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MULTI-AUTHOR REVIEW

Necrosome core machinery: MLKL

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Abstract In the study of regulated cell death, the rapidly expanding field of regulated necrosis, in particular necroptosis, has been drawing much attention. The signaling of necroptosis represents a sophisticated form of a death pathway. Anti-caspase mechanisms (e.g., using inhibitors of caspases, or genetic ablation of caspase-8) switch cell fate from apoptosis to necroptosis. The initial extracellular death signals regulate RIP1 and RIP3 kinase activation. The RIP3-associated death complex assembly is necessary and sufficient to initiate necroptosis. MLKL was initially identified as an essential mediator of RIP1/RIP3 kinase-initiated necroptosis. Recent studies on the signal transduction using chemical tools and biomarkers support the idea that MLKL is able to make more functional sense for the core machinery of the necroptosis death complex, called the necrosome, to connect to the necroptosis execution. The experimental data available now have pointed that the activated MLKL forms membrane-disrupting pores causing membrane leakage, which extends the prototypical concept of morphological and biochemical events following necroptosis happening in vivo. The key role of MLKL in necroptosis signaling thus sheds light on the logic underlying this unique "membrane-explosive" cell death pathway. In this review, we provide the general concepts and strategies that underlie signal transduction of this form of cell death, and then focus specifically on the role of MLKL in necroptosis.

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Introduction

Regulated cell death has long been appreciated as the built-in brake valve system for organisms to balance with cell survival during development, maintenance of tissue homeostasis and elimination of infected cells in pathological conditions. Building on the classic work in *C. elegans* and various animal models, the apoptosis pathway was the first evolutionarily conserved regulated cell death signaling pathway that was discovered. A large body of investigation has firmly established a central role of caspase activation and the following regulated proteolysis that leads to apoptosis execution. In contrast to apoptosis, which manifests with specific morphological and biochemical markers, necrotic cell death was initially regarded as a passive form of cell death that results from acute cellular injury or overwhelming stresses. The impressive body of knowledge about the molecular apparatus of apoptosis sets up expectations to getting a comparable clarity of understanding about the regulated necrosis signaling, which was observed independent of caspase activity [1]. Since the late 1980s, researchers have observed that, under certain conditions, caspase inhibition does not block cell death induced by members of the tumor necrosis factor (TNF) family of cytokines, but rather shifts cell fate to necrotic death [1–6]. These observations highlight a long-standing ambiguity about whether necrosis was also a form of regulated cell death like apoptosis.

The necrosome machinery closes the gap between the death signal and membrane disruption

Necrosome assembly

The complexity of necrosis is revealed by a string of defined biochemical signals that occur under tight regulation when the necrotic death complex (necrosome) is assembled. Receptor-interacting kinase 1 (RIP1, also known as RIPK1) was the first molecule identified as a necrosome component required for Fas ligand-induced caspase-independent necrotic cell death [7]. RIP1 plays pleiotropic roles in multiplying signaling processes, such as NF-κB activation and apoptosis [8–11]. Its kinase activity was found to be specifically required in death receptor activation-induced regulated necrosis, also termed necroptosis. Upon death ligand ligation, RIP1 binds with death receptors via their respective death domains (DD). Once FADD or caspase activity is inhibited by genetic or chemical methods, RIP1 forms the death complex with receptor-interacting kinase 3 (RIP3, also known as RIPK3) through their homotypic interaction motif (RHIM) domains, and activates their kinase activities [12–14]. Thereafter, the activated RIP kinases respond to and link the death signals to a downstream substrate, the pseudokinase mixed-lineage kinase domain-like protein (MLKL) [15].

Determinants of necrosome complex assembly

TNF receptor 1 (TNFR1) ligation leads to the recruitment of TRADD, RIP1, TRAF2 and cIAP1/2, which is known as complex I. The E3 ubiquitin ligases, cIAP1/2, block RIP1 transition to the necrosome by ubiquitination of RIP1 that mediates NF-κB activation [16]. Loss of cIAP1/2 function, caused by treatment with Smac mimetics, promotes RIP1 kinase activation and necrosome complex formation [17]. A20, an inhibitor of NF-κB signaling, was recently reported to restrict ubiquitination of RIP3 at Lys5 (K5) and protect from the formation of the necrosome [18].

Death receptor-activated apoptosis and necroptosis share a common regulatory signal complex (complex IIB), which includes RIP1, FADD, caspase-8 and cFLIP. Mice with deletions of any of the complex-IIB genes display severe embryonic developmental defects that lead to either embryonic ($Casp8^{-/-}$; $Fadd^{-/-}$; $cFlip^{-/-}$) or postnatal lethality ($Rip1^{-/-}$) [19, 20]. RIP3- or MLKL-deficient mice do not show developmental or homeostasis defects [13, 21–23]. The lethality caused by deficiency in caspase-8 or FADD can be rescued by loss of either RIP3 or MLKL [20, 24–27]. In addition, the neonatal lethality of $Rip1^{-/-}$ mice was rescued by concurrent loss of caspase-8 and RIP3 [28–30]. Therefore, RIP1 and caspase-8 work synergistically as regulatory components to prevent both forms of cell death during development. It is worth noting that kinase-dead RIP1 (K45A; D138N) knock-in mice develop normally into adulthood [29, 31–33]. These data implicate that the kinase activity of RIP1 is dispensable for instruction of these two death pathways in regulating multiorgan development.

Necrosome assembly provides a structural basis for RIP3 oligomerization

Necroptosis induced by the TNF family of cytokines (TNF, CD95/FasL, TRAIL) requires RIP1 for assembling the necrosome components. However, exceptions have been found. For instance, RIP3 and MLKL are required in Toll-like receptor signaling (TLR3/TLR4), but in an RIP1-dispensable way. Instead, another RHIM domain-containing protein, TRIF, is associated with RIP3 via its RHIM domain, which is analogous to the RIP1–RIP3 necrosome complex [30, 34–36]. Likewise, murine cytomegalovirus (MCMV)-induced necroptosis employs a DAI–RIP3–MLKL axis. In addition, the virus DNA sensor, DAI, was shown to be associated with RIP3 also through its RHIM domain [34, 37, 38]. Additionally, the herpes simplex virus (HSV)-1 effector protein, ICP6, binds with RIP1/3 through their virus–host RHIM interactions, which leads to either pro-necroptotic or anti-necroptotic effects in mice and humans, respectively [39–42]. Given this shared mode of necroptosis induction across physiological and disease processes in varying species, a simple working definition for necroptosis initiation includes the RHIM domain protein-mediated activation of RIP3 functionality (Fig. 1).

A complementary study to assess the mutual dependency of RIP1 and RIP3 has been to generate a death receptor-free environment by building up an artificial dimerization system for RIP1 and RIP3. Cook et al. found that necroptosis can be induced either by dimerization of RIP1 or RIP3 in immortalized mouse embryonic fibroblasts (MEFs) [43]. Wu et al. expressed combinations of wild-type and RHIM mutant RIP1 and RIP3 in 293T cells [44]. They emphasized that the necroptotic signal is propagated from the RIP1–RIP3 amyloid scaffold by recruiting additional free RIP3 molecules. Orozco et al. added a point that RIP1 might negatively regulate the spontaneous oligomerization of RIP3 when RIP3 is maintained at a relatively low level, although they faintly induced necroptosis by expressing RIP3 dimer in their system [45]. These discrepant data of dimerized RIP3 were explained by side effects of the different positions that the protein was tagged with the dimerization domains.

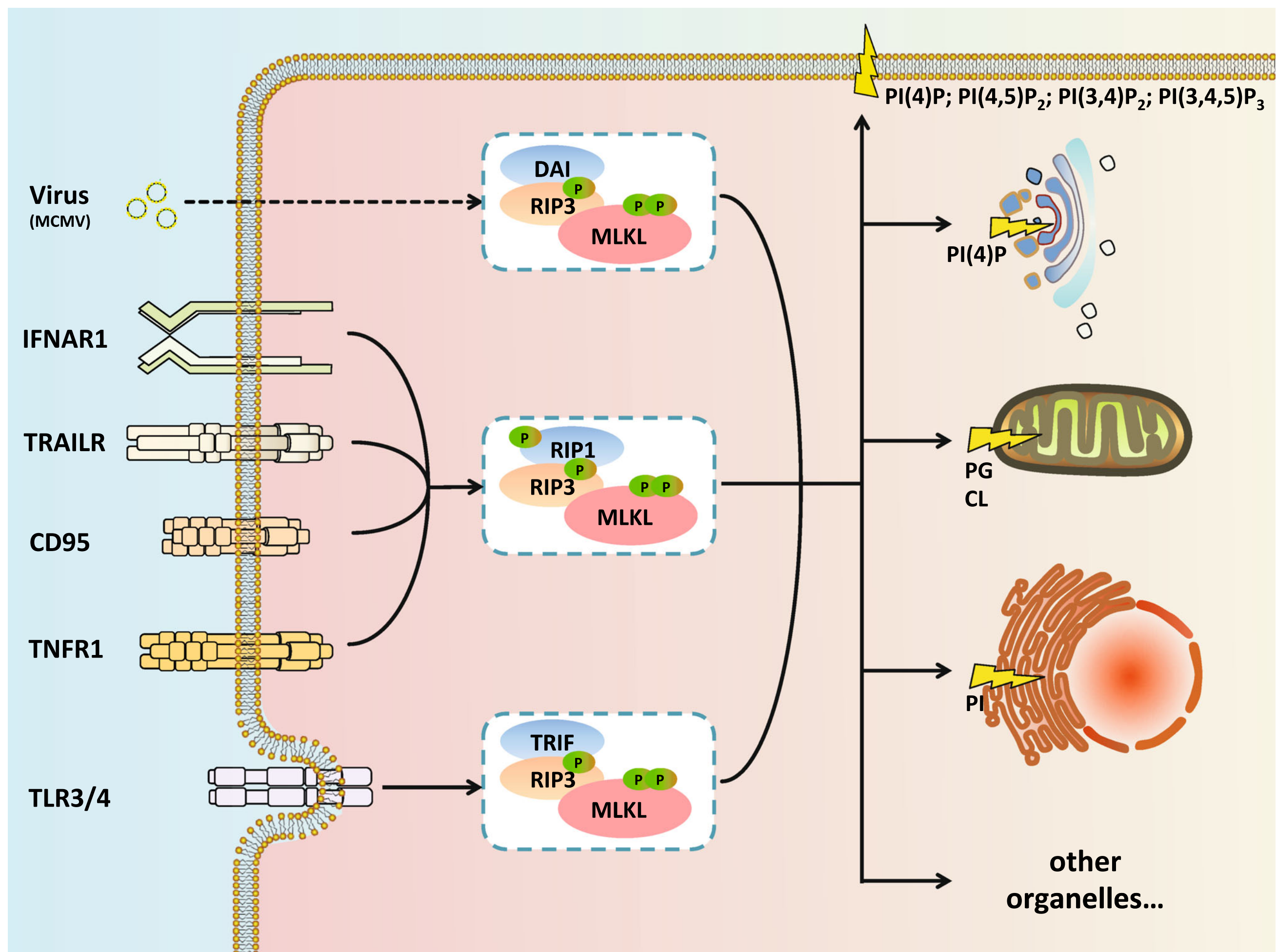

Fig. 1 Necrosome assembly and signal propagation. Ligation of members of the tumor necrosis factor receptor superfamily (TNFRSF, including TNFR1, CD95, TRAILR) leads to the recruitment of receptor-interacting protein kinase 1 (RIP1). RIP1 and RIP3 form a pro-necrotic death complex via their RHIM domains. RIP3 is then activated by auto-phosphorylation at Ser227 (Ser232 for mouse RIP3). The activated RIP3 recruits and phosphorylates its substrate MLKL at Thr357/Ser358 (Ser345 for mouse MLKL), a step which defines the formation of the functional necrosome. Besides TNFRSF, activation of type I interferon receptor (IFNAR1) also triggers the formation of a RIP1–RIP3–MLKL-containing necrosome. Moreover, pattern recognition receptors (PRRs) drive necrosis in an RIP1-independent manner. Toll-like receptor-induced TRIF–RIP3–MLKL necrosome formation (for TLR3, e.g., by sense dsRNA such as poly I:C; for TLR4 in response to LPS) also depends on RHIM interaction between TRIF and RIP3. Likewise, infection with M45 mutant murine cytomegalovirus (MCMV) leads to RHIM-mediated interaction between DAI and RIP3. Of all necrosome protein complexes reported to date, MLKL binds to activated RIP3 to propagate the necrotic death signal, regardless of what RHIM protein RIP3 utilizes for its self-activation. Activated MLKL binds phosphatidylinositol phosphates (PIPs), cardiolipin (CL) and phosphatidylglycerol (PG), which navigate necrosomes to different phospholipid-rich cellular compartments. Once targeted to membranes, MLKL disrupts membrane integrity and finally causes necroptotic cell death

Nonetheless, we assemble here a scenario where the death signal flows from RIP1 to RIP3 through their RHIM domains, and additional molecules of RIP3 would be recruited afterward for auto-phosphorylation-driven signal propagation. Meanwhile, an emerging theme is the highly conditional influence of these artificial systems, in that RIP1 is needed under some circumstances but not in others. Altogether, these data lend further support to the notion that RIP3 oligomerization is the minimal functional unit that is required to drive necrosome assembly.

How RIP3 activates MLKL

Mixed-lineage kinase domain-like protein was found to be specifically required for the RIP3-dependent necroptosis pathway. It was initially identified as a RIP3-binding protein via its C-terminal kinase-like domain. A series of kinase assays combined with proteomics studies revealed that MLKL is the natural target of RIP3 kinase. Recruitment of MLKL depends on auto-phosphorylation of RIP3 at S227 (S232 for mouse RIP3) [15]. In a following study,

Xie et al. presented the co-crystal structure of the mouse RIP3 kinase domain (residues 1–318) with the MLKL kinase-like domain (residues 182–464) at 2.5 Å resolution [46]. The binding interface between RIP3 and MLKL was depicted aligning in a parallel fashion. The phosphate group of the phosphorylated Ser232 in RIP3 was clearly visible, which accepts an H-bond from the hydroxyl group of Ser404 in MLKL. In addition, other observed phosphorylated residues, Ser184 and Thr231, of RIP3 were found not to be involved in the interface with MLKL, and had little impact on RIP3–MLKL complex formation. At the center of their interface, Phe27 of RIP3 and Phe234 of MLKL stack against each other through π–π interactions, providing the most prominent bond strength for RIP3 and MLKL [46]. A cochaperone complex, HSP90–CDC37, was discovered as RIP3-binding partner [47]. Knocking down CDC37 or using HSP90 inhibitors efficiently blocks necroptosis by preventing auto-phosphorylation on RIP3–Ser227 and RIP3 punctae formation. Besides, one of the negative regulators of this auto-phosphorylation on RIP3, protein phosphatase 1B (Ppm1b), was recently found to suppress necroptosis by dephosphorylation of RIP3 [48]. Taken together, the auto-phosphorylation of RIP3 at S227 (S232 for mouse RIP3) shapes up the interface of RIP3 in recognizing its substrate MLKL.

Evidence suggests that p-MLKL is the key for necroptotic signal transfer. When activated RIP3 binds to MLKL, it subsequently phosphorylates MLKL at T357/S358 (S345 for mouse MLKL) [15]. A study of doxycycline (DOX)-inducible MLKL mutants expressed in *mlkl*$^{-/-}$MEFs also confirmed that phosphorylation of Ser345 is critical for RIP3-mediated necroptosis, while other reported sites (Ser347 and Thr349) either play minor roles or seem to be irrelevant [49].

MLKL phosphorylation as a biomarker of necroptosis activation

Based on the knowledge that MLKL is phosphorylated by RIP3, a rabbit monoclonal antibody was developed to specifically recognize phosphorylated MLKL (p-MLKL) and serve as a marker for necroptosis [50]. This work advances the field by allowing to detect necroptosis in a more accurate way. Given the specificity of this phospho-antibody, an emerging picture of the properties of necrotic diseases has helped us gain a broader understanding of under what conditions this form of cell death occurs. For example, drug-induced liver injury (DILI) was the first reported clinical disorder that exhibits necrotic damage by showing strong p-MLKL signals in the diseased compartments [50]. After that, researchers also observed p-MLKL signals in human non-alcoholic steatohepatitis (NASH) samples. Recent studies implicated that MLKL-dependent necroptosis is highly prevalent in isolating and removing pathogens [39]. In addition, necroptosis is implicated in regulating chemical-induced cell injuries, such as cerulein-induced pancreatitis [21]. Furthermore, excessive necrotic cell death is associated with ischemia reperfusion-induced damage in the brain as well as in patients with neurodegenerative disorders [51]. In a study of human multiple sclerosis (MS) pathological specimens, robust p-MLKL signals were detected [52]. Apart from these clinical conditions, p-MLKL signals also occur in primate ovarian tissues (human and rhesus monkey), which may contribute to follicular atresia and luteolysis in females, eventually leading to menopause [53]. Application of this phospho-antibody as a biomarker to reflect necroptotic cell death in patients will provide us sufficient proof-of-principle support for the development of pharmaceutical agents that interfere with these necroptotic diseases.

Necrosome inhibitors

A range of chemical screens for necroptosis inhibitors has significantly increased our understanding of necrotic signaling; however, each has had its drawbacks. The first well-defined necroptosis inhibitor is necrostatin-1 (Nec-1), which blocks necroptosis by targeting RIP1 kinase activity [54, 55]. More in vivo tests of Nec-1 derivatives were explored in animal models, such as 7N-1 which blocks demyelination induced by cuprizone and encephalomyelitis EAE [34, 52]. However, since RIP1 also contributes to other processes beyond necroptosis, such as the regulation of inflammatory cytokine release and apoptosis, an independent focus has also been placed on RIP3 and MLKL inhibitors. But, the challenge for RIP3 inhibitors resides in the potential gain of function of triggering apoptosis. A collection of RIP3 inhibitors induced apoptosis while simultaneously blocking necroptosis, which could be explained by the discovery that overexpression of kinase-dead RIP3 (K51A), causes the cells to die from apoptosis [56]. In line with this, recently, RIP3 kinase-dead knock-in mice (D161N) were found to be embryonic lethal due to vast apoptosis [31]. A larger pool of RIP3 inhibitors is in the process of being further characterized for their apoptotic toxicity.

All of these data above exemplify the urgent need for finding less-toxic necroptosis inhibitors. So far, the MLKL inhibitor, NSA, shows the least amount of toxicity to cells [15]. NSA blocks necroptosis by targeting Cys86 on MLKL, thus interfering with MLKL oligomerization. In the studies of necroptosis signaling, NSA was proven to be a unique tool for dissecting the downstream process of necroptosis without disturbing necrosome death complex assembly. However, because NSA has an exclusive recognition of human MLKL, this limits its application in

mouse models. In 2014, another mouse MLKL inhibitor, referred to as compound 1 by Hildebrand et al., was identified to inhibit the necroptosis pathway by delaying MLKL translocation to the membrane, but its toxicity and multiple targets on the other components of necrosome preclude the application of this MLKL inhibitor [57]. New necroptosis inhibitors that target mouse MLKL will expand our knowledge for the in vivo significance of MLKL-dependent necroptosis. In addition, a series of chemical tools also anchor our interpretation of the underlying logic of MLKL activation.

Emerging concepts in the study of necroptosis execution

Membrane pore-forming machinery by MLKL

Phosphorylation drives MLKL oligomerization and membrane translocation

Within the past few years, it has become clear that the phosphorylation of MLKL builds on the most formidable engine of necrosome machinery, which ignite and propel the death signal toward membranes. As we review here, there is now much clearer understanding of the crucial role of MLKL in necroptosis execution.

Downstream necroptosis signals work through MLKL. Accordingly, phosphorylated MLKL would be expected to transduce death signals. Both in vivo and in vitro biochemical analyses characterized the oligomerization nature of MLKL. Necroptosis induction causes a molecular weight shift of MLKL, which appears on non-reducing PAGE gels. Wang et al. demonstrated that phosphorylation of MLKL turns on its oligomerization [50]. However, researchers have not reached a consensus on the minimal units of the MLKL oligomer [50, 58–60]. Interpretations of these data, using crosslinkers or gel filtration, should be regarded with caution, given the present technical limitations. Only alternative approaches with higher resolution, such as NMR and crystallography technology, could provide a conclusive physical picture of MLKL oligomerization.

Biochemical fractionation revealed that MLKL translocates to the plasma membrane after necroptosis is induced. By following the phospho-MLKL signals, it was found that this translocation was not limited to the plasma membrane; organelle membranes were also targeted by p-MLKL. This targeting to membranes is facilitated by the N-terminal coil–coil domain of MLKL, which possesses a patch of positively charged amino acid that enables MLKL to interact with phospholipids. Pleckstrin homology (PH) domains are well known for their binding to phosphatidylinositol lipids (PIPs)-containing lipids within biological membranes. Different PH domains possess specificities for different lipids [61–64]. It is noteworthy that MLKL has a broader affinity for PIPs than PH domains. Both Wang and Dondelinger found that the N-terminal MLKL directly binds with phosphatidylinositol phosphates (PIPs) [50, 65]. In addition, Wang et al. found that cardiolipin (CL) could also bind to MLKL.

Different PIPs may direct MLKL to different cellular compartments. The lipid composition of these membranes varies from organelle to organelle even though these distinct membrane systems are in communication through intracellular trafficking by vesicles. Most membranes are rich in phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI). PC and PI are enriched in the ER; phosphatidylglycerol (PG) and CL are synthesized in and confined to mitochondria [66, 67]. For instance, plasma membranes have the most abundant PI(4)P and PI(4,5)P2. This could explain the vast damage on cell membranes after necroptosis induction. Cardiolipin (CL) is mostly distributed in the mitochondrial inner membrane. During necroptosis, interference with mitochondrial fusion has been described. This was well timed for CL cytosolic exposure, and the CL-enriched microdomain provides a signaling platform for MLKL-mediated death signals on mitochondria [68–70], which would strengthen the intrinsic amplification of death signals. In the same spirit, other PIPs have their distinctive organelle distribution, which would give MLKL the proper guidance to the designated membrane compartments.

Evidence that MLKL punches membranes

Membrane rupture and organelle swelling have remained cornerstone features of necrosis. Investigators have generally regarded membrane damage as a basic morphological criterion for necrosis. The released cellular content of necrotic cells will trigger an immune response. Thus, the exposure of DAMPs (damage-associated molecular pattern) [71] has been considered as a signature indicator for necrosis.

Using the classic liposome leakage assay, MLKL was found to translocate to PIPs- or CL-containing membranes and to disrupt the membrane integrity in a dose-dependent manner [50] (Fig. 2a, adapted from [72]). NSA can block these membrane disruptions. Since NSA is targeted to the N-terminus of MLKL, this discovery is consistent with the finding that the N-terminal domain mediates liposome damage and cell death. Moreover, when the intracellular levels of MLKL-binding PIPs were downregulated by interfering with their production processes, MLKL-dependent necroptosis was largely attenuated [65]. These experiments extensively characterized the pore-forming

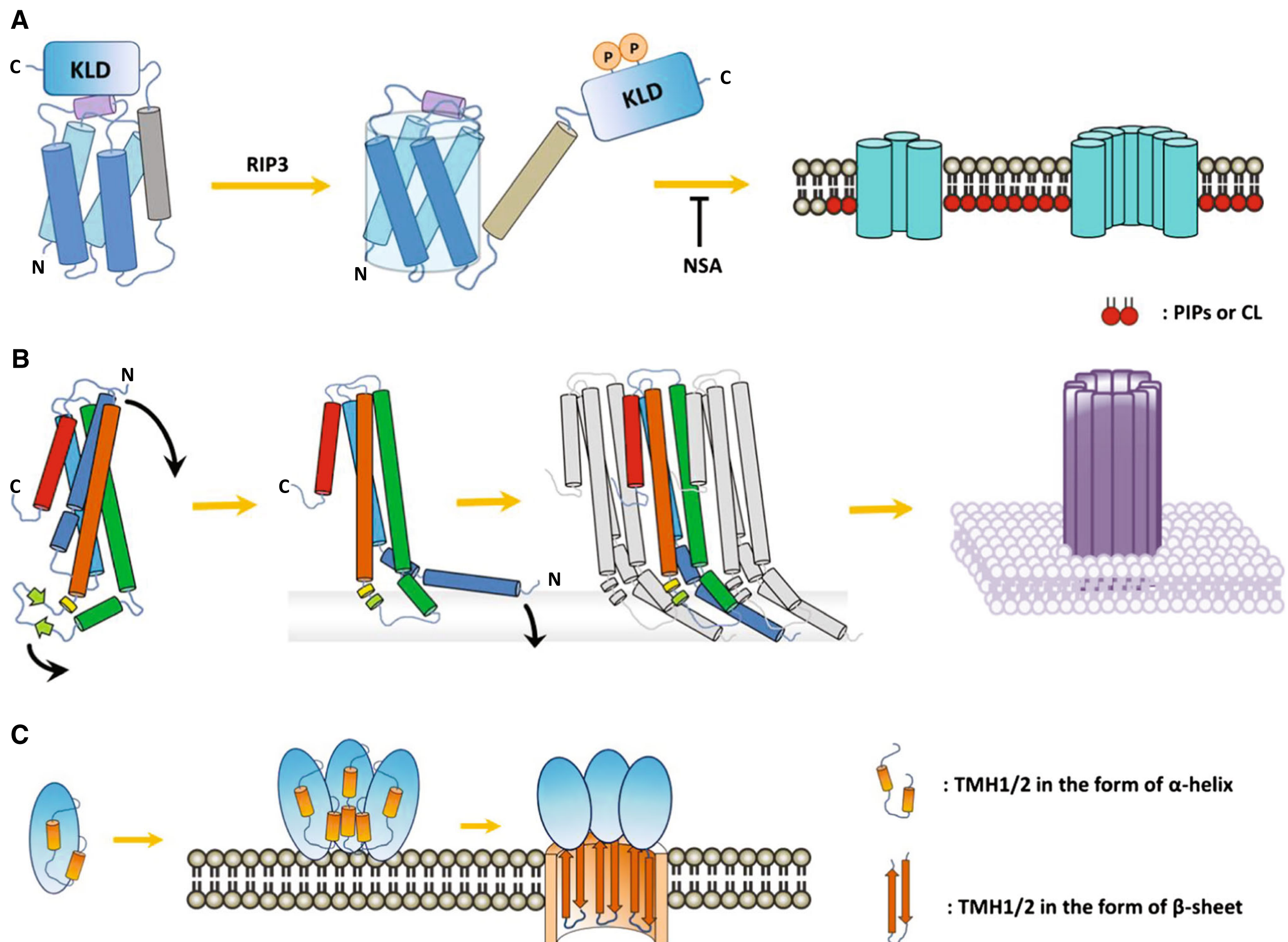

Fig. 2 Overview of the membrane-punching mechanisms of pore-forming proteins. **a** Oligomerized MLKL punches membranes. The MLKL monomer is sequestered in an inactivated state by its C-terminal kinase-like domain (KLD). Phosphorylation of MLKL releases the auto-inhibition on the amino-terminal MLKL and enables MLKL to bind with PIPs or CL. NSA blocks MLKL from oligomerization and membrane translocation. **b** A representative α-PFT protein, cytolysin A (ClyA, also known as HlyE), forms a pore on a target membrane. The β-tongue, consisting of two β-strands between the third and fourth helices, locks ClyA in a compacted soluble state. Once ClyA binds to the membrane, the C-terminal helix is released from the bundle, touches the membrane and further recruits other molecules. After assembled into oligomers, the aggregated C-terminal helices insert into the membrane to form pores. **c** Structural transitions of CDC pore-forming proteins. Beta-PFTs are assembled into oligomeric pre-pores and bind to the membrane. The tandem transmembrane helical (TMH) units that are initially buried in the core of the pre-pore intermediate will be exposed and inserted into the membrane as a β-barrel. Perforin and the complement share similar primary structures of both monomer and oligomer, whether there is a transition station remains unclear

property of MLKL on membrane structures. It puts forth the possibility that MLKL is the executioner of necroptosis by punching holes in cellular membranes. It has become increasingly clear that the pore-forming properties of MLKL are further influenced by the interplay between the N-terminus of MLKL and phospholipids.

The structure of the MLKL N-terminal region has been determined by nuclear magnetic resonance spectroscopy, which reveals that the four-helix bundle (4HBD) with an additional helix at the top is likely to be the key for MLKL function. Further, fluorescence spectroscopy measurements indicate that much of the 4HBD inserts into membranes, but not the intermediate helix [72]. Moreover, 4HBD is sufficient to induce liposome leakage, while the C-terminal helix inhibits this activity [50, 72]. It has also been reported that expressing truncated forms of MLKL lacking the pseudokinase domain can lead to constitutive cell death in both *Mlkl*$^{-/-}$ and WT MEF cells or HEK293T cells [57, 65], indicating an essential role of the MLKL N-terminal domain in necroptosis signaling and implicating the C-terminal pseudokinase domain as a suppressor to restrain the N-terminal 4HBD function. A recent work by Quarato et al. has further confirmed that the interaction between the brace and N-terminal 4HBD exerts the “inhibitory plug” regulation. Moreover, they also showed once MLKL integrates with the membrane, its N-terminal helix bundle

utilizes a "rolling over" mechanism to expose additional high-affinity PIP-binding sites, which added another layer of distinct PIP-binding sites responsible for robust association to the membrane [73].

Apart from the aforementioned MLKL activation pathways, artificial systems can also lead to the activation of MLKL and to execution of necroptosis. Taking advantage of the HBD*-4-OHT dimerization system, Chen and colleagues demonstrated that activation of MLKL by forcing the protein or its N-terminus together (tetramerization here) directly triggers necroptosis, which can bypass RIP3 signals [58]. In accordance with the artificial system, they also observed MLKL tetramers in TNF-induced necroptosis. Using the FKBPv-AP20187 dimerization system, Wang and colleagues reported that polymerization of phosphomimic-MLKL leads to more necroptosis than WT-MLKL in RIP3-deficient cells [50].

Controversies: channel theories

A range of experimental criteria has been employed to monitor ion disturbances, which potentially contribute to necroptosis. In a cell-based experiment, Cai et al. found that TRPM7-mediated Ca^{2+} influx is required for necroptosis execution [59]. This finding suggested an activation process of non-voltage-sensitive Ca^{2+} channels downstream of MLKL. Adding to this, Chen et al. provided an experimental indication of a potential Na^{+} channel downstream of MLKL, suggesting that the membrane translocation of MLKL is truly associated with membrane disruption [58]. These perspectives, however, conflict with the phenomenon that Ca^{2+}/Na^{+} depletion blocks necroptosis, which largely depends on the experimental settings and the specific backgrounds of cell lines. Nevertheless, more exploration is required on how such channels mesh with membrane rupture happening, which involves many biological processes. The main doubt about these data hinges on the presumption that either Ca^{2+}/Na^{+} influx is the cause or one of the consequences that the osmotic pressure posed by the MLKL perforator elicits.

Other pore-forming proteins

Apart from MLKL, Mother Nature makes judicious use of finite pore-forming mechanisms to control cell death, of which there are a certain number of pore-forming toxins (PFTs) produced typically by bacteria, complement proteins or perforins released by cytotoxic T lymphocytes or natural killer cells. All these proteins share the ability to form pores in the plasma membrane. According to the secondary structure involved in pore formation, PFTs can be classified into α-PFTs and β-PFTs [74, 75]. α-PFTs form pores using helices (Fig. 2b, adapted from [76]) while β-PFTs, such as cholesterol-dependent cytolysins (CDCs), insert into membranes by their β-sheet structure [77–79]. Their membrane-damaging processes can be divided into three steps: oligomerization, membrane insertion, and pore formation. The membrane attack complex (MAC) of complement is a part of the mammalian innate immune system, which is formed by sequential assembly of C5b with C6, C7 and C8, and polymerization of C9, resulting in transmembrane pore formation and loss of membrane integrity [80, 81]. Recent findings provided some insights into the similarities between PFTs and MAC in the process of pore formation [82, 83]. Aside from the humoral cytotoxicity that is caused by the complement system, the lesion caused by cytotoxic T lymphocytes (CTLs) is also formed by polymerization of a pore-forming protein, called perforin, to form cylindrical pores on the target-cell membrane. The pores formed by perforin are similar to those observed in complement-mediated lysis, which mechanistically form pores in a CDC-like manner [84–87] (Fig. 2c adapted from [88]).

The pore-forming proteins mentioned above share general characteristic principles. Thus, the impact of the pore-forming mechanism spans many fields of study, such as the conversion from water-soluble monomers to intramembranous oligomers, membrane insertion by specific secondary structure motifs, selective recognition of membrane lipids, and induction of cell lysis. The finding that MLKL does not fit into any of these known pore-forming protein categories also, in turn, sheds light on new paradigms for defining pore-forming proteins.

In addition, gasdermin D (GSDMD) was recently identified as a critical component in pyroptosis, another kind of necrosis driven by inflammatory caspases, for which the membrane-damaging mechanism has remained a mystery for years. GSDMD can be functionally divided into gasdermin-N and gasdermin-C domains. Once inflammatory caspases (caspase-1, -4, -5 and -11) are activated, they specifically cleave GSDMD behind D257 (D276 in mouse) and enable the gasdermin-N domains to sufficiently drive pyroptosis [89, 90]. However, the biophysical properties by which the N-terminal fragment elicits pyroptotic cell death remains unknown, and it remains to be explored whether it shares a similar membrane-damaging mechanism with the known pore-forming proteins or whether it has a requirement for a receptor on the target membrane.

Concluding remarks and perspectives

Necroptosis can be triggered by various death signals. We have deliberately focused on mechanisms that convey the signal from RIP kinases to MLKL. We believe that wide variations in the RIP1/RIP3 kinases interplay/hierarchy

reflect the differences in experimental settings, which will ultimately be traced to variations in the potential modulatory factors for these death kinases. Recent progress on necroptosis signaling advances our knowledge on the core machinery, most importantly on MLKL activation and its highly ordered organization on liposome structures leading to membrane disruption. More biophysical analysis about the membrane-binding characteristics of MLKL is needed, which will further our understanding of the executioner role of MLKL during necroptosis.

Apart from the notion that MLKL receives death signals from RIP3 kinase by direct phosphorylation, it was not at all obvious how MLKL transduces signals to its downstream effectors. The phenomenon of MLKL nuclear translocation before cell death was found by immunofluorescence and biochemical fractionation [91]. Although the functional relevance of this population of p-MLKL for necroptosis execution is still unclear, the consequences of different subcellular translocations of p-MLKL remain to be systemetically clarified. We do not claim that this summary provides a complete explanation of how necrotic damage appears on membranes. Even if the pore forming of MLKL is not the whole story of membrane rupture, it will be fruitful to pursue the regulators of RIP1/RIP3, and the potential co-factors of MLKL. Though many aspects of necrosome signaling are still shrouded in mystery, it is clear that new techniques such as a combination of cellular analysis and biophysical investigation can illuminate the unique complexity of the necrosome machinery.

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