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Genetic Regulation of RIPK1 and Necroptosis

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Keywords

RIPK1, RIPK3, MLKL, TNFR1, apoptosis, necroptosis, neurodegeneration, inflammation

Abstract

The receptor-interacting protein kinase 1 (RIPK1) is recognized as a master upstream regulator that controls cell survival and inflammatory signaling as well as multiple cell death pathways, including apoptosis and necroptosis. The activation of RIPK1 kinase is extensively modulated by ubiquitination and phosphorylation, which are mediated by multiple factors that also control the activation of the NF-κB pathway. We discuss current findings regarding the genetic modulation of RIPK1 that controls its activation and interaction with downstream mediators, such as caspase-8 and RIPK3, to promote apoptosis and necroptosis. We also address genetic autoinflammatory human conditions that involve abnormal activation of RIPK1. Leveraging these new genetic and mechanistic insights, we postulate how an improved understanding of RIPK1 biology may support the development of therapeutics that target RIPK1 for the treatment of human inflammatory and neurodegenerative diseases.

INTRODUCTION

RIPK1: receptor-interacting protein kinase 1

RHIM: receptor-interacting protein homotypic interaction motif

TNFR1: tumor necrosis factor receptor 1

TRADD: tumor necrosis factor receptor 1–associated death domain protein

FADD: FAS-associated death domain protein

LUBAC: linear ubiquitination assembly complex

HOIP: HOIL1-interacting protein

HOIL1: heme-oxidized IRP2 ubiquitin ligase 1

SHARPIN: SHANK-associated RH domain interactor

TAK1: transforming growth factor-β-activated kinase 1

IKK: inhibitor of NF-κB kinase

cIAP: cellular inhibitor of apoptosis

cFLIP: cellular FADD-like IL-1β converting enzyme inhibitory protein

RIPK1 and RIPK3 are members of the receptor-interacting protein (RIP) family of protein serine/threonine kinases. RIPK1 has emerged as a master upstream regulator that controls cell survival and inflammatory signaling as well as multiple cell death pathways, including apoptosis and necroptosis. The RIPK1 protein has a molecular weight of about 76 kDa and is composed of a serine/threonine kinase domain in the N terminus (1–330a.a. hRIPK1), a death domain (DD) in the C terminus (583–669a.a. hRIPK1), and an intermediate domain between the kinase domain and DD (331–582a.a. hRIPK1) with a receptor-interacting protein homotypic interaction motif (RHIM) (137). RIPK3 is a 50-kDa protein that bears an amino-terminal kinase domain (21–287a.a. hRIPK3) and a carboxy-terminal region (288–518a.a. hRIPK3) and harbors a RHIM but lacks a DD, which makes it unable to be recruited into complex I in the tumor necrosis factor receptor 1 (TNFR1) signaling pathway (141, 168). RIPK3 is a downstream mediator of RIPK1 in promoting necroptosis.

The N-terminal kinase domain of RIPK1 is important in cell death induction and has become an important drug target for small molecule inhibitors. The prototype small molecular RIPK1 inhibitor is Necrostatin-1s (Nec-1s), an allosteric inhibitor of RIPK1 kinase activity (23). The intermediate domain of RIPK1 is critical for NF-κB activation and RHIM-dependent signaling. The RIPK1 RHIM domain is involved in mediating its interaction with other RHIM-containing proteins, including RIPK3 (142); TIR-domain-containing adaptor-inducing interferon β (TRIF) (60); Z-DNA-binding protein 1 (ZBP1), also known as DAI or DLM1 (125); and the RHIM-containing murine cytomegalovirus (MCMV) protein M45 (155). The C-terminal DD is homologous to the DD of receptors such as TNFR1, Fas, DR4, and DR5. The DD provides the basis for RIPK1 to be recruited to these receptors at the cytoplasmic membrane via DD-mediated homotypic interactions upon stimulation by the corresponding cognate ligands (117). The RIPK1 DD can also bind to other DD-containing proteins, such as tumor necrosis factor receptor 1–associated death domain protein (TRADD) (117) and FAS-associated death domain protein (FADD) (118), to form intracellular complexes that mediate downstream signaling. The RIPK1 DD is also important for its homodimerization, which promotes the autophosphorylation of the N-terminal kinase domain in *trans* to promote its activation (86, 164).

The TNFR1 signaling pathway is the most extensively characterized signal transduction process that regulates RIPK1 functions. The pivotal role for RIPK1 in regulating the outcomes from TNFR1 signaling is modulated by a transient membrane signaling complex associated with the intracellular DD of TNFR1, named complex I (**Figure 1**). Complex I is the key checkpoint that determines whether the cell will survive and activate NF-κB or die by apoptosis or necroptosis. TRADD and RIPK1 are the first two components recruited to complex I upon stimulation of TNFR1. The recruitment of TRADD and RIPK1 to complex I is mediated by the homotypic interaction of their DDs with the intracellular DD of trimerized TNFR1 (88). Upon recruitment into complex I, TRADD organizes the recruitment of the adaptors tumor necrosis factor receptor-associated factor 2 (TRAF2), TRAF5 (52, 53), and the E3 ubiquitin ligases cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2 (129, 135), which in turn perform K63-linked ubiquitination of RIPK1 (6) in complex I. The linear ubiquitination assembly complex (LUBAC) containing HOIL1-interacting protein (HOIP), heme-oxidized IRP2 ubiquitin ligase 1 (HOIL1), and SHANK-associated RH domain interactor (SHARPIN), recruited by binding to K63-linked ubiquitin chains in complex I, performs M1-linked ubiquitination of RIPK1 (40). Ubiquitinated RIPK1 then recruits and activates transforming growth factor-β-activated kinase 1 (TAK1) and inhibitor of NF-κB kinase (IKK) complexes via the K63-linked and M1-linked ubiquitin chains, respectively (55, 151). A20, encoded by gene *TNFAIP3*, is a ubiquitin-editing enzyme that plays

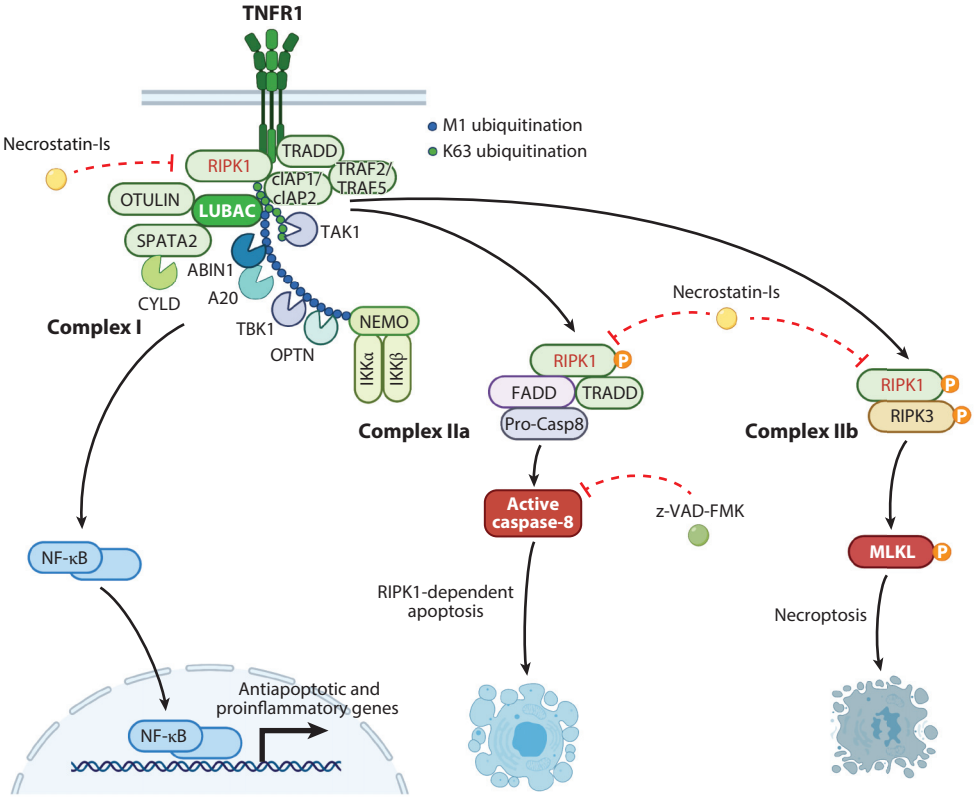


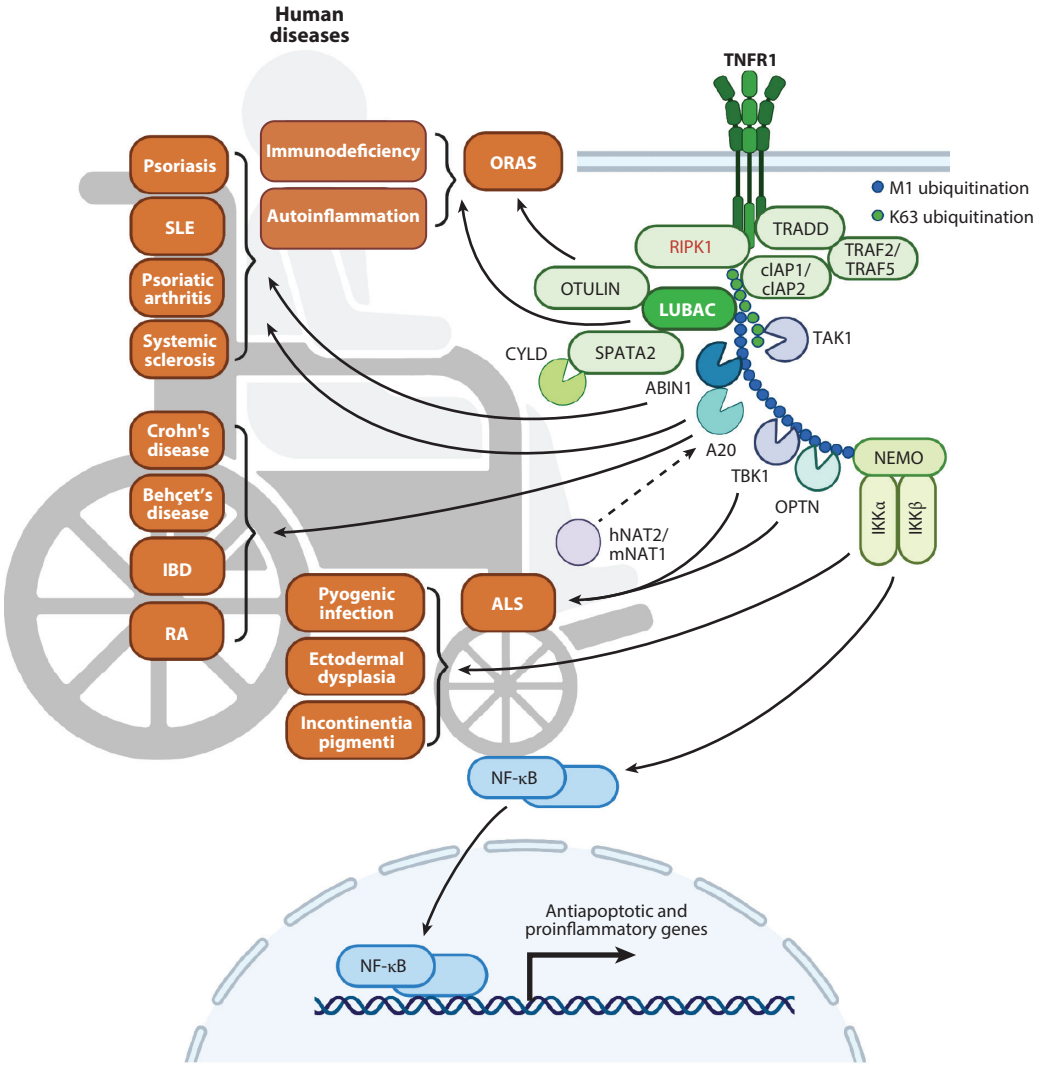
Figure 1

TNFR1 activation may promote NF-κB activation, RDA, or necroptosis. TNFα stimulates the trimerization of TNFR1 to form complex I in association with the intracellular death domain of TNFR1. The adaptor protein TRADD and RIPK1 both contain a death domain and are recruited first to the trimerized TNFR1. TRADD then recruits cIAP1/cIAP2 to perform K63 ubiquitination of complex I components, which in turn recruits LUBAC components, composed of HOIP, HOIL1, and SHARPIN. The LUBAC performs M1-linked ubiquitination of RIPK1. OTULIN is a deubiquitinase that can reduce LUBAC autoubiquitination to promote LUBAC activity. The M1 ubiquitinating chains in complex I recruit ubiquitin-binding protein ABIN1, which in turn promotes A20 recruitment to complex I. A20 is a suppressor of RIPK1 activity and NF-κB activation. Two ubiquitin-binding proteins, NEMO and OPTN, can both bind to ubiquitinated RIPK1. TBK1 is recruited into complex I to suppress the activation of RIPK1 by direct inhibitory phosphorylation. Activated RIPK1 (marked by phosphorylation at S166 RIPK1) can bind with FADD, TRADD, and caspase-8 to form complex IIa, which promotes RDA. When caspases are inhibited by z-VAD-FMK, activated RIPK1 binds to RIPK3 to form complex IIb, which results in MLKL activation and necroptosis. Pharmacological inhibition of RIPK1 kinase activity by Necrostatin-1s blocks the activation of RIPK1 and the formation of both complex IIa and complex IIb to inhibit RDA and necroptosis, respectively. Abbreviations: ABIN1, A20 binding inhibitor of NF-κB 1; cIAP, cellular inhibitor of apoptosis; CYLD, cylindromatosis lysine 63 deubiquitinase; FADD: FAS-associated death domain protein; HOIL1, heme-oxidized IRP2 ubiquitin ligase 1; HOIP, HOIL1-interacting protein; IKK, inhibitor of NF-κB kinase; LUBAC, linear ubiquitination assembly complex; MLKL, mixed lineage kinase domain-like pseudokinase; NEMO, NF-κB essential modulator; NF, nuclear factor; OPTN, optineurin; OTULIN, OTU deubiquitinase with linear linkage specificity; Pro-Casp8: pro-caspase-8; RDA, RIPK1-dependent apoptosis; RIPK, receptor-interacting protein kinase; SHARPIN: SHANK-associated RH domain interactor; SPATA, spermatogenesis associated; TAK1, transforming growth factor-β-activated kinase 1; TBK1, TANK-binding kinase 1; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TRADD, tumor necrosis factor receptor 1–associated death domain; TRAF, tumor necrosis factor receptor-associated factor. Figure adapted from images created with BioRender.com.

ABIN1: A20 binding inhibitor of NF-κB 1

a critical role in modulating the activation of RIPK1. The activation of RIPK1 in complex I is also regulated by ubiquitin-binding proteins, including ABIN1 (A20 binding inhibitor of NF-κB; encoded by gene *TNIP1*) (31), NEMO (157), and OPTN (57). Activated TAK1 and IKK then stimulate the NF-κB pathway to transcriptionally induce the expression of antiapoptotic and proinflammatory genes (47). TAK1 (39) and IKK (27) also directly inhibit the activation of RIPK1 by phosphorylation. Cellular FADD-like IL-1β-converting enzyme inhibitory protein (cFLIP), which encodes the catalytically inactive homolog of caspase-8 (87), is a transcriptional target of NF-κB and a key regulator of caspase-8 activation. Caspase-8 negatively controls the activation of RIPK1 by cleavage (78). Mutations in the genes that encode the multiple components in complex I lead to multiple inflammatory and autoimmune diseases in humans and activation of RIPK1 in experimental systems (**Figure 2**).

Current models for tumor necrosis factor α (TNFα) signaling indicate that RIPK1 plays very important roles in the four branches of the TNFR1 response: to inhibit apoptosis via



(*Caption appears on following page*)

Figure 2 (*Figure appears on preceding page*)

Mutations and polymorphisms in regulators of complex I lead to human diseases involving RIPK1 activation. Complex I is a key checkpoint in cells stimulated by TNFα that decides if RIPK1 is to be activated. The genetic variants in the components of complex I that lead to human diseases are indicated by the brown boxes on the left. Loss-of-function mutations in the components of LUBAC, OTULIN, ABIN1, A20, NEMO, TBK1, OPTN, and hNAT2/mNAT1, a modulator of A20, have been shown to promote the activation of RIPK1 to mediate apoptosis and necroptosis in experimental systems. Genetic deficiency in LUBAC components leads to immune deficiency and autoinflammatory conditions in humans. OTULIN is a deubiquitinase that modulates the activation of the LUBAC. OTULIN deficiency leads to ORAS. The M1 ubiquitinating chains (*blue circles*) in complex I recruit ABIN1, which is encoded by the gene TNIP1. TNIP1 variants are known to be the most common genetic cause in humans for psoriasis, SLE, psoriatic arthritis, and systemic sclerosis. A20 is a ubiquitin-editing enzyme that can suppress RIPK1 and NF-κB activation. Genetic variants of A20 encoded by the gene TNFAIP3 are associated with Crohn's disease, Behçet's disease, IBD, and RA. Genetic hypomorphic variants of NEMO, a ubiquitin-binding protein that serves as a scaffold to recruit IKKα and IKKβ into complex I, drive NEMO syndrome with diverse immunodeficient phenotypes. Reduction-of-function variants of TBK1 and OPTN promote ALS and FTD. hNAT2/mNAT1 deficiency reduces the acetylation of A20, which promotes its lysosomal degradation. Genetic variants of hNAT2 are associated with type 2 diabetes in humans. Reduced expression of hNAT2/mNAT1 is found in the cerebrovascular endothelial cells of AD patients and AD mouse models. The mutations in IKKs promote ectodermal dysplasia, incontinentia pigmenti, and pyogenic infection. The dotted arrow represents hNAT2/mNAT1 metabolic regulation of A20 protein expression. Abbreviations: ABIN1, A20 binding inhibitor of NF-κB 1; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; cIAP, cellular inhibitor of apoptosis; CYLD, cylindromatosis lysine 63 deubiquitinase; FTD, frontotemporal degeneration; hNAT2, human arylamine *N*-acetyltransferase 2; IBD, inflammatory bowel disease; IKK, inhibitor of NF-κB kinase; LUBAC, linear ubiquitination assembly complex; mNAT1, mouse arylamine *N*-acetyltransferase 1; NEMO, NF-κB essential modulator; NF, nuclear factor; OPTN, optineurin; ORAS, OTULIN-related autoinflammatory syndrome; OTULIN, OTU deubiquitinase with linear linkage specificity; RA, rheumatoid arthritis; RIPK, receptor-interacting protein kinase; SLE, systemic lupus erythematosus; SPATA, spermatogenesis-associated; TAK1, transforming growth factor-β-activated kinase 1; TBK1, TANK-binding kinase 1; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; TNIP1, TNFAIP3-interacting protein 1; TRADD, tumor necrosis factor receptor 1–associated death domain; TRAF, tumor necrosis factor receptor-associated factor. Figure adapted from images created with BioRender.com.

NF-κB-mediated production of pro-survival genes (scaffold function, RIPK1-kinase-independent); to inhibit necroptosis via restraining ZBP1 and TRIF (scaffold function, RIPK1-kinase-independent); to promote apoptosis and necroptosis (RIPK1-kinase-dependent); and to induce NF-κB-mediated inflammation (RIPK1-kinase-dependent). With prolonged lipopolysaccharide (LPS) stimulation, activated RIPK1 can bind to the NF-κB complex to mediate sustained inflammation in cells (54). These diverse functions of RIPK1 are tightly controlled by different cellular contents and are described in detail in this review.

GENETIC REGULATION OF RIPK1 KINASE ACTIVITY AND SCAFFOLD FUNCTION

As a central mediator of cell death and cell survival, the activation of RIPK1 can dictate cell survival independently of its kinase activity or promote cell death and/or inflammation mediated by its kinase activity (**Figure 1**). The importance and dual functions of RIPK1 in regulating inflammation and cell death have been underscored by human genetic variants that lead to either loss- or gain-of-function mutations in RIPK1 (**Figure 3**).

The Scaffold Function of RIPK1 in Promoting Cell Survival

Homozygous loss-of-function mutations of RIPK1, including frameshift, missense, and nonsense mutations in humans, cause severe immunodeficiency, inflammatory bowel diseases (IBDs), arthritis, and intestinal inflammation (19, 75, 154). The loss of RIPK1 in patients can promote the pediatric onset of primary immunodeficiency with chronic enteropathy (154). In human skin fibroblasts, RIPK1 deficiency impairs the activation of NF-κB and promotes RIPK3/mixed lineage kinase domain-like pseudokinase (MLKL)-dependent necroptosis.

LPS: lipopolysaccharide

IBD: inflammatory bowel disease

MLKL: Mixed lineage kinase domain-like pseudokinase

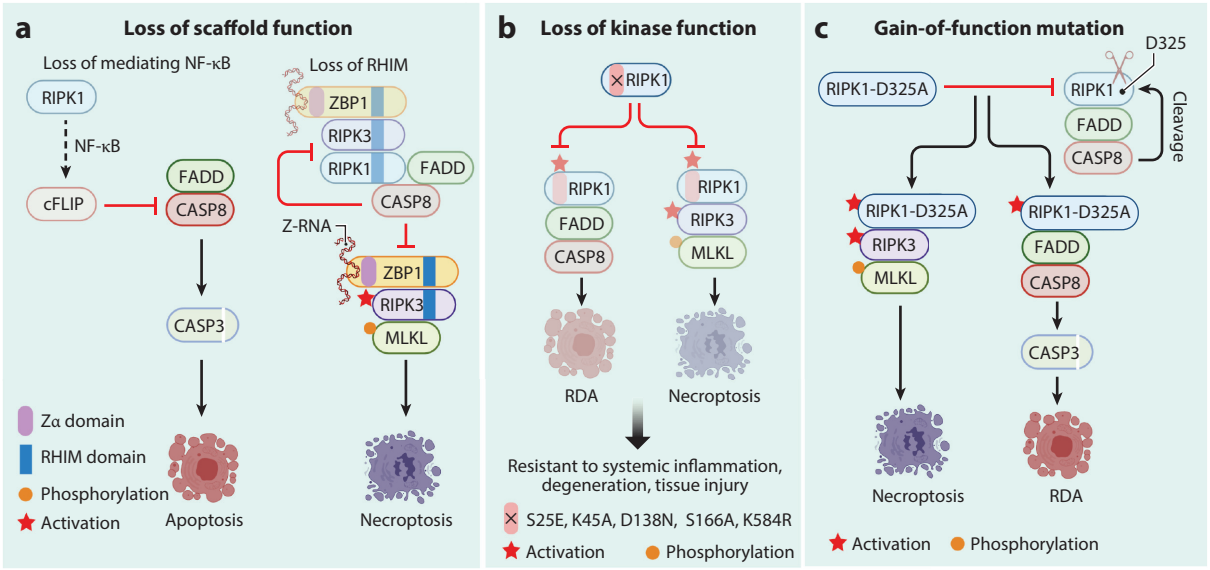


Figure 3

Genetic regulation of RIPK1 kinase activity and scaffold function. (*a*) Loss of scaffold function. In mice, loss of RIPK1 scaffold function promotes apoptosis and necroptosis. The scaffold of RIPK1 mediates ubiquitination- and RHIM-dependent signaling. The RIPK1-associated ubiquitination-dependent signaling is required for NF-κB activation to mediate the transcription of pro-survival genes, such as cFLIP (as indicated by the *dotted arrow*). cFLIP inhibits apoptosis by interacting with caspase-8 to prevent its activation. RIPK1 deficiency reduces the levels of cFLIP, which promotes apoptosis mediated by FADD/caspase-8. In addition, the RHIM-dependent signaling is important for RIPK1 to restrict ZBP1 in mediating necroptosis. Loss of RIPK1 RHIM function leads to ZBP1-activated necroptosis in response to endogenous retrovirus-derived Z-RNA. (*b*) Loss of kinase function. Genetic and pharmacological inhibition of RIPK1 kinase suppresses both RDA and necroptosis. Kinase-dead knock-in mutant mice, including D138N, K45A, and S166A, are resistant to systemic inflammation associated with chronic infection, sepsis, neurodegenerative conditions, and other types of acute tissue injury. (*c*) Gain-of-function mutation. Activating mutations that block the caspase-8-mediated cleavage of RIPK1 promote RDA and necroptosis. Caspase-8 negatively regulates the activation of RIPK1 by cleaving human and mouse RIPK1 after residues D324 and D325, respectively. D325A knock-in RIPK1 mutation in mice leads to mouse embryonic lethality due to RDA and necroptosis. In humans, rare familial variants of RIPK1, such as D324V and D324H, lead to an autoinflammatory disease characterized by increased production of proinflammatory cytokines, recurrent fevers, and lymphadenopathy in an autosomal dominant manner. Abbreviations: CASP, caspase; cFLIP, cellular FADD-like IL-1β-converting enzyme inhibitory protein; FADD, FAS-associated protein with death domain; MLKL, mixed lineage kinase domain-like pseudokinase; NF, nuclear factor; RDA, RIPK1-dependent apoptosis; RHIM, receptor-interacting protein homotypic interaction motif; RIPK, receptor-interacting serine/threonine protein kinase; ZBP1, Z-DNA-binding protein 1. Figure adapted from images created with BioRender.com.

While human individuals with RIPK1 loss-of-function mutations survive and develop inflammatory disease, *Ripk1*$^{-/-}$ mice are born at the expected Mendelian ratios but die around postnatal days 1–3 (22, 26, 59, 126, 144). Genetic inhibition of both apoptosis and necroptosis in *Ripk1*$^{-/-}$*Fadd*$^{-/-}$*Ripk3*$^{-/-}$, *Ripk1*$^{-/-}$*Casp8*$^{-/-}$*Ripk3*$^{-/-}$, *Ripk1*$^{-/-}$*Fadd*$^{-/-}$*Mlkl*$^{-/-}$, or *Ripk1*$^{-/-}$*Tradd*$^{-/-}$*Ripk3*$^{-/-}$ mice fully rescues the perinatal lethality of *Ripk1*$^{-/-}$ mice to adulthood (2, 22, 26, 29, 59, 126, 144). Thus, the loss-of-function mutation in RIPK1, independent of its kinase activity, promotes FADD/caspase-8-dependent apoptosis and RIPK3/MLKL-dependent necroptosis (**Figure 3**).

The RHIM in the intermediate domain of RIPK1 may also contribute to its scaffold function in promoting survival (**Figure 3**). ZBP1 and TRIF, two RHIM-containing proteins, are involved in mediating RIPK3/MLKL-dependent necroptosis in *Ripk1*$^{-/-}$ mice (77, 104). Combined ZBP1 and TRIF deficiency can inhibit necroptosis in *Ripk1*$^{-/-}$ mice and rescue perinatal lethality in

Ripk1$^{-/-}$*Casp8*$^{-/-}$ mice (104). Mice with *Ripk1*-RHIM mutations also die around birth due to massive necroptosis but not caspase-8-dependent apoptosis (77, 104), suggesting that the RHIM of RIPK1 is an essential part of the RIPK1 pro-survival scaffold function during development. The perinatal lethality and the histological lesions in *Ripk1*-RHIM mutant mice can be prevented by either RIPK3 or MLKL deficiency (77, 104). Interestingly, genetic ablation of ZBP1 also protects *Ripk1*-RHIM mutant mice from perinatal lethality and inhibits RIPK3 activation (104), suggesting that the RIPK1 RHIM prevents ZBP1 from activating RIPK3/MLKL-dependent necroptosis.

RDA: RIPK1-dependent apoptosis

The scaffold function of RIPK1 may also be important in mediating mTORC1 inhibition by AMPK during energetic stress (98). RIPK1 deficiency results in elevated mTORC1 activity that promotes lysosomal dysfunction, leading to the accumulation of RIPK3 and caspase-8 and to sensitization to cell death. RIPK1 may act as a scaffold to mediate the interaction between AMPK and TSC2 and facilitate TSC2 phosphorylation by AMPK.

RIPK1 Kinase Activity Promotes Apoptosis and Necroptosis

The kinase function of RIPK1 is required for both RIPK1-dependent apoptosis (RDA) and necroptosis. Genetic inactivation of RIPK1 kinase by introducing kinase-dead knock-in mutations in mice, including D138N (124) and K45A (5, 59), results in no gross or histological abnormalities (**Figure 3**). In fact, these kinase mutant mice are highly resistant to systemic inflammation associated with chronic infection, sepsis, degenerative conditions, or other types of tissue injury. The resistance of RIPK1-kinase-dead mutant mice to TNFα-induced systemic inflammatory syndrome can be phenocopied by RIPK1 kinase inhibitors, such as Nec-1s and other small molecule inhibitors of RIPK1 (24, 30).

Additional point mutations that block the activation of RIPK1 kinase can also protect mice from TNFα-induced systemic inflammatory syndrome. Mutant mice with a K584R knock-in mutation in the DD, which blocks the dimerization-mediated activation of RIPK1, are normal (86). K584R mutant mice are protected from TNFα-induced septic shock and hypothermia (86). Autophosphorylation of S166 in the kinase domain of RIPK1 has been widely used as a marker of RIPK1 activation (110). Genetic study reveals that RIPK1 autophosphorylation at S166 is important for the induction of RIPK1-kinase-dependent cell death and inflammation (69). *Ripk1*$^{S166A/S166A}$ mice appear normal and are resistant to TNFα-induced hypothermia and lethality, as well as other types of tissue injury and inflammation (69). Thus, the kinase activity of RIPK1 promotes cell death and inflammation, which drives the onset and progress of inflammatory and degenerative diseases.

Activating Mutations in RIPK1 Promote Apoptosis and Necroptosis

In contrast to the kinase-dead knock-in mutations in RIPK1, genetic activating mutations that block caspase-8-mediated cleavage of RIPK1 promote the activation of RIPK1 as well as apoptosis and necroptosis (**Figure 3**). Caspase-8 negatively regulates the activation of RIPK1 by cleaving human and mouse RIPK1 after residues D324 and D325, respectively, which separates the RIPK1 kinase domain from the intermediate domain and DD (78). The D325A knock-in RIPK1 mutation leads to mouse embryonic lethality that can be suppressed by the *cis*-inactivation of RIPK1 by the D138N mutation, loss of TNFR1, and inactivation of both necroptosis and apoptosis by the double knockout of *Ripk3* and *Fadd* or *Mlkl* and *Fadd* (102, 172). Consistently, rare familial human variants of RIPK1, such as D324V and D324H (68, 147), lead to an autoinflammatory disease characterized by increased production of proinflammatory cytokines, recurrent fevers, and lymphadenopathy in an autosomal dominant manner. Patient peripheral blood mononuclear cells

show increased activation of RIPK1, as well as apoptosis and necroptosis induced by TNFα, which can be blocked by the RIPK1 inhibitor Nec-1s. Thus, the kinase activity of RIPK1 is an important mediator of cell death and inflammation.

TLR: Toll-like receptor

REGULATION OF RIPK1 ACTIVATION BY UBIQUITINATION

RIPK1 is extensively modulated by ubiquitination. In control unstimulated mouse embryonic fibroblasts (MEFs), many lysine residues in RIPK1 are already highly ubiquitinated, including 11 lysine residues in the kinase domain (K20, K30, K45, K65, K115, K137, K140, K153, K163, K167, and K307), 4 lysine residues in the intermediate domain (K376, K392, K395, and K429), and 4 lysine residues in the DD (K589, K612, K627, and K633) (74). Moreover, the ubiquitination of RIPK1 in TNFα-stimulated cells is dynamically regulated. In wild-type MEFs stimulated by TNFα for 5 min, the ubiquitination levels of 19 lysine residues (K20, K45, K46, K65, K105, K137, K140, K153, K163, K167, K376, K392, K395, K429, K550, K589, K612, K619, and K633) are increased, while the ubiquitination levels of 4 lysine residues (K30, K115, K307, and K627) are decreased.

The ubiquitination of RIPK1 is functionally important in regulating the activation of RIPK1. Genetic or pharmacological inhibition of cIAP1/cIAP2 reduces K63-linked ubiquitination of RIPK1 and promotes RDA or necroptosis (92, 160). K377 is the main residue providing a docking site in human RIPK1 for K63-linked polyubiquitin chains mediated by cIAP1 and cIAP2 (32, 72). K376R knock-in mutant mice are embryonic day (E)12.5 lethal with RIPK1 activation and excessive cell death in the embryos and yolk sacs (146, 173). Double knockout of *Fadd* and *Ripk3* or *Fadd* and *Mlkl* rescues $Ripk1^{K376R/K376R}$ mice from embryonic lethality, suggesting that the embryonic lethal phenotype of $Ripk1^{K376R/K376R}$ mice is mediated by FADD/caspase-8-dependent apoptosis and RIPK3/MLKL-dependent necroptosis (146, 173). However, knockout of *Tnfr1* only rescues $Ripk1^{K376R/K376R}$ mice at the embryonic stage and fails to prevent the postnatal systemic inflammation. Interestingly, *Ripk3* deficiency prevents the lethal inflammation of $Ripk1^{K376R/K376R}Tnfr1^{-/-}$ mice, suggesting that K63-linked ubiquitination of RIPK1 also suppresses inflammation during postnatal development.

K627 in human RIPK1 (K612 in murine RIPK1) is also a key ubiquitination site that regulates the overall ubiquitination pattern of RIPK1, including the ubiquitination of K377 (74). K612 ubiquitination in murine RIPK1 (K627 in humans) has distinct roles in regulating its pro-death kinase activity in response to TNFα and pro-survival activity in response to Toll-like receptor 3 (TLR3) and TLR4 signaling. The K612R mutation inhibits the activation of RIPK1 and blocks both apoptosis and necroptosis mediated by TNFR1 signaling. But unexpectedly, the K612R mutation sensitizes cells to necroptosis and caspase-1 activation in response to TLR3 and TLR4 activation. $Ripk1^{K612R/K612R}$ mice are viable but develop age-dependent intestinal inflammation and splenomegaly, which can be reduced by antibiotic treatment and partially reduced by *Ripk3* ablation. Mechanistically, the ubiquitination of K612 in murine RIPK1 is important for its interactions with FADD, which contributes to the suppression of RIPK3 activation mediated by TLR3 and TLR4 signaling. Together, these genetic studies provide functional evidence that K376-mRIPK1/K377-hRIPK1 and K612-mRIPK1/K627-hRIPK1 are critical residues for ubiquitin-dependent regulation of RIPK1.

GENETIC REGULATION OF RIPK1 ACTIVATION BY THE COMPONENTS OF COMPLEX I

Complex I, associated with TNFR1 upon TNFα stimulation, provides a critical checkpoint that decides if RIPK1 kinase is to be activated (**Figure 1**). Many known key regulators of NF-κB

activation, such as TAK1, IKKs, NF-κB essential modulator (NEMO), A20, and ABIN1, are also recruited into complex I to directly regulate the activation of RIPK1 by modulating its ubiquitination and phosphorylation patterns. Mutations in the genes that encode these key components of complex I lead to inflammatory and neurodegenerative diseases in humans (**Figure 2**). In addition, the transcriptional targets of the NF-κB pathway also regulate the activation of RIPK1 by controlling the expression of TNFAIP3, which encodes A20, and cFLIP. Here, we review the genetic evidence that supports the dysregulated activation of RIPK1 in complex I in human inflammatory diseases.

NEMO: NF-κB essential modulator

A20 Deficiency–Associated Immunopathologies

A20, encoded by the *TNFAIP3* gene, is an important suppressor of RIPK1 activity and NF-κB activation. *TNFAIP3* gene variants that are heterozygous loss-of-function mutations are linked with early-onset systemic inflammation and autoimmune diseases (**Figure 2**).

A heterozygous C234Y A20 mutation has been identified to be responsible for chronic inflammation in autosomal dominant Behçet's disease (BD) (134). Mononuclear cells from these patients produce large amounts of inflammatory cytokines and show impaired suppression of NF-κB activation. The reduced effect of C234Y A20 on suppressing inflammatory cytokine production suggests that it is a loss-of-function mutation. Similarly, patients with truncated A20 mutations suffer from early-onset systemic inflammation, a syndrome that resembles BD (177). These truncated A20 mutants are likely to act by haploinsufficiency, with defective K63 deubiquitinating activity of TRAF6, NEMO, and RIPK1 upon TNFα stimulation.

A genome-wide association study (GWAS) has identified *TNFAIP3* as a susceptibility gene for Crohn's disease, a common form of chronic IBD (163). To further support this finding, three de novo heterozygous A20 mutations, including one nonsense and two frameshift mutations, are recognized to be linked with infantile-onset intractable IBD (175). *TNFAIP3* variants are also associated with rheumatoid arthritis, which is another common chronic inflammatory disease characterized by increased proinflammatory cytokines, such as TNFα, interleukin 1(IL-1), and IL-6 (10). Multiple *TNFAIP3* polymorphisms, including two noncoding and one coding polymorphism, have been revealed to be independently associated with systemic lupus erythematosus (SLE), an autoimmune disease (96). Interestingly, the two noncoding single-nucleotide polymorphisms (SNPs) are perfect proxies for the two rheumatoid arthritis–associated SNPs (122).

Tnfaip3$^{-/-}$ mice display systemic inflammation and neonatal lethality that can be rescued by inhibition of RIPK1 kinase activity or deletion of RIPK3 (100, 114). A20 deficiency promotes the activation of RIPK1 and necroptosis (31), while elevated A20 has also been noted to promote TNFα-induced RIPK1 activation that mediates intestinal epithelial cell (IEC) death in IBD (36). Given the importance of A20 in regulating the activation of RIPK1, patients with dysregulated A20 may provide a genetically defined population for clinical studies of RIPK1 inhibitors for the treatment of inflammatory and autoimmune diseases.

ABIN1 Gene Variants Associated with Inflammatory and Autoimmune Diseases

ABIN1, encoded by the *TNFAIP3* interacting protein 1 (*TNIP1*) gene, is a ubiquitin-binding protein recruited into complex I (31) (**Figure 1**). Recruitment of ABIN1 to complex I is mediated by binding to M1 ubiquitinating chains modulated by the LUBAC; the association of ABIN1 in complex I in turn promotes the recruitment of A20. Similar to the studies of *TNFAIP3*, GWASs have identified *TNIP1* polymorphisms strongly associated with multiple inflammatory and autoimmune diseases, including psoriasis, psoriatic arthritis, SLE, and systemic sclerosis (SS) (41). The rs17728338 SNP of *TNIP1* is a shared genetic variant that is associated with susceptibility

to psoriasis and psoriatic arthritis (9, 97). The *TNIP1* variant rs7708392 is associated with SLE patients of Caucasian and Asian origins (37, 62, 169) (**Figure 2**).

Tnip1$^{-/-}$ mice die embryonically with live necrosis (31). Inhibition of RIPK1 kinase activity or deletion of RIPK3 rescues the embryonic lethality of *Tnip1*$^{-/-}$ mice. *Tnip1*$^{-/-}$ MEFs are sensitized to RIPK1 activation and RIPK3-dependent necroptosis. *TNIP1* heterozygosity sensitizes the innate immune response in *Tnip1*$^{+/-}$ mice by promoting the production of proinflammatory cytokines, which can be blocked by inhibiting RIPK1 kinase activity (139). *Tnip1*$^{+/-}$ MEFs also show a hyperactive antiviral response due to NF-κB-dependent and RIPK1-independent expression of pattern recognition molecules, including TLR3, RIG-I, and MDA5. These data suggest that RIPK1 inhibitors may ameliorate excessive inflammation in patients with *TNIP1* variants while maintaining their host defense responses.

NEMO Deficiency Syndrome

NEMO, encoded by the X-linked *IKBKG* gene, is a ubiquitin-binding protein that serves as a scaffold to recruit IKKα and IKKβ into complex I to regulate the activation of NF-κB as well as that of RIPK1 (32) (**Figure 2**). Hypomorphic mutations of NEMO lead to diverse immunodeficient phenotypes, known as NEMO syndrome. Most patients with NEMO syndrome are associated with ectodermal dysplasia, serious pyogenic infection, mycobacterial infection, incontinentia pigmenti (IP), and inflammatory diseases (46, 136). NEMO gene hypomorphisms include missense, splice-site, frameshift, and nonsense mutations. More than 50% of these mutations are located within the zinc finger domain, which forms a specific complex with ubiquitin and is required for NF-κB signaling in response to TNFα stimulation (18). Other subsets of NEMO syndrome patients carry mutations that affect the regions important for interacting with the IKK complex, promoting NEMO oligomerization, or binding with K63-linked polyubiquitin (46). A distinct group of patients harboring a C-terminal deletion of NEMO develop inflammatory skin and intestinal disease, in addition to ectodermal dysplasia with anhidrosis and immunodeficiency (180). The NEMO C-terminal deletion mutants interact poorly with A20 and lead to enhanced K63-ubiquitinated RIPK1 upon TNFα stimulation.

Ikbkg-deficient mice reproduce the human genetic disorder IP, which is characterized by male embryonic lethality and heterozygous female skin lesions (81, 130). IEC-specific ablation of NEMO induces Paneth cell and colonocyte apoptosis, impaired antimicrobial factor expression in the ileum, and microbiota-driven chronic inflammation in the colon (157). In these mutant mice, inhibition of RIPK1 kinase activity or the combined deletion of FADD and RIPK3 prevents IEC death, Paneth cell loss, and development of colitis. NEMO-deficient cells are highly sensitive to RIPK1-associated apoptosis, independent of NF-κB activation (70). These data collectively suggest that RIPK1 inhibitors could be strong candidates for the treatment of colitis and excessive inflammation in individuals with NEMO mutations.

LUBAC Deficiency Syndrome

Genetic deficiency in LUBAC components, including HOIL1, HOIP, and SHARPIN, drives immunodeficiency and autoinflammation in patients with amylopectinosis of cardiac and skeletal muscle (7, 8) (**Figure 2**). Biallelic loss of HOIL1 via a single-copy loss of 31.799 kb on chromosome 20p13, which contains intron 4 of *HOIL1*, or a homozygous deletion of CT at positions 121 and 122 in exon 2 of *HOIL1* impairs the stability of the LUBAC and compromises NEMO recruitment to cytokine receptors (8). While lower levels of NF-κB activation in response to IL-1β are observed in patient fibroblasts, patient mononuclear leukocytes are hypersensitive to IL-1β in

upregulating the expression of proinflammatory cytokine genes, which may be responsible for the clinical autoinflammation seen in these patients.

A homozygous germline HOIP mutation, L72P, has been identified in a patient with multiorgan autoinflammation combined with immunodeficiency (7). Similar clinical features are also observed in a second case of patients harboring biallelic variants in close proximity to splice sites in HOIP (108). Both disease-associated mutations of the *HOIP* gene decrease HOIP protein expression and destabilize the whole LUBAC.

Hoil1$^{-/-}$ mice and cells derived from these mice show defective NF-κB signaling induced by IL-1β or TNFα, and *Hoil1*$^{-/-}$ hepatocytes are hypersensitized to RDA (152). In addition, the loss of SHARPIN, another LUBAC component, has been shown to lead to severe skin and multiorgan inflammation in transgenic mice (5). Inhibition of RIPK1 kinase activity ameliorates all chronic proliferative dermatitis-related pathology in *Sharpin*-deficient mice. Collectively, these data suggest that RIPK1 kinase represents a potential therapeutic target for LUBAC deficiency–related syndromes.

OTULIN: OTU deubiquitinase with linear linkage specificity

TBK1: TANK-binding kinase 1

OTULIN-Related Autoinflammatory Syndrome

Ovarian tumor (OTU) deubiquitinase with linear linkage specificity (OTULIN) is a deubiquitinase that promotes LUBAC activity by preventing its autoubiquitination (63). M1 ubiquitination of RIPK1 in response to TNFα is regulated by OTULIN. Patients with biallelic hypomorphic mutations in OTULIN develop a potentially fatal autoinflammatory disease, termed OTULIN-related autoinflammatory syndrome (ORAS) (20, 21, 178) (**Figure 2**).

A homozygous missense substitution L272P in the OTU domain of OTULIN has been detected in a family with three premature newborns displaying severe idiopathic inflammatory symptoms (21). The L272P OTULIN mutant protein is less stable and much less active toward M1-linked di- and tetraubiquitination compared to the wild-type protein. Y244C OTULIN is another missense mutation located in the OTU domain of OTULIN, which has been identified in a patient with early-onset fevers as well as pustular and scarring rashes (178). The mutant cells from these patients show accumulated linear-ubiquitinated RIPK1, NEMO, TNFR1, and ASC, and accumulated high molecular linear ubiquitin aggregates, compared to healthy individuals (178). Loss-of-function mutations of OTULIN increase linear ubiquitination of TNFα signaling molecules and therefore lead to TNFR1-, RIPK1-, NF-κB-, and ASC-dependent inflammation. Consistent with the important role of TNFα signaling in ORAS, anti-TNFα treatment ameliorates systemic inflammation in this disease (20, 21, 178).

Consistent with OTULIN as an activator of the LUBAC, *Otulin*$^{-/-}$ and catalytically inactive *Otulin*$^{C129A/C129A}$ mice die during embryonic development as a result of RIPK1-dependent cell death mediated by TNFR1 (51, 120). In adult mutant mice, the inactivation of OTULIN also promotes proinflammatory cell death. Embryonic lethality and adult autoinflammation are prevented by inhibiting RIPK1 kinase activity or the double knockout of caspase-8 and RIPK3, suggesting that RIPK1-dependent necroptosis is activated by OTULIN deficiency–related autoinflammatory syndrome.

TBK1 Haploinsufficiency Sensitizes Cells to RIPK1 Activation in Aging Brains

Heterozygous variants of TANK-binding kinase 1 (TBK1) have been found to be associated with amyotrophic lateral sclerosis (ALS) and frontotemporal degeneration (FTD) in humans (17, 34) (**Figure 2**). Rare individuals who carry familial homozygous loss-of-function mutations in TBK1 develop chronic and systemic autoinflammation, which can be ameliorated by the treatment with

NAT: arylamine N-acetyltransferase

anti-TNF (143). TBK1 contains an N-terminal kinase domain, a ubiquitin-like domain, and two C-terminal coiled-coil domains (CCD1 and CCD2). *Tbk1*$^{-/-}$ mice are embryonic lethal, and the lethality is blocked by inactivating RIPK1 in *Tbk1*$^{-/-}$;*Ripk1*^{D138N} mice and partially blocked in *Tbk1*$^{-/-}$;*Ripk3*$^{-/-}$ mice (164). TBK1 is recruited into complex I like TAK1 is recruited in TNFα-stimulated cells. TBK1 deficiency also sensitizes cells to RDA in the presence of TNFα. Reduced expression of *Tbk1* by the mutant alleles associated with ALS and FTD has led to the proposal that the haploinsufficiency of *Tbk1* is a pathological mechanism (34, 107).

Aging is a major risk factor in the development of all chronic neurodegenerative diseases, including ALS and FTD (105). The mean onset of ALS and FTD is between 50 and 65 years of age. Individuals with familial mutations that predispose them to ALS and FTD, such as carriers of TBK1 mutations, may live asymptomatically until middle age. The levels of TAK1, an important suppressor of RIPK1 (39), are decreased in the aged brains (>60 years old) of individuals who passed away due to non-neurological causes (164). A genetic model of the aging-dependent interaction of TBK1 and TAK1 with one copy of a loss-of-function mutation in TBK1 and myeloid-specific 50% reduction of TAK1 expression is sufficient to promote all key hallmarks of ALS and FTD, including neuroinflammation, TDP-43 aggregation, axonal degeneration, neuronal loss, and behavior deficits—all of which are rescued by inhibition of RIPK1 (164). The reduction of TAK1 expression in the aging human brain may provide a common sensitization mechanism underlying a variety of aging-related neurodegenerative diseases, including ALS and FTD, to promote the activation of RIPK1 and the onset of neurodegeneration.

Human NAT2 Deficiency–Induced Cerebral Vascular Endothelial Necroptosis in Alzheimer's Disease

Increasing evidence suggests that RIPK1 activation and necroptosis play an important role in the pathogenesis of Alzheimer's disease (AD) (11, 65, 111, 181). Human arylamine *N*-acetyltransferase 2 (*hNAT2*) has recently been discovered as a genetic regulator of necroptosis in AD (181) (**Figure 2**). *hNAT2* was initially identified as an insulin sensitivity gene (64). Deficiency of its murine homolog, *mNat1*, in mice leads to insulin resistance and type 2 diabetes associated with mitochondrial dysfunction and metabolic defects (12). Human *hNAT2* variants have also been suggested to be associated with an increased risk for developing AD (42, 113, 128). Recent findings show reductions of hNAT2 and mNAT1 in the cerebral vascular endothelial cells of AD patients and transgenic AD mouse models, respectively (181). The decrease of hNAT2/mNAT1 levels drives endothelial necroptosis and therefore may promote the breakdown of the blood–brain barrier (BBB) and cognitive decline, contributing to the pathogenesis in AD.

Mechanistically, decreased hNAT2/mNAT1 expression leads to mitochondrial and metabolic dysfunctions, which in turn reduce the production of acetyl-CoA, the acetyl donor for posttranslational protein acetylation. A decreased level of acetyl-CoA results in deacetylation of A20 and correspondingly increased A20 lysosomal degradation (181). Inhibition of cerebral endothelial necroptosis mediated by *mNat1* deficiency prevents BBB dysfunction in AD mice. Therefore, *hNAT2/mNat1* serves as a regulator of A20 and necroptosis in cerebral endothelial cells of AD.

RIPK1-Mediated Neuroinflammation in Alzheimer's Disease

A genetic variant of *SHARPIN* has been identified as a risk factor for developing late-onset AD (LOAD) (3). Since *Sharpin*-deficient *cpdm* mice develop RIPK1-mediated dermatitis and multiorgan inflammation (5, 119, 162), the reduced SHARPIN activity may increase the risk of LOAD by promoting RIPK1-mediated neuroinflammation.

Supporting the role of RIPK1 and necroptosis in AD, activated RIPK1, RIPK3, and MLKL have been found in the insoluble fractions of sporadic AD postmortem brain tissue (11, 111) and in granulovacuolar degeneration lesions in degenerating neurons both in AD and in preclinical stages of AD associated with Tau pathology (65). The activation of RIPK1 may play an important role in driving neuroinflammation mediated by microglia in AD (111). A single-cell RNA sequencing study identified a subclass of microglia in mouse models of ALS, termed RIPK1-regulated inflammatory microglia (RRIMs), which show significant upregulation of classical proinflammatory pathways, including increased TNFα and IL1β mRNA and protein levels (89). The prevalence of these microglia can be suppressed by inhibiting RIPK1 activity downstream of TNFR1. It would be interesting to further investigate the involvement of RRIMs in AD.

CASPASE-8: THE COMMON CHECKPOINT FOR CELL DEATH AND INFLAMMATION

Caspase-8 has been established as a critical mediator of death receptor–mediated apoptosis (153). Unexpectedly, caspase-8 has also emerged as a key suppressor of necroptosis and inflammation. Thus, regulation of caspase-8 is important in promoting cell survival and selecting the cell death modality, as well as in controlling inflammation.

Regulation of cFLIP by NF-κB Signaling

Apoptosis is another possible outcome of TNFα stimulation. NF-κB-dependent transcription of pro-survival genes puts a brake on apoptosis. *cFLIP* is a prominent example of such a pro-survival gene. The inactivation of NF-κB or deleting cFLIP unleashes TNFα cytotoxicity and induces apoptosis (116). This is mediated by the formation of complex IIa, a cytosolic complex that includes FADD and caspase-8, which drives the activation of caspase-8 and the subsequent cleavage of RIPK1 and execution of apoptosis (78, 150, 167).

Catalytic Activity of Caspase-8 Suppresses Necroptosis

When caspase-8 activation is blocked, for example, by large DNA viruses encoding caspase-8 inhibitors (176), RIPK1 activation promotes the formation of a complex called the necrosome (or complex IIb), which includes RIPK1, RIPK3, and MLKL (167) (**Figure 1**). In the necrosome, RIPK3 is activated, which in turn mediates the phosphorylation of MLKL to drive its activation and oligomerization for the execution of necroptosis (140, 174). The requirement of caspase-8 inhibition for triggering necroptosis is due at least in part to the cleavage of RIPK1 by caspase-8. Caspase-8 inhibition leads to the accumulation of activated RIPK1, which is sufficient to recruit RIPK3 and promote RIPK3 activation to mediate downstream events of necroptosis. The role of caspase-8 activity in preventing necroptosis is also supported by genetic studies. *Casp8*$^{C362A/C362A}$ knock-in mice expressing catalytically inactive caspase-8 exhibit embryonic lethality between E10.5 and E12.5, due to both RIPK1 and RIPK3 activation (103). Hence, MLKL knockout in *Casp8*$^{C362A/C362A}$ *Mlkl*$^{-/-}$ mice prevents the embryonic lethal phenotypes, establishing that the proteolytic activity of caspase-8 normally limits necroptosis during embryogenesis.

Inhibition of Caspase-8 Promotes RIPK1-Kinase/NF-κB-Dependent Inflammation

RIPK1 kinase activity has been linked to driving inflammation in the absence of cell death. When caspase-8 activity is inhibited, treatment with LPS induces two waves of proinflammatory cytokine production without inducing cell death in microglia-like BV2 cells (54). The first wave is

mediated by an early RIPK1-scaffold-dependent but RIPK1-kinase-independent mechanism, which is followed by a prolonged and robust inflammatory cytokine production mediated by a RIPK1 kinase/NF-κB dual-dependent mechanism. The autocrine production of TNFα is responsible for promoting the activation of RIPK1 and the formation of the secondary signaling complex with canonical mediators of NF-κB signaling. This study provides an example in which the kinase activity of RIPK1 drives the scaffold function of RIPK1 to promote inflammation in a novel NF-κB pathway, which is negatively regulated by caspase-8 and FADD.

Homozygous caspase-8 deficiency has also been detected in patients with immunodeficiency or IBD (16, 71). At residue 248 of human caspase-8 in the p18 subunit, an arginine is changed into a tryptophan in certain human individuals who exhibit autoimmune lymphoproliferative syndrome (ALPS)-like clinical symptoms and defects in the activation of T lymphocytes, B lymphocytes, and natural killer cells (16). The R248W caspase-8 mutant shows reduced protein stability and enzymatic inactivity. Another homozygous caspase-8 mutation, Q237R, which reduces caspase-8 protein expression levels, has been identified in two patients who presented with early-onset IBD and immune dysfunction (71). One of the patients died of septic complications. Caspase-8 deficiency in humans may promote the activation of RIPK1 and may have additional effects on adaptive immunity, which is not known to be associated with autoinflammatory disease with noncleavable RIPK1 (68, 147).

THE CONSEQUENCE OF RIPK1 ACTIVATION IN COMPLEX I: RDA

Defects in the specific components of complex I lead to the activation of RIPK1 kinase and subsequent RDA (1) (**Figure 1**). Excessive activation of RIPK1 leads to the formation of a distinct detergent-insoluble, highly ubiquitinated species (iuRIPK1) that can act as a platform to form complex IIa (1). The formation of complex IIa requires RIPK1 kinase activity and drives robust caspase-8-mediated apoptosis. RDA is initiated by RIPK1 kinase activation in complex I following cIAP1/cIAP2 depletion or inhibition of TAK1 or by mutating RIPK1 ubiquitination site K377 (K376 in mouse) (39, 109, 160). In addition to ubiquitination, multiple phosphorylation events have been implicated in regulating the activation of RIPK1 in complex I. Mass spectrometry studies revealed multiple phosphorylation events in RIPK1 that can either promote or inhibit RIPK1 kinase activity (25). RIPK1 ubiquitination and phosphorylation are intimately linked regulatory events. Many kinases that regulate RIPK1 activity and RDA are recruited to the ubiquitin chains of RIPK1 in complex I to perform phosphorylation on specific sites of RIPK1 (27, 39, 67, 164). For example, TAK1 can phosphorylate RIPK1 on multiple sites, including S321, to inhibit the activation of RIPK1 (39). A combination of TNFα and TAK1 inhibitor (5Z)-7-oxozeanol induces robust RDA of MEFs (1).

Downstream of TAK1, IKKs can also perform important suppressive phosphorylation on RIPK1. IKKα and IKKβ are recruited to M1-linked ubiquitin modification in complex I. The recruitment of IKKα and IKKβ in complex I promotes their activation, which subsequently mediates the phosphorylation of RIPK1 at S25 (27, 28). Depletion of IKKα and IKKβ drives RIPK1 activation and RDA (28). S25E knock-in mutation protects mice from TNFα-induced cell death and inflammation by inhibiting RIPK1 kinase activity (27). Apart from IKKα and IKKβ, the related IKK kinases TBK1 and IKKε are also recruited to complex I to phosphorylate RIPK1(67, 164). Deletion of TBK1 results in robust RIPK1 activation in complex I and RDA. The embryonic lethal phenotype of *Tbk1*$^{-/-}$ mice is mediated by RDA in the fetal liver (164). In addition, adaptors in complex I, which are required for the recruitment of these kinases, can also act as important brakes restricting the activation of RIPK1. For example, IKKα and IKKβ are recruited to complex I by NEMO binding to the ubiquitin network. NEMO deficiency also sensitizes to RDA upon TNFα stimulation (28).

TRADD, a 34-kDa adaptor protein with an N-terminal TRAF2-binding domain and a C-terminal DD, is known to regulate extrinsic apoptosis mediated by TNFR1 (52, 53, 123). In TNFα-stimulated cells, TRADD is rapidly recruited to TNFR1, mediated by the direct binding of its DD with the TNFR1 intracellular DD (**Figure 1**). The recruited TRADD acts as the primary scaffold to organize the formation of complex I by regulating an intricate set of ubiquitination modifications, including both K63-linked ubiquitination mediated by TRAF2/cIAP1 and M1-linked ubiquitination mediated by the LUBAC, as well as phosphorylation events acting on RIPK1, to decide whether cells should activate NF-κB to survive or die by apoptosis or necroptosis (29, 43, 123). Downstream of complex I, TRADD plays an important role in mediating the formation of complex IIa by interacting with RIPK1, FADD, and caspase-8, which promotes the activation of caspases to mediate apoptosis (88). Thus, TRADD is a key mediator of RDA. TRADD is also involved in mediating TLR signaling by binding with TRIF and RIPK1 in response to viral and bacterial stimulation. *Tradd*$^{-/-}$ mice are alive; normal in development and reproduction; and highly resistant to systemic inflammatory responses induced by TNFα, poly(I:C), and LPS (13, 33, 123). In addition, TRADD also regulates the binding of TRAF2 and cIAP1 to regulate the ubiquitination of Beclin1, which in turn regulates autophagy (165). Thus, pharmacological targeting of TRADD may provide a strategy to not only inhibit RDA but also activate autophagy.

RIPK3 AND NECROPTOSIS

Necroptosis is a genetically regulated form of necrotic cell death that has emerged as an important pathway mediating the pathology of human diseases (112). Necroptosis can be activated by RIPK1 kinase, which in turn promotes the activation of RIPK3 (**Figure 1**), or by the interaction of RIPK3 with other RHIM-containing proteins, such as ZBP1 or TRIF (**Figure 4**).

RHIM-Dependent Activation of RIPK3 by Activated RIPK1

Necroptosis can be induced when cells are stimulated by TNFα in the caspase-8 inhibition condition. The RHIM-mediated interaction between activated RIPK1 and RIPK3 during necroptosis results in the formation of a large amyloid-like complex that serves as a platform for RIPK3 activation (73, 90) (**Figure 1**). The activation of RIPK3 is a key step in the initiation of necroptosis, which leads to the autophosphorylation of murine RIPK3 at T231 and S232 and human RIPK3 at S227 (14, 49, 170). RIPK3 in turn phosphorylates pseudokinase MLKL to induce a conformational change, homo-oligomerization, and translocation to the plasma membrane to promote membrane permeabilization and cell lysis (140, 159).

Activation of RIPK3 by Other RHIM-Containing Signaling Molecules

TLR3 or TLR4, or the virus-sensing adaptor protein ZBP1, is triggered by the corresponding ligand (50). While the activation of RIPK1 in the TNFR1 signaling pathway promotes its interaction with RIPK3 via respective RHIMs to mediate necroptosis, the RHIM of RIPK1 performs an inhibitory function in the TLR3/TLR4 signaling pathway by binding with the RHIM of TRIF and ZBP1, as RIPK1 knockout promotes the activation of RIPK3 by removing its inhibitory interaction with TRIF and ZBP1 (50) (**Figure 4**). The RHIM-mediated interaction between RIPK3 and TRIF leads to RIPK3 activation in TLR3 and TLR4 pathways under conditions of caspase-8 inhibition (48, 61).

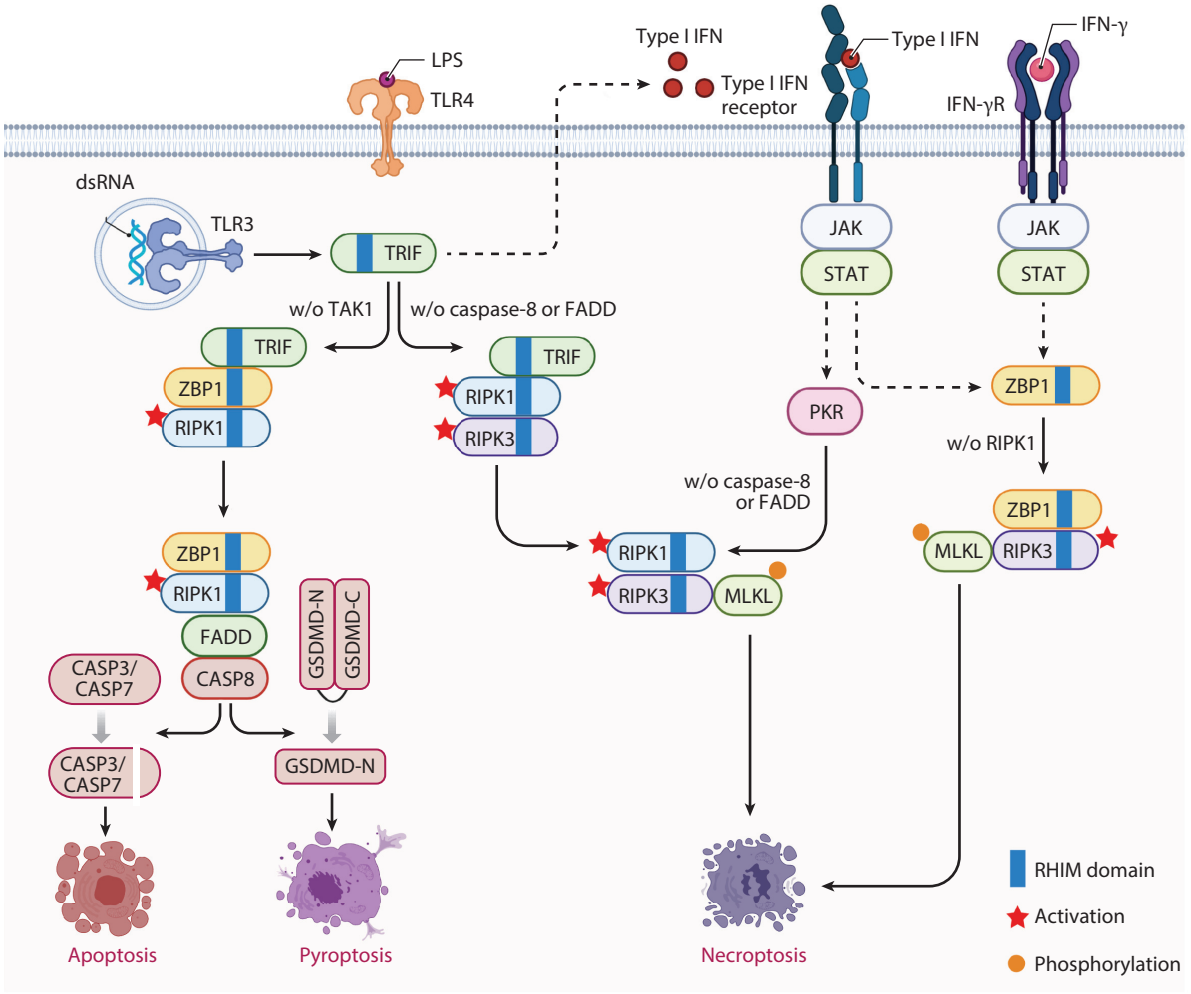


Figure 4

Regulation of RIPK1 and necroptosis by TLR3 and TLR4 pathways and type I and II IFN pathways. (*Left*) Necroptosis can be activated by the stimulation of TLR3 or TLR4 by dsRNA or LPS, respectively, in conditions when caspase-8 or FADD is inhibited or depleted. TRIF is required for the activation of RIPK1 and RIPK3 via RHIM-mediated interaction. In conditions when TAK1 is inhibited, the activation of TLR4 by LPS leads to RIPK1- and caspase-8-dependent apoptosis via cleavage of caspase-3 and pyroptosis via cleavage of GSDMD. In this condition, TRIF binds to ZBP1 via an RHIM domain interaction. This binding recruits RIPK1, which in turn mediates the recruitment of FADD and caspase-8 to promote apoptosis and pyroptosis. (*Right*) Both type I and II IFNs activate RIPK1- and RIPK3-dependent necroptosis when caspase-8 or FADD is depleted. IFNs induce JAK/STAT-dependent transcriptional activation of the latent kinase PKR, which initiates the formation of the necrosome. In addition, TLR4-induced production of type I IFN also contributes to necroptosis triggered by LPS/zVAD. In the absence of RIPK1, the ISG product, ZBP1, mediates the pro-necroptotic signal. Dotted arrows represent transcription of the indicated target gene. Abbreviations: CASP, caspase; dsRNA, double-stranded RNA; FADD, FAS-associated death domain protein; GSDMD, gasdermin D; IFN, interferon; ISG, IFN-stimulated gene; JAK, Janus kinases; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like pseudokinase; PKR, protein kinase R; RHIM, receptor-interacting protein homotypic interaction motif; RIPK, receptor-interacting serine/threonine protein kinase; STAT, signal transducer and activator of transcription proteins; TAK1, transforming growth factor-β–activated kinase 1; TLR, Toll-like receptor; TRIF, TIR-domain-containing adaptor-inducing IFNβ; w/o, without; ZBP1, Z-DNA-binding protein 1; zVAD, z-VAD.fmk. Figure adapted from images created with BioRender.com.

RIPK3 can be activated by another cellular RHIM-containing protein, ZBP1 (148, 156) (**Figure 4**). ZBP1 was identified as a sensor of double-stranded DNA (dsDNA) involved in the activation of innate immune responses (145). ZBP1 is unique among cell death proteins since it contains a RHIM domain to mediate cell death and also a Zα domain, which binds Z-nucleic acids, including Z-DNA (44, 131) and Z-RNA (80, 121, 148). dsDNA typically adopts the B-conformation, while dsRNA is usually in the A-conformation. Both DNA and RNA can adopt a Z-conformation that is characterized by a left-handed helical arrangement (45, 158). ZBP1 can sense Z-form nucleic acids from influenza A viruses (IAVs) to trigger necroptosis of macrophages (171). IAV is a single-stranded negative-sense segmented RNA virus that can induce cell death in a RIPK3-dependent manner (106). Subsequently, it has been shown that IAV activates RIPK3-mediated necroptosis through ZBP1 (66, 148). The ligand that triggers ZBP1-mediated RIPK3 activation upon IAV infection is somewhat disputed (66, 148). However, more recent data demonstrate that replication of IAV during infection generates Z-RNAs, which activate ZBP1 in infected cells and eventually cause RIPK3-dependent necroptosis (171). In addition to necroptosis, RIPK3 also contributes to apoptosis during IAV infection. In the absence of necroptosis executor MLKL, IAV-induced activation of RIPK3 instead triggered apoptosis in a RIPK1-, FADD-, and caspase-8-dependent manner, indicating a compensatory mechanism for the loss of necroptosis in eliminating IAV (106). Consistently, *Mlkl*$^{-/-}$*Fadd*$^{-/-}$ double knockout mice are more susceptible to IAV infection than either wild-type or *Mlkl*$^{-/-}$ mice alone (106). The presence of RIPK3-dependent apoptosis in IAV infection suggests a reverse signaling from the necrosome, which is also found in the settings of RIPK3 inhibition or conformation change.

Genetic studies have shown that ZBP1 can also induce necroptosis in sterile settings when the scaffold function of RIPK1 is disrupted, as described above (77, 104). However, the ligand that triggers ZBP1 in this setting is unclear. ZBP1 can cross-link to endogenous RNA in uninfected cells (80). Cells expressing high levels of ZBP1 undergo necroptosis when caspase-8 is blocked, suggesting the possibility that ZBP1 continuously senses self-RNA and triggers necroptosis when sufficient levels of downstream signaling component RIPK3 become available, as is the case when caspase-8 is blocked (80). However, the exact nature of the endogenous RNAs that are sensed by ZBP1 remain unknown at present. Two groups recently demonstrated that Z-RNA derived from endogenous retroelements, especially endogenous retroviruses (ERVs), is the Zα domain ligand that triggers the activation of ZBP1 in sterile conditions (58, 161). ERVs are Z-form retrovirus-like elements with long repeats that, in humans, comprise 5–8% of the genome (4). Derepressed endogenous retroviral RNA derived from double-stranded RNAs (dsRNAs) in cells may act as a ligand to prime ZBP1 to mediate necroptosis (58, 161). The ERV-derived Z-RNA could therefore instruct the assembly of cell death signaling scaffolds, which facilitate RIPK3 activation via the RHIM-induced interaction of RIPK3 and ZBP1 (58, 161, 171). ZBP1 can also drive RIPK3-mediated cell death induced by interferon in the absence of RIPK1 (56). Thus, RIPK3 can act as a signal-integrating molecule for necroptosis by receiving signals from diverse pathways via a homotypic RHIM interaction.

Genetic Regulation of RIPK3 Kinase Activity and Adaptor Function

Genetic studies of disease models have demonstrated the involvement of necroptosis in exacerbating pathology. RIPK3- or MLKL-deficient mutant mice have been used as genetic models to explore the functional roles of RIPK3 and MLKL as pathogenic determinants and biomarkers of disease progression and severity in models of human diseases, including inflammatory diseases, as well as hepatic, renal, cardiovascular, skin, pulmonary, and neurodegenerative diseases (15, 91, 133, 167).

Unlike *Ripk3*$^{-/-}$ mice, which develop normally with no gross or histological abnormalities, mice engineered to express a version of catalytically inactive kinase, *Ripk3*-D161N mutant mice, die at E11.5 (101). Interestingly, the *Ripk3*-D161N knock-in mutation promotes lethal RIPK1- and caspase-8-dependent apoptosis. *Ripk3*$^{D161N/D161N}$*Casp8*$^{-/-}$ mice are viable, demonstrating the role of caspase-8-mediated apoptosis in promoting the embryonic lethality of RIPK3-D161N mice (101). However, mice with a different knock-in version of catalytically inactive RIPK3-K51A with reduced RIPK3 expression not only survive to birth, but are viable and fertile (82). These genetic studies suggest the possibility that the pharmacological or genetic inhibition of RIPK3 kinase may alter the conformation of RIPK3, which can then act as a platform and recruit RIPK1 and FADD to promote the activation of caspase-8 and, subsequently, apoptosis. Treatment with a RIPK3 inhibitor in cells expressing a catalytically inactive RIPK3-K51A mutant can also unleash a proapoptotic activity of RIPK3 (82); this suggests a kinase-independent function of RIPK3 in promoting apoptosis. Thus, in the context of RIPK3 inactivation by chemical inhibition or D161N mutation, RIPK3 serves as a predominantly proapoptotic adaptor to promote apoptosis by recruiting RIPK1, FADD, and caspase-8.

REGULATION OF RIPK1 AND RIPK3 TURNOVER

The protein levels of RIPK1 and RIPK3 in cells can be a critical determinant of their sensitivity to the activation of apoptosis and necroptosis. In particular, the inhibition of proteasome or lysosome activity, which affects the levels of RIPK1, can promote the activation of RIPK1 (110, 111). Given the role of RIPK1 as an upstream regulator of cell death, the status of RIPK1 may act as a sensor that monitors cellular homeostasis.

Autophagy Deficiency–Induced Accumulation of RIPK1 and RIPK3

The degradation of RHIM-domain proteins, including RIPK1 and RIPK3, is mediated by autophagy (76). Autophagy deficiency as the result of autophagy gene autophagy-related 16-like 1 (*Atg16L1*) mutation leads to accumulated insoluble forms of RIPK1 and RIPK3 and enhancement of necroptosis. Variants of Atg16L1 and other autophagy-related genes have been identified in GWASs as risk factors of Crohn's disease, a debilitating IBD (138). The T300A *Atg16L1* mutant introduces a neo-caspase cleavage site that promotes its degradation by caspase-3 and increases the incidence of Crohn's disease (95). TNFα stimulation of *Atg16L1*$^{-/-}$ intestinal organoids induces necroptosis (84). More importantly, pharmacological inhibition of RIPK1 kinase activity by treatment with Nec-1s ameliorates IBD-related pathological features in *Atg16L1* IEC conditional knockout mice (84). These findings suggest that RIPK1-dependent necroptosis is a therapeutic target for autophagy-deficiency-induced IBD.

Proteasomal Turnover of RIPK1 and RIPK3

The expression levels of RIPK1 and RIPK3 are increased in the spinal cords of mice with optineurin (OPTN) deficiency, and RIPK1 activation is observed in a novel inflammatory microglial state in *Optn*$^{-/-}$ mice (57, 89). OPTN interacts with RIPK1 and promotes its K48 ubiquitination—a key signal for proteasomal degradation. The proteasomal turnover rate of RIPK1 is therefore decreased in *Optn* $^{-/-}$ MEFs (179).

Three types of OPTN mutations have been identified in ALS patients, including a homozygous deletion of exon 5, a homozygous Q398X mutation that causes a premature stop during translation, and a heterozygous E478G mutation (83). The first two mutations of OPTN lead to a decrease in OPTN expression via nonsense-mediated messenger RNA decay. The heterozygous

E478G mutation increases the level and changes the cellular distribution of OPTN, which may disturb its neuronal functions and promote inclusion body formation in ALS.

Regulation of RIPK1 and RIPK3 Degradation by CHIP

Both RIPK1 and RIPK3 expression levels are negatively regulated by carboxyl terminus of Hsp70-interacting protein (CHIP; also known as STUB1) E3 ligase-mediated ubiquitination. *Chip*$^{-/-}$ MEFs and CHIP-depleted L929 and HT-29 cells exhibit higher levels of RIPK1 and RIPK3 expression, resulting in increased sensitivity to necroptosis induced by TNFα (132). Both RIPK1 and RIPK3 can be ubiquitinated by CHIP, which leads to their lysosomal degradation. *Chip*$^{-/-}$ mice die within a few weeks after birth, displaying severe inflammation in the thymus and massive cell death in the small intestinal tract. These phenotypes can be rescued by RIPK3 deficiency. Thus, CHIP deficiency promotes necroptosis.

OTHER SIGNALING TRANSDUCTION PROCESSES THAT REGULATE RIPK1 AND NECROPTOSIS

Beyond the critical role of RIPK1 in mediating TNFR1 signaling pathways, other inflammatory signaling pathways have also been found to be able to regulate RIPK1 activation and RIPK3-dependent necroptosis (**Figure 4**).

Toll-Like Receptor 3 and Toll-Like Receptor 4 Pathways

RIPK1 activation can also be mediated by TLR3 and TLR4 pathways under conditions of caspase-8 inhibition (35, 48, 61) or TAK1 inhibition (93, 94). TLR3 recognizes viral dsRNA or the synthetic dsRNA polyinosinic:polycytidylic acid [poly(I:C)], while TLR4 is activated by the LPS of Gram-negative bacteria (38). In macrophages, when caspase-8 activity is inhibited by the pan-caspase chemical inhibitor z-VAD.fmk, both poly(I:C) and LPS can induce RIPK1-dependent necroptosis and inflammation via the adaptor protein TRIF (48, 61, 99). TRIF is an adaptor downstream of TLR3 and TLR4 that initiates the activation of NF-κB and transcription factor IRF3-mediated type I interferon response (38). Like RIPK1 and RIPK3, TRIF also contains a RHIM that can recruit RIPK1 and RIPK3 via their own RHIM domains following the activation of TLR3 and/or TLR4. Interestingly, the requirement for RIPK1 kinase activity in TLR3- and TLR4-mediated necroptosis is cell-type dependent. In macrophages, both TLR3- and TLR4-induced necroptosis can be prevented by the inhibition of the kinase activity of RIPK1, while in mouse fibroblasts, TLR3-induced necroptosis does not require RIPK1 kinase activity (61), suggesting that RIPK3 can be activated by the binding of TRIF, independent of RIPK1.

When TAK1 is inhibited by chemical inhibitor (5Z)-7-oxozeaenol, LPS can induce RIPK1-kinase-dependent apoptosis and pyroptosis (94). The effector protein YopJ in *Yersinia* species bacteria can also block the activation of TAK1 and activate RIPK1 (85). The activation of caspase-8 in *Yersinia*-infected cells mediated by RIPK1 triggers apoptosis via cleavage of caspase-3 and pyroptosis via cleavage of GSDMD (94, 115). In addition, ZBP1 may also be involved in LPS/TAK1 inhibition or *Yersinia*-induced macrophage cell death (93).

Type I and Type II Interferon Pathway–Mediated Necroptosis

Salmonella enterica serovar Typhimurium can induce massive macrophage necroptosis that leads to a compromised innate immunity response and lethality in mice (79, 127). The death of

macrophages induced by *S.* Typhimurium is reduced by the knockout of the receptor for type I interferons (*Ifnar1*$^{-/-}$ mice) but is not affected by TNFR1/TNFR2 double knockout, suggesting the involvement of type I interferons but not TNFα. Inhibition of RIPK1 kinase by Nec-1s and by RIPK3 deficiency reduces the death of macrophages induced by *S.* Typhimurium (127). IFNAR binds to RIPK1 in *S.* Typhimurium-infected macrophages, suggesting that *S.* Typhimurium infection induces the production of type I interferon, which drives necroptosis of macrophages mediated by RIPK1 activity and RIPK3. The pathway mediated by IFNAR to promote necroptosis should be further investigated.

Both type I and type II interferons activate RIPK1- and RIPK3-dependent necroptosis in MEFs when the adaptor protein FADD is absent or inactivated by phosphorylation, or when caspase-8 is inactivated (149) (**Figure 4**). Mechanistically, interferons induce JAK/STAT-dependent transcriptional activation of the latent kinase PKR. Once activated, PKR initiates the formation of the necrosome, comprising PKR, RIPK1, and RIPK3, that executes necroptosis. FADD and caspase-8 inhibit the formation of the PKR-containing necrosome, and this inhibition is relieved when FADD is phosphorylated and/or when caspase-8 is inactivated (149). Interferons also induce necroptosis in the absence of RIPK1 in L929 fibroblast cells, which requires interferon-induced *Zbp1* gene expression. In this condition, the ZBP1 RHIM domain interacts with RIPK3 to initiate RIPK3-dependent necroptosis (166).

Taken together, signaling pathways activated by TLRs and interferons can also activate necroptosis under specific conditions.

CONCLUDING REMARKS

Necroptosis was discovered as a regulated necrotic cell death mechanism in a chemical biological screening study (24). Understanding the molecular mechanisms by which the activation of RIPK1 kinase mediates necroptosis and inflammation has played an important role in defining human genetic conditions that may benefit from the therapeutic application of RIPK1 inhibitors. In particular, such studies have led to the characterization of human genetic variants that lead to loss- or gain-of-function mutations in RIPK1. In addition, the characterization of complex I has implicated the critical roles of multiple key mediators of innate immunity, including TAK1, NEMO, A20, ABIN1, OTULIN, and the LUBAC, in regulating the activation of RIPK1 and connections to RIPK1 activation in patients carrying disease variants of these genes. Genetic studies of mutant mice bearing loss-of-function alleles of TAK1, NEMO, A20, ABIN1, OTULIN, and the LUBAC have further verified the participation of RIPK1 and necroptosis as an important contributor to the pathology of these autoinflammatory human diseases. These studies have also implicated the involvement of RIPK1-mediated inflammation and cell death in human diseases that may or may not have a strong genetic predisposition, including neurodegenerative diseases such as ALS and AD. With ongoing advances that increase the number of RIPK1 inhibitors in human clinical studies for the treatment of many different human inflammatory and degenerative diseases, we expect that genetic studies of RIPK1-regulated human diseases will continue to provide helpful guidance and new, exciting directions.

FUTURE ISSUES

1. The activation of receptor-interacting protein kinase 1 (RIPK1) is extensively regulated by the components of complex I, which mediate complicated posttranslational modifications of RIPK1 to control its activation. It will be interesting to define the precise

modification patterns that dictate the activation or inhibition of RIPK1 to control cell death and survival.

2. Extensive research has documented the role of dysregulated RIPK1 in mediating autoinflammatory diseases in patients with genetic variants of complex I components. Given the role of TNFα in mediating inflammation in general, it will be interesting to investigate the role of RIPK1 in human inflammatory diseases without a direct link to complex I components.
3. RIPK1 kinase has been implicated in multiple aging-related neurodegenerative diseases, including amyotrophic lateral sclerosis and Alzheimer's disease. It will be interesting to investigate the role and mechanism of aging that regulate RIPK1 kinase activation.
4. RIPK1 kinase activity has been implicated in mediating neuroinflammation in microglia, which promotes the degeneration of neurons and oligodendrocytes non-cell-autonomously. RIPK1 kinase activity may also induce the death of neurons or oligodendrocytes cell autonomously. The mechanisms of such microglia-neuron or microglia-oligodendrocyte crosstalk are currently unclear, and the underlying mechanisms also need to be elucidated.
5. RIPK1 has been implicated in mediating the transcriptional induction of proinflammatory cytokines. The mechanism by which RIPK1 coordinates the transcription to promote an inflammatory response needs to be investigated.
6. RIPK1 inhibitors are currently being tested in multiple human clinical trials for the treatment of neurodegenerative and inflammatory diseases. It will be important to identify additional clinical biomarkers and perform image-based analysis to define the status of RIPK1 in vivo.

DISCLOSURE STATEMENT

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