

# The potential role of necroptosis in clinical diseases (Review)

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**Abstract.** As an important type of programmed cell death in addition to apoptosis, necroptosis occurs in a variety of pathophysiological processes, including infections, liver diseases, kidney injury, neurodegenerative diseases, cardiovascular diseases, and human tumors. It can be triggered by a variety of factors, such as tumor necrosis factor receptor and Toll-like receptor families, intracellular DNA and RNA sensors, and interferon, and is mainly mediated by receptor-interacting protein kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like protein. A better understanding of the mechanism of necroptosis may be useful in the development of novel drugs for necroptosis-related diseases. In this review, the focus is on the molecular mechanisms of necroptosis, exploring the role of necroptosis in different pathologies, discussing their potential as a novel therapeutic target for disease therapy, and providing suggestions for further study in this area.

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## 1. Introduction

Necroptosis, an emerging field closely related to apoptosis, is a non-caspase-dependent cell death that has been implicated in the pathological processes of various diseases. It is regulated by various genes that cause regular and ordered cell death. Through activating specific death signaling pathways, it shares typical characteristics of necrosis, including loss of metabolic function and subcellular changes (1,2). Receptor-interacting protein kinase 1 (RIP1) was the first signaling molecule identified in the necrosome (3). RIP1 and RIP3 interact with the receptor protein, transducing death signals, and further recruiting and phosphorylating mixed lineage kinase domain-like protein (MLKL) (4-7). Necroptosis can be involved in the regulation of several signaling pathways, including the caspase-8-dependent apoptotic pathway, the mitogen-activated protein (MAP) kinase cascade, and activation of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway.

To explore the potential role of necroptosis in human diseases, researchers have developed various methods, such as gene knockdown and knockout, and pharmacological inhibitors. By using these methods, it has been found that necroptosis plays an important role in pathophysiological processes of several clinical diseases, including infections, liver diseases, kidney injury, neurodegenerative diseases, cardiovascular diseases, and human tumors (8). In the current review, we aimed to explore the potential role of necroptosis in various clinical diseases.

## 2. Overview of the molecular mechanism of necroptosis

Necroptosis can be triggered by a variety of factors, such as tumor necrosis factor receptor (TNFR) and toll-like receptor (TLR) families, intracellular DNA and RNA sensors, and interferon (IFN) (9-11). TNF-dependent TNFR1 stimulation has three consequences that depend on the assembly of regulatory proteins. These different pathways ultimately stimulated NF- $\kappa$ B-dependent inflammation, caspase-8-dependent apoptosis, or selective activation of necroptosis under caspase-8 inhibition (Fig. 1) (12). TNF-dependent necroptosis is regulated by RIP1 and RIP3, which interact through unique RIP homotypic-interacting motifs (RHIMs) (Fig. 2) (13,14).

The interaction of RIP1 and RIP3 results in autophosphorylation, transphosphorylation, and assembly of 'necrosome' complex (5). RIP3 and MLKL are essential for necroptosis, whereas RIP1 is only sometimes involved in this process.

RIP3 and MLKL knockout mice do not show deficiency in embryogenesis, homeostasis and development, indicating the role of necroptosis may be not essential in non-challenged conditions (15,16).

### 3. Difference of the key characteristics between apoptosis and necroptosis

Although necroptosis is characterized by caspase independence, the molecular pathway involved is similar to and shares features of apoptosis. However, the immunological and morphological consequences of necroptosis are vastly different (Fig. 3). Necroptosis shares the major morphological features of necrosis, such as the swelling of organelles, gradually translucent cytoplasm, and rupture of the cellular membrane (12). By contrast, apoptosis is characterized by membrane blebbing, cell shrinkage, nuclear fragmentation, and chromatin concentration (17). The rupture of the cellular membrane results in the release of cellular contents, leading to the exposure of damage-associated molecular patterns (DAMPs), triggering a strong inflammatory response in necroptosis, suggesting necroptotic cells are more immunogenic than apoptotic cells, which is relatively intact, with DAMP restricted to the plasma membrane, or encapsulated in the apoptotic bodies (17). It has also been shown that necroptosis was associated with maintenance of T-cell homeostasis, as it has been found to be able to clear excess and abnormal T cells in the absence of caspase-8 (18), which can prevent abnormal proliferation of lymphocytes (19).

### 4. Identification of necroptosis

As there is currently no specific marker for necroptosis, multiple methods are usually required to identify necroptosis (Fig. 4). In cultured cells, transmission electron microscopy can be used to identify necroptotic cells (20). Detection of key molecular, including RIP1, RIP3 and MLKL activation, necrosome formation, MLKL oligomerization, and membrane translocation can also be used to identify necroptosis (21). Activation of RIP3 and MLKL can be monitored by western blot analysis to assess phosphorylation status (22,23). Phosphorylation of MLKL at Ser358 and Thr357 and RIP3 at S227 indicates the activation of necroptosis (24). In particular, MLKL phosphorylation has been used as a biomarker for certain disease diagnosis and prognosis (25). In addition, several pharmacological inhibitors such as the necrostatin (Nec)-1, GSK872, and necrosulfonamide (NSA) have also been used to detect necroptosis (7,26). *In vivo*, the activation of necroptosis can be identified by the elevated levels of RIP1, RIP3, or MLKL mRNA or protein. Additionally, previous findings suggested that RIP3 and MLKL are more specific molecular biomarkers than RIP1 for the detection of necroptosis (27).

### 5. Potential role of necroptosis in clinical diseases

**Physiological functions.** Over the last decade, researchers have put a lot of effort into the development of effective RIP1, RIP3, and MLKL inhibitors, and created mouse models that lack one or more components of the necroptotic pathway at systemic level or in specific tissues (28). Due to the existence of these models, the physiological function of proteins of necroptosis

have been investigated. Conditional deletion of RIP1 in keratinocytes or intestinal epithelial cells suggested RIP1 plays an essential role in maintaining epithelial homeostasis (29,30). It is worth noting that the role of RIP1 in maintaining the intestinal barrier is similar to caspase-8 (31). In addition, mice with Birc2, Birc3, and Xiap codeletion in the myeloid lineage have high levels of circulating inflammatory cytokines, sterile inflammation, and granulocytes, which can be partially corrected by the lack of RIP1 or RIP3 (32). Tamoxifen-induced systemic *RIP1* gene knockout in adult mice is fatal due to a surge in cell death and intestinal bone marrow failure, which accumulates and causes fatal systemic inflammation (33,34). Fetal hepatocytes that received tamoxifen-induced RIP1 deletion or *RIP1*<sup>-/-</sup> progenitor cells are unable to repopulate irradiated receptors. This defect can be partially corrected by the concomitant lack of RIP3, indicating that RIP1 plays a key role in the survival of hematopoietic stem and progenitor cells (33,34). Furthermore, systemic inflammation caused by *RIP1*<sup>-/-</sup> can be restricted in *RIP1*<sup>-/-</sup>*RIP3*<sup>-/-</sup>*Casp8*<sup>-/-</sup> hosts (34,35). These hosts show age-related lymphoproliferative disorders similar to those developed by *RIP3*<sup>-/-</sup>*Casp8*<sup>-/-</sup> mice (11,34,36). In addition, compared to control animals, *RIP1*<sup>+/-</sup> mice, mice treated with intravenous siRNA targeting RIP1, and Nec-1-treated mice showed a higher rate of physiological intestinal epithelial cell regeneration in the small intestine (37). In addition, the negative effects of Nec-1 on the regeneration of intestinal epithelial cells are also present in *RIP3*<sup>-/-</sup> mice (37). Findings of those studies suggest that there may be a delicate balance between different cell deaths in maintaining homeostasis in adults.

#### *Infectious diseases*

**Viral infections.** Findings have shown the crucial role of necroptosis in inflammation during viral infection (Table I). The viruses use the host's signaling pathways, such as anti-apoptotic proteins, to enhance infection, thereby increasing its ability to replicate in the host cell. It has been reported that viral encoding protein involving the RHIM domain interacts with RIP1 and RIP3 to inhibit virus-induced cell death (38). Viral inhibitor of RIP activation (vRIA) disrupts the combination of DAI and RIP3, thereby suppressing cytomegalovirus-mediated necroptosis (9). By contrast, human cytomegalovirus differs in protein, which does not disrupt RIP3 binding with DAI; it works via blocking signaling downstream of MLKL (39). Experimental studies in mice lacking RIP3 have shown impaired virus-induced necroptosis and increased susceptibility to viral infections such as vaccinia virus, influenza A virus, and HSV-1 (5,25,38,40) (Fig. 5).

**Bacterial infections.** Necroptosis also plays an important role in the inflammation caused by bacterial infections. Enteropathogenic *E. coli* (EPEC) has been shown to synthesize and secrete large amounts of the immunogenic effector protein NleB1 and modify the arginine residues of the Fas-associated death domain (FADD) and RIP1 death domains to prevent apoptosis and necroptosis (41,42). EPEC lacking NleB1 fails to colonize intestinal epithelial cells, indicating that bacterial necroptosis is a protective mechanism of the organism (41,42). Similarly, the absence of RIP3 sensitizes host cells to *Yersinia*. Moreover, the simultaneous knockout of FADD or caspase-8 could make cells more sensitive (43,44).

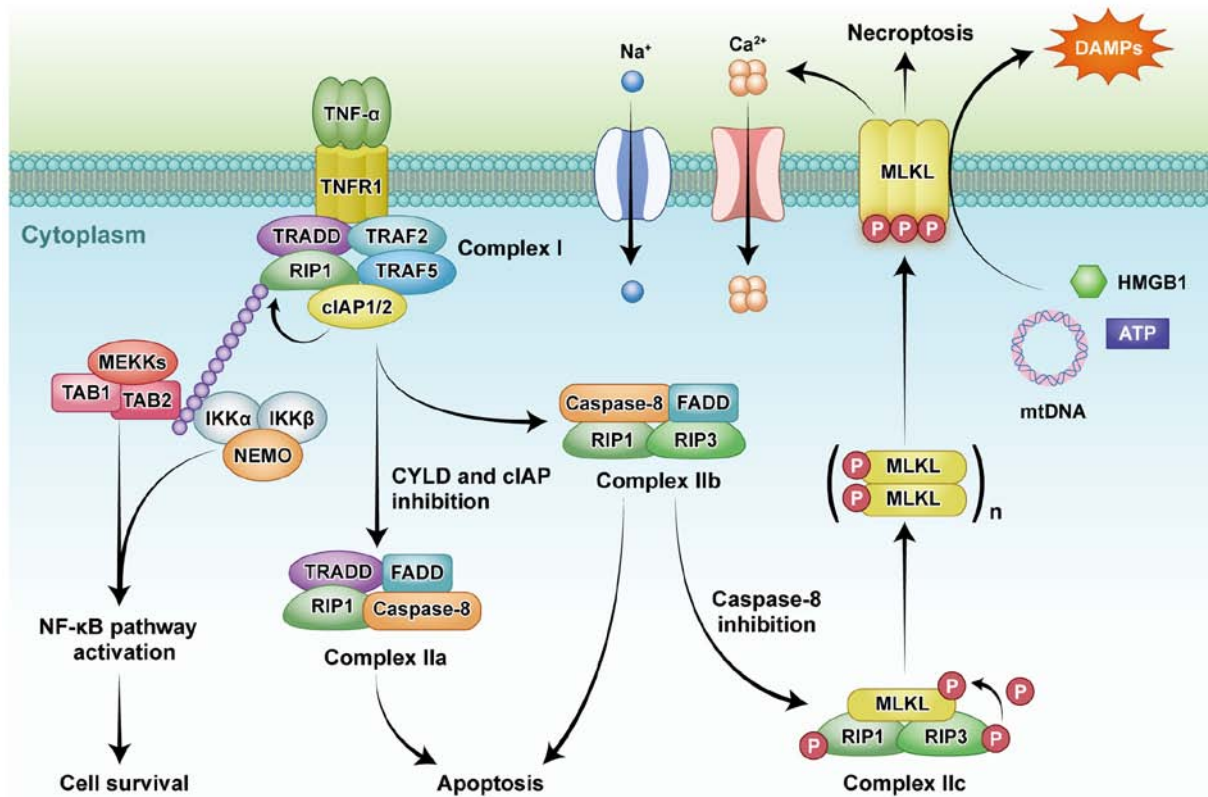


Figure 1. TNFR1-mediated survival and cell death pathways. After TNF binding, TNFR1 recruits TRADD and RIP1 to complex I via their respective death domains. TRADD recruits TRAF2 and cIAP1/2, after which cIAP1/2 ubiquitinate components of complex I. The ubiquitination of RIP1 promotes the formation and activation of the TAK1/TAB complex and the IKK $\alpha$ /IKK $\beta$ /NEMO complex, which induced the NF- $\kappa$ B pathway and cell survival. Deubiquitination of RIP1 by cylindromatosis (CYLD) induces the dissociation of TRADD and RIP1 from TNFR1, which leads to the formation of either complex IIa or complex IIb. FADD and pro-caspase-8 are recruited to TRADD and RIP1 to form complex IIa, resulting in the activation of caspase-8 by oligomerization and cleavage. In the absence of cIAP1/2, TAK1 or IKK complex, complex IIb, which contains RIP1, FADD and pro-caspase-8 except TRADD, is formed and then activates caspase-8, after which caspase-8 induces apoptosis. When caspase-8 activity is blocked, for example by zVAD-fmk, complex IIc/necrosome is formed, and RIP3-dependent necroptosis is induced. In the necrosome, RIP3 phosphorylates MLKL, and translocation of phosphorylated MLKL to the cell membrane leads to direct pore formation with the release of DAMPs. In spite of pore formation, MLKL also mediates its effect after interacting with ion channels.

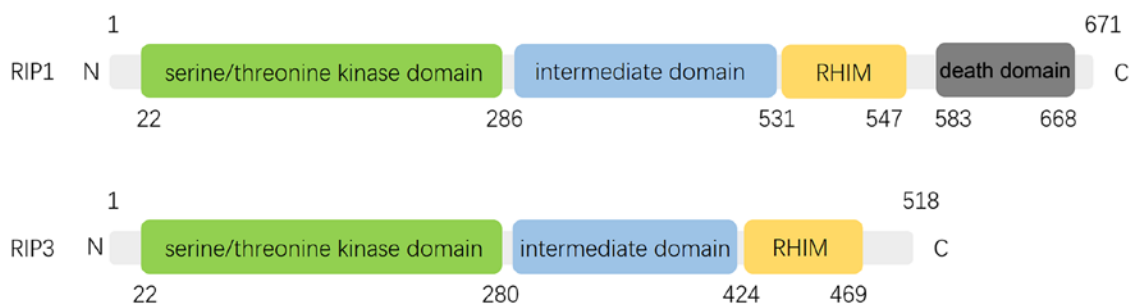


Figure 2. Domain structure of RIP1 and RIP3. The intermediate domain of RIP1 contains the RIP homotypic interaction motif (RHIM) that enables the protein to combine with the RHIM in RIP3 to activate necroptosis. Length is indicated in a number of amino acids.

*In vitro*, *Salmonella typhimurium* is able to escape TNF $\alpha$ , causing RIP1- and RIP3-dependent necroptosis in infected macrophages. In a model of *Salmonella typhimurium* venous infection, RIP3 knockout significantly reduces splenic macrophage death, thereby reducing bacterial numbers and prolonging mouse survival (45,46). Findings focusing on oral *Salmonella typhimurium* infection have also shown that outer protein B was downregulated during infection, which resulted in promoting bacterial translocation, increasing macrophage necroptosis, and exacerbating bacterial infection (Table I) (47).

**Parasite infections.** Parasitic diseases such as malaria and leishmaniasis usually cause hemolysis, anemia, and bleeding. These are due to the release of hemoglobin (Hb) into the circulation by the rupture of red blood cells. When Hb is oxidized, heme is generated, the Fenton reaction starts, and peaks with the generation of reactive oxygen species (ROS). Heme is also involved in the activation of TLR4, causing autocrine secretion of ROS and TNF, and synergistically activating RIP1/3-dependent necroptosis (48). In addition, it has been shown that 10 ng/ml TNF $\alpha$  can induce infected

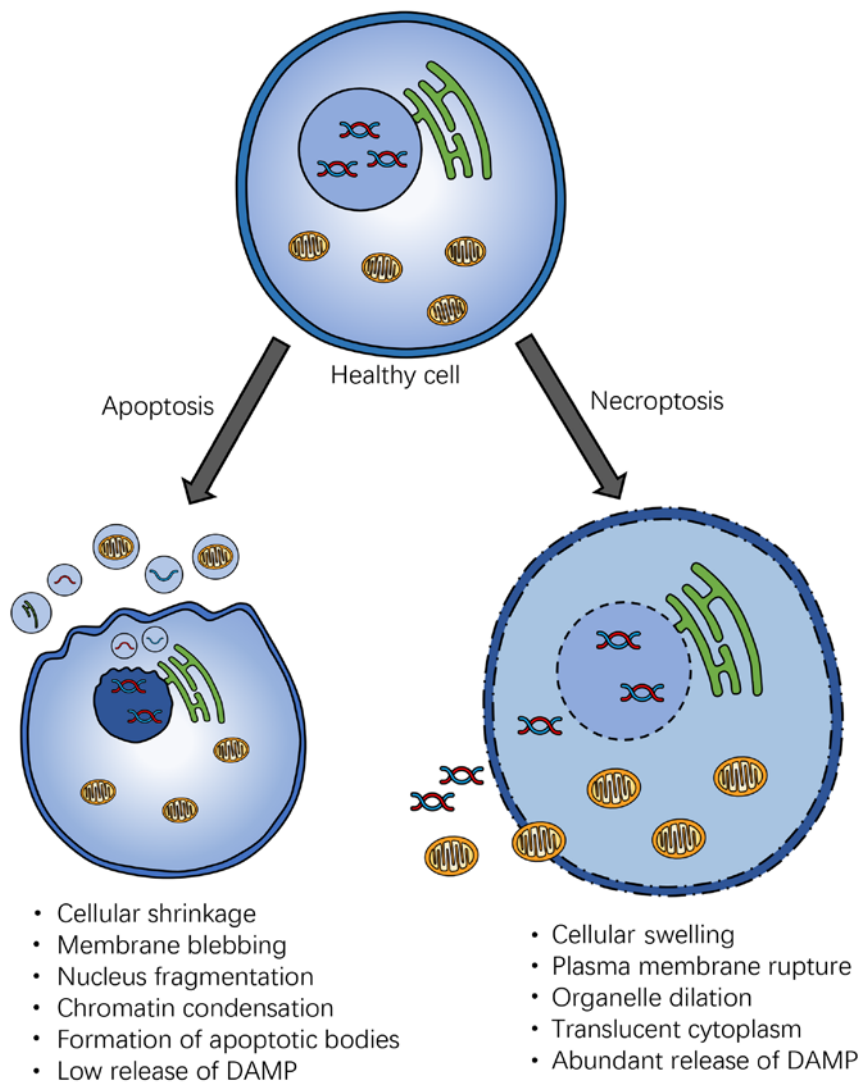


Figure 3. Difference of the key characteristics between apoptosis and necroptosis. Necroptosis is characterized by the swelling of organelles, gradually translucent cytoplasm, and rupture of the cellular membrane. The rupture of the cellular membrane results in the release of cellular contents, leading to the exposure of DAMPs, triggering a strong inflammatory response in necroptosis. Apoptosis is characterized by membrane blebbing, cell shrinkage, nuclear fragmentation, and chromatin concentration, with DAMPs restricted to the plasma membrane, or encapsulated in the apoptotic bodies.

human foreskin fibroblasts egressing *Toxoplasma gondii* (Table I) (49).

#### Cancers

**Type of cancers.** Studies on necroptosis highlight its role in cancer because of its necroptosis-inducing function (Table II) (50). Chen *et al* suggested that necroptosis is an important cell death mechanism for blocked apoptosis, and has been proposed as an alternative cell death procedure to prevent cancer (20). Previous studies have shown that a decreased expression of RIP3 or MLKL is associated with worse prognosis and poor survival in breast cancer (51,52), colorectal cancer (53-55), acute myeloid leukemia (56,57), melanoma (58,59), head and neck squamous cell carcinoma (60), gastric cancer (61), ovarian cancer (62), and cervical squamous cell carcinoma (63). However, increased RIP3 or RIP1 expression was also correlated with cancer development, including glioblastoma (64), lung cancer (65), and pancreatic cancer (66,67). SN38, the topoisomerase inhibitor, was found to be able to promote necroptosis progression, inhibit cell

proliferation, and induce DNA damage accumulation in colon cancer (68). These findings indicate that inhibiting activities of necroptosis components may be a strategy in the treatment of cancers.

**Metastasis.** Metastasis is the most common cause of cancer-related death. Researchers have found that metastasis involves a complex interaction between cancer cells and the microenvironment. By promoting inflammation, necroptosis may be able to promote metastasis (69). It has been shown that TNF $\alpha$  plays a critical role in cancer progression. However, the exact mechanism of this process has not been fully understood. Increased expression of TNF $\alpha$  in cancer is a key characteristic in numerous malignancies and is usually associated with a poor prognosis and decreased survival (69). Consistent with the pro-inflammatory properties of necroptosis and the cancer-promoting effect of inflammation, Nec-1 was able to reduce inflammation and colitis-related tumor formation (70), indicating that targeting necroptosis may be a strategy for preventing cancer metastasis.

Table I. The role of necroptosis in infectious diseases.

Type of infections	Observations		(Refs.)
Viral infections	HSV-1	HSV-1-Induced necroptosis is partially dependent on RIP1, and fully dependent on RIP3 and MLKL	(38)
	Influenza A virus	Mice deficient in RIPK3 is more susceptible to influenza A virus than wild-type counterparts	(40)
	MCMV	RIP3 <sup>-/-</sup> murine embryonic fibroblasts were resistant to MCMV-induced necrosis	(9,156)
	HIV-1	Necrostatin-1 restrains HIV-1-induced cytopathic effect and inhibits the formation of HIV-induced syncytia in CD4 <sup>+</sup> T-cell lines	(157)
	Reovirus	Cell death following reovirus infection was sensitive to inhibition of RIP1	(158)
	Vaccinia virus	RIP1 <sup>-/-</sup> mice cells infected with Vaccinia virus was resistant to TNF- $\alpha$ induced death RIP3 <sup>-/-</sup> mice exhibited severely impaired virus-induced tissue necrosis and inflammation	(159) (5)
Bacterial infections	<i>Clostridium prefringens</i>	RIP1 or RIP3 inhibitors reduced both bacteria-induced apoptosis and necrosis	(160)
	<i>Salmonella typhimurium</i>	Inhibition of the RIP1 or RIP3 prevented the bacteria-induced death of wild-type macrophages Deletion of MLKL rescued severity of bacteria-induced tissue inflammatory	(47) (45)
	<i>M. tuberculosis</i>	RIP1 and RIP3 morpholino knockdown reduced susceptibility of zebrafish to <i>Mycobacterium marinum</i>	(161)
	<i>Yersinia pestis</i>	Deficiency of both RIP3 and caspase-8 completely abrogated <i>Yersinia</i> -induced cell death	(44)
	<i>Staphylococcus aureus</i>	RIP3 <sup>-/-</sup> mice exhibited significantly improved staphylococcal clearance	(162)
	<i>Toxoplasma gondii</i>	Blocking necroptosis by necrostatin-1 has little impact on TNF- $\alpha$ -induced egress of <i>T. gondii</i>	(49)
Parasite infections	Leishmaniasis and Malaria	Inhibition of the RIP1 or RIP3 protected macrophages from heme-induced cell death	(48)

HSV-1, herpes simplex virus type 1; RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein; MCMV, murine cytomegalovirus; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; HIV-1, human immunodeficiency virus type-1.

#### Neurodegenerative diseases

**Parkinson's disease.** Researchers have shown that necroptosis was activated in Parkinson's disease (PD), and may be associated with mitochondrial defects which led to necroptosis (Table III) (71). Compared with healthy brain, the level of necroptosis components, including RIP1, RIP3 and MLKL, was significantly increased in the substantia nigra of PD brain (72). Moreover, researchers have found Nec-1 could protect PC12 cells from death in PD models (73). This suggests that the activation of RIP1 may be a risk factor for dopaminergic neurons lost in PD patients. In addition, leucine-rich repeat kinase 2, which was identified in a systematic RNAi screen, is encoded by a gene that is frequently mutated in PD and is able to promote activation of RIP1 (74).

**Alzheimer's disease.** Alzheimer's disease (AD) is a degenerative brain disease featured by loss of neurons. Previous studies have found that there were activated necroptosis in both human (75) and mouse (76) AD brain. In the AD brain, the

levels of necroptosis components, such as RIP1 and MLKL, were significantly higher than the normal brain (Table III) (75). Treatment of AD in brain of mice with the necroptosis inhibitor can significantly suppress necroptosis and prevent neuronal loss (75). This indicates that targeting necroptosis may be a new therapeutic strategy for AD treatment.

**Amyotrophic lateral sclerosis.** Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that is characterized by loss of motor neurons. In a previous study, the ALS spinal cord was shown to have a significant increase in necroptosis components including RIP1, RIP3, and MLKL in the ALS mouse model compared to healthy mouse spinal cord (77). In addition, loss of optineurin, an ALS-related gene, resulted in susceptibility to necroptosis. Nec-1 inhibition of RIP1 or knockout of RIP3 could prevent demyelination and reduce axonal pathological hallmarks in ALS mouse models (Table III) (77). Those findings suggested that targeting necroptosis may have potential therapeutic value in ALS patients.



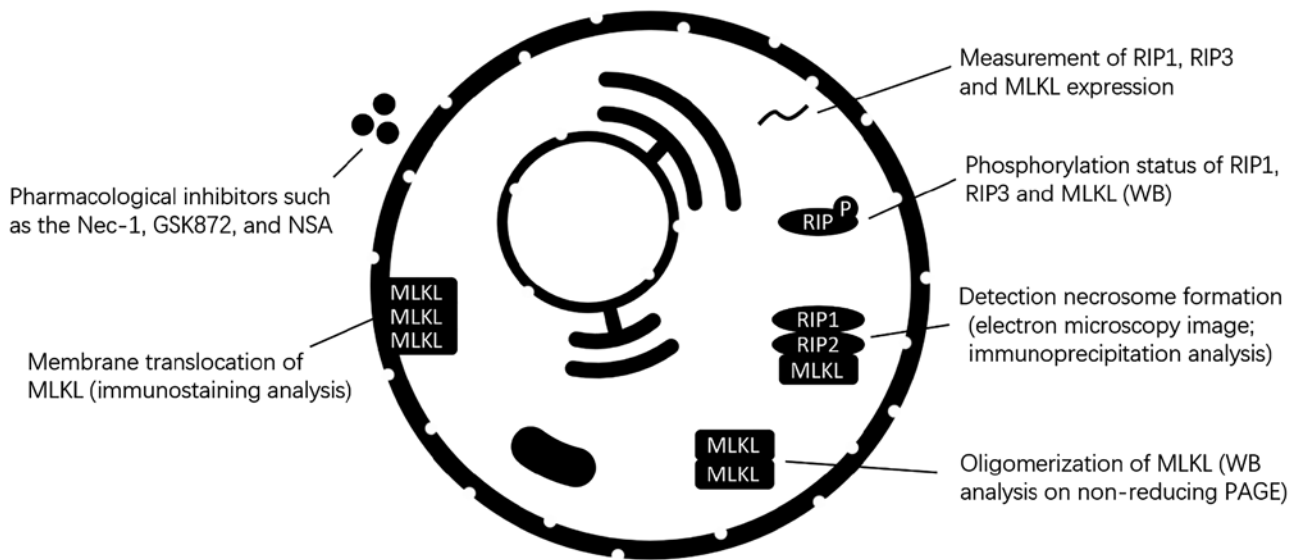


Figure 4. Methods to identify necroptosis. In cultured cells, transmission electron microscopy can be used to identify necroptotic cells. Detection of key molecular, including RIP1, RIP3 and MLKL activation, necrosome formation, MLKL oligomerization, and membrane translocation can also be used to identify necroptosis. Activation of RIP3 and MLKL can be monitored by western blot (WB) analysis to assess phosphorylation status. Several pharmacological inhibitors such as the Nec-1, GSK872, and NSA have also been used to detect necroptosis. *In vivo*, the activation of necroptosis can be identified by the elevated levels of RIP1, RIP3, or MLKL mRNA or protein.

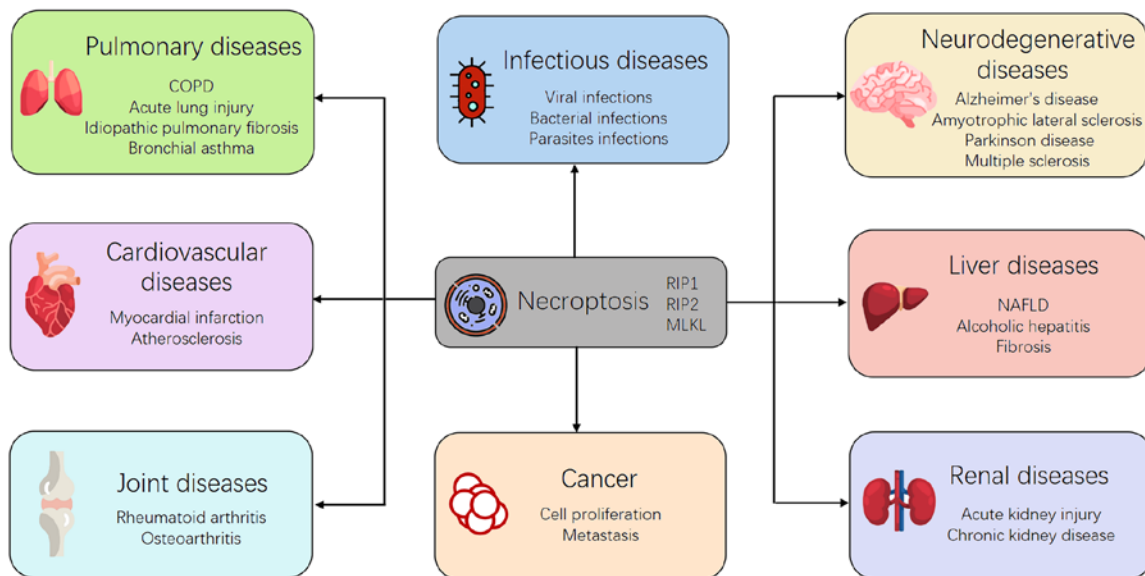


Figure 5. The potential role of necroptosis in clinical diseases. Necroptosis has been implicated in pathophysiological processes of several clinical diseases, including infectious diseases, neurodegenerative diseases, liver diseases, pulmonary diseases, renal diseases, cardiovascular diseases, joint diseases, and human tumors.

**Multiple sclerosis.** Multiple sclerosis (MS) is degenerative disease characterized by oligodendrocyte loss and demyelination. Previous findings have shown a significant increase in necroptosis components including RIP1, RIP3, and MLKL in MS patients. In addition, MLKL oligomers were significantly increased in MS pathology samples compared with controls (Table III) (78). This suggests necroptosis is activated in the pathogenesis of MS. In MS mouse model, oral administration of RIP1 inhibitor can suppress oligodendrocyte degeneration and reduce disease severity (78). Moreover, researchers have shown that inhibition of RIP1 reduced demyelination and disease progression in an MS

model (79). Notably, MLKL was shown to be involved in the MS process (79).

**Liver diseases.** *Non-alcoholic fatty liver disease (NAFLD)*, Non-alcoholic fatty liver disease (NAFLD) is a chronic disease characterized by excess triglyceride accumulation in the liver. Several studies have used different models to assess the effects of necroptosis on NAFLD (Table IV). Studies have found necroptosis components, including RIP1, RIP3 and MLKL, were increased in NAFLD models, as well as in RIP3KO mice (80-85). In addition, an increased expression of RIP3 and MLKL in the human NAFLD was

Table II. The role of necroptosis in cancer.

Cancer type	Observations	(Refs.)
Breast cancer	Decrease of RIP3 expression was associated with worse prognosis	(51,52)
Colorectal cancer	Decrease RIP3 and MLKL expression were associated with decreased overall survival	(53-55)
Gastric cancer	Low MLKL expression was significantly associated with decreased overall survival	(61)
Ovarian cancer	Decrease of MLKL expression was associated with worse prognosis	(62)
Pancreatic cancer	Increase of RIP1, RIP3, FADD and MLKL expression were associated with worse prognosis	(66,67)
Lung cancer	Increased RIP1 expression was associated with worse prognosis	(65)
Acute myeloid leukemia	Decrease of RIP3 expression was associated with worse prognosis	(56,57)
Melanoma	Decrease of RIP3 expression was associated with worse prognosis	(58,59)
Head and neck squamous cell carcinoma	Decrease of RIP1 expression was associated with worse prognosis	(60)
Cervical squamous cell carcinoma	Decrease of MLKL expression was associated with worse prognosis	(63)
Glioblastoma	Increased RIP1 expression was associated with worse prognosis	(64)

RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein; FADD, Fas-associated death domain.

Table III. The role of necroptosis in neurodegenerative diseases.

Neurodegenerative diseases	Observations	(Refs.)
Parkinson's disease	RIP1 inhibition improved survival of optic atrophy 1-mutant human induced pluripotent stem cell-derived neurons <i>in vitro</i> . RIP1 inhibition attenuated MPTP-induced dopaminergic neuronal loss	(72)
Alzheimer's disease	RIP1 inhibition reduced A $\beta$ burden, levels of inflammatory cytokines, and memory deficits in a mouse model of Alzheimer's disease	(76)
Amyotrophic lateral sclerosis	RIP1 inhibition or RIP3 deficiency blocked oligodendrocyte death, microglial inflammation, and axonal degeneration	(77)
Multiple sclerosis	Cortical lesions in human multiple sclerosis brain samples showed increased activation of RIP1, RIP3 and MLKL	(78)
	Inhibition of RIP1 inhibited the progression of demyelination and disease development in a cuprizone-induced model for multiple sclerosis	(79)

RIP, receptor-interacting protein kinase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MLKL, mixed lineage kinase domain-like protein.

identified (83,85). Increased level of RIP3 and p-MLKL was also found in visceral adipose tissue of obese patients. Furthermore, RIP3 expression correlated with p-MLKL and metabolic serum markers including blood insulin levels and Hemoglobin A1c (84).

**Alcoholic hepatitis.** Similar to the pathologies of NAFLD, alcoholic hepatitis is an inflammatory syndrome in liver, which can result in high morbidity and mortality. Several studies have found that RIP3 was increased following ethanol feeding, and RIP3 deletion could protect the liver from ethanol-mediated injury (Table IV). In addition, p-JNK was regulated by RIP3 in a model of alcoholic hepatitis, and RIP3 deletion reduced ethanol-induced p-JNK expression (86). Another study found pharmacological inhibition of proteasome and liver-specific

PSMC1 KO mice could increase RIP3 expression, indicating RIP3 expression was post-translationally regulated in ethanol-mediated liver injury (87). Additionally, when RIP3 was deleted, the steatosis and inflammatory effects of ethanol in hepatocyte could be reduced (86). However, the inflammatory and steatosis effects of high fat diet for hepatocyte were increased when RIP3 was deleted (80).

**Fibrosis.** Hepatic fibrosis is one of the most common liver diseases, which is closely related to liver failure and hepatocellular cancer. RIP3 deletion reportedly aggravated hepatic fibrosis by increasing insulin resistance (80). Furthermore, inhibition of RIP3 did not result in protective effect in carbon tetrachloride (CCL4)-induced fibrosis (83) (Table IV). Additionally, curcumin suppressed serum inflammatory

Table IV. The role of necroptosis in liver diseases.

Liver diseases	Observations	(Refs.)
NAFLD	MLKL deficiency and necrostatin-1 administration improves insulin sensitivity without affecting inflammation	(81)
	RIP3KO mice had increased hepatic steatosis but reduced inflammation	(82)
	MCD diet-fed RIP3KO mice were protected, but CCL4-induced fibrosis model mice were not protected	(83)
	RIP3 maintains WAT homeostasis and has a role in WAT insulin signaling	(84)
	RIP3 deficiency protects from steatosis, inflammation, and fibrosis	(85)
Alcoholic hepatitis	RIP3 expression increased following ethanol feeding	(163)
	RIP3 but not RIP1 inhibition protects from ethanol-induced hepatic injury and steatosis	(86)
	RIP3 ablation and necrostatin-1 decreased hepatic inflammation	(87)
Fibrosis	Curcumin reduced ethanol-induced necroptosis in Nrf/p53-dependent mechanism	(164)
	RIP3KO mice were not protected against steatosis, inflammation and fibrosis	(80)
	RIP3 deficiency protects from steatosis, inflammation, and fibrosis	(85)
	RIP3KO MCD diet-fed mice were protected from inflammation and fibrosis, while CCL4-induced fibrosis was not reduced in RIP3KO mice	(83)
	RIP3 deficiency reduced inflammation, oxidative stress, and fibrosis in 3-day CBD ligation model	(165)
	Melatonin protects from CCL4-induced fibrosis	(166)
	Curcumol-mediated decreased fibrosis is associated with increased necroptosis in hepatic stellate cells	(88)

NAFLD, non-alcoholic fatty liver disease; RIP, receptor-interacting protein kinase; KO, knockout; MCD, methionine and choline-deficient; CCL4, carbon tetrachloride; WAT, white adipose tissue; CBD, common bile duct.

markers, transaminases, and fibrosis in a dose-dependent manner by inducing necroptosis of hepatic stellate cells in a liver fibrosis model (88). Curcumol-induced increased necroptosis was mediated by increased expression of p-RIP3 and p-JNK (88). Those results indicated that pharmacotherapy which induced increased necroptosis may be a notable strategy for the treatment of hepatic fibrosis in the future.

#### *Pulmonary diseases*

**Chronic obstructive pulmonary disease.** Chronic obstructive pulmonary disease (COPD) is characterized by persistent and progressive airway inflammation and narrowing, and is a major source of the high healthcare expenditure in the elderly (89). An increasing number of studies have shown that necroptosis is associated with the etiology of COPD (Table V). In addition, necroptosis of epithelial cell is associated with COPD (90). Cigarette smoking (CS)-related necroptosis and DAMP release could cause neutrophil inflammation in mice, and Nec-1 could reduce the inflammation (91). In addition, researchers have found that in airway epithelial cells, endoplasmic reticulum chaperone protein GRP78 could promote CS-induced inflammation. This may be due to the upregulation of necroptosis and subsequent activation of the NF- $\kappa$ B pathway (92).

**Acute lung injury.** Acute lung injury (ALI) is one of the most common complications in critically ill patients (93). Recent findings have shown the involvement of RIP3-mediated necroptosis in neonatal mice with hypoxia-induced lung injury, which can be attenuated by gene deletions in RIP3 (94). In additional, inhibition of RIP3 could significantly reduce inflammatory activation

and lipopolysaccharide-induced necroptosis (95). Researchers have also found that mice lacking RIP3 were protected from ventilator-induced lung injury (96). Additionally, inhibition of RIP1 can reduce systemic and pulmonary inflammation and increase survival rate of septic neonatal mice (Table V) (97).

**Idiopathic pulmonary fibrosis.** Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, fibrotic lung disease characterized by the usual interstitial pneumonia pattern at histopathologic examination (98). In a previous study using alveolar epithelial cells, RIP3-mediated necroptosis was associated with IPF development by releasing DAMP (99). Additionally, RIP3 and p-MLKL levels in the lungs of IPF patients are significantly higher than those in healthy lungs (99). Mice with RIP3 knockout showed a reduced cell death, with a decrease of p-MLKL level in alveolar epithelial cells (99). RIP3 knockout could effectively suppress the DAMP releasing, cell death, and pulmonary fibrosis without reducing the expression of cleaved caspase-3 (Table V) (99). These indicate that inhibiting activities of necroptosis components may be a strategy in the treatment of IPF.

**Bronchial asthma.** Bronchial asthma is the most common chronic respiratory disease characterized by bronchial hyper-responsiveness and airway obstruction (100). Viral-induced bronchial asthma exacerbation mimicked by IFN- $\beta$  knockout mice treated with house dust mite is associated with increased necroptosis components, including p-MLKL and LDH in the bronchoalveolar lavage fluid (101). As a major inflammatory cytokine in bronchial asthma, IL-33



Table V. The role of necroptosis in pulmonary diseases.

Pulmonary diseases	Observations	(Refs.)
COPD	CS-induced necroptosis and the release of DAMPs trigger neutrophilic inflammation in mice that was reduced with Nec-1 treatment	(91)
Acute lung injury	RIP3-mediated necroptosis is observed in neonatal mice with HALI, which is attenuated by genetic deletion in RIP3	(94)
	RIP3-deficient mice are protected against ventilator-induced lung injury	(96)
Idiopathic pulmonary fibrosis	RIP3 and p-MLKL expression levels are significantly higher in the lungs of IPF patients than in healthy control lungs	(99)
	Bleomycin-treated AECs isolated from RIP3 knockout mice show attenuation of cell death with decreased p-MLKL expression	
Bronchial asthma	GW806742X can abrogates IL-33 reaction <i>in vitro</i> and attenuates eosinophilia in a mouse model of asthma	(102)

COPD, chronic obstructive pulmonary disease; CS, cigarette smoking; DAMPs, damage-associated molecular patterns; RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein; HALI, hypoxia-induced lung injury; IPF, idiopathic pulmonary fibrosis; AECs, alveolar epithelial cells; IL-33, interleukin 33.

Table VI. The role of necroptosis in renal diseases.

Renal diseases	Observations	(Refs.)
Acute kidney injury	RIP3 <sup>-/-</sup> and MLKL <sup>-/-</sup> mice are less sensitive to oxalate crystal-induced and cisplatin-driven AKI than are their wild-type counterparts	(107,110)
	The RIP3 <sup>-/-</sup> genotype confers considerable protection against mild IRI, and such a protection can be extended to severe IRI by the concomitant deletion of Ppif	(104)
Chronic kidney disease	RIP1 and RIP3 participate in the loss of renal cells of subtotal nephrectomized rats	(112)
	Gene deletion of RIP3 or MLKL ameliorated renal tubular cell necroptosis, and then finally reduced interstitial fibrogenesis in the long term after IRI	(113)

RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein; AKI, acute kidney injury; IRI, ischemia-reperfusion injury.

is released in response to necroptosis and causes eosinophil and basophil activation (102). Moreover, in a mouse model of asthma induced by *Aspergillus fumigatus* extract, the necroptosis inhibitor GW806742X can eliminate necroptosis and IL-33 response, and attenuates eosinophilia (102). Additionally, The TNF $\alpha$ -induced necroptosis enhanced by mucin 1 can be reduced by Nec-1 in human bronchial epithelial cells (103) (Table V).

#### Renal diseases

**Acute kidney injury.** Acute kidney injury (AKI) is a common and severe clinical disease that often requires renal replacement therapy (Table VI). Signs of an ongoing necroptotic response have been found in AKI caused by ischemia-reperfusion injury (IRI) (104-106), urolithiasis (107), cisplatin-based chemotherapy or radiocontrast (104,108-111). Previous findings have shown that compared with wild-type counterparts, RIP3<sup>-/-</sup> and MLKL<sup>-/-</sup> mice are less sensitive to oxalate crystal-induced AKI, and are associated with reduced plasma creatinine levels, neutrophil infiltration, and limited tubular injury (107,110). Moreover, the RIP3<sup>-/-</sup> mice confer protection from mild IRI, and

the protection can be extended to severe IRIs with the deletion of Ppif (104). Similar results are also found when Nec-1, SfA, and 16-86 are employed alone or in combination (104,109). The abovementioned results suggest that inhibition of necroptosis may be a therapeutic option for AKI treatment.

**Chronic kidney disease.** Similar to the AKI, necroptosis was also found in chronic kidney disease (CKD) after unilateral nephrectomy (Table VI) (112). Researchers have shown that necroptosis and the highest levels of RIP1 and RIP3 occurred 8 weeks after subtotal nephrectomy (112). Notably, the renal pathological changes and renal function could be significantly improved after Nec-1 treatment, and the overexpression of RIP1, RIP3, MLKL could be significantly reduced (112). These results suggest that necroptosis contributes to the loss of renal cells in subtotal nephrectomized rats. Furthermore, during the AKI to CKD process, upregulation of expression and interaction between RIP3 and MLKL can induce necroptosis in proximal renal tubular cells and promote inflammasome activation under IRI conditions (113). RIP3 or MLKL knockout could protect the renal tubular cells from necroptosis and

Table VII. The role of necroptosis in cardiovascular diseases.

Cardiovascular diseases	Observations	(Refs.)
Myocardial infarction	RIP3 deficiency protects mouse hearts from IR-induced necroptosis and significantly reduces infarct size	(22)
Atherosclerosis	Necrostatin-1 prevents both short and long-term effects of myocardial ischemia Ox-LDL deposited in the endothelium can upregulate the expression of RIP3 and ox-LDL-related genes, resulting in the necroptosis of macrophages	(118)

RIP, receptor-interacting protein kinase; IR, ischemia-reperfusion; ox-LDL, oxidized-low-density lipoprotein.

Table VIII. The role of necroptosis in joint diseases.

Joint diseases	Observations	(Refs.)
Rheumatoid arthritis	RIP1, RIP3 and MLKL were potently increased in the synovium of a collagen-induced RA mouse model	(120)
	RIP1 inhibitor significantly decreased the expression of RIP1, RIP3 and MLKL and suppressed the expression of IL-17, IL-1 $\beta$ , IL-6 and TNF $\alpha$ in a RA mouse model	(121)
Osteoarthritis	Gene expression levels of RIP3 and MLKL were elevated in highly degenerated cartilage tissue	(123)
	Trauma induced cell death and subsequent release of pro-inflammatory mediators could be largely attenuated by necrostatin-1 or N-acetylcysteine	

RA, rheumatoid arthritis; RIP, receptor-interacting protein kinase; MLKL, mixed lineage kinase domain-like protein; IL, interleukin; TNF $\alpha$ , tumor necrosis factor- $\alpha$ .

inflammasome activation, which prevent kidney from interstitial fibrogenesis after IRI (113).

#### Cardiovascular diseases

**Myocardial infarction.** Myocardial infarction, characterized by regional myocardial ischemia and hypoxia, is one of the leading causes of death worldwide (114). Previous findings have shown that compared to wild-type mice, the levels of RIP1 and RIP3 were significantly higher in hearts of ischemic mice (22,115). In an acute IRI mouse model, RIP3 deficiency was able to protect heart from IRI-induced necroptosis and reduce the infarct size (116). Notably, researchers have found that Nec-1 could protect heart against short-term and long-term effects of myocardial ischemia, including reduced necrotic cell death and size of myocardial infarction, which helped to maintain long-term cardiac function (22) (Table VII).

**Atherosclerosis.** Atherosclerosis, a chronic inflammatory disease, is frequently observed in middle-aged individuals and the elderly, and is a major cause of cardiovascular death (117). It has been demonstrated that necroptosis may promote the inflammatory response and atherosclerosis development. Oxidized low-density lipoprotein (LDL) is able to upregulate RIP3 and oxidized LDL-related gene expression in macrophages, leading to macrophage necroptosis (118). It triggers an inflammatory response, which leads to atherosclerosis. During the progression of the disease, some cytokines are released and monocytes accumulate in the lesion, exacerbating the accumulation of inflammation. Additionally, necroptosis can lead to the death of foam cells, which in turn aggravates the progression of the disease (Table VII) (118). These results

suggest that inhibition of necroptosis may be a therapeutic option for atherosclerosis treatment.

#### Joint diseases

**Rheumatoid arthritis.** Rheumatoid arthritis (RA), characterized by synovial membrane inflammation, is a chronic systemic inflammatory autoimmune disease that affects 0.5-1% of the population worldwide (119). Previous findings have shown a significant increase in necroptosis components including RIP1, RIP3, and MLKL in the synovium of an arthritis mouse model (120). Additionally, researchers have also found in an arthritis mouse model, Nec-1 could significantly reduce these key components of necroptosis and IL-17, IL-1 $\beta$ , IL-6, and TNF $\alpha$  (Table VIII) (121). These results suggested that inhibiting activities of necroptosis components may be a strategy in the treatment of RA.

**Osteoarthritis.** Osteoarthritis (OA) is the leading cause of pain and disability among chronic disease, which affects about 10% of men and 18% of women older than 60 years (122). A significant increase in necroptosis components including RIP3, and MLKL was found in highly degenerated cartilage tissue (123). Moreover, it has been shown that Nec-1 could significantly reduce cell death and subsequent release proinflammatory mediators in the OA model (Table VIII) (123).

### 6. Drugs and agents that regulate necroptosis

As necroptosis not only participate in the maintenance of organismal homeostasis, but also constitute etiological determinants of diverse human pathologies (124), at least two

Table IX. Drugs and agents that regulate necroptosis.

Drugs and agents	Target	Disease condition	(Refs.)
Drugs and agents that induce necroptosis			
Interferons	RIP3 and MLKL	Different diseases	(127)
Valproic acid	RIP1	Epilepsy and mood disorders	(128)
Decitabine and 5-azacytidine	RIP3	Breast cancer	(51)
Shikonin	RIP1 and RIP3	Pancreatic and non-small cell lung cancers, osteosarcoma	(129)
Emodin	RIP1, RIP3 and MLKL	Renal cancer	(130)
Bufalin	RIP1 and RIP3	Pancreatic and breast cancers	(131)
Resibufogenin	RIP3 and MLKL	Pancreatic and colorectal cancers	(132)
Radiotherapy	Caspase-8	Different cancers	(147)
5-fluorouracil	RIP1 and RIP3	Different cancers	(148)
Cisplatin	RIP1, RIP3 and MLKL	Different cancers	(149)
Anthracyclines and oxaliplatin	RIP3 and MLKL	Lung cancer	(150)
Obatoclox	RIP1, RIP3 and MLKL	Different cancers	(151)
Neoalbacinol	RIP1 and RIP3	Nasopharyngeal carcinoma	(152)
Tanshinone	RIP1 and RIP3	Hepatocellular carcinoma	(153)
Drugs and agents that inhibit necroptosis			
Cyclosporine A	RIP1 and RIP3	Immunosuppressive drug	(133)
Rapamycin	RIP1	Restenosis in coronary arteries, transplant rejection in lymphangiomyomatosis, and retinal detachment	(134)
Patchouli alcohol	RIP3 and MLKL	Colitis	(139)
Pazopanib	RIP1	Renal cell carcinoma and advanced soft tissue sarcoma	(140)
Ponatinib	RIP1 and RIP3	Leukemia	(140)
GSK2982772	RIP1	Inflammatory diseases (colitis, rheumatoid arthritis, psoriasis)	(167)
GSK3145095	RIP1	Pancreatic cancer	(141)
Dabrafenib	RIP3	Melanoma	(88)
Carfilzomib	RIP3 and MLKL	Multiple myeloma	(142)
Sorafenib	RIP1 and RIP3	Thyroid and renal cell cancers, hepatocellular carcinoma	(143)
Phenytoin	RIP1	Epilepsy and breast cancer	(144)
Aucubin	RIP1 and MLKL	Epilepsy	(145)
Wogonin	RIP1	Acute kidney injury	(146)

therapeutic paradigms can be envisioned: i) the activation of necroptosis, as a means to bypass the accrued resistance of most tumors to apoptosis (125); ii) the inhibition of necroptosis, as a strategy to limit the loss of post-mitotic cells in pathologies such as inflammatory, ischemic, and toxic syndromes (126). Therefore, drugs affecting either the expression or the activity of necroptosis mediators may have therapeutic potential (Table IX).

Several drugs have been found to upregulate the expression of the key molecules of necroptosis, including interferons (127), histone deacetylase inhibitor valproic acid (128), and hypomethylating agents such as decitabine and 5-azacytidine (51). Additionally, several traditional Chinese medicine drugs such as shikonin (129), emodin (130), bufalin (131), and resibufogenin (132) were also found to upregulate RIP1 and RIP3, which finally induced necroptosis. By contrast, various drugs have been documented to downregulate necroptosis, including immunosuppressive drug Cyclosporine A (133) and

Rapamycin (134), inhibitors of the HSP90 [(G-TPP) (135), Kongensin A (136), 17-demethoxy-reblastatin (137), DHQ3 (137), gamitrinib (18), and geldanamycin (138)], as well as traditional Chinese medicine such as patchouli alcohol (139).

Promising specific inhibitors are also being developed for the central molecules of necroptosis. Currently, several drugs with anti-necroptotic activity have been used for the treatment of different types of cancer [Pazopanib (140), Ponatinib (140), GSK3145095 (141), Dabrafenib (26), Carfilzomib (142), and Sorafenib (143)]. Moreover, a clinically used anti-convulsant, Phenytoin (144) as well as components found in different plants [aucubin (145) and wogonin (146)] could inhibit RIPK1 activity. By contrast, radiotherapy (147), chemotherapeutic agents such as 5-fluorouracil (148), cisplatin (149), oxaliplatin (150), and anthracyclines (150), pan-BCL-2 inhibitor Obatoclox (151), or traditional Chinese medicines such as neoalbacinol (152) and tanshinone (153) have been documented to upregulate

necroptosis. Although these drugs did not affect the expression of necroptotic component, these medicines may increase the effect of the drugs affecting the expression of the necroptotic molecules in combination therapy in cancer cells.

## 7. Conclusions and perspectives

During the last decade, necroptosis has been recognized as an alternative to apoptosis when cells are exposed to various stimuli under specific conditions (154). The necrosome components, RIP1, RIP3, and MLKL, are critical regulators of necroptotic cell death. RIP1 functions as a traffic cop for mechanisms of cell death. MLKL acts as the executioner of necroptosis, based on the phosphorylation, oligomerization and membrane translocation (155). Current understandings demonstrated a pathway in which RIP3 activation, possibly mediated by RIP1, induces MLKL activation, and finally results in permeabilization of the plasma membrane and cell death.

Recent studies have revealed a complex role for necroptosis in diverse clinical diseases, such as ischemia-reperfusion injury, neurological and inflammatory diseases, retinal disorders, acute kidney injury or bacterial infections. On the one hand, it functioned as a cell-death mechanism activated by various signal transduction cascades in the same cell or the same tissue; on the other hand, it acted as an inflammation inducer through the release of DAMPs. Cross-regulation between necroptosis and other modes of cell death increase the complexity of these pathways. The major necroptosis-regulating proteins exert pleiotropic signaling functions that culminate in necroptotic cell death and have cell death-independent functions, such as regulation of inflammasome activation, mitochondrial function and integrity, and cellular metabolic activities (96).

As necroptosis constitutes etiological determinants of multiple human pathologies, targeting the necroptotic pathway is a potential therapeutic approach for multiple diseases, and several activators or inhibitors of the necroptosis pathway have been developed, such as dabrafenib, pazopanib, and ponatinib. These small-molecule activators or inhibitors of necroptosis may be useful as therapeutics in a specific clinical disease. However, most studies investigating the therapeutics targeting necroptosis are based on *in vitro* experiments or animal models, thus the feasibility of the clinical use of these compounds and agents remains to be assessed *in vivo* and clinical trials. Additionally, the off-target effects of the necroptosis-targeting therapeutics should be scrutinized, and novel approaches that conjugate necroptosis inducers and disease-guiding agents should be developed to enhance selectivity and safety.

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## Availability of data and materials

Not applicable.

## Authors' contributions

YFA and WLD conceived the study; JC and XQH were involved in data curation; XL, WLD and JC were involved in collection of references as well as writing and editing; YFA, XQH and JC supervised the study. All authors have read and agreed to the published version of the manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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