Ars Moriendi; the art of dying well – new insights into the molecular pathways of necroptotic cell death

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Abstract

When our time comes to die most people would probably opt for a quick, peaceful and painless exit. But the manner and timing are rarely under our direct control. Hence the *Ars moriendi*, literally, “The Art of Dying”, two texts written in Latin around the 15th century that offered advice on how to die well according to the Christian ideals of the time. In contrast, for individual cells, the death process is frequently under their control and several signaling pathways that cause cell death, including apoptosis, pyroptosis and necroptosis, have been described. Furthermore the manner in which cells die can have good or bad consequences for the organism. In this review we will discuss how cells die via the necroptotic signaling pathway, with emphasis on recent structural work and place this work in a biological context by discussing relevant studies with knock-out animals.

Keywords necroptosis; RIP kinases; MLKL; cIAP; TNF

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See the Glossary for abbreviations used in this article.

What is necroptosis?

Necroptosis is commonly considered to be as a back-up cell death pathway that comes into play if apoptosis is inhibited. The scenarios where necroptosis has been most consistently observed are therefore when apoptosis, by definition a caspase mediated process, is induced by an extracellular signal such as “death” ligands of the TNF superfamily (TNFSF^{DL}); and simultaneously inhibited, usually by caspase inhibitors or by knock-out of caspases. Apoptosis, the orchestrated and defined destruction of a cell by caspases, has been considered to be an immunologically silent form of cell death. This makes sense for developmentally regulated cell death where

inflammation could interfere with correct development. However, apoptosis is also believed to be a host response to infection by viruses and in this case it is less obvious why the cell death should be ignored by the immune system. It has therefore been suggested that it is the context in which the cell death occurs that determines the inflammatory nature of the cell death [1]. Because apoptosis can limit viral replication, viruses have evolved mechanisms to inhibit caspases [2] and therefore viral infection may be a physiologically relevant situation where apoptosis inhibition results in necroptosis. Consistent with the potential threat associated with this nefarious situation, necroptosis is believed to be inflammatory in nature (Fig 1A).

Naming new species

Paleontologists name new species with incomplete information and therefore it happens that apparently different species turn out to be the same. In paleontology the first name is the one that counts and the reason that the genus *Brontosaurus* no longer exists, is because it is a “deprecated” genus. Of course *Apatosauri*, the first and therefore “correct” name for this genus, no longer exist either... but for a different reason. Similarly molecular biologists have named the protein complexes that induce necroptosis with incomplete information and different complexes may turn out to be the same. Ontologically the first of these complexes is a plasma membrane receptor signaling complex that is formed in response to extracellular TNFSF^{DL}. Historically this has been called complex 1, or the receptosome and it is unlikely to induce cell death directly [3]. In the case of TNFR1, complex 1 is involved in activation of NF- κ B and MAP kinases. Because of the experimental focus on TNF signalling much of the nomenclature strictly relates to complexes downstream of TNFR1, but there is evidence to suggest, and it is the authors’ opinion, that these complexes will be very similar in other TNFRSF and TLR induced complexes [4–6]. Complex 1 can mature into complex 2 that is no longer associated with the plasma membrane. The preponderance of evidence suggests it is cytosolic but it may be

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Glossary

B-RAF	B- rapidly accelerated fibrosarcoma
c-Cbl	Casitas B-lineage lymphoma
cFLIP	cellular FLICE-like inhibitory protein
ciAP	cellular Inhibitor of Apoptosis protein
EGFR	Epidermal growth factor receptor
FADD	Fas associated via death domain
IAPs	Inhibitor of apoptosis proteins
IDO	indoleamine 2,3-dioxygenase
JAK	Janus kinase
MAPK	Mitogen activated protein kinase
MLKL	Mixed lineage kinase domain-like
Nec-1	Necrostatin-1
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
PKA	Protein kinase A
PKR	double stranded RNA-dependent protein kinase
RHIM	RIPK homotypic interaction motif
RIPK	Receptor interacting protein kinase
TLR	Toll like receptors
TNF	Tumor necrosis factor
TNFR1	TNF receptor 1
TNFRSF	TNF receptor superfamily
TNFSF	TNF superfamily
TNFSF^{DL}	TNF superfamily death ligand
TRADD	TNF Receptor 1A associated via death domain

membrane associated [3,7]. Complex 2 was originally identified as a caspase-8 containing complex downstream of TNFR1 and contains TRADD, FADD and RIP [3] and it may exist in different flavours. If levels of cFLIP_L, driven by complex 1 activity, are sufficient,

complex 2 does not have any killing activity [4,5]. It is possible that the heteromer of caspase-8 and cFLIP_L has a limited or distinct activity that contributes to signaling in an as yet undefined manner and it also may cleave RIPK1 [8]. Certainly over-expression of cFLIP_L limits recruitment of RIPK1 into complex 2 [5]. If cFLIP levels are low and therefore caspase-8 activity is above an upper threshold, caspase-8 induces apoptosis by promoting caspase-3 and/or Bid cleavage. At the same time it can cleave RIPK1 within this complex preventing RIPK1 activation and necroptosis; we can call this complex 2(a)poptotic. If caspase-8 activity is below a lower threshold (for example inhibited by a chemical caspase inhibitor, or in a caspase-8 knock-out) RIPK1 is no longer disabled by cleavage. If other conditions are right (for example ciAPs are disabled by a small molecule smac-mimetic) RIPK1 levels become high enough within this complex to, presumably, auto-activate and initiate a necroptotic cell death by phosphorylating RIPK3. This complex has been called complex 2(n)ecroptosis, or the necrosome. The composition of complex 2n is somewhat contentious, it certainly contains RIPK1 and RIPK3 but whether this complex activates MLKL in a hit and run type manner or whether MLKL is more stably associated with RIPK1 and RIPK3 in complex 2n is unclear [9–12]. Recently an additional complex, the Ripoptosome, has been described. This is probably indistinguishable from complex 2a and, depending upon the conditions, complex 2n [4,5]. The distinction is, we think, meant to emphasize the formation of a 2a or 2n complex in the absence of a TNFSF^{DL} stimulus for example upon genotoxic stress [6] or stimuli that deplete IAPs [4,5]. An interesting speculation is whether IAPs constitutively inhibit complex 2 formation (in much the same way they target NIK kinase [13,14]) and that one of the functions of TNFSF^{DL} signalling is to reassign them.

Figure 1. Connections between the apoptosis and necroptosis pathways at the physiological and molecular level.

(A, B) Schematic indicating how different cell death signaling pathways are connected. (C) Sequence homology amongst RHIM motifs of RIPK1, RIPK3, RIPK4 and TICAM (TRIF) and DAI (ZBP1) from *Homo sapiens*, *Mus musculus*, *Gallus gallus* (Chicken), *Pelodiscus sinensis* (Turtle), *Anolis carolinensis* (Chameleon), *Xenopus laevis*, *Oryzias latipes* (Medaka), *Takifugu rubripes* (Puffer fish), *Equus ferus*, *Bos taurus*, *Felis catus*, *Pteropus alecto* (Flying Fox), *Salmo salar* (Salmon), *Gasterosteus aculeatus* (Stickleback) and the M45 protein from murine cytomegalovirus. Searching with the motif [IYFMLV]-x-[IYFMLV]-x(5)-[IVL]-Q-[IVFLV]-G-x-[HNYGS]-N-x-[ML] identifies RHIM motifs in RIP Kinases, TRIF and DAI proteins.

Inflammation and necroptosis, chicken or egg

A major question in this nascent area of cell death research is a chicken and egg problem. Namely, does inflammation cause necroptosis (it certainly can) or can necroptosis be an initial insult to induce inflammation. This question is particularly pertinent when we consider the phenotype of the caspase-8 and FADD knock-out mice. FADD, caspase-8, RIPK1 and RIPK3, (Fig 1B), form the core of complex 2n [15], an approximately 2MDa multimeric platform from which apoptosis can be induced by TNFSF^{DL} and TLR ligands and, in the absence of FADD or caspase-8, can induce necroptosis [4–6]. In addition, the formation of complex 2n is negatively regulated by the cellular Inhibitors of Apoptosis (cIAPs) and cFLIP_L [4–6] to thwart the initiation of cell death pathways and favour signaling via Complex 1 to induce proinflammatory cytokine synthesis. RIPK3 and RIPK1 are two of the several proteins that are required for the induction of necroptosis in response to TNFSF^{DL} ligands. Remarkably, the embryonic lethality associated with the loss of caspase-8 and FADD is rescued by loss of RIPK3 or RIPK1 [8,16,17], suggesting that necroptotic death is the result of the embryonic phenotype (Fig 2). cIAPs are ubiquitin E3 ligases that are required for induction of NF- κ B by TNF [18–21], and hence are required for the production of inflammatory cytokines by

TNF. They are also essential to protect cells from TNF induced cell death [14,22]. Similarly to the caspase-8, FADD and cFLIP knock-out animals, cIAP1 and cIAP2 double knock-out mice are embryonic lethal at approximately E10. Surprisingly, given that TNFR1 is not required for embryonic development, the death of cIAP1/cIAP2 double knock-out mice at E10 can be prevented by simultaneously deleting *Tnfr1* [20].

Together these observations suggest that there is a normal but developmentally insignificant TNF signal at the E10 stage of embryonic development. If there is a developmentally appropriate, stage specific, production of TNF it is clearly not required for normal development because TNFR1 and TNF knock-out mice are viable. And this TNF signal is only revealed if it is not regulated properly, because in the absence of caspase-8, FADD, cFLIP or cIAPs, the consequences are disastrous. However, an alternative explanation is that these proteins are directly involved in limiting cytokine production in the absence of appropriate stimuli [23–25] and therefore in the absence of these proteins, inappropriate inflammatory cytokines, including TNF, are produced at this developmental stage. Because the caspase-8, FADD, cFLIP and cIAP1/cIAP2 double knock-out animals are all susceptible to TNF induced necroptosis this could then provoke necroptotic cell death that stimulates production of more inflammatory cytokines and embryonic death.

Figure 2. Analysis of the role of key molecules in the necroptotic pathway in mouse development.

Due to the lack of good markers for necroptosis and the technical limitations of looking for the initial event in stage E10 embryos we do not yet have data that would allow us to distinguish between these two possibilities. Another major question in the field is the mechanism of necroptotic cell death. In this regard there is recent progress in obtaining structural insight and the rest of this review is devoted to these developments.

Putting flesh on structural bones

Either the necroptosis signaling pathway is simple, or our knowledge is rudimentary. It is probably a combination of both things: as a backup pathway it probably can't be overly complicated and if it is a pathway that has evolved to counter pathogen infection it would need to be executed rapidly. At the heart of this pathway lies the activation of RIPK1, usually by TNFSF^{DL}/TLR signaling. While the kinase activity of RIPK1 does not appear to play a role in TNF induction of NF- κ B, it has been reported to regulate phosphorylation of a number of downstream kinases, such as ERK and Akt, that can lead to increased TNF production [26,27]. Within the necroptosis pathway the kinase activity of RIPK1 is essential to phosphorylate and activate RIPK3, as determined by Necrostatin-1 inhibition, although it has not been shown that RIPK1 phosphorylates RIPK3 directly [28,29].

RIPK3 principally regulates necroptosis by phosphorylating both the upstream kinase, RIPK1 [28,30], and the downstream effector, MLKL [9,10,31], and also regulates apoptosis via a poorly understood mechanism [32]. How TNFSF^{DL}/TLR signaling activates the kinase activity of RIPK1 to induce necroptosis, however, is not clear. It is possible that RIPK1 levels increase to a point where it can become auto-activated, as is the case for other kinases such as EGFR [33]. In this scenario, caspase-8 mediated cleavage and cIAP ubiquitylation may prevent the increase in RIPK1 protein levels so that it cannot become auto-activated. RIPK1 might also be activated by an upstream kinase and recently it was shown that knock-down, or inhibition of PKR (dsRNA-dependent protein kinase) activity reduced RIPK1 phosphorylation and necroptosis induced in *Fadd*^{-/-} MEFs treated with IFN γ [34].

While RIPK1 is considered to function upstream of RIPK3 in necroptotic signaling, under some circumstances it is expendable to the pathway. For example, overexpression of RIPK3 can alleviate the requirement of RIPK1 for initiation of necroptosis [35] this time, presumably, because RIPK3 auto-activates. Additionally, the RHIM domain-containing TLR3/4 effector, TRIF, has recently been identified as a direct activator of RIPK3 that can initiate necroptotic signaling in the absence of RIPK1 [36]. Again, RIPK3 does not normally appear to play a role in non-necroptotic TNFSF^{DL}/TLR signaling and is not present in complex 1 or complex 2a signaling complex or required for their activity [37]. The RHIM domains of RIPK1 and RIPK3 are required for this interaction.

The RHIM domain and higher order oligomers

The RHIM domain is an approximately 16 amino acid motif that is highly conserved between RIPK1 and RIPK3 ([30], Fig 1C). Examples of this motif have subsequently been identified in viruses, such

as viral M45-encoded inhibitor of RIP activation (vIRA) [38–41], enabling these proteins to subvert the normal necroptotic signal [39,42]. It has been shown that the RHIM domain is required for the interaction between RIPK1 and RIPK3 and recently, RIPK1 and RIPK3 oligomers were found to form amyloid structures in cells [43]. These amyloid structures could be replicated *in vitro* using recombinant proteins encompassing the RHIMs of RIPK1 and RIPK3 [43]. The physiological function of such a high molecular weight complex is unclear, but one explanation may be that the requirement for such an assembly reduces the potential for unintended “noisy” activation of the kinases and provides a threshold of activation, resulting in a “digital”, all-or-nothing, read-out [44]. The association kinetics of these assemblies may prevent transient fluctuations in concentration etc. from initiating signaling so that cells only respond when the stimulation is persistent and strong. Clearly for a non-reversible process, such as cell death, the correct interpretation of signals is essential. Furthermore, such complexes may provide a uniform maximal response once a signal is above a certain threshold thereby generating a digital signal that is necessary in a process such as cell death. Li *et al* [43] were able to characterize the amyloid structure of the RIPK1:RIPK3 RHIM domain multimeric complex using a combination of electron microscopy, solid-state NMR spectroscopy and X-ray diffraction. However, deducing whether each RHIM domain adopts a structured, stably folded interaction module, and thereby defining the exact composition of the domain, has proven technically challenging. The multimerization of RHIM domains *in vitro* has precluded obtaining atomic level insights thus far, although meeting this challenge using innovative X-ray crystallography or solution NMR spectroscopy in the future will play an important role in advancing our understanding of these interesting domains. Indeed, such insights might inform the development of small molecules that could be utilized therapeutically as necroptosis blockers by inhibiting the RIPK1:RIPK3 RHIM domain interaction.

RIPK1

RIPK1 was the first effector molecule identified in a non-apoptotic cell death pathway induced by a TNFSF^{DL} pathway [45]. Subsequently it was identified as the target of the compound, Necrostatin-1 (Nec-1) [46], that was originally identified from a screen to identify small molecules that inhibited TNF- and Z-VAD-FMK-induced cell death [47]. The precise mechanism of inhibition by Nec-1 was, however, unclear although it was shown that Nec-1 competed with ATP for binding to RIPK1. The recent atomic structure of the RIPK1 kinase domain in complex with a Nec-1 analog, Nec-1a (or the Nec-3 analog, Nec-3a, and Nec-4) has shown that the inhibition mechanism is distinct from classic ATP competitive kinase inhibitors [11]. Nec-1a does not occupy the adenine-binding pocket in a classical ATP-competitive inhibitor binding mode, but binds remotely to the ATP binding site in the position usually occupied by Mg²⁺ residues in an active kinase:ATP:Mg²⁺ complex ([11], Fig 3A and B). Unexpectedly, Nec-1a binding leads to displacement of the helix α C, forcing the RIPK1 kinase domain into a catalytically inactive conformation (Fig 3A and B). The role of helix α C, the only helix in a conventional kinase N-lobe, is to contribute a Glu residue (E63 in RIPK1; E91 in the archetypal

Figure 3. Schematic indicating the key structural elements of (A) a prototype kinase, PKA, (B) human RIPK1 kinase bound to Nec-1, motifs MIMK⁴⁵, HKD¹³⁸ and D¹⁵⁶LG are highlighted, and (C) murine pseudokinase MLKL, with motifs VTIK²¹⁹, HRN³¹⁸ and G³³⁶FE highlighted. (D) Human pseudokinase MLKL, with motifs VAIK²³⁰, HGK³³¹ and G³⁴⁹FE highlighted. (E) Cartoon representation of the structure of MLKL generated with Pymol.

protein kinase, Protein Kinase A (PKA); Fig 3A) to an ion-pair with the catalytic lysine residue in the N-lobe β3 strand (K45 in human RIPK1; K72 in mouse PKA; Fig 3A). Not only does displacement of the helix αC perturb the capacity of the helix αC Glu (E63) to position the β3 Lys in a catalytically competent conformation, but Nec-1a is also engaged in key hydrogen bonds with the D156 and S161 of the activation loop (D¹⁵⁶LGLAS¹⁶¹) that would be deleterious to catalytic activity. Nec-1a binding to D156 of the “DFG” motif would critically compromise RIPK1’s engagement of Mg²⁺, a necessary co-factor for phosphoryl-transfer, and furthermore, leads to perturbations within the activation loop that preclude formation of the hydrophobic “R” (regulatory) spine, a stack of hydrophobic side chains that is believed to organize a protein kinase domain into a catalytically active conformation [11,48].

Despite marked chemical heterogeneity amongst the Necrostatins; Nec-1a, Nec-3a, and Nec-4, all occupy overlapping positions within the RIPK1 kinase domain active site. This suggests the existence of unexpected plasticity within the hydrophobic pocket in RIPK1 that accommodates the Necrostatins. As noted by Xie *et al* [11], the conformation of the inhibited RIPK1 kinase domain resembles that of B-Raf bound to PLX4032 [49]. PLX4032 is a much larger molecule than the Necrostatins and binds to an extended pocket in the B-Raf kinase domain, including the pocket

that would bind the adenine ring of ATP during phosphoryl-transfer catalysis. The comparison with B-Raf: PLX4032 raises the prospect that even greater specificity and potency against RIPK1 might be imparted by design of larger, extended Necrostatin analogs in future.

Nec-1 binds RIPK1 with a K_d in the low nanomolar range and the rather unique mode of binding of the Necrostatins to RIPK1 probably accounts for the high specificity of Nec-1 which has little inhibitory potential against other kinases at 10 μM [50]. It is therefore more specific than a number of other well-known kinase inhibitors including Imatinib (Gleevec) [51]. However, this specificity against kinases is misleading because Nec-1 is structurally very similar to L-Tryptophan, a substrate for a key enzyme in inflammatory signaling, IDO (indoleamine 2,3-dioxygenase) [52,53]. For this reason, Nec-1, or 5-((1H-indol-3-yl)methyl)-3-methyl-2-thioxoimidazolidin-4-one to give it its full name, is known in the inflammatory signaling field as MTH-Trp [53]. Although the K_i of Nec-1 is around the 10 μM mark [54] this is unfortunately the concentration at which Necrostatin-1 is often used in *in vitro* assays. While this may have led to a mistaken assignment of RIPK1 kinase activity in certain processes that are in fact regulated by IDO fortunately, now that the community is aware of this overlap it is possible to use compounds with more

selectivity, such as Necrostatin-1s that inhibits only RIPK1 and 1-MT that inhibits only IDO, to distinguish between these two possibilities [52].

MLKL – The dead kinase regulating cell death

The pseudokinase domain-containing protein, Mixed Lineage Kinase domain-Like (MLKL), was recently implicated in the necroptosis pathway by two independent reports [10,12]. Initially, MLKL was identified as the target of a small molecule inhibitor in a high throughput chemical screen for necroptosis inhibitors, leading to the development of the tool compound, necrosulfonamide [10]. A complementary approach using a shRNA-mediated gene knockdown screen for necroptosis effectors also identified MLKL [12]. In both studies, MLKL was reported to operate downstream of TNFR1 > RIPK1 > RIPK3 to induce necroptosis. Studies in MLKL knock-out cells by ourselves and others have since demonstrated beyond doubt that MLKL is an essential component of the necroptosis machinery, since the genetic deletion of MLKL renders these cells insensitive to death when exposed to necroptotic stimuli, whilst not interfering with their capacity to undergo apoptosis [9,55]. MLKL comprises a C-terminal pseudokinase domain and an N-terminal region, for which no known structural homologs could be identified based on sequence homology (Fig 3E). Pseudokinase domains have sequence homology to protein kinase domains, but are predicted to be enzymatically-dead owing to the absence of one of three key catalytic motifs: the β 3 strand Lys from the VAIK motif involved in positioning the α - and β -phosphates of ATP for phosphoryl transfer; the Mg^{2+} binding Asp from the DFG motif in the activation loop; or the catalytic Asp from the catalytic loop's HRD motif (Fig 3A) [56,57]. Pseudokinase domains have been largely ignored as signaling molecules, however the pseudokinase domain of JAK2 is an essential negative regulator of its adjacent tyrosine kinase domain [58]. The subsequent discovery that mutations within the JAK2 pseudokinase domain cause human hematopoietic malignancies, and account for 95% of polycythemia vera cases [59–63], a clonal myeloproliferative disorder characterized by increased production of red cells, platelets and granulocytes, has ignited interest in these signaling domains. While the JAK2 pseudokinase domain has recently been shown to exhibit a weak catalytic activity [64], the prevalence of catalytic activities among pseudokinases remains a matter of controversy [56,65,66].

To probe the mechanism by which MLKL mediates necroptotic signaling, we recently solved the X-ray crystal structure of full length mouse MLKL [9] the first full length structure of a protein within this pathway (Fig 3E). The structure revealed that the N-terminal region adopts a four-helix bundle fold, rather than the coiled-coil domain predicted from the sequence. Although not detected from the amino acid sequence, the N-terminal four-helix bundle shows some structural homology to the four-helix bundle subdomain within the tyrosine kinase binding (TKB) domain of c-Cbl [67], but the functional consequences of this similarity remain unclear. In MLKL, the N-terminal four-helix bundle domain (residues 1–125) is fused to the C-terminal pseudokinase domain (179–464) via a two-helix brace. The brace:four-helix bundle interaction is largely mediated by

charged interactions, whereas the brace:C-terminal pseudokinase domain interaction is predominantly hydrophobic.

MLKL and RIPK3 have been reported to directly interact, with the MLKL pseudokinase domain proposed to function as the RIPK3 binding domain [10,12,68]. However, endogenous RIPK3 was not immunoprecipitated by endogenous MLKL from wild-type mouse dermal fibroblasts undergoing necroptotic cell death, nor could we detect an interaction between recombinant mouse RIPK3 kinase domain and mouse MLKL using isothermal titration calorimetry [9]. These observations are reinforced by a recent study in which recombinant mouse MLKL pseudokinase domain and mouse RIPK3 kinase domain did not stably interact *in vitro*, but, nevertheless formed a stable complex when co-expressed from insect cells [31]. Coupled with another recent study in which phosphorylation of mouse RIPK3 at S232 and T233 as key determinants that govern whether RIPK3 forms a stable complex with mouse MLKL [68], it appears the phosphorylation status of RIPK3, rather than MLKL, underlies the propensity for the two proteins to form a stable complex [31]. Whilst the necessity of a stable MLKL:RIPK3 complex for necroptotic signaling remains unclear at this point, it is evident that RIPK3 phosphorylates the pseudokinase domain of MLKL within its activation loop to activate the necroptotic signaling function of MLKL and that this is a crucial step in propagating necroptotic cell death downstream of TNFR > RIPK1 > RIPK3 activation necroptotic signaling function [9,10,31]. Together, these data suggest that the RIPK3:MLKL interaction is not as robust as that observed for the RIPK1:RIPK3 complex 2n and are most consistent with the idea that MLKL is a downstream effector of necroptotic signaling that is a transient enzymatic substrate of RIPK3.

The structure of the mouse MLKL pseudokinase domain revealed an unexpected conformation [9]. The two key structural features that distinguish the MLKL kinase domain from that of conventional kinases are that its activation loop forms a helix instead of an unstructured loop and that this activation loop helix is buttressed against the helix α C in the pseudokinase domain N-lobe, thereby preventing the domain from adopting a conformation typical of an active protein kinase, like PKA (Fig 3A and C). Intriguingly, this disposition of the helix α C shows a striking resemblance to that seen in the Nec-1a inhibited structure of the RIPK1 kinase domain (Fig 3B and C). Another peculiar feature of the murine MLKL pseudokinase domain structure is a Gln residue within the atypical activation loop helix that substitutes for the helix α C Glu residue as a binding partner of the Lys in the VTIK motif (Fig 3C and E). Surprisingly, this non-canonical activation loop helix occupies the position that is normally occupied by helix α C in a conventional kinase structure and contributes a highly conserved residue, Q343, to a hydrogen bond with K219 of the VTIK motif (Fig 3C and E). The unconventional K219:Q343 interaction underpins the integrity of the “pseudoactive” site and, as discussed below, positions K219 to participate in nucleotide binding.

Another striking difference between the MLKL pseudokinase domain and a conventional kinase is that MLKL binds ATP but this is diminished in the presence of Mg^{2+} ! Superimposition of this structure with the ATP Mg^{2+} complex of PKA indicates that there is no structural reason why MLKL should be unable to engage ATP. Indeed, a thermal shift assay demonstrated that MLKL is able to bind ATP, ADP or AMPPNP in a divalent cation-independent manner [9]. The inability to engage divalent cations

can be explained by the absence of the conventional Mg^{2+} binding motif DFG. Instead of this motif, almost all known MLKL orthologs contain the sequence GFE. Another important observation from comparisons of sequences between orthologs is that the conventional protein kinase catalytic motif, HRD, is highly divergent between MLKL orthologs. The inability to bind divalent cations and the poor sequence conservation within the catalytic loop makes it exceedingly unlikely that MLKL is capable of catalyzing a phosphoryl transfer reaction. Supporting this conclusion, MLKL was unable to autophosphorylate in ^{32}P - γ -ATP *in vitro* kinase assays [9].

The cleft between the N- and C-lobes can be termed the “pseudo-active” site, since this region corresponds to the active site in a *bona fide* kinase, even though in MLKL it lacks essential catalytic residues. There is a novel interaction between K219 of the VTIK motif and Q343 from the activation loop helix within the pseudoactive site of MLKL. K219M and Q343A MLKL mutants exhibited stimulus-independent necroptotic signaling when expressed in *Mklk1*-deficient fibroblasts, with even more pronounced constitutive cell death signaling in the absence of necroptotic stimuli in wild-type fibroblasts [9], indicating that the integrity of the pseudoactive site is intrinsically intertwined with MLKL activation. Furthermore, when introduced into *Ripk3*^{-/-} fibroblasts, these mutants caused cell death independent of necroptotic stimuli. On the other hand, overexpression of wild-type MLKL could not overcome a requirement for RIPK3 for necroptotic signal transduction even in the presence of necroptotic stimuli. K219 and Q343 are in proximity to the RIPK3-substrate residues within the MLKL activation loop, S345, S347 and T349, indicating that mutation of K219 or Q343 is likely to emulate the structural changes that are induced upon MLKL activation loop phosphorylation. Consistent with this idea, when the S345 was substituted with the negatively-charged, phosphomimetic residue, Asp, to generate S345D MLKL, it induced cell death in the absence of necroptotic stimuli. These data support a model in which, under normal circumstances, RIPK3-mediated phosphorylation of MLKL activation loop at S345 and/or S347/T349 leads to a structural perturbation that switches MLKL from being an inert molecule to an active effector of necroptotic signaling.

In addition to the mouse MLKL structure described above, the human MLKL pseudokinase domain was recently reported [31,69]. Like mouse MLKL, the human pseudokinase domain was shown to bind ATP in a divalent cation independent fashion [9,66,69], although in contrast to mouse MLKL, apo human MLKL was crystallised in a closed conformation (Fig 3D) synonymous with an active protein kinase, such as PKA (Fig 3A). Additionally, unlike mouse MLKL, the human structure does not contain the atypical activation loop helix, but instead contains a predominantly unstructured activation loop. Because phosphorylation of the MLKL activation loop by RIPK3 is known to be the cue for activating MLKL and the initiation of necroptosis [9,10], it is possible that the two orthologous MLKL structures might represent snapshots of an inactive and activated form of MLKL. In contrast to mouse MLKL, the human MLKL structure and accompanying mutational analyses [69] implicate the Lys residues in the HGK sequence (present in place of the conventional kinase catalytic motif, HRD, Fig 3D) and the VAIK motif as key ATP binding determinants. This is notable, since the counterparts of the HRD motif in MLKL orthologs are highly divergent,

Sidebar A. In need of answers

- (i) What is the source and purpose of TNF that kills caspase-8, cFLIP, FADD or cIAP1&2 deficient mouse embryos at E10.5?
- (ii) What is the precise composition of death inducing complexes?
- (iii) How does RIPK1 kinase become activated in them?
- (iv) Is MLKL part of a RIP Kinase containing complex and how does it kill cells?
- (v) Can necroptotic cell death initiate auto-inflammatory diseases?
- (vi) What evolutionary pressure has selected for necroptotic cell death?

which provides further evidence opposing the possibility that MLKL might serve a catalytic function.

The big question remains as to what MLKL does once it becomes activated. The K219M mutant is unable to bind ATP yet is an activated form of MLKL that is able to induce cell death. Aside from the structural arguments given above, this seems to rule out any role for even limited kinase activity being required for MLKL to kill cells. One possibility is that MLKL has retained the phosphorylation dependent conformational switch common to kinases but lost the ability to phosphorylate substrates. But what the conformational switch might activate remains unknown. Other possibilities are that MLKL modulates the activity of another downstream kinase or serves as an “elastic” scaffold, as other pseudokinases have been shown to do. Preliminary studies have, however, failed to find readily associated proteins (JMM & JS, unpublished), suggesting that activated MLKL may have a hit and run mode of action.

There is no doubt that cells have mastered the art of dying. Apoptotic cell death, the subject of thousands of research papers, is used throughout development to sculpt the embryo and fine tune the adult. If this process goes wrong, cells may fail to die, development may go awry and diseases such as cancer may develop. An alternative fate to survival is that cells undergo a necroptotic cell death. The necroptotic cell death pathway utilises some of the same proteins involved in the apoptotic response but bifurcates from this pathway at the level of activation of RIPK3 and MLKL. One significant difference between the two pathways remains that we think we understand the biological purpose of the apoptotic pathway very well but are unsure of the evolutionary advantage to maintaining the necroptotic pathway. On the contrary, the *in vivo* consequences of necroptosis appear to be, in many cases, deleterious to the organism, and for example mice without RIPK3 survive systemic inflammatory response syndrome or kidney ischemia-reperfusion injury better than wild type mice [70,71]. One possibility is that necroptosis has been selected as a back-up cell death mechanism to thwart devious pathogens that block apoptosis. The idea that viruses are engaged in an “arms race” with cell death defences is supported by the observation that murine cytomegalovirus (MCMV) encodes inhibitors of caspase-8 and RIPK3 and that a RIPK3 inhibitor deficient MCMV strain is attenuated in wild type but not in *Ripk3*^{-/-} mice [40]. Thus it is possible that the deleterious consequences in certain disease scenarios are tolerated because of the advantages conferred in resisting pathogen infection. Whatever the ultimate reason, greater insight into the evolutionary forces selecting for necroptosis cell death pathways will help us understand why cells have also perfected the art of dying well.

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Author contributions

JMM and JS contributed equally to writing this review.

Conflict of interest

JS is on the SAB of TetraLogic Pharmaceuticals.

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