Journal V, e1; Month/s 2013; © 2013 Landes Bioscience

Phosphorylated MLKL causes plasma membrane rupture

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Keywords: MLKL, Necroptosis, RIPK1, RIPK3, Regulated necrosis

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Submitted: 07/02/2014;

Accepted: 07/03/2014;

Published Online: 08/13/2014

http://dx.doi.org/10.4161/mco.29915

Necroptosis, cell death caused by uncontrolled swelling (oncosis) and rapid plasma membrane rupture, exerts detrimental pathogenic effects.[1,2] According to recent reports by Wang et al.[3] in Molecular Cell and Dondelinger et al.[4] in Cell Reports, the underlying mechanism of necroptosis might involve the assembly of pore-forming oligomers of phosphorylated mixed lineage kinase domain-like protein (pMLKL).

Wang et al.[3] followed a straight line of investigation after their group had identified phosphorylation of MLKL as a downstream target of receptor-interacting protein kinase 3 (RIPK3).[5] Within a supramolecular platform referred to as the necrosome RIPK3 phosphorylates a number of proteins to exert currently uncharacterized functions, but the only one shown to be critically involved in the execution of necroptosis to date is MLKL. The authors report the first tool to directly detect an active necroptosis pathway: a highly specific rabbit anti-human pMLKL antibody that was raised against the MLKL phosphorylation motif. In addition to its value as a scientific tool, this antibody may potentially be used for clinical diagnostics. In the commonly used necroptosis-sensitive human adenocarcinoma cell line HT29, the pMLKL-antibody detected a specific signal within 6 h after induction of necroptosis by the classic combination of TNFα, Smac mimetics, and the pan-caspase inhibitor zVAD-fmk. Because the execution of necroptosis includes cellular swelling (oncosis), the authors hypothesized that pMLKL itself might form pores in membranes or might associate with membranes as preformed complexes. In an elegant series of centrifugation and phase shift assays, Wang et al. demonstrate that pMLKL, in contrast to cytosolic non-phosphorylated MLKL, is almost exclusively detected in membrane fractions such as plasma membranes and heavy or light intracellular organelles, but not in the cytosol. Furthermore, protein-lipid overlay assays revealed association of the MLKL coiled-coil region with phosphatidylinositol phosphates (phosphoinositides) and with high concentrations of cardiolipids. Importantly, recombinant MLKL phosphomimic mutants were sufficient to disintegrate the membranes of liposomes, especially when the liposomes contained PtdIns(4,5)P2, raising the possibility that MLKL itself, which forms supramolecular clusters, creates a hole in the lipid bilayer to facilitate oncosis. Certainly, this is not a cellular model and is far from direct in vivo proof, but this report proposes a novel concept for the downstream execution of necroptosis (**Fig. 1**). Importantly, necrostatin-1, a compound that keeps RIPK1 in a closed configuration and thereby inhibits tumor necrosis factor (TNF)-mediated RIPK3 activation,[6,7] prevents phosphorylation of MLKL and bursting of the plasma membrane, whereas necrosulfonamide (NSA), a compound that was shown by the same group to inhibit necroptosis downstream of MLKL, prevents cell death but not MLKL phosphorylation.[5] Given that NSA prevents cell death despite the presence of pMLKL, it cannot be concluded that detection of pMLKL equals detection of necroptosis, as proposed from immunohistochemical analysis of pMLKL in human sections of patients with drug-induced liver injury (DILI). However, the pMLKL IHC signal provides a very useful tool suggesting ongoing necroptosis.

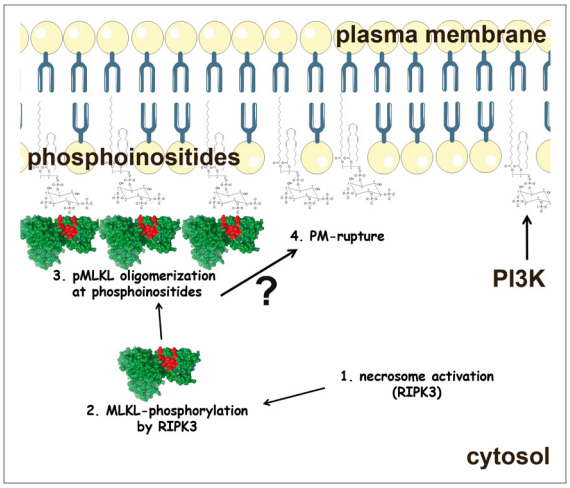

Figure 1. pMLKL binds phosphoinositides to cause plasma membrane rupture. The inner side of the lipid bilayer contains, among other lipids, various concentrations of phosphatidylinositol that can be phosphorylated to become phosphoinositides by members of the PI3K family. Based on experiments in liposomes, the concentration of phosphoinositides fine-tunes the probability of undergoing RIPK3-MLKL–dependent necroptosis. Within this pathway, various stimuli such as death receptor signals, protein kinase R, and activation of Toll-like receptors (not shown) activate the necrosome, which phosphorylates the pseudokinase MLKL. Upon phosphorylation, MLKL undergoes a conformational change that exposes an N-terminal "bundle-brace" motif (4HBD-BR) sufficient to induce cell death. Within this motif, 9 charged residues (shown in red) are responsible for binding to phosphoinositides. pMLKL is detected exclusively in membrane fractions as higher order oligomers. pMLKL mediates plasma membrane rupture by a currently unknown mechanism. MLK1, mixed lineage kinase domain-like protein; PM, plasma membrane; RIPK3, receptor-interacting protein kinase 3.

Independent of the study by Wang et al., Dondelinger et al.[4] found that the 4 helical bundle-brace region (4HBD-BR) of MLKL is both critical for translocation of the pseudokinase to the plasma membrane and sufficient to induce necroptosis upon overexpression in human embryonic kidney cells (HEK293T). Protein–membrane interactions often depend on electrostatic interactions between positively charged protein residues and the negatively charged membrane. This led the authors to further investigate the positively charged amino acids in the 4HBD-BR region (shown in red in **Figure 1**), where they found 9 positively charged amino acids between positions 22 and 35 that have been evolutionary conserved between species. Intriguingly, changing these positively charged amino acids to glutamate residues completely prevented both cell death and oligomerization of MLKL. In experiments using similar lipid arrays to those of Wang et al., a recombinant 4HBD-BR protein engaged PtdIns(4)P, PtdIns(4,5)P2, PtdIns(3,4,5)P3, and cardiolipin whereas the negatively charged mutant did not bind any of the lipids. Importantly, the authors subsequently tested SF1670, an inhibitor of phosphatase and tensin homolog (PTEN); P5i, an inhibitor of PIKfyve5; and the PI(3,4,5)P3 antagonist PITenin-7 (PIT-7). Remarkably, the combination of P5i and SF1670 drastically reduced necroptosis in both L929sAhFas and FADD-deficient Jurkat cells. This important piece of information identified such inhibitors as potential novel drugs for therapeutic applications, which now obviously should be tested in vivo.

As with most important studies, and despite the answers provided by these investigations, several important questions remain to be answered. For example: How does NSA prevent necroptosis? If pMLKL directly forms pores in the plasma membrane, why can this process be altered by ion channel inhibitors?[8] How are such channels involved at all? Are there pMLKL-phosphatases and, if yes, how are they activated? How precisely is pMLKL entering the plasma membrane? Which other intracellular organelles, (although not mitochondria[9,10]) are important for the execution of necroptosis? What is the role of constitutively active MLKL in human cancers?[11] Can we apply NSA to humans, and which disorders are the most promising targets?[2] Can PI3K inhibitors have beneficial effects for diseases that involve necroptosis? How are the PI3Ks regulated, activated, and inactivated for fine-tuning the sensitivity of cells to necroptosis?[12] Which preclinical condition might mimic such sensitization?

Necroptosis is currently believed to play a role in several clinically relevant settings, including ischemia-reperfusion injury, dermatitis, atherosclerosis, and cancer.[13] The studies by Wang et al. and Dondelinger et al. contribute to our understanding of this important pathway. Whereas the study by Wang et al. provides a novel potentially diagnostic anti-pMLKL antibody, Dondelinger's report identifies the PI3K pathway as a novel therapeutic target for modulation of the sensitivity to necroptosis.

The necroptotic pathway is by no means completely understood, and well-designed studies are required for the ultimate clinical translation of our knowledge of necroptosis. The studies by Wang et al. and Dondelinger et al. provide nice examples of how the field can be moved forward.

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