The pathogenesis in organ fibrosis: focus on necroptosis

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Abstract

Fibrosis is a common process of tissue repair response to multiple injuries in all chronic progressive diseases, which featured with excessive deposition of extracellular matrix. Actually fibrosis can occur in all organs and tends to be nonreversible with the progresses of the diseases. Different cells types in different organs are involved in the occurrence and development of fibrosis, i.e. hepatic stellate cell, pancreatic stellate cell, fibroblasts, myofibroblasts. Present studies have shown that several programmed cell deaths including apoptosis, autophagy, ferroptosis, and necroptosis were closely related to organ fibrosis. Among these programmed cell deathes type, necroptosis, an emerging regulated cell death type were regard as a huge potential target to ameliorate organ fibrosis. In this review, we summarized the role of necroptosis signaling in organ fibrosis, and collected the present small molecule compounds targeting necroptosis. In addition, we have discussed the potential challenges, opportunities and open questions in using necroptosis signaling as a potential target for antifibrotic therapies.

1. Introduction

Fibrotic diseases with high morbidity and mortality all over the world (Klinkhammer, Floege and Boor 2018, Henderson, Rieder and Wynn 2020) are characterized by excessive deposition of extracellular matrix (ECM) (Distler et al. 2019). It can potentially affect all organs and tissues in the body including multisystem diseases such as systemic sclerosis as well as individual organs containing pulmonary, kidney, hepatic, cardiac and bladder fibrosis(shown in Fig 1) (Rosenbloom, Ren and Macarak 2016). At present, there are few effective drugs for the direct treatment of fibrosis in clinic. A large number of diseases related to fibrosis urgently need specific drugs to improve the survival status of patients.

Fibrosis is the common outcome of tissue repair response which tends to be dysregulated following kinds of trigger and tissue injury, most significantly during chronic inflammatory diseases (Henderson et al. 2020). The excessive accumulation of ECM components including collagen and fibronectin, is a common and significant stage of tissue repair process in all organs. When tissues are damaged, local tissue fibroblasts tend to activate and occurred a series of changes including enhance their contractility, secrete inflammatory mediators, and excessive generated of ECM components, which sponsor the wound-healing response. When the injury reduced or vanished, the wound-healing response tend to be effective, exhibiting in only a short-lived enhance of the accumulation of ECM components and promoting the renewal of functional tissue structure. In contrast, when the injury persistent existence, the wound-healing response transformed into microenvironmental changes including ECM generating cells raise in amount or become immoderately active, which eventually leads to the formation of substantial scar formation and injury of normal organ structure (Pakshir and Hinz 2018).

In the different organs, a good deal of clinical research and animal experiments' evidence has obviously built the strong connection of cell death with fibrosis (Weiskirchen, Weiskirchen and Tacke 2019). Over the last decade, it has become definite that there are many forms of cell death, and diverse forms of cell death can be triggered in diseases, initiating different replies and biological results (Sauler, Bazan and Lee 2019). Regulated cell death (RCD) is a primary approach for human body to cleanup injured, infected, or surplus cells. In the past, apoptosis was considered the unique form of RCD. However, recent studies have discovered several new modes of RCD including necroptosis, pyrolysis, ferroptosis and autophagic cell death (Stockwell et al. 2017, Vande Walle and Lamkanfi 2016, Choi et al. 2019, Tang et al. 2020). With the in-depth understanding of the RCD mechanism, more and more evidence indicated that these RCD mechanisms are closely related to organ fibrosis (Shojaie, Iorga and Dara 2020, Schwabe and Luedde 2018). By straightly obstructing the mediators that regulate each mode of RCD or promote the mediators that response to certain types of RCD may bring us new opportunities to treat fibrotic diseases. The latest research shows that apoptosis and necroptosis emerge higher research value in the process of fibrosis, compared with pyrolysis, ferroptosis and autophagy(Schwabe and Luedde 2018). In addition, compared with apoptosis, necroptosis can cause cellular contents to flow out, further trigger a large amount of inflammation, accelerate the process of fibrosis, and may lead to the irreversibility of fibrosis. Therefore, the aim of this review is to summarize our understanding of the necroptosis in organ fibrosis, so as to provide certain research ideas for the therapy of organ fibrosis or the development of new drugs.

2. An overview of Necroptosis.

2.1 Necroptosis signal pathway

Cellula demise is a key factor in keeping homeostasis in organisms, and it is also a way to remove impaired, infected or degraded cells (Vaux and Korsmeyer 1999). In an adult, approximately ten to one hundred billion cells die per day and are substituted by new healthy cells to keep the homeostasis of the entire body (Glucksmann 1951). Therefore, it is obvious that when the balance between cell death and cell proliferation is disrupted, various abnormal reactions and diseases of the body will be caused (Renehan, Booth and Potten 2001). In the past, apoptosis has always been regarded as the only form of RCD, until the first genetic determinant of death receptor-induced necroptosis, receptor-interacting serine/threonine-protein kinase 1 (RIPK1), was found (Holler et al. 2000). Whereas the term of necroptosis used to express this nonapoptotic RCD form was created until 2005 (Degterev et al. 2005). Apoptosis is a caspase-dependent RCD which characterized by cell membrane blistering, cell contraction, nuclear fracturing, chromosome concentration and chromosomal DNA fragmentation(Kerr, Wyllie and Currie 1972). Different from apoptosis, necroptosis is a type caspase-independent RCD form which characterized by increased cell membrane permeability, plasmalemma ruptures, general swelling of cytoplasm and organelles, and overflow of cell components into the microenvironment (Galluzzi et al. 2017) (show in Fig 2). Present Studies have shown that necroptosis is mainly mediated by RIPK1, RIPK3, and mixed lineage kinase-like protein (MLKL) (Frank and Vince 2019).

2.2 Core components of Necroptosis

2.2.1 RIPK1

RIPKs are a seven-member family with the common characteristic of a homologous serine-threonine kinase domain. The family includes RIPK1-RIPK7. RIPK1 was first identified by Stranger et al. in 1995 as a death domain (DD)-including protein coactions with the DD of the Fas receptor, which is capable of inducing apoptosis (Stanger et al. 1995). RIPK1 include an amino-terminal kinase domain, a carboxy-terminal DD and a bridging intermediate domain (ID) that contains a RIP homotypic interaction motif (RHIM) (He and Wang 2018). RIPK1 can connect with several innate immune receptors containing tumor necrosis factor receptors (TNFRs), interferon alpha/beta receptor 1 (IFNAR1), toll-like receptors (TLRs), stimulator of interferon genes protein, mitochondrial antiviral-signaling protein and others to exert important value in innate immune regulation (Vanden Berghe, Hassannia and Vandenabeele 2016). RIPK1 has related to multiple signal reaction containing transcription and translation of inflammatory genes (Najjar et al. 2016, Muendlein et al. 2020), apoptosis, necroptosis and pyroptosis(Degterev et al. 2008, Amin et al. 2018,

Sarhan et al. 2018, Wegner, Saleh and Degterev 2017).

2.2.2 RIPK3

Compared with RIPK1,RIPK3 bears a carboxy-terminal region that harbors a RHIM but lacks a DD (He and Wang 2018). In addition, RIPK3 has the similar kinase-dependent function with RIPK1 including necroptosis, pro-inflammatory gene expression and sustained translation (Cho et al. 2009, Zhang et al. 2009, Newton et al. 2014). Activation of RIPK3 is a vital procedure in the enablement of necroptosis. The activated RIPK1 interact with RIPK3 to form a heterodimeric amyloid structure named necrosome complex by their respective RHIM domains (Li et al. 2012). This process can phosphorylate RIPK3 at Ser227 for human RIPK3 or at Thr231 and Ser232 for mouse RIPK3 and leading to activation of RIPK3. Caspase-8 can cleave RIPK3 at Asp328, leading to its inactivation of kinase-dependent activities (Feng et al. 2007). In addition, RIPK3 kinase triggers necroptosis through a pseudo-kinase MLKL.

2.2.3 MLKL

In the human genome, exceed five hundred protein kinases have been found and authenticated, in which about 10% of the protein kinases seems have no enzyme activity and having been classified as pseudokinases (Manning et al. 2002). MLKL, as one of pseudokinase, is made up of a C-terminal pseudokinase domain, a two-helix brace or linker, and an N-terminal four-helix bundle (4HB) (Murphy et al. 2013). It was originally recognized as a RIP3-binding protein via its C-terminal kinase-like domain. It is reported that phosphorylation of MLKL can cause the change of the pseudokinase domain conformation, and resulting in exposure of the 4HB domain (Petrie et al. 2018). The phosphorylation of MLKL activated by RIPK3 is a symbol of necroptosis. Recruitment of MLKL relies on auto-phosphorylation of RIPK3 at Ser227 for human RIPK3 or Ser232 for mouse RIPK3 (Sun et al. 2012b). The late formation of micro pores with an approximately 4 nm diameter is a key process in necroptosis (Ros et al. 2017). Evidences have shown that activated MLKL can form membrane destruction pores through the interaction between its N-terminus and phospholipids, leading to membrane leakage. This concept expands researchers' understanding of morphological changes following necroptosis occurs in vivo (Zhang et al. 2016a).

2.3necroptosis, a developmental signaling pathway

In general, necroptotic signal pathway could be activated by several stimuli covering ambient pressure, variety of chemotherapy medicines, mechanical damage, inflammation, and infection et al (Lalaoui et al. 2015). Current studies show that lipopolysaccharide (LPS) can promote necroptosis through TLRs (Kim and Li 2013). The necroptosis-promoting activities of type I IFN sectionally depends on TRIF and derives from the continuous provoke of signal transducer and activator of transcription 1 (STAT1), STAT2, and interferon regulatory factor 9 (IRF9) (McComb et al. 2014a). IFNAR1 and interferon gamma receptor 1 (IFNGR1) can also induce necroptosis in macrophages (Robinson et al. 2012, Thapa et al. 2013). Among these stimulus factors, TNFR superfamily was regarded as the most intensively studied (Grootjans, Vanden Berghe and Vandenabeele 2017). Therefore, the activation of the necroptotic signaling pathway can be summarized by the events triggered by TNF-α/TNFR. When the organism is subjected by various external stimuli, the tissue microenvironment will release lots of inflammatory factors including TNF-α. Then, the TNF-α combined with TNFR1 induces comformational change of TNFR1 trimers, which further recruit variety of proteins, covering RIPK1, tumor necrosis factor receptor type 1-associated death domain (TRADD), cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2) and TRAF5, forming complex I (Moriwaki, Balaji and Ka-Ming Chan 2020). Particularly worth mentioning is the protein of RIPK1 in complex I, which is a powerful cytokine regulatory factor determines the life and death of cell. RIPK1 can polyubiquitinated by cIAP1/2, which induce classical nuclear factor kappa-B signaling pathway and promote cell survival (Gong et al. 2019). When the continuous activation of nuclear factor NF-kappa-B (NF-ϰB) is blocked, the apoptotic pathway is tending to be activated. Therefore, RIPK1, caspase-8, TRADD and FAS-associated death domain protein (FADD) recruit each other to forming Complex II and activating caspase-8 (Hitomi et al. 2008). Then, the activated caspase-8 can start apoptosis-promoting caspase activation cascade and finally contribute to the occurrence of cell apoptosis (Wu, Liu and Li 2012). When caspase-8 is inhibited

due to certain physiological changes or external stimuli, the cell death mode will converted from apoptosis to necroptosis. At this time, RIPK1 is activated by phosphorylated, which result from the serine residue 161(S161) autophosphorylation at its N-termini (Degterev et al. 2008). The active RIPK1 will interact with RIPK3 to cause its phosphorylation and forming a necrosome complex (Bedoui, Herold and Strasser 2020). In addition, RIPK3 can also be triggered by TLR through a process of TIR domain-containing adapter molecule 1 (TRIF) inducing interferon-β. Active RIPK3 phosphorylates its well-featured functional substrate MLKL pseudokinase. Then, MLKL oligomerizes and transfers to the plasmalemma to trigger necroptosis and destroy the integrity of the plasmalemma by forming micro pores. The resulting inrush of water and sodium and potassium outflow cause cell swelling, destruction of membrane potential, and ultimately cell death characterized by loss of cell and organelle integrity (Sun et al. 2012a). Another study showed that Zα domains of ZBP1 can sense endogenous Z-form nucleic acids to activite RIPK3-dependent necroptosis (Jiao et al. 2020). At present, the downstream target of MLKL is still unclear. Research has shown that phosphorylated RIPK3 activate MLKL to form a homotrimer by its amino-terminal coiled-coil domain and locates to the cell plasmalemma, further mediate transient receptor potential melastatin related 7 induce Ca(2+) influx (Cai et al. 2014). Another research demonstrated that activated MLKL outcomes in the producing of broken, plasmalemma "bubbles" with uncovered phosphatidylserine that are liberated from the outside of the otherwise intact cell. The ESCRT-III machinery is required for forming these bubbles and acts to maintain survival of the cell when MLKL activation is limited or reversed (Gong et al. 2017).

(Show in Fig 3).

2.4 Necroptosis related signaling pathway

The different kinds of RCD can be regard as an individual, concerted cell death system, in which the single pathways are closely interrelated and mutual coordinate for each other. These interaction describes a complicated molecular signal network can be consider as a homeostatic mechanism for the organism (Galluzzi et al. 2017) (show in Fig 4). Therefore, it is not difficult to understand that necroptosis signal pathway is closely related with several other forms of RCD containing apoptosis, autophagy and ferroptosis. It has mentioned above, casepase 8 is seems like a key switch between apoptosis and necroptosis, due to its cleaving capacity of RIPK1 and RIPK3. In addition, apoptosis and necroptosis share the upstream TNFR pathway, mainly including FADD, cellular FLICE-inhibitory protein (cFLIP), caspase 8, cylindroma protein (CYLD) and various inhibitor of apoptosis protein (IAP) family members that interact with TNFR1(Oberst et al. 2011, Dillon et al. 2012). Moreover, caspase 6 is the downstream regulator of apoptosis and cathepsins can leading to lysosomal injury by accelerates permeation of mitochondrial outer membrane (Galluzzi, Bravo-San Pedro and Kroemer 2014). Both caspase 6 and cathepsins can proteolytic inactivate RIPK1 to exert necroptosis-inhibitory effects (van Raam et al. 2013, McComb et al. 2014b). Researches have shown that, in transformed cells, autophagy can promote necroptosis by ROS accumulation (Chen et al. 2011). In addition, autophagy-related protein 16-1 can interdicts necroptosis in the intestinal epithelium (Matsuzawa-Ishimoto et al. 2017). Moreover, it is reported that glutathione peroxidase 4, the primary endogenous inhibitor of ferroptosis, can mediates powerful necroptosis inhibition effects in erythroid precursor cells (Canli et al. 2016). Besides different forms of RCD mentioned above, necroptosis also intimately connected with inflammation, oxidative stress and many other physiopathological processes (Galluzzi et al. 2017). Ca(2+)-calmodulin-dependent protein kinase is a RIPK3 substrate mediating ischemia- and oxidative stress-induced myocardial necroptosis (Zhang et al. 2016b). Recent literature indicated that necroptosis is a primary mechanism of retinal pigment epithelial (RPE) cell death in response to oxidative stress (Hanus, Anderson and Wang 2015). It is well known that reactive oxygen species (ROS) can be regarded as a driving force of necroptosis. ROS can trigger RIPK1 autophosphorylation on S161 by modify three crucial cysteine residues which is necessary for RIP3 recruitment into necrosome complex of necroptosis (Zhang et al. 2017). In addition, studies show that TNFα-mediated necroptosis accelerates increased transcription of inflammatory cytokine gene by a cell-autonomous mechanism involving NF-ϰB and p38 (Zhu et al. 2018a). In a word, it has been demonstrated that necroptosis participate in cell death in diverse disease conditions, including organ fibrosis, viral infection, acute kidney injury, and cardiac I/R (Galluzzi et al. 2017, Schwabe and Luedde 2018). In this review, we aim to focus on the relationship between the pathological process of necroptosis and

tissue fibrosis, as well as potential drug targets of necroptosis for the clinical treatment of fibrotic diseases.

3. Roles of necroptosis in tissue fibrosis

Fibrosis represents excessive scar formation which occurs when the normal wound healing response exceeds the normal tolerance of the tissue in many organs. Both clinical manifestation and experimental study on animal models indicated that fibrosis is a two-way and reversible process (Weiskirchen et al. 2019). At present, the principles to reverse fibrosis mainly include changeover the chronic tissue damage and phlogosis, the demise of myofibroblasts and fibrolysis of excess matrix scaffold. The in-depth comprehension of the molecular mechanisms of various tissue fibrosis is the prerequisite for finding effective biomarkers or and novel antifibrotic treatment options.

3.1 Necroptosis in Cardiac fibrosis

Cardiac fibrosis is featured by excessive accumulation of ECM proteins in the myocardium, distorts the architecture of the myocardium, and contributes to arrhythmia and cardiac dysfunction in many cardiac pathophysiologic situations involing myocardial infarct, I/R injury and cardiac failure (Li, Zhao and Kong 2018). Recent research shows that necroptosis plays an important role in these cardiac diseases. Literatures show that targeting necroptosis by RIPK3 gene knockout in mice can reduce susceptibility of myocardial failure triggered by I/R or doxorubicin (a cardiotoxic chemotherapeutic agent) (Zhang et al. 2016b, Luedde et al. 2014). Experiments with isolated cardiomyocytes found that activated RIPK3 in myocardium seems to trigger a CaMKII-reliant signal pathway, outcomes of mitochondrial permeability transition and consequent necrosis (Zhang et al. 2016b). Another research found that *S. pneumoniae* can intrudes the myocardium and generates cardiac damage with necroptosis and apoptosis, in a nonhuman primate model of severe pneumonia. Once cardiomyocytes die, heart tissue is replaced with myofibroblasts that produce an ECM that is rich in collagen and leads to scar formation (Souders, Bowers and Baudino 2009). Necrostatin-1 (Nec-1), a small molecule inhibitor targeting RIPK1 of necroptosis, can keep off infaust heart remodeling after myocardial I/R in vivo. This protective effect of Nec-1 on heart features the value of necroptosis in myocardial ischemia disease (Oerlemans et al. 2012). Besides RIPK1, RIPK3 also shows an important regulatory role in myocardial injury. Experiment study shows that the obviously increased expression of RIPK3 in mice hearts suffered to I/R injury was positively correlated to the infarct area enlargement, cardiac insufficiency and expansive cardiomyocytes necroptosis. Further mechanistic studies indicated that gene knockout of RIPK3 eliminated the endoplasmic reticulum stress and prevent the ([Ca2+]c) overload-XO-ROS-mPTP pathways, outcomes to a pro-survival state by inhibition of cardiomyocytes necroptosis in cardiac IR injury (Zhu et al. 2018b). Another interesting research found that conditional gene knockout of COP9 signalosome complex subunit 8 in mice cardiomyocytes appears plenty of myocardial cell necroptosis followed by acute heart failure and premature death. Cardiac Cops8/COP9 signalosome obstacle shows RIPK1-RIPK3 dependent in myocardial cell in mice, Thus, researchers speculated that COP9 signalosome plays an important part in restraining myocardial cell necroptosis (Xiao et al. 2020). The above research shows that Inhibition of necroptosis of cardiomyocytes may improve cardiac fibrosis and further to protect heart function.

3.2 Necroptosis in Liver fibrosis

As a basic biological process, cell demise controls the results and long term sequelae in nearly whole liver disease. Acute hepatic failure is featured by mass mortality of hepatic parenchymal cell, and is generally followed by restitution previous condition. However, cell demise in chronic liver disease (CLD) usually happens at lesser degree but it can induce to long-term transforms in tissue architecture and function, leading to chronic hepatic cell renewal, immunocyte enlistment and activation of hepatic stellate cells (HSCs), giving rise to the progress of hepatic fibrosis, liver cirrhosis and liver cancer. Animal experiment indicate that RIPK3 has lower expression in heathy mice liver compared with other organs (Luedde et al. 2014), but has higher expression in cells that are activated to go through necroptosis (Vucur et al. 2013). RIPK3-deficient mouse reveal descended hepatocyte death after acetaminophen poisoning and longtime ethyl alcohol feeding, indicating an participation of necroptosis in liver injury (Ramachandran et al. 2013). Nonalcoholic steatohepatitis (NASH) characterized by hepatocyte steatosis, inflammation, hepatocyte cell death and often

fibrosis (Diehl and Day 2017). Evidences have shown that the effect of apoptosis as the unique cell demise form in NASH seems to be overrated, and necroptosis might be the fundamental factor in the process of NASH. It is reported that Nec-1s, another necroptosis inhibitor, can reverse the high expression of P-MLKL, MLKL, RIPK3 and P-RIPK3 protein levels in the livers of Sod1KO mice (Mice deficient in the antioxidant enzyme Cu/Zn-superoxide dismutase) which indicated that inflammation induced by necroptosis conduces to fibrosis in a mice model of increased oxidative stress and accelerated aging (Mohammed et al. 2021). Evidences show that inflammation and hepatic fibrosis by a RIPK3 mediates signal pathway restrained by Caspase-8 in human NASH and a steatohepatitis mice model. Further experiment study manifested that the activation of JNK mediated by RIPK3 contribute to the liberate of pro-inflammatory factors such as MCP-1, and appealing macrophages to the injured liver and further expansion RIPK3-mediate signal pathway, cell death, and hepatic fibrosis. According to the above results, there is enough reason to believe that RIPK3-dependent necroptosis play an important role in hepatic fibrosis which induced by NASH (Gautheron et al. 2014). In addition, instead of improvement effect, suppression of caspase 8 significantly increased liver injury and fibrosis in the (methionine- and choline-deficient) MCD diet-induced mouse model(Gautheron et al. 2014), showing that coincide with its assumption role in development-a dominating character of caspase 8 in NASH is to prevent excessive -activation of necroptosis. By comparison, suppress RIPK3 in the MCD NASH model can improve liver injury and fibrosis (Gautheron et al. 2014), which indicating that necroptosis, rather than apoptosis, is the driving force of liver injury and fibrosis in this well-established mouse model of NASH. Besides with these discoveries in mice, a Western blot assay of frozen hepatic tissue from a group of patients with biopsy-proven NASH indicated that these patients had lower levels of intrahepatic caspase 3 cleavages but higher hepatic RIPK3 expression than healthy persons (Gautheron et al. 2014), showing a convert from apoptosis to necroptosis in the livers of these patients. Moreover, researchers found that RIPK3 knockout mice were also protected in a model of alcoholic hepatic injury (Roychowdhury et al. 2013), further demonstrated a remarkable function of necroptosis as a metabolic cell death pathway in the hepatic tissue. These studies illustrated that besides apoptosis, necroptosis is a critical siginal pathway in human metabolic liver disease. Targeting necroptosis may provide a specific treatment method for human metabolic liver disease. However, further clinical studies are needed to evaluate which parts of the pathway can be used as targets for the treatment of chronic liver disease.

3.3 Necroptosis in kidney fibrosis

Fibrosis, characterized by loss of capillary networks, excessive deposition of fibrillary collagens, activated myofibroblasts and inflammatory cells (Zeisberg et al. 2003, Humphreys et al. 2010), is the eventual common pathway of chronic kidney disease (CKD) (Brosius et al. 2009). Present studies have shown that necroptosis signal responses are widely exist in various acute and chronic kidney diseases caused by different reasons including I/R (Linkermann et al. 2013a, Linkermann et al. 2012), cisplatin-based chemotherapy or radiocontrast (Linkermann et al. 2013b, Xu et al. 2015) and unilateral nephrectomy(Zhu et al. 2015). Evidence show that RIPK1and RIPK3 deficiency in kidney I/R injury can improve systemic inflammation associated with A20 deficiency or high-dose TNF model, which is consistent with necroptosis-independent functions for RIPK1 and RIPK3 (Newton et al. 2016). Ying Shi et al found that RIPK3 can mediate renal fibrogenesis by the domain-like receptor family pyrin domain-containing 3 (NLRP3) inflammasome. Diabetic RIPK3$^{-/-}$ mice and RIPK3 inhibitor dabrafenib can obviously reduce collagen deposition and myofibroblast activation in kidney (Shi et al. 2020b). Moreover, increased expression and interactions between RIPK3 and MLKL induced necroptosis of renal proximal tubular cells under the conditions of renal I/R injury. Gene deletion of RIPK3 or MLKL improved renal tubular cell necroptosis, macrophage infiltration and NLRP3 inflammasome activation with a reduction in caspase-1 activation and maturation of IL-1β, and then eventually decreased interstitial fibrogenesis in the long term after IRI. These studies indicate that necroinflammation activate by RIPK3-MLKL-dependent necroptosis plays an important role in the progression of IRI to CKD (Chen et al. 2018a). In clinical studies, researchers also found that in human oxalate crystal-related AKI, dying tubular cells stain positive for p-MLKL. Deficiency of RIPK3 or MLKL prevents oxalate crystal-induced AKI. The inhibitor of human MLKL (Nec-1 and necrosulfonamide) can suppress crystal-induced cell death in human renal progenitor cells (Mulay et al. 2016). The above studies indicate that inhibit the key proteins

of necroptosis may be a potential target for the treatment of acute and chronic kidney disease.

3.4 Necroptosis in pulmonary fibrosis

Idiopathic pulmonary fibrosis(IPF) is a chronic, progressive, and fibrotic lung disease which characterized by multiple genetic and environmental risk factors that cause micro-damage to the aging alveolar epithelial cells, in turn, leads to abnormal communication of epithelial-fibroblast, induces excessive deposition of ECM which produced by myofibroblasts and lung interstitial remodeling, ultimately leads to the destruction of alveolar structure, reduced lung compliance, interruption of gas exchange, until respiratory failure and death (Richeldi, Collard and Jones 2017). Evidences show that RIPK3 and p-MLKL levels in the lungs of IPF patients are obviously higher than those in healthy lungs. RIPK3 deficient mice could efficiently restrain the (damage associated molecular pattern) DAMP releasing, cell demise, and lung fibrosis which manifests that core components of necroptosis may be a specific target in the treatment of IPF (Lee et al. 2018). Acute lung injury (ALI), one of the most common complications in severe patients (Rubenfeld et al. 2005), is results in early inflammation, respiratory distress, and later fibrosis (Cui et al. 2019). Mice experiment indicate that RIP3-mediated necroptosis in hypoxia-induced lung injury neonatal mice, which can be improved by knockout of RIPK3 (Syed et al. 2019). Moreover, significant increases of necroptosis components were observed in the lungs of ALI mice induced by lipopolysaccharide (LPS). GSK872, a RIPK3 inhibitor can obviously restrained the activation of necroptosis and amelioration of lung injury (Chen et al. 2018b). In patients requiring ventilator support, mechanical ventilation may induce ALI (ventilator-induced lung injury, VILI). Interestingly, in clinical studies, researchers discovered that RIPK3 was increased in patients with mechanical ventilation. Moreover, RIPK3 deficiency mice were protected from VILI (Siempos et al. 2018). Present studies indicate that necroptosis have an important role in ALI and lung fibrosis.

Inhibit the key proteins of necroptosis may improve acute and chronic lung diseases.

3.5 Necroptosis in pancreatitis fibrosis

Acute pancreatitis (AP), characterized by acinar cell necroptosis and phlogosis responses, can progress into chronic pancreatitis accompanied with fibrosis. Recent study discovered that the main components of necroptosis , RIPK1 and RIPK3 exert dual function in AP. RIPK3 through phosphorylating MLKL promote acinar cell necroptosis, but RIPK1 maybe inhibit acinar cell necroptosis by activating the NF-ϰB signaling pathway in AP animal model (Wu et al. 2017). Another experiment found that RIP3 knockout can avoid tissue damage which induced by inflammation and further improve AP in an AP animal model (He et al. 2009, Zhang et al. 2009). Moreover, MLKL deficiency can improve the severity of cerulean-induced AP in mice (Wu et al. 2013). Chronic pancreatitis (CP) is defined as a pathological fibro-inflammatory syndrome which characterized by acinar cell injury and stress responses, duct dysfunction, persistent or altered inflammation (Kleeff et al. 2017). Pancreatic fibrosis is a significant characteristic of chronic pancreatitis, which contributes to sustaining and eternal injury in the pancreas. Pancreatic stellate cells serves as a primary source of ECM deposition in the period of pancreatic injury, and sustaining activation of pancreatic stellate cells plays an important part in the process of pancreatic fibrosis (Xue et al. 2015). Experiment with Atg7 deficiency Mice indicate that RIPK3, the core protein involved in necroptosis, can attenuates the chronic pancreatitis induced by deletion of ATG7 (Zhou et al. 2017). In short, current studies show that necroptosis plays an important role in acute and chronic pancreatitis and its fibrosis, the core proteins may be a potential target for clinical treatment of pancreatic diseases, but the specific mechanism remains to be further studied.

3.6 Necroptosis in other fibrosis

Duchenne muscular dystrophy (DMD), a severe degenerative disease triggered by dystrophin gene mutations,is traited with progressive myofibre necrosis. Researchers found that RIPK1, RIPK3 and MLKL are increased in dystrophic mouse myofibres which indicated that necroptosis maybe the potential mechanism of myofibre death in DMD (Morgan et al. 2018). Atherosclerosis is a lipoprotein-driven disease that results in plaque formation at particular locations of the arterial tree through intimal inflammation, fibrosis, and calcification (Bentzon et al. 2014). RIPK3 and MLKL are increased in humans with unstable carotid atherosclerosis, meanwhile, phosphorylated MLKL is detected in advanced atheromas. Inhibition of macrophage

necroptosis by Nec-1 can reduce lesion size of atherosclerotic plaques in Apoe (-/-) mice (Karunakaran et al. 2016). Targeting necroptosis may be effective strategies for novel drug discovery for atherosclerosis (Coornaert et al. 2018). Long term inflammatory bowel diseases (IBD) can trigger intestinal fibrosis (Rieder, Fiocchi and Rogler 2017). Previous literature reported that RIPK3, MLKL were up-regulation in children with IBD, which indicated that necroptosis is closely related with intestinal inflammation in children with IBD and contributes to strengthen the inflammatory process. Therefore, researchers speculate that RIP3 and MLKL can be the represent powerful targets for the treatment of human IBD (Pierdomenico et al. 2014).

4. Therapeutic potential of targeting necroptosis in therapies of organ fibrosis

For a long time, fibrosis in multiple organs/tissues seriously affects human physical and mental health. Despite diversified treatment strategies have been attempted to suppress and treat fibrosis, the therapeutic effects still poor (Hu et al. 2020, Han et al. 2021). As a consequence, finding effective therapeutic drugs for treating organ fibrosis is an urgent matter to be solved. In view of the vital function of necroptosis, this review collected the present molecules that aimed at necroptosis and could be considered as promising therapy in treating organ fibrosis. Here, the natural and/or synthetic necroptosis inhibitors for the prevention and treatment of organ fibrosis were highlighted in Table 1. Norberto et al reported that RIPK1 inhibitors Necrostatin-1s, -1, -5, or -7 all can reverse the necroptosis of macrophage. However, Necrostatin-1s, and -1 were demonstrated unsuitable for application in humans due to further drawbacks such as short half-life in vivo and poor metabolic stability (Berger et al. 2015). In addition, RIPK3 inhibitor GSK'872 can completely blocked mouse alveolar macrophages death following *S . marcescens* infection (Gonzalez-Juarbe et al. 2015). Dabrafenib is a noted inhibitor of B-Raf, which has been approved for clinical use for melanoma and thyroid cancers expressing B-Raf V600E mutations (Salama et al. 2020). Interestingly, recent studies found that dabrafenib is also a RIPK3 inhibitor, which has been proved in multiple models including human hepatocytes, hepatic injury mice models, and ischemic brain injury (Shi et al. 2020a). Primidone, a FDA-approved aromatic antiepileptic drug was found also an effective inhibitor of RIPK1 in vitro and in a murine model of TNFα-induced shock (Riebeling et al. 2021). So far, new drugs targeting key proteins of necroptosis are mainly inhibitors, and agonists haven't been found yet. In total, the above studies have shown that inhibiting necroptosis has broad development prospects for the treatment of various diseases including organ fibrosis. Given that necroptosis pathway is an effective target for treating organ fibrosis, RIPK1/3 or MLKL inhibitors are expected to be a candidate drug for ameliorate fibrosis and should be further explored.

5. Open questions, translation and future directions

The research history of necroptosis pathway has last decades of years from the term of necroptosis first created to express this nonapoptotic RCD form at 2005. Up to now, RIPK1/3 and MLKL as the main proteins of necroptosis have reached an agreement in necroptosis research field. However, the specific upstream/downstream protein targets of necroptosis have not been fully elucidated. Therefore, the pathophysiology effect of necroptosis in diseases was still unclear and even remains controversial. For example, acetaminophen poisoning is the most correlative factor for drug-induced liver injury (DILI) in the clinic (Krenkel, Mossanen and Tacke 2014). Several studies came to different and controversial conclusions on the effect of necroptosis in DILI, probably due to this pathway was modulated by different technical methods, including small molecule protein inhibitor Nec-1, anti-sense oligonucleotides against RIPK1/3 mRNA, or conditional knockout of RIP kinases(Takemoto et al. 2014, Dara et al. 2015, Li et al. 2014, Ramachandran et al. 2013). Though the physiological effect of necroptosis has many pending issues, the current plenty studies have already showing its broad outlook on organ fibrosis and even other dieases. Given that necroptosis is a method for treating organ fibrosis, RIPK1/3 and MLKL inhibitors are expected to be a candidate drug for improving organ fibrosis and should be further explored.

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Fig 1. Schematic diagram of mechanisms and cellular events involved in organ fibrosis. After suffering sustaining and diverse stimuli including drugs, mechanical damage, inflammation, and infection, complicated cellular signaling transduction related with fibrosis will happen in several organs. Multiple cell types including quiescent hepatic stellate cells, pancreatic stellate cells are activated into myofibroblasts, and outcomes to the excessive deposition of ECM, reflecting the early formation of fibrosis.

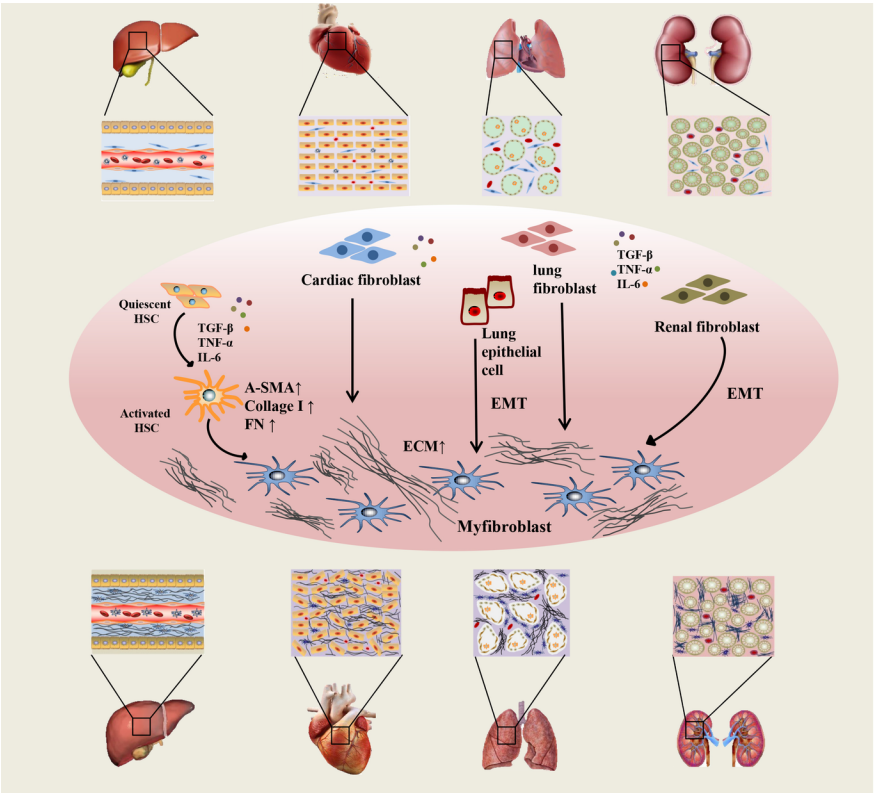
Fig 2. Difference between apoptosis and necroptosis. Apoptosis is a caspase-dependent RCD which characterized by cell membrane blistering, cell contraction, nuclear fracturing, chromosome concentration and chromosomal DNA fragmentation. Different from apoptosis, necroptosis is a type caspase-independent RCD form which characterized by increased cell membrane permeability, plasmalemma ruptures, general swelling of cytoplasm and organelles, and overflow of cell components into the microenvironment.

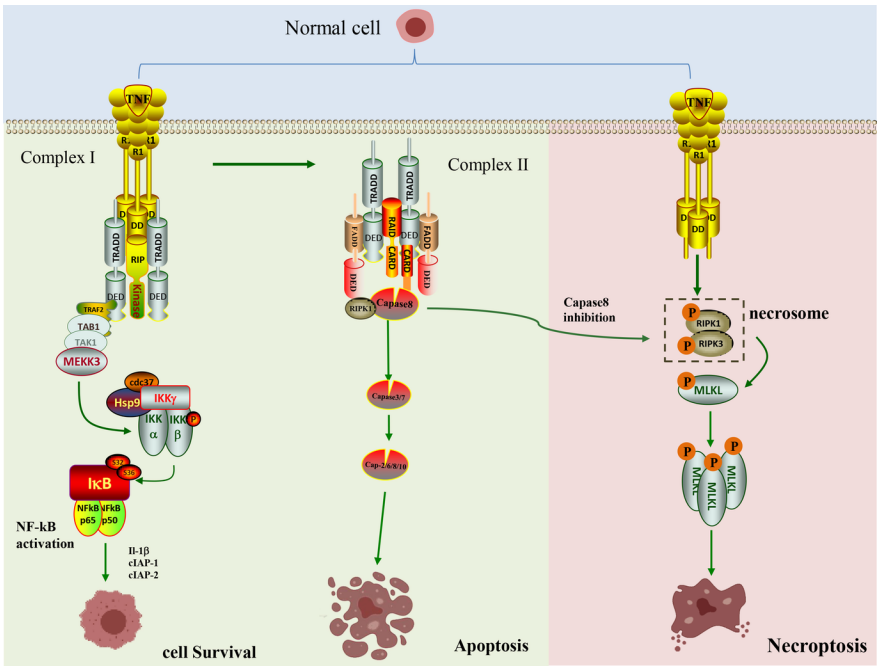
Fig 3. The detailed diagram of Necroptosis Signal pathway. When caspase-8 is inhibited, the active RIPK1 will interact with RIPK3 to cause its phosphorylation and forming a necrosome complex. Phosphorylated RIPK3 activate MLKL to form a homotrimer by its amino-terminal coiled-coil domain, further mediate transient receptor potential melastatin related 7 induce Ca(2+) influx and locates to the cell plasmalemma to destroy the integrity of the plasmalemma by forming micro pores. Zα domains of ZBP1 can sense endogenous Z-form nucleic acids to activite RIPK3-dependent necroptosis .

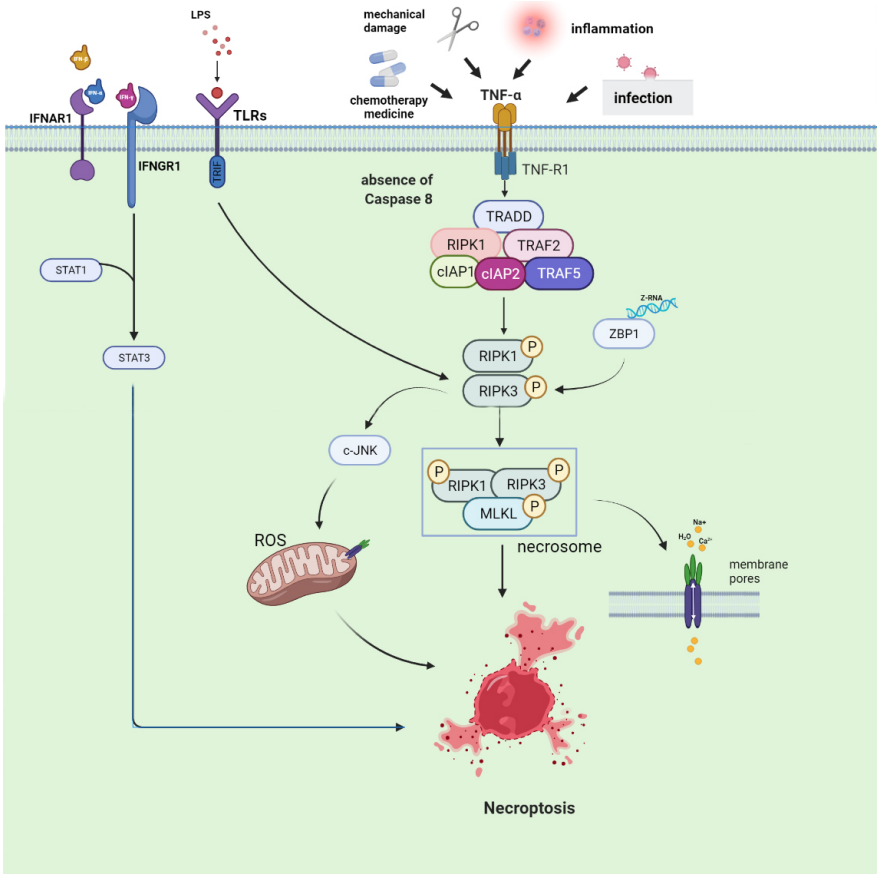
Fig 4. Necroptosis related signal pathway. Necroptosis signal pathway is closely related with several other forms of RCD containing apoptosis, autophagy and ferroptosis. Casepase 8 is a key switch between apoptosis and necroptosis, due to its cleaving capacity of RIPK1 and RIPK3. Autophagy-related protein 16-1 can

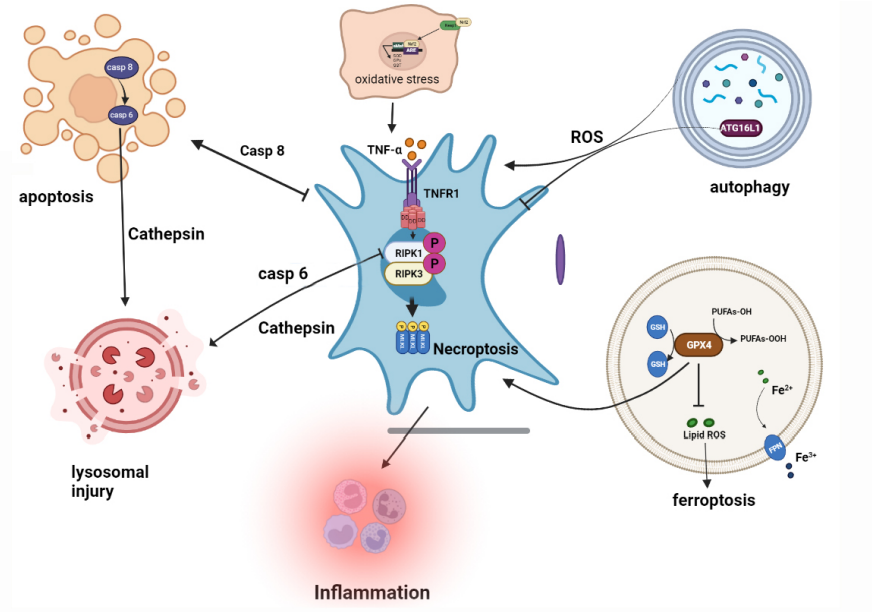
interdicts necroptosis. As the primary endogenous inhibitor of ferroptosis, glutathione peroxidase 4 can mediates powerful necroptosis inhibition effects. Besides different forms of RCD mentioned above, necroptosis also intimately connected with inflammation, oxidative stress and many other physiopathological processes.

Table 1. The small molecular inhibitor of necroptosis









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