Review

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A new kind of cell suicide: mechanisms and functions of programmed necrosis

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Classically, there are two major forms of cell death: necrosis, an unregulated digestion of cellular components; and apoptosis, a programmed mechanism that is promoted by caspases. However, another form of cell death has recently been identified that is inhibited by caspases, and yet occurs through a regulated mechanism, termed programmed necrosis or necroptosis. The biochemical basis of this program has begun to emerge, with the discovery of the receptor-interacting kinase RIP3 and its substrate, the pseudokinase mixed lineage kinase domain-like protein (MLKL), as core components. Furthermore, animal models have revealed significant functions for RIP3/MLKL-mediated necrotic cell death in immune responses against microbial infection and in the etiology of diseases involving tissue damage. This review discusses recent advances in our understanding of the mechanistic details and physiological functions of programmed necrosis.

Working definitions of necrosis

The term 'necrosis' was first coined by surgical pathologists to describe tissue destruction from the early 19th century [1,2]. Ever since, researchers in this field have long been working under ambiguity as to whether necrosis is controlled by defined genetic programs or whether it simply happens due to environmental circumstances.

Membrane rupture and organelle swelling have long been considered the characteristic features of necrosis. This form of cell death releases the cellular contents of the dead cells, resulting in immune reactions in the host animal. Therefore, exposure to damage-associated molecular pattern molecules (DAMPs) [3], such as nuclear high mobility group box-1 proteins (HMGB1) [4,5] and mitochondrial DNA [6,7], in the blood stream is now considered an indicator of cellular necrosis.

A hint that necrotic cell death might also occur through defined biochemical programs came from observations that, in certain cell lines, both apoptotic and necrotic forms of death were observed when these cells were treated with tumor necrosis factor alpha (TNF-α) [8–11]. Apoptotic cell death can be inhibited by a caspase inhibitor. Treatment with a caspase inhibitor did not save the TNF-treated cells from dying; rather, such treatment shifted the balance away from apoptosis and toward necrotic death. Moreover, caspase inhibition strongly exacerbated necrotic death, indicating that caspase activity actually negatively regulates necrosis [8,9,12]. Receptor-interacting kinase 1 (RIP1, also known as RIPK1) was the first signaling molecule to be identified in the pathway that controls caspase-inhibited necrotic cell death [13]. The requirement for RIP1 in necrotic cell death was further verified by a study that screened for a chemical inhibitor of RIP1 kinase activity, which the authors named necrostatin-1 (Nec-1) [14,15], that blocks this form of necrosis. Because this pathway shares components with the pathway that induces apoptosis, the term 'necroptosis' has been proposed to describe this form of cell death [14,16].

RIP1 is a pleotropic signaling kinase that triggers the activation of NF-κB (nuclear factor-κB), MAP kinase cascade, and caspase 8-mediated apoptosis, and thus cannot be accurately used as an activation marker for necrosis. Rather, it was the discovery of a related kinase RIP3 (also known as RIPK3) [17–19] and its substrate, the mixed lineage kinase-like domain protein MLKL [20,21], both of which function specifically in necrosis, that has allowed the recent determination of the molecular components of this pathway (Figure 1), which are the subject of this review.

Signaling for programmed necrosis

Analyses of the intracellular signal transduction of programmed necrosis have revealed the existence of a common mechanism for the signaling that began with activation of RIP kinases. Programmed necrosis can be activated when cells are treated with TNF family cytokines, including Fas/CD95 and TNF-related apoptosis-inducing ligand (TRAIL) [13]. The activated receptors interact with RIP1 through homophilic interactions between their carboxyl death domain (DD) and the DD domain of RIP1, and recruit cellular inhibitor of apoptosis proteins (cIAPs) to stabilize the RIP1-containing plasma membrane associated complex that activates NF-κB and mitogen-activated protein (MAP) kinases [22]. This function of RIP1 causes inflammation and shifts cells toward survival programs. Interestingly, this function of RIP1 in NF-κB signaling is independent of its kinase activity and, in this case, RIP1 functions more like a scaffold protein for downstream signaling [23–25]. Concomitant to the assembly of RIP1 with the death receptor complex, RIP1 is also rapidly

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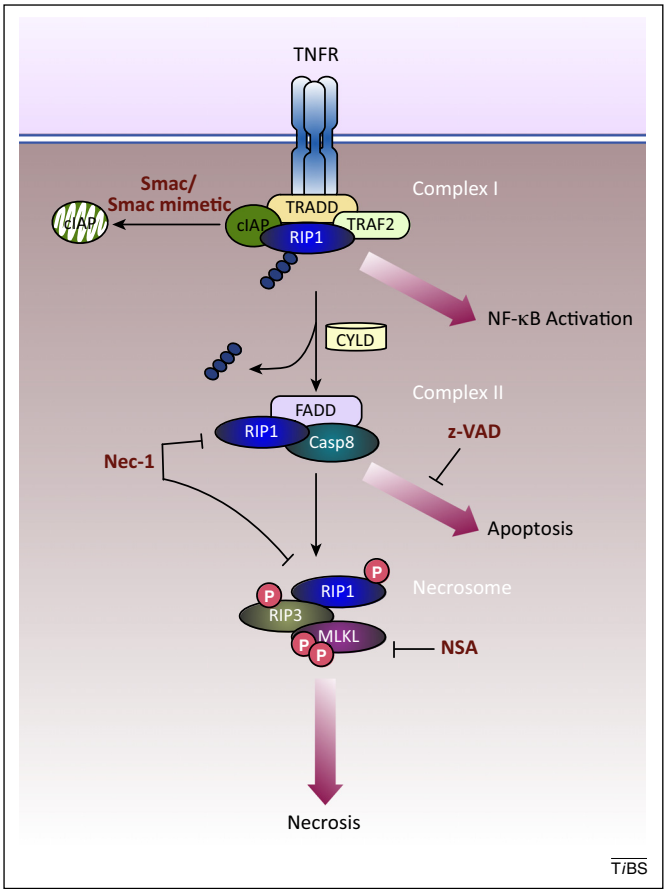

Figure 1. The programmed necrosis signaling pathway. Ligation of tumor necrosis factor receptor 1 (TNFR1) leads to the formation of complex I, which is comprised of TNFR1, TNFR1-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2), receptor-interacting kinase 1 (RIP1), and cellular inhibitors of apoptosis (cIAPs). RIP1 is initially modified by Lys63-linked polyubiquitin chains within the complex I after recruitment to the membrane, and this form of RIP1 serves as a signaling platform for the activation of nuclear factor-kappaB (NF-κB). Upon release of second mitochondria-derived activator of caspases (Smac) from the mitochondria, or upon Smac mimetic treatment, cIAPs are autodegraded. RIP1 is subsequently de-ubiquitinated by the de-ubiquitinating enzymes cylindromatosis (CYLD) or A20, and dissociated from the death receptor, which induces the formation of the Fas-associated protein with death domain (FADD)–RIP1–caspase 8 pro-apoptosis complex II. Transformation from complex I to complex II indicates the conversion of cell fate from survival to death. Within complex II, activated caspase 8 cleaves downstream caspases such as caspase3/7 to induce apoptosis, and blocks necrosis by cleaving and inactivating RIP1 and RIP3. When caspase 8 is inhibited (e.g., by z-VAD), RIP1 and RIP3 form the necrotic death complex and their kinase activities become activated. RIP3 recruits its substrate, the mixed lineage kinase domain-like protein (MLKL), and phosphorylates MLKL at T357 and S358, which causes its activation. Necrostatin 1 (Nec-1) inhibits RIP1 dependent necrosis by inhibiting the kinase activity of RIP1 to prevent necrosome formation. Necrosulfonamide (NSA) inhibits MLKL mediated necrosis by blocking its N terminal coil–coil domain function, independent of MLKL phosphorylation.

polyubiquitinated, which is required for recruiting the IKK complex and leads to the activation of NF-κB. The TNFR-associated factor (TRAF) family E3 ligases, such as TRAF2/3/5/6, polyubiquitinate RIP1 to form the K63 chain. cIAPs also polyubiquitinate RIP1, but this is not restricted to the K63 linkage. When the auto-ubiquitinated cIAP1 and cIAP2 are degraded through their own RING-finger domain, a process accelerated by the second mitochondria-derived activator of caspases (Smac) protein or small molecule mimetics of Smac (Smac mimetics) [26–30], RIP1 dissociates from the plasma membrane. The K63-linked polyubiquitin chain on RIP1 is then removed by cylindromatosis (CYLD) [31] or the ubiquitin-modifying enzyme A20 [32]. RIP1 then binds to the FAS-associated death domain (FADD) [29], through which it recruits procaspase 8; this subsequently leads to the activation of caspases and apoptosis [33].

If caspase 8 is inhibited by either viral or chemical inhibitors, and/or the cell expresses high enough levels of RIP3, then a necrosis cell death pathway will be activated instead. In this case, RIP3 and RIP1 will bind through their respective homotypic interaction motif (RHIM) domains [17–19,34]. RIP3 then undergoes auto-phosphorylation at the serine 227 site [20], an event that leads to the recruitment of a pseudokinase MLKL. When caspase 8 is not inhibited, or if RIP3 is expressed at low enough levels, caspase 8 antagonizes necrosis by cleaving and inactivating RIP1, RIP3, and CYLD [35–39], effectively terminating the necrosis signal (Figure 1). These pro- and anti-cell death functions of RIP1 have recently been verified elegantly *in vivo* with the combinatory knockout of RIP1, RIP3, and caspase 8 or FADD in mice [40–44]. The negative role of caspase-8 or FADD on necroptosis was demonstrated by the rescuing of embryonic lethality of caspase-8/or *FADD* knockout with *RIP1* or *RIP3* knockout; the postnatal lethality observed in *RIP1* knockout mice, presumably due to loss of its cell protective function, was rescued by concurrent knockout of *caspase 8* and *RIP3*.

Several different pathways can also induce RIP3-dependent necrosis through distinct mechanisms. For instance, interferons (IFNs) can trigger RIP1–RIP3 complex-dependent necrosis [45], in this case through the transcriptional activation of the RNA-responsive protein kinase PKR [46,47]. Toll-like receptors 3 and 4 bypass RIP1, which mediates the activation of RIP3 by the TNF family cytokines, to instead directly activate RIP3 via another RHIM-containing protein known as TRIF [48,49]. RIP1, in this case, may even antagonize RIP3-mediated necrosis by concurrently activating caspase 8, which cleaves and terminates RIP3 signaling [46]. Although it is less well characterized, the RHIM-containing cellular protein DNA-dependent activator of interferon regulatory factors (DAI) [50] may also be able to activate RIP3 directly, following infection with murine cytomegalovirus (MCMV), presumably through a RHIM–RHIM interaction. A MCMV-encoded viral inhibitor of RIP activation (vIRA) [51] blocked such activity, effectively allowing the virus to avoid immune surveillance.

It is now clear that RIP3 kinase activation is the key initiation step for necrosis. The upstream signals are perceived by the RHIM domain of RIP3 through interaction with other RHIM-containing proteins (Figure 2). Activated RIP3 then transduces the necrosis signal by phosphorylating its substrate, MLKL.

Necrosis execution

Comprehensive biochemical, genetic, and chemical analyses have pointed to MLKL as the executioner of programmed necrosis (Figure 3). MLKL contains an N-terminal four α-helix bundle and a two α-helix linker that connects to the C-terminal kinase-like domain [52]. The α-helix bundle is the functional domain of MLKL; it is normally kept in an inactive state by the kinase-like domain [20,30]. Inactive MLKL exists as a monomer in the cytosol. When RIP3 is activated, it binds to the kinase domain of MLKL through its

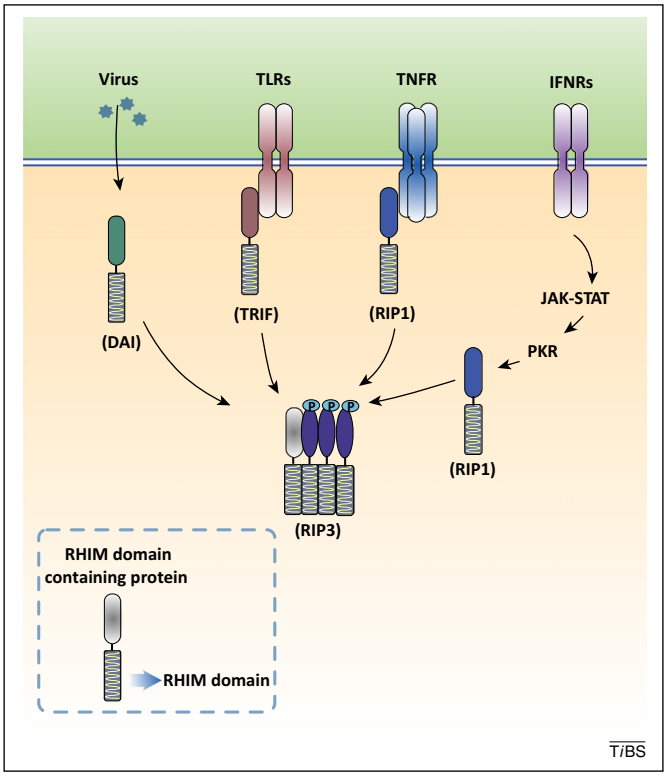

Figure 2. Various upstream stimuli activate receptor-interacting kinase 3 (RIP3) via pro-necrotic RHIM (RIP homotypic interaction motif)-containing proteins. Upon stimulation with tumor necrosis factor (TNF), TNF receptor 1 (TNFR1) recruits receptor-interacting protein kinase 1 (RIP1). When caspase 8 is inhibited by caspase inhibitors or by virally encoded proteins, the RHIM domains of RIP1 and RIP3 associate to form microfilament-like complexes. Other RIP3-activating stimuli also appear to function by promoting association between a RHIM domain containing-protein and RIP3. For example, the RHIM-containing cellular protein DNA-dependent activator of interferon regulatory factors (DAI; DLM-1/ZBP1) is a cytosolic DNA sensor that is activated by type I interferon (IFN-I) responses. Infection with mutant murine cytomegalovirus (MCMV) lacking functional viral M45-encoded inhibitor of RIP activation (vIRA/M45) led to RHIM-mediated interaction between DAI and RIP3 and necrosis of the infected cells. TIR-domain-containing adapter-inducing interferon-β (TRIF) is a TolI-like receptor (TLR3/TLR4) adaptor that mediates the induction of proinflammatory cytokines and type I interferons (IFNs) through the transcription factors NF-κB and interferon regulatory factor 3 (IRF3). Similar to TNF-induced necrosis, TLR-induced necrosis also requires RHIM-dependent binding between TRIF and RIP3. Type I or II IFNs induce RNA-responsive protein kinase PKR activation through the Janus kinase (JAK)-signal transducer and the activator of transcription (STAT) pathway. Activated PKR interacts with RIP1 and subsequently initiates RIP1/RIP3-dependent necrosis in the absence of Fas-associated protein with death domain (FADD) or caspase 8.

own kinase domain and phosphorylates the Thr357 and Ser358 sites of human MLKL [20] at an α-helix within the pseudokinase domain. This phosphorylation destabilizes the monomeric MLKL structure and drives its oligomerization, which enables the N-terminal helix bundle to bind phosphatidylinositol phosphate lipids (PIPs) [53,54] and the mitochondria-specific phospholipid, cardiolipin (CL) [53].

The oligomerized MLKL within the dying cells then translocates from the cytoplasm to the PIP- or CL-enriched membranes Consistent with this idea, a chemical inhibitor that disturbs the N-terminal domain functionality of MLKL blocks MLKL translocation to membrane compartments [20,53]. Association with PIP-enriched membranes is crucial for MLKL-dependent necrosis; when intracellular levels of PIPs were decreased by inhibiting the enzymes that generate them, MLKL-induced necrosis was attenuated [54].

Different PIPs may also direct MLKL to different cellular compartments, which may have important functional roles for necrosis induction. For example, plasma membranes have the most abundant PI(4)P and PI(4,5)P2. This could explain the vast damage on cell membranes after necrosis induction. CL is mostly distributed in the inner membranes of mitochondria, which can be exposed to (and, therefore, associate with) MLKL during necrosis. Despite the affinity of oligomerized MLKL for cardiolipin, the precise role of mitochondria in the execution of programmed necrosis has not been clearly defined. Since the mimetics of the mitochondrial intermembrane space protein Smac accelerate necrosis [17], and because a mitochondrial phosphatase PGAM5 interacts with the RIP3/MLKL containing necrosis death complex (necrosome) during necrosis, it is possible that mitochondria could function either upstream of necrosome formation or could participate in necrosis execution [55]. Induction of necrosis *in vitro*, using high doses of TNF, a caspase chemical inhibitor (z-VAD-FMK), plus Smac mimetics, may bypass or mask such functions of mitochondria. Similarly, other PIPs with distinctive distribution in organelles may recruit oligomerized MLKL to the corresponding membrane compartments. An antibody that specifically recognizes the phospho-Ser358 of human MLKL has been used to demonstrate that activated necrosomes translocate to multiple intracellular organelles and plasma membranes as cells undergo necrosis [53]. The identity of these organelles, as well as the consequences of MLKL targeting on necrosis, remain to be clarified.

Different modes of action were proposed to account for how oligomeric MLKL causes necrosis. It was reported that Ca^{2+} influx mediated by the calcium channel TRPM7 is required for necrosis execution [52]. A separate report indicated that a Na^{+} influx preceded necrotic cell death [56] is the death-inducing target of oligomerized MLKL. However, cells cultured in calcium- and sodium-free media were still able to undergo necroptosis, albeit a bit delayed [49]. Therefore, the calcium and sodium influx observed in necrotic cells could result from ion flow driven by normal salt gradients across the plasma membrane once membrane integrity has been compromised. In our opinion, although the observed ion influx potentially contributes to the necrotic phenotype, whether it is the trigger of cell death still needs further characterization.

The physiological and pathological roles of programmed necrosis

The specificity of RIP3 and MLKL in mediating necrotic cell death indicates that the physiological function(s) of this form of cell death are likely different to those of apoptosis. The fact that knockout of either gene in mice has little effect on animal development or fertility has allowed detailed analysis of the physiological and pathological roles of programmed necrosis.

Programmed necrosis is important in host antiviral responses. The first evidence of this was the demonstration that mice lacking the *RIP3* gene are much more susceptible to infection with vaccinia virus [18]. It appears that a viral-encoded caspase 8 inhibitor can switch the apoptotic response to necrosis in infected cells. If the cell expresses RIP3, the resulting necrosis will release DAMP molecules

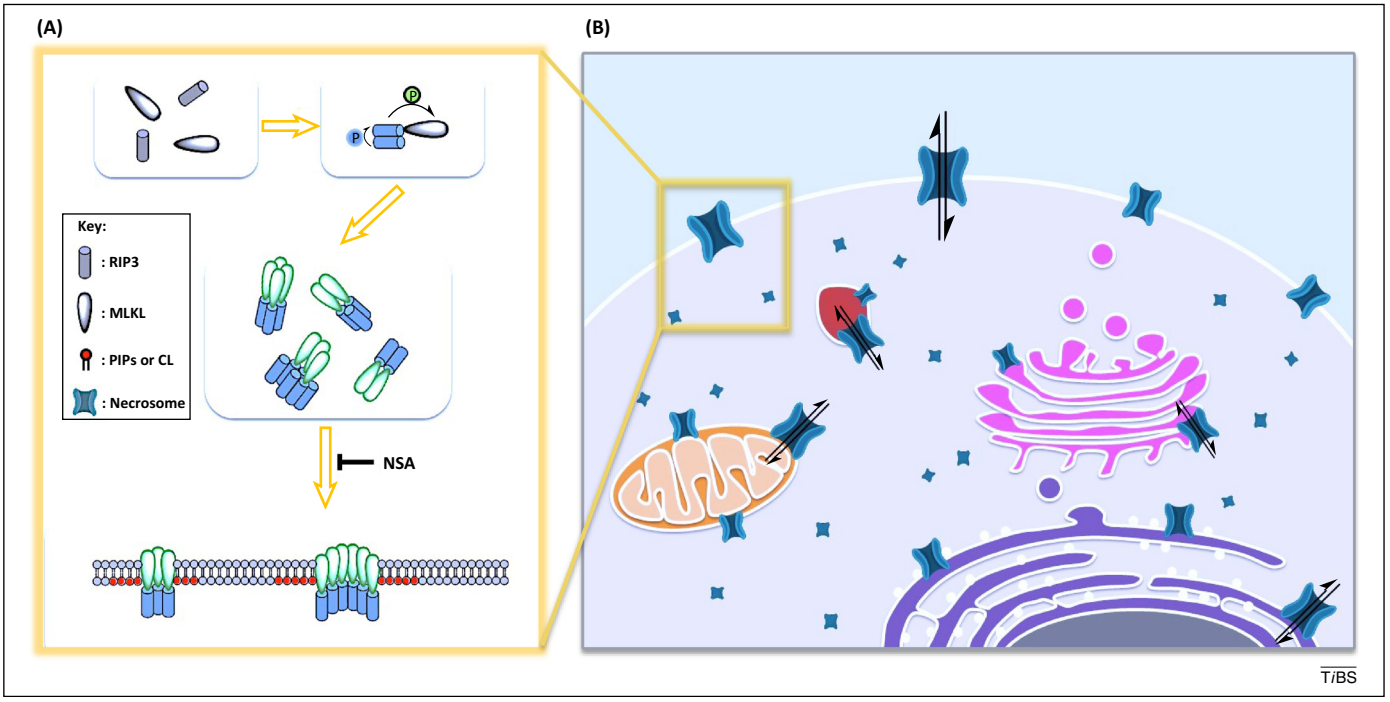

Figure 3. Mixed lineage kinase domain-like protein (MLKL)-containing necrosomes target membranes and disrupt membrane integrity. **(A)** Necrosome activation and membrane targeting. Upon induction of necrosis, cytosolic receptor-interacting kinase 3 (RIP3) aggregates to form microfilament-like complexes through its RIP homotypic interaction motif (RHIM) domains. Oligomerized RIP3 is then auto-phosphorylated at the Ser227 residue, increasing its binding affinity with its substrate MLKL. Once MLKL is recruited by RIP3 and is subsequently phosphorylated at Thr357/Ser358, the necrosomes are activated. Phosphorylation on MLKL promotes its oligomerization and releases the auto-inhibition of its N-terminal coil–coil domain that is imposed by the pseudokinase domain [70]. The oligomerization of MLKL enables MLKL to bind PIPs and CL. Necrosulfonamide (NSA) inhibits this process by targeting Cys86 of human MLKL. PIP, phosphatidylinositol lipids; CL, cardiolipin. **(B)** Necrosomes direct membrane rupture and organelle swelling. Different PIPs and CL orchestrate the translocation of MLKL to different membrane compartments, including the mitochondria (orange), the endoplasmic reticulum (purple), the Golgi (pink), and lysosome (red), where MLKL punches holes that ultimately lead to necrotic cell death.

that mobilize the host immune response against the virus. There could be multiple ways by which host cells activate necrosis when encountering a viral infection: for example, they could activate the autocrine TNF family of cytokines; they could activate RIP3 through toll-like receptors and TRIF; or they could directly activate RIP3 by sensing RHIM-containing viral proteins. Conversely, viruses can also counter necrosis through their own necrosis-inhibitory RHIM-containing proteins. For example, an interesting study showed that sustained infection by MCMV is dependent on a viral protein that interacts with RIP3 and blocks its necrosis-inducing function [51]. In addition to its function in antimicrobial infection, programmed necrosis may also be responsive to other forms of tissue injuries.

The *RIP3* knockout mice also provided an animal model to test the role of programmed necrosis in a variety of tissue-damage related diseases. So far, drug-induced liver injury [53], acute pancreatitis [17,19,57], ischemic reperfusion injury [58,59], TNF-induced systemic inflammatory syndrome [60], terminal ileitis [38,61], retinal detachment [62], cone cell degeneration [63], atherosclerosis [64], and neuronal loss in a mouse model of Gaucher's disease [65] have all manifested more severe morbidity in wild type animals compared to the *RIP3* knockout animals. These observations suggest that RIP3-mediated necrosis is part of the secondary damage inflicted by the immune system in response to initial chemical- or infection-caused injuries. With the development of a rabbit monoclonal antibody that can specifically recognize the phospho-Ser358 on human MLKL, it is now possible to probe human tissues from biopsy or autopsy samples using immunohistochemistry methods and specifically detect the activation of this pathway (Figure 4). Information gained from such studies will be valuable in efforts to develop therapeutic agents for the treatment of those diseases in which programmed necrosis may contribute to its etiology.

Necrosis inhibitors

A range of chemical screens for necrosis inhibitors have identified small molecule inhibitors that specifically block programmed necrosis (Figure 1). The first reported necrosis inhibitor was Nec-1, which blocks necroptosis by inhibiting RIP1 kinase activity [14,15,66]. Nec1 has been used to help researchers to distinguish whether or not an observed cell death is necroptotic. Newer versions of Nec-1 with improved target specificity and drug-like properties have since been developed [66,67]. Interestingly, Nec-1 also inhibits TLR3/4 mediated necrosis even when RIP1 kinase activity is not required. In this case, the kinase-inhibited RIP1 may function as a dominant-negative effector to compete binding to RIP3 with TRIF [47].

Given that RIP1 also contributes to other processes in addition to necroptosis, such as NF-κB signaling and apoptosis, the direct targeting of RIP3 or MLKL could potentially offer more specific inhibitors for the study and treatment of programmed necrosis. However, a recent report showed that a kinase-dead mutant form of RIP3 could activate apoptosis through RIP1-mediated caspase 8 activation [68], thus implying that inhibition of RIP3 kinase might result in unwanted apoptotic death. Although it is possible

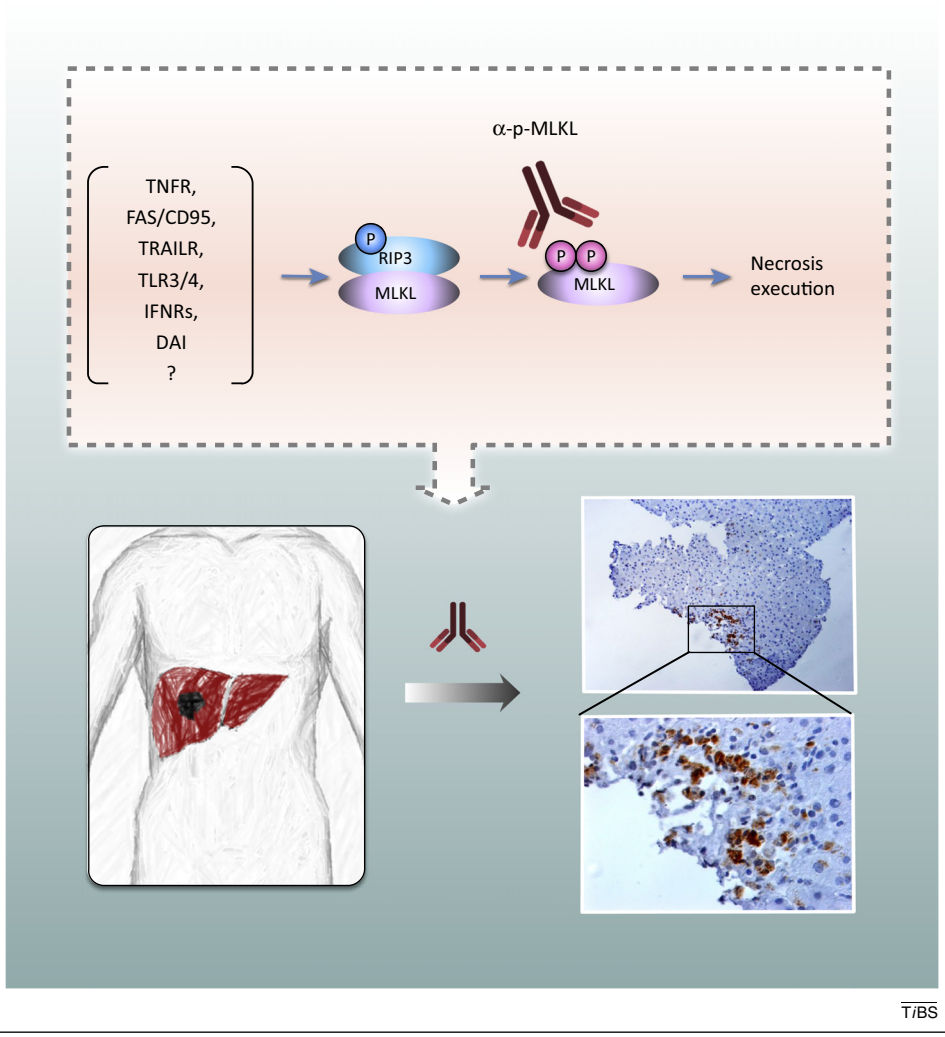

Figure 4. A monoclonal antibody against phospho-mixed lineage kinase domain-like protein (MLKL) provides physiological evidence of necrosis. Many death receptors involved in the initiation of necrosis have been identified, including TNFR (tumor necrosis factor receptor), CD95 (also known as FAS; which binds the ligand CD95L), TRAILR (TNF-related apoptosis-inducing ligand receptor also known as DR4 and DR5), TLR3/4 (TolI-like receptor 3 and 4), IFNR (IFNα/β receptor), and DAI (virus-mediated activation of DNA-dependent activator of IFN-regulatory factors, known as DNA protein sensors for short). Downstream of these receptors, receptor-interacting kinase 3 (RIP3)-dependent recruitment and phosphorylation of MLKL emerges as a common execution step in programmed necrosis when caspase 8 activity is compromised. Phosphorylated MLKL can directly disrupt membrane integrity, resulting in necrosis execution. Therefore, the monoclonal antibody against the phosphospecific form of MLKL (T357/S358) can be used as a necrosis marker in diseased human tissues *in vivo*. Immunohistochemical analysis using the antiphospho-MLKL antibody on liver samples from a patient with drug-induced liver injury shows positively stained cells (brown) in the injury region that group around the site of ensuing hepatocellular necrosis.

that the transgenically expressed kinase-dead form of RIP3 simply behaved as a gain-of-function mutant specific for that particular protein, if the conclusion generally holds true, the inhibition of RIP3 may cause severe side effects in animals.

MLKL may also be a possible target for the inhibition of necroptosis. Indeed, a chemical inhibitor called necrosulfonamide (NSA), which covalently modifies Cys86 of human MLKL [20], blocks necrosis without interfering in the RIP3-mediated phosphorylation of MLKL. NSA prevents necrosis by disrupting the proper oligomerization of MLKL; NSA-modified MLKL fails to move from the cytosol to the membranes. However, since NSA targets a surface cysteine residue rather than a specific binding pocket, there is not much room to further improve binding affinity and specificity. Therefore, it may only be used as a tool compound for research purposes. It will be interesting to see if targeting the kinase-like domain or the N-terminal helix bundle will yield more specific and/or more potent inhibitors of MLKL [52,69].

As the role of programmed necrosis in human diseases becomes more and more clear, the demand for inhibitors will increase. These inhibitors should also be helpful to researchers seeking to further characterize cell death *in vivo*.

Concluding remarks

Similar to the roles of Bax/Bak and caspases in apoptosis execution, MLKL seems to be the core biochemical machine that executes necrosis. The RIP3-mediated phosphorylation of MLKL converts MLKL from an innocuous cytosolic protein into membrane-disrupting oligomers that execute necrotic death. Future research should clarify if MLKL-mediated membrane disruption is sufficient to cause cell death or whether it needs any accomplices to accomplish this.

Apoptosis is negatively regulated at several levels. The best-characterized regulation of apoptosis involves anti-apoptotic Bcl-2 like proteins and the inhibitor of apoptosis proteins (IAPs). To date, knowledge about either the negative or positive regulators of programmed necrosis remains glaringly incomplete. The identification of such regulators should be a fertile ground for future discoveries.

Moreover, because there are animal models lacking RIP3 and MLKL, antibodies that specifically identify the activation of this pathway and small molecules that inhibit this pathway might be more easily developed, and the physiological and pathological roles of programmed necrosis can now be defined in greater detail. Therapeutic interventions for human diseases based on the knowledge of programmed necrosis may soon appear on the horizon.

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