

Review

Initiation and execution mechanisms of necroptosis: an overview

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Necroptosis is a form of regulated cell death, which is induced by ligand binding to TNF family death domain receptors, pattern recognizing receptors and virus sensors. The common feature of these receptor systems is the implication of proteins, which contain a receptor interaction protein kinase (RIPK) homology interaction motif (RHIM) mediating recruitment and activation of receptor-interacting protein kinase 3 (RIPK3), which ultimately activates the necroptosis executioner mixed lineage kinase domain-like (MLKL). In case of the TNF family members, the initiator is the survival- and cell death-regulating RIPK1 kinase, in the case of Toll-like receptor 3/4 (TLR3/4), a RHIM-containing adaptor, called TRIF, while in the case of Z-DNA-binding protein ZBP1/DAI, the cytosolic viral sensor itself contains a RHIM domain. In this review, we discuss the different protein complexes that serve as nucleation platforms for necroptosis and the mechanism of execution of necroptosis. Transgenic models (knockout, kinase-dead knock-in) and pharmacologic inhibition indicate that RIPK1, RIPK3 or MLKL are implicated in many inflammatory, degenerative and infectious diseases. However, the conclusion of necroptosis being solely involved in the etiology of diseases is blurred by the pleiotropic roles of RIPK1 and RIPK3 in other cellular processes such as apoptosis and inflammasome activation.

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Receptor-interacting proteins (RIPK1) exist in two major forms: as a ‘closed conformation’, that is, kinase-deficient, and activates NF- κ B and MAPK pathways, and as an ‘open conformation’, that is, kinase-active, and initiates apoptosis and necroptosis.

The open kinase-active form of RIPK1 recruits and activates RIPK3 through homotypic interaction involving the RHIM domain.

RIPK3 can also be activated through the interaction with RHIM domain-containing adaptor TRIF or sensor ZBP1/DAI.

RIPK3 recruits and phosphorylates the pseudokinase MLKL, which represents the executioner of the necroptotic pathway. Transgenic models (knockout, kinase-dead knock-in) and pharmacologic inhibition indicate that RIPK1, RIPK3 or MLKL are implicated in many inflammatory, degenerative and infectious diseases.

Open Questions

- Can RHIM-mediated interactions be targeted for therapeutic purposes?
- What are the functions of RIPK3 and MLKL beyond necroptosis and how are they differentially regulated?
- Is MLKL the only substrate of RIPK3 involved in the execution of necroptosis?
- Is necroptosis as such the sole initiator of inflammation?

Cell death is an essential part of homeostasis in multicellular organisms as a way of removing damaged, infected or degenerated cells.¹ Cell death is also a crucial factor in sculpting our bodies.² In adults, every day about 10–100 billion cells die and are replaced by new healthy cells to maintain homeostasis of the whole organism.³ Hence, it is no surprise that disruption of this delicate balance between cell death and cell proliferation results in disease. Indeed, resistance to cell death is one of the hallmarks of cancer cells,⁴ and it is well established that several viruses actively prevent the suicide of their host cell to ensure viral replication.⁵ On the other hand, too much cell death results in impaired organ function as is illustrated by heart- and brain infarct, or alcohol- or drug-induced liver injury.⁶ Historically, three morphologically distinct types of cell death were observed by Schweichel and Merker⁷ in embryonic tissues exposed to toxic drugs and were categorized based on phagocytic clearance (type I–III).⁸ Type I cell death was associated with heterophagy, while type II cell death featured autophagy and type III dead cells were not removed by hetero/autophagy.^{7,8} On the basis of the occurrence of type I cell death in healthy tissues and during embryonic development, it was proposed that type I cell death was controlled and hence termed ‘apoptosis’ by Kerr *et al.*⁹ (apoptosis = ‘falling off’ of leaves from a tree). Apoptotic cell death morphology is characterized by shrinkage of both nucleus (pyknosis) and cytoplasm, chromatin condensation (karyorrhexis), nuclear fragmentation, and the formation of apoptotic bodies. Intact apoptotic dying cells and apoptotic

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bodies are recognized and phagocytosed by surrounding cells.⁹ Type II cell death features extensive intracellular phagosome formation, retrospectively interpreted as autophagic digestion of cytosolic contents and organelles, and therefore referred to as autophagic cell death.⁸ However, it is better to call it cell death associated with autophagy, since autophagy does not contribute to the cell death process.¹⁰ Type III cell death, currently known as necrosis, is characterized by cellular rounding and swelling, increased cytoplasmic granularity, intact nuclei, and finally, membrane rupture and expulsion of cellular contents in a balloon-like morphology.¹¹ Originally, it was proposed by Kerr *et al.*⁹ that only apoptosis was genetically regulated, and that necrotic cell death was the result of irreversible damage. Research in *Caenorhabditis elegans* led to the discovery of the genetic program of apoptotic cell death that was confirmed and elaborated in biochemical details in vertebrates.^{12,13}

However, a pioneering publication in 1988 indicated that tumor necrosis factor (TNF) can induce both apoptotic and necrotic cell death depending on the cell line.¹⁴ Nowadays, it is clear that this necrotic cell death can be blocked by chemical inhibition, knockdown or knockout of specific genes, proving that necrosis can be regulated.^{15–19} The most studied subtype of regulated necrosis is TNF-induced necroptosis, which depends on RIPK1, RIPK3 and mixed lineage kinase domain-like (MLKL).^{15–18} Besides necroptosis, other types of regulated necrosis include caspase-1 and gasdermin D-dependent pyroptosis,²⁰ poly-(ADP ribose)-polymerase (PARP)-dependent parthanatos,²¹ iron-dependent ferroptosis,²² and NADPH oxidase (NOX2)-mediated NETosis (neutrophil extracellular trap).²³ Even secondary necrosis, which occurs when apoptotic cells are not removed by phagocytosis, appears to be regulated. In this case, proteolytic cleavage of deafness-associated tumor suppressor (DFNA5) by caspase-3 triggers secondary necrosis.²⁴ Figure 1 gives an overview of different types of regulated cell death, and of the core pathways involved in the initiation and execution of cell death. In the following sections, we outline the occurrence of necroptosis in embryonic development, homeostasis and disease, as a prelude to an overview of necroptotic signaling.

Biological Relevance of Necroptosis

The biological relevance of necrosis (and more specifically necroptosis) appears to be mainly related to diseases. Indeed, while apoptosis clearly occurs during embryonic development as a vital process sculpting body shape²⁵ and regulating cell populations,⁹ there are only a few indications that necrosis as a cell death process actually occurs during embryonic development and under physiological conditions. These are discussed below, followed by an overview of the implication of necroptosis in disease.

Necrosis occurring under physiological conditions.

Examples of necrosis during embryonic development have been observed in worms and chick embryos. During the development of *C. elegans*, the Linker cell helps to shape the gonads in male worms and then dies with a necrotic morphology.²⁶ During normal embryonic development of chicks, necrotic morphology of developing ciliary ganglia

and motor neurons of the spinal cord is observed.^{27,28} Another peculiar example of the occurrence of necrosis is found during development in pigs where the embryonic sacs in pigs show a necrotic cell morphology at the tips (necrotic apex) from halfway during pregnancy until birth, arguing for the occurrence of necrotic cell death under physiological conditions long before birth and expulsion of the placenta.²⁹ Of note, it cannot be excluded that these processes are due to secondary necrosis, occurring after an unnoticed apoptotic stage. Nonetheless, there have been a few reports describing the ability of the organism to bypass apoptosis by necrotic cell death when caspase activation is affected or absent. Indeed, mice deficient for apoptotic peptidase activating factor 1 (Apaf1) or where caspase activity is blocked, the interdigital webs of mouse embryos are removed by necrotic cell death instead of apoptosis,^{30,31} eventually resulting in the same limb morphology. Similarly, programmed cell death of spinal motor neurons in caspase-3 or caspase-9-deficient mice is delayed as compared to wild-type mice and displays typical necrotic features.³²

Altogether, it appears that necrosis during animal embryonic development only occurs sporadically and in rare instances, or as a backup method when apoptosis is impaired. In the context of this review on necroptosis signaling, it is important to mention that there is no information available on whether necrosis occurring during normal embryogenesis and physiological processes can be attributed to necroptosis. However, since neither the kinase-dead RIPK1 knock-in mice,³³ nor the RIPK3³⁴ or MLKL³⁵ knockout mice do have a spontaneous phenotype under non-challenged conditions, this strongly suggests that the core necroptotic pathway is mainly implicated in pathophysiological conditions of infection and disease.

Necroptosis in disease. In cells, necroptosis can be initiated by a variety of triggers, including TNF, Fas, TNF-related apoptosis-inducing ligand (TRAIL), interferon (IFN), lipopolysaccharide, dsRNA, DNA damage, endoplasmic reticulum stress, viral infection and anti-cancer drugs.^{36–44} Necrotic tissues *in vivo* have been described in the context of disease, such as gangrene, as early as the fifth century BC (Galen. *On the constitution of the art of medicine; The art of medicine; A method of medicine to Glaucon*). Indeed, long before the morphological definition of apoptosis, necrotic cells were linked to pathological conditions, such as alcohol abuse.⁹ The first proof that at least some of this disease-associated necrosis could be attributed to necroptosis, came with the discovery of necrostatin-1 (Nec-1),¹⁵ the first inhibitor of RIPK1.⁴⁵ It was shown that administration of Nec-1 could reduce cell death and/or mortality in several disease models, including brain infarct,¹⁵ cardiac infarct,^{46,47} TNF-induced systemic inflammatory respiratory syndrome (SIRS),⁴⁸ ConA- or acetaminophen-induced hepatitis,^{39,49} and ischemia-reperfusion injury (IRI) in kidneys.⁵⁰

However, Nec-1 was used at varying concentrations in these studies, which complicates their interpretation considering more recent findings. Indeed, Nec-1 is identical to methylthiohydantoin-tryptophan (MTH-Trp),⁵¹ a known inhibitor of indoleamine 2,3-dioxygenase (IDO), an important immune-regulatory enzyme. At low concentrations (0.6 mg/kg), Nec-1 can potentiate TNF-induced SIRS⁵² and cerulein-induced

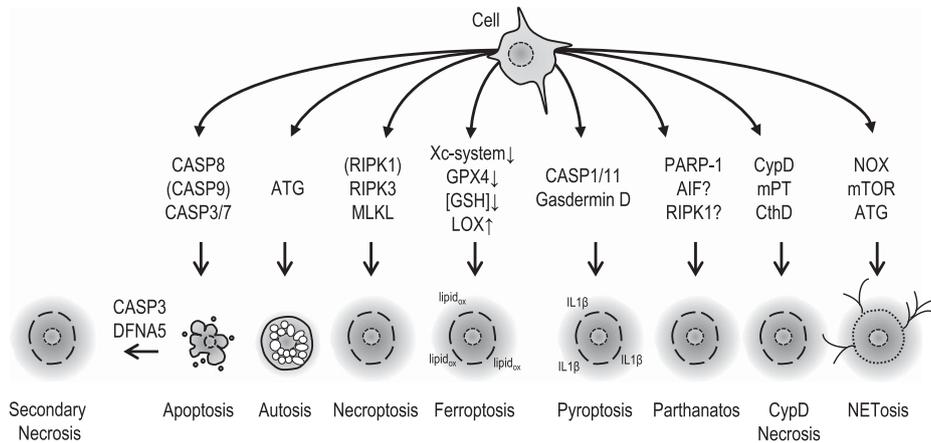


Figure 1 An overview of regulated cell death modalities and the core pathway proteins involved. Depending on cell type and intra/extracellular conditions, cells can activate different cell death modalities with different core pathway proteins involved. Cell death modalities with an apoptotic or an autophagic morphology are considered to be less immunogenic than cell death with a necrotic morphology, because the former two are consisting of containment programs aimed at preventing the release of intracellular content and associated with efficient phagocytosis, while necrotic cell death is essentially associated with membrane permeabilization resulting the rapid release of the cellular contents in the environment acting as danger or damage-associated molecular patterns (represented by a gray halo) and attracting immune cells. Note that in many conditions, induction of autophagy is not associated with cell death, but rather a homeostasis mechanism following cellular stress. However, in some conditions, autophagy may proceed to cell death and targeting Atg genes will retard in such cases the cell death process. AIF, apoptosis-inducing factor; ATG, autophagy related; CASP, caspase; CypD, cyclophilin D; DFNA5, deafness-associated tumor suppressor; GPX4, glutathione peroxidase 4; GSH, reduced glutathione; IL1 β , interleukin-1 β ; LOX, lipoxygenase; lipid_{ox}, peroxidized (phospho)lipids; MLKL, mixed lineage kinase domain-like; mPT, mitochondrial permeability transition pore; mTOR, mechanistic target of rapamycin; NOX, NADPH oxidase; PARP, poly-(ADP-ribose)-polymerase; RIPK, receptor-interacting protein kinase; Xc-system, cystin/glutamate antiporter

pancreatitis in mice.^{52,53} Given that Nec-1 can inhibit IDO *in vitro*, this contra-intuitive observation could be due to inhibition of IDO, or perhaps due to other yet unknown off-target effects. Hence, the data obtained by Nec-1 administration may complicate the interpretation of the *in vivo* results in those experimental disease models where IDO has been implicated such as inflammation-associated tumorigenesis.⁵⁴ Therefore, the initial findings established with Nec-1 should be challenged with experimental disease models using RIPK1 kinase-dead knock-in mice and RIPK3 knockout mice. RIPK3^{-/-} mice show a prolonged survival upon IRI in kidneys,⁵⁵ TNF-induced SIRS^{48,56} and cerulein-induced pancreatitis.^{18,53} Furthermore, RIPK3 ablation resulted in severely delayed mortality caused by atherosclerotic plaques in apolipoprotein E (ApoE) knockout mice.⁵⁷ RIPK3^{-/-} mice are also protected from poly(I:C)-induced necrosis in the retina,⁵⁸ and have reduced liver damage caused by alcohol.⁵⁹ As is in all these studies, RIPK3 is lacking entirely both as a scaffold and as a kinase, and the observed protective effect of RIPK3 ablation might in theory be attributed to RIPK3 either function or both. It should be said that many studies at the beginning of the availability of RIPK3 knockout mice were rather necroptosis biased. An interesting systematic study comparing RIPK1 kinase-dead knock-in mice, RIPK3 knockout and MLKL knockout in different models of inflammation and tissue injury⁶⁰ revealed that loss of RIPK3 had no effect on lipopolysaccharide-induced sepsis, dextran sodium sulfate-induced colitis, cerulein-induced pancreatitis, hypoxia-induced cerebral edema or the major cerebral artery occlusion stroke model.⁶⁰ This suggests that previous studies with Nec-1, which intended to reveal a role for necroptosis probably revealed the multiple targeting effects of Nec-1 such as apoptosis and IDO. Catalytically inactive RIPK1 knock-in mice showed reduced renal IRI and TNF-induced SIRS, while similar protection was

only received in case when RIPK3 or MLKL were combined with caspase-8 knockout, suggesting a role for RIPK1 kinase activity in both necroptosis and apoptosis.⁶⁰ Interestingly, one study revealed an enhanced sensitivity of RIPK3 knockout mice in a model of DSS-colitis, suggesting that RIPK3 may also have tissue regenerative functions.⁶¹

Necroptosis can also be beneficial in certain pathological settings: it is known that cellular suicide can prevent the completion of pathogen replication cycles, and thus prevent disease progression. This is for instance the case for vaccinia infection and murine cytomegalovirus (MCMV) infection. Indeed, RIPK3^{-/-} mice and RIPK1-D138N kinase-dead knock-in mice show less tissue damage during vaccinia infection, but suffer from increased viral titers and die after vaccinia infection, unlike their wild-type littermates.^{37,62} In the case of MCMV, its M45 protein blocks necroptosis of the host cell by disturbing RIPK homotypic interaction motif (RHIM)-dependent interactions of the necrosome.⁶³ It was shown that MCMV strains with a mutated RHIM domain in M45 fail to replicate in cells and *in vivo*.⁶⁴ In RIPK3^{-/-} mice however, the M45-mutant MCMV replicates like the wild-type strain, which indicates that RIPK3-dependent necroptosis prevents MCMV replication.⁶⁴ Other viruses such as herpes simplex virus 1 and 2 appear to contain similar RHIM-proteins.⁶⁵ Recently, it was discovered that during infection of its host, *Escherichia coli* injects the cysteine protease EspL, which is able to cleave the RHIM domain of all known RHIM-containing proteins.⁶⁶ Interestingly, ectopic expression of EspL prevented TNF- or poly(I:C)-induced necroptosis in cells, while EspL protease activity contributes to persistent colonization of mice by the bacterial enteropathogen *Citrobacter rodentium*.⁶⁶ These findings indicate that necroptosis may have an anti-infectious function by preventing specific viral and bacterial infections, or even contribute to tissue generation.⁶¹

Table 1 Overview of studies linking necroptosis to human pathologies

Disease	Major finding	Reference
Ovarian cancer	Low MLKL expression associated with poor prognosis	138
Inflammatory bowel disease	High RIPK3 and MLKL expression	139
Drug-induced liver injury	Phosphorylated MLKL in biopsies	99
Chronic obstructive pulmonary disease	Increased levels of RIPK3 in lung epithelial cells	140
Multiple sclerosis	Increased levels of RIPK1, RIPK3 and necrosome formation in lesions	141
Breast cancer	Low RIPK3 expression due to epigenetic modification	142
Melanoma	Melanoma cell lines lack RIPK3 expression, whereas primary melanocytes strongly express RIPK3.	143
HIV	Dysfunctional HIV-specific CD8 ⁺ T-cell proliferation is associated with increased caspase-8 activity and mediated by necroptosis.	144
Colon cancer	Low expression of RIPK1 and RIPK3 due to hypoxia	145
Small cell lung carcinoma	Loss of RIPK3 expression due to epigenetic modification	146
Non-Hodgkin lymphoma	SNPs in the RIPK3 are correlated with increased risk of non-Hodgkin lymphoma	147
Pancreatic adenocarcinoma	Low expression of MLKL	148
Gastric cancer	Low MLKL expression associated with poor prognosis	149
Cervical squamous cell carcinoma	Low MLKL expression associated with poor prognosis	150
Toxic epidermal necrolysis	Phosphorylated MLKL in biopsies	151
Leukemia	RIPK3 is downregulated	152

An additional complication is that RIPK1, RIPK3, MLKL, Fas associated via DD (FADD) and caspase-8 may have additional functions besides cell death. Indeed, several studies have shown that under particular conditions (caspase-8 inhibition or IAP inhibition), RIPK3 and MLKL can also activate inflammasomes, thus promoting IL1 β maturation and tissue inflammation^{67–70} (and reviewed in refs 71–72). In contrast to the inflammation associated with massive (necroptotic) cell death, recent studies hint that moderate and chronic necroptosis may perhaps serve to dampen an inflammatory response. Indeed, in caspase-8-deficient mice, RIPK3 loss provided greater protection to TNF-induced SIRS compared to MLKL deficiency.⁶⁰ This could mean that MLKL shuts down the pro-inflammatory signaling emerging from RIPK1 and RIPK3 by inducing necroptotic cell death, a notion supported by other studies.^{73,74} Furthermore, RIPK1, RIPK3 and MLKL may have unknown functions in the nucleus as they translocate to the nucleus early during necroptosis.⁷⁵ In this context, it is interesting that nuclear proteins involved in mRNA splicing were found to interact with RIPK3 during necroptosis^{18,76} and that several proteins involved in the cell cycle were reported to interact with RIPK1,⁷⁷ RIPK3⁷⁶ and MLKL⁷⁸ (summarized in Supplementary Table 1).

In conclusion, targeting RIPK1, RIPK3 or MLKL, could be beneficial in several inflammatory and degenerative diseases. Inhibition of RIPK1 or RIPK3 has proven to improve the outcome of a long list of pathological mouse models, including IRI in brain,¹⁵ heart^{46,47} and kidney,⁵⁰ SIRS,^{48,56,79} atherosclerosis,⁵⁷ pancreatitis,^{18,53} hepatitis^{39,49} and liver damage.^{49,59} It is now understood that not only necroptosis is targeted in such settings, but also RIPK1 kinase-mediated apoptosis. Moreover, due to the pleiotropy of RIPK3 in regulating other processes such as inflammasome activation,^{67–70} apoptosis⁵⁶ and intestinal tissue regeneration,⁶¹ it is not clear whether necroptosis is the only cell death modality or process implicated in these pathologies. Of note, necroptosis is not always a detrimental process: in some pathologies, it may be beneficial to boost necroptosis. This may be the case when apoptosis cannot be activated due to absence of FADD or caspase-8 in cancer cells or in virally

infected cells.^{37,42,44} Hence, a better understanding of the molecular signaling mechanisms that result in necroptosis and of the pleiotropy of the molecules involved, is guaranteed to have therapeutic implications. Indeed, necroptosis has been identified in several human pathologies (summarized in Table 1).

Necroptosis Signaling

In the following section, the three main proteins directly involved in necroptotic signaling are introduced, viz. RIPK1, RIPK3 and MLKL, and discussed in the context of the canonical and non-canonical necrosome formation.

Individual proteins and their domain structure. Receptor-interacting protein 1 (RIPK1) was the first protein shown to be essential for Fas, TNF- and TRAIL-induced necroptosis. RIPK1 kinase activity was required for these processes.³⁶ RIPK1 is an integrator of cellular stress signals and possesses several domains that can activate different cellular pathways: an N-terminal kinase domain, an intermediary domain (ID) and a C-terminal RHIM, and death domain (DD, Figure 2).⁸⁰ Its N-terminal kinase domain is required for canonical necroptosis,^{36,81,82} but also for RIPK1-dependent apoptosis under specific conditions.^{83,84} The C-terminal death domain of RIPK1 is involved in activation of apoptosis by recruitment of FADD and caspase-8.^{85,86} The ID of RIPK1 serves as a scaffold for K63 and linear M1 ubiquitylation allowing the recruitment of the TGF β activated kinase 1 (TAK1) complex and the I κ B kinase (IKK) complex. This results in activation of ERK, JNK, p38 and the nuclear factor κ B (NF- κ B).^{8,81} Furthermore, RIPK1 possesses a RHIM domain located at the C-terminal part of the ID, which is required for recruitment of RIPK3 and for binding to other RHIM-containing proteins, such as TRIF, (Toll/IL-1R domain-containing adapter-inducing IFN- β , also known as TICAM1) and Z-DNA-binding protein 1 (ZBP1, also known as DAI, Figure 2).^{87–89} While the kinase activity of RIPK1 promotes necroptosis and apoptosis, recent evidence indicates that the

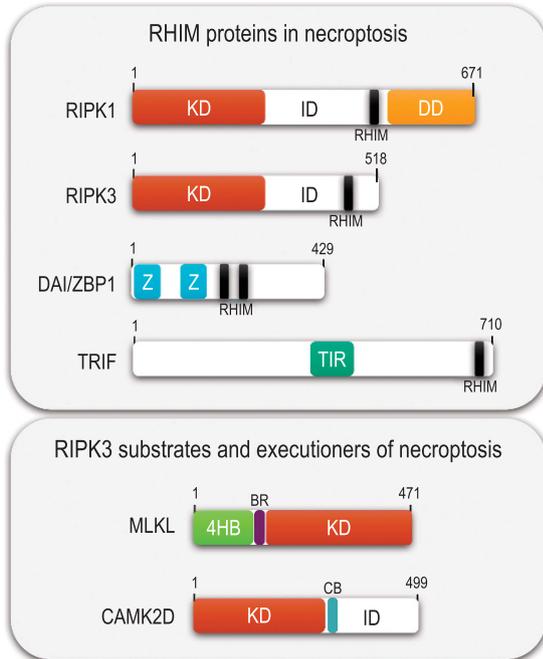


Figure 2 Domain structure of the key mediators of necroptosis. Length is indicated in number of amino acids. 4HB, four-helical bundle domain; BR, brace region; CAMK2D, calcium/calmodulin-dependent protein kinase type II subunit delta; CB, calmodulin-binding domain; DD, death domain; ID, intermediate domain; KD, kinase domain; MLKL, mixed lineage kinase domain-like; RHIM, RIP homotypic interaction motif; RIPK, receptor-interacting protein kinase; TIR, Toll/interleukin receptor domain; TRIF, TIR domain-containing adaptor-inducing IFN-beta (also known as TICAM1, TIR domain-containing adapter molecule 1); ZBP1, Z-DNA-binding protein 1 (also known as DAI, DNA-dependent activator of IFN-regulatory factors)

mere binding via its RHIM domain counteracts ZBP1/DAI and RIPK3-mediated necroptosis.^{90–92}

RIPK3 is a homologous kinase of RIPK1, with a similar N-terminal kinase domain, and an intermediate domain that can contribute to TNF-induced activation of NF- κ B if over-expressed (Figure 2). RIPK3 also contains a RHIM domain at its C-terminus, but lacks the C-terminal death domain compared to RIPK1.⁸⁰ RIPK3 kinase activity appears to be essential for necroptosis,^{18,37,38,79,93} although this was recently challenged by a single study demonstrating RIPK3-independent activation of MLKL in a model of RIPK1-dependent necroptosis (ConA-induced hepatitis).⁹⁴

Another key mediator of necroptosis is MLKL, a pseudokinase, which is currently seen as the sole and main effector of necroptosis.^{16,95–101} MLKL possesses an N-terminal four-helical bundle domain, which directly or indirectly results in membrane pore formation, but is kept inactive by its C-terminal pseudokinase domain (Figure 2).^{98,99,102} MLKL is in many cells constitutively associated with RIPK3, through an interaction of their kinase domains.¹⁶ Phosphorylation of human RIPK3 at Ser227 is required for its interaction with MLKL.¹⁶ Interestingly, the crystal structure of MLKL bound to RIPK3 revealed that MLKL forces RIPK3 in an inactive conformation,^{16,103} suggesting that RIPK3 and MLKL may exist as pre-assembled non-active complexes, while the RIPK3-mediated phosphorylation may release MLKL.

Different groups reported that MLKL either has no kinase activity,⁹⁶ or only a very modest kinase activity toward the artificial substrate myelin basic protein.⁹⁵ In contrast to RIPK1 and RIPK3, it appears that the kinase activity of MLKL (if any) is not required for necroptosis.^{95,96} Phosphorylation of MLKL by kinase-active RIPK3 induces a conformational shift, which exposes the four-helical bundle domain of MLKL.^{95,96,102}

More recently, calcium-dependent protein kinase II delta (CAMK2D) was identified as another potential substrate of RIPK3.¹⁰⁴ RIPK3 phosphorylates CAMK2D at Thr387, which results in the activation of CAMK2D.¹⁰⁴ Importantly, CAMK2D apparently executes necrotic cell death independently of MLKL.¹⁰⁴ CAMK2D regulates multiple ion channels by phosphorylation, including the L-type Ca²⁺ channel subunit beta-2 CACNB2,¹⁰⁵ the sarcolemmal cardiac Na⁺ channel SCN5A¹⁰⁶ and the K⁺ channel potassium channel, voltage gated Shal-related subfamily D (KCND3, also known as KV4.3).¹⁰⁷ Activation of these channels by CAMK2D results in an influx of extracellular ions,^{105–107} and in the case of necrotic cell death, most likely in eventual plasma membrane rupture. Interestingly, another group reported a reverse signaling cascade in neuroblastoma cells: first cytosolic calcium accumulation and subsequent CAMK2D activation resulting in RIPK1 activation and necroptosis.¹⁰⁸ The precise relation between CAMKs and necroptosis signaling remains to be determined.

Necroptosis initiation: necrosome formation

Canonical necrosomes: TNF-induced necroptosis is the best characterized necroptotic pathway. Upon TNF stimulation, RIPK1 and TNF receptor-associated DD (TRADD) are independently recruited to the TNF receptor by their DD domains (Figure 3a).^{109,110} At the TNF receptor complex (complex I), RIPK1 is ubiquitinated in its ID, allowing the recruitment of the I κ B kinase complex (NEMO, IKK α and IKK β).⁸¹ IKK α - and IKK β -dependent phosphorylation of RIPK1 prevents its dissociation from the receptor, and hence prevent the formation of a cytosolic pro-cell death complex (complex II).^{84,111} Different types of complex II can be distinguished (IIa and IIb), depending on the composition of complex II and the activity of the proteins therein (Figure 3b). While complex IIa and IIb induce apoptosis, they can also induce necroptosis if caspase-8 is inactive or absent.⁸¹ It is still unclear whether these complexes represent different physical entities or different compositions or post-translational regulations of essentially the same complex II. The phosphorylation and ubiquitylation of RIPK have been reviewed in detail elsewhere.^{112–115}

Complex IIa is formed after dissociation of TRADD from the TNF receptor 1 (TNFR1) and results in the recruitment of FADD, and subsequent recruitment and activation of caspase-8.^{111,116} The activation of caspase-8 and subsequent induction of apoptosis is independent of RIPK1 or its kinase activity.^{81,116} In case cIAPs, TAK1 or IKK α/β are inactive or absent, a similar complex is formed without TRADD (complex IIb, Figure 3b), where RIPK1 kinase activity is required for activation of caspase-8 and apoptosis.^{83,116,117} Hence, complex IIa formation results in RIPK1-independent apoptosis, while complex IIb promotes RIPK1 kinase activity dependent apoptosis.⁸¹

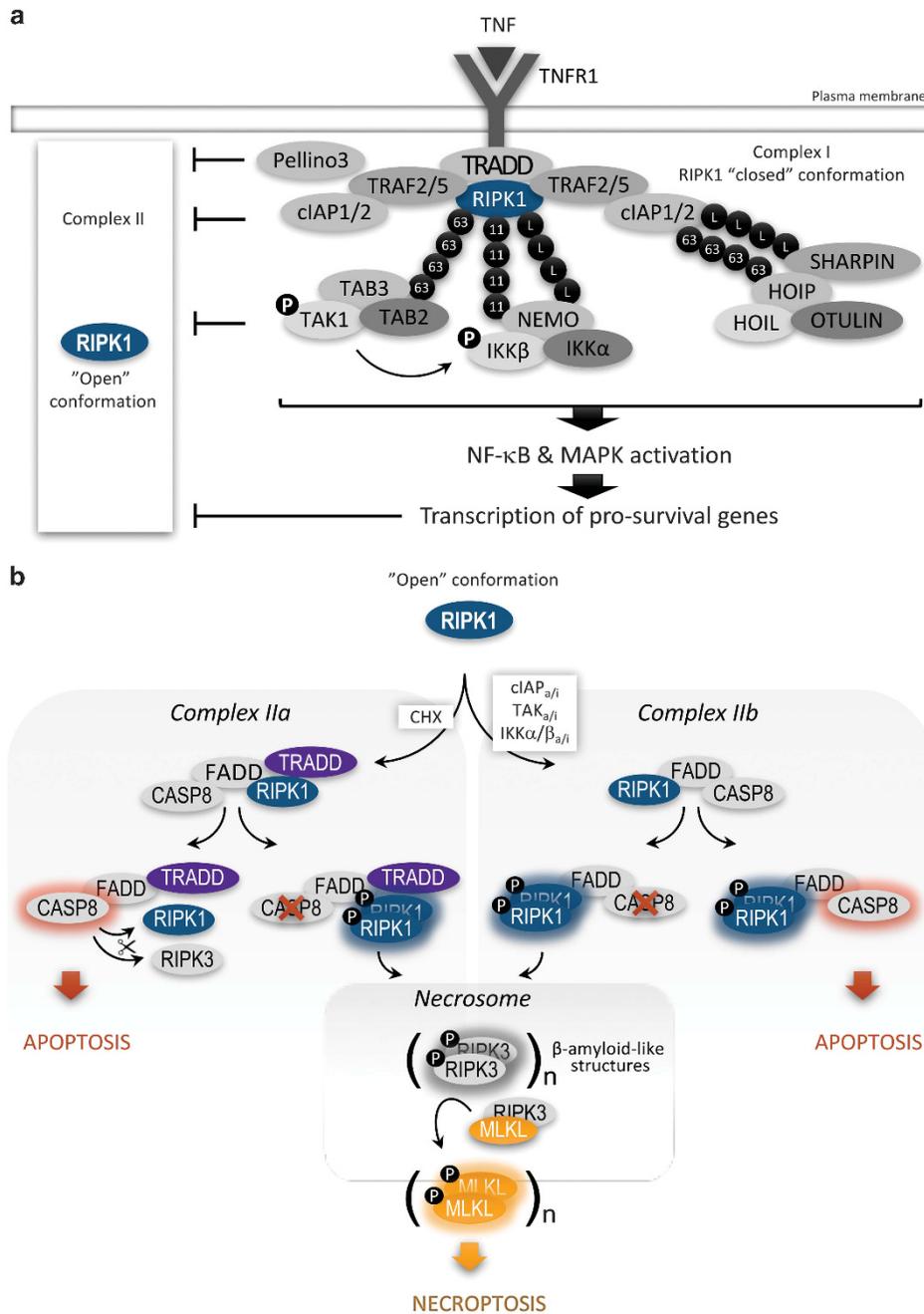


Figure 3 Signal transduction complexes in TNF-induced survival and cell death. **(a)** TNF stimulation results in formation of a receptor bound complex I, which essentially prevents cell death (see text). **(b)** TNF receptor dissociation of RIPK1 results in the formation of different pro-cell death complexes (complex IIa, IIb and the necrosome). Complex IIa contains TRADD and can be formed independent of the scaffold and kinase function of RIPK1. In contrast, complex IIb lacks TRADD and requires kinase-active RIPK1 for cell death induction. It is still unclear whether these complexes represent different physical entities or different compositions or post-translational regulations of essentially the same complex II. CASP, caspase; cIAP, cellular inhibitor of apoptosis protein; FADD, Fas-associated protein with death domain; HOIL-1 or RBCK1, RANBP2-type and C3HC4-type zinc-finger-containing 1; HOIP or RNF31, ring finger protein 31; IKK, inhibitor of NF-κB kinase; MLKL, mixed lineage kinase domain-like; NEMO, NF-κB essential modulator (sometimes misleadingly called IKK γ since it does not possess kinase activity); NF-κB, nuclear factor kappa B; Otulin, OTU deubiquitinase with linear linkage specificity; PELL1, pellino 1; RIPK, receptor-interacting protein kinase; Sharpin, SHANK-associated RH domain-interacting protein; TAB, TAK1-binding protein; TAK1, TGF β activated kinase 1; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNF-associated death domain

When caspase-8 is absent or inactivated, kinase-active RIPK1 in complex II recruits and activates RIPK3, resulting in the formation of the necrosome (Figure 3b). The necrosome formed from complex IIa consists of TRADD, RIPK1, RIPK3 and FADD, while the necrosome resulting from complex IIb

lacks TRADD.^{18,37,79} As a constitutive binding partner of RIPK3, MLKL is incorporated in the necrosome.¹⁶ Within the canonical necrosome, the kinase-active conformation of RIPK1 is required for the activation and autophosphorylation of RIPK3 at Ser199,⁷⁹ although transphosphorylation between

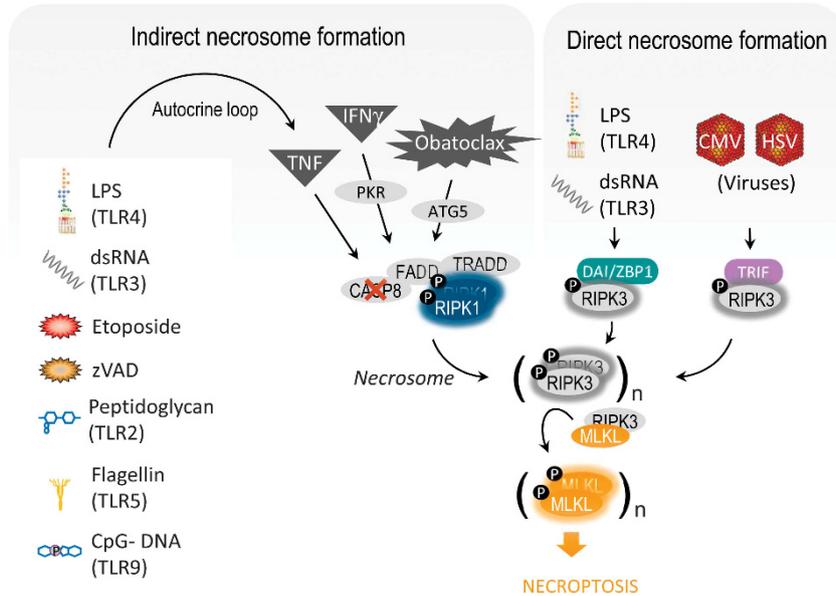


Figure 4 Direct and indirect necrosome formation by different stimuli. Many stimuli elicit necroptosis in a direct and indirect way. The direct necrosome formation involves the canonical pathway that requires RIPK1 kinase activity and the non-canonical pathway that is dependent on the TRIF adaptor or the ZBP1/DAI sensor. In the indirect way, stimuli elicit the production of TNF, which in its turn activates the canonical pathway. ATG5, autophagy related; CASP8, caspase; CMV, cytomegalovirus; FADD, Fas-associated protein with death domain; HSV, human Simian virus; IFN γ , interferon gamma; LPS, lipopolysaccharide; MLKL, mixed lineage kinase domain-like; PKR, dsRNA-activated protein kinase R (officially known as EIF2AK2); RIPK, receptor-interacting protein kinase; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRADD, TNF-associated death domain; TRIF, officially known as Toll-like receptor adaptor molecule (TICAM); ZBP1, Z-DNA-binding protein 1 (also known as DAI); zVAD, pan-caspase inhibitor

RIPK1 and RIPK3 is most probably not required since RIPK1 is unable to phosphorylate RIPK3 *in vitro*.^{16,37,87,117} Nonetheless, during TNF-induced necroptosis, RIPK3 is phosphorylated at Ser227 in a Nec-1 inhibitable manner,¹⁶ which led to the initial (wrong) idea of transphosphorylation between RIPK1 and RIPK3. Of note, pro-necrotic ubiquitylation of RIPK1 within the necrosome is also required for maintaining its kinase activity.¹¹⁸

Taken together, a working model on the interaction between RIPK1 and RIPK3 emerges where inactive RIPK1 initially exists in a closed conformation at the TNFR1, where its DD and RHIM are unavailable for the formation of pro-cell death complexes. De-ubiquitylation¹¹⁹ and particular dephosphorylation⁸⁴ may open up RIPK1, allowing it to form a cytosolic complex II. Activation of RIPK1 by a still to be discovered mechanism then results in its autophosphorylation and activation. Depending on the availability of caspase-8, activation of RIPK1 then allows either activation of caspase-8 or RIPK3 within complex II, which results in, respectively, apoptosis or necroptosis.

Several other stimuli result in necroptosis, which depends on the kinase activity of both RIPK1 and RIPK3, and the formation of a 'canonical' RIPK1–RIPK3–MLKL-containing necrosome as described for TNF (Figure 4). These stimuli include the activation of death receptors (TNFR, Fas and TRAILR), stimulation of pathogen and Toll-like receptors (TLR2, 3, 4, 5 or 9), mitochondrial antiviral signaling protein (MAVS), retinoic acid induced gene 1 (RIG-I/DDX58), activation of the T-cell receptor and anti-cancer drugs (Obatoclox, Shikonin and Smac mimetic), vaccinia infection, and IFN or DNA damage.^{15,36–38,41,42,44,97,120–125} Importantly, some of these types of necroptosis are dependent on autocrine

TNF secretion and subsequent activation of RIPK1 (Figure 4).^{77,97,120,126} This is the case for zVAD-fmk-induced necroptosis in L929 cells, where activation of AKT and cJun N-terminal kinase (JNK) result in TNF transcription mediated by transcription factors cJun and Sp1.^{77,120,126} Other examples of autocrine TNF secretion include activation of TLR2, 5 or 9 in macrophages by, respectively, peptidoglycan, flagellin or unmethylated CpG-DNA motifs in the presence of zVAD-fmk. Here, necroptosis depends on MyD88-dependent induction of TNF. Etoposide-induced necroptosis apparently also requires autocrine TNF secretion.¹²⁷ The requirement for autocrine TNF production in etoposide-induced necroptosis suggest that RIPK1 kinase activity and canonical necrosome formation are involved.⁹⁷ In conclusion, the type of necrosome formed may depend on trigger and cell type, but a key common feature is the activation of RIPK3.

Peculiar cases of 'direct' necroptosis induction have been described for IFN γ and Obatoclox, which are able to directly activate RIPK1, without the need for autocrine TNF secretion (Figure 4). Indeed, during IFN γ -induced necroptosis, RIPK1 is activated through phosphorylation by RNA-dependent protein kinase (PKR, officially known as EIF2AK2).⁴¹ In the case of Obatoclox treatment, autophagosome formation is induced, where autophagy gene 5 (ATG5) recruits RIPK1 to assemble a RIPK1–RIPK3–FADD-containing necrosome.⁴⁴ *Non-canonical necrosome formation:* Known exceptions to this canonical RIPK1–RIPK3 activation model are dsRNA-induced necroptosis in fibroblasts and endothelial cells, and CMV-induced necroptosis (Figure 4). CMV viral infection of primary MEF, 3T3-SA or SVEC4-10 cells results in RIPK3-dependent necroptosis, which does not involve RIPK1 kinase activity, but is dependent on the sensor ZBP1/DAI.⁶⁴

Similarly, in fibroblasts and endothelial cells, TLR3 stimulation results in RIPK3 and MLKL-dependent necroptosis, which can proceed independently of RIPK1 kinase activity, but requires the TRIF adaptor.⁹⁷ Interestingly, the RIPK3 activation platform may not only depend on the stimulus, but also on the cell type. Indeed, in HaCaT cells treated with Smac mimetic and in macrophages, TLR3-induced necroptosis requires the canonical RIPK1–RIPK3 necrosome instead of the TRIF–RIPK3 necrosome.^{97,128} It is not known how exactly RIPK3 is activated downstream TRIF or ZBP1/DAI, but it is possible that these RHIM domain-containing proteins can function as a scaffold allowing RIPK3 recruitment, activation and autophosphorylation, as was shown for ZBP1/DAI.⁸⁹ This model is supported by *in vivo* evidence, where the perinatal lethality of RHIM-deficient RIPK1 knock-in mice can be rescued by ZBP1/DAI deficiency, suggesting a protective role for the RIPK1 RHIM domain. Hence, ZBP1/DAI would bind and activate RIPK3, unless RIPK1 prevents this interaction by sequestering RIPK3 through homotypic RHIM–RHIM interactions.^{90–92}

Necroptosis propagation: RIPK3 oligomerization or amyloid formation? RHIM domain-containing proteins can aggregate in amyloid-like structures. It was claimed that these amyloid fibers are essential for TNF-induced

necroptosis, as they trap hyperphosphorylated RIPK1 and RIPK3 in a detergent insoluble fraction (Figure 5).¹²⁹ However, the function of amyloid formation during necroptosis is still unclear. The importance of RIPK1–RIPK3 amyloid formation for necroptosis was demonstrated by mutation of the RHIM domains of RIPK1 and RIPK3,¹²⁹ but this approach also prevents RIPK3 recruitment, activation and necroptosis.^{18,37,79,93,97} Furthermore, the protective effect of inhibitors of amyloid formation (such as Congo Red or Thioflavin T) on TNF-induced cell death was modest (~15% at 100 μ M).¹²⁹ Hence, the protective effect of the RIPK3 RHIM mutation on necroptosis could be due to interfering with homotypic interaction between RIPK1–RIPK3, RIPK3–RIPK3, TRIF–RIPK3 and ZBP1/DAI–RIPK3 interaction rather than disruption of extensive amyloid-fibril formation. Indeed, it was suggested that amyloid formation is not required for necroptosis as forced oligomerization of RIPK3 through an artificial interaction domain is sufficient for the induction of necroptosis, independent of the presence of the RHIM domain, TNF stimulation or RIPK1 activity.¹³⁰ Of note, these authors specifically showed that RIPK3 oligomerization rather than dimerization is required for necroptosis,¹³⁰ while another group claimed that forced dimerization of RIPK3 is sufficient for necroptosis.¹³¹ Nonetheless, necrosome formation

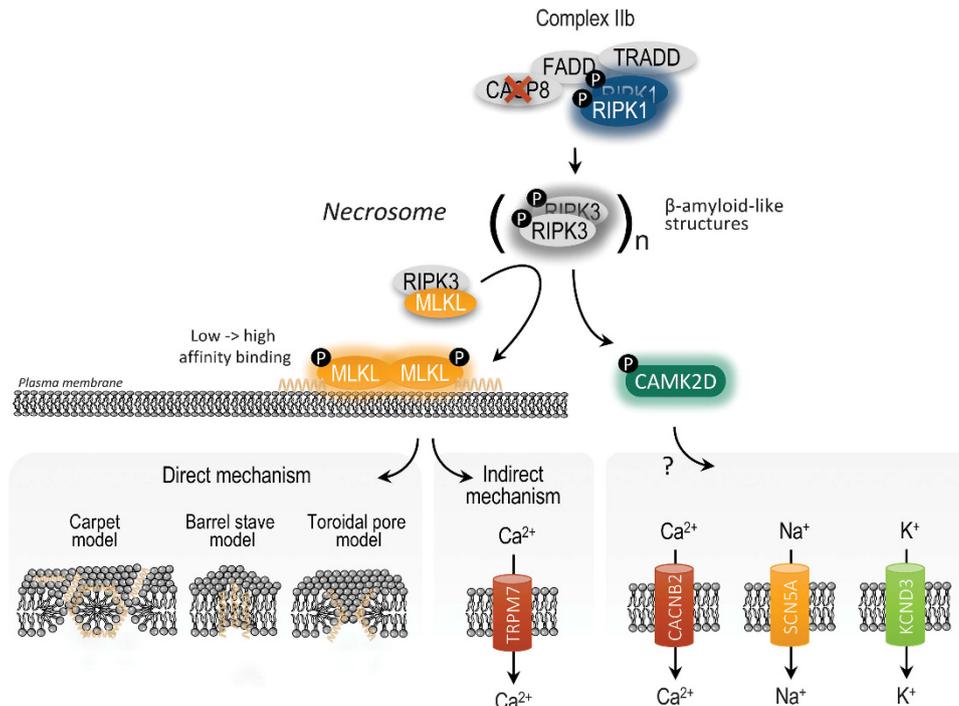


Figure 5 Necrosome formation and execution of necroptosis. RHIM-dependent necrosome formation results in phosphorylation and activation of MLKL or CAMK2 by RIPK3, which in turn activate ion influxes in the cell. MLKL is initially recruited to the plasma membrane by weak interactions with phospholipids (PIPs), but after a conformational change a second, stronger PIP interaction site is revealed in MLKL. MLKL can either directly form pores in the plasma membrane or activate ion channels, whereas CAMK2D is a known activator of several ion channels (without a currently demonstrated role in necroptosis). How MLKL induces permeabilization of the plasma membrane is unknown, but may be similar to other types of pore-forming proteins (carpet, barrel or toroidal model). 4HBD, four-helical bundle domain; CAC4NB, L-type Ca²⁺ channel subunit beta-2; CAMK2D, calcium-dependent protein kinase II delta; CASP, caspase; FADD, Fas-associated protein with death domain; KCND3, K⁺ channel potassium channel, voltage gated Shal-related subfamily D; MLKL, mixed lineage kinase domain-like; RHIM, RIPK homotypic interaction motif; RIPK, receptor-interacting protein kinase; SCN5A, sarcolemmal cardiac Na⁺ channel; TRADD, TNF-associated death domain; TRPM7, transient receptor potential cation channel, subfamily M, member 7; ZBP1, Z-DNA-binding protein 1 (also known as DAI)

promotes RIPK3 activation, dimer/oligo/ β amyloid-like fiber formation and activation of MLKL.

Necroptosis execution: MLKL activation. Once activated, RIPK3 phosphorylates human MLKL at Thr357, Ser358 and Ser345, Ser347, and mouse MLKL at Thr349 and Ser352 within the MLKL activation loop.^{16,96,103} Phosphorylation of these residues results in an open conformational shift of MLKL and exposure of its four-helical bundle domain.^{96,102} How MLKL exactly executes necroptosis is still a matter of debate (Figure 5). Overexpression of the N-terminal four-helical bundle domain alone is sufficient to induce membrane rupture in cells. There is consensus that during necroptosis, MLKL is phosphorylated at Thr357/Ser358, leading to oligomerization through the N-terminal domain (brace region) and translocation to the plasma membrane.^{98–100,102,132} However, the type of oligomers formed and the method of membrane recruitment is still under debate. Some have suggested that oligomerized MLKL can directly form a pore in the plasma membrane after binding to negatively charged phospholipids.^{98,99} A more advanced model was proposed, where MLKL binds to phosphorylated phosphatidylinositol phosphate (PIP) phospholipids with a low affinity site in its N-terminal bundle domain. Conformational rearrangement of MLKL at the plasma membrane then reveals a higher affinity site for PIPs in MLKL, and results in robust plasma membrane association of MLKL and displacement of the brace domain from its four-helical bundle domain.¹³³ These multiple interactions with phospholipids may cause different types of membrane permeabilizing mechanisms (toroidal, barrel stave and carpet, Figure 5),¹³⁴ which have not been properly examined yet. In contrast, others claimed that MLKL acts as a new type of highly selective Mg^{2+} channel and later on oligomerizes into a less selective, larger pore.¹³⁵ Yet another group found that trimerized MLKL is recruited with RIPK3 to the plasma membrane, where MLKL interacts with the kinase transient receptor potential cation channel, subfamily M, member 7 (TRPM7). In turn, the kinase/ion channel TRPM7 would be at least partially responsible for extracellular Ca^{2+} influx and subsequent plasma membrane damage.¹⁰⁰ Most recently, it was reported that MLKL tetramerizes within the necrosome, but requires further octamerization to translocate to the lipid rafts of plasma membrane, where it causes an Na^+ influx and cell death.^{132,136} Taken together, during necroptosis, phosphorylated MLKL unleashes its four-helical bundle domain, oligomerizes involving the brace domain and relocates to the plasma membrane, where it induces an influx of ions, either directly by channel/pore formation or indirectly through association with ion channels. Alternatively, RIPK3 may also activate CAMK2D, which in turn induces an ion influx by activating multiple ion channels, independently of MLKL¹⁰⁴ (Figure 5).

Conclusions

Over the years, our understanding of necroptotic signaling increased substantially. It is now clear that several triggers can induce necroptosis. Furthermore, several processes (e.g., mitochondrial ROS generation) can contribute to necroptosis,

but can be bypassed activating the necroptosis pathway downstream at the level of RIPK3¹¹⁷ or MLKL.⁹⁶ While originally RIPK1 kinase activity was a hallmark of necroptosis, recent findings indicate that RIPK3 and MLKL activation is universally required for necroptosis, both canonical and non-canonical. A core necroptotic pathway consisting of RIPK3 activation, followed by MLKL activation and membrane permeabilization has emerged. However, molecular signal transduction usually is far more complex, and the recent discovery of RIPK3- or MLKL independent necroptosis,^{94,104} and the unexpected function of ZBP1/DAI^{90–92} in the skin and thymus suggests that the necroptosis pathway did not yet yield all its secrets. And finally, to make the situation even more complex, phylogenetic analysis reveals that the necroptotic axis, except for RIPK1, is possibly poorly conserved in the animal kingdom, suggesting that completely alternative cell death mechanisms may bypass the need for necroptosis and question the universal role of necroptosis during innate immunity in the animal kingdom.¹³⁷

Conflict of Interest

The authors declare no conflict of interest.

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