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Flicking the molecular switch underlying MLKL-mediated necroptosis

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The pseudokinase domain of the necroptosis effector mixed lineage kinase domain-like (MLKL) functions as a latch to restrain the unleashing of its N-terminal 4-helix bundle (4HB) domain. Cell death mediated by the 4HB domain relies on membrane association and oligomerization, which can be inhibited by an ATP-mimetic small molecule that binds the pseudokinase domain of MLKL.

Our Study

While apoptosis is crucial to multicellular organism development and hence immunologically inert in most situations, in contrast the complementary necroptosis cell death pathway serves no apparent role in development and instead is a potent inducer of inflammatory responses. The necroptosis pathway is activated downstream of the tumor necrosis factor (TNF) receptor in scenarios when inhibitor of apoptosis proteins (IAPs) and proapoptotic caspase-8 are depleted or inhibited (Fig. 1). The pathway relies on receptor interacting protein kinase-1 (RIPK1) regulating the catalytic activity of RIPK3, which in turn phosphorylates the most terminal (known to date) effector in the pathway, the pseudokinase mixed lineage kinase domain-like (MLKL) protein,¹ leading to cell death (Fig. 1).

MLKL comprises 2 domains—an N-terminal 4-helix bundle (4HB) domain and a C-terminal pseudokinase domain—connected via 2 “brace” helices.¹ Our recent structural studies^{1,2} and functional analyses of structure-based mutants in fibroblasts derived from *Mkl^{-/-}* mice¹ led us to propose that the pseudokinase domain of MLKL functions as a molecular

switch that can be toggled by RIPK3-mediated phosphorylation. Consistent with this hypothesis, we³ and others⁴⁻⁷ have shown that deletion of the pseudokinase domain of MLKL de-represses the N-terminal 4HB domain to drive cell death. Furthermore, in keeping with the idea that the pseudokinase domain acts as a brake to prevent 4HB domain-mediated cell death, we found that ectopic expression of the C-terminal brace-pseudokinase domain of MLKL partially inhibited necroptosis in wild-type fibroblasts in a dominant-negative fashion.³ It has become clear from our work³ and that of others⁴⁻⁷ that MLKL's killer activity can be attributed to its 4HB domain. The consensus from these studies is that MLKL activation leads to membrane association; however, several different potential mechanisms of membrane disruption have been proposed (Fig. 1).

Although the mechanism by which the 4HB domain kills cells remains highly contentious (Fig. 1), by alanine-scanning mutagenesis we have established that there are 2 clusters of residues on opposite faces of the 4HB domain that are crucial to necroptosis. One cluster is essential for membrane localization and assembly of the 4HB domain into the high molecular weight

complexes that correspond to cell death. On the opposite face of the 4HB domain, a second cluster is essential for high-molecular weight complex formation, but not for membrane association.³ These findings argue that membrane association is necessary, but not sufficient, for 4HB domain-mediated necroptosis, and implicate either MLKL oligomerization (as previously proposed⁴⁻⁷) or formation of a complex of MLKL with other effectors as a precursor to cell death.

The stoichiometry of cell death-inducing MLKL complexes remains a matter of debate. MLKL has alternatively been proposed to form trimers⁴ or tetramers⁵ on the basis of migration in non-reducing SDS-PAGE, or hexamers based on non-reducing SDS-PAGE and analyses of recombinant MLKL.⁷ These oligomers were found to be stabilized by disulfide bonds that arose from oxidation during cell lysis,⁴ indicating that such complexes may not necessarily reflect complex composition *in vivo*. In our recent study,³ we performed cellular fractionations followed by Blue-Native (BN)-PAGE to monitor formation of high-molecular weight MLKL-containing complexes. This work demonstrated that cellular fractionation

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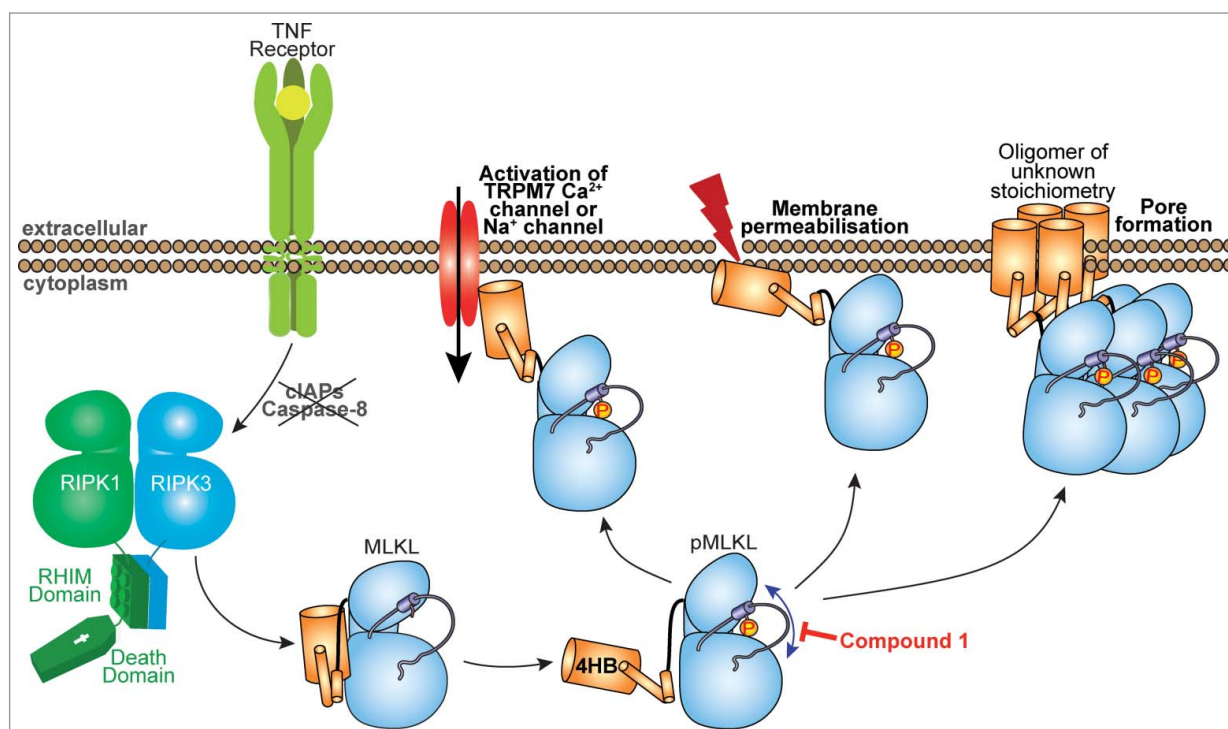


Figure 1. Molecular mechanism of MLKL-mediated cell death. Activation of TNF receptor 1 (TNFR1; lime) by tumor necrosis factor (TNF; yellow) induces necroptosis when cellular inhibitors of apoptosis proteins (ciAPs) and caspase-8 are inhibited or depleted from cells. Receptor interacting protein kinase-1 (RIPK1) and RIPK3 assemble via their RIP homotypic interaction motif (RHIM) domains into oligomers, within which RIPK1 regulates activation of RIPK3. Upon activation, RIPK3 phosphorylates mixed lineage kinase domain-like (MLKL), which resides in the cytoplasm in an inactive form, generating phospho-MLKL (pMLKL) in which the N-terminal MLKL 4-helix bundle (4HB) domain (orange) has been unleashed. Subsequently, MLKL translocates to membranes³⁻⁷ where it has been proposed to function variously as an activator of the ion channel transient receptor potential cation channel subfamily M member 7 (TRPM7),⁴ or an undefined Na⁺ channel,⁵ to perforate membranes,⁷ or form transmembrane pores of unknown stoichiometry.⁶

and BN-PAGE provides a robust method for examining the onset of necroptosis, especially in mouse cells. This is important because, unlike human studies, there is currently no antibody against mouse phospho-MLKL that can be used to monitor MLKL activation in mice. Following a necroptotic stimulus, endogenous MLKL moves to a crude membrane cell fraction that is insoluble in 0.025% digitonin and is detected as part of a >500 kDa protein complex that is not present in the cytoplasmic cell fraction.

Based on our earlier findings that the MLKL pseudokinase domain has retained an intact ATP binding site,^{1,2,8} we sought to determine whether we could modulate the conformation of the pseudokinase domain upon binding to an ATP-mimetic small molecule. We identified Compound 1 as an inhibitor that not only binds to the MLKL pseudokinase domain, but also prevents cell death by delaying MLKL translocation to

membranes.³ This suggested that, in the presence of Compound 1, MLKL is locked in a conformation that restrains the necroptotic activity of the 4HB domain (Fig. 1).

Until recently, targeting pseudokinases with small molecule inhibitors was considered highly challenging, mainly because of a lack of understanding of their true biological functions. Our study clearly highlights new avenues for drug development against MLKL using ATP mimetics. Recent studies have elegantly demonstrated that the biological function of the pseudokinase HER3 (ErbB3) as a modulator of the catalytically-active receptor tyrosine kinase HER2 (ErbB2) can be tuned by targeting the HER3 pseudokinase domain with ATP mimetic small molecules.^{9,10} As their biological functions become unraveled, we anticipate that the potential of pseudokinases as therapeutic targets will continue to be realized.

Outstanding Questions

Several distinct mechanisms have been proposed to describe MLKL-mediated cell death (Fig. 1), but precisely how MLKL functions to induce membrane breaches remains the subject of ongoing studies. It is presently unclear how the pseudokinase domain restrains the activity of the 4HB domain in a healthy cell prior to phosphorylation by RIPK3 and how phosphorylation of MLKL is affected by Compound 1 in cells. Future studies will play an important role in deducing the stoichiometry of MLKL within the pro-necroptotic high-molecular weight membrane-associated complex and whether engagement of additional effectors is required for necroptosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

- Murphy JM, Czabotar PE, Hildebrand JM, Lucet IS, Zhang JG, Alvarez-Diaz S, Lewis R, Lalaoui N, Metcalf D, Webb AJ, et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity* 2013; 39:443-53; PMID:24012422; <http://dx.doi.org/10.1016/j.immuni.2013.06.018>
- Murphy JM, Lucet IS, Hildebrand JM, Tanzer MC, Young SN, Sharma P, Lessene G, Alexander WS, Babon JJ, Silke J, et al. Insights into the evolution of divergent nucleotide-binding mechanisms among pseudokinases revealed by crystal structures of human and mouse MLKL. *Biochem J* 2014; 457:369-77; PMID:24219132; <http://dx.doi.org/10.1042/BJ20131270>
- Hildebrand JM, Tanzer MC, Lucet IS, Young SN, Spall SK, Sharma P, Pierotti C, Garnier JM, Dobson RC, Webb AJ, 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 U S A* 2014; 111:15072-7; PMID:25288762; <http://dx.doi.org/10.1073/pnas.1408987111>
- Cai Z, Jitkaew S, Zhao J, Chiang HC, Choksi S, Liu J, Ward Y, Wu LG, Liu ZG. Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. *Nat Cell Biol* 2014; 16:55-65; PMID:24316671; <http://dx.doi.org/10.1038/ncb2883>
- Chen X, Li W, Ren J, Huang D, He WT, Song Y, Yang C, Li W, Zheng X, Chen P, Han J. Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. *Cell Res* 2014; 24:105-21; PMID:24366341; <http://dx.doi.org/10.1038/cr.2013.171>
- Dondelinger Y, Declercq W, Montessuit S, Roelandt R, Goncalves A, Bruggeman I, Hulpiau P, Weber K, Schon CA, Marquis RW, et al. MLKL compromises plasma membrane integrity by binding to phosphatidylinositol phosphates. *Cell Rep* 2014; 7:971-81; PMID:24813885; <http://dx.doi.org/10.1016/j.celrep.2014.04.026>
- Wang H, Sun L, Su L, Rizo J, Liu L, Wang LF, Wang FS, Wang X. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell* 2014; 54:133-46; PMID:24703947; <http://dx.doi.org/10.1016/j.molcel.2014.03.003>
- Murphy JM, Zhang Q, Young SN, Reese ML, Bailey FP, Evers PA, Ungureanu D, Hammaren H, Silvennoinen O, Varghese LN, et al. A robust methodology to subclassify pseudokinases based on their nucleotide-binding properties. *Biochem J* 2014; 457:323-34; PMID:24107129; <http://dx.doi.org/10.1042/BJ20131174>
- Littlefield P, Moasser MM, Jura N. An ATP-Competitive Inhibitor Modulates the Allosteric Function of the HER3 Pseudokinase. *Chem Biol* 2014; 21:453-8; PMID:24656791; <http://dx.doi.org/10.1016/j.chembiol.2014.02.011>
- Xie T, Lim SM, Westover KD, Dodge ME, Ercan D, Ficarro SB, Udayakumar D, Gurbani D, Tae HS, Riddle S M, et al. Pharmacological targeting of the pseudokinase Her3. *Nat Chem Biol* 2014; 10(12):1006-12; PMID:25326665; <http://dx.doi.org/10.1038/nchembio>