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MINI-REVIEW

# New components of the necroptotic pathway

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## ABSTRACT

**Programmed necrosis, also known as necroptosis, has recently drawn great attention. As an important cellular regulation mechanism, knowledge of its signaling components is expanding. Necroptosisis demonstrated to be regulated by the RIP1 and RIP3 kinases, and its pathophysiological importance has been confirmed in a number of disease models. Here we review the new members of this necroptosis pathway, MLKL, PGAM5, Drp1 and DAI, and discuss some of their possible applications according to recent findings.**

**KEYWORDS** necrosis, necroptosis, necrosome

## INTRODUCTION

Necrosis is a type of cell death morphologically characterized by cell rounding, cell volume increase, organelle swelling, and the bursting of the cytoplasmic membrane (Laster et al., 1988). Necrosis is different from apoptosis not only in its morphologic definition, but also in the general belief that it is an unregulated cell death. However, evidence that the receptor-interacting protein kinase 1 (RIP1) and RIP3-regulated cell deaths possess necrotic phenotypes completely changes this misconception (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Based on the convention that special names, such as anoikis, entosis, and pyroptosis etc. (Galluzzi et al., 2012), are used for different types of cell deaths, "necroptosis" has recently been used for programmed necrosis (Galluzzi et al., 2012) despite the fact that it was originally used to describe only RIP1-dependent necrosis (Degterev et al., 2005).

Much of our understanding of necroptosis has been formed by studying tumor necrosis factor (TNF)-induced necroptotic cell death (Vandenabeele et al., 2010) (Fig. 1). Upon TNF and TNF receptor 1 (TNFR1) ligation, TNFR1 recruits TNFR-associated death domain (TRADD), receptor-interacting protein 1 (RIP1), and TNFR-associated factor 2 (TRAF2) (Harper et al., 2003; Micheau and Tschopp, 2003).TRAF2 then binds to cellular inhibitor of apoptosis proteins 1 and 2 (cIAP1/2), allowing for the recruitment of the linear ubiquitin chain assembly complex (LUBAC), which acts as a scaffold to recruit the TAB-transforming growth factor-activated kinase 1 (TAK1) complex and IκB kinase (IKK) subunit NEMO to form TNFR1 complex 1 (Ea et al., 2006; Wu et al., 2006; Bertrand et al., 2008; Haas et al., 2009). After deubiquitination by cylindromatosis (CYLD) (Wright et al., 2007), RIP1 recruits TRADD, the Fas-associated protein with a death domain (FADD) and caspase-8 combine to form TNFR1 complex II (Micheau and Tschopp, 2003; Wang et al., 2008; Cho et al., 2009), which mediates apoptosis. When RIP3 is present, it can incorporate itself into complex II to form complex IIb (also called necrosome), containing FADD, caspase 8, RIP1, and RIP3 (Holler et al., 2000; Cho et al., 2009). Both an inhibition of caspase 8 and increase in RIP3 can promote the activity of the necrosome and result in necroptosis (Vercammen et al., 1998a; Zhang et al., 2009; Oberst et al., 2011). Because both a dominant negative FADD expression and FADD depletion promote necroptosis, FADD appears to inhibit the necrosome (Galluzzi et al., 2011). The deubiquitinating enzyme CYLD has been shown to promote necroptosis in intestine epithelial cells, most likely by deubiquitinating RIP1 (Welz et al., 2011). However, ubiquitin editing enzyme A20 negatively regulates necroptosis (Vanlangenakker et al., 2011a). Other signaling molecules that promote NF-κB activation, such as TAK1 and cIAP1, also negatively regulate necroptosis (Vanlangenakker et al., 2011a, 2011b).

The necroptotic and apoptotic pathways can compete against each other since both the inactivation of the necrosome by the caspase 8-mediated RIP3 cleavage and the conversion of apoptosis to necroptosis by RIP3 upregulation have been reported (Zhang et al., 2009; Han et al., 2011; Oberst et al., 2011). FLICE-like inhibitor protein long ($cFLIP_L$) binds with caspase 8 in the resting stage, and the basal activity of caspase 8 is believed to be responsible for the inac-

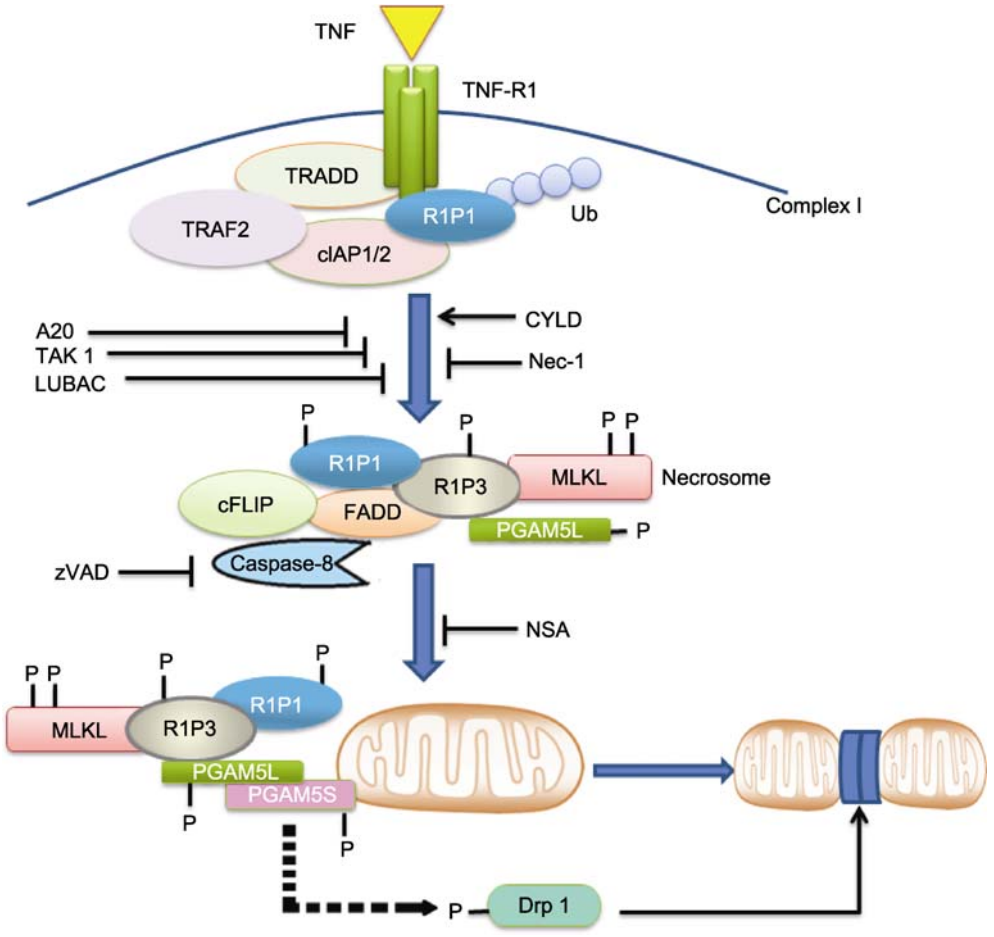


**Figure 1. TNF-induced programmed necrosis pathway.** Upon TNF induction, TNF receptor 1 recruits TRADD, RIP1, TRAF2 and cIAP1/2 to form TNFR1 complex I. Within this complex, A20, an ubiquitin-editing enzyme, LUBAC, a linear ubiquitylating enzyme complex, and TAK1 negatively regulate TNF-induced programmed necrosis. Necrostatin-1 blocks necroptosis by targeting RIP1 kinases. The deubiquitination of RIP1 by CYLD and the inhibition of caspase-8 are critical for the assembly of necrosome. Within the necrosome, the apoptosis machinery FADD, cFLIP, and caspase-8 suppress the induction of necroptosis. The kinase activities of RIP1 and RIP3 are necessary for necrosome formation. After RIP3 is phosphorylated, it phosphorylates MLKL and PGAM5L, and then engages PGAM5S on the mitochondrial membrane, during which the engagement is inhibited by NSA. Once activated, PGAM5L/PGAM5S then activate the mitochondrial fission regulator Drp1 by dephosphorylation, thus leading to mitochondrial fission.

tivation of the RIP1/RIP3 necrosome (Kaiser et al., 2011; Oberst et al., 2011; Zhang et al., 2011). However, it's currently unknown how RIP3 suppresses the apoptosis pathway. Necroptosis also functions as a backup for apoptosis (Vercammen et al., 1998a; Holler et al., 2000; Han et al., 2011; Kaiser et al., 2011; Oberst et al., 2011; Welz et al., 2011; Zhang et al., 2011). For example, in the absence of caspase 8, the death of T cells isnot blocked, but uses a necroptosis pathway instead (Salmena and Hakem, 2005; Ch'en et al., 2011).

The increasing attention on necroptosis is largely due to recent findings on its pathological and physiological importance. RIP1 kinase activity contributes to ischemic brain injury (Degterev et al., 2005; Northington et al., 2011) and myocardial ischemia-reperfusion injury (Smith et al., 2007; Oerlemans et al., 2012). RIP3-mediated necroptosis is proven to be involved in pancreatitis (He et al., 2009), photoreceptor cell loss (Trichonas et al., 2010), skin inflammation (Bonnet et al., 2011), and defense mechanisms against some virus infections, such as the vaccinia virus (He et al., 2009) and the murine cytomegalovirus (Upton et al., 2010). Necroptosis also participates in the host defense against *Salmonella Typhimurium* infections (Robinson et al., 2012). One developmental role of necroptosis is demonstrated by rescuing the embryonic lethality of FADD deficient mice with RIP1knockout, or Caspase 8 knockout withRIP3 gene deletion (Kaiser et al., 2011; Zhang et al., 2011). In addition, necroptosis is demonstrated to take part in lymphoproliferative disease (Ch'en et al., 2011; Kaiser et al., 2011), Crohn's

 

disease (Gunther et al., 2011; Welz et al., 2011) and acute liver injury (Liedtke et al., 2011).

Despite great progress in the past few years in the understanding the molecular mechanisms of programmed necrosis, necroptosis is still a new field in cell death study. Since a number of excellent reviews in the past couple of years have already summarized the basic RIP1/RIP3 necrosis pathway information, we briefly review the RIP1/RIP3 complexes and then focus on the recently identified components of the necroptosis pathway.

## CURRENT INSIGHT INTO THE RIP1/RIP3 SIGNALING COMPLEX

RIP1 and RIP3 play a central role in TNF-induced necroptosis (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Like the formation of complex II in apoptotic processes, necrosome formation appears to be facilitated by the deubiquitination of RIP1 (O'Donnell et al., 2011; Vanlangenakker et al., 2011b). While it is clear that the RIP1/RIP3 complex is the core of the necrosome, the formation and function mechanisms of the necrosome are still largely unknown. Necrosomes are large protein complexes with an estimated molecular weight of over 5 MDa (Feoktistova et al., 2011; Tenev et al., 2011). In addition to RIP1 and RIP3, the necrosome contains many other components and could be heterogeneous (Han et al., 2011). Recent studies have shown that RIP1 and RIP3 form an oligomeric amyloid signaling complex (Li et al., 2012), which could be the backbone of the large necrosome complex. RIP homotypic interaction motif (RHIM), found in both RIP1 and RIP3, is a key structure domain in both that mediates the formation of the amyloid complex. The scaffolds of amyloids may function as a crucial platform for recruiting other components, such as MLKL, and stimulating the downstream execution mechanisms of necroptosis (Li et al., 2012). Amyloid fibers, oligomers and aggregates can be found in various diseases, including Alzheimer's. Since prion-like amyloid aggregation itself can be cytotoxic, it would be very interesting to know if the cell can sense an amyloid-like necrosome as a dangerous signal or if it can initiate a specific signal for cell death.

## MLKL AS A NECESSARY COMPONENT FOR NECROSOME FUNCTION

By screening a chemical library, Xiaodong Wang's group found that necrosulfonamide ((E)-N-(4-(N-(3-methoxypyrazin-2-yl) sulfamoyl) phenyl)-3-(5-nitrothiophene-2-yl) acrylamide), referred to as NSA, can effectively block TNF-induced necroptosis in human cells. They further identified the mixed lineage kinase domain-like protein (MLKL) as a target of this compound (Sun et al., 2012). MLKL was also identified in the immunoprecipitation of RIP3, demonstrating that it interacts with RIP3 and is thus a component of the necrosome (Fig. 1). The importance of MLKL in necroptosisis also supported in later work by another group who identified it as a required molecule for TNF-induced necroptosis in a screening of a kinase/phosphatase shRNA library in human colon adenocarcinoma HT-29 cells (Zhao et al., 2012).

Similar to the effects of RIP3 knockdown on necroptosis, the knockdown of MLKL blocked cell death, showing that MLKL is required for TNF-induced necroptosis (Sun et al., 2012). To interact with MLKL, RIP3 needs to be phosphorylated at Ser227. The phosphorylation at Ser227 is likely to be autophosphorylation since it occurs when RIP3 is over-expressed and abolished in a kinase dead RIP3 mutant (Sun et al., 2012). In response to necroptosis induction, RIP3 phosphorylates MLKL at Thr357 and Ser358. Neither position's phosphorylation is sufficient for necroptosis, but both are required for necroptosis. Mutations at Thr357 and Ser358 do not affect the interaction between MLKL and RIP3, but has a dominant negative effect on the function of MLKL. In mice, Thr357 becomes an asparagine, while Ser358 is conserved. The regions around Ser227 in RIP3 and Thr357/Ser358 in MLKL are not conserved in humans and mice, which could be the reason for the undetectable interaction between murine RIP3 and human MLKL. Nevertheless, murine RIP3 interacts with murine MLKL, indicating that the functional relationship between RIP3 and MLKL is conserved in mammals.

The phosphorylations of MLKL at Thr357 and Ser358 have been proposed to be markers of necrosome activation because while a non-phosphorylated MLKL mutant can be incorporated into the necrosome, the phosphorylation of the two sites is required for cell death (Sun et al., 2012). Downstream necrosome activation events could be a sustained JNK activation and ROS generation (Zhao et al., 2012), but considering that the requirements of prolonged JNK activation and ROS induction are tissue and cell-type specific (He et al., 2009; Fortes et al., 2012), the other downstream events of MLKL could be more critical to programmed necrosis.

## DEPHOSPHORYLATION OF Drp1 BY PGAM5 AS A POTENTIAL CONVERGENT POINT OF DIFFERENT NECROTIC PATHWAYS

By separating and analyzing cell extracts containing RIP3, PGAM5, a mitochondrial phosphoglyceratemutase was identified to be another necrosome associated protein by Xiaodong Wang's group (Takeda et al., 2009; Wang et al., 2012). It was reported earlier that PGAM5 can use an alternative Ser/Thr phosphatase activity to dephosphorylate ASK1 (Takeda et al., 2009). Dynamin-related protein 1 (Drp1), a regulator of mitochondrial fission, could be regulated by PGAM5 through the dephosphorylation of Drp1 (Wang et al., 2012). Since mitochondrial fragmentation was observed during necroptosis, a model was proposed where PGAM5 promotes mitochondrial fission and subsequently cellular necroptosis through the dephosphorylation of Drp1 (Fig. 1).

 

Notably, the two variants of PGAM5, PGAM5S and PGAM5L, function differently during necroptosis, even though they are both required for necroptosis execution. After the core part of necrosome is formed, PGAM5L binds to the necrosome, unaffected by the necroptosis inhibitor NSA; the binding of PGAM5S, however, is blocked by NSA. What makes PGAM5 more interesting is that PGAM5S and PGAM5L are both required for intrinsic necroptosis induced by $H_2O_2$ or A23187. RIP1, RIP3, and MLKL, on the other hand, only affect extrinsic pathways induced by TNF-α and other extracellular ligands. Even though the ubiquitous involvement of PGAM5 needs more evidence, PGAM5 could fill the gap that is preventing investigators from studying the common execution mechanisms for different forms of necrosis. It needs to be noted, though, that the role of mitochondrial fission in apoptosis is not clear since both supporting and opposing data have been reported (Frank et al., 2001; Lee et al., 2004; Germain et al., 2005; Parone et al., 2006; Brooks et al., 2007; Estaquier and Arnoult, 2007; Sheridan et al., 2008). Whether the role of mitochondrial fragmentation is a cause or consequence of necrosis may need more investigation.

## DAI AS A NEW PARTNER OF RIP3 TO FIGHT AGAINST VIRUSES

Cellular necroptosis can be induced by some viral infections (Benedict et al., 2002). Cho et al showed that RIP3 was used to mediate vaccinia virus-induced necroptosis when cellular apoptosis machinery was inhibited (Cho et al., 2009). Murine cytomegalovirus (MCMV) infection induces a form of necroptosis that was shown to be RIP3-dependent by Upton et al (2010). While MCMV-induced cell death is controlled by RIP3 kinase activity and RHIM-dependent interactions, it does not depend on RIP1 or TRIF, two RHIM-containing members involved in regulated necrosis. Further studies have revealed that the DNA-dependent activator of IRF (DAI, also known as ZBP-1 or DLM-1), a RHIM domain containing protein and a potential DNA sensor, is the essential partner of RIP3 during MCMV infection induced necroptotic cell death (Upton et al., 2012).

DAI was first identified as a cytoplasmic DNA sensor capable of inducing type 1 interferon production (Takaoka et al., 2007), but was re-examined when $DAI^{-/-}$ mice showed the normal phenotype in both innate and adaptive immune responses to B-DNA and DNA vaccines (Ishii et al., 2008). While it's questionable whether DAI isa DNA sensor for an anti-viral IFN response, virus-induced DAI-RIP3 complexes indicate that it could be a type of specialized sensor that is responsible for necroptosis, but not for type I interferon response.

## CHEMICAL INHIBITORS OF PROGRAMMED NECROSIS

Small molecule compounds that inhibit programmed necrosis are not only effective tools in the study of necrotic cell deaths, but also have potential to become pro-drugs for developing treatments for necroptosis-related human diseases. Necrostatin-1 is the first necroptosis inhibitor to have been widely used *in vitro* or in animal models to study necroptosis. It was screened out from a small compound library as an inhibitor of necroptosis (Degterev et al., 2005). Based on structure modeling and prediction, necrostain-1 is believed to be a RIP1 inhibitor (Degterev et al., 2008). Although it is a RIP1 inhibitor, necrostain-1 does not inhibit RIP1-mediated apoptosis, which is believed to be due to the fact that necrostain-1 does not inhibit RIP1 kinase activity (Han et al., 2009). The inhibition of necroptosis by necrostain-1 is at least partly due to its inhibition of the association between RIP1 and RIP3 (Degterev et al., 2008; He et al., 2009). The application of necrostain-1 in mice disease models has been proven to be a very useful experimental approach and has provided much valuable information on the role of necroptosis in pathological conditions (Trichonas et al., 2010; Fortes et al., 2012). Currently, there is no RIP3 inhibitor available. Given the fact that RIP3 is involved in necroptosis but not apoptosis, a RIP3 inhibitor could be a more selective drug for necroptosis.

As is mentioned above, NSA was screened out to block necroptosis downstream of RIP3 activation (Sun et al., 2012). Biotin-NSA/streptavidin-conjugated beads pull down and structure-activity relationship (SAR) studies reveal that NSA targets the N-terminal fragment of MLKL and covalently modifies MLKL through a chemical reaction called a Michael addition at a reactive amino acid residue cysteine. NSA targets human MLKL but not mouse MLKL, so NSA cannot be used in animal models, but NSA could become a pro-drug for clinical applications in treating necrosis related human diseases.

## PERSPECTIVES

Evidence suggests that necroptosis is a tightly regulated process important in various physiological and pathological conditions (Han et al., 2011; Vanlangenakker et al., 2012). Our understanding of the necroptosis pathway is still in its infant stage, compared to that of apoptosis,. The most unclear part of necroptosis is how it is executed. To date, we still lack a biomarker for the *in vivo* detection of necroptosis. It is known that mitochondria play an important role in both apoptosis and necroptosis, and that Bcl-2 family proteins also have similar pro- and anti-roles for apoptosis and necroptosis (Tsujimoto et al., 1997; Kroemer et al., 1998; Meilhac et al., 1999; Irrinki et al., 2011), but how Bcl-2 family members function in influencing apoptosis and necroptosis is unclear. Mitochondrial membrane permeability transition pore opening is believed to be involved in both apoptotic and necroptotic cell death, and the pore component cyclophilin D was reported to be required for necroptosis (Nakagawa et al., 2005; Tsujimoto and Shimizu, 2007; Devalaraja-Narashimha et al.,

 

2009). However, there is still no data suggesting the involvement of cyclophilin D in RIP3-mediated necroptosis. It is highly possible that multiple necrosis pathways operate at the mitochondrial level. As more stimuli, including those via intrinsic pathways and extrinsic pathways, are discovered to trigger necrosis, RIP1/RIP3 dependent necrosis may be only one of many important necrotic pathways (Vercammen et al., 1998b; Petit et al., 2002; Meurette et al., 2007; Jouan-Lanhouet et al., 2012). In addition to mitochondrial ROS, mitochondrial fission and sustained JNK activation, the phosphorylation of STAT3 on Ser727 and the interaction between STAT3 and GRIM-19, a subunit of mitochondrial complex I, was found during necroptosis (Shulga and Pastorino, 2012). Finding the convergent point and the common executing mechanism of the different pathways of necrosis is still a challenge. Further understanding of the mechanisms of programmed necrosis should have a significant impact on the development of therapeutic intervention of many necrosis-related human diseases.

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## ABBREVIATIONS

$cFLIP_L$, FLICE-like inhibitor protein long; cIAP, cellular inhibitor of apoptosis protein; CYLD, cylindromatosis; Drp1, Dynamin-related protein 1; FADD, Fas-associated protein with a death domain; IKK, IκB kinase; LUBAC, linear ubiquitin chain assembly complex; MCMV, murine cytomegalovirus; MLKL, mixed lineage kinase domain-like protein; RIP, receptor-interacting protein; RHIM, RIP homotypic interaction motif; SAR, structure-activity relationship; TAK, growth factor-activated kinase; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNFR-associated death domain; TRAF, TNFR-associated factor

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