which in turn binds to the E3 ubiquitin ligases cIAP1 and cIAP2 (Shu et al. 1996; Vince et al. 2009). The cellular inhibitors of apoptosis proteins (cIAPs) ubiquitinate RIPK1, themselves, and possibly other components of complex I (Bertrand et al. 2008; Mahoney et al. 2008; Varfolomeev et al. 2008), forming polyubiquitin chains in which the carboxy-terminal glycine of one ubiquitin is conjugated predominantly to Lys11 or Lys63 of a second ubiquitin (Dynek et al. 2010). This polyubiquitin recruits several ubiquitin-binding proteins, including TAB2 and TAB3, adaptors for the kinase TAK1 (Cheung et al. 2004; Kanayama et al. 2004); NEMO, the regulatory subunit of the canonical IκB kinase (IKK) complex (Ea et al. 2006; Wu et al. 2006); and HOIL-1, HOIP, and Sharpin, which comprise the E3 ubiquitin ligase linear ubiquitin assembly complex (LUBAC) (Haas et al. 2009). LUBAC then modifies TNFR1, TRADD, RIPK1, and NEMO with Met1-linked polyubiquitin (Gerlach et al. 2011; Tokunaga et al. 2011; Draber et al. 2015), which further stabilizes complex I through interactions with NEMO (Lo et al. 2009; Rahighi et al. 2009). Hybrid polyubiquitin chains on RIPK1 containing both Lys63 and Met1 linkages are proposed to position TAK1 next to the IKK complex (Emmerich et al. 2016), so that TAK1 can phosphor-

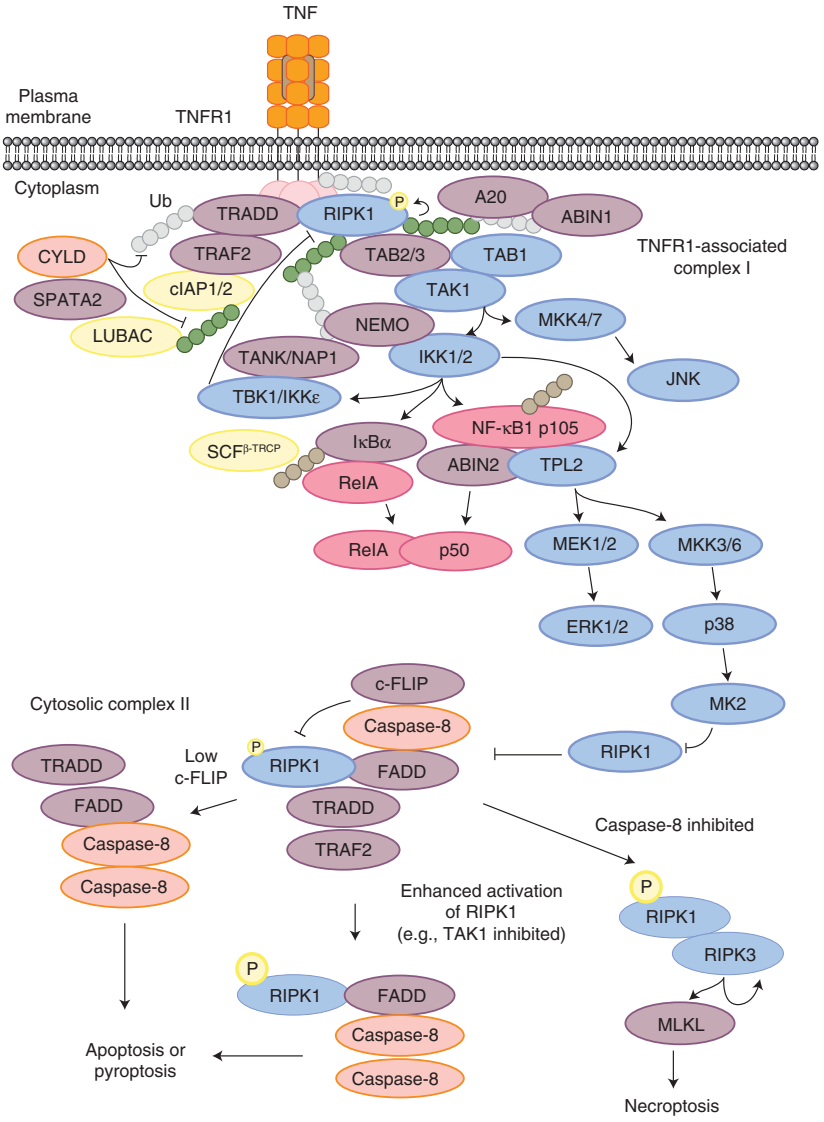

Figure 2. Receptor-interacting serine threonine kinase 1 (RIPK1) is a component of tumor necrosis factor receptor 1 (TNFR1) complex I and complex II. Binding of tumor necrosis factor (TNF) to TNFR1 triggers assembly of complex I, which activates nuclear factor (NF)-κB-dependent gene transcription and the mitogen-activated protein kinases ERK, JNK, and p38. Autophosphorylation of RIPK1 is detected in complex I in mouse cells, but it is unclear whether this is also true in human cells. Activation of the kinases TAK1, IKK, and TBK1/IKKε in complex I limits activation of RIPK1 and suppresses formation of cytosolic complex II. The translational inhibitor cycloheximide is thought to promote TNF-induced apoptosis by depleting c-FLIP from complex II, which facilitates the emergence of caspase-8 homodimers that drive cell death. Inhibitors of RIPK1 do not block this death, but they do prevent caspase-8-dependent cell death that is driven by perturbations of complex I that enhance activation of RIPK1, such as cIAP1/2 deficiency or inhibition of the IKK complex. The kinase activity of RIPK1 is also needed for RIPK3/MLKL-dependent necroptosis when the proteolytic activity of caspase-8/c-FLIP heterodimers is inhibited. Inhibitory phosphorylation of cytosolic RIPK1 by MK2 is thought to limit recruitment of additional RIPK1 into complex II. Kinases are colored blue, ubiquitin ligases are yellow, proteases are orange, adaptor proteins are purple, and transcription factors are pink. Met1-linked polyubiquitin (Ub) is colored gray, K63- or K11-linked chains are green, and K48-linked chains are brown.

ylate and activate the IKK2 catalytic subunit (Zhang et al. 2014). The IKK complex phosphorylates the NF-κB inhibitory proteins NF-κB1/p105 and IκBα, leading to their ubiquitination and proteasomal processing (Zhang et al. 2017). Degradation of IκBα and processing of NF-κB1/p105 to NF-κB1/p50 liberates NF-κB dimers composed largely of RelA and NF-κB1/p50. These dimers enter the nucleus and promote expression of their target genes. Processing of NF-κB1/p105 also liberates the kinase TPL2 to stimulate the MEK–ERK and MKK3/6–p38 kinase cascades (Eliopoulos et al. 2003; Pattison et al. 2016). Besides activating the IKK complex, TAK1 also stimulates the MKK7–JNK kinase cascade (Tournier et al. 2001; Shim et al. 2005).

NEMO and LUBAC in complex I are also needed to recruit TANK and NAP1, the ubiquitin-binding adaptors for the kinases TBK1 and IKKε (Lafont et al. 2018; Xu et al. 2018b). Phosphorylation of TBK1 and IKKε by the canonical IKK complex stimulates their activation (Clark et al. 2011). The major function of TBK1 and IKKε is to suppress the activation of RIPK1 and prevent TNF-induced cell death. Accordingly, lethal apoptosis in TBK1-deficient mouse embryos is prevented by TNFR1 deficiency (Bonnard et al. 2000), TNF deficiency (Matsui et al. 2006), or catalytically inactive RIPK1 (Xu et al. 2018b). TBK1 is proposed to phosphorylate RIPK1 directly on multiple residues to suppress RIPK1 autophosphorylation (Lafont et al. 2018; Xu et al. 2018b). TAK1 also suppresses RIPK1 activation after TNF treatment and this is independent of its role in activating NF-κB (Vanlangenakker et al. 2011; Dondelinger et al. 2013; Lamothe et al. 2013). Whether TAK1 phosphorylates RIPK1 directly is controversial (Dondelinger et al. 2015; Geng et al. 2017), but suppression of RIPK1 activation by TAK1-dependent IKK and MK2 activation is well documented (Dondelinger et al. 2015; Jaco et al. 2017; Menon et al. 2017).

IKK appears to phosphorylate RIPK1 in complex I directly on Ser25 (Dondelinger et al. 2015, 2019), but it could also influence phosphorylation of RIPK1 indirectly via the activation of TBK1 (Clark et al. 2011; Lafont et al. 2018; Xu et al. 2018b). Consistent with multiple IKK-dependent phosphorylation events restraining activation of RIPK1, mutation of RIPK1 Ser25 to Ala does not mimic inhibition of IKK (Dondelinger et al. 2019). MK2 is activated by the TAK1–MKK3/6–p38 kinase cascade and it phosphorylates cytosolic RIPK1 on murine Ser321/human Ser320 and possibly other residues (Jaco et al. 2017; Menon et al. 2017). Consistent with IKK and MK2 inhibiting RIPK1 by distinct mechanisms, combined IKK and MK2 inhibition sensitizes cells to TNF more than inhibiting just one of the kinases (Menon et al. 2017).

Genetic studies in mice support the idea that NEMO is critical for inhibiting the activation of RIPK1 and does so independent of its role in promoting NF-κB-dependent gene transcription. For example, *Nemo* deletion in intestinal epithelial cells causes colitis that is prevented by catalytically inactive RIPK1, whereas colitis is not induced by the combined loss of RelA, RelB, and c-Rel, the three NF-κB subunits with transactivation domains (Vlantis et al. 2016). Similarly, *Nemo* deletion in hepatocytes causes hepatocellular carcinoma that is prevented by catalytically inactive RIPK1, whereas hepatocellular carcinoma is not observed on deletion of *RelA*, *RelB*, and *c-Rel* (Kondylis et al. 2015). Although these experiments highlight an NF-κB-independent mechanism for RIPK1 inactivation by NEMO, perhaps via the combined actions of IKKα/β, TBK1, and IKKε, there is also evidence for NF-κB-dependent inactivation of RIPK1. For example, mice lacking either NEMO or RelA die during embryogenesis, but survive to birth if they express catalytically inactive RIPK1 (Vlantis et al. 2016; Xu et al. 2018a). Several prosurvival proteins that could impact RIPK1 activation are encoded by NF-κB target genes, including the cIAPs (Wang et al. 1998), X-linked inhibitor of apoptosis protein (XIAP) (Stehlik et al. 1998), A20 (Krikos et al. 1992), and cellular FLICE inhibitory protein (c-FLIP) (Kreuz et al. 2001).

The cIAPs contribute to the assembly of TNFR1 complex I as described above, but may also limit activation of RIPK1 by modifying RIPK1 with Lys48-linked polyubiquitin that targets it for proteasomal degradation (Varfolo-

meev et al. 2008; Dynek et al. 2010; Annibaldi et al. 2018). Genetic studies in mice implicate XIAP and A20 in the suppression of RIPK1 activation (Wong et al. 2014; Onizawa et al. 2015; Newton et al. 2016a; Kattah et al. 2018; Rijal et al. 2018), but the underlying biochemical mechanism in each case is still being elucidated. The binding of A20 to Met1-linked polyubiquitin in TNFR1 complex I appears to be critical to limiting TNF-induced cell death (Draber et al. 2015; Yamaguchi and Yamaguchi 2015; Polykratis et al. 2019). A20 also appears to act in concert with the ubiquitin-binding protein ABIN-1 (Oshima et al. 2009; Dziedzic et al. 2018; Kattah et al. 2018). c-FLIP is the catalytically inactive paralog of the cysteine protease caspase-8 (previously called FLICE) (Irmler et al. 1997) and is discussed in more detail in the section on activation of RIPK1.

TLR3 and TLR4 Signaling

RIPK1 recruitment to TLR3, which detects double-stranded RNA (dsRNA) in the endosomal compartment, or to TLR4, which detects bacterial lipopolysaccharide (LPS) at the cell surface and is then endocytosed, is mediated by the adaptor TRIF (Fig. 3; Meylan et al. 2004). TRIF binding to RIPK1 and to the E3 ubiquitin ligase TRAF6 promotes TAK1- and LUBAC-depen-

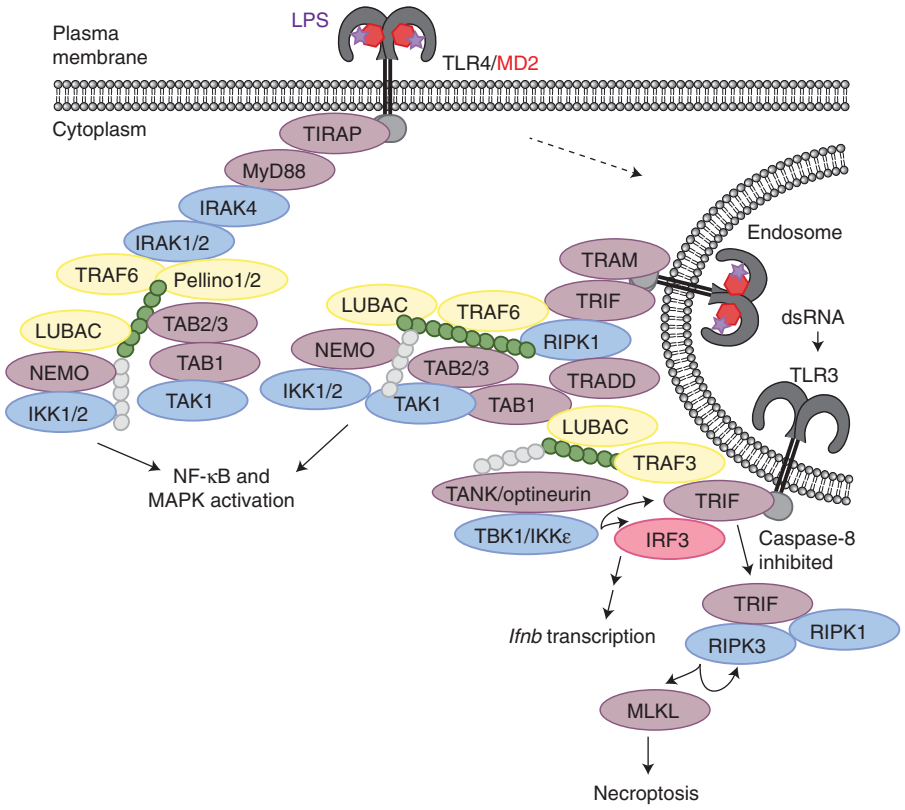

Figure 3. Receptor-interacting serine threonine kinase 1 (RIPK1) mediates TRIF-dependent NF-κB and mitogen-activated protein kinase (MAPK) activation by TLR3 and TLR4. The adaptor protein TRIF has a RHIM that recruits RIPK1 to TLR3 and TLR4 signaling complexes on endosomes. RIPK1 contributes to the activation of TAK1 and the IKK complex, but appears dispensable for TRIF-dependent activation of IRF3 and the expression of type I interferons, as well as TRIF-dependent activation of RIPK3/MLKL-dependent necroptosis. Inhibited RIPK1 can, however, block TRIF-dependent necroptosis. Both TLR3 and TLR4 trigger these different TRIF-dependent signals. RIPK1 is dispensable for TLR4 signaling via the adaptor MyD88.

dent IKK activation that leads to the expression of proinflammatory genes (Jiang et al. 2003, 2004; Sato et al. 2003; Meylan et al. 2004; Cusson-Hermance et al. 2005; Zinngrebe et al. 2016; Bakshi et al. 2017). Presumably, TRAF6 builds Lys63-linked polyubiquitin chains on RIPK1 that recruit TAK1, IKK, and LUBAC, but this has not been confirmed experimentally. Another E3 ubiquitin ligase, Pellino1, was implicated in TLR3-induced RIPK1 ubiquitination and IKK activation (Chang et al. 2009), but results obtained with cells expressing ligase inactive Pellino1 indicate that the E3 activity of Pellino1 is not required for either signaling event (Enesa et al. 2012). The adaptor TRADD, perhaps through binding to the DD of RIPK1, contributes to TLR3-induced NF-κB and mitogen-activated protein kinase (MAPK) activation in fibroblasts, but appears less critical in myeloid cells (Chen et al. 2008; Ermolaeva et al. 2008; Pobezinskaya et al. 2008). TLR3 and TLR4 also use TRIF to engage TANK- or optineurin-containing TBK1 complexes that promote activation of the transcription factor IRF3 and expression of *Interferon beta* (*Ifnb*) (Fitzgerald et al. 2003; Hemmi et al. 2004; McWhirter et al. 2004; Perry et al. 2004; Liu et al. 2015; Bakshi et al. 2017). However, neither TRAF6 nor RIPK1 is essential for TRIF-induced IFN-β expression (Sato et al. 2003; Jiang et al. 2004; Meylan et al. 2004; Cusson-Hermance et al. 2005).

Other Signaling Pathways Involved in Pathogen Defense

Recent genetic studies in mice have illuminated a role for RIPK1 in promoting proinflammatory gene expression during development, although the nature of the upstream activating signal(s) is unclear. For example, RIPK1 causes modest proinflammatory gene expression in mouse embryos lacking caspase-8 and the kinase RIPK3 (Kang et al. 2018). This aberrant gene expression is greatly exacerbated if LUBAC is compromised (Heger et al. 2018; Peltzer et al. 2018). Indeed, mice lacking caspase-8 and RIPK3 are viable (Kaiser et al. 2011; Oberst et al. 2011), whereas mice lacking caspase-8, RIPK3, and either HOIL-1 or Sharpin die in the perinatal period (Rickard et al. 2014a; Peltzer et al. 2018). Mice lacking caspase-8, RIPK3, and OTULIN activity also die in the perinatal period, OTULIN being a deubiquitinating enzyme that preserves LUBAC activity (Heger et al. 2018). Depending on the mouse model, cytokine and chemokine expression is normalized by either complete loss of *Ripk1* (Peltzer et al. 2018) or by loss of a single allele of *Ripk1* (Heger et al. 2018), and perinatal lethality is either delayed (Peltzer et al. 2018) or prevented (Heger et al. 2018). A small molecule inhibitor of RIPK1 was unable to rescue mice lacking caspase-8, RIPK3, and HOIL-1 (Peltzer et al. 2018), so the kinase activity of RIPK1 may be dispensable for this proinflammatory gene expression.

IFN-β and IFN-inducible chemokines such as CXCL10 were expressed in a RIPK1-dependent manner in both of the aforementioned studies (Heger et al. 2018; Peltzer et al. 2018). Although RIPK1 is dispensable for TLR3- or TLR4-induced IFN-β production (Meylan et al. 2004; Cusson-Hermance et al. 2005), RIPK1, TRADD, and FADD are implicated in IFN-β production triggered by cytosolic RLRs (RIG-I-like receptors) (Balachandran et al. 2004; Michallet et al. 2008; Rajput et al. 2011). RLRs RIG-I and MDA5 recognize dsRNA and engage the mitochondrial antiviral signaling (MAVS) protein, which in turn recruits TRAF2, TRAF5, and TRAF6 to promote NEMO-dependent activation of IKK and TBK1 (Liu et al. 2013). Precisely how RIPK1 fits into this signaling pathway and whether its kinase activity is needed is unclear. It is tempting to speculate that aberrant RIPK1-dependent IFN-β signaling drives the lethal phenotype of mice lacking LUBAC activity, RIPK3 and caspase-8, but exploring this further will require genetic crosses that eliminate the IFN-α/β receptor or MAVS.

ACTIVATION OF RIPK1 PROMOTES CELL DEATH

The enzymatic activity of RIPK1 was first shown to promote cell death in Jurkat T cells treated with FasL (Fas ligand) and the pan-caspase inhibitor Z-VAD-FMK (Holler et al. 2000). Fas-induced apoptosis mediated by the adaptor

FADD and caspase-8 was blocked, but now the cells died a necrotic death requiring the kinase activity of RIPK1. Jurkat T cells treated with TNF, CHX (cycloheximide), and Z-VAD-FMK also died in a manner requiring the kinase activity of RIPK1 (Holler et al. 2000). Although Jurkat T cells (and many other cell types) are not killed by TNF alone, addition of the translational inhibitor CHX is thought to promote TNFR1-dependent apoptosis by eliminating the labile protein c-FLIP (Micheau and Tschopp 2003). Caspase-8 then forms homodimers rather than caspase-8/c-FLIP heterodimers in a secondary, cytosolic complex II that also contains FADD, TRADD, TRAF2, and RIPK1 (Fig. 2). Activation of caspase-8 within complex II initiates the apoptotic caspase cascade that dismantles the cell (Micheau and Tschopp 2003). Note that the kinase activity of RIPK1 is dispensable for apoptosis induced by TNF and cycloheximide (Wang et al. 2008). It is only required when Z-VAD-FMK is incorporated into the death stimulus (Holler et al. 2000).

Z-VAD-FMK and other pan-caspase inhibitors block caspase-8-dependent apoptosis, but cell death is not prevented if cells express the kinase RIPK3 and the pseudokinase MLKL (mixed lineage kinase domain-like) (Cho et al. 2009; He et al. 2009; Zhang et al. 2009; Sun et al. 2012; Zhao et al. 2012; Murphy et al. 2013; Wu et al. 2013). Instead of dying by apoptosis, cells unleash a caspase-independent death program termed necroptosis (Degterev et al. 2005). The enzymatic activity of RIPK1, which is essential for TNFR1-induced necroptosis (Holler et al. 2000), is required for interactions between RIPK1 and RIPK3 in complex II (Cho et al. 2009; He et al. 2009). The only known substrate of RIPK1 is itself, with Ser166 and other residues within the kinase domain, being autophosphorylated in *trans* (Ting et al. 1996; Degterev et al. 2008). In primary mouse embryo fibroblasts (MEFs), autophosphorylated RIPK1 is detected in TNFR1 complex I within minutes of TNF stimulation (Newton et al. 2016b). Dimerization of RIPK1 via its DD promotes autophosphorylation (Meng et al. 2018), whereas the RHIM in RIPK1 is dispensable (Newton et al. 2016b). Autophosphorylation may induce conformational changes that expose the RHIM of RIPK1 for interactions with the RHIM of RIPK3. RHIM–RHIM interactions between RIPK1 and RIPK3 in complex II are required for oligomerization and autophosphorylation of RIPK3 (Cho et al. 2009; He et al. 2009; Sun et al. 2012; Chen et al. 2013; Wu et al. 2014; Mompeán et al. 2018). Autophosphorylated RIPK3 then phosphorylates the carboxy-terminal pseudokinase domain of MLKL (Sun et al. 2012; Murphy et al. 2013; Xie et al. 2013; Rodriguez et al. 2016) and conformational changes in MLKL trigger its translocation to membranes and cell lysis (Fig. 2; Cai et al. 2014; Hildebrand et al. 2014; Su et al. 2014; Wang et al. 2014; Petrie et al. 2018).

Necroptosis is considered a host-defense mechanism for eliminating cells infected with viruses that encode inhibitors of caspase-8-dependent apoptosis (Chan et al. 2003; Cho et al. 2009; Guo et al. 2015). Death ligands such as FasL, TRAIL, and TNF are not the only triggers of necroptosis. Provided caspase-8 is inhibited, RIPK3-dependent necroptosis can also be triggered by TLRs, RIG-I, the cytosolic DNA sensor cGAS, the RHIM-containing protein ZBP1, or the T-cell receptor (Ch'en et al. 2008, 2011; He et al. 2011; Upton et al. 2012; Kaiser et al. 2013; Schock et al. 2017; Brault et al. 2018). It is unclear how the T-cell receptor engages the necroptosis machinery, but TLRs that signal using the adaptor MyD88, RIG-I, and cGAS all appear to induce necroptosis via autocrine TNF production (Kaiser et al. 2013; Brault et al. 2018). In contrast, TLR3 and TLR4 can engage RIPK3 directly via TRIF (Fig. 3; He et al. 2011; Kaiser et al. 2013; Buchrieser et al. 2018). ZBP1, which appears to sense certain virus infections by binding to viral RNA transcripts (Thapa et al. 2016; Maelfait et al. 2017; Guo et al. 2018), can also engage RIPK3 (Upton et al. 2012; Lin et al. 2016; Newton et al. 2016b; Nogusa et al. 2016). ZBP1 and TRIF can both activate RIPK3 in the absence of RIPK1 (Lin et al. 2016; Newton et al. 2016b), so it is interesting that inhibited RIPK1 blocks TLR3-induced necroptosis in macrophages (He et al. 2011; Kaiser et al. 2013; McComb et al. 2014). The ability of RIPK1 to suppress RIPK3 activation by ZBP1 and TRIF is

discussed further in the section on the prosurvival function of RIPK1.

Many studies now indicate that activation of RIPK1 is not solely associated with necroptosis. For example, TNF induces excessive activation of RIPK1 in cells lacking cIAP1 and cIAP2 (Wang et al. 2008; Moulin et al. 2012; Dondelinger et al. 2013; Polykratis et al. 2014), TAK1 (Dondelinger et al. 2013; Lamothe et al. 2013), LUBAC (Berger et al. 2014; Kumari et al. 2014; Peltzer et al. 2014, 2018; Rickard et al. 2014a; Heger et al. 2018), IKK (Dondelinger et al. 2015), or ABIN-1 (Dziedzic et al. 2018; Kattah et al. 2018), and this results in caspase-8-dependent cell death. Active RIPK1 promotes the assembly of complex II, using its DD to interact with FADD. Activation of caspase-8 via FADD typically engages the apoptotic machinery, but a recent study showed that macrophages exposed to TNF and TAK1 inhibitor display a lytic form of cell death termed pyroptosis (Orning et al. 2018). In this setting, caspase-8 cleaves the protein gasdermin D (GSDMD) to produce an amino-terminal fragment that forms pores in the plasma membrane. Consequently, cell death that is prevented by RIPK1 inhibitors, such as Nec-1s (Degterev et al. 2008), could be caused by apoptosis, pyroptosis, or necroptosis. The type of cell death will depend on the activity of caspase-8 and the substrates that are available to it. Pyroptosis mediated by gasdermin pores is more rapid than apoptosis (Wang et al. 2017b), so it might be the dominant form of cell death in cells expressing both caspase-8 and GSDMD.

CASPASE-8 SUPPRESSES CELL DEATH BY CLEAVING RIPK1

Pan-caspase inhibitors such as Z-VAD-FMK are used to induce necroptosis in cultured cells, but exactly how inhibition of caspase-8 promotes activation of RIPK3 is not well understood. In apoptotic cells, caspase-8 cleaves RIPK1 after murine Asp325/human Asp324 (Lin et al. 1999) and this might limit interactions between RIPK1 and RIPK3 that would lead to necroptosis. It should be noted, however, that caspase-8 can suppress necroptosis even in the absence of apoptosis. For example, inhibition of caspase-8 with Z-VAD-FMK in mouse bone marrow–derived macrophages is sufficient to convert TNF from a nonlethal stimulus into a lethal necroptosis stimulus (Newton et al. 2016b). Indeed, necroptosis suppression appears to be mediated by the proteolytic activity of caspase-8/c-FLIP heterodimers rather than autoprocessed caspase-8 homodimers (Kang et al. 2008; Oberst et al. 2011). Consistent with c-FLIP suppressing both caspase-8-dependent apoptosis and RIPK3-dependent necroptosis, mice lacking c-FLIP die around embryonic day 11 (E11) (Yeh et al. 2000), but are viable if they also lack caspase-8 and RIPK3 (Dillon et al. 2012). Mice lacking caspase-8 or FADD also die around E11 (Varfolomeev et al. 1998; Yeh et al. 1998), but only need to lose RIPK3 or MLKL to be viable (Kaiser et al. 2011; Oberst et al. 2011; Alvarez-Diaz et al. 2016; Zhang et al. 2016). TNFR1- and RIPK1-dependent necroptosis, particularly in endothelial cells, appears to drive the lethal phenotype of FADD- or caspase-8-deficient embryos at E11 (Kang et al. 2004; Zhang et al. 2011; Dillon et al. 2014; Kaiser et al. 2014; Rickard et al. 2014b), although embryos lacking caspase-8 and TNFR1, or FADD and RIPK1, or caspase-8 and RIPK1 still succumb later in development (Zhang et al. 2011; Dillon et al. 2014; Kaiser et al. 2014; Rickard et al. 2014b) because ZBP1 and TRIF then drive RIPK3-dependent necroptosis (Newton et al. 2016b).

What do caspase-8/c-FLIP heterodimers cleave to prevent necroptosis? In mice, mutation of RIPK1 Asp325 to Ala to prevent RIPK1 cleavage by caspase-8 is lethal around E11 (Zhang et al. 2019a), which is what one would expect if RIPK1 has to be cleaved to prevent necroptosis. However, lethality is prevented by the combined loss of FADD and RIPK3, but not by the loss of RIPK3 alone (Zhang et al. 2019a). Therefore, RIPK1 D325A appears to trigger apoptosis mediated by FADD and caspase-8, rather than necroptosis. Perhaps transient assembly of TNFR1 complex II is sustained in the absence of RIPK1 cleavage and, as a consequence, the capacity of c-FLIP to curb caspase-8-dependent apoptosis is overwhelmed.

Caspase-8 can also cleave the deubiquitinating enzyme CYLD after Asp215 (O'Donnell

et al. 2011). CYLD is recruited to TNFR1 complex I by its adaptor SPATA2, which binds to HOIP (Elliott et al. 2016; Kupka et al. 2016; Schlicher et al. 2016; Wagner et al. 2016). Cleavage of Lys63- and Met1-linked polyubiquitin by CYLD then promotes complex II assembly and cell death (Hitomi et al. 2008; Wang et al. 2008; Moquin et al. 2013; Draber et al. 2015; Callow et al. 2018). Genetic studies in mice are consistent with CYLD facilitating necroptosis. For example, catalytically inactive CYLD delays RIPK3-dependent skin lesions when FADD is deleted from keratinocytes (Bonnet et al. 2011), and prevents some, but not all, RIPK3-dependent lesions when FADD is deleted from intestinal epithelial cells (Welz et al. 2011). Nonetheless, cleavage of CYLD after Asp215 does not appear to be a critical function of caspase-8 because mutation of CYLD Asp215 to Ala is not sufficient for TNF-induced necroptosis in macrophages (Legarda et al. 2016).

CELL DEATH–INDEPENDENT OUTCOMES OF RIPK1 ACTIVATION

Activation of RIPK1 may also promote inflammation independent of its role in triggering cell death, although the underlying mechanistic details are less clear. For example, mice expressing catalytically inactive RIPK1 show increased paresis after infection with Zika virus (Daniels et al. 2019) and increased mortality after infection with West Nile virus (Daniels et al. 2017). These phenotypes appear cell death–independent because they are not observed in mice lacking both caspase-8 and MLKL. Instead, activation of RIPK1 in neurons appears to be important for inducing antiviral transcriptional programs (Daniels et al. 2019). In a similar vein, MLKL-deficient mouse macrophages treated with LPS plus Z-VAD-FMK up-regulate proinflammatory genes such as *Tnf* and *Ifnb* without dying by necroptosis, and this transcriptional response is blocked by inhibitors of RIPK1 (Najjar et al. 2016; Saleh et al. 2017). In another study, RIPK1 inhibitor Nec-1 blocked transcription of *Tnf* in RIPK3-deficient macrophages treated with compound A, a pan-IAP antagonist (Wong et al. 2014). Collectively, these data suggest that inhibitors of RIPK1 could block several proinflammatory mechanisms, rather than just proinflammatory cell death.

THE PROSURVIVAL FUNCTION OF RIPK1

Despite being required for TNF-induced necroptosis, and in some contexts, TNF-induced apoptosis or pyroptosis, there is compelling genetic evidence that RIPK1 can also suppress caspase-8-dependent apoptosis and MLKL-dependent necroptosis (Fig. 4; Dannappel et al. 2014; Dillon et al. 2014; Kaiser et al. 2014; Orozco et al. 2014; Rickard et al. 2014b; Roderick et al. 2014; Takahashi et al. 2014; Raju et al. 2018). For example, mice lacking RIPK1 die around birth (Kelliher et al. 1998), unless both the apoptotic and necroptotic death programs are disabled by the loss of caspase-8 and RIPK3 (Dillon et al. 2014; Kaiser et al. 2014; Rickard et al. 2014b). RIPK1 may suppress caspase-8-dependent cell death by preventing interactions between TRADD and FADD (Anderton et al. 2019), but it is unclear whether this is because the DD of RIPK1 binds to and sequesters TRADD or FADD. Mice expressing RIPK1 with a K584R DD mutation are viable (Meng et al. 2018), but the role of the DD in the prosurvival function of RIPK1 remains ambiguous because this mutation is unlikely to block all DD interactions. For example, although human RIPK1 with the equivalent K599R mutation is unable to form homodimers, it can still interact with TNFR1, FADD, and TRADD (Meng et al. 2018). RIPK1 that completely lacks the carboxy-terminal DD is expressed poorly in mice (K Newton and VM Dixit, unpubl.), so lethality in the homozygous state cannot be attributed to a prosurvival function of the DD specifically.

Mutating the RHIM of RIPK1 causes ZBP1/RIPK3/MLKL-dependent perinatal lethality in mice without compromising the expression of RIPK1 (Lin et al. 2016; Newton et al. 2016b). Therefore, the RIPK1 RHIM is a critical brake on ZBP1-induced necroptosis. In macrophages, the RIPK1 RHIM also prevents TRIF from activating RIPK3 (Newton et al. 2016b). Biochemical evidence for RIPK1 simply sequestering the

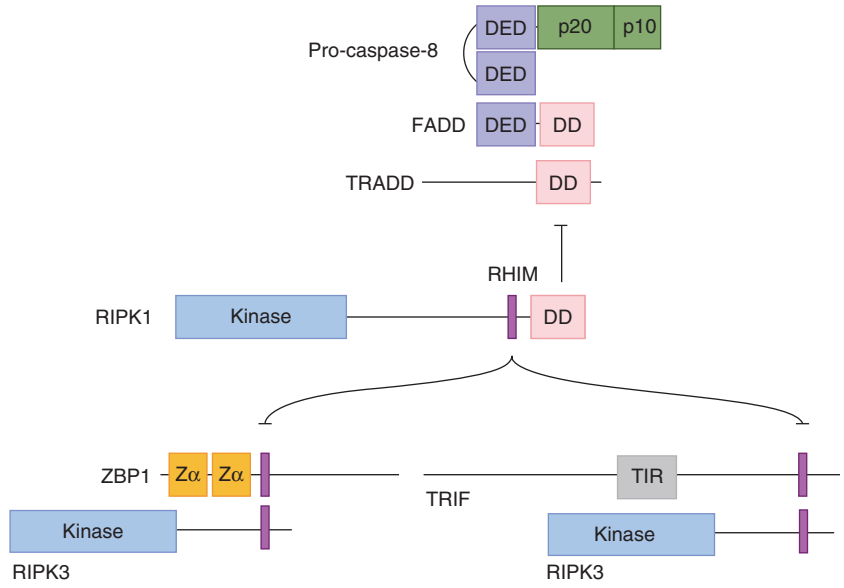

Figure 4. Model for the prosurvival function of receptor-interacting serine threonine kinase 1 (RIPK1). Mouse RIPK1 uses its RIP homotypic interaction motif (RHIM) to suppress activation of RIPK3-dependent necroptosis by TRIF and ZBP1. RIPK1 also suppresses FADD/caspase-8-dependent cell death, but whether this is dependent on the death domain (DD) of RIPK1 is unclear. The nature of the RHIM-dependent interactions that allow RIPK1 to limit activation of RIPK3 are also unknown.

other RHIM-containing proteins has proven elusive, prompting speculation that transient RHIM–RHIM interactions might hold RIPK3 in check. TNF-induced activation of MAPKs and NF-κB is normal in cells expressing RHIM mutant RIPK1 (Lin et al. 2016; Newton et al. 2016b), so the perinatal lethality of RHIM mutant RIPK1 mice does not appear to be caused by impaired expression of prosurvival factors such as c-FLIP, A20, and c-IAP1/2. ZBP1/RIPK3/MLKL-dependent inflammation is detected as early as E17.5 in RIPK1 RHIM mutant mice (Newton et al. 2016b), so there is also the question of what ZBP1 might be sensing in utero. Given that interactions between RIPK1 and other RHIM-containing proteins are usually not detected without a stimulus, identification of the ZBP1 ligand is probably key to unraveling how RIPK1 prevents ZBP1-induced necroptosis.

The enzymatic activity of RIPK1 is dispensable for the prosurvival function of RIPK1 because mice expressing catalytically inactive RIPK1 are viable (Kaiser et al. 2014; Newton et al. 2014; Polykratis et al. 2014). It may be that inhibition of RIPK1 enzymatic activity prevents cell death in certain contexts (e.g., necroptosis induced by TLR3 agonist plus z-VAD-FMK) by enforcing this ill-defined brake function of RIPK1. Therefore, a better understanding of how RIPK1 suppresses activation of caspase-8 and RIPK3 might help identify disease settings in which inhibitors of RIPK1 could offer a therapeutic benefit.

In humans, RIPK1 deficiency is associated with immunodeficiency, very early-onset inflammatory bowel disease, and arthritis (Cuchet-Lourenço et al 2018; Li et al 2019). Although inflammation appears less widespread than in RIPK1-deficient mice, cells from RIPK1-deficient patients display similar defects in signaling to cells from RIPK1-deficient mice. For example, fibroblasts fail to activate MAPK and NF-κB signaling normally in response to TNF or a TLR3 agonist, and they show increased cell death. Blood cells treated with LPS also show impaired cytokine production (Cuchet-Lourenço et al. 2018). Therefore, aberrant cell death

owing to RIPK1 deficiency might drive disease in humans as well as mice.

PRECLINICAL DISEASE MODELS IN WHICH INHIBITION OF RIPK1 IS BENEFICIAL

The ability of catalytically inactive RIPK1 or small molecule inhibitors of RIPK1 to prevent inflammatory skin disease and multiorgan inflammation in mice lacking Sharpin (Berger et al. 2014), colitis in mice lacking NEMO in intestinal epithelial cells (Vlantis et al. 2016), or inflammation and neurodegeneration in mice heterozygous for both TAK1 and TBK1 in microglia (Xu et al. 2018b) is consistent with the notion that cell death can be a potent driver of inflammatory disease. These genetic disease models reduce or remove known brakes on the activation of RIPK1, but in what other settings might excessive activation of RIPK1 be deleterious and promote disease? Activation of RIPK1 appears to contribute to ischemia-reperfusion injury because mice expressing catalytically inactive RIPK1 show improved survival in a kidney ischemia-reperfusion injury model (Newton et al. 2016a), as do mice dosed with the RIPK1 inhibitor Nec-1 (Linkermann et al. 2012). Nec-1 also reduces reperfusion injury after myocardial infarction in mice and pigs (Oerlemans et al. 2012; Koudstaal et al. 2015). Which cell types activate RIPK1 in these ischemia-reperfusion injury models is unclear. Autophosphorylated RIPK1 is detected in endothelial cells in human heart samples showing acute myocardial infarction (Patel et al. 2019), so the benefits of RIPK1 inhibition may stem from its effects on the vasculature. Mice expressing catalytically inactive RIPK1 also have improved outcomes in an intracerebral hemorrhage stroke model (Lule et al. 2017), plus RIPK1 inhibitors can reduce infarct size in a major cerebral artery occlusion stroke model (Degterev et al. 2005).

Besides ischemia-reperfusion injury, inhibition of RIPK1 pharmacologically or genetically is reported to ameliorate disease in mouse models of retinitis pigmentosa (Murakami et al. 2012), multiple sclerosis (Ofengeim et al. 2015; Zhang et al. 2019b), acetaminophen hepatotoxicity (Dara et al. 2015), atherosclerosis (Karunakaran et al. 2016), amyotrophic lateral sclerosis (Ito et al. 2016), aortic aneurysm (Wang et al. 2017a), and Alzheimer's disease (Ofengeim et al. 2017). More recent studies have investigated the effect of inhibiting RIPK1 on tumor development, progression, and metastasis, but discrepant results do not produce a clear picture. One group has reported that inhibition of RIPK1 in a mouse model of pancreatic ductal adenocarcinoma slows tumor progression by reprogramming tumor-associated macrophages to promote tumor immunity (Seifert et al. 2016; Wang et al. 2018). An independent study, however, observed no benefit from inhibiting RIPK1 in an intervention setting, and the changes in gene expression that were reported for macrophages expressing catalytically inactive RIPK1 were not replicated (Patel et al. 2019). Contradictory results have also been reported regarding the ability of inhibited RIPK1 to limit tumor cell metastasis (Strilic et al. 2016; Hänggi et al. 2017; Patel et al. 2019), which purportedly acts by either blocking the death of endothelial cells (Strilic et al. 2016) or altering vascular permeability (Hänggi et al. 2017). Unexpectedly, in a mouse model of liver cancer, rather than limiting tumor development, MLKL deficiency or Nec-1 skewed tumor development toward hepatocellular carcinoma instead of intrahepatic cholangiocarcinoma (Seehawer et al. 2018). Necroptosis-associated cytokines were proposed to drive the development of cholangiocarcinoma, but exactly how these cytokines achieve this needs clarification. It is also unclear what triggers hepatocyte necroptosis in this setting.

EFFECT OF INHIBITING RIPK1 ON INNATE IMMUNITY

Cell death can be an important immune defense against infection, so there are studies that have examined whether inhibition of RIPK1 compromises pathogen clearance. Results have differed depending on the pathogen and the route of infection. Activation of RIPK1 appears beneficial to the host in some infections. For example, mice expressing catalytically inactive RIPK1 are

less able to clear the Gram-negative bacteria *Salmonella typhimurium* (Shutinoski et al. 2016) and *Yersinia pseudotuberculosis* (Peterson et al. 2017). Inhibition of RIPK1 with Nec-1 has also been shown to increase the susceptibility of mice to the fungus *Candida albicans* (Cao et al. 2019). In other infections, however, activation of RIPK1 appears deleterious to the host. For example, inhibition of RIPK1 with Nec-1s is reported to reduce the number of bacteria in the bronchoalveolar lavage fluid of mice at 4 h after being dosed intranasally with Gram-positive *Staphylococcus aureus* (Kitur et al. 2015). In the same study, mice lacking RIPK3 were analyzed at 24 h after infection, and results were consistent with RIPK1/RIPK3-dependent proinflammatory signals contributing to lung damage and impeding bacterial clearance (Kitur et al. 2015). In contrast, in *Staphylococcus aureus* skin infection or sepsis models, inhibition of RIPK1 or MLKL deficiency increased bacterial counts and was deleterious (Kitur et al. 2016). Falling into yet a third category, some infections are unaffected by the inhibition of RIPK1. For example, neither Nec-1s nor MLKL deficiency altered bacterial burdens in a humanized mouse model of *Mycobacterium tuberculosis* infection (Stutz et al. 2018).

In terms of viral infections, mice expressing catalytically inactive RIPK1 are reported to have elevated virus titers in the spleen and liver after intraperitoneal injection of vaccinia virus (Polykratis et al. 2014). It was unclear, however, when after infection the mice were analyzed. Whether the mice eventually cleared the virus or succumbed to infection was not reported. As mentioned earlier, mice expressing catalytically inactive RIPK1 are reported to be more susceptible to infection with West Nile virus or Zika virus (Daniels et al. 2017, 2019). MEFs or mouse macrophages expressing catalytically inactive RIPK1 are killed by influenza A virus just like wild-type cells (Kuriakose et al. 2016; Nogusa et al. 2016), but whether mice clear the virus normally has not been reported. It will be important to explore the role of RIPK1 during infection further as inhibitors of RIPK1 are considered for therapeutic purposes (Harris et al. 2017).

CONCLUDING REMARKS

Experiments in preclinical disease models highlight the potential therapeutic benefit of inhibiting RIPK1 to limit inflammation and tissue injury, but still there is much we do not understand about this kinase. The kinase-independent prosurvival scaffolding functions of RIPK1, although clear from genetic experiments, are poorly understood at a biochemical level. We would also benefit from further insights into how activation of RIPK1 induces expression of proinflammatory genes independent of cell death. Other areas that merit further examination are the role of RIPK1 in signaling by RIG-I, the role of ubiquitination and proteins such as A20, ABIN1, and XIAP in modulating activation of RIPK1, and the role of RIPK1-dependent signaling in cancer.

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