REVIEW ARTICLE

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Necroptosis

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As early as the mid-19th century, Rudolf Virchow taught that necrosis is a recognizable form of cell death; since then, pathologists have identified necrosis as both a cause and a consequence of disease. A century later, another form of cell death, apoptosis, was defined, and we now understand that this process is driven by a set of molecular mechanisms that "programs" the cell to die. It has often been assumed that necrosis is distinct from apoptosis, in part because of the belief that necrosis is not programmed by molecular events. It is now clear, however, that in some contexts, necrotic cell death can be driven by defined molecular pathways. Here, we discuss one such process, a type of necrotic cell death called "necroptosis,"[1-4] and its role in disease (see Glossary). Although some investigators have used this term to refer to any form of active necrosis, we follow recent recommendations,[5] using it to denote necrotic cell death dependent on receptor-interacting protein kinase 3 (RIPK3) (Fig. 1). With our understanding of the molecular basis of necroptosis and other forms of regulated necrosis, along with the availability of inhibitors of some forms of necrosis (including necroptosis), a neglected therapeutic option emerges: it is possible to therapeutically interfere with necrosis.

Although necroptosis may have evolved as a line of defense against intracellular infection,[6,7] recent studies implicate it in a variety of disease states. Necroptosis is of central pathophysiological relevance in myocardial infarction and stroke,[8,9] atherosclerosis,[10] ischemia–reperfusion injury,[11,12] pancreatitis,[2,4,13] inflammatory bowel diseases,[14,15] and a number of other common clinical disorders. At the molecular level, intracellular assembly of a highly regulated complex, the necrosome, can be triggered by death receptors (e.g., tumor necrosis factor [TNF] receptor 1 [TNFR1]),[16-18] by cell-surface toll-like receptors,[19-21] by DAI (which may act as a cytoplasmic viral RNA sensor),[22,23] and probably by other signals.

Induction of Necroptosis

Apoptotic cell death involves the engagement of pathways that lead to the activation of caspase proteases, which ultimately cause the morphologic features of this type of cell death. In contrast, necroptosis was first recognized as a caspase-independent form of cell death that can be triggered by treatment with TNF only in the presence of a pan-caspase inhibitor, such as zVAD fluoromethyl ketone.[24] Before that time, we understood that TNF causes apoptosis through the induction of protein interactions that result in the activation of caspase 8; however, necroptosis requires that the function of caspase 8 be inhibited or disrupted. Several of the upstream signaling elements of apoptosis and necroptosis are shared, and sensitivity to each death pathway is regulated (sometimes in opposing ways) by an overlapping cluster of regulatory molecules, such as FLIP,[25] the deubiquitinases A20 and cylindromatosis, and the cellular inhibitors of apoptosis proteins cIAP1 and cIAP2.[26,27]

Other death receptors[28] and toll-like receptors[29] were shown to induce necrop-

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Glossary

Cyclosporine: Best known for its immunosuppressive quality. In addition to conferring immunosuppression, cyclosporine is a potent inhibitor of mitochondrial permeability transition.

DAI (DNA-dependent activator of interferon regulatory factors): A trigger of intracellular assembly of the necrosome. DAI acts as a cytoplasmic viral RNA sensor.

Damage-associated molecular pattern (DAMP): DAMPs are released from necrotic cells, presumably in any type of necrosis. Therefore, DAMP release is not restricted to necroptosis and triggers most components of the immune response — for example, in solid-organ transplantation.

FLIP (Fas-associated death domain–like interleukin-1β–converting enzyme [FLICE]–like inhibitory protein): Forms a heterodimer with caspase 8, and the heterodimer prevents caspase 8–mediated apoptosis and mediates the antinecroptotic properties of caspase 8.

Mitochondrial permeability transition: A common increase in permeability of both the inner and outer mitochondrial membrane, which may result in mitochondrial swelling, production of reactive oxygen species, nicotinamide adenine dinucleotide depletion, and subsequent necrotic cell death. Necroptosis occurs independently of mitochondrial permeability transition.

MLKL (mixed lineage kinase domain–like): A pseudokinase that is phosphorylated by RIPK3 and has a causal role in necroptosis.

Necroptosis: RIPK3-dependent regulated necrosis.

Necrosome: Supramolecular complex that consists of RIPK3 and other cell death–mediating molecules, such as RIPK1, dependent on the necroptotic trigger.

Receptor-interacting protein kinase 1 (RIPK1): One of the upstream triggers of the necrosome.

Receptor-interacting protein kinase 3 (RIPK3): The key molecule in necroptotic cell death.

RHIM (receptor-interacting protein homotypic interacting motif): A protein domain that is typically involved in necroptosis and that is found in DIA, TRIF, RIPK1, and RIPK3. The RHIM domain therefore plays a role in virus recognition and allows RIPK3 to interact with DAI, TRIF, and RIPK1, its upstream partners in the necroptotic pathway.

TRIF (toll–interleukin-1 receptor [TIR]–domain–containing adapter-inducing interferon-β): A RHIM-containing protein capable of triggering necroptosis downstream of toll-like receptors.

TUNEL (terminal deoxynucleotidyl transferase dUTP biotin nick end labeling): An assay for the detection of DNA double-strand breaks, such as those that occur during apoptosis. Although TUNEL staining is often used as an assay for apoptosis, it can also be used to detect cells that have died by other mechanisms, especially in vivo.

zVAD fluoromethyl ketone (benzyloxycarbonyl-Val-Ala-Asp[OMe] fluoromethyl ketone): An unspecific pan-caspase inhibitor.

tosis, and intracellular triggers of necroptosis, such as DAI[23] and protein kinase R,[30] were subsequently identified (Fig. 2). TNFR1 ligation by TNF induces signaling through the nuclear factor κB (NF-κB) pathway that involves the polyubiquitination of receptor-interacting protein kinase 1 (RIPK1) and of NF-κB essential modulator (NEMO).[31] On deubiquitination of the K63 and linear ubiquitin chains of RIPK1[31] by deubiquitinases,[32,33] RIPK1 loses its default prosurvival function and promotes cell death. Ligated TNFR1 recruits the adapter protein TNF-receptor–associated death domain (TRADD) to associate with the adapter Fas-associated death domain (FADD),[34] which then binds to procaspase 8, a protease that is autocatalytically activated on homodimer formation. Conversely, FLIP, a protein that is structurally related to caspase 8 but has no protease activity, forms a heterodimer that prevents both caspase 8–mediated apoptosis and necroptosis.[17,35] The caspase 8–FLIP heterodimer predominates in cells with FLIP expression, which is induced on NF-κB activation. When either caspase 8 or FLIP is lost or there is interference with the activation or function of caspase 8, RIPK1 forms an intracellular complex with RIPK3 to assemble the necrosome,[13,36,37] an amyloid-like structure[38] that acts as the transducer of the necroptotic signal. Downstream of RIPK3 is another protein, MLKL,[13,36,37,39,40] which is a pseudokinase that causes necroptosis in a manner that has not been unambiguously elucidated.

Deletion of Fadd,[41] Flip,[42] or caspase 8[43] in

mice results in embryonic death on day 10.5; embryonic death is prevented if the deletion is applied to mice that are already deficient in Ripk3.[35,44] Tissue-specific deletion of Fadd or caspase 8 also causes disease (depending on the type of tissue) that involves cell death, and this is also prevented by ablation of Ripk3.[14,15] Therefore, it appears that a critical function of the FADD–caspase 8–FLIP complex is the prevention of RIPK3-mediated necrotic cell death (although alternative interpretations are discussed below). It is on this basis that we have defined necroptosis as a form of regulated necrosis that follows an intracellular signaling cascade dependent on RIPK3. However, the downstream mediators in the necroptotic pathway are incompletely understood, although it is tempting to speculate that plasma-membrane channels are involved in the rapid swelling of necroptotic cells that results in plasma-membrane rupture.

Unlike apoptosis, in which several of the highly immunogenic intracellular proteins are sequestered in the dead cell, necroptosis is a strong trigger of innate and adaptive immune responses.[45] But why would immunogenic cell death be preserved in higher organisms? The answer may involve the recognition of and response to microbes. RIPK3 can interact with other proteins through a RHIM domain, which is present in both RIPK1 and RIPK3. To date, only four known RHIM-containing proteins — RIPK1, RIPK3, DAI, and TRIF — have been identified in the human genome (although this may be a function of our limited ability to recognize this motif). TRIF is capable of triggering necroptosis after ligation of toll-like receptors 3 and 4,[21] and DAI integrates viral signals into the necroptotic pathway.[7] Indeed, infection with vaccinia virus, which expresses a viral caspase inhibitor, was found to be lethal in Ripk3-deficient mice but not in wild-type mice.[1,46] In addition, both apoptosis and necroptosis can be induced by type I and type II interferons, which promote the death and removal of virally infected cells.[30] Several viruses and intracellular bacteria express proteins that interfere with the activation of caspase 8, sensitizing cells to necroptosis.[47]

We can therefore hypothesize that necroptosis provides higher vertebrates with a defense mechanism against such intracellular invaders, and this hypothesis is further supported by the identification of viral inhibitors of necroptosis.[7,35] Similarly, loss of FLIP leads to cell death by both apoptosis and necroptosis. Because FLIP undergoes rapid protein turnover and is expressed in response to NF-κB activation, anything that blocks protein synthesis or interferes with NF-κB might sensitize cells to die. Regu-

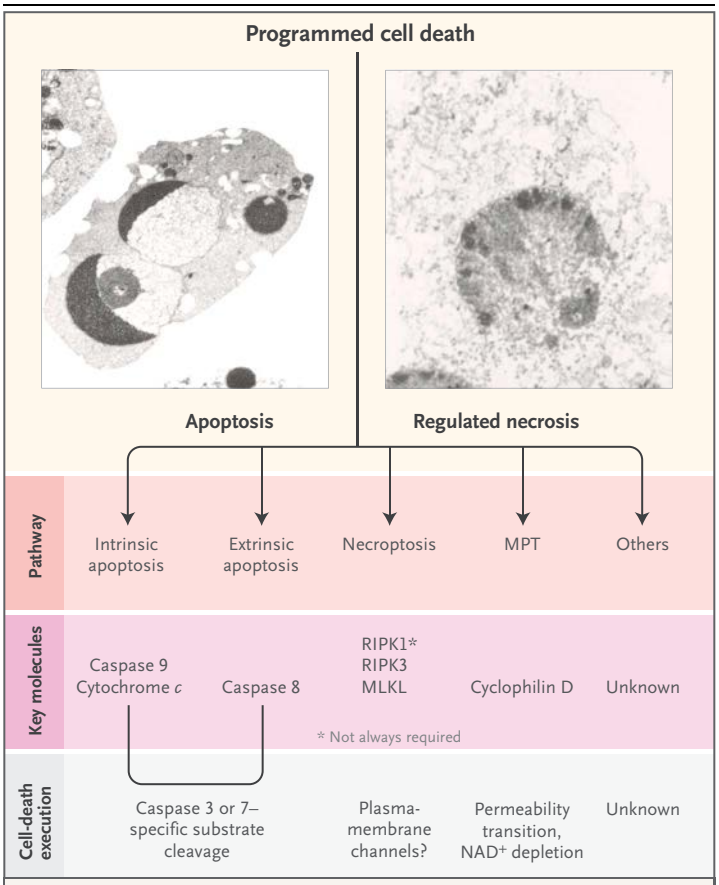

Figure 1. Mediation of Programmed Cell Death by Apoptosis or Regulated Necrosis.

The term "programmed cell death" was widely used synonymously with "apoptosis" until necrotic cell death was shown to depend on genetically defined signaling pathways. Although the role of caspase-mediated apoptosis in diseases has been revealed in detail over the past three decades, the contribution of regulated necrosis to the pathophysiological basis of diseases was investigated only recently. Pathways of regulated necrosis include necroptosis, which is dependent on receptor-interacting protein kinase 3 (RIPK3), and regulated necrosis mediated by mitochondrial permeability transition (MPT), which involves cyclophilin D–dependent opening of the MPT pore. Although MPT and necroptosis have been shown to represent two distinct pathways, other emerging signaling cascades of regulated necrosis have been described, and it remains unclear to what extent such pathways may have overlapping mechanisms. NAD^+ denotes nicotinamide adenine dinucleotide.

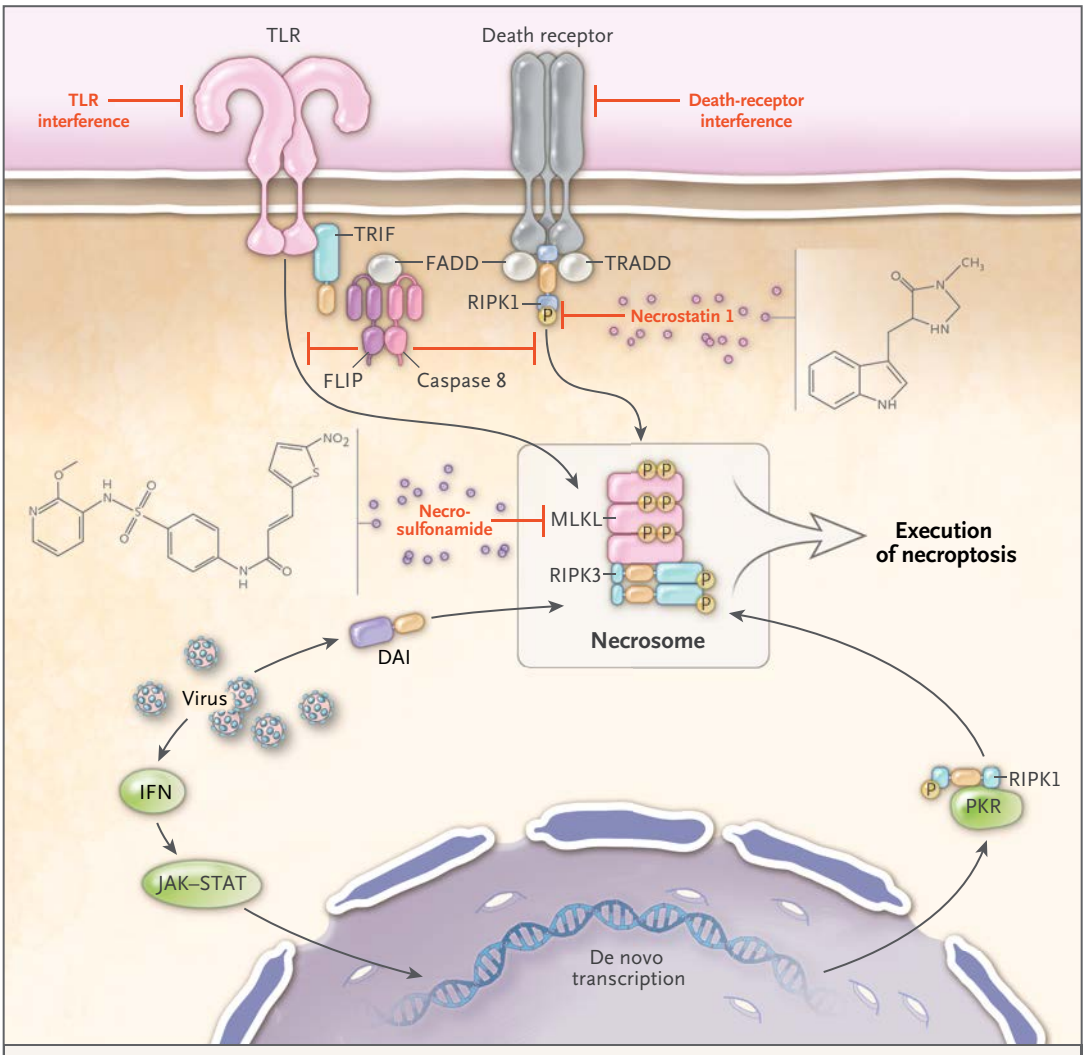

Figure 2. Activators of the Necrosome as Therapeutic Targets.

Various stimuli lead to activation of the supramolecular necroptosis-inducing complex, referred to as the necrosome. Initially, studies of necroptosis used models of death-receptor stimulation in the presence of caspase inhibition (not shown). The intracellular adapter molecules Fas-associated death domain (FADD) and TNF-receptor–associated death domain (TRADD) recruit receptor-interacting protein kinase 1 (RIPK1), which subsequently undergoes an incompletely understood series of ubiquitination, deubiquitination, and phosphorylation (P) events before exposing its RHIM domain to recruit RIPK3. RIPK1, RIPK3, and MLKL are phosphorylated during the assembly of the necrosome. Within the human genome, RIPK1, RIPK3, and two other proteins have RHIM domains. One of these is TRIF, an intracellular signal transducer that is capable of activating the necrosome downstream of toll-like receptors (TLRs), which are triggered by microbial molecules. The fourth RHIM-domain protein, DAI, integrates signals from viral RNA sensors into the necrosome. Finally, viral infection is accompanied by production of interferon (IFN), triggering new protein kinase R (PKR) synthesis that is dependent on Janus kinase (JAK) and signal transducer and activator of transcription (STAT); PKR phosphorylates FADD, which in turn directly interacts with RIPK1, inducing necrosome formation. Death-receptor–mediated necroptosis involves deubiquitination of RIPK1, the kinase domain of which is targeted by necrostatin 1. Second-generation RIPK1 inhibitors, such as necrostatin 1s, a stable version of necrostatin 1 that is more potent at lower concentrations, might have less severe side effects. Necrosulfonamide inhibits MLKL and prevents the activity of the necrosome in human cells. In addition, RIPK3 inhibitors, death-receptor antagonists, and plasma-membrane channel blockers might be attractive therapeutic targets.

lation of the necroptotic pathway by proteins such as acid sphingomyelinase[48] and the mitochondrial phosphatase phosphoglycerate mutase 5[40] may also contribute to the control of this process under a variety of conditions, but further evidence of their involvement is needed. Although the necroptotic pathway in humans may be beneficial in providing a defense against some infections, this process may have harmful effects in a number of pathologic states.

CONTRIBUTION OF NECROPTOSIS TO PATHOPHYSIOLOGICAL PROCESSES

The development of the RIPK1 inhibitor necrostatin 1 (a compound that was later found to be identical to a previously reported indoleamine 2,3-dioxygenase inhibitor[49]) has stimulated research on necroptosis. The activity of this agent was taken as evidence supporting a role of necroptosis in neurologic disorders (Table S1 in the Supplementary Appendix, available with the full text of this article at NEJM.org), and the first disease model in which the role of necroptosis was investigated was ischemic brain injury.[50] Neuronal tissue is also associated with necrostatin 1–inhibitable cell death in clinically related models of brain damage, including controlled cortical impact[51] and neonatal hypoxia–ischemia, in which necrostatin 1 was found not only to provide protection from oxidative damage but also to prevent the subsequent debilitating immune-cell infiltration.[52,53] Necroptosis has been observed in microglia treated with caspase inhibitors,[54] and this mechanism was interpreted as a strategy for preserving neurons; however, detailed mechanisms in vivo are not understood. Necrotic cell death is also a hallmark of retinal detachment[55] and retinal ischemic cell death,[56] and it has been suggested that in this specialized compartment, apoptosis and necroptosis are triggered simultaneously.[55] More recently, it was proposed that cones, but not rods, undergo necroptosis in a genetic model of retinitis pigmentosa.[57] These findings provided preliminary support for the concept that apoptosis and necroptosis are not mutually exclusive programs and may occur in the same organ.

Several independent studies have related the effects of necrostatin 1 to genetic ablation of Ripk3 in mouse models of ischemic organ damage and ischemia–reperfusion injury in the heart and the kidney (Table S1 in the Supplementary Appendix). Necrostatin 1 was found to have a striking protective effect in models of brain ischemia[50]; its effect in the prevention of cardiac remodeling after myocardial infarction[12] and the prevention of myocardial[9] and renal[11] ischemia–reperfusion injury was marked but not as pronounced. Administration of necrostatin 1 in mice 30 minutes after reperfusion did not provide protection from kidney ischemia–reperfusion injury,[11] suggesting the presence of off-target effects of necrostatin 1, a rapid assembly of the necrosome, or another role for Ripk1 in endothelial cells of peritubular capillaries. The last of these might be of particular interest, because necroptosis has not been observed in primary kidney cells, neurons, retinal cells, or cardiomyocytes. Nevertheless, Ripk3-deficient mice are protected from ischemia–reperfusion injury, and treatment of Ripk3-deficient mice with necrostatin 1 does not provide further protection.[58] Changes in parenchymal blood flow might explain the benefit of blocking necroptosis in models of ischemia–reperfusion injury, because necrostatin 1 may influence capillary diameters.[59] It has been demonstrated that glomerular endothelial cells, in contrast to renal tubular cells, mesangial cells, and podocytes, express high levels of Ripk3,[11] which may correlate with the likelihood that cells will undergo necroptosis on death-receptor ligation.[2] It will be of interest to determine whether the involvement of the necroptotic pathway in ischemia–reperfusion injury can be confirmed in Mlkl-deficient mice.[13] Ultimately, it will be important to develop tissue-specific Ripk3 or Mlkl deletion models to identify the tissues that are most relevant to the role of necroptosis in ischemia–reperfusion injury.

Necroptosis may also be associated with disorders of the skin and intestinal epithelium. A Ripk3-dependent dermal chronic inflammatory phenotype results from conditional deletion of Fadd[60] or caspase 8[61] from keratinocytes in mice, a phenomenon that is partially prevented when the cells are also Tnfr1 deficient.[60] Although other dermatologic disorders have not yet been linked with necroptosis, it is tempting to speculate that regulated necrosis triggers skin infections, such as atopic dermatitis. Apart from atopic

dermatitis, chronic proliferative dermatitis was described in mice with a deficiency of the Ripk1 regulator Sharpin,[62] a component of the linear ubiquitin chain–assembly complex, and the dermatitis was prevented when Sharpin deficiency was combined with Tnfr1 deficiency.[31] However, it is not yet clear how Ripk3-dependent necroptosis is involved in this inflammatory reaction.

As with epithelial cells of the skin, specific depletion of caspase 8 or Fadd from intestinal epithelium results in spontaneous necroptosis and pathologic changes that are morphologically similar to those seen in inflammatory bowel diseases, especially Crohn's disease[14,15] (Table S1 in the Supplementary Appendix). In contrast, crossing mice that had Fadd or caspase 8 depletion in the intestinal epithelium with Ripk3-deficient mice completely prevented these pathologic changes. Similarly, ablation of the Ripk1 deubiquitinase A20 sensitizes mice to lethal colitis, and this effect is associated with TUNEL-positive Tnf-mediated death of the intestinal epithelial cells.[14,15] Although this cell death has been interpreted as apoptosis, it remains possible that necroptosis is involved. Mechanistically, these inflammatory conditions may be due to the high immunogenicity of necrotic cells, the loss of barrier function that occurs on such cell death, or both.[45] In contrast to Crohn's disease, which is characterized by chronic inflammation, necrotizing pancreatitis is clinically characterized by acute inflammation. The preclinical model of pancreatitis induced by ceruletide (formerly cerulein) was the first description of necroptosis in the gastrointestinal tract.[2,4] Because necrotizing pancreatitis is a devastating disorder for which conventional treatment is limited to intensive care and the administration of fluids and anesthetics, the potential to interfere with necroptosis has raised hopes for therapy. However, whereas marked protection from ceruletide-induced pancreatitis was seen in *Ripk3*$^{-/-}$ mice,[2,4] necrostatin 1 administration increased serum lipase and amylase levels as well as histologic damage scores.[63] Recently, *Mlkl*$^{-/-}$ mice were reported to be protected from ceruletide-induced pancreatitis,[13] yet another finding that sustains the debate about treatment strategies. A possible explanation for this recent finding is the short half-life of necrostatin 1, and studies of second-generation RIPK1 inhibitors or MLKL inhibitors are expected to clarify this issue.

NECROPTOSIS IN SOLID-ORGAN TRANSPLANTATION

One clinical situation in which interference with necroptosis is predicted to have therapeutic relevance is solid-organ transplantation. Damage-associated molecular patterns (DAMPs) are released from necroptotic cells and trigger a strong immune response.[45,64] It is conceivable that DAMPs, by their ability to activate both innate and adaptive immunity, promote many of the harmful immunologic responses observed in solid-organ transplantation; interference with necroptosis might be beneficial because the prevention of necrotic cell death minimizes the loss of functional parenchymal cells in the transplanted organ and minimizes the release of DAMPs, which would reduce proinflammatory responses that activate rejection pathways. In support of this concept, a recent study showed protection of Ripk3-deficient kidneys in a mouse model of allotransplantation, with a strong survival benefit.[65] In that model, inhibition of caspase 8 by small interfering RNA up-regulated necroptosis and reduced renal allograft survival, whereas in comparison with kidneys from wild-type mice, Ripk3-deficient allografts had better function and longer rejection-free survival.[65] These experiments might be relevant to clinical transplantation, pointing the way toward use of machine perfusion systems to saturate donor organs with necroptosis-inhibiting drugs as a potential strategy for preventing organ rejection before implantation. However, a protective effect of necrostatin 1 or its derivatives has not been analyzed thus far in a transplantation model, and blockade of necroptosis may have side effects in patients who are receiving immunosuppressive therapy after undergoing transplantation, since they are susceptible to viral infection — for example, cytomegalovirus infection[66] — even without blockade of necroptosis. Nevertheless, the potential to inhibit necroptosis will probably have an effect on organ transplantation.[67] Further investigations are needed before clinical trials can begin to specifically target necroptosis and to elucidate the mechanisms by which RIPK3 deficiency benefits transplanted organs. Given the protection from ischemia–reperfusion injury that is also conferred by RIPK3 deficiency, it will be important to clearly separate primary cell death from secondary organ damage mediated by infiltrating immune cells.[45] Conventional clinical strategies are focused exclusively on the latter.

BALANCE BETWEEN NECROPTOSIS AND INFLAMMATION

TNF-shock models have suggested that Ripk1 and Ripk3 may be involved in Tnf-induced signaling in endothelial cells (Table S1 in the Supplementary Appendix). In vivo intravenous injection of Tnf causes Tnfr1-dependent apoptotic detachment of enterocytes and kills mice within 48 hours.[63,68] Addition of the pan-caspase inhibitor zVAD–fluoromethyl ketone does not have a protective effect; in fact, it accelerates the lethal effects in this model, referred to as hyperacute Tnf shock; all mice die within 24 hours. Ripk3 deficiency provides partial protection from hyperacute Tnf shock.[63,69] The hyperacute Tnf-shock model does not accurately mimic sepsis; the more widely used sepsis model is that of cecal ligation and puncture.[70] However, whereas one study showed that necrostatin 1 was protective in the cecal ligation and puncture model,[69] others showed that necrostatin 1 further accelerated the time to death in the hyperacute Tnf-shock model,[63,71] and mice deficient in Ripk3 or Mlkl appear to have no benefit in the cecal ligation and puncture model.[13,63] The role of necroptosis in sepsis therefore remains an open question.

The involvement of RIPK3 in pathophysiological processes is not an unequivocal demonstration of a role for necroptosis, even if our definition of the term is used. RIPK3 activation and its regulation by RIPK1 and the FADD–caspase 8–FLIP complex may have direct inflammatory effects that are independent of necroptotic cell death. It has been suggested that activation of RIPK3 directly participates in inflammation mediated by the DNA sensor retinoic acid–inducible gene 1 (RIG-I)[72] and by the NLRP3 inflammasome.[39] At present, it is not possible to formally separate such putative effects from those of necroptosis and the release of DAMPs, especially since in at least one case, the proinflammatory effect was observed to also depend on MLKL.[39] Ultimately, determination of the direct contribution of RIPK3 to inflammation versus its indirect contribution through necroptosis may have to await elucidation of the final effector mechanism in necroptosis. Therefore, although we believe that the effects of RIPK1 inhibition and RIPK3 ablation in pathophysiological contexts are most likely due to effects on necroptosis, we recognize that alternative explanations are possible. Nevertheless, this distinction may ultimately be irrelevant to clinicians, because interference with RIPK3 activation, function, or both is likely to have therapeutic benefits regardless of the ultimate pathologic mechanism. It is in this context that we continue our discussion of therapeutic intervention.

THERAPEUTIC STRATEGIES FOR THE PREVENTION OF NECROPTOTIC DISEASES

In theory, interference with necroptosis is possible at the levels of the receptor, RIPK1, RIPK3, MLKL, the assembly of the necrosome, and undefined intermediate and downstream mechanisms (Fig. 2) that may ultimately lead to cellular swelling and plasma-membrane rupture. With these targets only recently recognized, the major focus has been on necrostatin 1. Because of the outstanding specificity of necrostatin 1 to RIPK1,[73] some authors have referred to the inhibition by necrostatin 1 as a definition of necroptosis.[21] As the structural interaction of necrostatin 1 with the RIPK1 kinase domain has been elucidated,[74] novel interpretations of the inhibition of necroptosis by necrostatin 1 have emerged. Ripk1-deficient mice die perinatally,[75] and it remains formally possible that RIPK1 acts as an inhibitor of necroptosis unless its kinase activity is engaged and that necrostatin 1 might stabilize RIPK1 in its inhibitory state.

Apparently, there are effects of necrostatin 1 that are not related to cell death,[59] and drawbacks regarding the clinical applicability of necrostatin 1 have been reported, because it appears to accelerate death in some models in which RIPK3 ablation is beneficial (discussed above). Fortunately, second-generation RIPK1 kinase inhibitors with higher affinity and specificity, such as necrostatin 1s, have been identified,[71] and the acceleration of disease progression was not observed when necrostatin 1s was used as treatment for Tnf shock in mice.[71] Further studies in other models are expected to clarify the mechanisms of this dichotomy.

Another attractive treatment approach emerged from studies of a direct inhibitor of human MLKL, necrosulfonamide.[36] Although the clinical application of necrosulfonamide will depend on further analysis of its specificity and pharmacokinetic properties, its activity provides evidence that MLKL may serve as a drug target in principle. RIPK3 inhibitors may also have therapeutic poten-

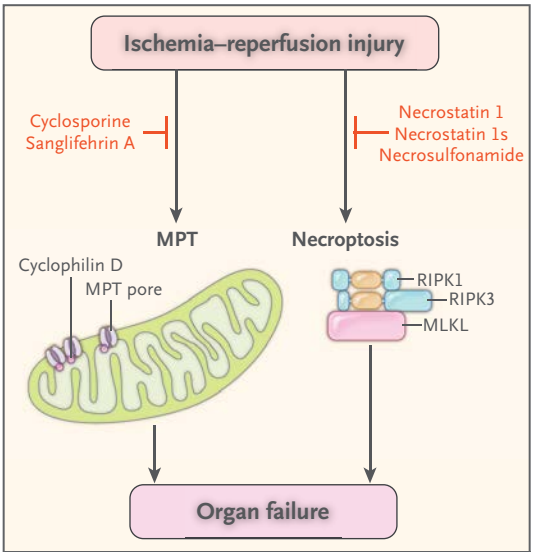

Figure 3. Combination Therapy Targeting Regulated Necrosis.

In ischemia–reperfusion injury, independent pathways of regulated necrosis contribute additively to overall organ damage. Whereas formation of the necroptotic pathway involves RIPK1-triggered assembly of the necrosome (MLKL–RIPK3), MPT independently contributes in a cyclophilin D–dependent manner. Necroptosis may be blocked by necrostatin 1, second-generation necrostatin 1s, or the MLKL inhibitor necrosulfonamide. MPT is inhibited by cyclosporine or sanglifehrin A. In experimental models, combination therapy with necrostatin 1 and sanglifehrin A confers significantly stronger protection than does either agent alone.

tial, but apart from phosphorylation of MLKL, relevant targets of RIPK3 remain unknown. Identification of the putative necroptosis-mediating plasma-membrane channels downstream of the necrosome might define promising targets. In addition to small molecules and inhibition of plasma-membrane channels, successful strategies in clinical use include specific inhibition of an event-triggering receptor. Although in vitro studies have indicated that TNFR1 is the primary inducer of necroptosis, this has not been confirmed in vivo in studies that investigated the renal ischemia–reperfusion injury model with the use of mice deficient in Tnfr1[76] or deficient in both Tnfr1 and Tnfr2.[77] Therefore, receptors and signals other than TNFR1 are actively being explored for their ability to induce necroptosis. Promising candidates for such alternatives include other death receptors, toll-like receptors, and intracellular receptors.

OTHER PATHWAYS OF NECROSIS WITH THERAPEUTIC POTENTIAL

Apart from necroptosis, other pathways of regulated necrosis have been identified, but only a few of these have been mechanistically separated from the necroptotic core machinery. Mitochondrial permeability transition is a process that induces necrotic cell death dependent on the mitochondrial matrix protein cyclophilin D, an intracellular target of cyclosporine. Mice deficient in cyclophilin D are partially protected from ischemia–reperfusion injury in various organs, and cyclosporine has been shown to prevent ischemic myocardial organ damage in humans.[78] Although recent studies in zebra fish suggest that cyclophilin D might be a downstream target of the necrosome,[48] mitochondrial permeability transition and necroptosis are now clearly understood to be separate pathways — as demonstrated, for example, by studies of ischemia–reperfusion injury in mice deficient in both cyclophilin D and Ripk3.[58] Therapeutically targeting these two pathways of regulated necrosis with the use of combination therapy (Fig. 3) has resulted in strong additive protection from ischemia–reperfusion injury in initial experiments,[58] and future strategies to inhibit regulated necrosis might best be based on interference with multiple pathways.

Undoubtedly, cyclosporine has revolutionized solid-organ transplantation and has been widely accepted because of its immunosuppressive properties. However, studies of isolated mitochondria clearly show that cyclosporine prevents mitochondrial permeability transition.[58,79-81] The immunosuppressive capacity of cyclosporine, when administered after reperfusion, is clearly less potent than other immunosuppressive agents, such as tacrolimus, sirolimus, and mycophenolate mofetil.[82] However, with the exception of tacrolimus,[83] none of these compounds prevented graft loss as effectively as cyclosporine in clinical trials.[84] One might therefore speculate that clinicians have already been exploiting the capacity of cyclosporine to block mitochondrial permeability transition as a means of reducing the inflammatory response and regulated necrosis and thus improving overall graft survival. Taken together, studies of cyclosporine indicate that regulated necrotic cell death — that is, necroptosis, mitochondrial permeability transi-

tion, or both — occurs in solid-organ transplantation, rendering this clinical field an ideal area for further investigations of the clinical potential of preventing necrosis.

Semantically, it is important to understand "regulated necrosis" as an umbrella term that encompasses mitochondrial permeability transition, necroptosis (Fig. 1), and other pathways that have been identified, such as ferroptosis,[85] pyroptosis,[86] PARP1-mediated regulated necrosis,[87] NADPH-oxidase–mediated regulated necrosis,[88] and lysosomal membrane permeabilization.[89] However, it is not clear to what extent these pathways of necrosis are distinct, nonoverlapping cell-death programs. Specific biomarkers must be identified in order to unravel them. The continuing elucidation of the molecular subroutines of various forms of regulated necrosis, including necroptosis, and the efficient design of combination therapies hold promise for our ability to control regulated necrosis in clinical settings.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

REFERENCES

1. Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell 2009;137:1112-23.
2. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009;137:1100-11.
3. Vandenabeele P, Galluzzi L, Vanden Berghe T, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol 2010; 11:700-14.
4. Zhang DW, Shao J, Lin J, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science 2009;325: 332-6.
5. Galluzzi L, Vitale I, Abrams JM, et al. Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. Cell Death Differ 2012;19:107-20.
6. Cho Y, McQuade T, Zhang H, Zhang J, Chan FK. RIP1-dependent and independent effects of necrostatin-1 in necrosis and T cell activation. PLoS One 2011;6(8): e23209.
7. Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. Curr Opin Virol 2013;3:296-306.
8. Degterev A, Hitomi J, Germscheid M, et al. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat Chem Biol 2008;4:313-21.
9. Smith CC, Davidson SM, Lim SY, Simpkin JC, Hothersall JS, Yellon DM. Necrostatin: a potentially novel cardioprotective agent? Cardiovasc Drugs Ther 2007;21:227-33.
10. Lin J, Li H, Yang M, et al. A role of RIP3-mediated macrophage necrosis in atherosclerosis development. Cell Rep 2013; 3:200-10.
11. Linkermann A, Bräsen JH, Himmerkus N, et al. Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. Kidney Int 2012;81:751-61.
12. Oerlemans MI, Liu J, Arslan F, et al. Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. Basic Res Cardiol 2012;107:270.
13. Wu J, Huang Z, Ren J, et al. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. Cell Res 2013;23:994-1006.
14. Günther C, Martini E, Wittkopf N, et al. Caspase-8 regulates TNF-α-induced epithelial necroptosis and terminal ileitis. Nature 2011;477:335-9.
15. Welz PS, Wullaert A, Vlantis K, et al. FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. Nature 2011;477:330-4.
16. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. Cell Mol Life Sci 2010;67: 3241-53.
17. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. Nat Rev Mol Cell Biol 2011;12:757-63.
18. Weinlich R, Dillon CP, Green DR. Ripped to death. Trends Cell Biol 2011; 21:630-7.
19. Kim SJ, Li J. Caspase blockade induces RIP3-mediated programmed necrosis in Toll-like receptor-activated microglia. Cell Death Dis 2013;4:e716.
20. Seya T, Shime H, Takaki H, Azuma M, Oshiumi H, Matsumoto M. TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis. Oncoimmunology 2012;1:917-23.
21. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature 2011;471: 368-72.
22. Welz PS, Pasparakis M. A way to DAI. Cell Host Microbe 2012;11:223-5.
23. Upton JW, Kaiser WJ, Mocarski ES. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. Cell Host Microbe 2012;11:290-7.
24. Vercammen D, Beyaert R, Denecker G, et al. Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. J Exp Med 1998;187:1477-85.
25. Silke J, Strasser A. The FLIP side of life. Sci Signal 2013;6(258):pe2.
26. Vanlangenakker N, Vanden Berghe T, Bogaert P, et al. cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ 2011;18:656-65.
27. Vanlangenakker N, Bertrand MJ, Bogaert P, Vandenabeele P, Vanden Berghe T. TNF-induced necroptosis in L929 cells is tightly regulated by multiple TNFR1 complex I and II members. Cell Death Dis 2011;2:e230.
28. Holler N, Zaru R, Micheau O, et al. Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat Immunol 2000;1:489-95.
29. Kim SO, Ono K, Han J. Apoptosis by pan-caspase inhibitors in lipopolysaccharide-activated macrophages. Am J Physiol Lung Cell Mol Physiol 2001;281: L1095-L1105.
30. Thapa RJ, Nogusa S, Chen P, et al. Interferon-induced RIP1/RIP3-mediated necrosis requires PKR and is licensed by FADD and caspases. Proc Natl Acad Sci U S A 2013;110:E3109-E3118.
31. Gerlach B, Cordier SM, Schmukle AC, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. Nature 2011;471:591-6.
32. Mevissen TE, Hospenthal MK, Geurink PP, et al. OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. Cell 2013;154:169-84.
33. O'Donnell MA, Perez-Jimenez E, Oberst A, et al. Caspase 8 inhibits programmed necrosis by processing CYLD. Nat Cell Biol 2011;13:1437-42.
34. Wilson NS, Dixit V, Ashkenazi A. Death receptor signal transducers: nodes of coordination in immune signaling networks. Nat Immunol 2009;10:348-55.

35. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature 2011;471:363-7.

36. Sun L, Wang H, Wang Z, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell 2012;148:213-27.

37. Zhao J, Jitkaew S, Cai Z, et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A 2012;109:5322-7.

38. Li J, McQuade T, Siemer AB, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. Cell 2012;150:339-50.

39. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. Immunity 2013;38:27-40.

40. Wang Z, Jiang H, Chen S, Du F, Wang X. The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell 2012;148:228-43.

41. Yeh WC, de la Pompa JL, McCurrach ME, et al. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 1998;279:1954-8.

42. Yeh WC, Itie A, Elia AJ, et al. Requirement for Casper (c-FLIP) in regulation of death receptor-induced apoptosis and embryonic development. Immunity 2000;12:633-42.

43. Varfolomeev EE, Schuchmann M, Luria V, et al. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity 1998;9:267-76.

44. Dillon CP, Oberst A, Weinlich R, et al. Survival function of the FADD-CASPASE-8-cFLIP(L) complex. Cell Rep 2012;1:401-7.

45. Kaczmarek A, Vandenabeele P, Krysko DV. Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity 2013;38:209-23.

46. Moquin D, Chan FK. The molecular regulation of programmed necrotic cell injury. Trends Biochem Sci 2010;35:434-41.

47. Li S, Zhang L, Yao Q, et al. Pathogen blocks host death receptor signalling by arginine GlcNAcylation of death domains. Nature 2013;501:242-6

48. Roca FJ, Ramakrishnan L. TNF dually mediates resistance and susceptibility to mycobacteria via mitochondrial reactive oxygen species. Cell 2013;153:521-34.

49. Vandenabeele P, Grootjans S, Callewaert N, Takahashi N. Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. Cell Death Differ 2013;20:185-7.

50. Degterev A, Huang Z, Boyce M, et al. Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol 2005;1:112-9. [Erratum, Nat Chem Biol 2006;1:234.]

51. You Z, Savitz SI, Yang J, et al. Necrostatin-1 reduces histopathology and improves functional outcome after controlled cortical impact in mice. J Cereb Blood Flow Metab 2008;28:1564-73.

52. Chavez-Valdez R, Martin LJ, Flock DL, Northington FJ. Necrostatin-1 attenuates mitochondrial dysfunction in neurons and astrocytes following neonatal hypoxia-ischemia. Neuroscience 2012;219:192-203.

53. Northington FJ, Chavez-Valdez R, Graham EM, Razdan S, Gauda EB, Martin LJ. Necrostatin decreases oxidative damage, inflammation, and injury after neonatal HI. J Cereb Blood Flow Metab 2011;31:178-89.

54. Fricker M, Vilalta A, Tolkovsky AM, Brown GC. Caspase inhibitors protect neurons by enabling selective necroptosis of inflamed microglia. J Biol Chem 2013;288:9145-52.

55. Trichonas G, Murakami Y, Thanos A, et al. Receptor interacting protein kinases mediate retinal detachment-induced photoreceptor necrosis and compensate for inhibition of apoptosis. Proc Natl Acad Sci U S A 2010;107:21695-700.

56. Lee YS, Dayma Y, Park MY, Kim KI, Yoo SE, Kim E. Daxx is a key downstream component of receptor interacting protein kinase 3 mediating retinal ischemic cell death. FEBS Lett 2013;587:266-71.

57. Murakami Y, Matsumoto H, Roh M, et al. Receptor interacting protein kinase mediates necrotic cone but not rod cell death in a mouse model of inherited degeneration. Proc Natl Acad Sci U S A 2012;109:14598-603.

58. Linkermann A, Bräsen JH, Darding M, et al. Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. Proc Natl Acad Sci U S A 2013;110:12024-9.

59. Linkermann A, Heller JO, Prókai A, et al. The RIP1-kinase inhibitor necrostatin-1 prevents osmotic nephrosis and contrast-induced AKI in mice. J Am Soc Nephrol 2013;24:1545-57.

60. Bonnet MC, Preukschat D, Welz PS, et al. The adaptor protein FADD protects epidermal keratinocytes from necroptosis in vivo and prevents skin inflammation. Immunity 2011;35:572-82.

61. Li C, Lasse S, Lee P, et al. Development of atopic dermatitis-like skin disease from the chronic loss of epidermal caspase-8. Proc Natl Acad Sci U S A 2010;107:22249-54.

62. Seymour RE, Hasham MG, Cox GA, et al. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. Genes Immun 2007;8:416-21.

63. Linkermann A, Bräsen JH, De Zen F, et al. Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor-α-induced shock. Mol Med 2012;18:577-86.

64. Ladoire S, Hannani D, Vetizou M, et al. Cell-death-associated molecular patterns as determinants of cancer immunogenicity. Antioxid Redox Signal 2013 March 20 (Epub ahead of print).

65. Lau A, Wang S, Jiang J, et al. RIPK3 mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. Am J Trans 2013;13:2805-18.

66. De Keyzer K, Van Laecke S, Peeters P, Vanholder R. Human cytomegalovirus and kidney transplantation: a clinician's update. Am J Kidney Dis 2011;58:118-26.

67. Linkermann A, Hackl MJ, Kunzendorf U, et al. Necroptosis in immunity and ischemia-reperfusion injury. Am J Trans 2013;13:2797-804.

68. Piguet PF, Vesin C, Guo J, Donati Y, Barazzone C. TNF-induced enterocyte apoptosis in mice is mediated by the TNF receptor 1 and does not require p53. Eur J Immunol 1998;28:3499-505.

69. Duprez L, Takahashi N, Van Hauwermeiren F, et al. RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. Immunity 2011;35:908-18.

70. Dejager L, Pinheiro I, Dejonckheere E, Libert C. Cecal ligation and puncture: the gold standard model for polymicrobial sepsis? Trends Microbiol 2011;19:198-208.

71. Takahashi N, Duprez L, Grootjans S, et al. Necrostatin-1 analogues: critical issues on the specificity, activity and in vivo use in experimental disease models. Cell Death Dis 2012;3:e437.

72. Zou J, Kawai T, Tsuchida T, et al. Poly IC triggers a cathepsin D- and IPS-1-dependent pathway to enhance cytokine production and mediate dendritic cell necroptosis. Immunity 2013;38:717-28.

73. Biton S, Ashkenazi A. NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-α feedforward signaling. Cell 2011;145:92-103.

74. Xie T, Peng W, Liu Y, et al. Structural basis of RIP1 inhibition by necrostatins. Structure 2013;21:493-9.

75. Kelliher MA, Grimm S, Ishida Y, Kuo F, Stanger BZ, Leder P. The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. Immunity 1998;8:297-303.

76. Burne MJ, Elghandour A, Haq M, et al. IL-1 and TNF independent pathways mediate ICAM-1/VCAM-1 up-regulation in ischemia reperfusion injury. J Leukoc Biol 2001;70:192-8.

77. Ko GJ, Jang HR, Huang Y, et al. Blocking Fas ligand on leukocytes attenuates

kidney ischemia-reperfusion injury. J Am Soc Nephrol 2011;22:732-42.
78. Piot C, Croisille P, Staat P, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. N Engl J Med 2008;359:473-81.
79. Baines CP, Kaiser RA, Purcell NH, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 2005;434:658-62.
80. Nakagawa T, Shimizu S, Watanabe T, et al. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005;434:652-8.
81. Schinzel AC, Takeuchi O, Huang Z, et al. Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 2005;102:12005-10.
82. Yang B, Jain S, Pawluczyk IZ, et al. Inflammation and caspase activation in long-term renal ischemia/reperfusion injury and immunosuppression in rats. Kidney Int 2005;68:2050-67.
83. Ekberg H, Tedesco-Silva H, Demirbas A, et al. Reduced exposure to calcineurin inhibitors in renal transplantation. N Engl J Med 2007;357:2562-75.
84. Merion RM, White DJ, Thiru S, Evans DB, Calne RY. Cyclosporine: five years' experience in cadaveric renal transplantation. N Engl J Med 1984;310:148-54.
85. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. Cell 2012;149:1060-72.
86. Cookson BT, Brennan MA. Pro-inflammatory programmed cell death. Trends Microbiol 2001;9:113-4.
87. Sosna J, Voigt S, Mathieu S, et al. TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. Cell Mol Life Sci 2013.
88. Yazdanpanah B, Wiegmann K, Tchikov V, et al. Riboflavin kinase couples TNF receptor 1 to NADPH oxidase. Nature 2009;460:1159-63.
89. Boya P, Kroemer G. Lysosomal membrane permeabilization in cell death. Oncogene 2008;27:6434-51.