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Necroptosis in cardiovascular disease - a new therapeutic target

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Abstract

Contrary to the apoptosis-necrosis binary view of cell death, recent experimental evidence demonstrates that several forms of necrosis, represented by necroptosis, are regulated or programmed in nature. Multiple death stimuli known to be associated with cardiovascular disease are capable of causing either apoptosis or necroptosis. Whether a cell dies from apoptosis or necroptosis has distinct consequences on inflammation. It is known that apoptosis, a non-lytic form of death mediated by the caspase family of proteases, does not generally evoke an immune response. Necroptosis, on the other hand, is a lytic form of cell death. Due to the rapid loss of plasma membrane integrity, cells dying from necroptosis release proinflammatory intracellular contents and subsequently cause inflammation. Our review delineates various genetic and biochemical evidence that demonstrates a compelling role of necroptosis in the pathogenesis and/or progression of cardiovascular disease including myocardial infarction, atherosclerosis, and aortic aneurysm. Through recent studies of necroptosis in cardiovascular diseases, we attempt to discuss the role of necroptosis in vascular inflammation as well as the potential of necroptosis inhibitors in future clinical management of cardiovascular events. Inhibiting necroptosis in the vasculature has an overall protective role and necroptosis may represent a new therapeutic target to prevent the development and progression of cardiovascular diseases.

Keywords

Apoptosis; atherosclerosis; aneurysm; cardiovascular; inhibitors; necroptosis; RIP3; RIP1

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Disclosures

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Introduction

A fine balance between cell proliferation and cell death is essential for healthy physiological processes. Disruption to this life and death balance is frequently observed in pathological events including major cardiovascular diseases (CVDs). For decades, apoptosis was depicted as the only mode of programmed cell death whereas necrosis was viewed as incidental. While sequential activation of caspases is the molecular signature of apoptosis, non-lytic nature is the morphological characteristic of apoptosis. Based on studies in various animal models, the importance of apoptosis in health and disease is well recognized [1–3]. Contrasting to apoptosis, necrosis involves rapid loss of plasma membrane-integrity and is frequently associated with extreme environmental stress (Figure 1). Major morphological differences between apoptotic and necroptotic cells are also found in intracellular organelles like mitochondria, Golgi and the nucleus (Figure 1) [4]. In addition to the morphological differences, apoptosis and necrosis are regulated through distinct signaling mechanisms and have drastically different impacts on inflammation [5], as discussed in detail in the review.

The concept of necrosis being passive and unregulated was first challenged about fifteen years ago [6]. Several seminal works reported forms of cell death with morphological features of necrosis and yet regulated by well-orchestrated signaling networks that are distinct from the caspase cascade [7] [8] [6] [9] [10] (Table 1). Ever since, studies of programmed necrosis have led to description of several mechanisms of regulated necrosis including necroptosis, cyclophilin D-mediated mitochondrial permeability transition [11] [12], glutamate-induced oxytosis [13], parthanatos [14], ferroptosis [15], NETosis [16], pyronecrosis and pyroptosis [17]. Among them, necroptosis, which is defined as necrosis mediated by receptor interacting protein kinase-3 (RIP3), has been most extensively studied [18–20]. Results generated from animal models suggest that inhibiting necroptosis may serve as a novel strategy for the treatment of many pathological processes such as ischemia-reperfusion injury [21], neurodegenerative diseases [22] and cardiovascular diseases [23–26].

1. Molecular and Cellular Basics of Necroptosis

Triggers of Necroptosis

The precise stimuli of necroptosis across various human disease conditions remain obscure, although several chemical “triggers” of necroptosis have been suggested (Figure 2). Necroptosis triggers include but are not limited to ligands to death receptors (such as TNFα, FAS ligand) [27] [28] [8], genotoxic stress [29], pathogen-derived double strand DNAs/RNAs [30] and interferons [31] (Figure 2). Necroptosis can also be triggered by damage associated molecular patterns (DAMPs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RGRs), ripoptosome and protein kinase R (PKR) complexes as reviewed in detail by Berghe et al. [32]. Those triggers are thought to individually or jointly induce necroptosis in complicated disease conditions. Regardless of the upstream triggers, RIP3 plays a decisive role in necroptosis and its expression is directly linked to the susceptibility of cells to undergo necroptosis. The expression of RIP3 occurs basally in many tissue types as reported by databases like ENCODE, the human protein atlas and also in primary literature. RIP3

expression becomes noticeably elevated during infection, tissue injury or disease [33] [34]. Structurally, RIP3 is a RHIM domain containing protein. Unlike other members of the RIP kinase family which includes RIP1, RIP3 lacks a death-domain [35] [36]. The death domain is required for interacting with the death-domain containing adaptors like FADD and TRADD. Many pro-necroptosis triggers are also capable of inducing apoptosis. How does a cell decide whether to die through apoptosis or necroptosis? In addition, why do we need multiple cell death mechanisms? Thus far, these fundamental questions are best addressed in the context of host defense. The importance of necroptosis in host defense was first demonstrated by Cho and colleagues who described severely impaired virus-induced tissue necrosis, inflammation, and control of viral replication in *Rip3* gene deficient mice [27]. The current view is that necroptosis functions as an alternative mechanism to eliminate infected cells when the default apoptotic mechanism is compromised [37]. It remains unclear how the necroptosis cell fate is decided in non-sterile conditions. Experimentally, one can “reveal” or “unleash” necroptosis activities in cells or tissues by deleting caspase 8, which normally suppresses necroptosis by cleaving RIP3 [38]or by using caspase inhibitors such as Z-Val-Ala-Asp-fluoromethylketone/carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (zVAD-fmk) [39].

Morphological Features of Necroptotic Cells

In textbooks, the term “necrosis” frequently refers to cells that fail to maintain membrane integrity. Histologically, necrotic cells appear swollen and show increased eosinophila in hematoxylin and eosin (H&E) stains [40]. Electron microscopy reveals many ultrastructure changes of necrosis, exemplified by disrupted plasma membrane and fragmentation or swelling of organelles [4]. The disrupted plasma membrane allows large molecules such as Evan’s blue or propidium iodide (PI) to enter the intracellular compartment. Excluded from healthy cells and apoptotic cells, these dyes are commonly used to identify cells dying from necrosis [41].

Because cells dying from necroptosis display typical necrotic features, they are currently indistinguishable under standard histological inspections from cells that die truly incidentally. Apoptotic cells, on the other hand, are recognized by their shrinkage, membrane blebs, and chromatin condensations (Figure 1 and Table 1). There are also many reliable biochemical approaches to detect apoptosis in vitro and in vivo such as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), which identifies apoptotic cells by detecting DNA fragmentation. Biochemical markers for necroptosis are under development. Currently, there are no commonly used definite biomarkers for necroptosis. Our lab uses the proximal ligation assay to detect the formation of pronecroptosis complexes also called necrosome as a method of identification of necroptosis in cells and tissues in which protein-protein interaction between RIP3 and RIP1 is required for the onset of necroptosis (unpublished work). Phosphorylation of RIP3 substrate is also used to measure necroptosis by multiple investigative groups [42] [43] [44].

Intracellular Signaling during Necroptosis

Parallel to the caspase activation cascade, activation of RIP3 is the molecular signature of necroptosis. However, comparing to the extensive information in regards to caspase

signaling and regulation, our knowledge of RIP3 activation, its interacting partners, and downstream substrates is primitive. Since the necroptosis initiation mechanism has been discussed in details by multiple review articles including the recent ones by Liu [45] and Grootjans [46], we will be brief in this regard. Essentially, the molecular mechanisms underlying necroptosis initiation (by initiator proteins (Figure 2A) is largely derived from studies of tumor necrosis factor α (TNFα)-mediated necroptosis. TNFα is a potent inducer of cell death but may also promote survival under certain conditions. Additionally, it has a major role during inflammation in infection or tissue injury. The divergent functions of TNFα are accomplished through distinct intracellular signaling pathways triggered by binding of TNFα to TNF receptor 1 (TNFR1), illustrated as three signaling complexes (Figure 2B) that may lead cell survival, apoptosis or necroptosis [19, 20]. Complex I is pro-survival and includes TNF-R1, TNFR-associated death domain (TRADD), cellular inhibitors of apoptosis, and RIP1 among others. Complex IIa is pro-apoptotic and results from the de-ubiquitination of RIP1. Complex IIa also contains caspase-8 that activates downstream caspases but also suppresses the initiation of necrotic events by cleaving RIP1 and RIP3. Under pathological or experimental conditions in which either RIP3 expression is upregulated or caspase-8 is inhibited, Complex IIb also called necrosome is formed (Figure 2B) [47]. Whether TNFα produces pro-survival, pro-apoptotic or pro-necroptotic protein complexes is influenced by cell type, cellular contents and regulatory mechanisms such as ubiquitination and phosphorylation. Phosphorylation of RIP1 and RIP3 is crucial to the assembly and activity of the necrosome. Mass spectroscopy data suggest RIP1 may be autophosphorylated upon necrosis initiation [48] [49]. Inhibiting the kinase activity of RIP1 can successfully block necrosome assembly [27, 50]. *In vitro*, RIP3 can directly phosphorylate RIP1, and *Rip3* gene deficiency abolishes necrosis-associated RIP1 phosphorylation [27]. The phosphatase Ppm1b interacts with phosphorylated RIP3 and dephosphorylates it, preventing necroptosis in this capacity [51].

Mouse knock-out models have contributed substantially towards our understanding of the necroptosis pathway and the interplay between RIP kinases and caspases. Mice deficient in the *Rip3* gene are viable and fertile [52]. However, under pathological conditions such as mouse models of atherosclerosis [23] and photoreceptor degeneration [53] [54], RIP3 deficiency prevents necrosis but not apoptosis. Moreover, cells harvested from $Rip3^{-/-}$ mice display similar sensitivity to a variety of apoptotic stimuli as the wild-type cells [55], suggesting that apoptosis and necroptosis are somewhat modular in their signaling. Contrary to this notion, the kinase dead mutant D161N mice ($Ripk3^{D161N/D161N}$) die at the embryonic state due to the spontaneous induction of Caspase 8-dependent apoptosis [52]. Apoptosis can also be triggered when RIP3 kinase activity is perturbed by high concentrations of certain inhibitors [19]. Mandal *et al.* postulated that RIP3 exists in two conformations: a kinase-dependent form leading to necroptosis and a kinase-independent form that recruits RIP1, FADD, and Caspase 8 which subsequently activates apoptosis [19]. However, not all RIP3 kinase dead mutants cause apoptosis. Unlike the pro-apoptotic activity of $RIP3^{D161N}$, RIP3 kinase dead mutants K51A, D143N and D161G did not trigger apoptosis *in vitro* [19]. Furthermore, $Rip3^{K51A/K51A}$ kinase-mutant knock-in mice are viable and fertile.

Unlike *Rip3* knock-out mice, mice lacking the *Rip1* gene die shortly after birth [56] [57]. However, this perinatal lethality is independent of RIP1 kinase activity. Several groups have

generated RIP1 kinase-dead knock-in mice *Ripk1*^{K45A} (which bear a point mutation in exon 3 corresponding to the catalytic lysine of the Ripk1 gene) [56] and *Ripk1*$^{D138N/D138N}$ (where conserved aspartate is mutated to asparagine at position 138) [57]. These RIP1 kinase dead mutant mice are viable and healthy, indicating that RIP1 kinase activity is dispensable for development. However, both strains of RIP1 kinase dead mutants show abrogated pro-necroptotic activities. A growing body of evidence also suggests that the kinase activity of RIP1 underlies some pathological events independent of RIP3, such as IL-1α production in hematopoietic cells [50]. Therefore, RIP1 kinase dead mouse line is an important tool to dissect such functions of RIP1 kinase in disease models.

RIP3 may regulate certain physiological and pathological events independent of RIP1. Although RIP1 and RIP3 often act in synergy to promote cell death, RIP3-dependent necroptosis can occur in the absence of RIP1 under certain conditions such as DAI-induced necroptosis triggered by mouse cytomegalovirus infection [58] and Toll like receptor 3 (TLR3)-induced necroptosis triggered by poly (I:C) in the presence of pan-caspase inhibitor [59]. The pseudokinase mixed lineage kinase domain-like (MLKL) was identified by Wang's group in 2012 as a substrate of RIP3 in necroptosis pathway [9] (Figure 2A). Phosphorylation of MLKL by RIP3 is essential for necroptosis execution with different executioner mechanisms of MLKL proposed. Vandenabeele's group and Wang's group independently report that phosphorylated MLKL forms an oligomer that binds to negatively charged phosphatidylinositol phosphates and acts as a pore-forming complex, directly causing necrotic membrane disruption [60, 61]. Other studies suggest that following its phosphorylation, MLKL translocates to the plasma membrane causing an influx calcium or sodium which leads to necrotic membrane rupture due to the increase in osmotic pressure [55, 62]. Consistent with the role of MLKL in the execution of necroptosis, MLKL knock-out mice display resistance to necroptosis in vitro and in-vivo using a pancreatitis model, without affecting apoptosis.

Mitochondria together with ROS is known to be associated with cell death [63] and several mechanisms implicate these in necroptosis. Zhang [64] reported a lack of phosphorylation of MLKL during myocardial necroptosis in response to ischemia and oxidative stress. The authors demonstrated Ca^{2+}-calmodulin-dependent protein kinase (CaMKII) being an alternative effector. RIP3 activates CaMKII by phosphorylation and oxidation triggering the opening of the mitochondrial permeability transition pore (mPTP) and myocardial necroptosis [64] (Figure 2A). Whether and how MLKL and CaMKII may functionally interact with one another remains unclear. ROS was also found to be produced in RIP3 expressing 3T3 cells but not RIP3 lacking cells and was required for the induction of necrosis; in this report, it was demonstrated that the decision to execute necroptosis over apoptosis was closely tied with mitochondrial ROS production [65]. The mitochondrial phosphatase PGAM5 is also involved in the execution of necroptosis and is found to be associated with RIP1/RIP3 complex (Figure 2A) [66]. It functions downstream of the necrosome as well as intrinsic triggers like ROS. PGAM5 serves to recruit the mitochondrial fission protein Drp1, which is required to fragment the mitochondria. This appears to be a pre-requisite for necroptosis, as attenuating PGAM5 inhibits necroptosis [66]. Others have reported a dispensable role of Drp1 in a RIP3 dimerization-dependent model for necroptosis,

which may be owing to the difference in the trigger for necroptosis in the latter system (i.e. independent of TNFα) [67].

2. Pharmacological targeting of necroptosis

With the discovery of a unique set of proteins that constitute the necroptosis pathway, great efforts have been devoted towards developing small-molecule inhibitors that target these proteins. The loss-of-function phenotypes in mice as discussed above have provided a proof of concept that inhibiting the necroptotic machinery can have across the board benefits for many diseases, including CVDs. In the following section, we discuss the development of various inhibitors targeting necroptosis in general while the application in CVDs is discussed later in a disease-specific manner.

RIP1 inhibitors

In an effort to define the non-apoptotic pathway triggered by activation of death receptors, Yuan and her group screened a chemical library of 15,000 compounds for chemical inhibitors of the necrotic death of human monocytic U937 cells induced by TNFα and zVAD.fmk. The effort led to the discovery of Necrostatin 1s (Nec-1s) [6]. Since this hallmark discovery in 2005, Nec-1 has been applied widely to necroptosis in various cell types and disease process. Later, the same group identified RIP1 as the primary intracellular target of Nec-1 [68]. Using a comprehensive loss and gain of function mutant study and computational modelling of RIP1, Degterev et al established Nec-1's mode of action as an activation loop based inhibition [68]. The crystal structure of Nec-1 bound to the RIP1 kinase domain also suggests that Nec-1 interacts with conserved amino acids in the activation loop thus stabilizes RIP1 in an inactive conformation [35]. The potential use of Nec-1 to treat human disease has been investigated in mouse models of neuronal loss, photoreceptor loss, ischemic brain injury, myocardial infarction, and most recently atherosclerosis [6, 18, 23, 33] [69]. Necrostatin-1s (Nec-1s) is a modified version of Nec-1 with significant improvement in target specificity and stability [70]. Interestingly, low doses of Nec-1 sensitizes mice to lethality during TNF-induced systemic inflammatory response syndrome (SIRS) [71] [72]. This toxicity has not been observed with Nec-1s, suggesting Nec-1 may sensitize systemic inflammation through targets other than RIP1.

In addition to Nec-1 and other necrostatins, several other RIP1 inhibitors have been identified in chemical screens. Among the RIP1 inhibitors, GSK2982772 is in phase 2a clinical trial for psoriasis, rheumatoid arthritis, and ulcerative colitis [73]. GSK2982772 shows excellent selectivity for RIP1 [73] and it blocks inflammation induced by TNFα through blocking cytokine production [73]. The potential application of GSK2982772 has not been tested in CVDs, although in principle it should be beneficial since inflammation is a large component of CVDs. As with Nec-1, excellent structure activity relationship (SAR) data for GSK2982772 may lead to the development of analogs for additional pre-clinical and clinical applications [73].

RIP3 inhibitors

Conceptually, RIP3 is a better target than RIP1 because *Rip3* gene knockout mice are viable and displayed disease protection even with deletion of one gene copy at least in some pathological models [23, 41]. In addition, RIP3 has been shown to regulate inflammatory gene-expression [74] [41] [75]. Through chemical library screening with human recombinant RIP3 kinase domain, Mocarski's group identified several compounds including GSK'840, GSK'843 and GSK'872 that bind and inhibit RIP3 at sub-nM concentrations [76, 77]. While effective in suppressing necroptosis, these inhibitors alone induce apoptosis in a concentration dependent manner. The cytotoxicity of GSK'840 and other related inhibitors led to the appreciation of RIP3 kinase-independent function in apoptosis [76]. Despite the known cytotoxicity, GSK'872 was applied to a mouse cerebral ischemic-reperfusion model for 72 hours and found to produce a ~30% reduction in infarct volume and reduced phosphorylation of MLKL [78]. Recently, a class of inhibitors for B-Raf (V600E), traditionally used in treating metastatic melanoma, has demonstrated promising results for RIP3 inhibition where several in-class compound (represented by dabrafenib) show RIP3 binding [79]. Dabrafenib potently inhibited RIP3 activity in-vitro and in-vivo in a B-Raf independent manner by competing with ATP binding. Dabrafenib also showed activity in protecting against necroptosis in ex-vivo and in-vivo models, emerging as a leading inhibitor for necroptosis although its effect on CVDs remains untested.

Hybrid inhibitors

FDA-approved anti-cancer drugs ponatinib and pazopanib are recently found to inhibit necroptosis *in vitro* [80]. Ponatinib inhibits both RIPK1 and RIPK3, while pazopanib preferentially inhibits RIP1. However, tyrosine kinase inhibitors ponatinib and pazopanib are also associated with cardiovascular toxicities, which precludes *in vivo* studies [81].

Najjar et. al. from the Degeterev group reported a "hybrid" inhibitor that combined features of Nec-1 and ponatinib [82]. The investigators asserted that the "hybrid" inhibitors target RIP1 with a high potency and inhibited TNF-induced injury in-vivo, which was caused via tail vein injection of 5 ug/mouse with TNFα [82].

MLKL inhibitors

Relative to RIP1 and RIP3, few inhibitors have been reported for MLKL despite of the fact that MLKL is essential for the execution of necrosis and therefore may serve as an excellent candidate target for preventing necroptosis and the subsequent non-sterile inflammation. To this, necrosulphonamide (NSA) is an inhibitor of human MLKL that was found to bind to MLKL in an affinity probe and blocked necroptosis [9]. NSA may be a promising drug candidate since it has found to prevent necroptosis in a variety of disease, although mostly using ex-vivo/cell-culture based systems. Its potential application in pre-clinical animal models is prevented by its specificity for human MLKL.

3. Necroptosis and inflammation

Necroptosis and inflammation are intimately associated. Inflamed tissues often contain necrotic cells. Many pro-inflammatory cytokines such as TNFα are also capable of

triggering necroptosis. The disruption of cellular membranes during necroptosis leads to massive release of intracellular contents. Some of these cellular components are danger-associated molecular patterns (DAMPs) which are pro-inflammatory when present outside the cells. By contrast, the preserved plasma membrane and orderly disassembly of intracellular contents prevents or limits apoptotic cells from releasing DAMPs [83, 84]. The chromatin associated protein high-mobility group box 1 (HMGB1) is a well-established DAMP that acts on several immune cells to trigger inflammatory responses. Known receptors for HMGB1 include toll like receptor 2 (TLR2), TLR4 and receptor for advanced glycation end products RAGE [85]. HMGB1 can induce dendritic cell maturation [86], induce production of other pro-inflammatory cytokines (IL-1, TNFα, IL-6, IL-8) [87] as well as upregulate expression of cell adhesion molecules (ICAM-1, VCAM-1) on endothelial cells [88]. Other DAMP molecules include DNA and RNA outside of nuclei or mitochondria, S100 proteins and purine metabolites [89].

The intracellular mediators of necroptosis such as RIP1 and RIP3 can promote pathological inflammatory processes independent of their functions in cell death. Since Wallach and colleagues provided an extensive overview of this topic in their recent review [90], we will discuss only a few developments. For example, inflammasome activation and IL-1 maturation in LPS-primed macrophages requires RIP3 [20]. While caspase 8 deficiency in dendritic cells depends on the function of RIP3, they also require RIP1, MLKL and PGAM5 [91]. Lawlor *et al.* confirm the role of RIP3 in inflammasome and IL-1 maturation in vivo using in a rheumatoid arthritis mouse model [92]. Interestingly, the authors found that RIP3 promotes the inflammatory responses without needing MLKL [92], further underscoring the presence of additional RIP3 substrates or effectors. In our own study in vascular smooth muscle cells (VSMCs), we demonstrated that RIP3 regulates the expression of pro-inflammatory cytokines and adhesion molecules through the NF-κB pathway [41]. We also noted that inhibition of RIP1 with Nec-1s attenuated macrophage migration toward MCP1 however failed to influence cytokine expression [93], indicating diverse functions of RIP1 and RIP3 in regulation of inflammatory response,

4. Targeting necroptosis in Cardiovascular Diseases

Accumulating animal work proves the involvement of necroptosis in pathogenesis and progression of cardiovascular diseases including atherosclerosis, myocardial infarction, restenosis and abdominal aortic aneurysms. In the following paragraphs, we attempt to offer a brief summary of findings regarding necroptosis in several major cardiovascular events.

Myocardial infarction

Acute myocardial infarction, commonly referred as heart attack, is a leading cause of death worldwide. Myocardial infarction occurs when blood flow to a section of heart suddenly becomes blocked. If the blocked coronary circulation is not restored on a timely fashion, cardiomyocytes in the affected area die and result in irreversible cell loss [94]. Timely administration of thrombolytic medicines or/and percutaneous coronary interventions is effective to minimize heart muscle damage. However, the process of restoring blood flow to ischemic myocardial tissue can also induce cell injury and death, a process that is termed as

ischemic reperfusion (I/R) injury. Therefore, prevention of ischemic reperfusion injury is a pressing concern for modern cardiology.

The involvement of necroptosis in myocardial infarction was first suggested by Luedde *et al,* who discovered up-regulated levels of RIP3 protein in ischemic mouse hearts. The same authors reported that mice deficient for RIP3 recovered from an experimental infarction resulted from permanent left anterior descending coronary artery (LAD) ligation with a significantly higher ejection fraction and less hypertrophy compared to their wildtype counterparts. The functional improvements were accompanied by diminished infiltrating T cells in the *Rip3*$^{-/-}$ hearts [18]. A more recent study by Zhang *et al.* using an acute I/R injury model (30 min ischemia/4 h reperfusion) affirms that RIP3 deficiency protects mouse hearts from IR-induced necrosis and significantly reduces infarct size [64]. The benefit of *Rip3* knockout extended to long term cardiac remodeling reflected by improvements in fibrosis, hypertrophy, ejection fraction, and mortality measured 8 weeks after a temporary LAD ligation. Mechanistically, Zhang and colleagues asserted that (1) elevated RIP3 is responsible for myocardial necroptosis, as well as apoptosis and inflammation and (2) RIP3 signals through CaMKII instead of MLKL in cardiomyocytes (Figure 3B) [64].

The concept that pharmacological targeting of the necroptosis pathway is cardio-protective against reperfusion injury was first established by Smith *et al.* who demonstrated that the RIP1 inhibitor Nec-1 inhibits myocardial cell death and reduces infarct size in mice [95], thus establishing pharmacological inhibition of the necroptosis pathway as a potential cardio-protective therapy against I/R induced cardiac injury. It is worthwhile to note while Nec-1 at low concentration (30 μM) significantly reduced infarct size, administration of high concentration of Nec-1(100 μM) increased infarct size, potentially through off target effects [95]. A study by Dmitriev *et al.* also revealed that intraperitoneal injections of Nec-1 or Nec-5 prior to reperfusion of isolated heart from rats also reduced the infarction zone caused by 30 min global ischemia and 120-min reperfusion [96]. Importantly, Nec-1 prevents both short and long-term effects of myocardial ischemia, including diminished necrotic cell death and reduced myocardial infarct size, as well as preservation of long-term cardiac function and preventing adverse cardiac remodeling [97]. Mechanistically, Oerlemans showed an increased RIP1/3 phosphorylation after myocardial ischemia and reperfusion [97]. Administration of Nec-1 significantly decreased RIP1 phosphorylation, RIP1-RIP3 binding, and MLKL phosphorylation, the necessary signaling steps leading to necrotic cell death. Results from Oerlemans' study in regards to MLKL appears to be inconsistent from what reported by Zhang *et al.* who showed that siRNA-mediated knockdown of MLKL in cardiac myocytes produced little effect on necroptosis [64]. The precise role of MLKL remains to be further tested.

The aforementioned studies do not exclude the critical role of apoptosis. The apoptotic pathway has long been established as an underlying mechanism responsible for the irreversible loss of cardiomyocytes due to infarction [98]. The notion that both caspase and RIP pathways contribute to cellular damages caused by I/R injury was supported by finding that combined administration of a pan caspase inhibitor zVAD-fmk and Nec-1 leads to an even further reduction in infarct size, compared to Nec-1 alone [99]. Since RIP1 participates in the signal-cascade leading to apoptosis [59] as well as necroptosis, Nec-1-mediated RIP1

inhibition is found to reduce both necroptosis and apoptosis of cardiac myocytes [99] [97]. Of note, cardiac apoptosis, resulting from either ischemic injury or I/R injury, involves both intrinsic (mediated by caspase 9) and extrinsic (mediated by caspase 8) pathways [99, 100]. The precise role of RIP1 in cardiac apoptosis remains elusive.

Atherosclerosis

The formation of atherosclerotic plaques in arteries reduces blood flow to major organs and contributes to ischemia of major organs as well as limbs. Rupture of atherosclerotic plaque leads to the formation of an occluding thrombus on the surface of the plaque which is the most common cause of myocardial infarction and stroke [101]. Various lipid-lowering drugs represented by statins have been used successfully in reducing the major cardiovascular events [102, 103]. However, detection of unstable plaques and preventing them from ruptures continues to be a challenge in the management of millions of atherosclerotic patients.

Histologically, atherosclerotic plaques that are prone to rupture are characterized by thinning of the fibrotic cap coupled with expanded necrotic core. VSMC apoptosis as well as secondary necrosis resulted from untimely clearance of apoptotic cells has been actively studied in the context of plaque rupture [101, 104]. In comparison, investigations of necroptosis in atherosclerosis are emerging only recently. Using murine models of atherosclerosis, Lin *et al.* demonstrated the presence of necroptotic cells and elevated RIP3 in the necrotic core of advanced lesions[23]. Meng and colleagues subsequently showed phosphorylated RIP3 (serine 232) in atherosclerotic lesions of ApoE knockout mice fed with high fat diet [105]. More recently, Karunakaran *et al.* reported enhanced RIP3 and MLKL as well as levels of phosphorylated MLKL (used as an indication of activation of RIP3) in human carotid atheroma [33].

To more directly test the role of RIP3 thus necroptosis in atherosclerosis, Lin and colleagues bred *Rip3*$^{-/-}$ mice into atherosclerosis-prone *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ backgrounds. The resultant mice were fed with a Western-type diet for either 8 or 16 weeks. The lack of RIP3 was found to dose-dependently reduce the lesion areas and diminish the features of plaque vulnerability including the necrotic core size, disruption of collagen, thinning of the fibrous cap, and elastin depletion in mice that was on the Western diet for 16 weeks [23]. A similar athero-protective effect of *Rip3* gene deficiency was also reported by Meng et al. Using the bone marrow transplantation approach, Lin and co-authors showed that loss of RIP3 expression from bone-marrow-derived cells is responsible for the reduced plaque maturation. Interestingly, *Rip3* gene deficiency produced little effect on lesion sizes at the 8-week time point [23], suggesting that necroptosis is dispensable during the formation atherosclerotic plaques. This notion that necroptosis may drive plaque instability is further highlighted by Karunakaran who reversed plaque maturation of established atherosclerosis in Apoe$^{-/-}$ mice with the RIP1 inhibition Nec-1 (Figure 3A) [33].

A major functional distinction of necroptosis from apoptosis is its pro-inflammatory property. Indeed, mouse bone marrow derived macrophages responded to necroptosis inducing-conditions such as TNFα combined with Smac mimetic and the pan-caspase inhibitor z-VAD-fmk (TSZ) with profound release of IL-1α and Il-1β in a RIP3-dependent

manner [105]. This RIP3-sensitive production of cytokines is thought to be secondary of necroptosis because *Rip3* knockout has little effects on cytokine release triggered by stimuli that do not cause necroptosis [105]. Along the same line of argument, the diminished vascular inflammation detected in the lesion prone areas of fat-fed Rip3 KO mice in $Ldlr^{-/-}$ or $Apoe^{-/-}$ backgrounds is most likely resulted from inhibition of necroptosis. However, lineage tracing studies demonstrate that as high as 40% of foam cells in the atherosclerotic plaques originated from VSMCs [106]. Whether RIP3 has a direct role in regulation of inflammatory response of VSMC-derived macrophage like cells remains to be tested. Pertinently, our group showed that RIP3 may regulate cytokine expression in VSMCs through a cell death-independent mechanism [41].

Contrasting to the cardiomyocyte study which suggests that RIP3 contributes to both necroptosis and apoptosis [64], Lin's work indicates the role of RIP3 in macrophages is primarily necrosis. The authors believe that the high necrotic activity in advanced atherosclerotic lesions is most likely a consequence of elevated RIP3 expression lesion macrophages because RIP3 overexpression in cultured macrophages skewed the effect of oxidized LDL from apoptosis to necroptosis [23]. We believe whether high RIP3 drives necroptosis or both necroptosis and apoptosis depends on the nature and context of the cell. In support of this notion, adenovirus-mediated overexpression of RIP3 triggered VSMC apoptosis (and suppressed VSMC proliferation) in balloon injured rat carotid arteries [107]. The same investigative group also demonstrated RIP3 gene silencing in cultured VSMCs potentiated mitogen-induced Akt activation and cell proliferation [107].

Abdominal aortic aneurysm

Abdominal aortic aneurysm (AAA) is defined clinically as a full-thickness dilatation of the vessel wall of the abdominal aorta exceeding the normal vessel diameter by 50% [108]. Ruptured AAAs frequently lead to lethality [109, 110]. Although small aneurysms do occasionally rupture, the risk of rupture is found to be associated with larger aneurysm size [111]. Currently, surgical interventions, including open surgical and endovascular aneurysm repairs, are the only effective treatments to prevent rupture in patients with AAAs larger than 5.5cm (male) or 5.0cm (female) [111]. However, the majority (90%) of AAA cases identified by diagnostic screening is small, asymptomatic aneurysm (<3.5cm in diameter) [112] and is left untreated due to the low benefit-to-risk ratio of surgical interventions in this patient population [113, 114]. The clinical need of pharmacological treatments for small AAAs has motivated active investigations which have identified several key pathophysiological processes, including chronic vascular inflammation, excessive local production of matrix-degrading proteases, progressive destruction of structural matrix proteins, particularly elastin and collagen, as well as depletion of medial VSMCs [108, 115]. VSMC apoptosis has been noted in human aneurysmal tissue for a long time [116–120]. Several anti-inflammatory strategies, such as removing mast cells[120], deletion of TNFα [121], or blocking angiotensin II (Ang II) [122], were found to prevent VSMC loss, suggesting that inflammation is an important cause of VSMC death at least in experimental models of aortic aneurysm. To determine whether cell apoptosis is a contributing, pathological event in aortic aneurysm, our group treated *Apoe*−/− mice with a pan caspase inhibitor (z-QVD-fmk) in an Angiontensin II-induced aortic aneurysm model. While the

administration of caspase inhibitor at the onset of Ang II infusion successfully prevented aneurysm formation, administration of the inhibitor one week after Ang II pump installation failed to stop aneurysm growth [93]. We postulated that the contribution of apoptosis is somewhat limited to the early phase of aneurysm formation in aneurysm models. Indeed, time course studies indicate that TUNEL positive cells (a marker of apoptosis) as well as caspase 3 activation peaked before aortic dilation reaches aneurysmal threshold in multiple models including Ang II/*Apoe*−/− model, $CaCl_2$ and elastase models [41, 93, 100, 123]. In human, early stages of aneurysm tissues are not accessible. Although apoptosis markers were detected in advanced human aneurysmal tissues, it is difficult to appreciate the prevalence of apoptosis in the absence of tissue specimens from various disease stages of AAAs.

Since inhibiting apoptosis is not sufficient to reduce aneurysm growth, we turned to necroptosis. We found protein levels of necroptosis mediators RIP1 and RIP3 were elevated in human AAA tissues compared to normal aortae [41]. The upregulation of RIP3 as well as complex formation between RIP1 and RIP3 in VSMCs was also detected in mouse aneurysm models. The direct experimental evidence supporting the role of necroptosis in this preclinical model was provided by *Rip3* gene deficient mice. Deletion of a single or both copies of *Rip3* genes protected mice from aneurysm formation [41]. Two weeks after the elastase-perfusion, the aortic walls of $Rip3^{-/-}$ mice appeared normal, populated by contractile protein-expressing VSMCs and free of infiltrated inflammatory cells. The protection appears to be intrinsic to the vessel wall because a $Rip3^{-/-}$ aorta retains its aneurysm resistant property even when transplanted to the abdominal aortic circulation of a wild type mouse [41], which suggests that the lack of RIP3-mediated necroptosis and inflammatory response of vascular cells most likely VSMCs is a major mechanism underlying aneurysm protection provided by Rip3 gene deficiency.

More recently, we used the optimized RIP1 inhibitor necrostatin-1s (Nec-1s) to test the concept that pharmacologically targeting necroptosis may serve as a new and effective way for treating small aneurysm progression [93]. We started treatment with RIP1 inhibitors 7 days after elastase perfusion, a time point at which the mean aortic dilation is slightly below the aneurysm threshold (defined in this model as 100% over the diameter prior elastase perfusion). Mice were randomized to three groups to receive daily IP injections of either DMSO (post-elastase+DMSO), Nec-1 (3.2 mg/kg/day, post-elastase+Nec-1) or Nec-1s (1.6mg/kg/day, post-elastase+Nec-1s), respectively. Upon sacrifice 14 days after elastase perfusion, mice in the post-elastase + DMSO group exhibited a mean aortic expansion of 172.80±13.68% (1.408±0.068mm) as compared to the day 7 measurement of 100.70±4.37%, indicating progressive aneurysm growth. In contrast, mice in the post-elastase+Nec-1s group had a mean aortic expansion of 64.12±4.80% (0.864±0.032 mm), indicating no aneurysm growth.

However, mice in the post-elastase + Nec-1 group displayed the mean aortic expansion of 121.60±10.40% (1.133±0.052mm), which was smaller than mice treated with DMSO, though this difference was not statistically significant. While it remains to be determined whether Nec-1 failed to block aneurysm growth in our study due to insufficient dosing, results produced by Nec-1s are encouraging (Figure 3C) [93].

Histologically, Nec-1s treated aortic wall showed resolved inflammation and improved tissue integrity [93], which is consistent with what was observed in RIP3 knockout mice. In cultured VSMCs, inhibition of RIP1 with Nec-1 or Nec-1s or siRNA attenuate necroptosis triggered by TNFα in the presence of zVAD, indicating the essential role of RIP1 in regulation of necroptosis of VSMCs. However, RIP3 appears to regulate the cytokine expression by VSMCs independent of RIP1 because inhibition of RIP1 does not affect TNFα-induced cytokine expression. Intriguingly, Nec-1 prevents macrophage from migrating toward MCP-1, suggesting a potential role of RIP1 in regulation of inflammatory infiltration, an area warrants further investigations.

Summary and future perspective

Primary necrotic cell death has long been observed in human pathological conditions, however, its relevance and importance in human diseases has only recently been highlighted. In contrast to the conventional thought that primary necrosis is passive and unregulated, recent discoveries revealed different types of regulated necrosis including necroptosis. The discovery and use of Nec-1 in small animal studies prove the concept that intervening necroptosis is efficacious in treating cardiovascular disease at least in mice. However, similar studies need to be repeated particularly with inhibitors against RIP3.

Although the experimental data are strong in establishing the pathological involvements of RIP1, RIP3 and MLKL in cardiovascular disease, the majority of these studies were performed using a “preventive” approach, i.e. inhibitors were administered or genes were deleted prior to disease induction, conditions that are far away from clinical situation. Even in the few “treatment” studies such as those performed by Karunakaran and by our own group young and healthy mice were used, which is unlike the typical cardiovascular patients. Therefore, future studies are warranted not only in the areas of discovering new components in the necroptosis signaling but also in the areas of translational studies that focuses on development of new inhibitors with high specificity/low toxicity as well as new animal models that are better replicates of clinical realities.

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Highlights

- RIP3-mediated necrosis (necroptosis) is implicated in cardiovascular diseases
- Necroptosis eliminates essential tissue and causes non-sterile inflammation
- VSMC, cardiomyocytes and macrophages are major targets of necroptosis in CVD
- Many proteins in the necroptosis pathway can be targeted by inhibitors
- Targeting necroptosis is beneficial in CVD; clinical efficacy of inhibitors unknown

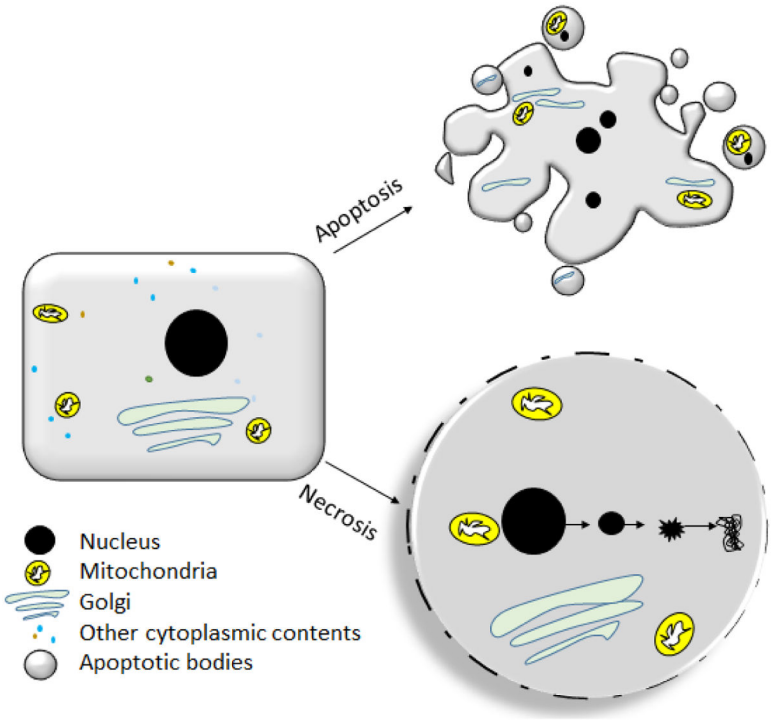

Figure 1.

Schematic diagram depicting the key morphological differences between apoptosis and necrosis. Apoptotic cells have shrunken nucleus, un-ruptured plasma membrane, shrunken mitochondria and golgi. Necrotic cells have ruptured plasma membrane, enlarged organelles and an overall increase in cell-volume.

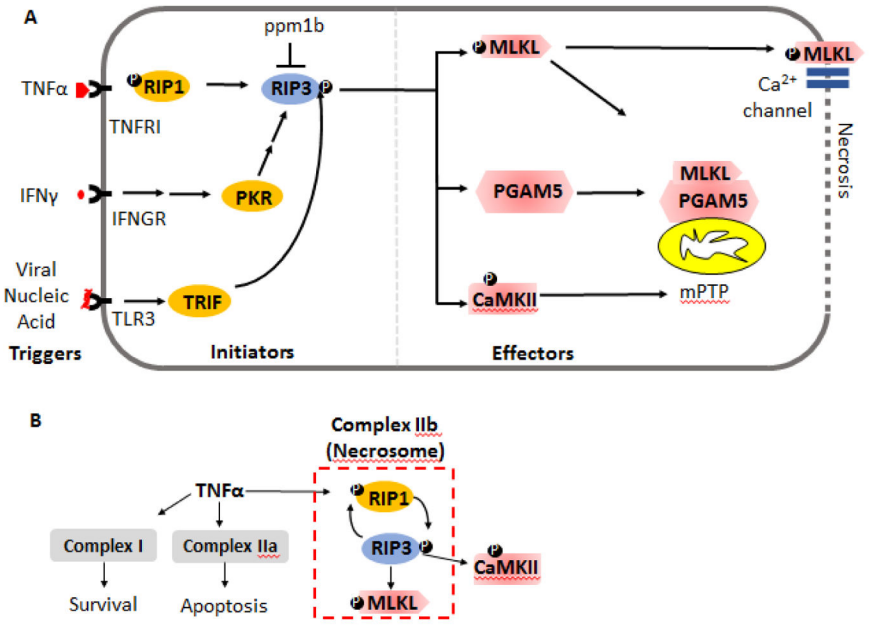

Figure 2.

Signal-transduction events in necroptosis. A) The necroptosis pathway may be categorized into the triggers, initiators and effectors of necroptosis. The initiator molecules involve cytokines like TNFα, IFNγ or viral nucleic acids which via their cognate receptors activate the initiator molecules. These initiators converge on activating RIP3, for instance via phosphorylation. Activated RIP3 is currently known to function via at least three downstream effectors-- MLKL PGAM5 and CaMKII, which are the effector molecules leading to necrosis. Protein phosphatase ppm1b is found to de-phosphorylate RIP3 and prevent necroptosis. Ⓟ denotes phosphorylation. B) The complexes involved in cell-death are depicted. Depending on the cell-type and cellular-context, TNFα may promote formation of a pro-survival complex (complex I), a pro-apoptotic complex (complex IIa) or depending on the inhibition of caspase 8 and presence of RIP3, a pro-necroptotic complex (complex IIb). The components of the pro-necroptosis complex II are depicted.

PKR: Protein Kinase R; TRIF: TIR-domain-containing adapter-inducing interferon-β, MLKL: Mixed Lineage Kinase domain Like pseudokinase; CaMKII: Ca2+/calmodulin-dependent protein kinase II; ppm1b: Protein Phosphatase, Mg2+/Mn2+ Dependent 1B; mPTP: mitochondrial Permeability Transition Pore complex; PGAM5: Phosphoglycerate Mutase Family Member 5

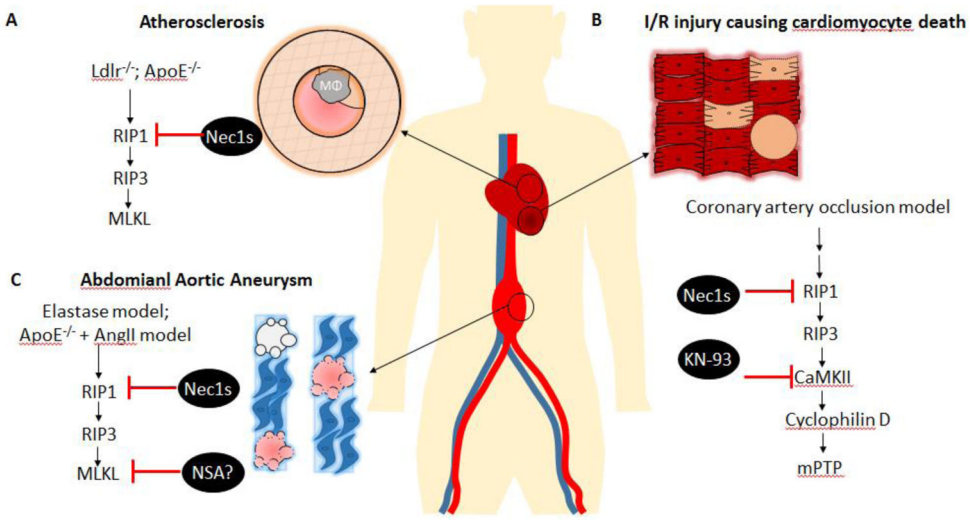

Figure 3.

Schematic outline of the involvement of necroptosis in elimination of cells of the cardiovascular system and reported inhibition strategies based on necroptosis pathway. A) Role of Nec1s in treatment of atherosclerosis in response to inflammation and necroptotic death of cells including macrophages and VSMCs. B) Ischemia-reperfusion related injury has been found to eliminate cardiomyocytes, notably via necroptotic machinery somewhat different from the conventional signaling pathway. C) Treatment of abdominal aortic aneurysms by Nec1s and potentially NSA by prevention of VSMC-necroptosis as a therapeutic strategy. ApoE−/−, apolipoprotein E knockout; Ldlr−/−, low-density lipoprotein receptor knockout; Mφ, macrophage; I/R, ischemia reperfusion; mPTP, mitochondrial permeability transition pore

Table 1

Comparison of the biochemical, morphological and immunological features accompanying apoptosis and necroptosis.

	Apoptosis	Necroptosis
Biochemical Features	Caspase activation; intrinsic or extrinsic pathways	Activation of RIP1/RIP3/MLKL
	Exposure of PS to the cell-exterior	ROS production
	Loss of mitochondrial membrane potential	Cathepsin activation
	Cytochrome c release	Ceramide and Sphingosine overproduction
	ROS production and formation of DNA/Protein adducts	RIP3 dependent ROS production
	Activation of DNases	Unknown
	ATP consumption decreases; production unchanged	ATP production decreases; consumption unchanged
Cell-Morphology	Plasma membrane blebbing	Rupture of plasma membrane
	Overall shrinkage in cell-volume; increased opacity	Overall swelling; increased transparency
	Condensation and fragmentation of chromatin	Overall no known change in chromatin condensation or nuclear shrinkage
	Formation of apoptotic bodies and shedding of intracellular organelles	
	Mitochondria usually normal	Swelling of Mitochondria occurs
Immunogenecity	Negligible release of DAMPs like ATP, HMGB1, ROS or dsDNA	Considered highly immunogenic due to the release of DAMPs
	Efficient clearance by phagocytosis	Poor clearance
	Extracellular PS exposure facilitates clearance	Clearance mechanisms include macropinocytosis
	Non-amplificatory in nature	Amplificatory in nature

PS, phosphatidylserine; ROS, reactive oxygen species; DAMPs, damage associated molecular patterns; HMGB1, High mobility group box 1 protein; dsDNA, double stranded DNA