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Location, location, location: A compartmentalized view of TNF-induced necroptotic signaling

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ABSTRACT

Necroptosis is a lytic, pro-inflammatory cell death pathway, which has been implicated in host defense and, when dysregulated, the pathology of many human diseases. The central mediators of this pathway are the receptor-interacting serine/threonine protein kinases RIPK1 and RIPK3 and the terminal executioner, the pseudokinase mixed lineage kinase domain-like (MLKL). Here, we review the chronology of signaling along the RIPK1-RIPK3-MLKL axis and highlight how the subcellular compartmentalization of signaling events controls the initiation and execution of necroptosis. We propose that a network of modulators surrounds the necroptotic signaling core and that this network, rather than acting universally, tunes necroptosis in a context-, cell type- and species-dependent manner. Such a high degree of mechanistic flexibility is likely an important property that helps necroptosis operate as a robust, emergency form of cell death.

THE CORE AXIS OF NECROPTOTIC SIGNALING

Necroptosis is a caspase-independent form of programmed cell death (*1*). Although necroptosis likely arose as a host response to counter pathogens that block apoptosis (*2-9*), dysregulated necroptosis also plays a role in the pathologies of numerous non-communicable diseases, including inflammatory diseases (*10-12*), inflammatory bowel disease (*13*) and kidney ischemia-reperfusion injury (*14, 15*). Accordingly, there is widespread interest in the pharmacological inhibition of necroptotic cell death, with several pathway inhibitors having progressed into early phase clinical trials (as reviewed in (*16*)). Necroptosis can be induced by inflammatory stimuli that activate death receptors, Toll-like receptors (*17, 18*), interferon receptors (*19, 20*), or the cytosolic nucleic acid sensor, ZBP-1 (*21, 22*) (Figure 1A). There is a growing appreciation for the mechanistic cross-talk and signal amplification that exists between different pro-necroptotic stimuli (*23-26*) (Figure 1A). However, of the various initiating stimuli, necroptosis triggered by ligation of the death receptor, TNFR1 (tumor necrosis factor receptor 1) is the prototypical form of necroptosis and thus is the focus of this review. Two intracellular mediators are essential for TNF-induced necroptosis: RIPK3 (receptor-interacting serine/threonine protein kinase-3) and MLKL (mixed lineage kinase domain-like). RIPK3 was identified as a key mediator in 2009 (*27-29*), while MLKL was shown to be the terminal effector of necroptosis in 2012 (*30, 31*). RIPK1 is another critical mediator of TNF-induced necroptosis; however, under certain conditions, TNF-induced necroptosis can proceed in the absence of RIPK1 (*32*). Nonetheless, RIPK1-RIPK3-MLKL is considered the core signaling axis of TNF-induced necroptosis. Signaling along this axis culminates in the activation of MLKL, which in turn kills cells by triggering membrane lysis (*33-38*). Thus, necroptosis is a lytic form of cell death that promotes inflammation through the unmitigated release of intracellular contents (*39-41*). Much attention has been put towards expanding our understanding beyond the core actions of RIPK1, RIPK3 and MLKL. For example, many screens have been performed to identify additional mediators of necroptosis (*27, 28, 31, 42-47*). Although these efforts have uncovered myriad interactors, post-translational modifications and epigenetic regulators that control necroptotic signaling, little overlap exists between the regulators identified in genetic screens (Figure 1B). This observation suggests that, besides the core RIPK1-RIPK3-MLKL axis, many of the identified interactors influence necroptosis in a context-dependent manner. Accordingly, rather than discuss the various regulators that tune necroptosis (reviewed in (*48*)) or the role of necroptosis in human disease (reviewed in (*49, 50*)), here we instead focus on the sequential interactions (Figure 1C), intracellular movements and activation of RIPK1, RIPK3 and MLKL that occur during TNF-induced necroptosis.

TNFR1 INTERNALIZATION: THE FIRST COMPARTMENTALIZATION EVENT IN NECROPTOSIS

The binding of TNF to TNFR1 induces an outside-in allosteric signal (*51, 52*) that promotes the recruitment of numerous proteins including TRADD (TNFR-associated death domain), RIPK1 (receptor-interacting serine/threonine protein kinase 1), TRAF2 (TNFR-associated factor 2), LUBAC (linear ubiquitin chain assembly complex) and cIAP1 and cIAP2 (cellular inhibitors of apoptosis-1 or -2; cIAP1/2) to the cytoplasmic tail of TNFR1 (*53, 54*). This TNF-induced membrane-bound assembly is known as Complex I (*53*) (Figure 2). The signaling capacity of Complex I is tightly controlled by ubiquitination, phosphorylation and other post-translational modifications (reviewed in (*55*)). For example, cIAP1/2 add K63-linked polyubiquitin chains to RIPK1 and other proteins within Complex I, whereas LUBAC catalyses the addition of ubiquitin to the N-terminal methionine (Met1) of RIPK1 and other proteins within Complex I, which in turn stimulates downstream NF-κB activation and cell survival (*56-58*) (Figure 2). NF-κB signaling does not promote necroptotic cell death or the accompanying release of damage-associated molecular patterns (DAMPs), but rather induces synthesis of pro-inflammatory cytokines, including IL-6 and IL-8 (as reviewed in (*59*)). Signaling is diverted from NF-κB when cIAP1/2 activity is low during TNFR1 stimulation, which in turn reduces K63-linked ubiquitylation of RIPK1 within Complex I, promotes the CYLD-mediated removal of Met1-linked ubiquitin from Complex I, and thereby favours the formation of a potent death-induced signaling complex (*60-63*) (Figure 2). Complex I signaling is also governed by its subcellular location (Figure 2). For instance, stimulation of Complex I within lipid rafts favours subsequent NF-κB or RhoA signaling (*64, 65*), whereas activation of Complex I outside of lipid rafts promotes TNF-induced cell death (*65*). Moreover, clathrin-mediated endocytosis of TNFR1 is critical for proper Complex I formation and downstream cytotoxic signaling (*66*). Our finding that inhibiting clathrin-mediated endocytosis blocks the induction of TNF-induced necroptosis, but does not block the terminal steps of necroptosis (*67*), is consistent with the notion that TNFR1 internalisation is an important early event for TNF-induced cell death.

PERINUCLEAR NECROSOME CLUSTERING: THE SECOND COMPARTMENTALIZATION EVENT IN NECROPTOSIS

TNF is best known for its role in the induction of programmed cell death through the extrinsic apoptosis pathway. TNF-induced apoptosis arises when the activities of c-FLIP$_L$ (an endogenous caspase-8 inhibitor) and/or cIAP1/2 are suppressed during TNFR1 stimulation. Under these conditions, deubiquitylated RIPK1 within internalized Complex I transforms into a multiprotein assembly known as Complex IIa or Complex IIb (Complex IIa/b) (*53, 68*) (Figure 3). These physically- and biochemically-distinct complexes are variably comprised of TRADD, FADD (Fas-associated via death domain), RIPK1, RIPK3 and Caspase-8 (*53, 68*) (Figure 3). Caspase-8 activity within Complex IIa/b skews downstream signaling towards an apoptotic outcome by activating Bid and Caspase-3 (*69, 70*) and simultaneously prevents necroptosis by cleaving RIPK3 (*71*). Caspase-8-mediated cleavage of RIPK1 also occurs during TNFR1 stimulation (*72*), which then broadly impairs TNF-induced cell death (*73-75*).

Necroptosis is an alternative form of TNF-induced cell death that arises when cellular conditions favour the amyloid-like assembly of RIPK1 and RIPK3 through their RHIM (RIP homotypic interaction motifs) regions (*76, 77*). In this scenario, internalised Complex I instead transforms into a ~2 megadalton cytosolic complex known as the necrosome (*78*) (Figure 3). FADD, Caspase-8, MLKL and various other proteins are also recruited to the necrosome, although the chronology and underlying mechanisms of these different recruitment events are largely unknown (*27-30, 79-81*). Caspase-10, a homolog of Caspase-8 that is conspicuously absent from rodent species, is also recruited to RIPK1-containing complexes following death receptor activation (*53, 82-84*). However, the role of Caspase-10 in TNF-induced necroptosis is largely unexplored. In the laboratory, TNF-induced necroptosis can be induced by chemical depletion of cIAP1/2 and chemical inhibition of Caspase-8:cFLIP heterodimer catalytic activity (Figure 3). The use of chemical cocktails to stimulate necroptotic cell death in cultured cells enables the necroptosis pathway to be studied in a focused manner. In vivo, however, it is more complicated, with multiple cell death modalities likely operating in tandem and with crosstalk between different programmed cell death pathways (*11, 14, 15, 41*). Notwithstanding the contribution of other death modalities, pathophysiological settings that favour necroptosis arise in the absence of pharmacological agents, such as during *Mycobacterium tuberculosis* infection (*85*), during inorganic crystal deposition (*86*), or during TNF-induced systemic inflammatory response syndrome (*87, 88*) when the proteolytic activity of Caspase-8 can be inhibited by regulatory phosphorylation events (*81*).

As with other TNF-induced signaling complexes, the necrosome is strictly controlled at the post-translational level by phosphorylation and ubiquitination (*60, 78, 89*). Trans- and/or auto-phosphorylation of RIPK1 and RIPK3 within the necrosome is critical for TNF-induced

necroptosis (*27, 28, 90, 91*) (Figure 1C). In contrast, TNF-induced apoptosis does not typically rely upon the kinase activities of RIPK1 and RIPK3 (*92-94*), although there are examples where the kinase activity of RIPK1 promotes apoptosis (*95, 96*).

Despite the importance of compartmentalization to TNF-induced signaling, no study has addressed whether the physical segregation and subcellular localisation of the necrosome influences its activity. Multiple studies show that during TNF-induced necroptosis, RIPK1, RIPK3 and MLKL relocate from the cytosol into discrete perinuclear clusters which are presumably necrosomes (*27, 30, 67, 76, 80, 89, 97-101*) (Figure 3). Despite considerable efforts, no study has been able to show substantial colocalization between necrosomes and any membrane-bound organelle (*27, 67, 102*), a point that leads us to propose that the necrosome may instead be a membraneless organelle. Membraneless organelles are formed by phase separation – a process in which multivalent interaction between biomolecules drives the formation of reversible higher-order assemblies (*103, 104*). In turn, this liquid-like coalescence of interacting biomolecules allows highly specialised signaling events to occur (*103, 104*). Akin to other membraneless organelles, necrosomes are composed of a defined subset of proteins, are the site of highly-specialised signaling events, have a reproducible size range, have a consistent subcellular location and can be biochemically fractionated (*27, 30, 67, 76, 78, 80, 89, 97-100*). We further propose that liquid-like compartmentalization could be accomplished by the amyloid-like nature of RIPK1-RIPK3 RHIM interactions (*76, 77*), by association with a known phase separating protein such as ZO-1 (*67*), and/or through the intrinsically disordered regions predicted to connect the RIPK1 and RIPK3 kinase domains to their C-terminal RHIMs (Figure 1C). In vitro analyses with full-length RIPK1 and RIPK3, rather than studies using isolated RHIM motifs will prove invaluable for defining the propensity of RIPK1 and RIPK3 to assemble into phase separated entities.

The canonical function of the necrosome is to facilitate RIPK3-mediated phosphorylation of MLKL (*30, 105*). MLKL is comprised of an N-terminal four-helix bundle (4HB) domain, an intermediary two-helix 'brace' region and a regulatory pseudokinase domain (*105*) (Figure 1C). The RIPK3-mediated phosphorylation of the pseudokinase domain in MLKL is an obligatory event in necroptosis that triggers a conformational change in MLKL (*30, 35, 105-108*). This phosphorylation-induced conformational switch relieves auto-inhibition of the 4HB domain to promote the oligomerisation of MLKL (*35, 38, 105, 107*).

The formation of MLKL oligomers is necessary, but insufficient, to trigger necroptosis (*107, 109*). We and others have noted that phosphorylated MLKL exists almost exclusively in an oligomeric state, which implies that these two events happen in quick succession at the necrosome

(*67, 110*). Nonetheless, RIPK3-mediated phosphorylation of MLKL and ensuing MLKL oligomerisation may be divorceable steps. This idea is supported by the observation that the induction of necroptotic signaling in cells that lack the inositol phosphate kinases IMPK or ITPK1 leads to MLKL phosphorylation without MLKL oligomerisation (*42, 111*).

The second brace helix of MLKL facilitates its oligomerization (*33, 112*). Despite this mechanistic understanding, the stoichiometry of necroptotic MLKL oligomers remains contentious, with reports of MLKL trimers (*33, 107, 112, 113*), tetramers (*35, 114*), hexamers (*38*), octamers (*100*) and high-order amyloids (*115*). Multiple studies on this topic have drawn conclusions using recombinant oligomers of MLKL (*33, 35, 38, 107, 112*), but how these findings relate to oligomers formed in cells during necroptosis remains to be determined. This is an important consideration given the impact that RIPK3-mediated phosphorylation and tertiary interactors may have on oligomerization. Despite these caveats, the current consensus is that oligomerization results in the formation of human MLKL tetramers and mouse MLKL trimers (*48*). After formation of this initial pro-necroptotic oligomer, MLKL is then thought to further assemble into a higher-order species (*35, 67, 107*). Although the nature of these higher-order oligomers is truly unknown, smearing of activated MLKL on non-reducing gels suggests it adopts a range of supramolecular states (*100*). Undoubtedly, to fully define MLKL's oligomeric species during necroptosis, new technologies that resolve oligomers both in vitro and in vivo will be essential.

It is noteworthy that intermolecular disulfide bonding between MLKL protomers can occur during necroptosis (*38, 114*). Because MLKL basally resides within the reducing environment of the cytosol, it is unclear whether disulfide crosslinking of MLKL occurs in a more oxidative environment such as the Golgi or the plasma membrane, or may even occur after cell death given that cytosolic proteins are prone to disulfide crosslinking upon necrotic cell death (*116, 117*). Although one study suggests that MLKL disulfide crosslinking can be prevented by including the thiol-reactive agent N-ethylmaleimide in the cell lysis buffer prior to immunoblotting (*113*), our data suggest that MLKL can be disulfide-crosslinked prior to cell death (*101*). The functional importance of this disulfide crosslinking is also ambiguous given that individual cysteine-to-serine substitutions within mouse MLKL have no major impact on TNF-induced necroptosis (*100*). Furthermore, there are conflicting reports as to whether combined mutation of three 4HB domain cysteines impacts necroptotic function (*100, 107, 115*), and whether combined cysteine-to-serine mutation influences the 4HB domain structure has not yet been examined. Irrespective of how, when or where MLKL is disulfide bonded, it is salient that only a fraction of the phosphorylated MLKL pool is crosslinked during necroptosis, whereas Blue-Native PAGE show that virtually all

phosphorylated MLKL exists in an oligomeric state (*35, 101, 107*). This disconnect suggests that, rather than reflecting the full extent of bona fide MLKL oligomerization in necroptotic cells, disulfide crosslinking merely catches a small portion of oligomeric MLKL "in the act". Thus, although disulfide crosslinking of MLKL is a useful diagnostic for necroptotic signaling, its overall physiological relevance warrants further investigation.

TRANSLOCATION TO MEMBRANES: THE THIRD COMPARTMENTALIZATION EVENT IN NECROPTOSIS

The next checkpoint in TNF-induced necroptotic signaling is the association of MLKL with internal cell membranes (*97, 107, 113, 114*) (Figure 4). This compartmentalization event is a routinely used marker of necroptosis, because it involves the easy-to-measure transition of MLKL from its basal form in the aqueous phase into its activated membrane-bound form in the detergent phase (*97, 107*). Multiple lines of evidence show that the 4HB domain is primarily responsible for the association of MLKL with membranes (*34-37, 97, 107, 112*). For example, inhibitors or protein ligands that specifically bind the 4HB domain do not prevent MLKL phosphorylation or oligomerisation, but instead selectively block the translocation of MLKL to membranes and thereby prevent necroptosis (*39, 67, 97, 109*). During TNF-induced necroptosis, activated MLKL is thought to associate with multiple internal membranes, including those of the endoplasmic reticulum (*97*), mitochondria (*97*), (autophago)lysosomes (*97, 118-121*), endosomes (*122*), exosomes (*119, 122-124*), the nucleus (*98, 125*) and the plasma membrane (*67, 97, 113, 114*). Of these membranes, the plasma membrane is thought to be a critical site of MLKL accumulation for the execution of necroptosis (*67, 113, 114*).

The movement of MLKL from the cytosol to cellular membranes is not a passive process, but instead relies on highly-orchestrated trafficking events (*67, 109*). The partner proteins that traffic MLKL to the membrane are currently unknown, but their existence is supported by several lines of evidence. Firstly, occluding or mutating a site centered on the α4 helix of the 4HB domain prevents activated MLKL from translocating to membranes and triggering cell death in human (*35, 109*) and mouse (*107*) cells. These data implicate this site in mediating interactions with auxiliary proteins that facilitate MLKL trafficking to the plasma membrane. Secondly, MLKL trafficking to the plasma membrane relies on the Golgi, microtubule and actin machinery (*67*), because chemical inhibition of these pathways slows the translocation of MLKL from cytoplasmic necrosomes, which attenuates cell death. There are many interactors that modulate MLKL oligomerization, including HSP90 (*28, 126-128*), HSP70 (*129*), Thioredoxin-1 (*130*), TAM kinases (*131*) and

inositol phosphate kinases (*42, 111, 132*). It is an intriguing possibility that these auxiliary interactors may also influence the trafficking of MLKL during necroptosis.

The 4HB domain confers MLKL with the ability to disrupt membrane integrity. Although the recombinant 4HB domain can lyse liposomes (*36, 37*), and although forced expression of the isolated mouse 4HB domain or the dimerised human 4HB domain is toxic to cells (*37, 107, 112*), the translocation of full-length activated MLKL to internal membranes is insufficient to trigger necroptosis (*35, 107, 109*). Thus, there is a clear distinction between the association of MLKL with internal membranes during necroptotic signaling and subsequent MLKL-mediated perturbation of membranes which ultimately causes cell death. In line with this notion, the translocation of MLKL to membranes often precedes necroptosis by several hours (*67, 110, 113*). This temporal gap between MLKL's membrane translocation and membrane disruption has been suggested to imply the existence of necroptotic effectors downstream of MLKL (*113*), although definitive evidence for an obligatory necroptotic effector that acts downstream of MLKL has not been obtained. In contrast, our data suggest that this temporal gap reflects the time needed to (i) traffic membrane-bound MLKL from the necrosome to the plasma membrane, and (ii) accumulate sufficient amounts of MLKL at the cell periphery to surpass the threshold for plasma membrane lysis (*67*). These are two newly described rate-limiting steps in the necroptotic pathway (Figure 4). The accumulation of MLKL as micron-sized "hotspots" in the plasma membrane (*42, 67, 133*) raises the prospect of a final compartmentalization event that spatially concentrates MLKL at the plasma membrane and thereby potently controls the threshold for necroptotic cell lysis (Figure 4).

MANY ROADS LEAD TO PLASMA MEMBRANE LYSIS

MLKL-mediated permeabilization of the plasma membrane is, by definition, necessary for necroptotic cell death. Indeed, the plasma membrane is the foremost destination for MLKL during TNF-induced necroptosis (*67, 113, 114*). Nonetheless, a growing body of work shows that MLKL also translocates to other internal membranes and that multiple organelles are disrupted during necroptosis. Here, we discuss if, when, and how the recruitment of MLKL to different membrane-bound organelles contributes to necroptotic cell death.

Mitochondria

Mitochondria are bioenergetic organelles that are pivotal for numerous cell death subroutines (*134*). Accordingly, the contribution of mitochondrial dysfunction to necroptosis has received

considerable attention over the years. It is now well-established that increased mitochondrial reactive oxygen species (ROS) is a common, but not a universal, feature of TNF-induced necroptosis (*27, 29, 99, 135-137*). For example, although antioxidants protect many cell-types from TNF-induced necroptosis, they do not prevent the commonly-used human HT29 colorectal adenocarcinoma cells from MLKL-mediated death (*99*). Nonetheless, when necroptotic signaling does increase ROS production, this phenomenon is MLKL-dependent (*31*), coincides with the translocation of MLKL to mitochondrial membranes (*102*), arises prior to plasma membrane lysis (*136*), and is accompanied by both mitochondrial hyperpolarisation (*136*) and mitochondrial fragmentation (*138, 139*). This series of events directly implicates MLKL as the inducer of mitochondrial dysfunction during necroptosis.

There is evidence supporting the notion that mitochondrial perturbation exacerbates necroptosis. For instance, studies show that regulators of mitochondrial membrane integrity – namely cyclophilin D, BID, BAX, BAK, and PUMA – can promote TNF-induced necroptosis (*140-143*). Although further mechanistic studies are required on this topic, mitochondrial amplification of TNF-necroptosis has been suggested to involve MLKL-mediated herniation of mitochondrial DNA, which in turn activates cytosolic DNA sensors to orthogonally propagate necroptotic signaling (*140*).

Compelling evidence against a key role for mitochondria in necroptosis has also been reported. For instance, PGAM5- and Drp1-mediated mitochondrial fission was proposed to be critical for necroptosis (*138*), but subsequent studies have unequivocally showed that necroptosis is not affected by the silencing or deletion of PGAM5 or Drp1 (*105, 139, 144-147*). Tait and colleagues also showed that the experimental depletion of mitochondria from cells does not alter the rate or extent of necroptotic death (*139*). In this study, antioxidants still protected against necroptosis in cells depleted of mitochondria, suggesting that non-mitochondrial ROS may be important for necroptosis (*139*). However, because these mitochondrial depletion experiments were performed in only two cell models of necroptosis, whether mitochondrial dysfunction is superfluous during TNF-induced necroptosis remains an open question.

Autophago(lyso)somes

Autophagosomes are double-membrane enclosed structures that deliver cytoplasmic constituents to lysosomes, which then catabolise their cargo for recycling into biosynthetic processes. This resource management system, otherwise known as autophagy, helps maintain homeostasis and can

promote cell survival during stress (*148*). Several studies investigating a role for autophagy during necroptotic signaling have showed increased lipidated LC3 and other markers of autophagosomes during TNF-induced necroptosis (*120, 137, 149, 150*). However, this accumulation of autophagosomes is not thought to be due to the stimulation of autophagy during necroptosis, but rather due to a failure of autophagosomes to undergo proper lysosomal degradation (*120, 150*). Consistent with this notion, lysosomal permeabilization and/or lysosomal exocytosis occurs prior to MLKL-mediated plasma membrane lysis (*97, 120, 136, 151*). To explain how necroptotic signaling may cause autophagolysosomal dysfunction, activated MLKL has been proposed to translocate to autophago(lyso)somes and disrupt their membrane integrity (*120*). However, contrary to this train of thought, only negligible amounts of activated MLKL relocate to lysosomes during necroptosis unless the toxic stimulus is removed or a checkpoint for membrane-bound MLKL is blocked (*10, 67, 97, 119*). There is also no consensus about whether autophagolysosomal dysfunction causally contributes to necroptotic cell death. For instance, in some studies, chemical modulation or knockdown/knockout of autophagolysosomal mediators protects against TNF-induced necroptosis (*118, 149, 152*), but not in other studies (*67, 120, 137*). Hence, broader studies that also take into account the autophagic turnover of necroptotic mediators are needed (*118*).

Nucleus

The nuclear envelope is a double lipid bilayer that separates the nucleus from the cytoplasm. Nuclear pores span the nuclear envelope. Although a subset of proteins that are 40-60kDa in size can freely pass through the nuclear pore, the transport of molecules >30kDa across the nuclear envelope is often tightly regulated by cis-acting sequences and trans-acting chaperones (*153*). MLKL-mediated disruption of the nuclear envelope may occur prior to necroptotic cell death (*39*). However, because the vast majority of RIPK1 (monomer of ~76kDa), RIPK3 (monomer of ~57kDa) and MLKL (monomer of ~54kDa) resides in the cytoplasm under basal conditions (*27, 30, 67, 76, 80, 89, 97-99*), an intranuclear role for necroptotic signaling appears counterintuitive. As a challenge to this assumption, Yoon and colleagues proposed that RIPK3-mediated phosphorylation of cytosolic MLKL exposes a nuclear localization signal in the pseudokinase domain of MLKL, triggering the nuclear import of MLKL in its non-membrane bound form, which may be important for necroptosis (*125*). A second study proposed a different mechanism, in which RIPK3-mediated phosphorylation and oligomerization of MLKL occurs in the nucleus (*98*). It was proposed that nuclear MLKL is then exported to seed assembly of higher-order cytosolic necrosome clusters and that blocking this nuclear export of MLKL protects against necroptosis

(*98*). A third study indicated that nuclear import of p65 and subsequent upregulation of pro-inflammatory cytokines relies on the activation of MLKL during TNF-induced necroptosis (*154*). Lastly, a fourth study suggests that export of retinoic acid receptor (RAR)-γ from the nucleus is important for the formation of Complex II during TNF-induced necroptosis (*46*). Thus, although compartmentalization of necroptotic signaling across the nuclear envelope is an emerging idea (*155, 156*), further assessment of the proposed import/export mechanisms and their impact on cell death is needed, especially given the strict size requirements for transport across the nuclear envelope and the lack of canonical conserved nuclear localisation sequences in RIPK1, RIPK3 or MLKL. In this context, we and others have noted that negligible amounts of endogenous phosphorylated MLKL arise in the nucleus during TNF-induced necroptosis (*67, 101, 156*), raising the possibility that overexpression of MLKL may cause spurious nuclear localization.

Plasma membrane

The plasma membrane is a single lipid bilayer that represents the outermost limiting structure of a cell. The selective permeability of the plasma membrane allows cells to maintain ionic-, pH- and redox-potentials within homeostatic norms. These essential transmembrane potentials are lost upon prolonged disruption of the plasma membrane, which commensurately triggers cell death (*157, 158*). It is therefore not surprising that perturbation of the plasma membrane by MLKL is the prevailing cause of necroptotic cell death – above and beyond damage to other organelles during TNF-induced necroptosis. This conclusion can be inferred from the finding that the cytosolic proteome, but not the proteomes of intracellular organelles such as the mitochondria or the endoplasmic reticulum, is selectively lost from necroptotic cells (*151*). This conclusion is also based on studies showing that considerable amounts of activated MLKL translocate to the plasma membrane prior to necroptotic cell lysis (*67, 97, 100, 113, 114, 119, 123, 159*). Furthermore, we showed that the subcellular location and timing of MLKL's accumulation at the plasma membrane correlates with the site and timing of plasma membrane damage (*67*). This spatiotemporal correlation argues that activated MLKL increasingly disrupts the plasma membrane until a threshold is surpassed. A similar conclusion can be drawn from the finding that the plasma membrane becomes more permeable as cells approach necroptotic death (*132, 160*). By extension, these observations imply that substantial, albeit sub-threshold, amounts of MLKL can be tolerated at the plasma membrane. In line with this notion, inhibitors can still block necroptosis when they are added after MLKL has begun to accumulate at the plasma membrane (*67, 123*).

Further support for a membranolytic threshold stems from the observation that phosphatidylserine (PS) exposure precedes necroptotic cell death (*67, 123, 124, 161-163*). Although PS normally resides in the inner leaflet of the plasma membrane, Ca^{2+}-dependent translocation of PS to the outer leaflet of the plasma membrane occurs during apoptosis (*164*). By comparison, PS externalisation during necroptosis occurs independently of Ca^{2+} flux (*123, 124, 160, 161*). Thus, the mechanisms of PS exposure differ between necroptosis and apoptosis. Indeed, because inner leaflet MLKL accumulation and outer leaflet PS exposure spatially correlate, PS exposure may be a direct, local and proportional consequence of MLKL-mediated plasma membrane disruption during TNF-induced necroptosis (*67, 123*). Notably, the functional impact of PS exposure also differs between necroptosis and apoptosis, with PS facilitating the engulfment of apoptotic, but not necroptotic cells (*162*).

Overt lysis of the plasma membrane is another feature that distinguishes necroptosis from apoptosis. Unlike apoptosis in which the integrity of the plasma membrane is largely preserved, necroptosis is characterised by a focal region of membrane rupture (*67, 124, 159, 165*). Our data suggest that the site of necroptotic membrane rupture closely approximates the site of PS exposure and MLKL accumulation (*67*). Such cataclysmic failure of the plasma membrane allows spillage of intracellular constituents – such as the HMGB1, ATP and LDH DAMPs – from necroptotic cells (Figure 4; (*39, 40, 151, 166*)). Indeed, as cytokine production is suppressed in cells undergoing TNF-induced necroptotic death (*151, 167*), the release of these DAMPs may be the main way that necroptosis triggers further inflammation and immune reactivity (*39, 40, 151, 166*). Nonetheless, even after lysis of the plasma membrane, low but measurable amounts of cytokine production by the endoplasmic reticulum persists (*168*), which once again intimates that the plasma membrane, rather than internal organelles, is the primary site of MLKL-mediated damage.

The importance of MLKL activity at the plasma membrane is underscored by the slew of mechanisms that regulate its abundance at the cell periphery. Both endocytic and exocytic events can lower the pool of MLKL residing at the plasma membrane during necroptotic signaling (*10, 119, 122-124, 132*). For instance, Flotillin-mediated endocytosis is thought to prevent necroptosis by removing MLKL from the plasma membrane (*119*) (Figure 4). Jettisoning of MLKL from the plasma membrane by ESCRT-, ALIX-, syntenin-1- and/or Rab27-mediated exocytosis is also thought to attenuate necroptotic cell death (*119, 122-124, 132, 160*) (Figure 4). However, the roles of endocytosis and exocytosis in necroptosis are convoluted. Not only are endocytosis and exocytosis mechanistically intertwined (*169*), but MLKL may also constitutively control endosomal trafficking (*122, 170*) and endocytosis is central to TNFR1 signaling (Figure 2). For

example, ALIX and ESCRT not only regulate MLKL abundance at the plasma membrane, but also govern upstream TNFR1 signaling and TNFR1 subcellular localisation (*171, 172*). In light of these roles, and given that exosome release variably occurs during necroptosis (*67, 119, 122*), a deeper understanding of the regulation of MLKL's plasma membrane abundance is needed.

Despite being the final event in necroptosis, the mechanism by which MLKL disrupts the plasma membrane remains a mystery. Proposed models include (i) the partial insertion of MLKL into the lipid bilayer to disrupt membrane integrity (*112*), (ii) MLKL forming a membrane-spanning pore by MLKL that directly triggers osmolysis (*36, 55, 100, 173, 174*), (iii) MLKL forming a membrane disrupting amyloid-like structure (*115*), or (iv) MLKL engaging a downstream cofactor that in turn causes cell lysis (*113*). We direct readers to other reviews for an in-depth evaluation of these proposed membranolytic models (*175-177*).

Another uncharacterised feature of late-stage necroptotic signaling relates to the findings that rather than associating uniformly with the plasma membrane, MLKL concentrates into supramolecular structures (*42, 67, 97, 119, 123, 133*). In some instances, these accumulations of MLKL grow into micron-sized structures that we have termed "hotspots" (*42, 67, 133*). These findings suggest that compartmentalization of MLKL at the plasma membrane is an important parameter of necroptotic cell lysis. Because MLKL preferentially binds specific lipid subtypes such as PS (*35, 97*), we speculate that the non-uniform distribution of these lipids controls the location of MLKL at the cell periphery. This however may be an oversimplified explanation, because MLKL preferentially translocates to cholesterol-rich lipid rafts in the plasma membrane (*114, 119*) despite having negligible affinity towards cholesterol (*34, 35, 97*). A complementary explanation is that the location of MLKL on the plasma membrane is controlled by a tertiary interactor such as Flotillin or ESCRT (*119, 122, 123*). In this context, our findings that activated MLKL colocalises and coaccumulates with tight junction proteins at the plasma membrane may provide insight into this final compartmentalization event during necroptosis (*67*). Tight junctions are large protein complexes that span the plasma membranes of abutting cells and thereby restrict both lateral diffusion of lipids in the plasma membrane and paracellular diffusion of water-soluble molecules (*178*). The coaccumulation of MLKL with tight junctions is functionally critical, because the onset of necroptosis is slowed by stabilising tight junctions and accelerated by destabilising tight junctions (*67*). Because the accumulation of MLKL at the plasma membrane is not changed by the presence of tight junction modulators, we hypothesised that tight junctions may be a rheostat that controls the threshold for MLKL-mediated membrane lysis (*67*). The coaccumulation of MLKL with tight junctions also has broader cell-extrinsic ramifications, because it leads to the cross-

junctional propagation of damage and the acceleration of necroptosis in neighboring cells (*67*). Collectively, the assembly of MLKL into large supramolecular structures at the plasma membrane likely represents the final regulatory checkpoint in necroptotic signaling. Future studies are needed to determine what controls the focal accumulation of MLKL at the cell periphery and to understand how such compartmentalization influences necroptotic cell lysis.

DIFFERENT CELL TYPES MODULATE NECROPTOTIC SIGNALING IN DIFFERENT WAYS

As emphasised above, the accumulation of threshold amounts of MLKL at the plasma membrane is required to trigger necroptotic cell death. Consequently, the threshold for necroptosis is more likely to be reached in cells that have relatively high MLKL abundance. This is an important consideration because RIPK1, RIPK3 and MLKL are variably expressed by different cell-types and across different tissues (*27, 105, 131, 179-181*). Within the repertoire of immortalised and primary cell lines that have been investigated, a subset lack the core necroptotic mediators and thus are unable to undergo MLKL-mediated death (*27, 131, 181*). For example, the commonly used HeLa and HEK293T cell lines are unable to undergo necroptosis due to absence of RIPK3 (*27*). Even when cells express all core mediators of TNF-induced necroptosis, there is substantial variability in their ability to undergo MLKL-mediated cell death. This variability likely relates to the plethora of modulators that tune signaling along the core necroptotic axis (Figure 1A-B). For instance, increased reactive oxygen species (ROS) generation during TNF-induced necroptosis often promotes cell death, yet ROS-dependent toxicity is not universally observed (*27, 99*). Similarly, the intracellular Ca^{2+} flux that occurs during TNF-induced necroptosis has been proposed to play an important role downstream of MLKL activation (*113*). However, it has since been shown that Ca^{2+} flux plays a modulatory role that accelerates necroptosis in some, but not all cell-types (*160, 174*). The multitude of MLKL trafficking mechanisms is another example in which the modulation of necroptosis is variably applied (*67*). Trafficking of MLKL to the cell periphery through Golgi, actin, and microtubule networks represents a late-stage checkpoint for necroptosis, with inhibition of this trafficking reducing the rate of MLKL-mediated death in many, but not all tested cell lines (*67*). Collectively, these studies highlight that the mechanisms that modulate necroptosis are not conserved across all cell-types. A prominent example of how core necroptotic signaling is flexibly modulated involves the endocytic and exocytic processes that remove phosphorylated MLKL from the plasma membrane (*119, 122-124*). In particular, ALIX-mediated exocytosis attenuates necroptosis in many cell types, but not in the commonly used L929 cell line

(*119*). These endocytic and exocytic clearance mechanisms are also functionally redundant, because exocytosis could remove activated MLKL from the plasma membrane when flotillin-mediated endocytosis was genetically or pharmacologically blocked (*119*). Thus, although RIPK1-RIPK3-MLKL signaling is essential for almost all forms of TNF-induced necroptosis, a surrounding suite of modulators flexibly regulates this core axis in a context-specific manner (*67, 119*).

RAPID SEQUENCE DIVERGENCE EMPHASISES THAT NECROPTOSIS IS MECHANISTICALLY AGILE

MLKL is a unique protein within the animal kingdom because it harbours the only known membrane-permeabilizing 4HB (or "HeLo") domain. However, several analogous 4HB-containing proteins have convergently evolved in plants and fungi (*182*). Similar to MLKL in animals, these 4HB-containing proteins have been attributed functions in host defense, consistent with the idea that MLKL's ancestral role lies in innate immunity (*183*). Proteins encoded by viral and bacterial pathogens inhibit necroptotic cell death by targeting RIPK1, RIPK3 and MLKL (*4, 6, 7, 183-185*). Accordingly, the selective pressures applied by these pathogens are thought to have driven marked sequence divergence in RIPK3 and MLKL between species. For example, rodent MLKL and human MLKL share only 62% sequence identity. Indeed, the sequences of the core necroptotic effectors have evolved so rapidly that MLKL orthologs from closely related species, like rat and mouse, cannot interchangeably reconstitute necroptotic signaling in the same cell type (*33, 35, 37, 106*). Moreover, the genomes of some species, including those of the Carnivora, do not encode MLKL (*186, 187*). The basis for this rapid interspecies divergence can be attributed to the hand-in-glove relationship between RIPK3 and MLKL, which in turn means that RIPK3 and MLKL have coevolved as a signaling cassette (*8, 33, 35, 106, 184*). More specifically, the coevolution of RIPK3 for MLKL likely involves selection-driven changes to the structure of the N-lobe and activation loop of the pseudokinase domain in MLKL from one species (*105, 106, 108*), with cognate changes to the kinase domain in RIPK3 from the same species. Validation of this hypothesis awaits the solving of additional RIPK3 structures (beyond the sole reported structure of mouse RIPK3 (*188*)).

The rapid co-evolution of the obligatory effectors RIPK3 and MLKL has resulted in important species-specific differences along the core necroptotic signaling axis. Despite this rapid divergence, the fundamental steps in necroptosis – namely RIPK3-mediated phosphorylation of MLKL, MLKL oligomerization, MLKL translocation and permeabilization of the plasma

membrane – are conserved. This conservation is telling, because it highlights that the underlying mechanisms of necroptosis are agile yet robust, and that completion of these core signaling events is sufficient to achieve necroptotic cell death. As discussed above, a network of ancillary modulators surrounds the RIPK1>RIPK3>MLKL axis and tunes necroptotic signaling. It is unlikely that this network of modulators has evolved at the same pace as RIPK3 and MLKL. Instead, we posit that through redundancy and flexibility, this network retains its ability to modulate necroptosis in a cell type-, context- and species-specific manner. Support for this idea can be inferred from an overview of the many screens that have been conducted to identify regulators of necroptosis: where the hits from each screen have very little overlap ((*27, 28, 31, 42-47*); Figure 1B). This nimble yet potent core mechanism perfectly equips necroptosis to be an effective "fail-safe" response during times of emergency.

CONCLUSIONS

The core necroptotic signaling pathway involves at least three major compartmentalization events. The cues and regulation underlying these compartmentalization events are poorly understood. Surrounding these core relocation events, the pathway seems to exhibit a tremendous degree of mechanistic agility, in terms of its executioner's destination (for example, which membranes are perturbed prior to plasma membrane rupture), its regulation (for example, between different cell-types) and its speciation. This flexibility supports the idea that necroptosis is an emergency form of non-apoptotic death that can be executed when sufficient amounts of activated MLKL are translocated from the necrosome to surpass the lytic threshold at membranes.

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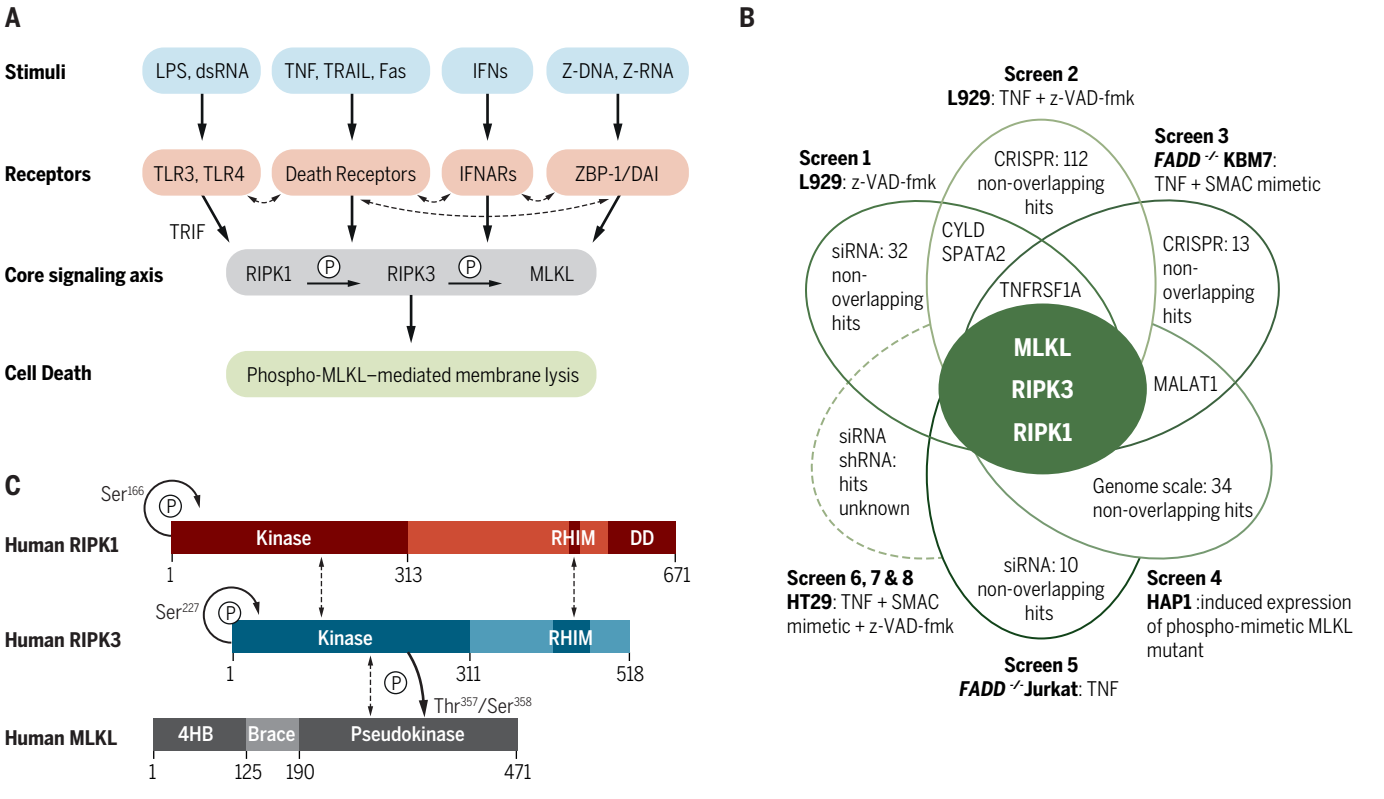
Figure 1. A variable network of modulators surrounds the core necroptotic signaling axis of TNF-induced necroptosis. (**A**) Summary of the different stimuli and receptors that signal MLKL-mediated death. Necroptotic triggers converge at the core signaling axis (RIPK1-RIPK3-MLKL) and result in phospho-MLKL-mediated membrane lysis. Dashed arrows represent the possible cross-talk between the receptors involved in necroptotic signaling (*23-26*). **(B)** Venn diagram depicting the results of eight genetic screens that identified regulators of TNF-induced necroptosis (*27, 28, 42-46*). The cell lines and necroptotic stimuli used for each screen is listed. The non-overlapping hits identified in each screen are also enumerated, whereas CYLD, SPATA2, TNFRSF1A, MALAT1 are hits that overlapped between two or more screens. Owing to its central role in TNF-induced necroptosis, the core signaling axis of RIPK1-RIPK3-MLKL has been superimposed over these screen results. (**C**) Summary of the domain architecture of human RIPK1, RIPK3 and MLKL. RHIM, RIP homotypic interaction motif; DD, death domain; 4HB, four-helix bundle; and Brace, brace helices. Grey arrows indicate critical pro-necroptotic phosphorylation events. Dashed black arrows indicate important protein:protein interactions.

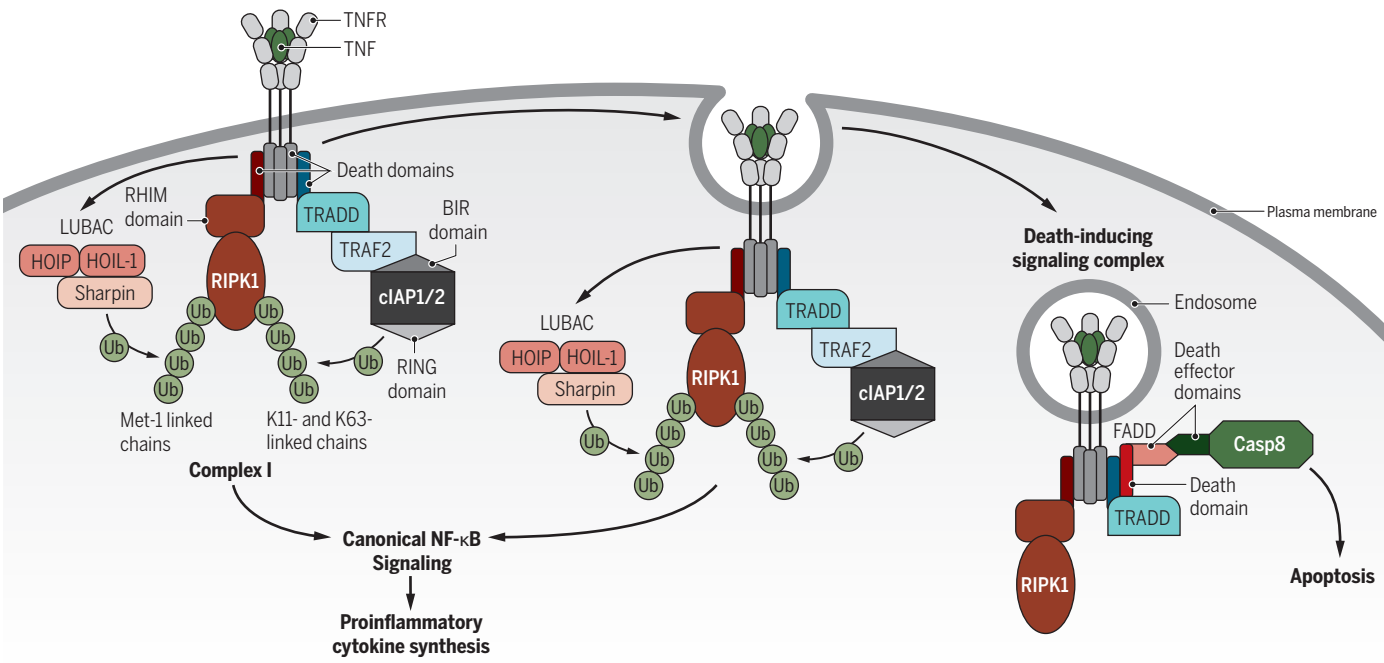
Figure 2. The first compartmentalization event in TNF-induced necroptosis. Schematic showing that TNFR1 activation leads to the formation of Complex I, which can then promote cell survival and pro-inflammatory cytokine production. Conversely, particularly when Complex I is internalized and when K11- and K63-linked ubiquitin chains are removed from RIPK1, potent cell death signals are transduced that trigger either extrinsic apoptosis or necroptosis.

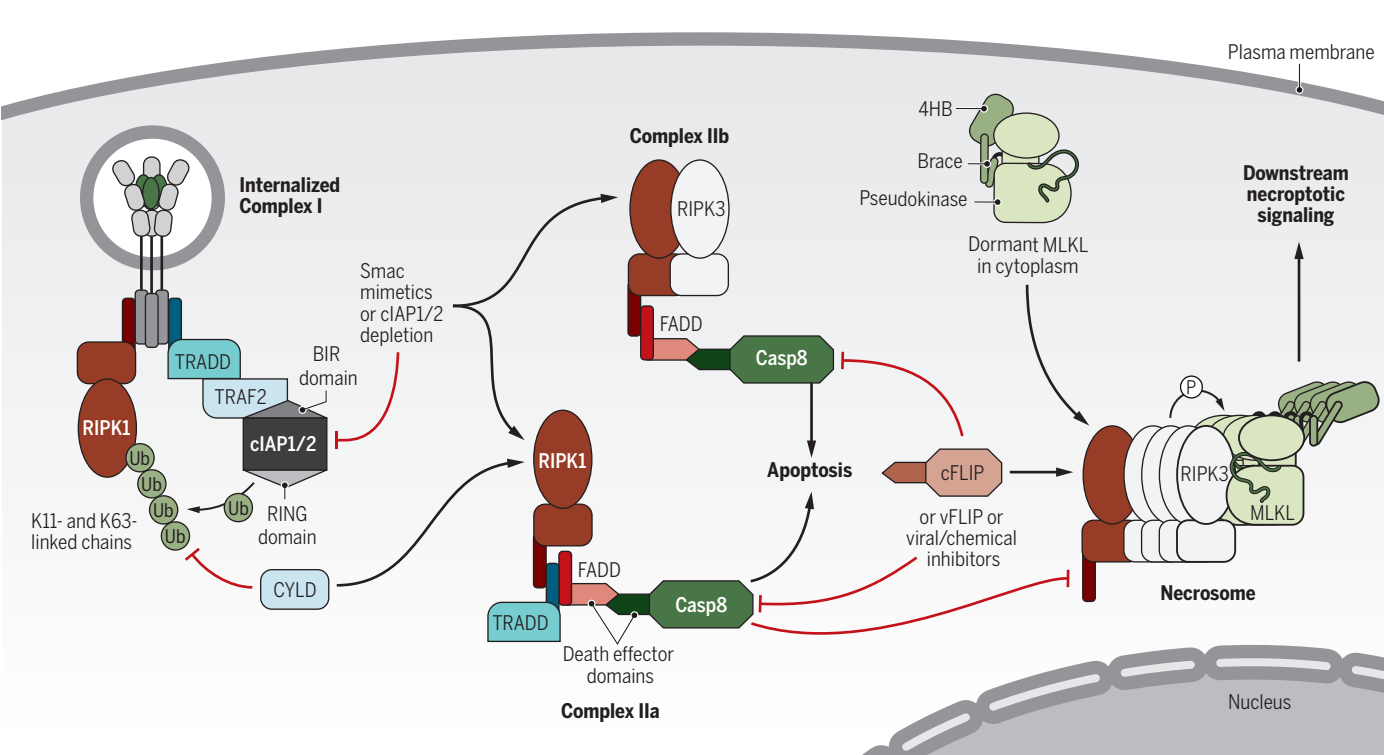
Figure 3. The second compartmentalization event in TNF-induced necroptosis. Internalized Complex I can transform into Complex IIa or Complex IIb when Met1-, K11- and K63-linked ubiquitin chains are removed from RIPK1 by the deubiquitinase CYLD (*189*). Whereas Caspase-8 activity within Complex IIa or Complex IIb drives apoptosis, its inhibition leads to the formation of the necrosome. The necrosome preferentially localizes to the perinuclear region of the cytoplasm and recruits multiple proteins, including MLKL. The activation of MLKL within the necrosome then allows it to mediate downstream necroptotic events.

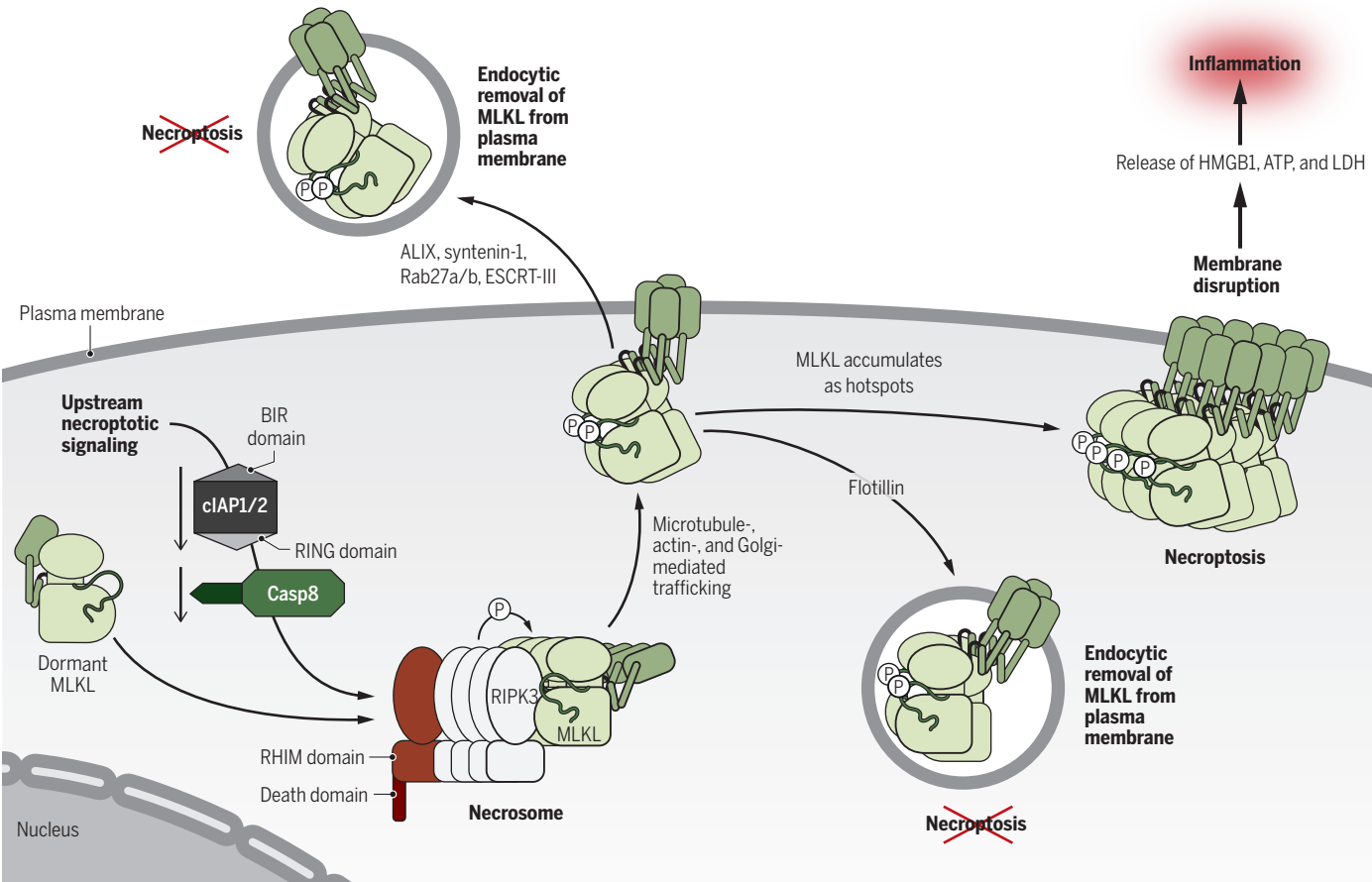
Figure 4. The third compartmentalization event in TNF-induced necroptosis. Necroptosis can be instigated in scenarios where cIAP1/2 and Casp8 abundance or activity are compromised. Downstream of pathway initiation, MLKL departs from the necrosome toward its primary destination, the plasma membrane. Here, MLKL accumulates into supramolecular structures

known as hotspots and eventually triggers necroptosis by lysing the membrane. Plasma membrane rupture promotes the release of DAMPs including high mobility group box protein 1 (HMGB1), adenosine triphosphate (ATP) and lactate dehydrogenase (LDH), which in turn are paracrine signals that induce ongoing inflammation and immune reactivity (*190*). In many, but not all cell types, MLKL is actively trafficked from the necrosome to the plasma membrane through Golgi-, microtubule- and actin-dependent mechanisms. Necroptosis can also be prevented by the endocytic or exocytic removal of activated MLKL from the plasma membrane. The former is mediated by Flotillin and the latter by Rab27a or Rab27b, ALIX, syntenin-1 and other components of the ESCRT-III complex. Whether MLKL resides within the lumen or associates with the external face of endosomes and exosomes is currently unclear.


A
Stimuli
LPS, dsRNA
TNF, TRAIL, Fas
IFNs
Z-DNA, Z-RNA
Receptors
TLR3, TLR4
Death Receptors
IFNARs
ZBP-1/DAI
TRIF
Core signaling axis
RIPK1
P
RIPK3
P
MLKL
Cell Death
Phospho-MLKL–mediated membrane lysis
B
Screen 2
L929: TNF + z-VAD-fmk
Screen 1
L929: z-VAD-fmk
Screen 3
FADD -/- KBM7:
TNF + SMAC mimetic
CRISPR: 112
non-overlapping
hits
CYLD
SPATA2
siRNA: 32
non-
overlapping
hits
CRISPR: 13
non-
overlapping
hits
TNFRSF1A
MLKL
RIPK3
RIPK1
MALAT1
siRNA
shRNA:
hits
unknown
Genome scale: 34
non-overlapping hits
siRNA: 10
non-overlapping
hits
Screen 6, 7 & 8
HT29: TNF + SMAC
mimetic + z-VAD-fmk
Screen 4
HAP1 :induced expression
of phospho-mimetic MLKL
mutant
Screen 5
FADD -/- Jurkat: TNF
C
Ser166
P
Human RIPK1
Kinase
RHIM
DD
1
313
671
Ser227
P
Human RIPK3
Kinase
RHIM
1
311
518
P
Thr357/Ser358
Human MLKL
4HB
Brace
Pseudokinase
1
125
190
471


TNFR
TNF
Death domains
RHIM domain
LUBAC
HOIP
HOIL-1
Sharpin
RIPK1
TRADD
TRAF2
BIR domain
cIAP1/2
RING domain
Ub
Met-1 linked chains
K11- and K63-linked chains
Complex I
Canonical NF-κB Signaling
Proinflammatory cytokine synthesis
Plasma membrane
Death-inducing signaling complex
Endosome
Death effector domains
FADD
Casp8
Death domain
Apoptosis


Plasma membrane
Internalized Complex I
TRADD
TRAF2
BIR domain
cIAP1/2
RING domain
RIPK1
Ub
K11- and K63-linked chains
CYLD
Smac mimetics or cIAP1/2 depletion
Complex IIb
RIPK3
FADD
Casp8
RIPK1
FADD
TRADD
Casp8
Death effector domains
Complex IIa
Apoptosis
cFLIP
or vFLIP or viral/chemical inhibitors
4HB
Brace
Pseudokinase
Dormant MLKL in cytoplasm
P
RIPK3
MLKL
Necrosome
Downstream necroptotic signaling
Nucleus


Inflammation
Release of HMGB1, ATP, and LDH
Membrane disruption
Necroptosis
Endocytic removal of MLKL from plasma membrane
ALIX, syntenin-1, Rab27a/b, ESCRT-III
Plasma membrane
MLKL accumulates as hotspots
Upstream necroptotic signaling
BIR domain
cIAP1/2
RING domain
Casp8
Dormant MLKL
Microtubule-, actin-, and Golgi-mediated trafficking
Flotillin
RIPK3
MLKL
RHIM domain
Death domain
Necrosome
Necroptosis
Endocytic removal of MLKL from plasma membrane
Necroptosis
Nucleus