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REVIEW

# The modulation of necroptosis and its therapeutic potentials

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## Abstract

**Purpose of review** Necroptosis is a form of cell death regulated by specific cellular protein machinery. Although the cell death is tightly controlled like apoptosis, another type of programed cell death, the biological features of necroptosis rather resemble necrosis that is defined as an uncontrolled accidental cell death. The pathway executing necroptosis relies on a protein kinase, RIPK3, and its downstream effector molecule, MLKL. Upon necroptosis initiating signals, both RIPK3 and MLKL undergo extensive post-translation modifications to construct a death complex called necrosome, finally leading to lysis of cell membrane. Preclinical mouse models demonstrated the physiological importance of necroptosis in the progress of various inflammation-associated diseases. The objective of this brief review is to introduce a new emerging concept in cell death biology and to provide a first entry into the research field of necroptosis.

**Recent findings** The uncovering of necroptosis pathway brought a fundamental change in the basic concept that necrotic cell death is passive and unregulated. Currently, multiple small molecules that can target necrotic cell death are under development and some of them are under clinical trials to evaluate their therapeutic potentials. Better understanding of the molecular mechanism leveraging necroptosis will provide an unprecedented opportunity to pathological necrosis-driven human diseases.

**Keywords** Cell death · Necroptosis · RIPK1 · RIPK3 · MLKL · Inflammation

## Introduction

Cell death is a final event to cease all biological functions in unicellular organisms, but the same cellular event is a dynamic process which controls homeostasis and immunity in multicellular organisms. In humans, as many as a hundred billion cells die each day and are replaced by other cells (Nagata 2018). Therefore, the equilibrium between cell death and proliferation is crucial for the maintenance of tissue homeostasis. For example, the continuous cell death-induced shedding of intestinal epithelial cells during normal tissue homeostasis is a crucial process to maintain intestinal barrier integrity (Vereecke et al. 2011). Cell death also plays important functions in inflammation and immunity. It can restrict viral replication (Danthi 2016), but at the same time, excessive cell death can cause tissue damage and inflammation (Wallach et al. 2014). Indeed, cell death has been acknowledged as an eminent pathological feature of various inflammatory diseases (Kist and Vucic 2021). Consequently, the cell death process during immune responses is required to be tightly coordinated to ensure beneficial immunological effects without resulting in detrimental tissue damage.

The role of cell death in tissue homeostasis, immunity and inflammation have been extensively discussed in the context of two different types of cell death: apoptosis and necrosis. Apoptosis is a programed cell death whereby a cell commits a suicide by utilizing its own cellular signaling cascades in response to specific stimuli (Nagata 2018). Apoptotic cell dies by a self-destruction program coded by caspase-dependent signaling, and the cellular dead body is then cleared by the neighboring tissue-resident phagocytes. Since the process does not permit leakage of intracellular components, apoptosis is believed as a noninflammatory form of cell death.

In contrast to apoptosis, necrosis is an accidental cell death occurring in a fatally damaged cell by an external fatal insult (Galluzzi 2007). The necrotic cell death was originally regarded as a simple failure of cell survival caused by toxic interference of essential cellular functions. A necrotic cell undergoes swelling, followed by uncontrolled rupture of the cell membrane, resulting in a massive release of cellular

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contents to surrounding tissue environment. The unleashed cellular contents function as danger-associated molecular patterns (DAMPs) and often create inflammation in tissues (Venereau et al. 2015).

Early studies considered apoptosis as the only form of regulated cell death and necrosis was seen as an unregulated and, therefore, uncontrollable accidental cell death. The traditional concept of necrosis, which was considered as a passive cell death, has been changed since the discovery of a specific form of regulated cell death called necroptosis. As in necrosis, necroptosis executes a highly inflammatory cell death, accompanying by release of DAMPs (Newton and Manning 2016). While the phenotype and the consequence of cell death similar to necrosis, collective biochemical and genetic evidence revealed that necroptosis occur as a programed cell death regulated by Receptor Interacting Protein Kinase 3 (RIPK3) and its substrate, Mixed Lineage Kinase Like (MLKL). During necroptosis, modified MLKL triggers plasma membrane permeabilization and therefore, promotes the release of proinflammatory substances from the dead cell (Grootjans et al. 2017).

Accumulating evidence suggests that necroptosis is a physiologically relevant cell death and it is critical for tissue homeostasis, immunity, and inflammation. In this brief review, I will discuss about our current understanding of necroptosis and its implication in tissue homeostasis and inflammation.

## Overview of necroptosis

Apoptosis, which is a form of caspase-dependent programed cell death, is characterized by several distinct biological features such as shrinkage of the cell, forming membrane blebs, and nuclear fragmentation (Nagata 2018). This cell death can be triggered by a wide variety of stimuli and conditions such as genotoxic stress, deprivation of growth factors, and occupation of death receptors by their specific ligands. Apoptosis is believed to be a non-inflammatory cell death since apoptotic cells generate "eat me" signals on their cell surface assisting rapid phagocytosis by surrounding tissue-resident immune cells. Because of this silent nature of cell death, apoptosis is utilized during normal development to balance cell populations in tissues (Voss and Strasser 2020).

The term, necroptosis was first introduced when a group of researchers recognized a cell death accompanied by necrotic morphological features was caspase-independent and inhibited by a small molecule called, necrostatin-1(Nec-1) (Degterev 2005). In contrast to apoptotic cell death, which is governed by caspase-mediated series of molecular events, necroptosis deploys apical protein kinases for its execution and does not require caspases. Although it was initially thought that RIPK1 is a mandatory driver of necroptosis, currently necroptosis is defined as RIPK3- and MLKL-dependent cell death (Galluzzi 2018). The main reason why RIPK1 was thought to be required for necroptosis is that Nec-1, a RIPK1 inhibitor, potently inhibits TNF-dependent necroptosis. In fact, many studies still utilize Nec-1 to define a cell death as necroptosis. However, it is now very clear that RIPK1 can function both as an inhibitor and a promoter of necroptosis depending on the cellular contexts (Voss and Strasser 2020) and therefore the absence of RIPK1 often results in necroptosis-driven inflammation in vivo due to lack of the kinase-independent survival function of RIPK1 (Ito 2016; Lin 2016; Newton 2016a).

The physiological role of RIPK1 in cell survival and death has been well-demonstrated by mouse genetic studies. RIPK1 deficient mice display postnatal lethality (Kelliher 1998). It was initially thought that the lethal phenotype is delivered by excessive apoptosis in the animals. But later, it was found that the deficiency of Fas-associated protein with death domain (FADD), which would inactivate apoptosis, did not rescue the lethal phenotype of RIPK1 KO mice (Zhang 2011). Furthermore, loss of RIPK3 also did not prevent the lethality resulted from RIPK1 deficiency in the animals. The postnatal death of RIPK1 KO mice was eventually rescued by the combined ablation of Caspase-8 and RIPK3, indicating that RIPK1 can repress both caspase-8-dependent apoptosis and RIPK3-dependent necroptosis (Dillon 2014; Rickard 2014). Interestingly, animals with a kinase-inactive form of RIPK1 are viable, unlike RIPK1 KO mice (Berger 2014; Polykratis 2014). The result demonstrates that a kinase-independent scaffolding function of RIPK1 is responsible for the pro-survival effect of RIPK1. Taken together, RIPK1 can either promotes apoptosis and necroptosis via its kinase activity or suppress cell death by its kinase-independent function. In this regard, it is important to keep it in mind that inhibition of a cell death by RIPK1 inhibitors does not necessarily indicate the mode of cell death is necroptosis.

## Activation of necroptosis

Necroptosis can be triggered by different initiating signals such as death receptors, some toll-like receptors, and unknown ligands sensed by protein, Z-DNA binding protein 1(ZBP1) (Grootjans et al. 2017). All these necroptotic signals employ proteins contain RIP Homotypic Interaction Motif (RHIM) and the RHIM provides a critical protein–protein interaction interface that regulates the formation of death-inducing protein complexes (Rebsamen 2009). There are only four proteins that contain RHIM in the mammalian system: RIPK1, RIPK3, ZBP1 and TIR-domain-containing adapter-inducing interferon-β (TRIF).

The molecular mechanism of necroptosis is most well-established in TNFR1 signaling pathway(Fig. 1) (Wajant and

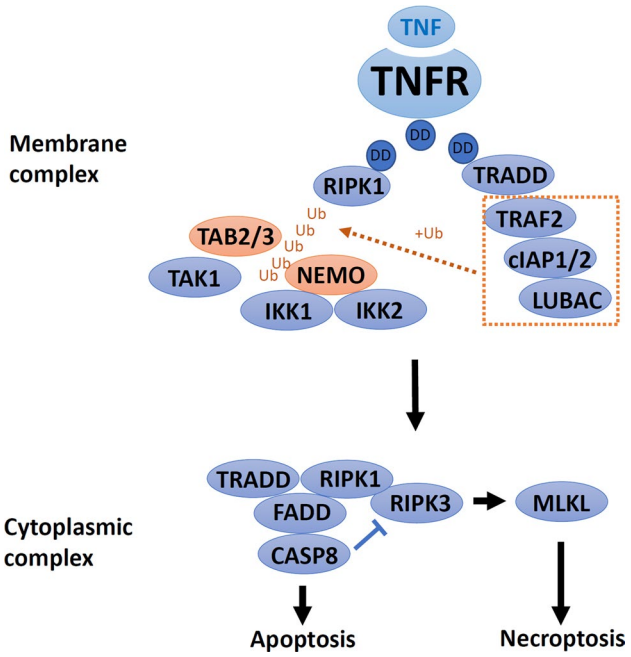


**Fig. 1** TNFR1 signaling pathway. Upon ligation of TNF, TNFR1 forms a large membrane-associated complex that is responsible for inflammation and cell survival-promoting cellular signaling pathways. During the process, if the survival-promoting signal is attenuated, death-inducing cytoplasmic complex can potentiate caspase-8 dependent apoptosis. RIPK3-dependent necroptosis is believed as an alternative backup cell death program, which occurs when apoptosis is compromised. The RIPK3-dependent modification of MLKL induces the translocation of MLKL to cell membrane resulting in lysis of a cell.

Siegmund 2019). TNFR1 is a member of the death receptor family. Upon the ligation of TNF, the death domain (DD) in TNFR1 enables to recruit DD-containing proteins including TNFR1-associated death domain protein (TRADD) and RIPK1 (Haas 2009). The ubiquitin ligases, cIAP1/2, which preexist as cytoplasmic complexes with TNF receptor-associated factor 2 (TRAF2) (Zheng et al. 2010), are then recruited to the TFNR1 membrane complex, followed by LUBAC, an E3 ubiquitin ligase complex composed of proteins, SHARPIN, HOIP, and HOIL-1 (Spit et al. 2019). The ubiquitin chains assembled by the coordination of cIAP1/2 and LUBAC allow the recruitment of ubiquitin binding proteins such as TAK1-binding protein 2/3 (TAB2/3) and NF-κB essential modulator (NEMO), resulting in the activation of NF-kB signaling pathway. Once TNF-mediated pro-inflammatory and cell survival signals are established, later the TNFR1 membrane complex transforms into a cell death-inducting cytoplasmic complex associated with FADD, caspase-8 and RIPK3 (Grootjans et al. 2017). The cytoplasmic death complex can induce caspase-8-dependent apoptosis and normally, RIPK3 is inactivated in the complex by its cleavage by caspase-8 (Feng 2007). Nevertheless, under caspase-inactive conditions, RIPK3-dependent necroptotic pathway begins by the autophosphorylation of RIPK3, which enables interaction with its effector, MLKL. The RIPK3-dependent phosphorylation of MLKL allows oligomerization of MLKL, leading to lysis cell membranes (Sun 2012; Petrie et al. 2019).

## Pathological implications of necroptosis

Necrotic cell death is found in various pathological conditions (Nieminen 2003). Despite its presence in a broad range of clinical states, necrosis in human disease was not perceived as a potential therapeutic target until very recently since it was thought that necrotic cell death is an uncontrollable process. However, the discovery of necroptosis, which is regulated by cellular machinery, provided a new opportunity to target pathological necrosis.

Recent studies performed in preclinical animal models have revealed the potential benefit of targeting necroptosis in various human diseases, including sepsis, tissue injuries, chronic inflammatory disease, and neurodegenerations (Spit et al. 2019; Khoury et al. 2020; Molnar 2019). Notably, some studies utilized only specific RIPK1 inhibitors or RIPK1-deficient mice in testing necroptosis as a potential therapeutic target. Importantly, as discussed before, RIPK1 can activate both apoptosis and necroptosis, and can potentiate NF-kB-dependent inflammation. Therefore, RIPK1-dependent phenotypes do not necessarily correlate with necroptosis-driven pathological outcomes. In this section, I will briefly discuss only studies that confirmed the pathological contribution of necroptosis in the absence of activity of RIPK3 or MLKL.

The inflammatory nature of necroptosis proposes its potential involvement in the pathologies of acute inflammation. A mouse model of TNF-induced systemic inflammatory response syndrome (SIRS) showed that loss of RIPK3 in animals were protected against lethal SIRS (Duprez 2011). In the same study, ablation of RIPK3 also prevented cecal ligation and puncture-induced sepsis.

In addition to acute inflammation models, necroptosis has been implicated in chronic inflammation-associated disease models. A model of nonalcoholic fatty liver disease (NAFLD) demonstrated that when RIPK3-deficient mice were fed with methionine- and choline-deficient (MCD), MCD diet-induced liver injury, steatosis and fibrosis were attenuated (Afonso 2015). Moreover, the high levels of RIPK3 expression in patient livers were correlated with poor prognosis of alcoholic cirrhosis (Zhang 2018). In another chronic inflammation-associated animal model, RIPK3-deficiency reduced atherosclerotic lesions, which were promoted by loss of LDL receptor (Lin 2013).

Necroptosis also appears to contribute to multiple acute tissue injury models. A study using a model of kidney I/R reported protection of RIPK3-deficient mice but interestingly, the protection was minimal in MLKL-deficient mice (Newton 2016b). Another acute kidney model induced by toxic folic acid demonstrated that RIPK3 or MLKL did not contribute to the early stage of renal injury, but later when the TWEAK signaling-dependent necroptosis occurred, ablation of RIPK3 or MLKL reduced the late injury of kidney in the model (Martin-Sanchez 2018). In addition to kidney models, RIPK3-deficient mice were protected from long-term adverse post-infarct remodeling following I/R injury-induced myocardial infarction (Luedde 2014). Furthermore, the contribution of RIPK3 to lung injury was reported in a murine model of acute lung injury (ALI). The study demonstrated that both necroptosis-dependent and -independent functions of RIPK3 can contribute to the phenotype of an LPS-induced ALI model. Importantly, RIPK3 inhibitor ameliorated lung injury and reduced inflammation in this model (Chen 2018).

Beside acute tissue injury models, necroptosis has been reported in neurodegenerative diseases. RIPK3 deficiency markedly improves neurological and systemic disease in a mouse model of Gaucher's disease (GD). When GD was induced by daily injection of a GlcCerase inhibitor, RIPK3-deficient mice showed considerably improved survival and motor coordination. In a mouse amyotrophic lateral sclerosis (ALS) model, the RIPK1, RIPK3 and MLKL–dependent necroptosis was observed in the central nerve system. In this optineurin-deficient ALS mice, the axonal pathology was rescued by RIPK3-deficiecy (Ito 2016).

Collectively, the studies discussed above synchronously propose crucial roles of necroptosis in the development of pathophysiology. It is worth to note that some studies suggested there are MLKL-independent, therefore necroptosis-independent roles of RIPK3 (Chen 2018; Alvarez-Diaz 2016). In this regard, when studying the role of necroptosis in vivo, it is important to confirm if the identified RIPK3-dependent phenotypes are also MLKL-dependent, therefore, ensuring *bona fide* necroptosis-driven pathology.

## Conclusion

Investigating the details of the mechanism regulating necroptosis is critical to understand physiological roles of necroptosis in the pathogenesis of human diseases. The key signaling molecules in necroptotic pathway are largely distinct from those involved in other pathways including apoptosis and inflammation. So far, therapeutic potentials of targeting necroptosis have been reported mainly in mouse models. It remains to be demonstrated if blocking necroptosis is a viable strategy to treat human diseases.

The development of specific inhibitors against RIPK3 was initially perused to achieve specific therapeutic benefits in the necroptosis-associated pathophysiology of the disease. But it turned out that the inhibition of RIPK3 either by specific inhibitors or mutations leading to inactivation of its kinase activity not only block necroptosis but promotes spontaneous apoptosis (Mandal 2014). Hence, it seems a specific inhibitor against MLKL will be a better strategy to target necroptosis in the clinic.

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## Declarations

**Conflict of Interest** The author declares that there is no conflict of interest.

**Human and animal rights** This review article contains published animal studies, which were performed under institutional and national guidelines.

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