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Programmed Necrosis in the Cross Talk of Cell Death and Inflammation

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Abstract

Cell proliferation and cell death are integral elements in maintaining homeostatic balance in metazoans. Disease pathologies ensue when these processes are disturbed. A plethora of evidence indicates that malfunction of cell death can lead to inflammation, autoimmunity, or immunodeficiency. Programmed necrosis or necroptosis is a form of nonapoptotic cell death driven by the receptor interacting protein kinase 3 (RIPK3) and its substrate, mixed lineage kinase domain-like (MLKL). RIPK3 partners with its upstream adaptors RIPK1, TRIF, or DAI to signal for necroptosis in response to death receptor or Toll-like receptor stimulation, pathogen infection, or sterile cell injury. Necroptosis promotes inflammation through leakage of cellular contents from damaged plasma membranes. Intriguingly, many of the signal adaptors of necroptosis have dual functions in innate immune signaling. This unique signature illustrates the cooperative nature of necroptosis and innate inflammatory signaling pathways in managing cell and organismal stresses from pathogen infection and sterile tissue injury.

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INTRODUCTION

Cell death is an important biological process that sculpts the development of multicellular organisms. In the immune system, cell death plays critical roles in immune cell development and pathogen defense. Apoptosis is an orderly form of cell death marked by chromatin condensation, DNA fragmentation, and membrane blebbing into apoptotic bodies. Apoptosis can be triggered by receptors in the tumor necrosis factor (TNF) superfamily (extrinsic pathway) or through direct activation of mitochondrial effectors (intrinsic pathway). Caspases are cysteine proteases that drive apoptosis. Effector caspases cleave and inactivate the flippase adenosine triphosphatase type 11C and scramblase X Kell blood group precursor related family member 8 (Xkr8) (1, 2). This results in exposure of phosphatidyl serine (PS) on the cell surface, which flags the dying cell for uptake and clearance by professional phagocytes such as macrophages (3). The rapid clearance of apoptotic cells ensures minimal risk of detrimental inflammation. This explains why apoptosis is the preferred and dominant pathway by which multicellular organisms eliminate unwanted cells during development. In contrast, necrosis is marked by rapid loss of plasma membrane integrity. Plasma membrane leakage in necrosis is widely thought to occur prior to or concomitant with exposure of PS and other eat-me signals. This early rupture of the plasma membrane releases endogenous danger signals or damage-associated molecular patterns (DAMPs), which are potent stimulants of inflammation (4). As such, necrosis is often detected in infections and inflammatory diseases. This association has led to the popular view that necrosis represents pathological cell death, whereas apoptosis is more central for development.

Pathologists have historically relied on morphology to distinguish between apoptosis and necrosis. Apoptosis is marked by cell shrinking, the appearance of membrane blebs called apoptotic bodies, and condensation of chromatin. In contrast, necrosis is associated with cell and organelle swelling and limited chromatin condensation. Biologists have long considered necrosis as the consequence of trauma or accidental injury. This view has now been revised with recent advances showing the existence of dedicated molecular pathways controlling necrotic cell death. Notably, the classical markers that define apoptosis can sometimes be detected in necrosis. For example, Annexin V staining is a commonly used method to detect exposure of PS on the outer leaflet of the plasma membrane in early apoptotic cells. In some necrotic cells, PS exposure can be detected without significant plasma membrane leakage (5). PS exposure is supposed to mark apoptotic cells for clearance by phagocytes. However, researchers have also described scavenger receptors that recognize necrotic cells (6, 7). Moreover, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, which detects DNA strand breaks in apoptosis, also detects such breaks in necrotic cells (8). These observations suggest that the morphological definition of apoptosis and necrosis is insufficient to distinguish between these two cell death modules. Instead, we favor a molecular definition based on genetic pathways. Terms such as programmed necrosis, regulated necrosis, and necroptosis are now used to describe necrosis induced by the receptor interacting protein kinases (RIPKs) (9). In addition, certain forms of regulated necrosis can occur without the RIPKs (10, 11).

Here, we focus our discussion on RIPK-driven necrosis. The term necroptosis is used throughout to distinguish RIPK-dependent necrosis from other forms of regulated necrosis. As is discussed below, pathways that control necroptosis and inflammation often use overlapping signaling adaptors. The sharing of common signal adaptors establishes an intimate link between inflammation and necroptosis that goes beyond their association in disease pathologies. Hence, necroptosis and inflammation can be mutually reinforcing processes that govern not only inflammatory diseases but also immune and organismal homeostasis.

THE MOLECULAR MACHINERY OF PROGRAMMED NECROSIS

Necroptosis can be activated by death receptors in the TNF superfamily, Toll-like receptor 3 (TLR3) and TLR4, and the interferon receptors (12). The signaling pathway for necroptosis is best characterized for TNF receptor 1 (TNFR1). TNFR1 is the prototypic member of a subfamily within the TNF receptor superfamily that contains an essential protein interaction domain called the death domain (DD). DD-containing death receptors include CD95/FAS/APO-1, TNF-related apoptosis-inducing ligand (TRAIL) receptor 1 and 2, death receptor 3 (DR3), DR6, and ectodysplasin A receptor. However, cell death is not the only signaling outcome for the death receptors. In fact, nuclear factor-κB (NF-κB) activation is often the dominant response emanating from these receptors. TNFR1 is a prime example of such a receptor. Ligation of the preassembled TNFR1 trimer (13) with TNF causes a conformation change that promotes formation of a short-lived membrane-signaling complex termed Complex I by Micheau & Tschopp (14). This membrane complex is composed of the adaptors TNF receptor–associated death domain (TRADD), TNF receptor–associated factor 2 (TRAF2), RIPK1, cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, and the linear ubiquitin chain assembly complex (LUBAC), which is made up of the subunits RanBP-type and C3HC4-type zinc finger containing 1 (aka HOIL-1), ring finger protein 31 (aka HOIP), and Shank-associated RH domain interacting protein (SHARPIN) (15). The E3 ligases cIAP1, cIAP2, and HOIL-1 within LUBAC critically control ubiquitination of many of the adaptors within Complex I. Ubiquitin linkages of different types have been found with various adaptors in Complex I. This ubiquitin network within Complex I is essential for recruitment and activation of the inhibitor of κB kinase (IKK) complex. The activated IKK phosphorylates IκBα, leading to IκBα degradation by the proteasome and nuclear translocation of NF-κB dimers.

NF-κB is a key transcription factor for many proinflammatory and survival genes. A proper NF-κB response is crucial for cell survival and to counteract the cytotoxic effects of TNF. The prosurvival effect of NF-κB is mediated in part by its transcriptional targets cIAP1, cIAP2, and the long form of cellular FLICE-like inhibitor protein ($cFLIP_L$) (16, 17). As we discuss below, $cFLIP_L$ and the cIAPs critically regulate cellular sensitivity to apoptosis and necroptosis. Hence, Complex I is a critical checkpoint for cell death versus cell survival signaling (**Figure 1**). In addition, Complex I adaptors appear to have NF-κB-independent survival functions. For example, mice lacking both cIAP1 and cIAP2 die at an earlier stage in embryonic development than RelA/p65-deficient mice (18). The adaptor TRAF2 stabilizes cIAP1 expression by preventing its autoubiquitination and proteasomal degradation (19). Consistent with this association, *Traf2*$^{-/-}$ mice also exhibit embryonic lethality (20). In contrast, *cpdm* mice that lack the LUBAC subunit SHARPIN show defective NF-κB activation but are nonetheless viable (21–23). These results demonstrate that the cytoprotective effects of TRAF2, cIAP1, cIAP2, and X-linked IAP (XIAP) are mediated through NF-κB-dependent and -independent functions.

Unlike conventional death receptors such as Fas or TRAIL receptors, Fas-associated via death domain (FADD) and caspase-8 are not recruited to the TNFR1-associated Complex I (14, 24). Instead, rapid receptor internalization is important for docking of the adaptor FADD and the initiator caspase, caspase-8, to the complex. Although evidence suggests that FADD and caspase-8 can be recruited to the TNFR1 complex (25), standard biochemical pull-down supports a model in which TNFR1 dissociation occurs prior to docking of FADD and caspase-8 (14). The cytosolic complex that contains FADD and caspase-8 is often referred to as Complex IIa (**Figure 1**). Normally, apoptosis is prevented by dimerization between caspase-8 and $cFLIP_L$, an enzyme-inactive homolog of caspase-8. The caspase-8/$cFLIP_L$ heterodimer inhibits full activation of caspase-8 and apoptosis but retains cleavage of essential necrosis regulators such as RIPK1, RIPK3, and

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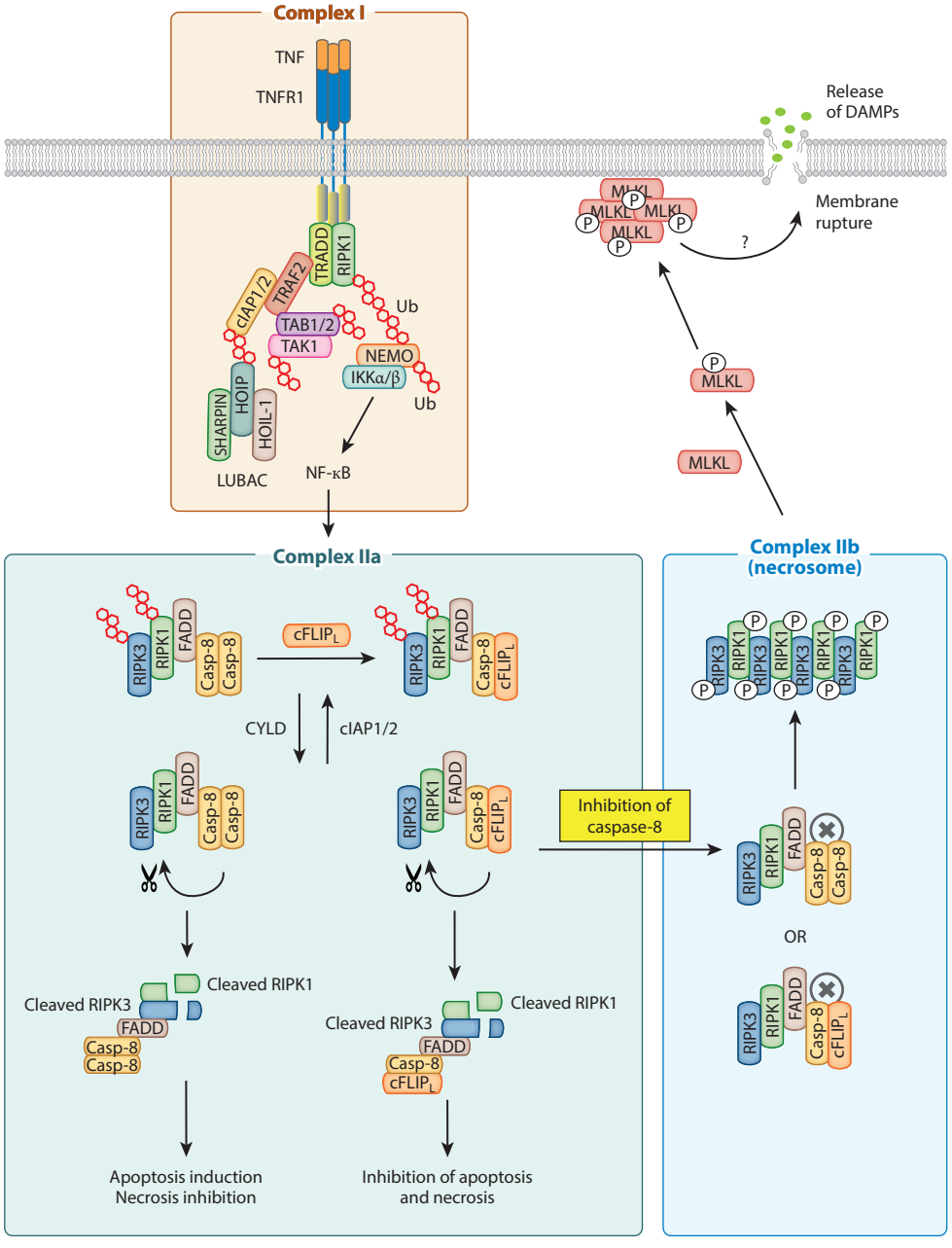
cylindromatosis (CYLD) (26–30). Hence, NF-κB-dependent induction of $cFLIP_L$ inhibits apoptosis as well as necroptosis.

Active caspase-8 in Complex IIa not only initiates the caspase cascade and the apoptotic program but also cleaves and inactivates essential necroptosis mediators such as RIPK1, RIPK3, and CYLD. Hence, inhibition of caspase-8 or its upstream adaptor FADD primes cells for necroptosis by preserving the integrity of RIPK1 and RIPK3. Stabilization of RIPK1 and recruitment of RIPK3 convert Complex IIa to Complex IIb or the necrosome (**Figure 1**). The



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cytosolic complexes that contain the RIPKs have also been referred to as the ripoptosome (31, 32), although this term does not make the distinction between apoptosis and necroptosis. RIPK1 and RIPK3 interact via the RIP homotypic interaction motif (RHIM) to form an amyloid-like complex that is essential for recruitment and activation of the downstream RIPK3 substrate mixed lineage kinase domain-like (MLKL) (33–35). RIPK3 phosphorylates MLKL at Thr357 and Ser358 to stimulate its oligomerization and translocation to intracellular and plasma membranes (34, 36–39) (**Figure 1**). The precise mechanism by which MLKL induces membrane rupture is controversial, with some reports implicating disruption of calcium or sodium ion channels (36, 37) and others showing direct binding to membrane phospholipids and disruption of membrane integrity (38, 39). In contrast to MLKL, another reported substrate of RIPK3, the mitochondrial phosphatase phosphoglycerate mutase family member 5 (PGAM5) (40), may not be crucial, as small hairpin RNA–mediated knockdown of *Pgam5* did not consistently confer protection against TNF-induced necroptosis (41, 42). In agreement with the notion that PGAM5 is not a core component of the necroptosis machinery, widespread depletion of mitochondria did not impair necroptosis (43). The differential requirement for mitochondrial signaling further distinguishes necroptosis from apoptosis.

Because excessive necrosis in FADD- or caspase-8-deficient mice was rescued by inactivation of RIPK1 or RIPK3 (28, 44, 45), FADD and caspase-8 are paradoxically prosurvival factors during development. This yin-yang relationship between the RIPKs and FADD/caspase-8 also plays out in the skin keratinocytes, intestinal epithelium, and T cells (46–49). Genetic evidence also provides a mechanistic explanation for the biochemical interaction between FADD, caspase-8, and the RIPKs. Intriguingly, although caspase-8-deficient Jurkat T cells are sensitized to necroptosis induced by TNF, Fas ligand (FasL), and TRAIL, FADD-deficient Jurkat cells are only sensitized to TNF-induced necroptosis (50). The molecular basis for the resistance of these cells to FasL- and TRAIL-induced necrosis is unknown. One possibility is that because FADD is the apical adaptor recruited to Fas and TRAIL receptors, downstream signaling will be completely blunted in its absence. Although this is certainly the case for FADD-mediated apoptosis, it is insufficient to explain RIPK3 signaling in T cells. *Fadd*$^{-/-}$ T cells undergo RIPK1- and RIPK3-dependent necroptosis in response to T cell receptor (TCR) stimulation (45, 48, 49). Because *Fadd*$^{-/-}$*Ripk3*$^{-/-}$ mice develop lymphoproliferation resembling that caused by the Fas mutant in *lpr* mice (51), one can argue that Fas triggers necroptosis of *Fadd*$^{-/-}$ T cells through RIPK3. Alternatively, necroptosis of *Fadd*$^{-/-}$ T cells could be the consequence of direct TCR signaling. As we explore further below, RIPK3 is also capable of signaling for necroptosis in the absence of RIPK1 under certain conditions. These perplexing results highlight the fact that the traditional model of Complex I to Complex II transition may not be adequate to account for signaling in necroptosis.

Figure 1

TNF-induced signaling complexes. The membrane-associated Complex I is chiefly responsible for NF-κB activation. The ubiquitin chains are represented by red hexagons. Induction of $cFLIP_L$ expression by NF-κB inhibits apoptosis and necroptosis. Active caspase-8 in Complex IIa promotes apoptosis and inhibits necroptosis by cleavage of RIPK1, RIPK3, and CYLD (*scissors*). When caspase-8 is inactive (*circled X*), RIPK1 and RIPK3 initiate Complex IIb assembly, amyloid conversion, and recruitment of MLKL. Both Complex IIa and Complex IIb are also regulated by protein ubiquitination. CYLD acts as the deubiquitinase that promotes Complex II activity by removing ubiquitin chains on RIPK1 and RIPK3. (Abbreviations: cIAP, cellular inhibitor of apoptosis 1; CYLD, cylindromatosis; DAMP, damage-associated molecular pattern; FADD, Fas-associated via death domain; IKK, inhibitor of κB kinase; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; NF, nuclear factor; P, phosphorylation; RIPK, receptor interacting protein kinase; TNF, tumor necrosis factor; TNFR1, TNF receptor 1; Ub, ubiquitin.)

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UBIQUITINATION: A CRITICAL CHECKPOINT FOR NECROPTOSIS

RIPK1 and other Complex I adaptors are key substrates of the E3 ubiquitin ligases cIAP1 and cIAP2. As discussed above, the ubiquitin network within Complex I functions to recruit the IKK complex and to promote survival through NF-κB-dependent and -independent mechanisms (52–56). Bivalent IAP antagonists or Smac mimetics (SMs) are often used to deplete cIAP1, cIAP2, and XIAP. SMs are small-peptide mimetics of second mitochondrial-derived activator of caspases (Smac) that trigger autoubiquitination and degradation of the IAPs. Because RIPK1 ubiquitination does not occur in the absence of the IAPs, SM tips the balance of TNF signaling toward cell death. Moreover, because the NF-κB-inducing kinase (NIK) is constitutively targeted for ubiquitination and degradation by the IAPs (57–60), SM can additionally stabilize NIK, leading to noncanonical NF-κB activation and autocrine TNF production. Thus, SM primes cells for cell death through two mutually reinforcing mechanisms: elimination of a cytoprotective ubiquitin network and induction of TNF. Given that many tumors overexpress cIAPs and are resistant to traditional chemotherapies, SM can provide a powerful one-two punch to trigger cancer cell death through either apoptosis or necroptosis (61, 62). Physiologically, IAP depletion occurs in response to stimulation of TNFR2, the TNF receptor whose expression is highly inducible (63). Although TNFR2 does not contain a cytoplasmic DD, it recruits TRAF2 and the cIAPs and triggers their proteasomal degradation. Hence, similar to the action of SM, TNFR2 also skews TNF signaling toward cell death (64–67). Hence, the IAPs and ubiquitination play important roles in fending off the cytotoxic effects of TNF.

The importance of cIAPs and the ubiquitin machinery in regulating RIPK activities and necroptosis is illustrated by the partial rescue of embryonic lethality of *ciap1*$^{-/-}$*xiap*$^{-/-}$ or *ciap1*$^{-/-}$*ciap2*$^{-/-}$ embryos by loss of *Ripk3* or a single *Ripk1* allele (18). Systemic autoinflammatory disease of mice with myeloid-specific deletion of cIAP1, cIAP2, and XIAP was also corrected by inactivation of RIPK1 or RIPK3 (68). These results provide strong evidence that the IAPs are crucial guardians that keep RIPK1 and RIPK3 in check to prevent deleterious cell injury and inflammation. Further evidence that the ubiquitin network within Complex I serves critical functions in limiting cell death and inflammation comes from mice lacking the LUBAC components SHARPIN or HOIL-1. Cells lacking SHARPIN or HOIL-1 are sensitized to apoptosis as well as necroptosis (21, 69), and mice lacking these components develop systemic autoinflammatory diseases (21–23, 70). Interestingly, the severe skin and multiorgan inflammation in SHARPIN-deficient *cpdm* mice was corrected by crosses to knock-in mice expressing kinase-inactive RIPK1 (*Ripk1-K45A*) (71). Fibroblasts and macrophages from *Ripk1-K45A* knock-in mice exhibit normal mitogen-activated protein kinase and NF-κB responses but are resistant to TNF-induced necroptosis (71, 72). Hence, excessive cell death appears to be the major driver for RIPK1-dependent inflammation in *cpdm* mice. However, because the kinase activity of RIPK1 is required for apoptosis under certain conditions (73), it remains to be determined if RIPK1-induced inflammation in *cpdm* mice is driven by apoptosis or necroptosis.

In addition to mouse models, human patients with mutations in the E3 ligase subunit of LUBAC HOIL-1 exhibit chronic inflammation, increased cytokine expression in response to IL-1β, cardiomyopathy, and susceptibility to pyogenic bacteria due to impaired NF-κB activation (74, 75). Consistent with the key role of the LUBAC complex for recruitment of the IKK complex, mutations in the IKK regulatory subunit NF-κB essential modulator (NEMO)/IKKγ cause incontinentia pigmenti (IP), a disease marked by skin lesions and multiorgan inflammation. Because *Nemo* is an X-linked gene, male mice lacking NEMO are embryonic lethal (76–78), and male patients of IP are rarely found. In the few rare cases of male patients harboring mild mutations in NEMO, patients develop a variant form of the disease called hypohidrotic ectodermal

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dysplasia, which is marked by abnormalities in the teeth, hair, and eccrine sweat glands (79). The chronic inflammatory phenotypes caused by mutations in the cIAPs, LUBAC, and NEMO seem to contradict the fact that cells lacking these adaptors are impaired in cytokine-induced NF-κB responses. Because cells lacking these components in the ubiquitin network are also highly sensitive to death signals, heightened cell death is likely the driver of the chronic inflammation. It will be interesting to determine if the kinase function of RIPK1 is similarly responsible for driving the lethal inflammatory disease of *Nemo*$^{-/-}$ mice as in *cpdm* mice (71).

The NF-κB transcriptional target A20 and the tumor suppressor CYLD are believed to facilitate Complex I transition to Complex II by promoting deubiquitination of RIPK1. Although both A20 and CYLD are recruited to Complex I, small interfering RNA (siRNA) knockdown of CYLD, but not A20, protects cells against TNF-induced necroptosis (69, 80, 81). Surprisingly, RIPK1 ubiquitination in Complex I was not altered in *Cyld*$^{-/-}$ cells. Rather, RIPK1 and RIPK3 ubiquitination within the necrosome was greatly elevated in *Cyld*$^{-/-}$ cells (81). Hence, rather than regulating RIPK1 ubiquitination in Complex I, CYLD acts within the necrosome to deubiquitinate RIPK1 and RIPK3. Moreover, these results suggest that in addition to regulating NF-κB activation within Complex I, the E3 ligases cIAP1 and cIAP2 may also control necrosome activation through ubiquitination of RIPK1 and RIPK3.

THE JANUS NATURE OF RIPK1

Besides acting as the upstream activator of TNF-induced, RIPK3-mediated necroptosis, RIPK1 is also required for SM-primed, TNF-induced apoptosis (73). Paradoxically, RIPK1 also functions as an inhibitor of RIPK3- and caspase-8-mediated cell injury and inflammation. *Ripk1*$^{-/-}$ mice suffer from perinatal lethality that was originally believed to be caused by defective NF-κB-mediated induction of survival genes (82). However, *Ripk1*$^{-/-}$ mice are born alive, whereas *RelA*$^{-/-}$ mice die in utero at E15.5. In addition, a recent report argues that RIPK1 does not play a significant role in NF-κB activation (83). These results suggest that defective NF-κB activity may not fully account for the perinatal lethality of *Ripk1*$^{-/-}$ mice. Mice that lack multiple cIAPs, such as *ciap1*$^{-/-}$*Xiap*$^{-/-}$ mice, suffer from embryonic lethality at E10.5. This lethality is eerily similar to that in mice lacking *Fadd*, *Casp8*, or *cFlip*. Strikingly, hemizygous *Ripk1* deficiency significantly prolonged survival of *ciap1*$^{-/-}$*Xiap*$^{-/-}$ mice until weaning age (18). Thus, researchers have proposed an alternative model in which RIPK1 ubiquitination sterically hinders recruitment of downstream cell death effectors (55, 56).

Recently, the molecular basis that underlies the perinatal lethality of *Ripk1*$^{-/-}$ mice was examined in further detail. *Ripk1*$^{-/-}$ mice exhibit extensive cleaved caspase-3 in multiple tissues and a systemic increase in inflammatory cytokines. The increase in caspase-3 activation and apoptosis appears to be partly due to a failure to upregulate $cFLIP_L$ expression (84). Cleaved caspase-3 and apoptosis were significantly reduced in *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ mice. However, these mice still succumbed to perinatal lethality (85), indicating that apoptosis is not the only driver for the lethal phenotype. Deletion of *Ripk3* also had minimal effect on the survival of *Ripk1*$^{-/-}$ mice (84). However, *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice survived until adulthood and developed an *lpr*-like autoimmune disease that is also observed in *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ mice (84–86). Tissue-specific deletion of *Ripk1* and bone marrow reconstitution experiments show that RIPK1 is essential for the survival of hematopoietic stem cells, skin keratinocytes, and intestinal epithelial cells (85, 87, 88). Inactivation of TNFR1 and the IFN receptor significantly increased survival of *Ripk1*$^{-/-}$ mice (84), suggesting that RIPK1 inhibits TNFR1- and IFN-induced cell death in multiple cell types (**Figure 2**). Surprisingly, knock-in mice expressing kinase-inactive RIPK1 are viable and do not exhibit the abnormalities found in *Ripk1*$^{-/-}$ mice (71, 72). Hence, although

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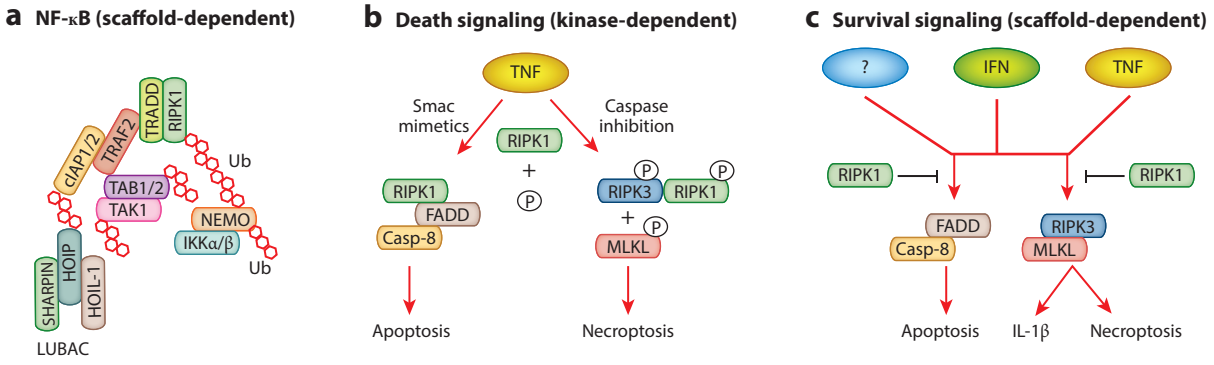

Figure 2

RIPK1 mediates cell survival and cell death through distinct mechanisms. (*a*) RIPK1 facilitates assembly of the ubiquitin scaffold that stimulates NF-κB activation. This function does not require the kinase activity of RIPK1. (*b*) The kinase activity of RIPK1 promotes apoptosis and necroptosis. (*c*) The scaffolding function of RIPK1 promotes survival and suppresses inflammation by inhibiting FADD–caspase-8 and RIPK3-MLKL activation. This RIPK1 function is required to neutralize deleterious signals from the interferon receptor, the TNF receptor, and other yet-to-be-identified receptors. The kinase activity of RIPK1 is dispensable for this survival function. [Abbreviations: cIAP, cellular inhibitor of apoptosis; FADD, Fas-associated via death domain; HOIL-1, heme-oxidized IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; IFN, interferon; IKK, inhibitor of κB kinase; LUBAC, linear ubiquitin chain assembly complex; MLKL, mixed lineage kinase domain-like; NEMO, NF-κB essential modulator; NF, nuclear factor; P, phosphorylation; RIPK, receptor interacting protein kinase; SHARPIN, SHANK-associated RH domain interacting protein (SHARPIN); Smac, second mitochondrial-derived activator of caspases; TAB, TAK1-binding protein; TAK, TGF-β-activated kinase; TNF, tumor necrosis factor; TRADD, TNF receptor–associated death domain; TRAF, TNF receptor–associated factor 2; Ub, ubiquitin.]

its kinase activity promotes cell death through apoptosis and necroptosis, RIPK1 has a separate scaffolding function that curbs the death signals emanating from multiple innate immune and death receptors (**Figure 2**).

RHIM-MEDIATED AMYLOID CONVERSION IN NECROPTOSIS

In addition to needing to avoid caspase-8-mediated cleavage and deubiquitination by CYLD, induction of necroptosis also requires a RHIM-mediated interaction between RIPK1 and RIPK3 (89, 90). The RHIM is defined by a highly conserved tetrapeptide core flanked by hydrophobic residues that predominantly form β-sheets (**Figure 3**). RHIM-like adaptors are found in viruses and in *Drosophila*, arguing for a critical role for RHIM-mediated interaction immunity throughout evolution (see below). Strikingly, RHIM-containing adaptors exhibit a strong propensity to adopt an amyloid-like conformation either alone or in complex with another RHIM-containing adaptor. This unique structural scaffold is important for signaling. In the case of RIPK1 and RIPK3, disruption of this amyloid scaffold severely impairs autophosphorylation and activation of RIPK1 and RIPK3, as well as downstream execution of necroptosis (33).

Although RHIM-mediated interaction is essential for RIPK1- and RIPK3-dependent necroptosis downstream of TNFR1, not all RHIM-mediated interactions lead to cell death. For example, Toll/Interleukin-1 receptor (TIR) domain–containing adaptor-inducing interferon-β (TRIF) and RIPK1 interact via their respective RHIMs to mediate NF-κB activation downstream of TLR3 or TLR4 (91, 92). By contrast, RIPK3 inhibits this response, apparently through disruption of RHIM-RHIM interaction between RIPK1 and TRIF. Similarly, the murine cytomegalovirus (MCMV) necrosis inhibitor M45/viral inhibitor of RIPK activation (vIRA) inhibits premature

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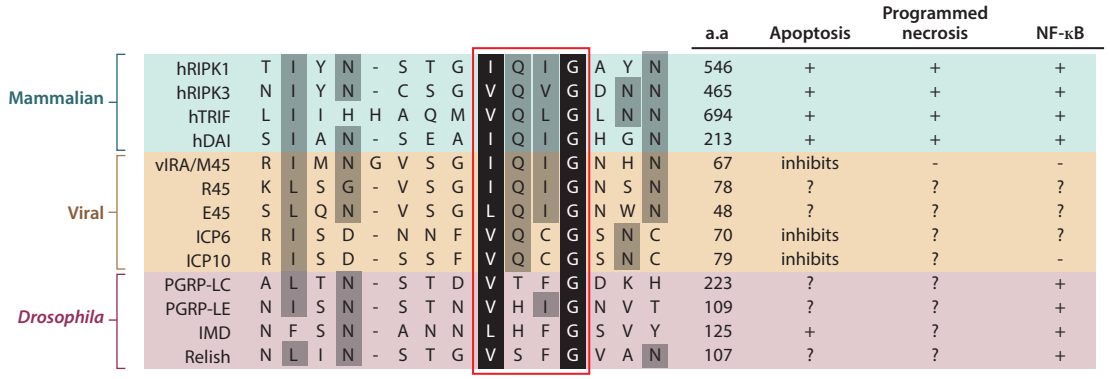

Figure 3

The RHIM is a conserved signaling motif in innate immune and death signaling adaptors. Sequence alignment of mammalian (human), viral, and *Drosophila* RHIM-containing adaptors is shown. R45 and E45 are the M45 homologs in the Maastricht and English isolates of rat CMV, respectively. ICP6 and ICP10 are the M45 homologs of HSV-1 and HSV-2, respectively. Although all the viral RHIM adaptors encode a ribonucleotide reductase domain, not all are active enzymes (149). The *Drosophila* RHIM-like adaptors and receptors are included for comparison, although there is currently no evidence to indicate that they function like the mammalian RHIMs. In addition to the PGRPs, the *Drosophila* IMD and Relish also contain RHIM-like motifs (A. Kleino & N. Silverman, personal communication). The red box indicates the tetrapeptide core of the RHIM. The black and gray shading represents highly and moderately conserved residues, respectively, as defined by functional side chains. (Abbreviations: +, positive inducer of the indicated response; –, unable to induce the indicated response; ?, undefined function for the indicated response; a.a., amino acid position of the last residue shown in the sequence alignment; CMV, cytomegalovirus; HSV, herpes simplex virus; IMD, immune deficiency; NF, nuclear factor; PGRP, peptidoglycan recognition protein; RHIM, receptor interacting protein homotypic interaction motif.)

necroptosis in infected cells by binding to RIPK3 and preventing it from interacting with its partner, DNA activator of interferon (DAI) (93, 94) (see below). Because all these RHIM-containing adaptors have the propensity to form amyloid fibrils in vitro (33), it will be vital to determine whether amyloid conversion also occurs in situations that do not result in cell death.

Amyloid complexes are widely perceived to be the etiological agents for age-related dementias such as Alzheimer's disease (AD) and Parkinson's disease. The neurological pathologies in these diseases are often marked by necrosis and inflammation. Recent evidence indicates that signaling through the nucleotide-binding oligomerization domain-like receptor, pyrin domain containing 3 (NLRP3) inflammasome drives the disease pathology in AD (95, 96). Interestingly, the NLRP3 inflammasome is activated by agonists that are aggregate in nature, including silica, uric acid crystals, and amyloid β (Aβ) peptides. This raises the tantalizing possibility that the RIPK1-RIPK3 amyloid fibrils may stimulate neuronal injury and inflammation by activating the NLRP3 inflammasome. Alternatively, released RIPK1-RIPK3 amyloid fibrils from dying neurons may seed the amyloid conversion of pathogenic amyloid proteins such as Aβ, α-synuclein, or tau. RIPK1 has been implicated in several models of ischemia-reperfusion-induced brain injury (97, 98). It will be interesting to determine if there is any interaction between the RIPK1-RIPK3 complex and any of the neurotoxic, amyloid-like peptides and whether the RIPKs contribute to age-related neurodegeneration.

NONCANONICAL NECROSOMES

As we alluded to in the above section, the RHIM is also found in the TLR3/4 adaptors TRIF and DAI. A common link for the mammalian RHIM-containing adaptors is that they share functions in innate immune signaling, cell death signaling, or both (**Figure 3**).

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In the presence of caspase inhibition, TLR3 and TLR4 stimulation causes necroptosis mediated by TRIF and RIPK3 (99, 100). Similar to RIPK1 and RIPK3, TRIF is a cleavage substrate of caspase-8. TRIF cleavage by caspase-8 inhibits its ability to stimulate NF-κB-dependent cytokine gene expression (101). However, researchers do not know whether caspase-8 cleavage of TRIF also inhibits necroptosis. TRIF-dependent necroptosis requires binding to RIPK3 via the RHIM. In contrast to that of TRIF and RIPK3, the role of RIPK1 in TLR3- and TLR4-induced necroptosis is enigmatic. The RIPK1 inhibitor necrostatin-1 (Nec-1) inhibited TLR3- and TLR4-induced necroptosis in primary bone marrow–derived macrophages, the macrophage cell line J774, and, to a lesser extent, the endothelial cell line SVEC4-10 (99, 100). However, *Ripk1*$^{-/-}$ fibroblasts or siRNA knockdown of RIPK1 in 3T3 fibroblasts and SVEC4-10 did not rescue TLR3-induced necroptosis. Because Nec-1 was able to enhance survival of TLR3- and TLR4-induced necroptosis in J774 macrophages with silenced expression of RIPK1 (100), the protection conferred by Nec-1 might be due to off-target effects (102, 103).

Unlike RIPK1, TRIF does not possess kinase activity. This implies that the mechanism by which TRIF activates RIPK3 is different from that used by RIPK1. We therefore propose the term noncanonical necrosome to distinguish pronecrotic RIPK3 complexes that do not contain RIPK1. In addition to TRIF, RIPK3 can also partner with DAI to induce necroptosis during MCMV infection (see below). Canonical necrosome activation requires RIPK1-dependent phosphorylation of RIPK3 at specific sites including Ser199, Ser357, and Ser358 (34, 104). Will these modifications also be required for noncanonical necrosome activation? If they are, what kinases mediate these events in the absence of RIPK1? These and other questions will need to be addressed in the future.

NECROPTOSIS IS CONTROLLED BY PHOSPHORYLATION

Both RIPK1 and RIPK3 are heavily phosphorylated in the necrosome. Mass spectrometry analyses have identified multiple phosphorylation sites on RIPK1 and RIPK3 (34, 105), with the majority of these phosphorylation sites localized within the N-terminal kinase domains. Interestingly, expression of truncated RIPK1 or RIPK3 lacking the kinase domain, but not full-length proteins, results in spontaneous formation of amyloid fibrils. Because alanine substitutions of individual serine or threonine residues on RIPK1 have little effect on RIPK1 kinase activity and TNF-induced necroptosis (104), these results are most consistent with a model in which the kinase domain inhibits RIPK activation by masking the RHIM. In this model, phosphorylation of RIPK1 in the kinase domain alters the conformation of the kinase, perhaps through charge repulsion, to allow RHIM-mediated interaction with downstream signal adaptors (**Figure 4*a***).

Although it is widely accepted that RIPK1 is the upstream kinase that activates RIPK3, evidence suggests that RIPK3 can signal for necroptosis independently of RIPK1. For example, inducible dimerization of RIPK3 drives RHIM- and MLKL-dependent necroptosis independently of RIPK1 (106–108). These results argue that the major function of RIPK1 is to initiate the nucleation event for RIPK3 oligomerization. Indeed, the phosphomimetic mouse RIPK3 mutant S204D (S199D in human RIPK3) restored TNF-induced necroptosis in *Ripk3*$^{-/-}$ fibroblasts that was no longer sensitive to inhibition by the RIPK1 kinase inhibitor Nec-1 or siRNA knockdown of RIPK1 (104). Moreover, overexpression of RIPK3 also leads to TNF-induced necroptosis that is independent of RIPK1 (109). Because RIPK3 expression is highly inducible by different activation signals (9, 90), the result from RIPK3 overexpression suggests that TNF-induced necroptosis can indeed proceed without RIPK1 under certain physiological conditions. The current model predicates that RIPK1 is essential for recruitment and activation of RIPK3; thus, these results do raise questions about

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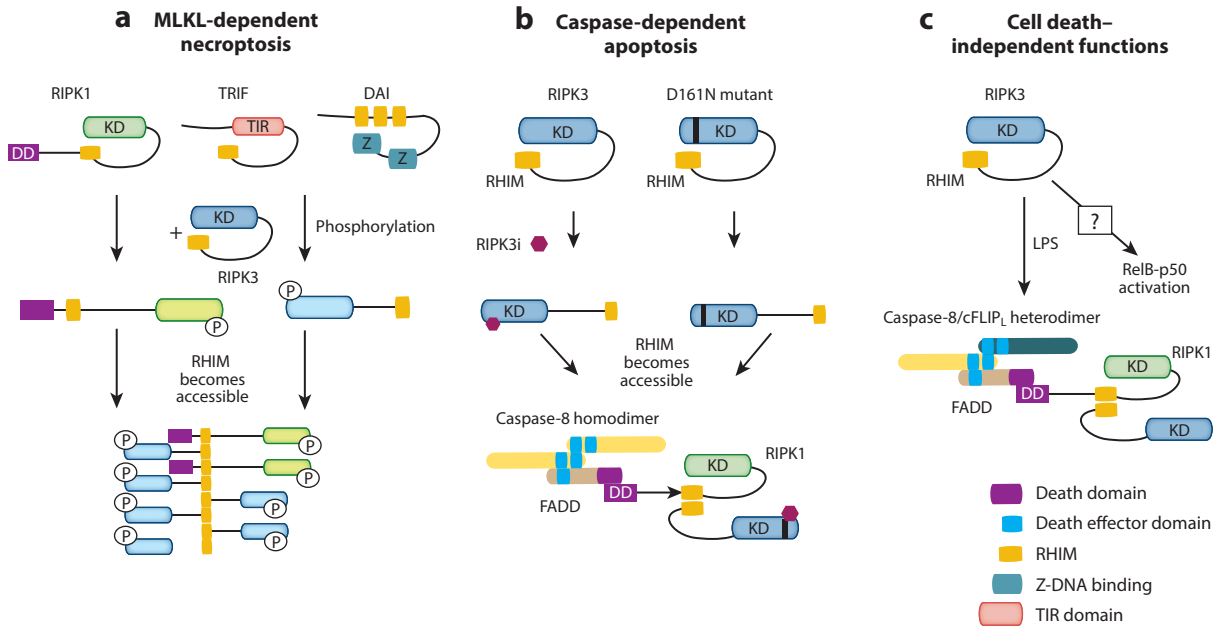

Figure 4

RIPK3 signals for cell death and inflammation through diverse mechanisms. (*a*) RIPK3 mediates necroptosis by binding to RIPK1 or other RHIM-containing adaptors. This causes amyloid conversion of RIPK3, which serves as a platform for docking and recruitment of the RIPK3 substrate MLKL. (*b*) Binding of the RIPK3 kinase inhibitor or introduction of the D161N mutation causes a conformational change that promotes a different form of RHIM-mediated interaction between RIPK1 and RIPK3 that leads to FADD and caspase-8 binding and apoptosis. (*c*) Although the mechanisms have yet to be defined, RIPK3 can also induce pro-IL-1β processing through caspase-1 and caspase-8. In overexpression studies, RIPK3 has also been shown to either enhance or inhibit NF-κB signaling. (Abbreviations: DAI, DNA activator of interferon; DD, death domain; FADD, Fas-associated death domain protein; KD, kinase domain; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like; NF, nuclear factor; P, phosphorylation; RHIM, RIP homotypic interaction motif; RIPK, receptor interacting protein kinase; RIPK3i, RIPK3 inhibitor; TIR, Toll/interleukin-1 receptor; TRIF, TIR domain–containing adaptor-inducing interferon-β.)

the veracity of the traditional Complex I to Complex II transition model downstream of TNFR1 signaling.

In addition to Ser204, phosphorylation of RIPK3 at Ser227 was reported to mediate MLKL binding (34). Although alanine substitution at this residue abrogated RIPK3 function (34), a phosphomimetic mutant was unable to restore TNF-induced necroptosis (104). Therefore, the negative charge that results from phosphorylation of RIPK3 at Ser227 is not crucial for MLKL recruitment. Rather, Ser227 phosphorylation may convert RIPK3 into a permissive conformation to interact with MLKL. This type of conformation-sensitive interaction involving RIPK3 is also found in the kinase-inactive RIPK3 mutant D161N. Mice and cells that express RIPK3-D161N undergo apoptosis following assembly of an alternative caspase-8-activating complex that contains RIPK1, RIPK3-D161N, FADD, and caspase-8 (72). However, not all kinase-inactive mutants of RIPK3 drive assembly of this apoptosis-inducing complex. For example, expression of RIPK3-K51A and RIPK3-D143N is not toxic to cells. Surprisingly, high doses of RIPK3-specific kinase inhibitors can drive assembly of this apoptosis complex in cells that express wild-type RIPK3 or the kinase-inactive RIPK3 mutants K51A or D143N (110) (**Figure 4*b***). Because an intact RHIM is also required to drive assembly of this caspase-8-activating complex, RHIM-mediated interaction alone is not sufficient to determine the cell death mode. Additional factors such as differences

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in conformation or recruitment of distinct adaptors are likely important in determining the cell death module being activated.

RIPKs, NF-κB ACTIVATION, AND IL-1β

The receptors that induce necroptosis are also potent inducers of the proinflammatory transcription factor NF-κB. NF-κB induces expression of prosurvival factors such as cFLIP and the cIAP genes and hence is generally considered to be a mutually exclusive signaling outcome from apoptosis or necroptosis. However, this is not always the case. For example, activated T cells upregulate expression of TNFR2 and are highly sensitive to cell death signals (111, 112). The sensitization to cell death can be recapitulated in Jurkat T cell leukemia by expression of TNFR2. Under these conditions, enhanced TNF-induced apoptosis or necroptosis is accompanied by strong NF-κB activation (64). In addition, SM, which sensitizes cells to death cytokines, also causes noncanonical NF-κB activation (57, 58, 113). Hence, NF-κB and necroptosis can synergize with each other to maximize the inflammatory response to stress signals.

To further highlight the cross talk between necroptosis and inflammation signaling, both RIPK1 and RIPK3 can promote NF-κB activation. As discussed above, RIPK1 facilitates NF-κB downstream of TNFR1 and other innate immune receptors such as TLR3 and TLR4 (114, 115). Because of its homology to RIPK1, early studies on RIPK3 also focused on its ability to modulate NF-κB signaling. Overexpression of RIPK3 either stimulates or inhibits NF-κB activation in a context-dependent manner (92, 116–119). However, embryonic fibroblasts and macrophages from *Ripk3*$^{-/-}$ mice were normal for TNF- and TLR4-induced IκBα phosphorylation and degradation, and cytokine expression was unaffected (90, 120). Although these results suggest that RIPK3 is not a core component of the NF-κB pathway, we found that RIPK3 can indeed modulate NF-κB signaling, especially that of RelB and p50, in certain dendritic cell subsets (121) (**Figure 4*c***). Taken together, these results indicate that RIPK1 and RIPK3 can promote inflammation in vivo through necrosis-dependent and -independent mechanisms.

Besides its role in necroptosis and NF-κB activation, RIPK3 has also been implicated in facilitating pro-IL-1β processing in macrophages and dendritic cells. IL-1β is an innate inflammatory cytokine that requires NF-κB-dependent de novo synthesis as well as cleavage and maturation by caspase-1. Caspase-1 cleavage of pro-IL-1β happens as a result of activation of a macromolecular complex termed the inflammasome, which consists of a sensor such as absent in melanoma 2 (AIM2) or NLRP3, the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1. This basic signaling scheme is eerily similar to that used by death receptors, suggesting that the apoptosis and inflammasome signaling pathways share common evolutionary ancestry. Although the necroptosis signaling pathway does not use a similar signaling scheme, the necrosome and inflammasome both require the assembly of a higher-order filamentous complex for activation. For the AIM2 and NLRP3 inflammasomes, cryoelectron microscopy revealed that activation of the inflammasome sensor causes a nucleation reaction driven by the pyrin domain of the adaptor ASC, leading to multimerization of caspase-1 and formation of an elongated, filamentous complex (122). This prion-like property again highlights the potential link between cell death, inflammation, and neurodegeneration. This multimerization model of caspase-1 activation contrasts with that of the widely accepted model of proximity-induced dimerization of apoptosis-inducing caspases (123). However, more recent work reveals that oligomerization is also important for caspase-8 activation by TNFR-like death receptors (124, 125). Interestingly, although the filamentous inflammasome complex is not amyloid in nature, it can apparently seed further polymerization reactions in neighboring cells as it is released from cells undergoing pyroptosis (126, 127). Higher-order oligomerization appears to be an emerging

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theme in innate and cell death signaling, as other intracellular pattern-recognition receptors and sensors including retinoic acid inducible gene-I and mitochondrial antiviral signaling protein are also activated by similar polymerization mechanisms (128–130).

In addition to processing by the caspase-1-associated inflammasome, pro-IL-1β can also be processed by caspase-8 in certain situations (131–136). For example, the chemotherapeutic agent doxorubicin exclusively induces caspase-8-mediated pro-IL-1β processing in bone marrow–derived dendritic cells (133). Moreover, in lipopolysaccharide (LPS)-primed macrophages that lack cIAP1, cIAP2, and XIAP, pro-IL-1β processing is mediated through caspase-1 and caspase-8 in a RIPK3-dependent manner (137). The mechanism by which RIPK3 promotes IL-1β processing is unclear at present. As we discussed above, RIPK3 inhibitors and the kinase-inactive RIPK3 mutant D161N can drive formation of an alternative caspase-8-activating complex. Could a similar complex be involved in caspase-8-mediated pro-IL-1β processing? In the case of the D161N mutant, this complex promotes apoptosis. However, if caspase-8 is paired with its inhibitor $cFLIP_L$, this complex may no longer promote apoptosis but instead facilitate pro-IL-1β processing. This model is consistent with published reports that the caspase-8/$cFLIP_L$ heterodimer exhibits altered substrate specificity compared with the caspase-8 homodimer (29). Because these effects are manifested when the cIAPs are depleted, the IAPs are crucial gatekeepers of RIPK3 activity in cell death and inflammation.

In addition to promoting caspase-8, RIPK3 can also promote caspase-1-mediated pro-IL-1β processing. *Fadd*$^{-/-}$ and *Casp8*$^{-/-}$ macrophages and dendritic cells produced greatly elevated levels of IL-1β that was reversed by deletion of *Ripk3* (138, 139). However, researchers disagree on whether the enhanced IL-1β production was due to increased necrosis-associated release of DAMPs or direct effects of RIPK3 on caspase-1 activation. Regardless of the mechanism, RIPK3 can clearly promote caspase-1- and caspase-8-mediated pro-IL-1β processing via distinct mechanisms.

The necrosis-independent effects of RIPK1 and RIPK3 on NF-κB and pro-IL-1β processing illustrate an important principle: The RIPKs facilitate inflammation through multiple means. They also reinforce the notion that death-signaling adaptors often have important functions beyond cell death. The multifaceted nature of death-inducing adaptors is not a novel concept. FADD, for example, has been implicated in regulating cell cycle entry (140–142), and caspases have important functions in cell differentiation, wound repair, and pruning of neuronal dendrites (143–145). The diverse functions of RIPK3 remind us that inhibition of necroptosis is not the only possible explanation for why *Ripk3*$^{-/-}$ mice often show protection in many inflammatory disease models.

NECROPTOSIS IN VIRAL INFECTIONS

Because the release of DAMPs can stimulate pattern-recognition receptors such as TLRs, necroptosis is widely recognized to be beneficial in innate immune responses against pathogens. However, studies also show that necrosis-dependent inflammation can lead to detrimental pathology in sterile injury–induced diseases (**Table 1**). Given the fact that caspase inhibition is a priming signal for necroptosis, perhaps it is not surprising that viruses that encode caspase inhibitors are susceptible to host cell necroptosis (146). Poxviruses are master evaders of the host cell death machinery. In the case of vaccinia virus, the viral serpin Spi2/B13R is a potent inhibitor of caspase-1 and caspase-8. As in the case of most pathogens, vaccinia virus infection causes an early wave of TNF expression, which triggers RIPK1/RIPK3-dependent necroptosis in different infected tissues (65, 90). In vitro experiments confirmed that wild-type cells infected with vaccinia virus were sensitized to TNF-induced cytotoxicity, but infected *Ripk1*$^{-/-}$ and *Ripk3*$^{-/-}$ cells were highly resistant to TNF-induced necroptosis (65, 90). *Ripk3*$^{-/-}$ mice had reduced necrosis and inflammation in

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Table 1 Necroptosis-related diseases

Cause of cell injury	Disease	Model	Reference(s)
Viral infection	Vaccinia virus	*Ripk3*$^{-/-}$ and *Ripk1-D138N* mice	65, 90, 147
	Murine cytomegalovirus	*Ripk3*$^{-/-}$ and *Dai*$^{-/-}$ mice	93, 94
Bacterial infection	*Mycobacterium tuberculosis*	Zebrafish	158
	Salmonella enterica serovar Typhimurium	*Ripk3*$^{-/-}$ mice and macrophages; necrostatin-1	157
Sterile injury–induced inflammation	Psoriasis	Keratinocyte-specific deletion of *Fadd* or *Casp8*; *cpdm* mice	47, 71, 187
	Inflammatory bowel disease	Intestinal epithelium–specific deletion of *Fadd* or *Casp8*	8, 46
	Myocardial infarction	Necrostatin-1	170
	Hypoxia-ischemia-induced brain injury	Necrostatin-1	97
	Ischemia-reperfusion kidney injury	Necrostatin-1; *Ripk3*$^{-/-}$ mice	161, 188
	Retinal degeneration	Retinal detachment; interphotoreceptor retinoid-binding protein–deficient mice; poly(I:C)-induced retinal injury; rd10 mice; pde6c mutant zebrafish	175–178
	Pancreatitis	Cerulein-induced pancreatitis in *Ripk3*$^{-/-}$mice	153, 168, 189
	Atherosclerosis	*Ripk3* deletion in apolipoprotein E or low-density lipoprotein cholesterol receptor–deficient mice	174
	Gaucher's disease	Conduritol B epoxide inhibition of glucocerebrosidase in *Ripk3*$^{-/-}$ mice	169

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infected tissues and ultimately succumbed to the infection because of failure to control viral replication. In agreement with these results, mice expressing a kinase-inactive RIPK1 (D138N) were also partially impaired in clearance of vaccinia virus (147). These results established necroptosis as an important antiviral response against certain viral pathogens. This innate immune defense mechanism may be important to tamp down viral replication before robust, virus-specific T cell responses are mobilized (**Figure 5*a***).

The results from vaccinia virus are surprising because they seem to indicate that by blocking caspase activation, viruses set themselves up for destruction by the host. Because ablation of necroptosis leads to rapid death of the host, blocking necroptosis may actually deprive the virus of the opportunity to disseminate and infect another host. From this perspective, one can argue that necroptosis is beneficial not only to the host but also to the invading virus (**Figure 5*a***). Moreover, when compared to other naturally occurring poxviruses, vaccinia virus contains large deleted gene segments (148). Thus, a tantalizing possibility is that the gene that inhibits necroptosis is lost in vaccinia virus because of these gene deletions. In this scenario, triggering of necroptosis may be an exception rather than a rule for vaccinia virus. To this end, it will be important to determine if other poxviruses also cause infected cells to undergo necroptosis.

Viral inhibition of apoptosis is widely perceived to give the virus an edge in its struggle with the host. Because of its antiviral effects, it stands to reason that some viruses may have also developed strategies to counteract necroptosis. Herpesviruses are highly adept at countering the

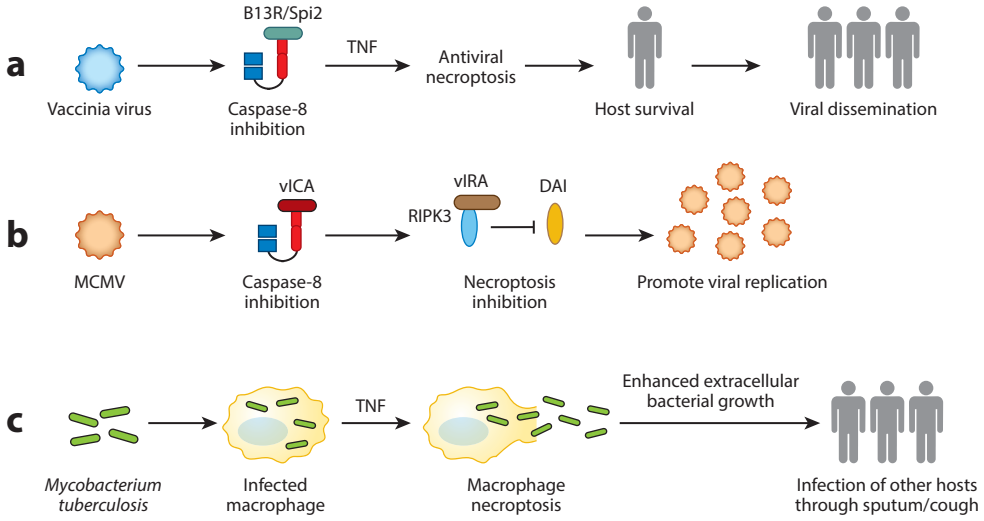

Figure 5

Necroptosis in host-pathogen interactions. (*a*) Vaccinia virus inhibits caspase-8 via the viral inhibitor B13R/Spi2. This primes the cells toward necroptosis. Although necroptosis and the ensuing inflammation have antiviral effects, they may in fact promote viral dissemination to another host by avoiding premature death of the infected host. (*b*) MCMV inhibits caspase-8 and necroptosis via vICA and vIRA. Genetic experiments show that vIRA is essential to prevent premature death of the infected cells. Hence, vIRA-mediated necroptosis inhibition is important for the virus to complete its replication cycle and to generate more viral progeny. (*c*) *Mycobacterium tuberculosis* uses RIPK3-dependent necroptosis to release the bacteria into a growth-permissive environment, which in turn enhances spread of the pathogen to uninfected hosts via the sputum. (Abbreviations: DAI, DNA activator of interferon; MCMV, murine cytomegalovirus; RIPK, receptor interacting protein kinase; TNF, tumor necrosis factor; vICA, viral inhibitor of caspase-8-induced apoptosis; vIRA, viral inhibitor of RIPK activation.)

host cell death machinery. MCMV encodes several viral cell death inhibitors, one of which is the viral inhibitor of caspase-8-induced apoptosis (vICA). Because inhibition of caspase-8 is a priming signal for necroptosis, one would expect that cells infected with MCMV would become susceptible to necroptosis. However, MCMV-infected cells are spared from necroptosis because the virus also encodes vIRA, the product of the *M45* gene. M45/vIRA is a RHIM-containing viral cell death inhibitor that binds to RIPK3 to prevent virus-induced necroptosis (94, 146). Recombinant MCMV expressing a tetra-alanine-substitution RHIM mutant of vIRA fails to inhibit RIPK3 and succumbs to rapid necrosis. Because of this premature cell death, the mutant virus fails to establish a productive infection in cells and mice. Productive infection was reestablished with the mutant virus in *Ripk3*$^{-/-}$ cells and *Ripk3*$^{-/-}$ mice (93).

In contrast to RIPK3, TNF signaling and RIPK1 are both dispensable for mutant MCMV-induced necroptosis. Instead, RIPK3 interacts with DAI, another RHIM-containing adaptor, to form a noncanonical necrosome that drives virus-induced necrosis (**Figure 5*b***). *M45* encodes a ribonucleotide reductase (RNR) with no enzymatic activity. Interestingly, many RNRs from other herpesviruses also encode a RHIM (**Figure 3**) (149). This suggests that viral inhibitors that target the RIPKs via the RHIM represent a common viral immune evasion strategy for herpesviruses. The results from vaccinia virus and MCMV highlight the importance of necroptosis in acute viral infections. Yet questions still remain on whether necroptosis can influence the quality and magnitude of adaptive immune responses, the generation of immunological memory, and viral latency.

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BACTERIAL AND PARASITIC INFECTIONS

TNF is a major driver of bacterial sepsis, a life-threatening condition marked by systemic cytokine storm and multiorgan failure. In agreement with the idea that RIPK-dependent necroptosis promotes damaging inflammation, *Ripk3*$^{-/-}$ mice are resistant to TNF-induced systemic inflammatory syndrome (SIRS) (150, 151). In contrast to RIPK3, the role of RIPK1 in TNF-induced SIRS is more controversial. Although several reports show that mice expressing kinase-inactive RIPK1 and wild-type mice treated with RIPK1 kinase inhibitors are protected from TNF-induced SIRS (71, 151, 152), another study found that Nec-1 exacerbates the disease (150). Furthermore, the response of *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice against cecal ligation and puncture–induced sepsis is also variable (151, 153). Because *Ripk3*$^{-/-}$ mice and *Ripk3*$^{-/-}$ macrophages exhibit a normal response to LPS (120, 121), RIPK1 and RIPK3 likely play minor roles in acute bacterial sepsis.

Although the role of the RIPKs in LPS-induced responses is ambiguous, they are nonetheless crucial in controlling certain bacterial pathogens. *Yersinia pestis*, the etiological agent of the black death pandemic, causes rapid RIPK1- and caspase-8-dependent macrophage apoptosis. As in TNF- and SM-induced apoptosis, *Y. pestis*–induced macrophage apoptosis requires intact RIPK1 kinase activity. In addition, RIPK1 is required for inflammatory cytokine production in response to *Y. pestis* infection (154, 155). However, RIPK3 appears to play a minimal role in *Y. pestis* infection. *Salmonella enterica*, a flagellated, gram-negative bacterium, is also a potent inducer of macrophage cell death. Although it is widely accepted that macrophage cell death induced by *Salmonella* is caused by inflammasome activation and caspase-1-mediated pyroptosis (156), a recent report argues that RIPK3-dependent necroptosis is also involved (157). These discrepant conclusions could be reconciled by the fact that RIPK3 can also modulate inflammasome and caspase-1 activation (137, 139).

Host control of *Mycobacterium tuberculosis* (Mtb) critically requires TNF. One of the major protective functions of TNF is to promote granuloma formation, which is thought to be crucial in containment of the bacteria. Using zebrafish as a model, Roca & Ramakrishnan (158) show that RIPK1 and RIPK3 are both required to trigger TNF-induced reactive oxygen species (ROS) production and necroptosis in response to tuberculosis infection. Although necroptosis of infected macrophages initially inhibits bacterial growth, it later enhances growth as bacteria are released into the growth-permissive extracellular environment. Hence, unlike the situation with vaccinia virus and MCMV, one can view Mtb as a pathogen that hijacks the host necroptosis machinery to promote its own growth and dissemination (**Figure 5*c***). Consistent with this thesis, necrosis is often associated with severe Mtb infection (159).

Mechanistically, TNF-induced necroptosis in Mtb-infected macrophages requires mitochondrial cyclophilin D (CypD) and acid sphingomyelinase–induced ceramide production. Ceramide has long been implicated in death receptor–induced apoptosis. However, its role in mammalian cell necroptosis has yet to be thoroughly tested. CypD is an inner mitochondrial protein and an important component of the mitochondrial permeability transition pore. It is required for certain forms of necrosis, such as that induced by calcium and ROS (160). CypD and RIPK3 act in synergy to mediate acute kidney injury in an ischemia-reperfusion model (161). However, CypD deficiency did not rescue excessive necroptosis of *Casp8*$^{-/-}$ T cells (48). Moreover, widespread elimination of mitochondria through induced mitophagy did not alter the cellular response to TNF-induced necroptosis (43). Hence, rather than being a core component of the necroptosis machinery, the CypD pathway appears to be uniquely involved in Mtb-induced necroptosis. It will be interesting to determine whether similar mechanisms involving RIPK1, RIPK3, CypD, and ceramide are involved in immune defense against Mtb infections in mammals.

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Parasitic diseases such as malaria and leishmaniasis target red blood cells, leading to anemia, hemolysis, and bleeding in some cases. These symptoms are caused by red blood cell lysis, which releases cell-free hemoglobin into the circulation. Oxidation of hemoglobin releases heme to trigger the Fenton reaction and generation of highly reactive oxygen radicals. The oxidative stress drives lipid and protein peroxidation, DNA damage, and other insults to the cell (162). Free heme greatly sensitizes hepatocytes to TNF-induced apoptosis in response to infection with *Plasmodium*, the etiological agent for malaria (163). In addition to hepatocytes, macrophages are also highly susceptible to heme-induced cytotoxicity. Through a poorly defined mechanism, heme directly activates TLR4, leading to autocrine TNF and ROS production, which synergize with each other to induce RIPK1- and RIPK3-dependent necroptosis (164). By inducing macrophage necroptosis, RIPK1 and RIPK3 may restrict the niche within which parasites can replicate. In vivo infections will be required to validate the biological role of necroptosis in parasitic infections.

NECROPTOSIS IN STERILE INFLAMMATION

Besides its role in pathogen infections, necrosis is also a hallmark of acute and chronic sterile inflammation. In agreement with induced expression of RIPK3 in response to acute and chronic exposure to alcohol, *Ripk3*$^{-/-}$ mice were protected from alcoholic liver disease (165). Moreover, *Ripk3*$^{-/-}$ mice were protected from acetaminophen-induced liver injury (166), and elevated phospho-MLKL signals were detected in drug-induced liver diseases (38). Repeated doses of cerulein led to a biphasic cell death reaction in the acinar cells that was partially dependent on TNF, RIPK3, and MLKL (167). As such, *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice are partially protected from cerulein-induced acute pancreatitis (153, 168). RIPK3 deficiency also improves the neurological manifestation of Gaucher's disease, a lysosomal storage disease caused by mutations in glucocerebrosidase (169).

Necroptosis appears to be an important mechanism of cell injury in ischemia-reperfusion-induced tissue injury. As discussed above, RIPK3-dependent necroptosis is partially responsible for ischemia-reperfusion-induced kidney injury (161). The RIPK1 kinase inhibitor Nec-1 is effective in alleviating hypoxia-ischemia-induced oxidative brain injury and inflammation in neonatal mice (97). Nec-1 also reduced mouse and rat models of ischemia-reperfusion-induced myocardial cell death and infarct formation (170). However, in a model of permanent left anterior descending coronary artery ligation, the resulting inflammation and tissue remodeling were impaired in *Ripk3*$^{-/-}$ mice (171). Because Nec-1 has been shown to exhibit off-target effects (102, 172), these results need to be interpreted with caution. As mice expressing kinase-inactive RIPK1 have recently been generated (71, 72), they will be useful in further dissecting the kinase-dependent necroptotic signaling versus scaffold-dependent non-necroptotic signaling in these disease models.

In addition to drug- and trauma-induced tissue injury and inflammation, RIPK3-dependent necroptosis also contributes to chronic inflammatory diseases such as atherosclerosis. Macrophage necrosis is widely viewed as a key factor in atherosclerotic plaque formation (173). Mice deficient in apolipoprotein E or low-density lipoprotein receptor (LDL-R) that are fed a high-fat diet developed atherosclerosis marked by macrophage necrosis in the atherosclerotic plaques. Strikingly, RIPK3 deletion ameliorates macrophage necrosis in the plaques and atherosclerosis in *ApoE*$^{-/-}$ and *Ldl-r*$^{-/-}$ mice (174). Given that *Ripk3*$^{-/-}$ macrophages are resistant to oxidized LDL–induced necroptosis, these results strongly suggest that RIPK3-dependent macrophage necroptosis is a direct driver of atherosclerotic plaque formation. Finally, investigators have also shown RIPK3-dependent necroptosis to be causative of mouse models of retinal injury (175–178). These examples point to the emerging role of the RIPKs in diverse inflammatory diseases. However, researchers

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need to consider both necroptosis-dependent and -independent effects of the RIPKs when interpreting these results.

EVOLUTIONARY PERSPECTIVES

Studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have contributed greatly to our knowledge of apoptosis signaling mechanisms. The conservation of apoptosis machinery through evolution illustrates its importance in the maintenance of organismal homeostasis. Is the mammalian necroptosis pathway also conserved in *C. elegans* and *Drosophila*? Interestingly, RHIM-like adaptors are found in *Drosophila* (**Figure 3**). In response to gram-negative bacteria, the innate immune receptors peptidoglycan recognition protein (PGRP)-LC and PGRP-LE stimulate antimicrobial peptide expression through immune deficiency (IMD), a RIPK1-like adaptor, and Relish, a *Drosophila* NF-κB (179). The tetrapeptide core sequences of *Drosophila* RHIM-containing adaptors differ from those in the mammalian RHIMs (**Figure 3**). Mutations of the RHIM-like motif in PGRP-LC and PGRP-LE compromise antimicrobial peptide expression in response to peptidoglycan stimulation (180), indicating that these variant RHIMs are functional. The structural similarity between mammalian and *Drosophila* RHIM adaptors argues that they may have evolved from a common primordial pathway. Although the PGRP-IMD-Relish pathway is generally not known to promote cell death, overexpression of IMD has been shown to result in cell death. Interestingly, IMD-induced cell death was only partially rescued by the caspase inhibitor p35 (181), suggesting the possibility that nonapoptotic cell death may be involved.

In addition to the IMD pathway, transgenic overexpression of the *Drosophila* TNF ortholog *Eiger* in the developing eye primordium leads to JNK-dependent necrosis-like cell death (182). Interestingly, suppression of genes involved in glycolysis and mitochondrial respiration inhibits *Eiger*-induced cell death (182). The apoptosis protease activating factor 1 (Apaf1) interacts with caspase-9 and cytochrome *c* to form the apoptosome, a macromolecular structure essential for mitochondria-mediated apoptosis. Surprisingly, an Apaf1 hypomorph mutant also exhibits progressive wing cell necrosis, which triggers a systemic inflammatory response, wasting, and expression of antimicrobial peptides (183). In both Eiger- and Apaf1-mediated necrosis, the cell death phenotype is associated with changes in energy metabolism. This is in contrast to mammalian necroptosis, which does not require JNK or mitochondria (43, 102). Other pathways of nonapoptotic cell death have recently been described in *Drosophila* nurse cells (184) and developing neuroblasts (185). It will be interesting to determine if similar principles that govern mammalian necroptosis are conserved in these situations.

CLOSING THOUGHTS

In considering the molecular machinery that controls necroptosis and its roles in different diseases, perhaps it will be helpful to step back and ponder why evolution has preserved this unique cell death module. Phylogenetic analysis indicates that modern-day RIPKs evolved through a series of gene duplication events. The relatively short branch between RIPK1 and the RIPK progenitor suggests that RIPK1 is probably the most ancient RIPK (**Figure 6**). This evolutionary model is appealing, as RIPK1 is the only RIPK that is crucial for embryonic survival and beyond. Although the *Drosophila* IMD was once thought to be a RIPK1 ortholog (181), it shares homology only in the DD and lacks the essential kinase domain. The absence of RIP-like kinases in lower organisms such as *Drosophila* or *C. elegans* argues that these kinases are relatively novel products of evolution. The earliest example of RIPK1-like kinases is found in bony fish. How are we supposed to make sense of this? One possible explanation is that necroptosis is the product of coevolution with certain

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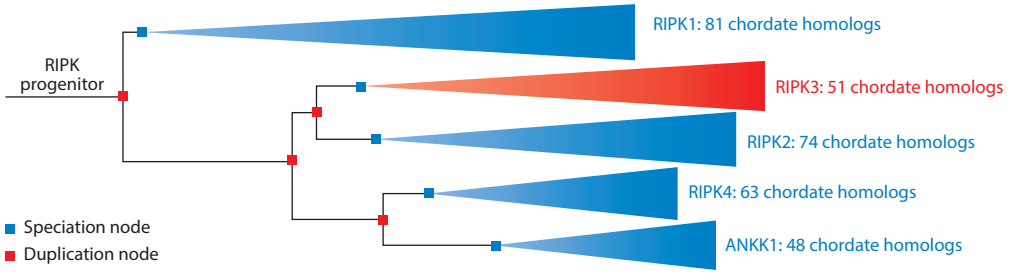

Figure 6

The RIPK gene family evolved through a series of gene duplication events. This reconstructed phylogeny was generated by the Ensembl genome browser (186). Internal nodes correspond to key speciation (*purple*) and gene duplication (*red*) events. Branch lengths correspond to rates of evolutionary change. ANKK1 does not function as a RIPK but is closely related to RIPK4 and other RIPKs. (Abbreviations: ANKK1, ankyrin repeat and protein kinase domain–containing protein 1; RIPK, receptor interacting protein kinase.)

viruses that target vertebrates. The strongest support for this argument comes from the examples of vaccinia virus and MCMV. According to this model, sterile injury–induced necroptosis is the price we pay in this evolutionary struggle with viruses. This is appealing because necroptosis tends to associate with detrimental pathologies in sterile inflammation. Studies of more viruses will be crucial to validating this hypothesis.

SUMMARY POINTS

1. The receptors that stimulate programmed necrosis or necroptosis are also apoptosis inducers.
2. Necroptosis is regulated by RIPK3 and MLKL.
3. Protein phosphorylation, ubiquitination, and FADD/caspase-8-mediated proteolytic processing are the three major post-translational mechanisms that control the induction of necroptosis.
4. RIPK3 promotes inflammation through necroptosis, NF-κB activation, and caspase-1- and caspase-8-mediated pro-IL-1β maturation.
5. RIPK-dependent necroptosis contributes to pathogen-induced and sterile inflammation.
6. Necroptosis can promote or suppress antiviral immune responses in a pathogen-specific manner.
7. The existence of viral inhibitors of necroptosis argues for an important role for necroptosis in host-pathogen interactions.

DISCLOSURE STATEMENT

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