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Review

Using drugs to target necroptosis: dual roles in disease therapy

Zhen Wang[1], Li-min Guo[1], Hong-kang Zhou[1], Hong-ke Qu[2], Shu-chao Wang[1], Feng-xia Liu[3], Dan Chen[1], Ju-fang Huang[1] and Kun Xiong[1]

[1]Department of Anatomy and Neurobiology, [2]Department of Forensic Science, School of Basic Medical Science, Central South University, Changsha, Hunan and [3]Department of Human Anatomy, School of Basic Medical Science, Xinjiang Medical University, Urumqi, Xinjiang, China

Summary. Necroptosis is programmed necrosis, a process which has been studied for over a decade. The most common accepted mechanism is through the RIP1-RIP3-MLKL axis to regulate necroptotic cell death. As a result of previous studies on necroptosis, positive regulation for promoting necroptosis such as HSP90 stabilization and hyperactivation of TAK1 on RIP1 is clear. Similarly, the negative regulation of necroptosis, such as through caspase 8, c-FLIP, CHIP, MK2, PELI1, ABIN-1, is also clear. Therefore, the promise of corresponding applications in treating diseases becomes hopeful. Studies have shown that necroptosis is involved in the development of many diseases, such as ischemic injury diseases in various organs, neurodegenerative diseases, infectious diseases, and cancer. Given these results, drugs that inhibit or trigger necroptosis can be discovered to treat diseases. In this review, we briefly introduce up to date concepts concerning the mechanism of necroptosis, the diseases that involve necroptosis, and the drugs that can be applied to treat such diseases.

Key words: Necroptosis, Drugs, Disease therapy, RIP1, RIP3

Offprint requests to: Kun Xiong and Ju-Fang Huang, Department of Anatomy and Neurobiology, Morphological Sciences Building, Central South University, 172 Tongzi Po Road, Changsha, Hunan 410013, China. e-mail: xiongkun2001@163.com, huangjufang@csu.edu.cn.

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Introduction

Cell death is generally divided into programed cell death and non-programmed cell death. Programmed cell death such as apoptosis and autophagy has been well studied and necrosis was once thought to be unregulated. However, in early 1988, necrotic death triggered by tumor necrosis factor (TNF) apart from apoptosis was clarified in some cell types (Laster et al., 1988). In 1998, Vercammen et al. (1998) found that both necrotic and apoptotic cell death can be regulated by the Fas receptor. And in 2003, Chan et al. (2003) found that TNFR2 and RIP could regulate programmed necrosis. Finally, in 2005, Yuan and her team found an inhibitor, Nec-1, that can block the non-apoptotic death, which was similar to necrosis in morphology, and termed this type of programmed cell death necroptosis (Degterev et al., 2005). Subsequently in 2008, Yuan et al identified the target of Nec-1 for blocking necroptosis. They found that Nec-1 was an inhibitor of the receptor-interacting protein kinase 1 (RIPK1), and that RIPK1 acted as a key upstream kinase that triggers necroptosis (Degterev et al., 2008). Afterwards, Han, Wang, and Chan, et al found RIP3 was a key molecule that regulates necroptosis as a downstream protein of RIPK1, and they interacted with each other by forming a complex involved in necroptosis after activation (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). Subsequently, studies of a mixed lineage kinase domain-like protein (MLKL) became another major advance in understanding necroptosis, as well as the identification of heat shock protein 90 (HSP90) as an important factor in mediating necroptosis (Sun et al., 2012; Zhao et al., 2012; Li et al., 2015) (Fig. 1).

The mechanism of necroptosis

Based on those milestone discoveries, hundreds of researchers were inspired to study the mechanism of necroptosis. Over the past decade, a number of molecules and processes have been identified as regulators of necroptosis, such as RIPK1, Toll/IL-1R domain-containing adapter-inducing interferon-β (TRIF), DAI, RIPK3, and MLKL homologs (Dondelinger et al., 2016; Wallach et al., 2016). RIPK1 was once thought to be the most upstream signal triggered by TNFR1, Fas, and TRAILR1/2 or pathogen recognition receptors. Recently, studies have shown that MAPKAP kinase-2 (MK2), as the direct upstream molecule of RIPK1, reduces necroptosis by phosphorylating its substrate RIP1 and inhibiting the autophosphorylation of RIPK1 (Dondelinger et al., 2017; Jaco et al., 2017; Menon et al., 2017). Hyperactivation of TAK1 leads to RIPK1 hyperphosphorylation in *TAB2* KO MEF, which has been shown to promote cells to necroptosis (Geng et al., 2017). Another RIP1 upstream molecule, Pellino 1 (PELI1), an E3 ubiquitin ligase, can mediate K63 ubiquitylation at the K115 site of RIPK1 during necroptosis by forming necrosomes, and PELI1 deficiency can block necroptosis efficiently (Wang et al., 2017a). The protein kinase RIPK3 acts downstream of RIPK1 in the necroptotic signaling pathway (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), which is activated by phosphorylated RIP1. Furthermore, ABIN-1, an ubiquitin-binding protein, plays an important role in mediating the activation of RIP1 by Met1 ubiquitylation and Lys63 deubiquitylation. ABIN-1-/- promotes cell death by necroptosis by Lys63 ubiquitylation of RIP1 (Dziedzic et al., 2018). Meanwhile, the autophosphorylation of RIP3 is mediated by ROS production from the mitochondria, which plays a key role in forming necrosomes (Zhang et al., 2017b). Activated RIPK3 interacts with RIPK1, TRIF or DAI *via* a RIP homotypic interacting motif (RHIM), and forms a necrosome which is a milestone on the pathway to necroptosis (Cho et al., 2009). RIPK1 and RIPK3 each contain more than one caspase-8 cleavage site, and are substrates for the procaspase-8-cFLIPL heterodimer. The procaspase-8-cFLIPL complex inhibits necroptosis by cleaving RIPK1 and RIPK3. Inhibition of caspase-8 breaks its ability to cleave RIPK1 and RIPK3 and leads to RIPK1-dependent TNF-induced RIPK3 activation and necroptosis (Tummers and Green, 2017). In addition, FLIP is thought to be a molecular switch for apoptosis and necroptosis (Gong et al., 2014). Apart from FLIP, the carboxyl terminus of Hsp70-interacting protein-mediated ubiquitylation (CHIP) is another negative regulator for necroptosis, by degrading the expression of RIP3 (Seo et al., 2016). In 2012, studies suggested that MLKL plays a role as a substrate of RIPK3, which mediates necroptosis by binding to

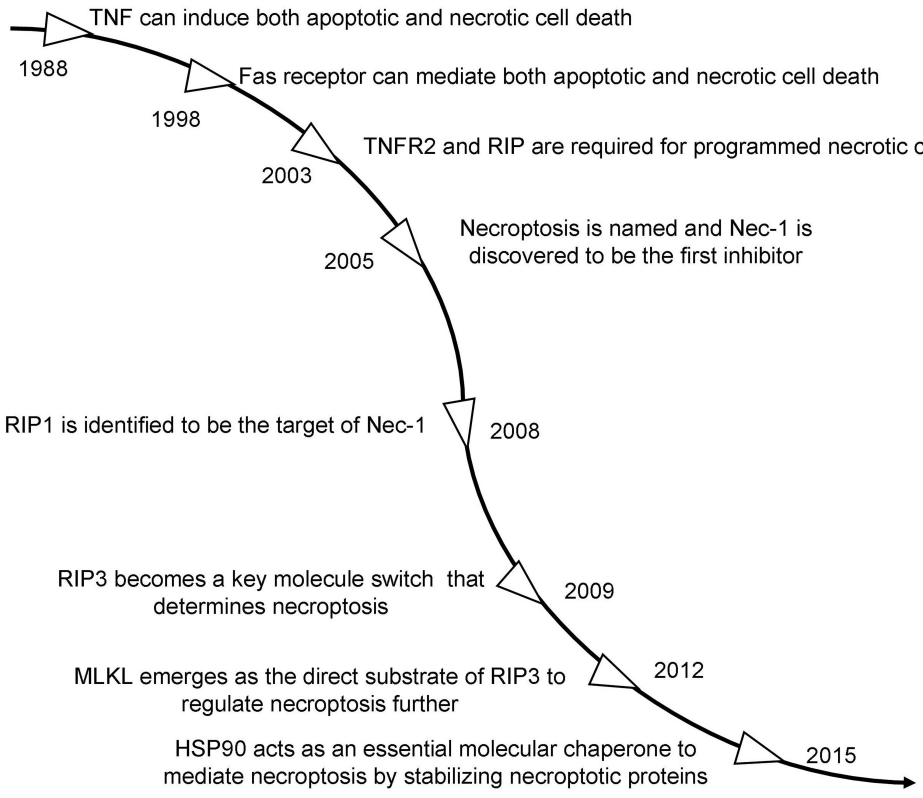

Fig. 1. Milestones in the history of necroptosis exploration.

RIPK3 and phosphorylation by RIPK3 (Sun et al., 2012; Zhao et al., 2012). MLKL undergoes a conformational change that allows the N-terminal region to oligomerize, and subsequently translocate to the plasma membrane and form the pores (Cai et al., 2014; Chen et al., 2014; Wang et al., 2014). Recently, Han et al further studied the morphology of the MLKL polymer and discovered that MLKL turned out to be an octamer depending on α-helices and penetrates the plasma membrane, leaving both N and C terminals inside the plasma membrane (Huang et al., 2017). Cation channels derived from those MLKL pores are permeable to Mg^{2+}, Na^{+}, and K^{+} instead of Ca^{2+}, which leads to ion disorder and finally to cell death (Chen et al., 2016b; Zhang et al., 2016a, 2017a). Recently, studies have shown that the function of MLKL differs according to a diurnal distribution. MLKL generates exosomes during the night and forms oligomers during daytime. Activated MLKL results in disruption of the plasma membrane (PM) and forms bubbles, and the formation of PM bubbles requires the ESCRT-III machinery. When confronting necroptotic stimuli, ESCRT-III maintains the integrity of the plasma membrane. MLKL together with the ESCRT machinery regulates the balance of membrane trafficking and necroptotic bubble formation (Gong et al., 2017; Guo and Kaiser, 2017; Vandenabeele et al., 2017; Yoon et al., 2017). Cellular contents, including lactate dehydrogenase (LDH), high-mobility group box 1 (HMGB1), mitochondrial DNA, N-formyl peptides, IL-1α and IL-33 act as damage-associated molecular patterns (DAMPs), are released outside, and give rise to inflammation accompanied with necroptosis (Krysko et al., 2011; Kaczmarek et al., 2013). Since 2015, HSP90 has been thought to be a chaperone molecule to mediate necroptosis by stabilizing necroptotic machinery proteins, and its co-chaperone molecule CDC37 acts in a synergistic role in regulating necroptosis. In addition, the inhibitors of HSP90 can easily disrupt necroptosis by disturbing the RIP1 and RIP3 complex as well as the phosphorylation of RIP3 and MLKL (Li et al., 2015, 2016a; Park et al., 2015; Jacobsen et al., 2016; Jacobsen and Silke, 2016; Yang and He, 2016; Zhao et al., 2016) (Fig. 2).

The identification of measurements for necroptosis

It is well known that necroptosis resembles necrotic cell death, apart from its classic regulation molecules. Therefore, most of the measurements for necroptosis are around the features of necrosis and its regulated mechanisms. In morphology, necroptosis presents as the swelling of organelles (such as the endoplasmic reticulum and mitochondria), the rupture of the plasma membrane, and the lysis of the cell, which resembles the typical presentation of necrosis (Dondelinger et al., 2016; Zhang et al., 2017a). Due to the disruption of the plasma membrane, the nucleus of necrotic cells can be stained by propidium iodide (PI) (Darzynkiewicz et al., 1992; Atale et al., 2014, Li et al., 2016b). Similarly, the cellular contents LDH released from necroptotic cells can be another index for the diagnosis of necrosis (Roe, 1977; Bahadar et al., 2016; Komolafe et al., 2017; Wang et al., 2018b). However, those methods cannot distinguish necrosis and necroptosis precisely and directly. Generally, investigators detect necroptosis by pretreating with specific drugs (such as Nec-1) for controlling injuries. The decreased number of necrotic cells after drug treatment following injuries compared to injury group was thought to be indirect evidence to prove the presence of necroptotic cells (Degterev et al., 2005, 2008; Rosenbaum et al., 2010; Shang et al., 2014; Ding et al., 2015; Chen et al., 2016a; Xiong et al., 2016; Liao et al., 2017; Shang et al., 2017). Additionally, the specific methods for detecting necroptosis are based on its biochemical features. Studies have identified RIP1/3, MLKL and their homologous phosphorylation forms as the specific indexes for detecting the incidence of necroptosis. At the same time, the RIP1/RIP3 and RIP3/MLKL complexes also play very important roles as markers of necroptosis (Vanden Berghe et al., 2013; Degterev et al., 2014; Jouan-Lanhouet et al., 2014; Zhao et al., 2015a; He et al., 2016; Feltham et al., 2017). Recently, studies found a novel flow cytometric assay to detect necroptosis by using anti-RIP-3, anti-active caspase-3 fluorescently tagged antibodies, and a fixable viability probe fluorescent dye. Necroptosis is recognized by the uptake of RIP3, while apoptotic cells are detected by an active-Caspase-3+ /RIP3- phenotype (Lee et al., 2017). However, the more accurate measurements for necroptosis still need further study and development.

Participation of necroptosis in acute and chronic diseases

Necroptosis induced by acute ischemia in brain

The diseases involved in necroptosis in the brain that are caused by ischemia were first reported in 2005. The RIPK1 inhibitor Nec-1 protects neurons from cerebral ischemia-reperfusion injury (IRI), and the infarct size following middle cerebral artery occlusion is reduced by pretreating with Nec-1 (Degterev et al., 2005; Linkermann et al., 2013). Ischemia insult leads to a toxic intracellular environment including increases in reactive oxygen species (ROS), calcium, and mitochondrial damage (Thornton and Hagberg, 2015; Zhang et al., 2016b; Yuan et al., 2017). These studies found that the expression of RIP3 in hippocampus increases following global ischemic insult *in vivo*. RIP3 knock-down experiments show a reduction of oxygen glucose deprivation (OGD)-induced necrotic neuronal death, while RIP3 over-expression sharpens the neuronal necrosis following OGD (Vieira et al., 2014). Another study showed that Nec-1 protects hippocampal neurons from IRI through the RIP3/DAXX pathway (Yang et al., 2017a). Further research showed that Nec-1 decreases necrotic neuronal death by inhibiting RIP3 up-regulation

and its translocation to the nucleus (Yin et al., 2015). In addition, RIP1/3 and the interaction between RIP1-RIP3 and MLKL increase following hypoxia-ischemia insult. Meanwhile, the oligomerized MLKL moves to the neuronal membrane, which leads to cellular membrane rupture. Furthermore, inhibiting MLKL by RNAi interference protects neurons from ischemia injury by attenuating the RIP1-RIP3-MLKL complex. Additionally, *in vivo* experiments further showed that MLKL inhibition reduces brain injury induced by ischemia. Therefore, MLKL may be a downstream molecular target for studying the mechanism of neuronal necroptosis (Qu et al., 2016). Other studies have shown that the RIP3-AIF complex and nuclear translocation are important for neuronal necroptosis induced by ischemia injury (Xu et al., 2016). A more interesting experiment has shown that HIF-1α, RIP1, RIP3, and MLKL are involved in OGD induced neuronal necroptosis (Yang et al., 2017b). All in all, necroptosis exerts an important role in ischemia injury induced neuronal cell death, and its necroptotic mechanisms contribute significantly to the process.

Necroptosis induced by acute and chronic injury in eyes

Apart from the brain, ischemia reperfusion (I/R) induced necroptosis occurs in many other organs. Our study found that the expression of RIP3 increases following acute high intraocular pressure (aHIOP) and ischemia injury in retinal ganglion cells (RGCs), which may trigger necroptosis (Huang et al., 2013b; Ding et al., 2015). Other studies showed that necroptosis is involved in I/R induced retinal injury, which is associated with the extracellular signal-regulated kinase-1/2- receptor-interacting protein kinase 3 (ERK1/2-RIP3) pathway (Gao et al., 2014). In one study, they proved that RIP1 and RIP3-dependent necroptosis turn up not only in the acute retinal injury mouse model, but also in the retinal degenerative mouse model (rd1 mice (*pde6β*^{rd1})) (Huang

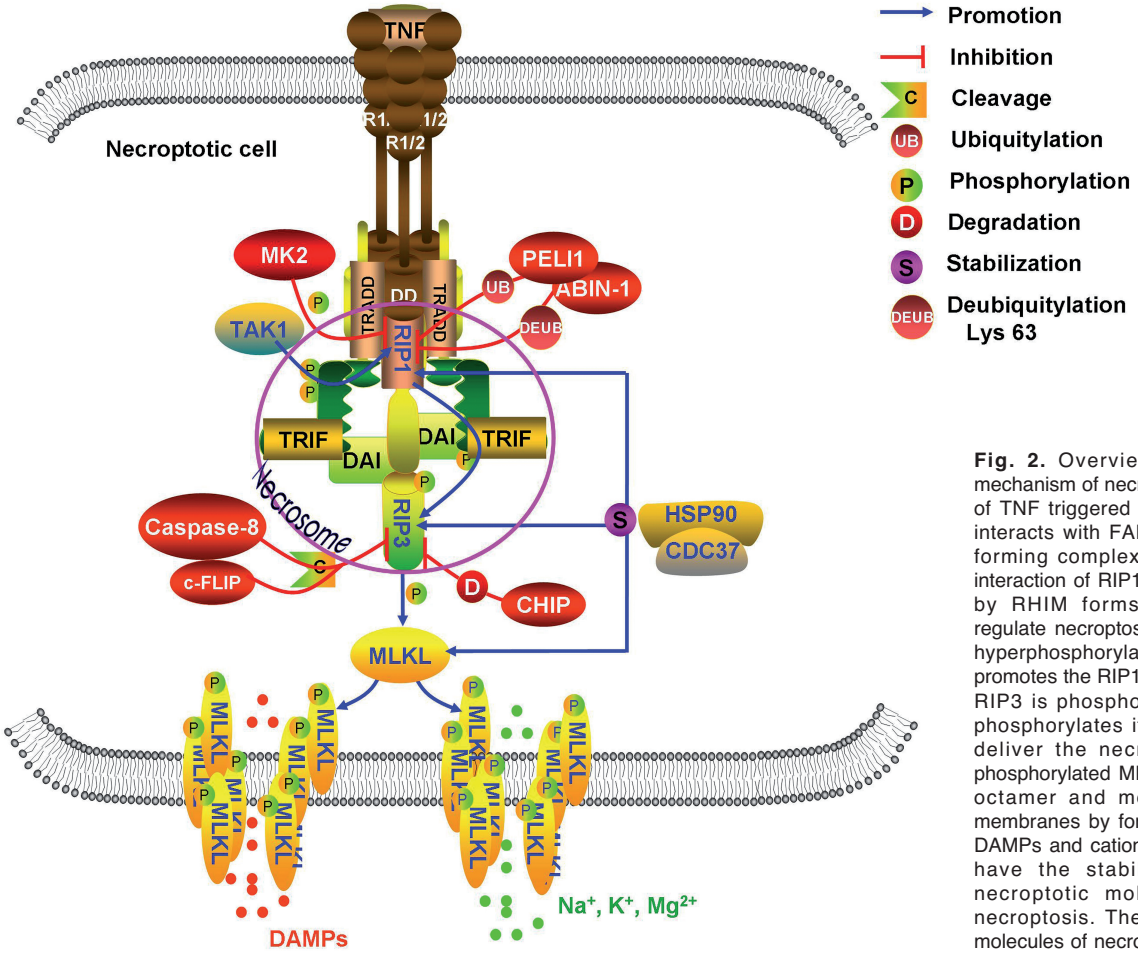

Fig. 2. Overview of the regulation mechanism of necroptosis. In the process of TNF triggered necroptosis, TNFR1/2 interacts with FADD, TRADD and RIP, forming complexIby DD domain. The interaction of RIP1, RIP3, TRIF, and DAI by RHIM forms the necrosome to regulate necroptosis. In the process, the hyperphosphorylation of RIP1 by TAK1 promotes the RIP1-mediated necroptosis. RIP3 is phosphorylated by RIP1 and phosphorylates its substract MLKL to deliver the necroptotic signal. The phosphorylated MLKL transforms into an octamer and moves to the plasma membranes by forming pores to release DAMPs and cations. HSP90 and CDC37 have the stabilization function of necroptotic molecules to regulate necroptosis. The negative regulation molecules of necroptosis are notable; the phosphorylation of RIP1 by MK2, ubiquitylation of RIP1 by PELI1, and deubiquitylation lys 63 of RIP1 by ABIN-1 can effectively inhibit necroptotic signals. Similarly, cleavage of RIP3 by caspase 8/ c-FLIP and degradation of RIP3 by CHIP are negative regulators of necroptosis.

et al., 2018). The pro-inflammatory cytokines and chemokines, such as TNF-α and chemokine ligand 2 released from the necroptotic microglia, direct the retinal inflammation and injury in the retina (Huang et al., 2018).

Necroptosis induced by ischemia in heart

In the heart, the programmed cell death morphology coincident with necrosis has been studied as necroptosis in many kinds of ischemia-induced injuries, which is dependent on activating RIP1/RIP3/MLKL machines (Adameova et al., 2017). Researchers have pointed out that necroptosis is involved in myocardial I/R injury, and a combination of necroptosis and apoptosis inhibition promotes the cardioprotective effect (Koshinuma et al., 2014). It is known that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) can initiate NF-ϰB upon I/R injury in cardiomyocytes by inflammatory responses, and is an important regulatory protein in I/R induced cardiac trauma (Ling et al., 2013). Soon after that, CaMKII inhibition was proved to inhibit necroptotic proteins to relieve the cardiac contractile dysfunction, which provided a new potential approach for treating heart ischemia injury (Szobi et al., 2014). Meanwhile, pretreatment with simvastatin, a common drug that protects the heart helps to reduce RIPK1 and RIPK3 protein activity in rat cardiac allograft I/R, which showed that the mechanism of necroptosis is involved in this model (Tuuminen et al., 2016). Studies have shown that miRNAs are very important to regulate cardiac cell death in I/R induced heart injury (Zhu and Fan, 2012). Among these miRNAs, miRNA223 is thought to play an important role in the necroptosis in the heart. Qin et al applied both transgenic and knock-out pre-miR-223 mouse models that underwent I/R to study the function of miR-223 on necroptosis in heart ischemia injury. The results from their experiments showed that hearts in pre-miR-223 mice were resistant to ischemia injury, while hearts in pre-miR-223 KO mice were sensitive to I/R-induced injury. Additionally, they found that RIP1/RIP3/MLKL and the NLRP3 inflammasome are inhibited in hearts of pre-miR-223 mice, however, they are significantly activated in hearts with pre-miR-223 KO mice upon I/R injury. Interestingly, inhibiting necroptotic signaling can effectively decrease the I/R-triggered cardiac injury status in pre-miR-223 KO mice (Qin et al., 2016).

Necroptosis induced by ischemia in kidney and intestines

In kidney, studies also show that necroptosis activated in ischemic injury is predominant over apoptosis, and Nec-1 has the therapeutic potential to prevent and treat I/R induced renal injury (Linkermann et al., 2012; Zhang et al., 2013). Apart from ischemia injury, tubular epithelial cells (TEC) will die in necroptosis mode when exposed to TNF-α *in vitro*, which can be reversed by Nec-1 or application of RIPK3(-/-) TEC. Following renal transplant, recipients who received RIPK3(-/-) kidneys had longer survival times and better renal function. On the other hand, RIPK3-mediated necroptosis in donor kidneys will worsen inflammatory injury (Lau et al., 2013). In intestines, RIPK1 is important for the homeostasis and survival of intestinal epithelial cells (IECs) by inhibiting Caspase 8-mediated IEC apoptosis and activating NF-ϰB (Takahashi et al., 2014). During injury conditions, studies show that RIPK 1/3 and MLKL mediate necroptosis in ischemia intestinal injury *in vivo*, which can be specifically blocked by Nec-1 (Wen et al., 2017).

Necroptosis in neurodegenerative diseases

Neurodegenerative diseases are severe nervous system diseases which present as the loss of many kinds of cells in the nervous system, such as Parkinson's disease (PD), Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Multiple sclerosis (MS) and so on. AD is one of the most common neurodegenerative diseases in the world. AD is commonly identified by the accumulation of β-amyloid peptide (Aβ) plaques and tau protein in the brain, as well as the features of neuronal degeneration, blood-brain barrier (BBB) pathology, neuroinflammation, oxidative stress, and microvascular, cytoskeleton, and mitochondrial changes which are responsible for AD development (Hubin et al., 2016; Pomorska and Ockene, 2017). Recent studies have shown that necroptosis is involved in the brains of AD patients, which is associated with Braak stage and brain weight as well as cognitive scores (Caccamo et al., 2017). In addition, a series of necroptotic genes such as *RIPK1* and *MLKL* are matched with AD mediators (Caccamo et al., 2017; Ofengeim et al., 2017). Furthermore, inhibiting necroptosis decreases the cell loss in the AD mouse model (Caccamo et al., 2017). Similarly, inhibiting caspase-8 and activating RIPK1, RIPK3, and MLKL promote the cortical lesions of human MS. It has been shown that necroptosis regulates TNF-α induced oligodendrocyte degeneration and that inhibition of RIPK1 helps reduce oligodendrocyte cell death in both *in vivo* and *in vitro* MS models (Ofengeim et al., 2015). PD is characterized by the loss of dopaminergic neurons and accumulation of modified α-synuclein (Lewy bodies) in striatum and substantia nigra (Taddei et al., 2017). It has been reported that the inhibitor of necroptosis, Nec-1, reverses the loss of human neuroblastoma SH-SY5Y cells in the PD model (Zhang et al., 2008; Wu et al., 2015). At the same time, Nec-1 protects dopaminergic neurons against injury together with apoptotic signaling (Jantas et al., 2014). Additionally, ALS is a neurodegenerative disease featuring the degeneration of motor neurons (MNs) in the spinal cord, cortex and brain stem, which results in the muscular atrophy and paralysis of patients.

Necroptosis of astrocytes is activated by embryonic stem-cell-derived MNs in the ALS model, accompanied by the activation of RIP1 and MLKL (Pirooznia et al., 2014; Re et al., 2014; Ikiz et al., 2015). Furthermore, RIPK1- and RIPK3-mediated axonal pathology is observed in both transgenic mice and samples from human ALS patients. These results indicate that RIPK1 and RIPK3 play important roles in regulating axonal degeneration (Ito et al., 2016; Politi and Przedborski, 2016). Further experiments proved that Nec-1 decreased the MNs death after co-culture with astrocytes from ALS patients, and delayed the function of MNs in the transgenic mouse model of ALS (Fan et al., 2017). Thus, RIP1 may be a mediator for axonal pathology in ALS, and blocking RIP1 may be an effective intervention for treatment of ALS. Up to now, there is no effective treatment for these degenerative nervous diseases. The chronic inflammation in neurons is another problem involved in these diseases. Studies show that necroptotic molecules such as RIP1, RIP3, MLKL can also mediate the development of inflammation, and from the data above we speculate that the necroptotic machine may promote the progression of neurodegenerative diseases, especially through inflammation.

Necroptosis in viral and bacterial infections

Viral infectious diseases that are involved in necroptosis

An early study demonstrated that RIPK3 plays a key role in regulating cell death by vaccinia virus (VACV) infection, followed by a study which showed that VACV infection leads murine fibroblasts to a necroptotic death after TNF treatment (Li and Beg, 2000; Cho et al., 2009; Upton et al., 2010). Additionally, Cho's group found that both the phosphorylated RIP1/RIP3 and RIP1-RIP3 complexes appear during VACV infection (Cho et al., 2009). Similar results were found in the fibrosarcoma L929 cell line infected by Sendai virus (SeV) and murine gammaherpesvirus-68 (MHV68), which is able to induce a significant amount of necroptosis. MHV68-induces cellular necroptosis in a TNF-dependent manner, while SeV-induced necroptosis is independent of TNF (Schock et al., 2017). The reasons are that MHV68-induced necroptotic death happens through the cytosolic STING sensor pathway, while SeV-induced necroptosis happens through the RNA sensing molecule RIG-I or the RIP1 de-ubiquitin protein, CYLD. Subsequently, many groups have demonstrated that Influenza A virus (IAV) in infected murine fibroblasts and lung epithelial cells (Nogusa et al., 2016) activates RIPK3 and triggers necroptosis through DAI and type I IFN in the necroptotic pathway. RIP3 interacts with mitochondrial anti-viral-signaling protein (MAVS), inhibits RIPK1 interaction with MAVS, and activates protein kinase R (PKR), which is independent of its conventional pathway in necroptosis (Downey et al., 2017). DAI is a recognition receptor which forms a complex with RIP3 during IAV infection, and leads to necroptosis (Upton et al., 2012; Thapa et al., 2016). In West Nile virus (WNV) encephalitis, studies showed that *Ripk3* deficient mice exhibit severe necroptosis in the central nervous system (CNS), which suggests the importance of RIPK3 in limiting virus spread by executing necroptotic cell death in response to WNV infection (Daniels et al., 2017; Gilley and Kaiser, 2017). Meanwhile, Japanese encephalitis virus (JEV) is also a prevalent cause of viral encephalitis worldwide, which can cause fatal neuroinflammation characterized by neuronal destruction accompanied with intense microgliosis and production of various inflammatory cytokines (Biswas et al., 2010; Chen et al., 2010). A study indicates that MLKL mediated necroptosis is activated in the pathogenesis of JEV. MLKL expression in neurons is upregulated during JEV infection and *MLKL* KO decrease inflammatory cytokines induced by JEV infection (Bian et al., 2017), as shown in Table 1.

It should be noted that not all viruses have the function of promoting necroptosis in the host. In fact, some viruses may inhibit necroptosis during infection, such as cytomegalovirus infection (MCMV). It has been reported that viral M45-encoded inhibition of RIP activation (vIRA) is essential for MCMV to block RIPK3-dependent necroptosis in 3T3-SA cells, by disrupting both the RIP3-RIP1 complex and RIP3 RHIM-dependent process while the M45 mutant phenotype of MCMV abolishes the activity noted above (Upton et al., 2010, 2012; Huttmann et al., 2015; Brune and Andoniou, 2017). Apart from MCMV, the N-

Table 1. Viruses that promote necroptosis.

Virus by promoting necroptosis	Infected host cells	Targeted molecules
Vaccinia virus	Murine fibroblasts	RIP1/RIP3
Sendai virus	Fibrosarcoma L929 cells	TNF-independent RNA sensing molecule RIG-I
Murine gammaherpesvirus-68	Fibrosarcoma L929 cells	TNF-dependent STING sensor pathway
Influenza A virus	Murine fibroblasts and lung epithelial cells	RIPK3, DAI and type I IFN
West nile virus	Neurons	RIPK3
Japanese encephalitis virus	Neurons	MLKL
Herpes simplex virus	Mouse cells	ICP6/10, RIP1/RIP3
RNA virus coxsackie virus B3	Intestinal epithelial cells (early infection)	RIP3

terminus E3 protein of VACV prevents DNA-dependent activation of interferon regulatory factors (DAI)-mediated induction of necroptosis in 293T cells in a similar fashion (Koehler et al., 2017). Herpes simplex viruses (HSV-1 and HSV-2) are examples of viruses that inhibit cell death pathways during infection in human cells. The protein ICP6/10 synthesized by HSV blocks necroptosis by inhibiting RIPK1 and RIPK3 interactions in human cells. Interestingly, the opposite occurs in mouse cells. RIP3 and its interaction with protein ICP6 induces necroptotic death in infected mouse cells by limiting viral propagation through its RIP1/RIP3 RHIM domain (Guo et al., 2015; Huang et al., 2015). Another interesting example demonstrated by Harris et al is that RIPK3 acts in dual roles when RNA virus coxsackie virus B3 (CVB) infects intestinal epithelial cells. In early infection, RIPK3 promotes CVB replication, and in later infection, the CVB-encoded cysteine protease 3 Cpro cuts RIPK3 into N- and C-terminals, making it unable to transduce the necroptotic signal. The C-terminal RIPK3 with RHIM has been proven to induce non-necrotic cell death, while the N-terminal kinase domain RIPK3 was unable to induce any form of cell death (Harris et al., 2015). In consideration of the inhibition of necroptosis by these viruses, we can hypothesize that the reconstructive or attenuated virus might be an ideal treatment target for diseases contributed by necroptosis, as shown in Table 2.

Bacterial infectious diseases involved in necroptosis

When encountered with bacterial infection, both apoptosis and necroptosis are involved in the process. necroptosis is a host innate response to bacterial infection, keeping pace with apoptosis (Chan et al., 2015; Orozco and Oberst, 2017; Pearson and Murphy, 2017). It has been found that *S. Typhimurium* destroys macrophages by activating type I interferon signaling to compromise innate immunity, and the type I interferon signaling activates RIPs-mediated necroptosis in macrophages (Robinson et al., 2012; Du et al., 2013; Hu and Zhao, 2013; Liang and Qin, 2013). Studies show that excess TNF can induce mitochondrial ROS in infected macrophages through RIPs dependent pathways. ROS induces necroptosis through modulation of mitochondrial cyclophilin D in mitochondrial permeability pore formation, and the production of acid sphingomyelinase-mediated ceramide in mycobacteria infected macrophages (Roca and Ramakrishnan, 2013). Recently, the TNF-RIP1-RIP3 signaling pathway mediates necroptosis in tuberculosis (TB)-infected macrophages through excess mitochondrial ROS production (Roca and Ramakrishnan, 2013; van Heijst and Pamer, 2013). When confronting TB infection, murine macrophages and murine fibroblasts will die of necroptosis in different signaling pathways, the former through RIP1 and RIP3 dependent manner and the latter *via* a RIP1 and MLKL dependent manner together with the production of mitochondrial ROS (Butler et al., 2017). Studies report that diverse bacterial pathogens, including *S. marcescens, Staphylococcus aureus, Streptococcus pneumoniae, Listeria monocytogenes,* uropathogenic *Escherichia coli* (*UPEC*), and purified recombinant *pneumolysin*, produce a pore-forming toxin (PFT) which can induce necroptosis of macrophages, and can be blocked by pretreating with inhibitors of RIP1, RIP3, and MLKL. Alveolar macrophages in *MLKL* KO mice and bone marrow-derived macrophages from *RIP3* KO are also protected against the bacterial infection, but not *caspase-1/11* KO *or caspase-3* KO mice, which indicates the dominance of necroptosis instead of apoptosis (Gonzalez-Juarbe et al., 2015; Kitur et al., 2016). Other studies have reported that RIPK1 increases the IFN-β production by LPS induction in bone marrow-derived macrophages. Also, RIPK1 and RIPK3 are required in the regulation of necroptosis induced by LPS, except for MLKL (Saleh et al., 2017). When infected with *Y. pestis*, the RIP3-dependent pathway in macrophages is activated to resist the infection. Mice without RIP3 are susceptible to infection and present with the decrease of proinflammatory cytokines (Weng et al., 2014). On the other hand, studies show that EPEC infection can inhibit TNF-induced activation of MLKL in intestines. EPEC encodes EspL, a type III secretion system effector, which can cleave the RHIM domains of the RIPK1-RIPK3 complex, TRIF, and DAI, and as a result inhibit necroptosis and inflammation (Li et al., 2013; Molloy, 2013; Pearson et al., 2017). The macrophages in bacterial infection function to defend the outside invasion. Therefore, the fate of macrophages after infection plays a significant role in treatment of patients with infections, by saving macrophages and strengthening immunity, as shown in Table 3.

Table 2. Viruses that inhibit necroptosis.

Viruses inhibiting necroptosis	Infected host cells	Targeted molecules
Cytomegalovirus	3T3-SA cells	vIRA, RIP1/RIP3
Vaccinia virus	293T cells	N-terminus E3 protein
Herpes simplex virus	Human cells	ICP6/10, RIP1/RIP3
RNA virus coxsackie virus B3	Intestinal epithelial cells (later infection)	Cysteine protease 3 Cpro, RIP3

Necroptosis in the development of tumors

Necroptosis acts in dual roles in the development of tumors. On the one hand, necroptosis can destroy cancer cells, as seen for apoptosis or autophagy, acting as an innate defender. For example, the necroptotic machine RIP3, as well as necroptosis, is reduced in acute myelocytic leukemia (AML) (Nugues et al., 2014). Similarly, it is reported that RIP3 is down-regulated in breast cancer which leads to the development and progression of tumors (Koo et al., 2015). On the other hand, necroptosis can also promote the development of tumors. A study in 2016 showed that RIPK1, RIPK3, and MLKL deficiency by CRISPR/Cas9 technology inhibits the tumor growth and tumor cell death, which proves that the three necroptotic genes are essential for tumor progression in MDA-MB-231 breast cancer cells (Liu et al., 2016). Studies show that the mechanism of promoting tumor progression may be the inflammation of necroptosis and the ROS from the mitochondria (Galadari et al., 2017; Wang et al., 2017c). The detailed data about necroptosis in tumors has been reviewed (Wang et al., 2017c). New approach to target necroptosis for treatment of tumors is a goal for future research.

Drugs targeted at necroptosis

Common inhibitors of necroptosis

Up to now, necroptosis has been studied for over ten years, after it was discovered and named for the first time in 2005 by Yuan and her team (Degterev et al., 2005). The first set of inhibitors that were identified to protect cells from necroptosis was Nec-1/Nec-1s/Nec-3/Nec-4/Nec-5 (Degterev et al., 2005; Wang et al., 2007), a specific inhibitor targeted at RIP1. Subsequently, the GSK2982772 (Harris et al., 2017), PN10 (Najjar et al., 2015), Cpd27 (Najjar et al., 2015), RIC (Do et al., 2017), and 6E11 (Delehouze et al., 2017; Wang et al., 2017a) turned out to be novel inhibitors of necroptosis, acting by targeting RIP1. When pERK inhibitors turned out to be new potent RIPK1 inhibitors, critical issues on the specificity and use of GSK963 (Berger et al., 2015), GSK2606414, GSK2656157, and Sibiriline (Le Cann et al., 2017; Rojas-Rivera et al., 2017) were discovered, as inhibitors of necroptosis, where the molecular target is RIP1. Since RIP3 is thought to be a very key mediator to determine the necroptosis, from a study in 2009, studies have been initiated to explore the specific inhibitors of necroptosis targeting RIP3. It was encouraging that GSK'843, GSK'840, GSK'872 (Kaiser et al., 2013), dabrafenib (Li et al., 2014), and ponatinib (Fauster et al., 2015) were discovered to be specific inhibitors of RIP3 in the prevention of necroptosis. In 2012, studies found the subtraction of RIP3, MLKL, which regulates necroptosis in an important manner, MLKL is phosphorylated by RIP3, forms oligomers, and is translocated to the plasma membrane forming pores that lead the rupture of the membrane. At the same time, studies found the inhibitors target MLKL of human-Necrosulfonamide (NSA) (Sun et al., 2012), TC13172 (Yan et al., 2017) and GW806742X (Gonzalez-Juarbe et al., 2015). Then in 2016, HSP90 was found to be a hot spot in chaperoning necroptotic machines to regulate necroptosis, and among the inhibitors of HSP90, kongensin A, 17-AAG, and geldanamycin (GA) are identified to prevent necroptosis (For our present work about GA inhibiting necroptosis in rat primary cultured cortex neurons following OGD *via* inhibiting HSP90α (Wang et al., 2017a-c, 2018a)) (Table 4). Most of the data above are reviewed (Degterev and Linkermann,

Table 3. Bacterial infection involved in necroptosis.

Bacteria	Affected host cells	Mechanisms
S. Typhimurium	Macrophages	Type I interferon signaling
Mycobacteria/ tuberculosis	Macrophages	ROS, TNF-RIP1-RIP3
S. marcescens, Staphylococcus aureus, Streptococcus pneumoniae, Listeria monocytogenes, pneumolysin, and uropathogenic *Escherichia coli* (UPEC)	Macrophages	RIP1-RIP3-MLKL, Pore-forming toxin
Y. pestis	Macrophages	RIP3
enteropathogenic *Escherichia coli* EPEC	Intestinal cells	EspL cleave the RHIM domains of RIPK1-RIPK3 complex, TRIF, and DAI

Table 4. Common inhibitors of necroptosis.

Necroptosis inhibitors	Targeted molecules
Nec-1/Nec-1s/Nec-3/Nec-4/Nec-5, GSK2982772, PN10, Cpd27, RIC, and 6E11 /GSK963, GSK2606414 and GSK2656157/Sibiriline	RIP1
GSK'843, GSK'840, GSK'872, dabrafenib, and ponatinib	RIP3
NSA, TC13172, and GW806742X	MLKL
kongensin A, 17-AAG, and GA	HSP90

2016; Galluzzi et al., 2017; Li et al., 2017), while in this review, we prefer to discuss the possible therapeutic drugs for diseases involved in necroptosis.

Anti-tumor drugs that target necroptosis

It is well known that drug resistant apoptosis is a problem in tumor treatment, and there are two methods to resolve the problem. One is to target the molecular machine that triggers necroptosis (Garg and Agostinis, 2017; Krysko et al., 2017). Caspase-8 inactivation sensitizes cancer cells to necroptosis and leads to RIPK3-induced necroptotic cell death (Gunther et al., 2011; Oberst et al., 2011). This finding could be very relevant for cancer treatment depending on the expression of the necroptotic core machinery, that is, RIPK3 and MLKL, such as the normal anti-tumor drugs, including interferon-ϰ (Balachandran and Adams, 2013; Deng et al., 2013), 5-fluorouracil (Grassilli et al., 2013; Su et al., 2014; Oliver Metzig et al., 2016) and Sorafenib (Petrini et al., 2012; Locatelli et al., 2014; Kharaziha et al., 2015; Ramirez-Labrada et al., 2015; Feldmann et al., 2017; Martens et al., 2017). These drugs kill tumor cells by increasing the rate of necroptosis. Another way to solve this problem is to target the immunogenicity of necroptosis, due to the release of DAMPs and cytokines by necroptotic cells. These factors may activate CD8+ T cells to recognize and kill tumor cells, and there is a study which points out that injecting necroptotic cells can help inhibit the development of tumors (Aaes et al., 2016). RIP3 and MLKL are important contributors to tumor immunogenicity and provide a hint into seeking more efficient methods to treat cancers (Yang et al., 2016).

Natural compounds for treatment of diseases by inhibiting or triggering necroptosis

Natural compounds that inhibit necroptosis

Early in 2014, our group found that the Timosaponin-BII extracted from the Chinese traditional herb *Rhizoma anemarrhenae* plays a role in protecting RGC-5 from hydrogen peroxide induced necroptosis, and the mechanism may be associated with the reduction of TNF-α (Jiang et al., 2014). And in 2016, a study involved extracting KA from croton, and discovered the function of KA on protection of cells from necroptosis. The target of KA turned out to be HSP90, a chaperon molecule which can maintain and stabilize the function of RIP1, RIP3, and MLKL (Li et al., 2016a). Recently a study also discovered that patchouli alcohol (PA) decreased dextran sulfate sodium (DSS)-induced cell death by down-regulating RIP3 and MLKL proteins as well as the inflammation (Qu et al., 2017). Another natural product, aucubin from *Eucommia ulmoides*, can ameliorate damage in lithium-pilocarpine induced status epilepticus (SE) in the hippocampus by reducing necroptotic neurons and inhibiting the expression of MLKL and RIP-1 in the hippocampus (Wang et al., 2017b). Baicalin, a flavonoid compound isolated from the dried roots of *Scutellaria baicalensis Georgi*, a plant-derived flavonoid (Gao et al., 1999), is the first recognized to be protective to neurons induced by ischemia injury, by blocking the phosphorylated CaMKII (Tu et al., 2009; Wang et al., 2016). Wogonin, a herbal active compound, can protect Acute kidney injury (AKI) by effectively inhibiting RIPK1 by occupying the ATP-binding pocket, which is a key regulator of necroptosis. In addition, RIPK1/RIPK3 inhibition reverses the protection of wogonin which indicates that wogonin works depending on RIPK1/RIPK3 (Meng et al., 2017). Additionally, baicalin is reported to alleviate oxidative stress and necroptotic cells by inhibiting the expression of RIP1/3 and the production of reactive

Table 5. Natural compounds inhibiting necroptosis.

Natural inhibitor of necroptosis	Targeted molecules
Timosaponin-BII,	TNF-α
Patchouli alcohol	RIP3 and MLKL
Kongensin A	HSP90
Aucubin	RIP1 and MLKL
Baicalin	CaMKII, RIP1/3
Wogonin	RIP1

Table 6. Natural triggers of necroptosis in tumor cells.

Natural triggers of necroptosis	Targeted molecules	Targeted cells
Shikonin	RIPK1 and RIPK3	MCF-7 and HEK293/osteosarcoma, glioma cells/U93/glioblastoma cells/MDA-MB-468/nasopharyngeal carcinoma cell line CNE-2Z/non-small cell lung cancer cells
Neoalbaconol	RIPK3-mediated ROS overproduction but also NF-κB-dependent expression of TNF-α	Human nasopharyngeal carcinoma C666-1 and HK1 cells
Tanshinone IIA	RIP1/RIP3	HepG2 cells
Piperlongumine	RIP1	Cancer stem cells
Eupomatenoid-5	ROS	Kidney cancer 786-0 cells
Papaya pectin	AGII	Cancer cells
Parthenolide	ROS, RIP1	Leukemia cells, human breast cancer MDA-MB231 cells

oxygen species in bEnd.3 cells induced by OGD (Luo et al., 2017), as shown in Table 5.

Natural compounds that trigger necroptosis in tumors

Early in 2007, shikonin, a naphthoquinone derived from *Lithospermum erythrorhizon* Siebold & Zucc. (Zicao), was initially found to induce necroptosis of MCF-7 and HEK293, which was characterized by morphology of necrotic cell death, loss of plasma membrane integrity, mitochondrial membrane potentials and elevation of ROS instead of apoptosis (Han et al., 2007). Most importantly, the function can be reversed by pretreating with Nec-1 (Han et al., 2007). Subsequently, studies found that shikonin can induce necroptosis in many kinds of cancer cells and act as an anti-cancer drug, for example in treating osteosarcoma (Fu et al., 2013), glioma cells (Huang et al., 2013a; Zhou et al., 2017), U937 (Piao et al., 2013), glioblastoma cells (Zhao et al., 2015b), MDA-MB-468 (Shahsavari et al., 2016), the nasopharyngeal carcinoma cell line CNE-2Z (Zhang et al., 2017c), and non-small cell lung cancer cells (Kim et al., 2017) by increasing the expression of RIPK1 and RIPK3. A series of studies showed that another natural compound, Neoalbaconol (NA), a constituent extracted from *Albatrellus* confluens can cause necroptotic death of cancer cells, which is identified by necrotic cell morphology, necrosome formation, and the up-regulation of RIP1/3 and protection by Nec-1 (Deng et al., 2013). In addition, it was also found that NA induced necroptosis, not only by RIPK3-mediated ROS overproduction but also through NF-κB-dependent expression of TNF-α in human nasopharyngeal carcinoma C666-1 and HK1 cells (Yu et al., 2015). Tanshinone IIA, a component of the traditional medicinal plant *Salvia miltiorrhiza bunge*, can cause both apoptosis and necroptosis in human hepatocellular carcinoma (HepG2 cells). The combination of Z-VAD-fmk turns apoptosis to necroptosis by inhibiting the activity of caspase 8 and promoting the formation of the RIP1/RIP3 necrosome complex (Lin et al., 2016). Piperlongumine, a natural product constituent of the fruit of the *Piper longum* and Taurolidine which is derived from the natural product taurine, can inhibit tumor cell growth both *in vitro* and *in vivo* by inducing apoptosis, necroptosis and autophagy of tumor cells, and even cancer stem cells. And the Nec-1 can block the part of necroptosis induced by Piperlongumine and Taurolidine (Mohler et al., 2014). Eupomatenoid-5 (Eup-5), a neolignan isolated from *Piper regnellii* (Miq.) C. DC. var. regnellii leaves, can induce necroptosis in kidney cancer 786-0 cells (Longato et al., 2015). Parthenolide can also induce rapid necrosis in leukemia cells by the interaction of parthenolide with the plasma membrane (Alpay et al., 2016). Also, parthenolide-mediated necrosis in human breast cancer MDA-MB231 cells can be reduced by Nec-1, by activating the production of ROS and RIPK1 (Carlisi et al., 2015). Pectin extracted from intermediate phases of *papaya* ripening can decrease cell viability and induce necroptosis in cancer cell lines. The possible reason is that papaya pectin extracted from the third day after harvesting disrupts the interaction between cancer cells and the extracellular matrix proteins, and enhances apoptosis/necroptosis. The anticancer function of papaya pectin depends on the presence of arabinogalactan type II (AGII) structure (Prado et al., 2017), as shown in Table 6.

Perspectives

The mechanism, related diseases and drugs that target necroptosis are reviewed in this paper. Necroptosis acts in more and more important roles in the development and treatment of diseases. Therefore, it was our goal to summarize the updated knowledge of necroptosis to inspire additional research. Although the mechanism of necroptosis is well studied, its application still needs to improve, especially in approaches to drug combinations for apoptosis resistant cancer drugs. Additionally, the immunity of necroptotic cells for treating tumors is another significant point for treating diseases. Apart from tumor treatment, the inhibitors of necroptosis for clinical drugs will require additional research.

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