MLKL ROLE IN NECROPTOSIS – a structural overview

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0 / INDEX

1 / INTRODUCTION TO CELL DEATH

Cell death is the biological process by which cells end carrying out their functions and die. Currently, it is widely accepted that cell death is an essential process precisely regulated and involved in homeostasis maintenance [1]. However, cell death was initially assumed as a damaging process induced by pathological factors. It was not until 1842 that Karl Vogt proposed the existence of physiological cell death in developmental processes [2]. The concept of *programmed cell death* appeared for the first time in 1964 when Lockshin and Williams described regulated cell death during insect metamorphosis [3]. Afterwards, Schweichel and Merker examined by electron microscopy cell death throughout the embryonic development of rodents treated with or without embryotoxic substances [4]. This study led them to report the existence of three different types of cell death regarding the morphology of the dying cells. Type I was related to heterophagy, type II was related to autophagy and type III did not include digestion events. In 1990, Clarke did a more detailed classification of cell death typologies based on Schweichel and Merker's work [5]. Clarke's studies allowed the generation of the current classification in which cell death type I, II and III are referred to as apoptosis, cell death associated with autophagy and necrosis, respectively. The current classification includes both morphological and biochemical features that are distinct in each cell death process.

APOPTOSIS

In 1972, Kerr, Wyllie and Currie coined the term *apoptosis* [6]. Cells undergoing apoptosis suffer nuclear condensation and fragmentation, along with extensive hydrolysis of nuclear DNA. They become rounded and retract from neighbouring cells. The plasma membrane starts blebbing and many of these blebs usually detach from the membrane, generating small free vesicles named apoptotic bodies, which contain cytoplasmic and nuclear fractions (Fig. 1A). Apoptotic bodies and cells are rapidly recognised and degraded by phagocytes, permitting to recycle the cellular contents and avoiding the cytoplasmic content leakage. This is thought to be essential to prevent neighbouring cell damage by limiting the release of intracellular molecules that are related either with the generation of direct cell damage or the activation of unwanted immune responses [7,8].

With regards to the molecular basis, there are different apoptosis activation pathways and all of them culminate in activating several members of a family of cysteine proteases known as caspases. The extrinsic apoptotic pathway is triggered by the binding of extracellular ligands, such as tumour necrosis factor-α (TNFα), to transmembrane death receptors. The signaling cascade culminates with the activation of caspase 8, which in turn activates effector caspases 3 and 7 [7,8,9]. The intrinsic apoptotic pathway can be initiated

by different stressing and damaging stimuli and involves the loss of mitochondrial membrane integrity. The intermembrane space protein cytocrome c and the caspase 9 are the leading molecules in this pathway [9,10].

CELL DEATH ASSOCIATED WITH AUTOPHAGY

As mentioned before, Lockshin and Williams introduced the expression programmed cell death more than 40 years ago. Remarkably, this first example of programmed cell death had the morphology of cell death associated with autophagy. This type of cell death occurs in the absence of chromatin condensation but is accompanied by large-scale autophagic vacuolisation of the cytoplasm [7,11] (Fig. 1B). The autophagic vesicles can be autophagosomes or autolysosomes. The former are double-membraned vesicles that contain cytosol or morpholo-gically intact cytoplasmic organelles. The latter arise from the fusion of autophagosomes and lysosomes. They are single membrane vesicles that contain degenerating organelles undergoing macroautophagy [11]. Even though there is no doubt that cells can manifest extensive autophagy shortly before or during their death, it is difficult to assess whether the autophagy is the inherent death cause or not [12].

NECROSIS AND REGULATED NECROSIS

For a long time, apoptosis was considered the standard programmed cell death process. Instead of that, necrosis was mostly considered an accidental cell death process that only occurs in pathological circumstances. Nonetheless, it is clear nowadays that necrosis can occur in a finely regulated manner during both physiological and pathological processes. This paradigm shift was due to the discovery of genetic evidence and chemical inhibitors for necrosis [1]. Necrotic cell death is morphologically characterised by cellular and organelle swelling, cytoplasmic granulation, plasma membrane rupture and subsequent loss of intracellular contents [1,11] (Fig. 1C). These morphological qualities are shared by multiple modes of regulated necrotic cell death that have distinct underlaying signalling pathways like necroptosis, parthanatos, oxytosis, ferroptosis, ETosis, NETosis, pyronecrosis or pyroptosis.

The current work will focus on necroptosis pathway. The term necroptosis was originally generated to indicate a specific case of regulated necrosis. Recently, the same term has been used as a synonym of regulated necrosis. The Nomenclature Committee on Cell Death (NCCD) encourages the use of this last acceptation in scientific publications. However, necroptosis can be used in its original meaning provided that this expression is explicitly defined at its first appearance and used consistently thereafter [12]. In the current work the word necroptosis will be used referring to its specific meaning.

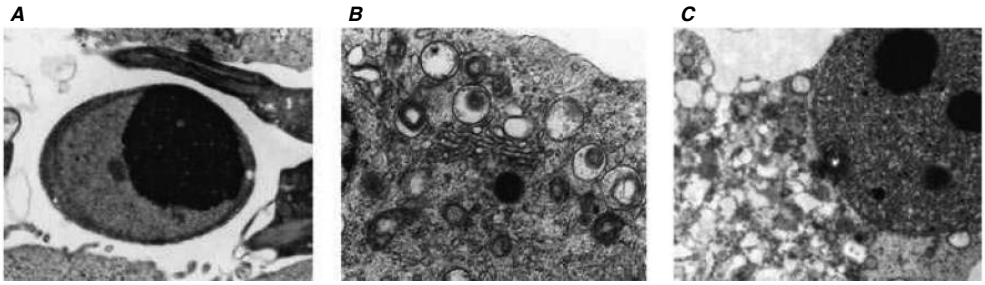

Figure 1. Cell death can happen in three different ways that show distinct morphologies. **(A)** Apoptosis in characterised by cell-rounding, nuclear fragmentation and membrane integrity maintenance, **(B)** cell death associated to autophagy shows cytoplasmic vacuolarization and **(C)** necrosis displays cytoplasmic granulation and membrane integrity loss.

2 / NECROPTOSIS

Necroptosis is a regulated type of cell death that occurs in cells in which the execution of apoptosis is inhibited. Therefore, necroptosis is an alternative mode of cell death that can be triggered by pro-apoptotic stimuli, but it is developed in a caspase-independent manner and shows morphological characteristics resembling necrosis [13]. Even though the molecular basis of necroptosis is not fully elucidated, it is known that it is executed in a highly regulated manner by a specific set of molecules — receptor-interacting protein 1 (RIPK1) and 3 (RIPK3), as well as mixed lineage kinase domain-like protein (MLKL) are the most relevant ones. A description of this molecular pathway is provided next.

INDUCING NECROPTOSIS

Necroptosis can be promoted by different stimuli. The best known way to induce necroptosis is through death receptors activation, which is also a common way to induce apoptosis. Death receptors is a plasma membrane integral protein family which includes CD95 (also known as FAS), TNF receptor 1 (TNFR1) and 2 (TNFR2), TNF-related apoptosis-inducing ligand receptor 1 (TRAILR1) and 2 (TRAILR2), amongst others [14]. Moreover, It has been discovered that necroptosis can also be triggered by several members of the pathogen recognition receptor (PRR) family. These receptors are expressed in cells of the innate immune system and are activated when they bind pathogen–associated molecular patterns (PAMPs). PRRs can be both plasma membrane or endosome membrane-associated proteins such as Toll-like receptors (TLRs). TLR3 and TLR4, activated by double-stranded RNA (dsRNA) and lipopolysaccharide (LPS) respectively, are two of the most studied PRRs related to necroptosis. Finally, there are also cytosolic PRRs that might be related to necroptosis induction [1, 14].

MAKING A CHOICE

Currently, the TNFR1 necroptosis-inducing pathway is the most extensively characterised necroptotic pathway. It is strikingly remarkable that cell exposure to TNFR1 ligand, TNFα, can result in cell survival, apoptosis or necroptosis, depending on many variables such as cell type, cell activation state and microenvironment factors. There are several key mechanisms that are involved in regulating the switch between cell survival, apoptosis or necroptosis responses. Particularly, the ubiquitin-editing system and the state of caspase 8 can modulate the final outcome of this pathway.

Initially, TNFR1 monomers spontaneously assemble forming inactive trimers. When the interaction between TNFR1 trimer and TNFα takes place, the receptor changes its conformation and it is therefore able to recruit several molecules through its cytosolic domain [15]. This recruitment allows the formation of a membrane-proximal supra-molecular structure known as complex I (Fig. 2A), which contains the TNFR-associated death domain (TRADD), the TNFR-associated factor 2 (TRAF2) and 5 (TRAF5), RIPK1, and the cellular inhibitor of apoptosis 1 (cIAP1) and 2 (cIAP2) [14].

The assembly of complex I can induce cell survival. cIAP1 and 2 are E3 ubiquitin ligases recruited to complex I by TRAF2, which avoids the polyubiquitination and degradation of cIAP1 and 2, making them more stable in the complex. In this situation, cIAPs can trigger polyubiquitination of RIPK1. This post-translational modification provides a platform for the interaction between transforming growth factor-β-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and 3 (TAB3), but also between IκB kinase α (IKKα), ß (IKKß) and γ (IKKγ). Thus, the polyubiquitination of RIPK1 favors the assembly of TAB2-TAB3-TAK1 and IKKα-IKKβ-IKKγ complexes that stimulate the canonical nuclear factor-κB (NF-κB) activation pathway [16]. This pathway is required for the transactivation of several cytoprotective genes and the induction of cell survival.

The activation of NF-κB pathway results in the upregulation of several genes including TNFα-induced protein 3 (A20) and FLICE-like inhibitory long isoform protein ($Flip_L$). The increased expression of these two proteins is believed to be involved in switching TNFR1 activation response to apoptosis [1,14]. A20 is thought to generate a negative feedback loop by removing RIPK1-polyubiquitination, which may restrict TNF induced NFκB signalling. In addition, cylindromatosis (CYLD), another enzyme with deubiquitylating activity, is also implicated in removing polyubiquitin chains from RIPK1. When deubiquitinated, RIPK1 can dissociate from complex I and takes part in the formation of the cytosolic death inducing signaling complex (DISC) also called complex IIa (Fig. 2B). Complex IIa includes TRADD, FADD, $Flip_L$ and caspase 8 [14,17]. Its formation is TRADD

dependent and is enhanced by the unregulation of $Flip_L$ by NFκB pathway. Within the complex, $Flip_L$ and caspase 8 generate heterodimers that recurit and cleave RIPK1, RIPK3 and CYLD, inactivating them and preventing necroptosis. Complex IIa also allows the formation of procaspase 8 homodimers that are thought to undergo rapid autoproteolysis driving caspase 8 activation. Activated caspase 8 dissociates from complex IIa and activates caspases 3 and 7 that execute apoptosis.

Alternatively, when cells are depleted of cIAPs, complex I is still formed but RIPK1 is not polyubiquitinated [1]. Under these circumstances, complex I induces the activation of the non-canonical NF-κB pathway. Furthermore, apoptosis can be triggered by the formation of a cytosolic complex named complex IIb or ripoptosome [1,14]. It is a TRADD independent complex that includes RIPK1, RIPK3, FADD and the $Flip_L$-caspase 8 heterodimer. Complex IIb promotes apoptosis in the same manner that complex IIa does it. In other occasions, caspase 8 can be inhibited by caspase inhibitors or viral proteins. When this happens, apoptosis cannot be carried out and complex IIa and IIb evolve to a different cytosolic complex called necrosome [17,18].

EXECUTING NECROPTOSIS — THE NECROSOME

Necrosome formation is required for necroptosis execution. The canonical necrosome deriving from complex IIa consists of TRADD, RIPK1, RIPK3 and FADD (Fig. 2C), while the canonical necrosome resulting from complex IIb lacks TRADD. As a constitutive binding partner of RIPK3, MLKL is incorporated in the necrosome [17]. In this situation, RIPK1 and RIPK3 are no longer degraded by $Flip_L$-caspase 8 heterodimer and they can interact through their respective RIP homotypic interaction motifs (RHIM) [18]. This interaction allows the activation and autophosphorylation of RIPK3. The activated RIPK3 aggregate in fiber-like complexes, probably ß amyloid-like complexes [17]. RIPK3 aggregates can recruit and activate MLKL, which is the sole and main effector of necroptosis pathway identified to date. It has been seen that phosphorylation of human RIPK3 at S227 and mouse RIPK3 at S232 is crucial for association with MLKL, but not for necrosome assembly [19].

The requirement for RIPK1 can be bypassed by direct activation of RIPK3 by other effectors which are RHIM containing proteins that can promote the formation of non-canonical necrosome forms (Fig. 2D). One of them is the Toll/IL-1 receptor domain-containing adaptor inducing IFN-b (TRIF) which is recruited by activated TLR3 or TLR4 and is able to produce RIPK3 aggregation. Likewise, the DNA-dependent activator of interferon regulatory factors (DAI) has been reported to enable RIPK3 aggregation in response to DNA viruses infection [18].

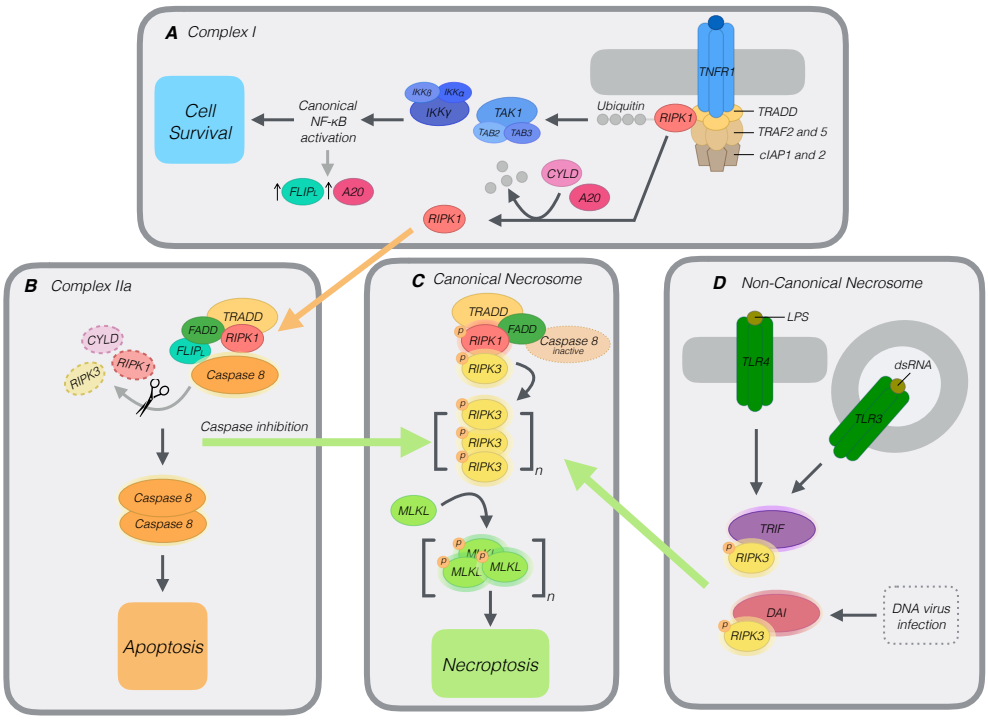

Figure 2. Necroptosis is a highly regulated pathway that occurs under caspase-inhibiting conditions. **(A)** Activation of TNFR1 promotes complex I formation, which is the canonical way to trigger necroptosis. After that, RIPK1 can be deubiquitinated and released from complex I to generate complex IIa or IIa. **(B)** When complex IIa is assembled and caspase 8 is active, apoptosis is initated. However, under caspase-inhibiting conditions complex IIa can lead to the canonical necrosome assembly. **(C)** Within canonical necrosome, RIPK3 is phosphorylated and therefore activated, being able to aggregate into fiber-like complexes that recruit MLKL. The interaction between RIPK3 and MLKL results in MLKL activation, oligomerisation and membrane translocation. Hence, the canonical necrosome formation induces necroptosis. **(D)** Nonetheless, necroptosis can be triggered through non-canonical pathways. The activation of the RHIM containing proteins such as TRIF and DAI can induce the formation of non-canonical necrosomes that promote RIPK3 phosphorylation and aggregation and the subsequent MLKL activation.

3 / MIXED LINEAGE KINASE DOMAIN-LIKE PROTEIN

MLKL is thought to be the chief effector and the most terminal protein in the signalling pathway that leads to necroptosis. Although how MLKL exactly executes necroptosis is still a matter of debate, it seems clear that it is through plasma membrane integrity disruption. This protein has two functional domains, an N-terminal four-helix bundle (4HB) and a C-terminal pseudokinase domain (PsKD), tethered together by a two-helix linker or *brace* helices (Fig. 3). The structure of full-length mouse MLKL was solved by X-ray crystallography [20]. After that, the human PsKD and N-terminal domain were solved separately by X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy, respectively [21,22]. Taken together, the results of these studies provide a good structural model of MLKL.

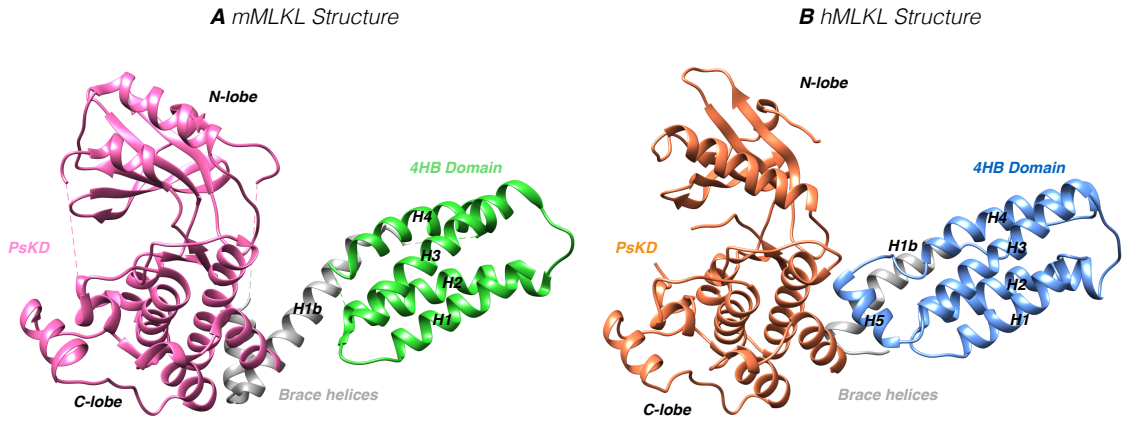

Figure 3. MLKL has two functional domains, the N-terminal 4HB domain and the C-terminal PsKD. They are joined together by two *brace* helices. **(A)** In the full-length mouse model the 4HB domain, PsKD and *brace* helices are represented in green, pink and grey respectively. **(B)** In the full-length reconstructed human model the 4HB domain, PsKD and *brace* helices are represented in blue, orange and grey respectively. Of note, the human 4HM domain presents an extra helix (H5).

N-TERMINAL DOMAIN — THE KILLER DOMAIN

The combined domain composed of the 4HB domain and the *brace* helices is called N-terminal domain (NTD). The 4HB domain consists of a four-helices core (H1-H4) which interacts with the *brace* helices but not with the PsKD. In the crystal structure of full-length mouse MLKL, electron density was missing for the segments between H3 and H4 and between H4 and the first helix of the *brace* (H1b), evincing the flexibility of these regions [20] (Fig. 3A). When the human NTD was solved by NMR spectroscopy an additional core helix was present at the site of the missing density between H3 and H4 in mouse full-length model [22] (Fig. 3B). This extra helix (H5) is thought to have a dynamic nature responding to the protein conformation [23].

The main function of NTD is to execute MLKL necroptotic effect, since expression of the MLKL 4HB domain or NTD induces necroptosis in MLKL$^{-/-}$ mouse fibroblasts [24,25]. It is generally accepted that plasma membrane is the primary site of MLKL action. This protein is known to translocate to plasma membrane after activation—the five core helices of the 4HB domain are thought to become transmembrane helices while the *brace* helices are not. The core helices are very amphipathic, with hydrophobic surfaces buried inside the domain and highly charged surfaces exposed to the solvent in the soluble form. The insertion of the 4HB domain seems to be driven by specific side-chain interactions between positive residues in the domain and negatively charged phospholipids, basically phosphoinositides (PIPs) (Fig. 4A). The 4HB domain-PIPs engagement is considered to happen in a two-step

mode: a first low-affinity recruitment followed by a second robust membrane association that leads to insertion (Fig. 5) [26].

The charged surfaces exposed in the soluble form cannot be inserted into the phospholipid bilayer. Nonetheless, there is a poorly packed interface between helices H2 and H4 that acts as a *natural gate* where the structure can open to expose the hydrophobic faces of the helices facilitating the insertion into the bilayer [22]. Related to that, it has been proposed a *plug* release mechanism by which H1b operates as an inhibitor of the 4HB domain insertion. H2 and H4 are packed against the N-terminal portion of H1b in human and mouse structural models of the soluble MLKL. Notably, some residues of H2 and H1b are close enough to suggest a dynamic interaction between these two segments [23] (Fig. 4B). The binding of H1b to the groove between helices H2 and H4 is expected to stabilize the soluble form of MLKL, hindering the opening of the 4HB domain and preventing the interaction with PIPs. The conformation changes in activated MLKL and its interaction with PIPs are the main forces that are thought to promote the H1b displacement and disordering, permitting membrane insertion (Fig. 5).

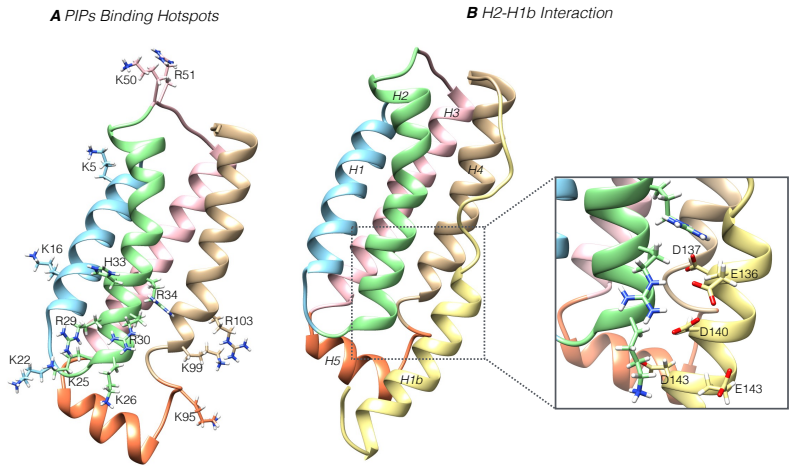

Figure 4. The solved structures of MLKL suggest that PIP binding site may involve clusters of positively charged K and R residues. **(A)** These basic residues are represented as sticks divided in two main PIP binding clusters, one comprising basic residues in H1 (blue) and H2 (green) and the other one comprising basic residues preceding H3 (pink) and some residues in H5 (orange). There are some residues that are thought to be involved in PIP binding in H4 (light brown). **(B)** The basic residues in H2 play a double role as they are involved in PIP binding but they also interact with acidic residues on the brace, which are represented as sticks. The brace displacement is required to obtain a total 4HB domain-membrane interaction because it releases H2 basic residues. However, this displacement is driven by the interaction between H2 and PIPs.

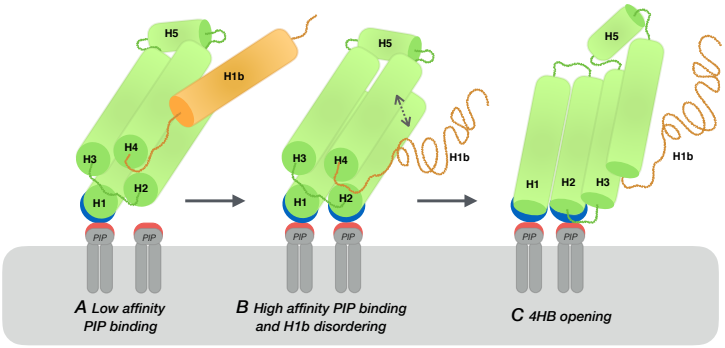

Figure 5. MLKL interaction with the membrane is thought to happen in a two-step way. **(A)** Firstly, H1 basic residues interact with the membrane PIPs. **(B)** This first step promotes H2-PIP engagement and brace disordering, which is the second step of the process. **(C)** When this happens H2-H4 interaction is disrupted and 4HB domain can open and insert into the bilayer.

Once translocated to the plasma membrane, MLKL generates cation fluxes increasing osmotic pressure, eventually causing membrane rupture. There is an ongoing debate on the precise mechanism by which it happens. The 4HB domain does not share significant sequence or structural homology to other protein families. This complicates the structural/functional study of MLKL, as it is not possible to infer how the 4HB domain permeabilises membranes studying homologous proteins [23]. Current models for MLKL membrane permeabilisation mechanism includes multi-step, partial insertion of 4HB domain to disrupt membranes (Fig. 6A) [24], insertion of 4HB to interact with ion channels including transient receptor potential melastatin-like 7 (TRPM7) to mediate osmolysis (Fig. 6B) [25], total insertion of NTD to form an ion channel itself (Fig. 6C) [26] or whole embedment of MLKL oligomers to form disrupting pores (Fig. 6D) [28]. Of note, none of these mechanisms exclude oligomerisation.

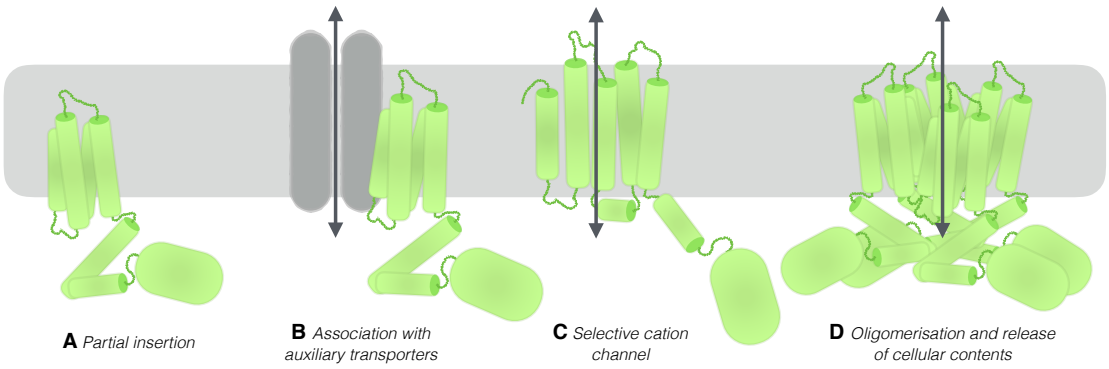

Figure 6. The mechanism by which MLKL provokes membrane integrity loss is still unknown. Different reports adduce different plausible modes which include: **(A)** MLKL partial insertion, **(B)** MLKL association and activation of auxiliary transporters, **(C)** MLKL insertion to act as a selective cation channel and **(D)** oligomer-pores formation. None of these mechanisms exclude oligomerisation.

Recent studies suggest that MLKL works as an oligomer. Although MLKL oligormes have been reported at the plasma membrane of necroptotic cells in several studies, the stoichiometry of oligomerisation remains unknown: trimers [24], tetramers [25] and even hexamers [28] or octamers [29] have been described. The interface through which MLKL self-associates is also unknown but the *brace* helices appear to be involved in the oligomerisation process [22,26]. Considering that 4HB domain alone is able to induce necroptosis without oligomerising, it is possible that *brace* mediated-oligomerisation serves to stabilize the active conformation of MLKL rather than govern MLKL killing activity [23].

C-TERMINAL PSEUDOKINASE DOMAIN — THE REGULATOR DOMAIN

PsKD is the C-terminal domain of MLKL and it topologically resembles protein kinase domains. However, it lacks critical residues that are necessary for phosphoryl-transfer, becoming catalytically-defective [20, 30]. Conventional kinase domains are bilobal, they present the N-terminal lobe (N-lobe) that packs against the larger C-terminal lobe (C-lobe). The N-lobe contains five antiparallel β-strands (ß1-ß5) and one α-helix (αC helix) while the C-lobe is almost entirely composed of α-helices [31] (Fig. 3). Kinase domains have a strictly organized internal architecture which is also very dynamic and allows regulation events. The main functional features of kinase domains rely on highly conserved sequence and structural motifs shared by most active kinases [31]. In fact, all conventional protein kinases have at least three essential conserved motifs: the VAIK motif, the DFG motif and the HRD motif. All of them play a paramount role in kinase catalytic activity (for a complete revision on kinase motifs see ref. [31]). The PsKD conserves the bilobal structure and the secondary structure of protein kinases. Despite that, most MLKL's PsKD from different species have modifications in the mentioned motifs. Whereas VAIK motif seems to be conserved, HRD motif is replaced by divergent sequences and DFG motif has been modified in most orthologs to GFE [32].

Over the last years, pseudokinase domains have been demonstrated to have relevant functions as signalling pathway regulators. An emerging idea is that pseudokinases might act as molecular switches, being able to modulate signalling pathways accordingly to upstream signals. Related to that, the PsKD is thought to function as a molecular switch that permits the structural rearrangement between the active and inactive conformations of MLKL. The upstream signal in this case is the phosphorylation of PsKD. Both human and mouse PsKD have been shown to be phosphorylated by RIPK3 at S/T residues, which are T357 and S358 in human and S345, S347, T349 and S352 in mouse [30]. The interaction of MLKL and RIPK3 takes place between the N-lobe of PsKD and the N-lobe of RIPK3—as it is

a kinase, it has N and C-lobes. The domains pack against each other through an interface dominated by Van der Waals interactions, H-bonds and π-stacking phenylalanine rings from RIPK3 and MLKL [30]. MLKL phosphorylation is known to correlate with its oligomerisation and membrane translocation. Hence, phosphorylation of PsKD is essential to MLKL activation. In addition, it is secondary to RIPK3 engagement since the alanine mutation of the phosphorylation-site residues does not preclude MLKL binding to RIPK3 [23].

These RIPK3-mediated phosphorylations are thought to induce conformational changes within the activation loop of the PsKD. The activation loop is a flexible regulatory element present in kinase proteins that allows kinase activation when phosphorylated. This loop is usually packed against the αC helix, which in turn interacts with the VAIK motif — the conserved E in αC helix ion pairs with the catalytic K of the VAIK motif [31]. Curiously, in PsKD this last interaction has been lost and it has been replaced by a new one. In the structure of full-length mouse MLKL K219 of the VAIK motif interacts with Q343 in the activation loop helix, a residue largely conserved amongst MLKL orthologs, while E239 of the αC helix faces the solvent (Fig. 7) [20].

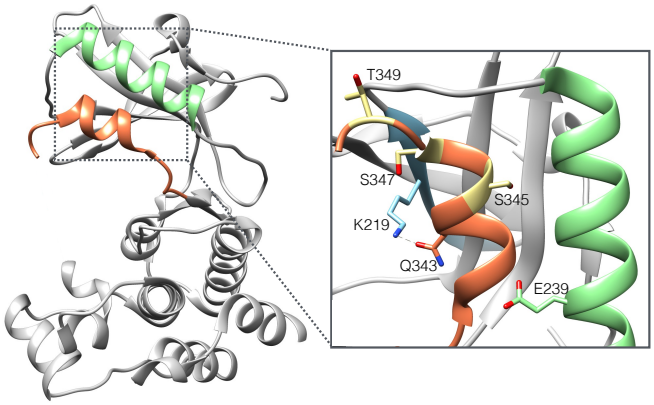

Figure 7. PsKD is believed to act as a switch mechanism that regulates the activation of MLKL and the release of 4HB domain. One of the most relevant structural features that support this hypothesis is the K219:Q343 interaction. The figure shows the αC helix (green), the activation loop helix (orange) and the VAIK motif (blue). Some of the phosphorylation sites are displayed (yellow) to demonstrate its proximity to the regulating interaction. Important residues are represented as sticks. The structure corresponds to mouse PsKD. Of note, the activation loop of mouse MLKL adopts an α-helix structure rather unusual [20].

The functional importance of the K219:Q343 interaction was corroborated by alanine mutation of either residue. The mutations induced necroptosis in absence of stimuli [20], indicating that the K219:Q343 interaction is important to mantain MLKL in an inactive state. Furthermore, these interacting residues are proximal to the RIPK3-mediated phosphorylation-sites. Taken together, these findings suggest that the phosphorylation of PsKD in the activation loop is a key step for MLKL activation via K219:Q343 interaction disruption,

supporting the molecular switch hypothesis. It is believed that these conformational changes are transmitted by the *brace* helices to the 4HB domain. Thus, the PsKD structural rearrangement is coupled to the 4HB domain release and MLKL total activation.

4 / CONCLUSIONS

Over the years, our understanding of cell death signalling has increased substantially. It is now clear that necrosis can take place in a programed manner via multiple molecular pathways such as necroptosis. Necroptosis is a regulated type of cell death that combines apoptotic and necrotic features and integrates many stimuli. The key necroptosis effector is MLKL. Simplistically, MLKL activation is triggered by RIPK3-mediated phosphorylation of the PsKD which induces a change in K219:Q343 interaction that is coupled with a structural rearrangement of the *brace* helices. This structural reorganization will cause the release of the 4HB domain that will induce the oligomerisation and the membrane translocation of MLKL, eventually provoking necroptosis execution (Fig. 8). Still, one has to take into account many other variables that can modulate MLKL activation. For instance, apart from the mentioned phosphorylations, MLKL has additional phosphorylation and ubiquitination sites and some of them can negatively regulate its activity [23]. Besides, MLKL has been recently identified as a client of Hsp90 and the protein kinase co-chaperone CDC37 [33] suggesting an extra regulating point. Indeed, Hsp90 is believed to ease the oligomerisation, the membrane translocation and the transmission of the phosphorylation-induced conformational changes from PsKD to 4HB domain.

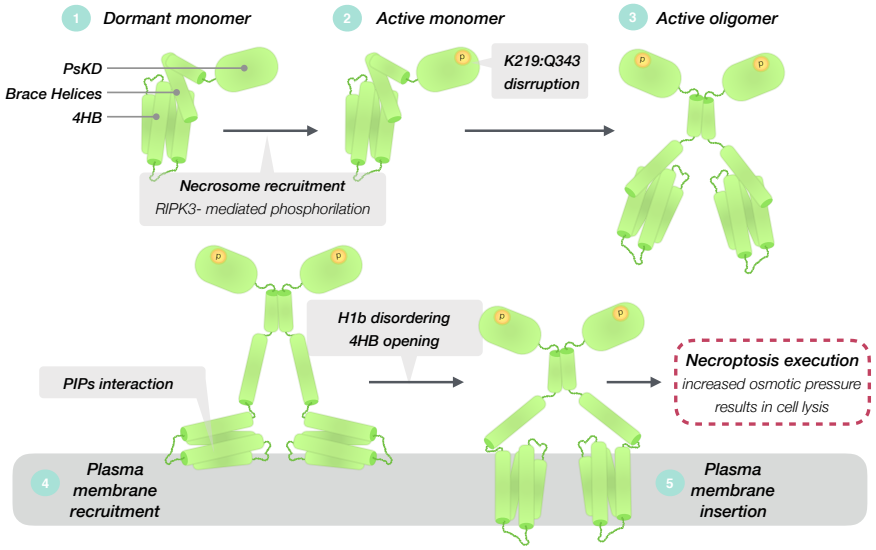

Figure 8. It is still difficult to asses how MLKL is activated and which are the steps that follow its activation. This figure presents a simple model that describes these events and seems to fit in with the current data.

Currently, we are on a start position for a more detailed examination of MLKL mechanism of action at the membrane. Obtaining knowledge about the MLKL and other molecules can help us to better understand the sequential interactions and events that ultimately lead to nectoptosis execution. To this purpose, the structural and biophysical technics can give us really valuable information. The use of high resolution structural techniques as cryo-electron microscopy in combination with biochemical and cellular assays will be crucial in resolving the debate about the precise mechanism of MLKL action and enlarging the current knowledge in necroptosis.

5 / REFERENCES

1. Berghe T Vanden, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: The expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol. 2014;15(2):135–47.
2. Vogt C. Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte (Alytes obstetricans). (Solothurn: Jent und Gassmann,1842).
3. Lockshin RA, Williams CM. Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silk moths. J. Insect. Physiol. 1964;10:643–649.
4. Schweichel JU, Merker HJ. The morphology of various types of cell death in prenatal tissues. Teratology 1973;7:253–266.
5. Clarke PG. Developmental cell death: morphological diversity and multiple mechanisms. Anat. Embryol.1990;181:195–213.
6. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972;26(4):239–57.
7. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, Agostinis P, et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ. 2018;25(3):486–541.
8. Taylor RC, Cullen SP, Martin SJ. Apoptosis: Controlled demolition at the cellular level. Nat Rev Mol Cell Biol. 2008;9(3):231–41.
9. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. Mol Cancer. 2015;14:48.
10. Yuan S, Akey CW. Apoptosome structure, assembly, and procaspase activation. Structure. 2013 Apr 2;21(4):501–15.
11. Kroemer G, Levine B. Autophagic cell death: The story of a misnomer. Nat Rev Mol Cell Biol. 2008;9(12):1004–10.
12. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, Blagosklonny M V., et al. Molecular definitions of cell death subroutines: Recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ. 2012;19(1):107–20.
13. Aki T, Funakoshi T, Uemura K. Regulated necrosis and its implications in toxicology. Toxicology. 2015;333:118–26.
14. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: An ordered cellular explosion. Nat Rev Mol Cell Biol. 2010;11(10):700–14.
15. Chan FK, Chun HJ, Zheng L, Siegel RM, Bui KL, Lenardo MJ. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. Science. 2000;288(5475):2351-4.
16. Bertrand MJM, Milutinovic S, Dickson KM, Ho WC, Boudreault A, Durkin J, et al. cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination. Mol Cell. 2008;30(6):689–700.
17. Grootjans S, Vanden Berghe T, Vandenabeele P. Initiation and execution mechanisms of necroptosis: An overview. Cell Death Differ. 2017;24(7):1184–95.

18. Vanden Berghe T, Hassannia B, Vandenabeele P. An outline of necrosome triggers. Cell Mol Life Sci. 2016;73(11–12):2137–52.

19. Chen W, Zhou Z, Li L, Zhong C-Q, Zheng X, Wu X, et al. Diverse sequence determinants control human and mouse receptor interacting protein 3 (RIP3) and mixed lineage kinase domain-like (MLKL) interaction in necroptotic signaling. J Biol Chem. 2013;288(23):16247–61.

20. Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang J-G, Alvarez-Diaz S, et al. The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism. Immunity. 2013;39(3):443–53.

21. Xie T, Peng W, Yan C, Wu J, Gong X, Shi Y. Structural Insights into RIP3-Mediated Necroptotic Signaling. Cell reports 2013; 5(1): 70-8.

22. Su L, Quade B, Wang H, Sun L, Wang X, Rizo J. A plug release mechanism for membrane permeation by MLKL. Structure 2014; 22(10): 1489-500.

23. Petrie EJ, Hildebrand JM, Murphy JM. Insane in the membrane: A structural perspective of MLKL function in necroptosis. Immunol Cell Biol. 2017;95(2):152–9.

24. Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P et al. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. Proc Natl Acad Sci USA 2014; 111(42): 15072-7.

25. Chen X, Li W, Ren J, Huang D, He WT, Song Y et al. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res 2014; 24(1): 105-21.

26. Quarato G, Guy CS, Grace CR, Llambi F, Nourse A, Rodriguez DA, et al. Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. Mol Cell. 2016;61(4): 589–601.

27. Xia B, Fang S, Chen X, Hu H, Chen P, Wang H, et al. MLKL forms cation channels. Cell Res. 2016;26(5):517–28.

28. Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, et al. Mixed Lineage Kinase Domain-like Protein MLKL Causes Necrotic Membrane Disruption upon Phosphorylation by RIP3. Mol Cell. 2014;54(1):133–46.

29. Deli H, Xinru Z, Zi-an W, Xi C, Wan-ting H, Yingying Z, et al. The MLKL Channel in Necroptosis Is an Octamer Formed by Tetramers in a Dyadic Process. Mol Cell Biol. 2017;37(5):1–17.

30. Xie T, Peng W, Yan C, Wu J, Gong X, Shi Y. Structural Insights into RIP3-Mediated Necroptotic Signaling. Cell Rep. 2013 Oct 17;5(1):70–8.

31. Taylor SS, Kornev AP. Protein kinases: evolution of dynamic regulatory proteins. Trends Biochem Sci. 2011;36(2):65–77.

32. Czabotar PE, Murphy JM. A tale of two domains - A structural perspective of the pseudokinase, MLKL. FEBS J. 2015;282(22):4268–78.

33. Jacobsen A V, Lowes KN, Tanzer MC, Lucet IS, Hildebrand JM, Petrie EJ, et al. HSP90 activity is required for MLKL oligomerisation and membrane translocation and the induction of necroptotic cell death. Cell Death Dis. 2016;7(1):e2051.

FIGURE REFERENCES

Figure 1. Took from reference [11].

Figure 2. Self-created based on references [1,18]

Figure 3. Self-created using UCSF Chimera. The structures were found in PDB and the codes are 4BTF, 4M67 and 2MSV for mouse full-length MLKL structure, human PsKD structure and human NTD structure, respectively. The human full-length model was build by overlapping the human partial structures to the mouse full-length structure.

Figure 4. Self-created using UCSF Chimera and Pymol. The structure was found in PDB and its code is 2MVS. Based on reference [26].

Figure 5. Self-created based on reference [26].

Figure 6. Self-created based on reference [23].

Figure 7. Self-created using UCSF Chimera. The structure was found in PDB and its code is 4BTF. Based on reference [32].

Figure 8. Self-created.