Control of Life-or-Death Decisions by RIP1 Kinase

Dana E. Christofferson, Ying Li, and Junying Yuan

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; email: jyuan@hms.harvard.edu

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Abstract

RIP1 kinase, a multifunctional protein that contains an N-terminal Ser/Thr kinase and a C-terminal death domain, has emerged as a key regulatory molecule involved in regulating both cell death and cell survival. When the proinflammatory cytokine TNFα stimulates its receptor, TNFR1, RIP1 regulates whether the cell lives by activating NF-κB or dies by apoptosis or necroptosis, two distinct pathways of programmed cell death that may be activated to eliminate unwanted cells. The kinase domain of RIP1 is involved in regulating necroptosis, and the death domain regulates RIP1 recruitment to the intracellular domain of TNFR1. The intermediate domain of RIP1 activates NF-κB and also interacts with RIP3 kinase, a downstream mediator of RIP1 in the execution of necroptosis. This review focuses on the functional roles of RIP1 in regulating multiple cellular mechanisms, the dynamic regulation of RIP1, and the physiological and pathological roles of RIP1 kinase in human health and disease.

INTRODUCTION

Regulated cell death is a complex and intricate process. It has now been recognized that there are multiple pathways through which a cell can die, each with different implications for the organism. Apoptosis was the first evolutionarily conserved cellular death pathway discovered. Initial upstream caspase activation unleashes a cascade of caspase proteolytic activity, breaking down cellular components by regulated proteolysis and resulting in cell death. Caspase inhibition protects cells against an extensive list of stress and cytotoxic stimuli. Consequently, apoptosis was long thought to be the predominant mechanism of regulated cell death. Necrotic cell death was also observed to occur, independently of caspase activation and with a morphology distinct from that of apoptotic cells, but was thought to be a form of passive cell death in response to overwhelming cellular stress. However, in the late 1990s and early 2000s, several groups described puzzling observations in certain cell lines. In L929 cells and Jurkat cells, caspase inhibition was unable to block cell death induced by tumor necrosis factor α (TNFα) and other death receptor agonists, and death occurred with a necrotic morphology (1, 2). Without mechanistic insights, however, one cannot rule out the possibility that these findings were due to peculiarities of specific cell lines.

Similar to the discovery of a programmed cell death pathway in the nematode *Caenorhabditis elegans* that propelled the establishment of apoptosis as a regulated cell death pathway, the demonstration of the requirement for a specific cellular activity and/or pathway was critical for overcoming the conventional belief that necrosis was a form of passive cell death and for establishing that necrotic cell death may instead be regulated in some instances. The kinase activity of receptor-interacting protein 1 (RIP1) was eventually found to be critical for mediating this caspase-independent necrotic cell death pathway (3–5). This finding played a critical role in the recognition of necroptosis, also termed programmed necrosis, as an alternative cell death pathway and stimulated active research in this area.

RIP1 was first identified as a Fas (CD95)-binding protein, another member of the death domain (DD) receptor family with TNFα receptor 1 (TNFR1), and subsequently RIP1 was also shown to be recruited into the TNFR1 signaling complex (6, 7). A 74 kDa protein, RIP1 has a C-terminal DD, which mediates the interaction of RIP1 with Fas and other DD-containing proteins; an N-terminal Ser/Thr kinase domain; and an intermediate domain that is involved in mediating nuclear factor-κB (NF-κB) activation. The intermediate domain also contains a RIP homotypic interaction motif (RHIM) that binds to the RHIM in RIP3 to activate necroptosis. From the past two decades of research on cell death, RIP1 has emerged as a central controller that is downstream of death receptor signaling that determines cell fate.

The application of chemical biology played an important role in the establishment of necroptosis as a regulated necrotic cell death mechanism and the role of RIP1 kinase in mediating necroptosis. Necrostatin-1 (Nec-1), a small molecule identified from a high-throughput screen for inhibitors of necroptosis in a cell-based assay, is a highly specific inhibitor of RIP1 kinase (4, 5). 7-Cl-O-Nec-1, an improved analog of the original Nec-1, binds to an allosteric site between the N-lobe and the C-lobe of the RIP1 kinase domain to keep the kinase in an inactive conformation (5, 8). The availability of a highly specific inhibitor of RIP1 kinase made it possible to conveniently examine the requirement of RIP1 kinase in different cell death and disease paradigms in vitro and in vivo (9) and to determine the role of necroptosis in human diseases. Establishment of the role of RIP1 kinase in mediating necroptosis overturned the dogma that necrotic cell death was passive and therefore unregulated, and opened the way for the development of new therapeutics for human diseases characterized by necrosis.

THE PHYSIOLOGICAL ROLE OF RIP1 IN IMMUNE SYSTEM DEVELOPMENT

RIP1 is important during the development of the immune system. *Rip1*$^{-/-}$ mice appear normal at birth; however, postnatally, *Rip1*$^{-/-}$ mice fail to thrive and die between one and three days of age, with extensive cell death in the lymphoid and adipose lineages (10). RIP1 is expressed predominantly in the lymphoid tissues, including the lymph nodes, thymus, and spleen. In lymphocyte populations, high levels of RIP1 are found in immature B cells in the bone marrow and in peripheral T and B cells (11). The postnatal death of RIP1-deficient cells may be partly due to the impairment of NF-κB activation in the absence of RIP1 (12).

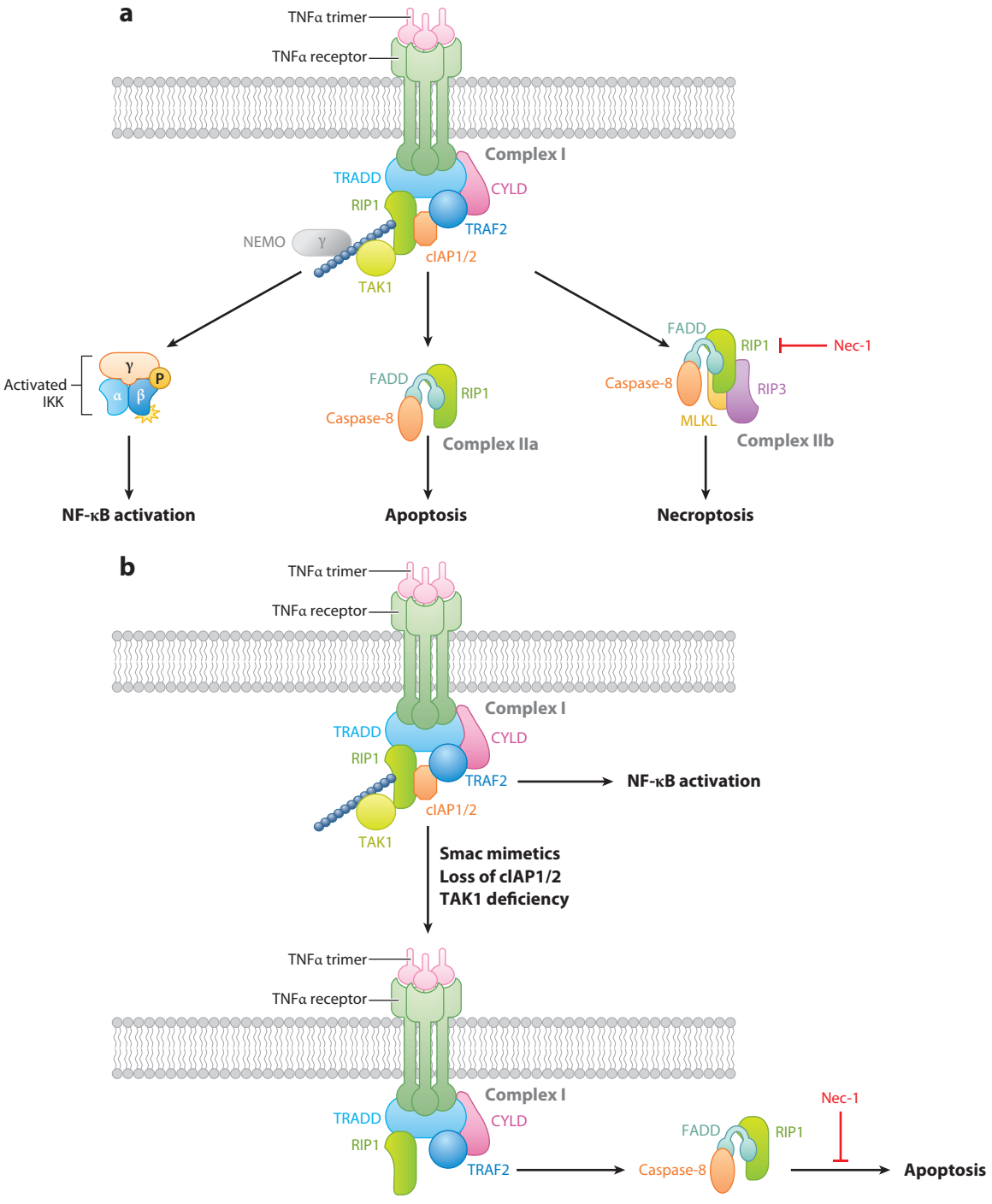
In addition to the role of RIP1 in maintaining the survival of lymphoid cells, RIP1 also plays a role in the development and maturation of T cells and B cells. Because *Rip1*$^{-/-}$ mice are viable only for several days after birth, lethally irradiated lymphocyte-deficient *Rag1*$^{-/-}$ or NSG (NOD *scid* gamma) immunodeficient mice were reconstituted with *Rip1*$^{-/-}$ fetal liver progenitor cells to study the role of RIP1 in lymphocyte development. In each of these models, *Rip1*$^{-/-}$ cells failed to reconstitute the T cell compartment of the recipient mice with reduced CD4^{+}CD8^{+} immature thymocytes, CD4^{+} T cells, and CD8^{+} T cells (12, 13). Reconstitution of immunodeficient NSG mice with *Rip1*$^{-/-}$*Fadd*$^{-/-}$ cells can partially correct the T cell deficiency of *Rip1*$^{-/-}$ cells: Whereas CD4^{+}CD8^{+} immature thymocytes are restored to wild-type (wt) levels, mature CD4^{+} T cells and CD8^{+} T cells are only partially restored to wt levels (13). *Rip1*$^{-/-}$ cells have an increased sensitivity to apoptosis after treatment with TNFα and a reduced ability to activate NF-κB (10). FADD deficiency blocks apoptosis in *Rip1*$^{-/-}$ cells after TNFα or anti-Fas treatment but has no effect on NF-κB activation, suggesting that the deficiencies in the T cell compartment in RIP1-deficient mice are due to increased apoptosis and reduced activation of prosurvival NF-κB signaling (13).

RIP1 is also required for B cell development. Initial development of *Rip1*$^{-/-}$ B cells is normal, but over time, there is a decline in the number of *Rip1*$^{-/-}$ B cells, including the pro-B (CD43^{+}B220^{lo}), pro/pre-B (B220^{lo}IgM^{-}), and immature (IgM^{+}IgD^{lo}) B cell populations. This finding suggests that RIP1 is required for maintenance of the peripheral B cell pool. Similar to the deficits in T cell development, FADD deficiency also rescues B cell development (13). Taken together, these findings show that RIP1 serves an important role in mediating the development and maintenance of the immune system. However, the kinase activity of RIP1 does not appear to be required in regulating normal functions of the immune system.

THE ROLE OF RIP1 KINASE IN MEDIATING TNFR1 SIGNALING

TNFR1, a death receptor containing an intracellular DD, mediates diverse signaling events activated by TNFα, including NF-κB activation and cell survival, apoptosis, and necroptosis. RIP1 is rapidly recruited to TNFR1 upon stimulation by TNFα (7) and plays a critical role in modulating the cellular decision between NF-κB activation, apoptosis, and necroptosis after TNFα stimulation (**Figure 1*a***). RIP1 is present in each of the three key complexes that mediate signaling downstream of TNFR1.

Trimerization of TNFR1 by TNFα brings together the TNFR1 cytoplasmic DDs to recruit TRADD, which in turn recruits RIP1 and TRAF2/5 (7, 14–17). TRAF2 binds to the cellular inhibitor of apoptosis proteins cIAP1 and cIAP2 and is responsible for their recruitment to TNFR1 (18–21). This complex of proteins, including TRADD, RIP1, TRAF2, and cIAP1/2, that is associated with the intracellular domain of TNFR1 at the cytoplasmic membrane is known as complex I (22). Some of the possible functional outputs of complex I signaling are NF-κB activation, cell survival, and cell proliferation. The first indication of the role of RIP1 in mediating NF-κB activation



came from a study that screened Jurkat cells after EMS (ethyl methanesulfonate) mutagenesis for mutants with deficits in NF-κB activation; this screen led to the identification of a RIP1-deficient Jurkat cell line that was defective in NF-κB activation. Reconstitution of wt or kinase-dead RIP1 restored the ability of RIP1-deficient Jurkat cells to respond to TNFα-induced NF-κB activation, suggesting that RIP1 regulates NF-κB activation in a kinase-independent manner (23).

In the absence of survival signaling, membrane-associated complex I transits to cytosolic complex IIa, which contains FADD, RIP1, and caspase-8. Caspase-8 is processed from its procaspase form to activate the caspase cascade and apoptosis (22, 24) (**Figure 1*a***). RIP1 is not essential for apoptosis to occur: RIP1-deficient Jurkat cells are just as sensitive to apoptosis as are wt Jurkat cells in response to TNFα stimulation (23). However, if apoptosis is blocked, for example, by caspase inhibition, cells may utilize an alternative complex II, complex IIb, that involves the interaction of RIP1 and RIP3 (25–27). The formation of complex IIb requires the kinase activity of RIP1; Nec-1 inhibits complex IIb assembly.

In the course of TNFα signaling, RIP1 is subject to a large array of regulatory modifications that include phosphorylation, acetylation, and multiple forms of ubiquitination, all with different implications for the activation of various downstream signaling pathways. A major challenge now is to understand how the interplay of these dynamic regulatory modifications on RIP1 regulates a cell's life-or-death decision.

UBIQUITINATION OF RIP1 PROMOTES CELL SURVIVAL

Within 5 min of TNFα stimulation, RIP1 is recruited to complex I and is polyubiquitinated by multiple forms of ubiquitin linkages, including K63, K48, K11, and linear ubiquitination (28, 29). Although the intracellular region of TNFR1 contains a DD, which appears sufficient to recruit RIP1 to TNFR1, association of the adaptor protein TRADD with TNFR1 plays an essential role in mediating RIP1 ubiquitination. *Tradd*$^{-/-}$ murine embryonic fibroblasts (MEFs) are deficient in RIP1 ubiquitination and in NF-κB activation (16, 17). Although not an E3 ubiquitin ligase, TRADD contains a TRAF2-binding domain that is critical for the recruitment of TRAF2 and its interacting E3 ubiquitin ligases cIAP1, cIAP2, and TRAF5, which mediate K11, K48, K63, and linear ubiquitination of RIP1.

TRAF2 and TRAF5 are RING domain–containing E3 ubiquitin ligases that have overlapping functions in NF-κB activation. In TRAF2 and TRAF5 double-knockout (DKO) cells, TNFα-induced NF-κB activation is attenuated more than in the wt control (20, 30, 31). TRAF2/5 DKO cells have markedly reduced RIP1 ubiquitination; normal levels of RIP1 ubiquitination can be restored by reexpression of wt TRAF2, but not of an E3 ligase–inactive, TRAF2 ΔRING construct, suggesting that E3 activity of TRAF2 is required for RIP1 ubiquitination (17, 20, 21,

Figure 1

Regulation and downstream pathways of RIP1 kinase in response to TNFα stimulation of TNFR1. (*a*) Activation of TNFR1 by TNFα leads to the trimerization of TNFR1 and to the formation of a cytoplasmic recruitment platform by its death domain, which recruits TRADD and RIP1. TRADD in turn recruits TRAF2 and cIAP1/2, E3 ligases that mediate the ubiquitination of RIP1. Ubiquitinated RIP1 then recruits the IKK complex and the TAK1-TAB1-TAB2 complex, which mediate the activation of NF-κB. In the absence of NF-κB activation, the cells may activate apoptosis by complex IIa, which mediates the activation of caspase-8 and apoptosis. Alternatively, in the absence of apoptosis, the cells may form complex IIb, which includes RIP1, RIP3, MLKL, caspase-8, and FADD, to mediate necroptosis by inducing mitochondrial damage through the production of reactive oxygen species. CYLD is involved in deubiquitinating components of complex I to promote downstream signaling. (*b*) In the absence of cIAP1/2 or TAK1, RIP1 is not ubiquitinated in complex I and, after TNFα stimulation, interacts with FADD and caspase-8 to mediate caspase activation and apoptosis.

32–34). However, it is not clear whether TRAF2 directly ubiquitinates RIP1. In one in vitro system, TRAF2 added K63-linked ubiquitin chains to RIP1, with sphingosine-1-phosphate as a cofactor, by using either UbcH5a or Ubc13/Uev1a as an E2 (35).

An important function of TRAF2 in complex I is to recruit cIAP1 and cIAP2, members of the IAP family of viral and cellular proteins that are characterized by baculovirus IAP repeat domains and that are direct mediators of RIP1 ubiquitination (18). cIAP1 and cIAP2 contain IAP repeats that facilitate their binding to caspases and other proteins and RING finger domains that function as E3 ubiquitin ligases. Cells deficient in both cIAP1 and cIAP2 by either knockout or Smac mimetic or IAP antagonist treatment are defective in RIP1 ubiquitination and NF-κB activation after TNFα stimulation (36–38). cIAP1 and cIAP2 display some redundancy in terms of NF-κB signaling: cIAP1 knockout mice, cIAP2 knockout mice, and cells with selective deficiency in one gene or the other do not have deficits in NF-κB signaling (36–40). The E2 ligase UbcH5a works with cIAP1 in vitro to promote the formation of ubiquitin chains with K63, K48, K11, and linear linkages, whereas Ubc13 does not have any significant activity with cIAP1 in vitro (38, 41, 42). Mass spectrometry analysis of RIP1 purified from cellular TNFα receptor complexes confirmed that RIP1 contains linear, K11, K48, and K63 ubiquitin linkages after TNFα stimulation in the cell (43).

In addition to TRAF2 and cIAP1/2, the linear ubiquitin chain assembly complex (LUBAC) also plays an important role in activation of NF-κB signaling (33, 44). Recruitment of LUBAC components to the TNFR1 complex requires TRAF2 and an active cIAP1 ubiquitin ligase, but RIP1 does not appear to be essential, suggesting that RIP1 ubiquitination does not recruit LUBAC to TNFR1 (33). Although knockdown of the LUBAC components HOIL-1 and HOIP reduces RIP1 ubiquitination after TNFα stimulation, it is unclear whether such reduction is due to direct ubiquitination of RIP1 or due to stabilization of the entire complex, as RIP1 does not appear to be a good substrate for LUBAC in vitro (33, 43). The linear ubiquitin chains reported on RIP1 (43) may be due to the activity of cIAP1, as cIAP1 adds linear chains to RIP1 in vitro (42).

The polyubiquitin chain(s) on RIP1 serves as a docking site for transforming growth factor (TGF)-β-activated kinase 1 (TAK1) and TAK1-binding proteins 2 and 3 (TAB2/3). TAB2 and TAB3 contain homologous novel zinc finger (ZnF) domains that bind preferentially to K63 polyubiquitin chains (45). TAK1 activates IKKβ to phosphorylate IκBα, promoting IκBα degradation and allowing translocation of NF-κB to the nucleus to activate transcription of target genes. NEMO (also known as IKKγ) also interacts with ubiquitinated RIP1 and has the highest affinity for linear polyubiquitin chains, suggesting that the linear ubiquitin linkages on RIP1 are important for recruiting NEMO and for NF-κB activation (41, 46–48). NEMO in turn forms a complex with IKKα and IKKβ, leading to the activation of IKKβ and to the subsequent activation of NF-κB.

The type(s) of polyubiquitin chain linkages required for activation of NF-κB is still under investigation. RIP1 is polyubiquitinated with a K63-linked polyubiquitin chain on K377. This polyubiquitination site is required for proper NF-κB activation and may mediate the recruitment of TAK1 and the IKK complex (49, 50). However, a later study questioned the functional relevance of K63-linked polyubiquitin chains in TNFα signaling on RIP1, showing that replacement of endogenous ubiquitin with a K63R mutant, which cannot form K63 linkages, completely restored RIP1 ubiquitination, NEMO binding, and NF-κB activation after TNFα stimulation (51). This finding, however, was specific to TNFα signaling, as K63 polyubiquitin chains were necessary for IL-1β-induced NF-κB activation (51).

Additionally, the E3 ubiquitin ligase(s) targeting K377 of RIP1 has not yet been definitively identified. Given the number of E3 ligases targeting RIP1 and the diversity of reported ubiquitin chain linkage types, other lysines on RIP1 are probably also ubiquitinated. In addition, there may be RIP1-independent mechanisms that activate the TAK1-TAB2-TAB3 complex, such as free K63 polyubiquitin chains. When not conjugated to any target protein, free K63 polyubiquitin

chains can directly activate TAK1 by binding to the ubiquitin receptor TAB2 in vitro (52). This finding may explain why RIP1 is not always essential in certain cell types for mediating NF-κB activation (53). Components of complex I besides RIP1 may also be able to recruit the IKK complex (54). There may be other mechanisms to compensate for RIP1 deficiency in inducing NF-κB activation after TNFα stimulation.

DEUBIQUITINATION REGULATES RIP1 AND COMPLEX II FORMATION

Whereas the ubiquitination of RIP1 in complex I is important for mediating NF-κB activation, deubiquitination of RIP1, and potentially other components of complex I, is required for the formation of complex IIa with caspase-8 and FADD to induce apoptosis or for the formation of complex IIb with RIP1 and RIP3 to induce necroptosis (25–27, 55–57) (**Figure 1*a***). Multiple deubiquitinating enzymes (DUBs), including CYLD and A20, modulate deubiquitination of RIP1.

RIP1 is rapidly ubiquitinated with K63 polyubiquitin chains upon TNFα stimulation. Over time, RIP1 K63 polyubiquitin chains are converted to K48 polyubiquitin chains through the action of A20/TNFAIP3 (58, 59). The conversion of K63 polyubiquitin to K48 polyubiquitin on RIP1 is proposed to be involved in downregulating RIP1 signaling to activate NF-κB. The N terminus of A20 contains an ovarian tumor (OTU) domain found in DUBs, and its C terminus contains seven ZnFs characteristic of E3 ubiquitin ligases (58). The OTU domain of A20 deubiquitinates RIP1, and the ZnF4 region adds K48 polyubiquitin chains to RIP1, promoting its proteasomal degradation (58). The C-terminal ZnF, ZnF7, binds ubiquitin chains to suppress NF-κB activation as well (60, 61). A20 plays an important role in the regulation of complex I, as RIP1 ubiquitination and recruitment to TNFR1 after TNFα stimulation are increased in *A20*$^{-/-}$ MEFs (61). However, RIP1 ubiquitination is still downregulated in A20-deficient cells, suggesting that other deubiquitinases also act on RIP1 (61).

CYLD and USP2a are deubiquitination enzymes that negatively regulate NF-κB signaling by specifically cleaving K63 polyubiquitin chains from target proteins. CYLD promotes deubiquitination of the ubiquitin-dependent NF-κB positive regulators TAK1, TRAF2, NEMO/IKKγ, and RIP1 (62–66). USP2a removes polyubiquitin chains from RIP1 and TRAF2 (67). CYLD preferentially depolymerizes unanchored polyubiquitin, but not conjugated polyubiquitin chains (52). This finding may indicate that adaptor proteins are involved in targeting CYLD to specific substrates to mediate their deubiquitination.

CYLD appears to be a key switch in regulating the activation of necroptosis over NF-κB activation and cell survival (**Figure 1*a***). Active caspase-8 is proposed to cleave CYLD to maintain cell survival after TNFα stimulation. Mutation of the caspase-8 cleavage site on CYLD promotes necroptosis after TNFα treatment (68). Furthermore, knockdown of CYLD can protect against apoptosis induced by TNFα/Smac mimetics as well as protect against necroptosis (69, 70). Polyubiquitinated RIP1 accumulates in *Cyld*$^{-/-}$ cells, suggesting that CYLD-mediated deubiquitination of RIP1 is important. CYLD also suppresses LUBAC-induced NF-κB activation. This finding suggests that CYLD may also deubiquitinate linear polyubiquitin chains, although this has not been explicitly tested (61).

Additional DUBs regulate ubiquitination of RIP1 in complex I. Cezanne, a member of the A20 family of deubiquitinating cysteine proteases, is recruited to TNFR complexes in response to TNFα, downregulates NF-κB, and may target RIP1 for deubiquitination (71). USP21 is a DUB that inhibits TNFα-induced NF-κB activation; loss of USP21 increases RIP1 ubiquitination and NF-κB activation after TNFα stimulation (72).

There are several apparently redundant mechanisms available for downregulation of RIP1 ubiquitination and NF-κB activation. Further study is needed to elucidate their specific roles and their cell type or stimulus specificity. The redundancies in downregulation of NF-κB activation do, however, indicate the importance of these pathways. NF-κB activation is generally proinflammatory, and prolonged activation of this pathway is associated with inflammatory disease and cancer.

REGULATION OF COMPLEX II FORMATION AND RIP1 KINASE ACTIVITY

In the absence of survival signaling, complex I transitions to cytosolic complex IIa, which contains FADD, caspase-8, and RIP1, to induce apoptosis (22, 24). If apoptosis is blocked, cells may utilize an alternative complex II—complex IIb, which requires RIP1 and RIP3—to induce necroptosis (25–27) (**Figure 1*a***).

The ubiquitination status of RIP1 regulates the formation of complex II. Loss of the complex I E3 ubiquitin ligases cIAP1 and cIAP2 in death receptor–stimulated cells promotes the formation of complex II, which contains RIP1, caspase-8, and FADD (36, 69, 73). Even in the absence of death receptor stimulation, loss of cIAP1 and cIAP2 with Smac mimetics or etoposide treatment was reported to spontaneously induce association of RIP1, FADD, and caspase-8 (74, 75). This death receptor–independent version of complex II has been termed the ripoptosome. Cells that express RIP1 K377R show a deficiency in polyubiquitination of RIP1, an increased sensitivity to TNFα-induced apoptosis, and increased association with caspase-8 after TNFα stimulation and doxorubicin stimulation in the formation of the ripoptosome (55, 75). This finding suggests that the polyubiquitination of RIP1 K377, in addition to its role in activating NF-κB, also inhibits the induction of cell death by preventing interaction with caspase-8. Consistently, the deubiquitinase CYLD is required for necroptosis, and its loss prevents the deubiquitination and transition of RIP1 to complex II (69, 70). These data suggest a model in which RIP1 ubiquitination by cIAP1/2 inhibits RIP1 transition to complex II and inhibits RIP1 kinase activity after TNFα stimulation.

A ROLE FOR RIP1 KINASE IN APOPTOSIS

Canonical apoptosis induced in cells by TNFα and the translation inhibitor cycloheximide does not require RIP1 kinase activity or even RIP1, as the loss of RIP1 does not block apoptosis (3, 69). However, RIP1 is required for apoptosis when cIAP1 and cIAP2 are inhibited during death receptor stimulation, as knockdown of RIP1 can completely block apoptosis under these stimuli (36, 53, 69, 73, 76, 77). In contrast to other forms of induced apoptosis, apoptosis induced by TNFα and Smac mimetics is blocked by Nec-1 inhibition of RIP1 kinase, suggesting that RIP1 kinase activity is activated under these conditions to induce apoptotic cell death (69) (**Figure 1*b***).

Ubiquitination of RIP1 by cIAP1/2 is important for the activation of NF-κB and for the recruitment of TAK1. *Tak1*$^{-/-}$ bone marrow–derived monocytes undergo constitutive cell death with caspase activation that can be blocked by TNFR1 deletion, suggesting that TAK1 deficiency sensitizes cells to TNFα-induced cell death. Unexpectedly, the treatment of Nec-1 alone is sufficient to block the death of *Tak1*$^{-/-}$ monocytes (78). Inhibition of caspases by zVAD.fmk in TAK1-deficient monocytes leads to increased necrotic cell death that can also be inhibited by Nec-1. Similarly, *Tak1*$^{-/-}$ MEFs are highly sensitive to TNFα-mediated cell death characterized by caspase activation (79). Although inhibition of caspase activity by zVAD.fmk blocked the activation of caspase-3, cell death of *Tak1*$^{-/-}$ MEFs induced by TNFα was not blocked. However, the addition of Nec-1 resulted in the block of both cell death and caspase-3 activation, suggesting that in the absence of TAK1, RIP1 kinase can mediate caspase activation (78, 79). Importantly, in *Tak1*$^{-/-}$ MEFs, interaction between RIP1 and FADD occurs rapidly in

response to TNFα stimulation (79), suggesting that in the absence of TAK1, RIP1 kinase may be involved in mediating the activation of caspase-8 by directly binding to FADD and caspase-8.

FADD AND CASPASE-8 SUPPRESS NECROPTOSIS

Caspase-8 and FADD, found in both complexes IIa and IIb, are critical mediators of apoptosis and suppressors of necroptosis (25, 26, 80). Whereas FADD-mediated caspase-8 activation is critical for apoptosis induced by the death receptor family (81–83), genetic deficiency in FADD or caspase-8 leads to early embryonic lethality with extensive necrotic cell death and vascular cardiac and hematopoietic defects, an unexpected finding for animals with deficiencies in key genes mediating apoptosis. Interestingly, whereas *Fadd*$^{-/-}$ mice die during midgestation stages (81, 82) and *Rip1*$^{-/-}$ mice die at birth (10), FADD deficiency–induced embryonic lethality is rescued in *Fadd*$^{-/-}$*Rip1*$^{-/-}$ DKO mice (13). *Fadd*$^{-/-}$ MEFs are highly sensitive to oxidative stress induced by H_2O_2, which is blocked by RIP1 deficiency as well as by Nec-1. Thus, FADD serves an important function during embryonic development to suppress RIP1 kinase–mediated necroptosis.

The interaction of FADD and RIP1 can also be observed in the immune system. FADD deficiency can rescue the inability of *Rip1*$^{-/-}$ fetal liver cells to reconstitute the peripheral T cell compartment in immunodeficient mice. Similarly, FADD deficiency can also partially rescue the deficits in B cell development in *Rip1*$^{-/-}$ mice. Whereas *Rip1*$^{-/-}$ thymocytes are readily killed by treatments with death receptor ligands such as anti-Fas antibodies and TNFα, *Fadd*$^{-/-}$*Rip1*$^{-/-}$ thymocytes are highly resistant to these death stimuli, suggesting that *Rip1*$^{-/-}$ thymocytes undergo apoptosis mediated by FADD (13).

Similar to that of *Fadd*$^{-/-}$ mice, the embryonic lethality of *Casp8*$^{-/-}$ mice can be blocked in the absence of necroptosis. Whereas *Casp8*$^{-/-}$ mice die during embryonic days 10–11, *Casp8*$^{-/-}$*Rip3*$^{-/-}$ mice are viable and mature into fertile adults with a full immune complement of myeloid and lymphoid cell types (84, 85). These mice are immunocompetent but develop lymphadenopathy by four months of age. The lymphadenopathy is marked by the accumulation of abnormal T cells in the periphery, similar to that of *lpr/lpr* mice with Fas deficiency (84). Thus, caspase-8 and its adaptor FADD negatively regulate necroptosis during embryonic development but contribute to homeostatic control in the adult immune system by mediating apoptosis to prevent autoimmunity. The signal(s) involved in activating necroptosis during the embryonic development of FADD- or caspase-8-deficient embryos is still unknown. Because *Fadd*$^{-/-}$ MEFs are highly sensitive to oxidative stress, elevated oxidative stress during embryonic development may trigger necroptosis under FADD- or caspase-8-deficient conditions. Alternatively, inhibition of caspase activity in certain cell lines activates TNFα production (see below), which may in turn activate necroptosis.

FADD and caspase-8 also play a prosurvival role during T cell activation. After antigen stimulation, T cells are activated and undergo clonal expansion to combat infection (86). Activated T cells deficient in FADD or caspase-8 do not proliferate and accumulate like wt activated T cells due to an increase in necroptosis (81, 87–91). Either Nec-1 treatment or deficiency in RIP1 can restore the normal T cell activation–induced proliferative response in FADD-deficient cells (13, 88–90). Correspondingly, the codeletion of *Rip3* reverses T cell activation–induced necroptosis in caspase-8-deficient cells (84, 85, 91). Thus, activation of *Fadd*$^{-/-}$ and *Casp8*$^{-/-}$ T cells leads to necroptosis mediated by RIP1 kinase and RIP3.

The proteolytic activity of caspase-8 may play a role in the suppression of necroptosis and in the promotion of cell survival. Knockdown of caspase-8 induces necroptotic cell death in a mouse T lymphoma–derived cell line (92). Reexpression of wt procaspase-8 as well as of cleavage-resistant procaspase-8 can correct this deficit and effectively complement the loss of

caspase-8. However, expression of a protease-inactive mutant, C362S, cannot complement the loss of caspase-8, and the cells undergo cell death, suggesting the critical importance of the protease activity of caspase-8 in promoting cell survival (92). This study was conducted in the absence of proapoptotic stimuli, raising the question of the role of basal procaspase-8 proteolytic activity in suppressing necroptosis versus the role of active caspase-8 after apoptotic death receptor stimulation. Activated caspase-8 cleaves both RIP1 and RIP3, which may be important for blocking necroptosis. The resulting C-terminal fragments of RIP1 and RIP3 promote apoptosis and inhibit TNFα-induced NF-κB activation (93–97). Caspase-8 also cleaves the deubiquitinase CYLD, which is required for necroptosis (68). Knockdown of CYLD can block necroptosis, and thus negative regulation of CYLD by caspase-8 may also play a role in the control of necroptosis (69, 70). However, the downregulation of RIP1 and RIP3 and the cleavage of CYLD have not been convincingly demonstrated in living cells, and therefore caspase-8 and FADD may have alternative mechanisms for negatively regulating cellular sensitivity to necroptosis.

PHOSPHORYLATION AND DEACETYLATION REGULATE COMPLEX IIB FORMATION

For RIP1 kinase to be active in complex IIb, it may need to be deubiquitinated, as the loss of the E3 ubiquitin ligases cIAP1 and cIAP2 promotes RIP1 kinase activation, and the loss of the deubiquitinase CYLD inhibits necroptosis (69, 70). Similar to many kinases, autophosphorylation of RIP1 plays an important role in its activation. Computational modeling of RIP1 suggests that its kinase structure may be similar to that of B-RAF, a major oncogenic kinase whose activity is regulated by the conformational change of its activation segment, or T-loop. When in the closed conformation, the T-loop blocks the kinase activity by obstructing the catalytic cleft. This inhibition can be relieved when the closed T-loop conformation is destabilized either (*a*) by phosphorylation or (*b*) by genetic mutations that promote an open conformation and significant increases in kinase activity (98, 99). RIP1 is autophosphorylated on S161; S161 on RIP1 corresponds to T598, the autophosphorylation site on B-RAF, which is important for regulation of the catalytic activity of B-RAF (5, 99). Interestingly, point mutations in RIP1 that are predicted to alter the conformation of the RIP1 kinase T-loop, specifically S161A and S161E/D, block the ability of Nec-1 to inhibit RIP1 kinase and necroptosis, suggesting that Nec-1 is an allosteric inhibitor of RIP1 kinase sensitive to the conformational change of its activation T-loop (5). Indeed, an X-ray crystallography study of RIP1 kinase complexed with *R*-7-Cl-O-Nec-1 showed that Nec-1 binds a hydrophobic pocket between the N- and C-lobes of the kinase domain, stabilizing RIP1 in an inactive conformation through interactions with highly conserved amino acids in the activation loop and the surrounding structural elements (8). The indole ring of *R*-7-Cl-O-Nec-1 contributes one H-bond between its nitrogen atom and the hydroxyl oxygen of S161 on the activation loop, explaining why S161 is critical for the inhibition of RIP1 kinase by Nec-1 (5). Interestingly, the S161E mutation, which promotes an open conformation of the RIP1 kinase T-loop, cannot induce necroptosis without additional stimulation, indicating that autophosphorylation and an open conformation of the activation loop are not sufficient for activation of RIP1 kinase–dependent necroptosis (5).

RIP1 kinase activity is required for the interaction of RIP1 with its homolog, RIP3, and for the formation of complex IIb. RIP3 has a kinase domain similar to that of RIP1, but the C terminus of RIP3 is unique and does not contain a DD (100, 101). RIP3 interacts with RIP1 via RHIM, a peptide motif that is found in the C-terminal domain of RIP3 and in the intermediate domain of RIP1 (102). RIP3 is specifically recruited to complex IIb during necroptosis by binding with RIP1 via a RHIM homotypic interaction (25, 26).

In addition to the RHIM domain of RIP1 for complex IIb assembly, the acetylation status of RIP1 is also important in complex IIb formation. SIRT2, a NAD-dependent cytosolic deacetylase that removes acetyl groups from their substrates, is required for necroptosis (103). Treatment with a SIRT2 deacetylase inhibitor, AGK2, can block RIP1-RIP3 complex formation and cell death after necroptotic stimulation, and *Sirt2*$^{-/-}$ cells are resistant to necroptosis. SIRT2 binds to the RHIM domain of RIP3, but RIP1 is the substrate of SIRT2. SIRT2 promotes the deacetylation of K530 of RIP1, which is directly involved in interacting with RIP3 and is adjacent to the RIP1 RHIM domain. RIP1 is basally acetylated, but the levels of acetylation are reduced in necroptotic cells. Although RIP1 K530A, a constitutively deacetylated mutant, forms a complex with RIP3, such formation is not sufficient for the cells to undergo necroptosis (103). Thus, the functional significance of complex IIb formation may be for RIP3 to deliver SIRT2 to RIP1 to mediate the deacetylation of RIP1.

The kinase activity of RIP1 is required for necroptosis, although a direct substrate of RIP1 has yet to be identified. RIP1 kinase activity is required for the activation of RIP3 autophosphorylation, but there is no direct evidence that RIP1 can directly phosphorylate RIP3 (25, 26). Several phosphorylation sites on RIP3, including S199 and S227, that may be involved in mediating downstream signaling important in necroptosis have been identified (26, 80).

RIP1- AND RIP3-ACTIVATED PATHWAYS FOR THE EXECUTION OF NECROPTOSIS

RIP1 and RIP3 form amyloid-like structures upon their interaction (104). This stable structure may act as a scaffold to activate multiple downstream pathways for necroptosis. The formation of this amyloid structure requires RIP1 kinase activity, as Nec-1 inhibits the formation of complex IIb. Execution of necroptosis has been proposed to involve multiple cellular pathways. MLKL, a substrate of RIP3, is found in complex IIb with RIP1 and RIP3, as is the mitochondrial protein phosphatase PGAM5 (80, 105). The short isoform of PGAM5, PGAM5S, recruits and activates the mitochondrial fission factor Drp1, leading to mitochondrial fragmentation (105). The role of Drp1 and mitochondrial fragmentation has so far been examined in only a few cellular models; additional studies are needed on the role of Drp1 in mediating necroptosis. In addition to PGAM5 and Drp1, a component of the mitochondrial permeability transition pore, cyclophilin D (CypD), has also been implicated in necroptosis; *CypD*$^{-/-}$ mice have a reduced infarct size in cardiac ischemia-reperfusion (I/R) injury. I/R injury is due in part to necroptotic cell death (4), and *CypD*$^{-/-}$ mice undergoing I/R cannot be further protected by treatment with Nec-1 (106).

In addition to activation of pathways of mitochondrial fragmentation, several metabolic enzymes, both cytosolic and mitochondria associated, bind to RIP3 after the induction of necroptosis (57). This finding raises the possibility of a mechanism whereby RIP3 directly modulates cellular metabolic activity to promote necroptosis. RIP3 kinase promotes the activity of the metabolic enzymes glycogen phosphorylase, glutamate-ammonia ligase, and glutamate dehydrogenase 1 (57). The activation of RIP3 and increased metabolic activity are associated with the production of reactive oxygen species, which are involved in necroptosis (25, 57). However, the mechanism by which RIP3 mediates the increased metabolic activity is not clear.

Autophagy is also activated downstream of RIP1 kinase in necroptotic cell death. Necroptosis stimulation increased autophagy in MEF cells downstream of RIP1 kinase activation (4). Tandem affinity purification of FADD indicated that FADD, RIP1, and caspase-8 complex with components of the autophagosome (Atg5, Atg12, and Atg16L), suggesting that FADD and caspase-8 may target RIP1 kinase to directly activate the autophagosome (88).

Growth factor signaling may also be involved in positively regulating necroptotic cell death, in contrast to the case of apoptosis, which is usually inhibited by growth factor signaling. Necroptosis

of L929 cells is sensitized by bFGF- and IGF-1-mediated Akt activation (107). In this cellular model, Akt is phosphorylated on T308 in a RIP1-dependent fashion. Akt activation in L929 cells requires independent signaling inputs from both growth factors and RIP1. Mammalian target of rapamycin complex 1 (mTORC1) mediates the sensitization of necroptosis by Akt (107). The physiological significance of the impact of selected growth factors on necroptosis remains to be understood.

RIP1-MEDIATED TNFα PRODUCTION

In addition to its role in regulating cell survival and cell death pathways downstream of TNFR1, RIP1 can also activate production of the cytokine TNFα. Various studies had observed that treatment with a caspase inhibitor alone induced cell types such as L929 to undergo necroptosis (108, 109), which was inhibited either (*a*) by Nec-1 or (*b*) by blocking TNFα signaling through a TNFα-neutralizing antibody or through knockdown of TNFR1. Such findings suggested that TNFα production mediates necroptosis (70). These reports led to the finding that in some cell types, caspase inhibition with zVAD.fmk induces TNFα transcription via a pathway dependent on RIP1 kinase activity, EDD/UBR5, c-Jun/AP-1, and the transcription factor Sp1. Importantly, this pathway is independent of NF-κB activation (110) (**Figure 2**). RIP1 kinase–dependent phosphorylation of Akt on T308 and the mTORC1 effector pathway may activate TNFα transcription (107). RIP1 kinase–dependent TNFα transcription can also be activated upon loss of cIAP1/2 with Smac mimetic treatment or upon loss of TRAF2 (110). This finding is consistent with reports that cIAP1 and cIAP2 inhibit RIP1 kinase activity (reviewed above) such that loss of cIAP1 and cIAP2 results in the activation of RIP1 kinase and, depending on the cell type and stimuli, may induce the activation of necroptosis, RIP1 kinase–dependent apoptosis, or (in this case) TNFα transcription.

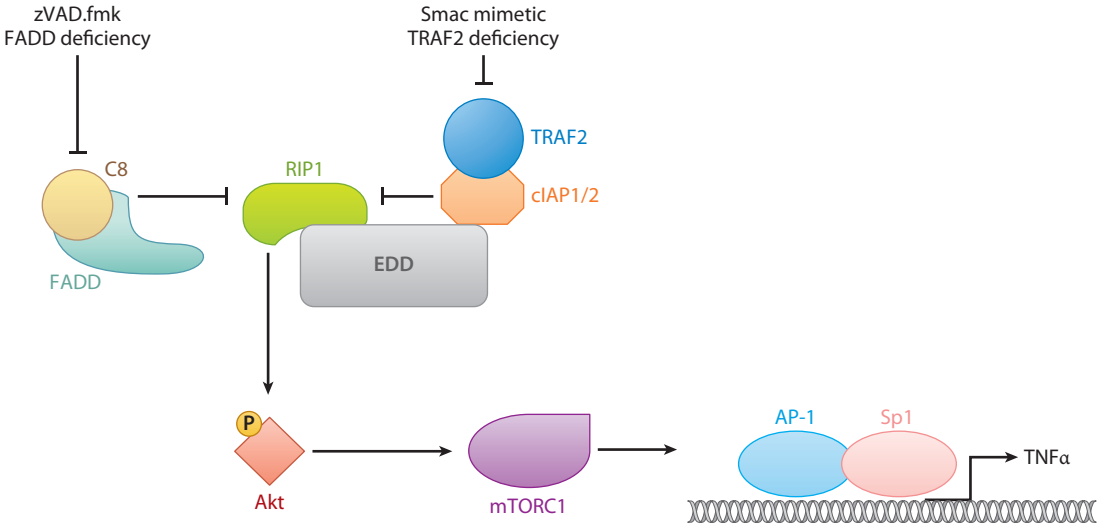


Figure 2

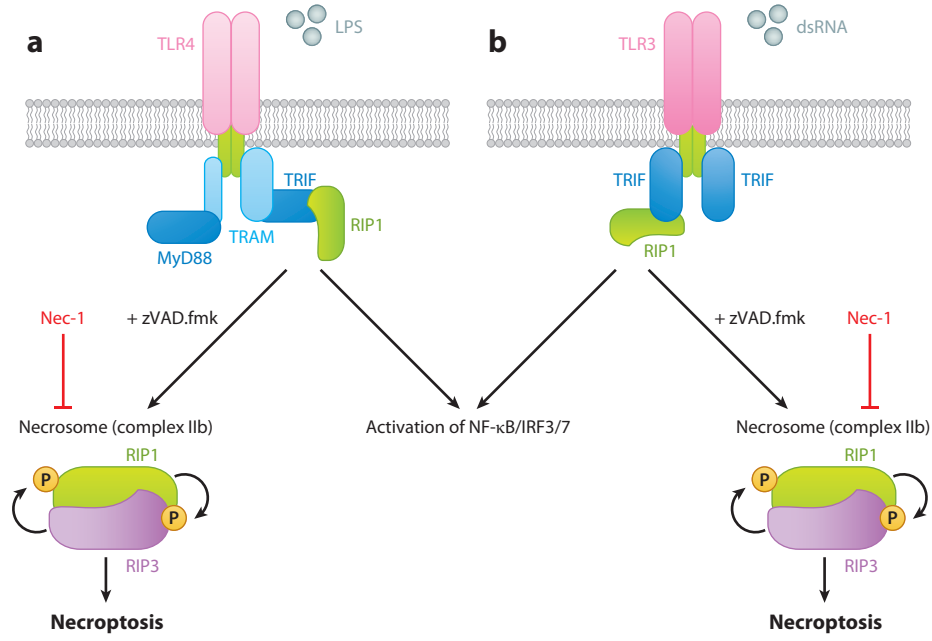
RIP1 kinase activates TNFα transcription. RIP1 kinase can be activated after caspases, including caspase-8, are inhibited by a caspase inhibitor such as zVAD.fmk, loss of cIAP1 and cIAP2 by Smac mimetic treatment, or deficiency in TRAF2 or FADD. RIP1 kinase mediates phosphorylation of Akt on T308, which promotes the activation of TNFα transcription through mTORC1. EDD, which binds to RIP1 and cIAP1, is also required for TNFα transcription. Transcription of TNFα requires the transcription factors c-Jun/AP-1 and Sp1.

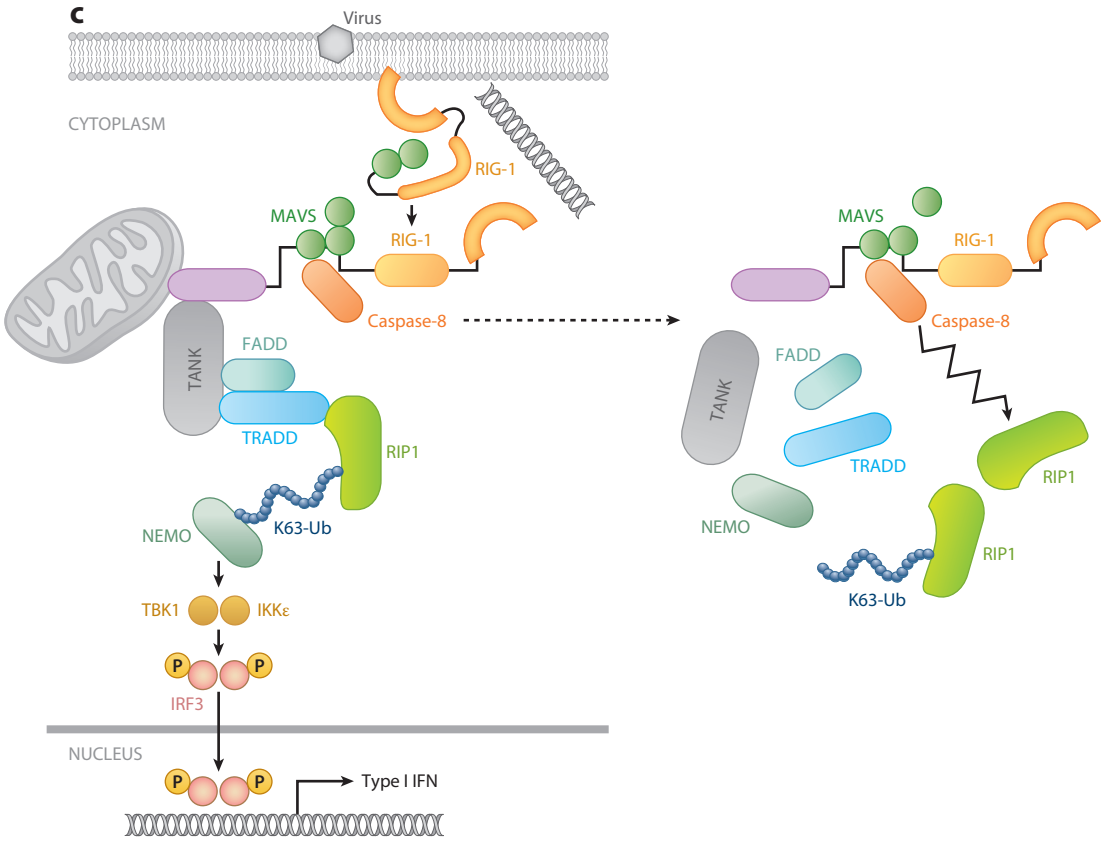
Deficiencies in caspase-8 and FADD, which promote necroptosis in some cases, may also play a role in inducing RIP1 kinase–dependent TNFα production. Some groups reported that specific knockdown of caspase-8 recapitulates the cell death seen with more general caspase inhibition, suggesting the importance of caspase-8 in mediating zVAD.fmk-induced necroptosis (109). Mice with caspase-8-deficient epidermal keratinocytes have severe inflammatory skin disease. This condition is partially ameliorated by crossing the mice with *Tnf*$^{-/-}$ mice, suggesting that aberrant TNFα signaling may play a role in the inflammatory condition caused by caspase-8 deficiency (111). Similarly, mice with FADD-deficient intestinal epithelial cells develop intestinal inflammation and spontaneous colitis (112). The FADD-deficient cells show increased expression of TNFα and undergo necroptosis dependent on CYLD, RIP3, and TNFα. Mice that express a catalytically inactive form of CYLD or that are deficient in either RIP3 or TNFα are protected against intestinal epithelial cell necroptosis, inflammation, and colitis (112). Deficiencies in either FADD or caspase-8 promote TNFα-dependent inflammation and necroptosis, similar to those observed in cellular models of caspase inhibition. In certain cell types and conditions, loss of FADD, caspase-8, and the E3 ligases cIAP1 and -2 and TRAF2 may promote the activation of RIP1 kinase and result in the activation of a downstream pathway of TNFα production, leading to inflammatory conditions, diseases, and necroptotic cell death.

THE ROLE OF RIP1 IN TOLL-LIKE RECEPTOR SIGNALING

In addition to its involvement in mediating NF-κB activation downstream of TNFR1, RIP1 regulates NF-κB activation induced by Toll-like receptor 3 (TLR3), which is activated by the binding of virus-derived double-stranded RNA, and by TLR4, which is activated after binding to lipopolysaccharide (LPS) (**Figure 3*a*,*b***). Stimulation of either receptor induces a potent interferon-β (IFN-β) response, as well as activation of mitogen-activated protein kinases and NF-κB. The intracellular signaling of TLR3 and TLR4 involves two parallel pathways mediated by the adaptor protein MyD88 and the TIR domain–containing adaptor protein TRIF/TICAM-1. TRIF directly associates with TLR3 and indirectly with TLR4 through another adaptor protein, TRAM (113, 114). The TIR domain of TRIF binds to TLRs and is flanked by an N-terminal domain and a C-terminal extension that includes a RHIM domain, similar to the RHIM domains of RIP1 and RIP3 (115). RIP1 binds to TRIF via a RHIM domain homotypic interaction and is ubiquitinated after stimulation with the TLR3 ligand polyinosinic-polycytidylic acid [poly(I:C)]. Stimulation of TLR3 leads to the recruitment and ubiquitination of RIP1, TRAF6, and the kinase TAK1 (116) (**Figure 3**). *Rip1*$^{-/-}$ MEFs are deficient in TLR3-mediated signals activating NF-κB but are not deficient in signals activating JNK or IFN-β, suggesting that RIP1 specifically mediates TRIF-induced NF-κB activation. Thus, RIP1 may serve an important function downstream of selected TLRs by mediating NF-κB activation, similar to its role in mediating NF-κB activation downstream of TNFR1.

Stimulation of macrophages and microglia with LPS or poly(I:C) in the presence of zVAD.fmk can lead to necroptosis, which is inhibited by Nec-1 (70, 117) (**Figure 3*a*,*b***). Because TLR ligands such as LPS, LTA, and PamC3sk4 induce the activation of caspase-8, caspase-3, and caspase-7 in microglia without cell death (118), caspases may play an important role in regulating the activation of microglia. However, the significance of caspase activation in microglia is controversial. Inhibition of caspase activation reduces TLR-mediated microglial activation, suggesting that caspases positively regulate microglial activation (118). In contrast, other researchers showed that inhibition of caspase activation in TLR-stimulated microglia leads to necroptosis that can be inhibited by Nec-1, suggesting that caspases are required for the survival of activated microglia (117). The in vivo physiological significance of caspase-mediated microglia survival remains





to be examined, as it is not clear whether necroptosis of microglia represents a mechanism for negatively regulating a microglia-mediated inflammatory response or whether necroptosis of activated microglia leads to additional inflammatory responses through leakage of the intracellular content of necrotic cells.

Necroptosis is also involved in mediating the innate immune antibacterial response early after infection. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is a pathogenic bacterium whose infection induces the rapid cell death of macrophages, which is an essential mechanism of its virulence. *S.* Typhimurium activates the type I IFN pathway and RIP1 and RIP3 to induce macrophage cell death. Nec-1 treatment or RIP3 deficiency strongly protected macrophages infected with *S.* Typhimurium in vitro, and *Rip3*$^{-/-}$ mice had significantly less bacterial load in vivo (119). Thus, *S.* Typhimurium promotes its own survival by exploiting the necroptotic cell death pathway in macrophages.

THE ROLE OF RIP1 IN RIG-I-MEDIATED ANTIVIRAL RESPONSE

The recognition of viruses by the innate immune system depends mainly on the ability to discriminate viral nucleic acids from host RNA or DNA through pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns. RIG-I, an important PRR required for the detection of specific RNA viruses, contains a central DExD/H-box RNA helicase domain and a C-terminal repressor domain with two caspase recruitment domains (CARDs). Upon stimulation with viral RNA, RIG-I undergoes an intramolecular conformational change that exposes the CARDs to allow for the association of RIG-I with the mitochondrial antiviral signaling (MAVS) adaptor and the formation of a large mitochondria-associated complex that includes RIP1, TRADD, FADD, and caspase-8 (120). This complex in turn recruits the regulatory IKK subunit NEMO to mediate downstream signaling to activate interferon regulatory factors (IRFs) and NF-κB. Similar to polyubiquitination of RIP1 associated with TNFR1 complex I after TNFα stimulation (46, 49), RIP1 polyubiquitination at K377 facilitates the assembly of the RIG-I complex, leading to IRF3 phosphorylation and downstream signaling (**Figure 3*c***). Interestingly, caspase-8 in this complex regulates the duration and magnitude of RIG-I signaling by cleaving RIP1 after D324 (121), resulting in a truncated 38 kDa RIP1 fragment, which is also found in the TNFR1 signaling pathway (93). Although there is no evidence that RIP1 kinase activity is involved in mediating the RIG-I response, this example showcases the antagonistic relationship of RIP1 and caspase-8 in mediating antiviral responses.

THE ROLE OF RIP1 KINASE IN PATHOLOGICAL CELL DEATH

Cell death in many pathologies occurs via multiple mechanisms, including both apoptosis and necrosis. I/R injury is due to a shortage of oxygen and glucose required for cellular metabolism as

Figure 3

RIP1-dependent TLR signaling pathways. (*a*,*b*) Activation of TLR3 and TLR4 receptor complexes leads to the recruitment of TRIF and RIP1, which in turn mediate transcriptional responses involving the NF-κB and interferon regulatory factor 3/7 (IRF3/7) pathways. These pathways result in immune responses and cytokine production in defense against invasion of microbial organisms. Inhibition of caspase activation with the stimulation of TLR3 or TLR4 receptors leads to necroptosis that can be inhibited by Nec-1 or RIP3 deficiency. (*c*) Recognition of viral RNA by RIG-I leads to the formation of a mitochondria-associated signaling complex including MAVS, TANK, RIP1, FADD, and TRADD. K63 polyubiquitination of RIP1 in this complex recruits NEMO and leads to the activation of an IRF3-mediated type I interferon (IFN) response. Cleavage of RIP1 by caspase-8 destabilizes the RIG-I complex and generates a short IRF3-inhibiting RIP1 fragment, which in turn downregulates the inflammatory response.

a result of restricted blood supply and is thus not the ideal setting for energy-dependent apoptosis, providing an opportunity to test the pathological relevance of necroptosis. Ischemic brain injury was the first pathological model tested to determine the role of necroptosis (4). 7-Cl-O-Nec-1, a highly specific RIP1 kinase inhibitor, significantly reduced ischemic brain injury, even when delivered 6 h after the onset of injury. Nec-1 has subsequently been used to demonstrate the involvement of necroptosis in an extensive list of animal models of acute ischemic brain, heart, kidney, and eye injuries, including neonatal I/R-mediated CNS injury (122, 123), cardiac I/R–induced myocardial infarction (106, 124), renal I/R injury (125), and retinal I/R injury (126, 127). In a neonatal hypoxia-ischemia model, Nec-1 prevented early oxidative injury, preserved mitochondrial function, and prevented secondary energy failure. Nec-1 treatment prevented the production of nitric oxide and 3-nitrotyrosine, blocked the expression of inducible nitric oxide synthase, and attenuated glutathione oxidation. In addition, Nec-1 also inhibited upregulation of hypoxia-inducible factor-1α and BNIP3 (BCL2/adenovirus E1B 19 kDa protein–interacting protein 3) expression and decline of mitochondrial complex I activity in astrocytes and in neurons (123). These studies suggest that RIP1 kinase is a critical mediator of ischemic injury. However, in addition to necroptosis, caspase-mediated apoptosis plays an important role in ischemic injury (128). The ability of Nec-1 to completely protect against cellular damage in models of ischemic injury suggests that, consistent with reports of Nec-1 inhibition of caspase activation in in vitro models, RIP1 kinase may activate caspases and apoptosis in addition to necroptotic cell death in ischemic injury.

RIP1 kinase and necroptosis also mediate tissue damage after controlled cortical impact (CCI), a model of traumatic brain injury (TBI). Administration of Nec-1 to mice after CCI decreased cell death and inflammation in the injured cortex and dentate gyrus and improved motor function and performance in the Morris water maze (129). In contrast to ischemic injury, Nec-1 treatment did not reduce caspase-3-positive cells in the dentate gyrus or cortex, suggesting that both apoptosis and necroptosis occur after CCI but that only necroptosis depends on RIP1 kinase activity. The protective effect of Nec-1 does, however, demonstrate the significant role that necroptosis plays in the pathogenesis of cell death and functional outcome after TBI.

The loss of photoreceptors in a retina detachment model demonstrates yet another paradigm of the activation of multiple forms of cell death after acute injury (127). In this model, neither caspase inhibition nor Nec-1 application is sufficient to prevent photoreceptor death. Instead, caspase inhibition leads to the activation of necroptosis, which is inhibited by either Nec-1 or RIP3 deficiency; simultaneous inhibition of apoptosis and necroptosis may be required for protecting photoreceptor cells upon retina detachment.

Taken together, these studies illustrate the varying significance of necroptosis and the multiple roles that RIP1 kinase may play in pathological cell death. Use of the RIP1 kinase inhibitor Nec-1 has been essential for demonstrating the important roles of RIP1 kinase in mediating acute tissue injury. Inhibition of RIP1 kinase may provide a novel therapeutic strategy for reducing neuronal cell death and functional loss in acute neurological injuries.

FUTURE QUESTIONS

RIP1 kinase has emerged as a central regulator that controls multiple cell fates, including apoptosis, necroptosis, and cell survival, by activating NF-κB. However, many questions remain regarding the functions and mechanisms of RIP1 kinase. For example, although Nec-1 has shown remarkable protective effects in multiple animal models involving different tissues and organs, the cell types that are protected by Nec-1 in these models have not been carefully characterized. It is not clear whether necroptosis always represents a backup cell death when apoptosis fails to occur or whether

certain cell types may choose necroptosis as the primary mechanism of cell death. The execution mechanism downstream of RIP1 kinase involves RIP3 kinase and its substrates and interacting proteins, such as MLKL and PGAM5, and may involve the formation of amyloid complexes. However, it is not clear how these protein complexes lead to necrotic cell death that includes disruption of cytoplasmic membrane integrity and swelling of intracellular organelles. In addition, because necroptosis has been established as a form of cell death mediated by death receptors, most of the studies on necroptosis have been limited to mammalian systems. It is still not clear whether the core mechanism of necroptosis is involved in organisms beyond vertebrate animals. Studies addressing these issues have the potential to increase our understanding of the cellular mechanisms that control programmed cell death during normal and pathological conditions. Such studies also provide rationales for developing inhibitors of RIP1 kinase for the treatment of human diseases.

DISCLOSURE STATEMENT

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