Nonapoptotic cell death in acute kidney injury and transplantation
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Acute tubular necrosis causes a loss of renal function, which clinically presents as acute kidney failure (AKI). The biochemical signaling pathways that trigger necrosis have been investigated in detail over the past 5 years. It is now clear that necrosis (regulated necrosis, RN) represents a genetically driven process that contributes to the pathophysiology of AKI. RN pathways such as necroptosis, ferroptosis, parthanatos, and mitochondrial permeability transition-induced regulated necrosis (MPT-RN) may be mechanistically distinct, and the relative contributions to overall organ damage during AKI in living organisms largely remain elusive. In a synchronized manner, some necrotic programs induce the breakdown of tubular segments and multicellular functional units, whereas others are limited to killing single cells in the tubular compartment. Importantly, the means by which a renal cell dies may have implications for the subsequent inflammatory response. In this review, the recent advances in the field of renal cell death in AKI and key enzymes that might serve as novel therapeutic targets will be discussed. As a consequence of the interference with RN, the immunogenicity of dying cells in AKI in renal transplants will be diminished, rendering inhibitors of RN indirect immunosuppressive agents.

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The kidney represents the best organ to study cell death in vivo. Unlike the brain or the heart, in which investigations of ischemic injury are hampered by the presence of collateral vessels, renal ischemia-reperfusion injury (IRI) affects the whole organ, resulting in a significantly lower SD with regard to histologic injury scores and more reliable markers of organ function than in the heart or brain. Therefore, in recent years, renal investigations have disproportionately contributed to our understanding of cell death, notwithstanding the tremendous clinical need to analyze stroke and myocardial infarction.

In addition, intravital microscopy can be easily used to investigate the kidney, and movies obtained from such analyses have improved our understanding of renal dynamics.¹–³ With a beating heart and the skull around the brain, this technique requires additional strategies to evaluate progression and intercellular development, such as synchronized cell death and necrosis-induced necrosis in heart and brain models of injury that are more simply monitored in kidneys and isolated renal tubules.¹ Understanding the cellular mechanisms of death in the kidney and developing small molecules to successfully interfere with regulated necrosis (RN) in such models are likely to illuminate renal injury as well as provide benefits in neurology and cardiology.

In addition, the clinical presentation of acute kidney injury (AKI) often manifests with easily accessible changes in the urine sediment that can be confirmed by kidney biopsy (especially in animal models) and that can facilitate translation of animal research to patients.

THE PHYSIOLOGICAL ROLE OF RN
Extrinsic death receptor-mediated apoptosis depends on caspase-8, 1 of the most prominent initiator caspases in mice described more than 2 decades ago.⁵–⁷ Because of the embryonic lethality of caspase-8–deficient mice, researchers misinterpreted apoptosis as a process required for vertebrate viability. Therefore, the reversal of the lethal phenotype of caspase-8–deficient mice on a receptor-interacting protein kinase 3 (RIPK3)–deficient background was both striking and surprising,⁸–¹¹ and other functions of caspase-8 also appear to be involved in the control of RIPK3.¹² This led to the conclusion that the single most important function of caspase-8 is the prevention of necroptosis,¹³–¹⁵ mediated by RIPK3,¹⁶–¹⁸ rather than promoting extrinsic apoptosis, the loss of which appears to be far less dramatic than its regulatory effects on RIPK3, because caspase-8/RIPK3–double deficient are viable and
fertile.8–10 C8/RIPK3-double knockout mice exhibit a phenotype similar to those previously recognized from mice that carry mutations in the death receptor Fas or Fas ligand, referred to as lpr and gld point mutations, respectively.8,11 Such phenotypes result in accumulation of B220+ cells, an enlarged spleen and lymph nodes, and an overall reduced life span, but these mutations are not as striking as those described for caspase-8–deficient mice.19 Importantly, loss of RIPK3, apart from a slight failure to gain weight,4 does not lead to any obvious phenotype.20 Therefore, the physiological nonredundant role for extrinsic apoptosis appears to be limited to the immune system, whereas RIPK3 deficiency is not essential for normal murine development. Currently, it has been suggested that the phylogenetic preservation of the dangerous RIPK3 molecule is intended to defend against microbes.

REGULATED NECROSIS FIGHTS MICROBES

The default process for the clearance of virally infected cells mostly involves extrinsic apoptosis through Fas (on virally infected cells)–Fas ligand (on CD8+ effector T cells), cells that are triggered by presenting MHC II to the T-cell receptor,1 and malfunction of this system has been associated with renal disease.22,23 This apoptotic cascade involves caspase-8 activation that then transduces the deadly signal though effector caspase-3, caspase-6, and caspase-7, resulting in clearance of these cells in an “immunologically silent manner.”24,25 Some viruses, however, express proteins that are inhibitors of caspses, such as vaccinia virus, cytomegalovirus, cowpox virus, and so on.26–28 In these viral infections, RIPK3 is no longer inhibited by caspase-8, and the effect is spontaneous initiation of necroptosis21,28 to clear the virally infected cell in an immunogenic manner. In a simple experiment, RIPK3-ko mice were shown to die after infection with murine vaccinia virus, whereas all wild-type littermates survived.19 Therefore, necroptosis is considered a “backup defense” mechanism against caspase-inhibitor–expressing viruses.29–34 Apart from viruses, bacteria such as Listeria monocytogenes are recognized by hepatic Kupffer cells that respond by undergoing necroptosis and thereby trigger (i) an antibacterial immune response and (ii) the release of the necroptotic cytokine IL-33 to actively initiate the replacement of Kupffer cells to restore hepatic homeostasis.35,36 In fact, it is plausible that bacteria are capable of inducing necroptosis because virtually every Toll-like receptor that binds to the intracellular adapter protein TIR-domain–containing adapter-inducing interferon beta (TRIF) is capable of inducing necroptosis by directly interacting with RIPK3 through a RIP homotypic interacting motif (RHIM) domain.33,34,37 In summary, bacteria (through Toll-like receptors), viruses (through caspase inhibition and other mechanisms), and cytokines (through death receptors) are capable of inducing necroptosis.

SIGNALING PATHWAYS OF REGULATED NECROSIS

Necrosis is a common feature of diverse disorders, including tissue infarction, cancer, atherosclerosis, pancreatitis, trauma, and the vitality of organ transplants.3,32,38 Unfortunately, even the possibility of interfering with necrosis has been neglected by clinicians over several centuries, because necrosis was interpreted as “good given” and therefore beyond therapeutic intervention. Currently, as it has become clear that necrosis is a regulated process (RN), the opportunity to prevent it has fascinated researchers, rendering rapid movement in the field of RN. In the transplanted organ, it is now appreciated that each additional hour of cold ischemia time increases the risk for graft failure and mortality,39 the pathophysiological basis of which may likely be the total amount of necrotic debris in a transplant. However, further development in this field requires that we clearly define RN subroutines, as recently suggested by the current version of the recommendation of the interpretation of cell death pathways.40–42

Necroptosis—“the leviathan of RN”

Necroptosis signaling. Necroptosis is the best-studied RN pathway. The backbone of this cell death pathway is the receptor-interacting protein kinase 3 (RIPK3). RIPK3-dependent phosphorylation of mixed lineage kinase domain-like (MLKL), a pseudokinase that mediates the deadly signal to the plasma membrane by unknown means, is critical for the membrane to burst,43 and 2 other phosphorylation sites are of minor relevance.43 During this process, a 4-helical bundle domain is unleashed from the closed conformation of MLKL to target the plasma membrane.44–48 RIPK3 activation, however, is not sufficient to signal necroptosis without stabilization of the so-called necosome, a structure that consists of RIPK3 proteins that may associate in amyloid-like structures that are stabilized by heat shock protein 90 and its co-chaperone CDC37.49,50 The phosphorylation of MLKL is antagonized by 1 of the most prominent intracellular phosphatases, Ppm1b,51 which regulates necroptosis downstream of necroosome formation (Figure 1). Three major physiological patterns result in necroosome assembly and activation. As pointed out earlier, and probably most important in vivo, every TRIF-binding Toll-like receptor functions as a necroptosis receptor, and therefore bacteria are inducers of necroptosis, as exemplified in the case of Listeria monocytogenes infection.35 TRIF binds directly to RIPK3 through its RHIM domain.33,53 Only 4 proteins in the human genome contain such a RHIM domain: TRIF, RIPK1, RIPK3, and DNA-dependent activator of interferon, a DNA sensor for viral proteins that engages RIPK1 and RIPK334,37 and thereby enables viruses to pull the necrotic trigger in a necroosome-dependent manner (Figure 1). The default pathway, which requires caspase inhibition in the presence of death receptor ligation (e.g., tumor necrosis factor receptor 1 [TNFRI]), results in deubiquitination of RIPK1, a process that allows translocation from the plasma membrane to the cytosolic kinase target RIPK3, again through the RHIM domains.16,17 TNFR family members are also capable of inducing apoptosis. The molecular switch that regulates cell
death is tightly controlled by Fas-associated protein with death (FADD) and cellular inhibitor of apoptosis proteins 1 and 2, and transforming growth factor beta-activated kinase, and the polyubiquitination status of RIPK1 (see further on). Loss of RIPK1 results in embryonic lethality at day 10.5 in utero and is caused by a combined action of both caspase-8 and RIPK3. Therefore, RIPK1-deficient mice are viable and fertile either on a combined caspase-8/RIPK3-double knockout background or after a kinase-dead RIPK1 mutant is reintroduced.

As with caspase-8 deficiency, loss of either the antia apoptotic protein cellular FLICE-like inhibitory protein or the adapter protein FADD results in embryonic lethality at day 10.5, which can be reversed on a RIPK3-deficient background in the case of FADD. Therefore, a tightly regulated complex of cellular FLICE-like inhibitory protein, FADD, caspase-8, and RIPK1 prevents spontaneous necroptosis. This complex is stabilized by polyubiquitination of both linear polyubiquitin linkages (mediated by the linear ubiquitin chain assembly complex) and K63 linkages (mediated by several E3 ligases). Mechanistically clearly separate means to regulated RIPK1 polyubiquitination. Other linkages also contribute to the stability of this complex. Similar polyubiquitination patterns may also be found in RIPK3. Interestingly, RIPK1 kinase-dead knock-in mice are viable and fertile and unexpectedly did not explain the failure to pass the embryonic checkpoint at day 10.5; therefore, the lethality-mediating structure in RIPK1 must be different from its kinase activity.
Recently, the release of IL-33 was associated with cells dying through necroptosis and was interpreted as a specific necroptotic damage associated molecular pattern.\textsuperscript{36,67} IL-33 may signal through the ST2 receptor to stabilize a regulatory T-cell response, as shown for the gut.\textsuperscript{68} It may be interpreted that interleukin (IL)-33 releasing necroptosis may therefore be relatively nonimmunogenic compared with other pathways of RN, such as pyroptosis,\textsuperscript{42} in which active production of IL-1ß and IL-18 amplifies the damage. This hypothesis is in line with recently reported inhibitory effects by necroptosis on the immune system.\textsuperscript{69} In addition, however, IL-1ß is actively produced during necroptosis.\textsuperscript{70–72} It is currently unclear and under debate how IL-1ß in necroptosis contributes to the necrotic damage \textit{in vivo},\textsuperscript{67,72} and what consequences this may have for renal models of inflammatory injury.\textsuperscript{83}

\textbf{In vivo relevance of necroptosis.} RIPK3-deficient mice \textit{per se} do not exhibit a dramatic phenotype, apart from slight failure to gain weight over months.\textsuperscript{4} Necroptosis deficiency in such animals—as well as the use of necroptotic inhibitors in diverse species including mice, rats, and pigs—shows protection in models of cisplatin-induced AKI,\textsuperscript{4,74} ischemia-reperfusion injury (IRI) in the kidney,\textsuperscript{3,75–81} and the heart,\textsuperscript{72,82–89} were protected from cerulean-induced pancreatitis,\textsuperscript{17,18,90} although this was strongly debated in other reports.\textsuperscript{4,80} Protection has also been observed in doxorubicin-induced lethality,\textsuperscript{91} TNF-induced shock models,\textsuperscript{92–95} and models of sepsis.\textsuperscript{96} Conversely, RIPK3 deficiency exquisitely promotes sensitivity to vaccinia virus infections.\textsuperscript{16} In addition, these mice are significantly protected in ethanol-induced liver injury,\textsuperscript{97} nonalcoholic steatohepatitis,\textsuperscript{98} acetaminophen-induced hepatic toxicity,\textsuperscript{99} a model of Gaucher’s disease,\textsuperscript{100} and inhibited hepatocarcinogenesis.\textsuperscript{101} RIPK3 additionally partially mediates the skin phenotype in SHARPIN-deficient mice, a detrimental skin disorder.\textsuperscript{102} In the absence of immunosuppression, kidneys from RIPK3-deficient C57Bl/6 mice that were transplanted into Balb/c mice exhibited improved organ function compared with wild-type littermate kidneys in overall survival by several months.\textsuperscript{3,103} In another study, hearts from RIPK3-deficient mice were transplanted in the presence of immunosuppression by rapamycin and exhibited delayed rejection later than mice with wild-type hearts.\textsuperscript{83} It has been published that MLKL-ko mice are protected from pancreatitis\textsuperscript{90} (see earlier discussion of “putative” RIPK3-ko–mediated protection in this model) and cisplatin-induced AKI\textsuperscript{74} and may be protected from renal IRI and in the model of TNF shock (Linkermann \textit{et al.}, unpublished data).

Apart from these analyses of genetically modified mice, necroptosis was investigated using the small molecule necrostatin-1 (Nec-1) by several groups of investigators. Unfortunately, Nec-1 not only inhibits necroptosis \textit{in vivo} but also affects ferroptosis.\textsuperscript{104} Therefore, conclusions drawn from experiments with Nec-1 in the absence of either a highly specific second-generation necrostatin (like Nec-1 stable or ponatinib) or a genetic model are problematic.

\textbf{Human relevance of necroptosis.} The detection of the human relevance of necroptosis has been a topic of pure speculation until very recently when an antibody directed against the specific phosphorylation site of MLKL became available. This antibody does not recognize murine phospho-MLKL but does recognize rat and human phospho-MLKL and should allow the detection of particular cell types affected by necroptosis in the near future.\textsuperscript{105} Given the pathway of necroptosis, it is important to consider that during this cell death program, cytokines are actively produced that account for a much higher immunogenicity compared with artificially damaged cells (e.g., freeze-thawed cells). Necroptosis may therefore contribute to diseases by (i) cytokine production and (ii) frank cellular necrosis. In this sense, it is important to consider the possibility that the RHIM domain of RIPK1 might specifically induce cytokine production on initiation of necroptosis \textit{in vitro} and that this mechanism might be specifically inactive in certain pathologic conditions while leaving the phospho-MLKL-mediated necrotic function intact. In this scenario, the detection of phospho-MLKL would consider only the cell death part of necroptosis, precluding conclusions regarding ongoing cytokine production, which may potentially contribute to many disease processes. However, there is no doubt about a relevance of the activity of this pathway in human disorders, with many more likely to be discovered. Additional information about cytokine production will be experimentally useful as well. Such considerations are equally relevant to nonnecroptotic RN signaling pathways.

\textbf{Mitochondrial permeability transition-mediated RN and parthanatos.} Poly(ADP-ribose) and mitochondrial permeability transition-mediated RN \textit{signaling}. Activation of poly(ADP-ribose) polymerase-1 (PARP-1) leads to reversible protein modification by poly(ADP-ribosylation), a process that additionally depends on the function of the poly(ADP ribose) (PAR) decomposing enzymes, such as PAR glycohydrolase and ADP-ribosyl hydrolase 3.\textsuperscript{106} Although poly(ADP-ribosylation) represents a common means of postranslational protein modification, uncontrolled PARP-1 activation (so-called PARP-1 overactivation\textsuperscript{107}) leads to caspase-independent, nonapoptotic cell death with a necrotic phenotype,\textsuperscript{106} classically induced by the compound N-methyl-N’-nitro-N-nitrosoguanidine.\textsuperscript{108} Importantly, N-methyl-N’-nitro-N-nitrosoguanidine treatment is not “off-target,” because PARP-1–deficient cells are entirely resistant to N-methyl-N’-nitro-N-nitrosoguanidine–induced parthanatos. This cell death subroutine is mediated by a mitochondrial protein, unfortunately named “apoptosis-inducing factor,” a clear misnomer.\textsuperscript{109} Until recently, it was unclear how PAR polymers translocate from the nucleus to mitochondria to release apoptosis-inducing factor, expressed on both the inner and outer mitochondrial membranes, into the cytosol. It is now clear that nicotinamide adenine dinucleotide depletion and mitochondrial permeability transition are required for apoptosis-inducing factor release from the mitochondria and...
subsequent parthanatos.\textsuperscript{40,110–112} Therefore, it has been suspected to overlap with mitochondrial-permeability transition-mediated RN (MPT-RN), which was previously thought to be a distinct form of parthanatos.\textsuperscript{40,42,111} However, the widely used immunosuppressant cyclosporine A (CsA), and its intrinsic activity to inhibit MPT-RN, has been widely recognized, but CsA also interrupts apoptosis-inducing factor release after N-methyl-N’-nitro-N-nitrosoguanidine treatment and parthanatos. Cyclophilin D (CypD), a regulatory component of the mitochondrial permeability transition pore is thought to represent the molecular target of CsA for the inhibition of MPT-RN, and it has not been excluded that CypD is also involved in parthanatos.\textsuperscript{32,113} Therefore, with respect to settings of AKI, it would be interesting to investigate PARP-1 deficiency in the presence of CsA and the effect of combined PARP-1 and CypD deficiency.

\textbf{In vivo relevance of MPT-RN and parthanatos.} Three independent laboratories have described protective effects for CypD deficiency in murine renal IRI,\textsuperscript{81,114,115} in line with a report that used a CypD small, interfering RNA in rats.\textsuperscript{116} CypD-ko mice are also protected from cisplatin-induced AKI.\textsuperscript{117} Consistent with these results, inhibitors of MPT, such as sanglifehrin A (SfA) and CsA protect wild-type animals from renal IRI.\textsuperscript{75,117} This effect does not necessarily depend on the immunosuppressive function of CsA and putatively sanglifehrin A, but might be mediated by inhibition of CypD, because such ko-mice phenocopy the extent of protection achieved with known inhibitors. In addition, CypD-deficient mice are protected from myocardial IRI\textsuperscript{118} and stroke.\textsuperscript{118,119} Similarly, PARP-1–mediated cell death has been demonstrated to contribute to IRI after renal, cerebral, and myocardial ischemia.\textsuperscript{120–129} In contrast to necroptosis, inhibition of MPT-RN also protects from cerulein-induced pancreatitis.\textsuperscript{130}

\textbf{Human relevance of MPT-RN.} One clinical trial has been performed by Piot et al.,\textsuperscript{131} investigating the potentially protective effect of CsA in humans undergoing myocardial catheterization. The preliminary trial yielded beneficial effects, but a larger follow-up study failed. This recently published trial, however, changed the lipid formulation of CsA, a decision that may well have influenced the efficacy of the treatment, especially because blood levels of CsA were not provided in this trial.\textsuperscript{131} In addition to the direct prospective nature of the initial Piot et al. study, CsA is frequently used in human solid organ transplantation and is part of the standard quadruple immunosuppressive regimen used in most transplantation centers. Given the moderately effective action of CsA on inhibition of T-cell proliferation compared with other immunosuppressive agents, it cannot be excluded that the frequent superiority in clinical trials is partially the result of inhibition of immunogenic cell death (see further on).

\textbf{Ferroptosis}

\textbf{The in vivo relevance of ferroptosis and its regulation.} The role of iron has been extensively investigated in kidney research over decades. It was noted that iron chelators, such as desferoxamine, have been shown to inhibit tubular cell death.\textsuperscript{132} However, because of the lack of mechanistic insights into this putative iron-dependent cell death pathway, the term “ferroptosis” was introduced by Dixon and Stockwell in 2012.\textsuperscript{133,134} Since then, the field has been rapidly unraveling some of the first molecules involved in this pathway and appreciated its profound pathophysiological relevance.

Screening for small molecules that kill Ras-transformed tumor cells, a unique compound was found and in 2007 was termed “erastin” for its ability to kill such cells.\textsuperscript{135} Erastin was then found to inhibit a cellular cystine/glutamate antiporter referred to as “system Xc,” that consists of the subunits SLC7A11 and SLC3A2 (solute carrier 3 member 2).\textsuperscript{136} This antiporter provides the cell with cystine required for the intracellular generation of glutathione (GSH), and therefore erastin or high levels of extracellular glutamate deplete cells of GSH. The heterodimer γ-glutamylcysteine synthetase, which consists of the 2 subunits gclc and gclm, and glutathione synthetase (GS), are required for ATP-dependent GSH synthesis.\textsuperscript{137} Glutathione peroxidase 4 (GPX4) requires GSH to be active in its function of antagonizing lipoxygenase activity and lipid peroxidation (phospholipids and cardiolipin).\textsuperscript{138} Either the depletion of GSH or the absence of GPX4 results in cardiolipin oxidation and subsequent phospholipid peroxidation. Using oxylipidomics, this unique signature of lipid peroxidation is typical of ferroptosis\textsuperscript{104} and also includes the disruption of the inner mitochondrial membrane, 1 of the production spots of oxygen radicals, especially in complex I of the respiratory chain.\textsuperscript{139}

SLC7A11, as referred to earlier, is a critical subunit of system Xc- that, by its expression, regulates and inhibits the activation of ferroptosis.\textsuperscript{140,141} Interestingly, SLC7A11 itself is inhibited by tumor suppressor p53, even if p53 is mutated and cannot exert other functions like induction of apoptosis and cell cycle arrest. This mutant, referred to as p53\textsuperscript{KR}, and wild-type p53 are therefore capable of inhibiting SLC7A11 expression and thereby promote ferroptosis\textsuperscript{140,141}. Loss of p53, therefore, might unleash the break on SLC7A11 that is highly expressed and results in resistance to ferroptotic tubular cell death, which is in line with the reported protection against AKI of p53-deficient tubular cells.\textsuperscript{142–144} Additionally, interference with p53 by small, interfering RNA\textsuperscript{45} may serve as a protective strategy in AKI because of this mechanism. However, in the process of regeneration after AKI, p53 may be involved in the efficient removal and regeneration of necrotic debris and adjacent dying cells that need to be removed quickly after the damage has occurred and therefore prevent “necroinflammation” (see further on).\textsuperscript{146} All these data are therefore in line with protection from IRI on application of ferrostatins and could explain why tubular deficiency for GPX4 results in spontaneous tubular necrosis and death of mice that are sensitive to ferrostatins.\textsuperscript{104} The rapidly emerging pathway of ferroptosis is depicted in Figure 2. In addition to this perspective, evidence accumulates that ferroptosis, and most probably associated innate immune signals, are regulated by lipid metabolism enzymes.\textsuperscript{147}
However, the integrative signal that links lipid metabolism and lipid modifying enzymes\(^{148}\) to the control of ferroptosis has not been identified.

**Synchronized necrosis—the detonation wave.** Studies of freshly isolated primary tubules recently revealed a synchronized necrotic event that occurred *ex vivo* on tubular perfusion with erastin.\(^4\) In this process, a complete tubule, as a functional unit, appears to succumb to ferroptosis. Such a “wave of death” may partially explain why the clinically observed phenomenon of nephron loss in diabetic patients may be relevant for the developing kidney, because p53-dependent mechanisms have been associated with metanephric development.\(^{149}\)

Intriguingly, intrarenal cast formation may be mediated by ferroptosis in AKI/IR\(^4,24\) and may represent a critical means for the initiation of the regenerative signal of the tubules.\(^{150}\) Therefore, future research should investigate the signals driven secondarily from the casts, such as met activation\(^{151}\) and macrophage recruitment\(^{152,153}\) after synchronized necrosis and how such signals affect tubular repair mechanisms.

**MEANS TO INVESTIGATE RN**

**Combining pathway deficiencies: “Criss-cross CRISPR-Cas”**

Genome editing by the clustered regularly interspaced short palindromic repeats-Cas9 technique facilitates investigation of multiple gene deficiencies in vertebrates without most of the major disadvantages of conventional crosses from 129 mice to C57Bl/6 or C57Bl/6N mice. These disadvantages include passenger mutations and extensive backcrossing.\(^{154}\) This technique, in combination with the ongoing development of small molecules that specifically target critical enzymes in

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*Figure 2 | Control of glutathione peroxidase 4 (GPX4) and the signaling pathway ferroptosis.* The glutamate/cystine antiporter system Xc in the plasma membrane consists of the 2 subunits SLC3A2 and SLC7A11, the expression of the latter of which is controlled by p53. The import of cystine into the cell is a critical step for glutathione (GSH) synthesis, driven by gamma-glutamylcysteine synthetase, which consists of the 2 subunits GCLC and GCLM, in an adenosine triphosphate (ATP)-dependent process. GSH is required for the function of GPX4, which inhibits lipoxygenase and prevents the accumulation of lipid reactive oxygen species (ROS). On ischemia, application of the system Xc-inhibitor erastin, high extracellular concentrations of glutamine (referred to as excitotoxicity in neurons), RSL3 application (which inhibits GPX4) and in the presence of L-buthionine sulfoximine (an inhibitor of gamma-glutamylcysteine synthetase), cellular stress results in iron-dependent (desferoxamine [DFO]-sensitive) accumulation of phospholipids and oxidized cardiolipin (lipid ROS). This unique lipid peroxidation signature leads to the loss of plasma membrane integrity by an unknown mechanism. Desferoxamine, ferrostatin-1 (Fer-1), Liproxstatin, and the compound 16-86 interfere with ferroptosis, which significantly contributes to tubular cell necrosis and synchronized death of renal tubules (see text for details). Therefore, ferrostatins are promising compounds for the treatment of acute kidney injury. ADP, adenosine diphosphate; CYS-2, cysteine-2; GLU, glutamic acid; GLY, glycine.
Figure 3 | The matrix to determine the relative contribution of several pathways of regulated necrosis (RN) to the overall organ damage. Several pathways of RN have been shown to be clearly separated (like necroptosis and mitochondrial permeability transition-induced regulated necrosis [MPT-RN] (Linkermann et al.) and necroptosis and ferroptosis (Dixon et al.)) standardized. We will use 4 different genetically deficient mice (MLKL-ko, CypD-ko, PARP1-ko, and GPX4-tg), all carefully backcrossed to C57BL/6N background, to generate an 8-fold transgenic animal (MLKL-ko/CypD-ko/PARP1-ko x GPX4-tg), which should in theory be devoid of necroptosis, MPT-RN, parthanos, and ferroptosis, respectively. At the date of submission of the proposal, we have MLKL-CypD-PARP1-tko mice which are far overweight but are viable. For each of these pathways, inhibitors are available for in vivo use, such as necrostatin-1 stable, sanglifehrin A (SFA), alaparib, and 16-86 for the prevention of necroptosis, MPT-RN, parthanos, and ferroptosis, respectively. This matrix will help identify the relative contribution of each of these independent pathways of RN to the overall organ damage after certain types of preclinical disease induction. In addition, Table 1 lists the key molecules, inhibitors, and definitions of the regulated cell death pathways. CyPd-ko, cyclophycin D knockout; CsA, cyclosporine A; Fer-1, ferrostatin 1; GPX4-transgenic, glutathione peroxidase 4; MLKL-ko, mixed lineage kinase domain-like knockout; Nec-1, necrostatin-1; Nec-1s, necrostatin-1 stable; NSA, necrosulfonamide; PARP1-ko, poly(ADP-ribose) polymerase-1 triple knockout; RIPK3-ko, receptor-interacting protein kinase 3 knockout.

Table 1 | Definitions of the pathways of regulated cell death, key molecules, and selected inhibitors

| Apoptosis | Typical morphologic appearance (shrinkage, nuclear chromatin condensation, blebbing) caused by caspase activation | Initiator caspases (caspase-8, caspase-10) and executor caspases (caspase-3/caspase-6-caspase-7) | zVAD-fmk, q-VD|79 |
|-----------|---------------------------------------------------------------|-------------------------------------------------|-----------------|
| Necroptosis | Cellular necrosis caused by RIPK3-mediated phosphorylation of MLKL | RIPK3 and MLKL (necosome) | Necrostatin-178,89,93 |
| Ferroptosis | Cellular necrosis caused by a specific oxylipidomics signature | Glutathione peroxidase 4 (GPX4) | Necrostatin-1s80 |
| MPT-RN | Cellular necrosis caused by MPT | Cyclophilin D (CyPd) | Necrosulfonamide (NSA)79 |
| Parthanos | Cellular necrosis caused by PARP-1 hyperactivation | Parp-1 | Ferrostatins4 |

MLKL, mixed lineage kinase domain-like; MPT, mitochondrial permeability transition; MPT-RN, mitochondrial-permeability transition-mediated regulated necrosis; q-VD, N-((2-Quinolyl)-1-valyl-1-asparyl-(2,6-difluorophenoxy)methylketone; RIPK3, receptor-interacting protein kinase 3; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone.

Combination therapy of RN

Inhibitors of necroptosis

When inhibitors of necroptosis were screened from small molecule libraries, Nec-1 was found to inhibit IRI in a stroke model. Subsequently, this original compound has successfully prevented IRI in murine kidneys,74,79,80 rat kidneys,75 hearts,85 of mice and pigs,75 in renal transplantation,171 and several other models of non-IRI diseases such as cisplatin-induced AKI176,89,156 and others.4,117 However, there are several drawbacks from the data obtained with Nec-1, because it is not specific for necroptosis as initially thought but also inhibits ferroptosis,104 and it is entirely unclear to what extent the in vivo effects seen result from inhibition of ferroptosis or necroptosis. However, a second-generation necrostatin, referred to as Nec-1 stable, interferes with necroptosis in TNF-shock models,94 but those are obviously different from the much more important IRI models and even from models of cecal ligation and puncture. In fact, in our hands, Nec-1 accelerated time to death in those models,93 whereas others have described striking in vivo effects that are obviously dependent on the efficacy of RIP1 kinase inhibition.95 This was recently highlighted by RIPK1 kinase-dead knock-in mice,96,97 which are strongly protected from the TNF-shock model. It will be interesting to evaluate those mice in renal IRI, and such studies are ongoing. In the same direction, ponatinib was recently described as an effective inhibitor of the kinase domains of RIPK1, RIPK2, and RIPK3.93,157 Ponatinib efficiently inhibits the TNF-shock

Each pathway of RN, provides an ideal tool to study necrotic cell death, as depicted in Figure 3 (in addition, Table 1 lists the key molecules, inhibitors, and definitions of the regulated cell death pathways). Any in vivo model of AKI, toxicities, sepsis, autoimmunity, and so on may be investigated by such a matrix. The critical controls are naturally being included because of the required use of inhibitors in consistent gene-deficient mice. With such an approach, the relative contribution of each of the pathways of RN to the overall organ damage/pathologic readout can be determined. It is important to identify the most redundant single pathway, because this will be the most promising therapeutic target. In preparation for phase I clinical trials, it appears virtually impossible to include more than 1 compound, especially because these inhibitors define a novel class of drugs. However, despite these first-in-class compounds being in their “fledgling stages,” the first RIPK1 kinase inhibitors have recently entered clinical trials. Preclinical research using the modern tools of combined inhibitor screens and multiple gene deficiencies in vivo will hopefully inspire commercial companies to continue investigations with even newer agents.
model but may not have effects on IRI (Linkermann et al., unpublished data).

**Inhibitors of parthanatos**

PARP-1 activation has been demonstrated to significantly contribute to IRI in the kidney by several groups, both by means of PARP-1–deficient mice (see earlier discussion) and by inhibitors of PARP-1.75,120,121 PARP inhibitors were primarily developed for cancer, and several of them have been approved by the US Food and Drug Administration as anticancer therapy. PAR polymers have been thought to functionally contribute to necroptosis,138 but this has recently been disproved in *in vitro* assays.108 However, although parthanatos is clearly distinct from necroptosis, mitochondrial permeability transition may be downstream of PARP-1 activation; at least this has not been experimentally excluded. In this sense, it is remarkable that inhibitors of parthanatos and MPT-RN yield comparable levels of protection in renal AKI models.80,114,120,121 Parthanatos affects the outer mitochondrial membrane, and PARP-1 inhibitors prevent this downstream feature. A combination therapy of PJ34 (one of the first PARP-1 inhibitors) and CsA (an inhibitor of MPT-RN, not only an immunosuppressant!) was applied to rats after renal transplantation; the additive protection might be explained by the intrinsic immunosuppressive impact of CsA. Therefore, investigation of CypD-PARP-1-double knockout mice in comparison with each single knockout along with the addition of PARP-1-inhibitors like PJ34 or olaparib to CypD mice and CsA to PARP-1-ko mice in those models of injury is urgently awaited.

**Inhibitors of ferroptosis**

Ferroptosis is induced by erastin, a compound initially found in a screen for the killing of Ras-transformed tumor cells that have been erased by this compound, thus the name.135 In a subsequent screen for inhibitors of erastin-induced cell death, none of the traditional inhibitors of apoptosis—MPT-RN, necroptosis, PARP-1, and so on—achieved any protection, but desferoxamine, an iron chelator, and a novel small molecule did afford protection.133,134 This molecule was named ferrostatin-1, and based on pharmacologic evaluation should be unstable in serum and plasma. Therefore, second- and third-generation ferrostatins have been constructed that strongly protect against IRI and also against *ex vivo* hydroxychloroquine/iron–induced tubular damage, as does desferoxamine.4,159

**REMOTE ORGAN INJURY**

“I lit the small fire – I don’t know who lit the big one!”

– Mick Flannery

IRI, as it happens in transplants, causes damage-associated molecular patterns to be released from necrotic cells (see earlier discussion).24 Such damage-associated molecular patterns, for example, from transplanted rat kidneys, follow the venous blood flow to cause parthanatos, necroptosis, and MPT-RN in pulmonary epithelial cells, a process referred to as “remote organ injury.”76,160 Similarly, pathogen-associated molecular patterns, such as bacterial virulence factors, also induce pulmonary damage in septic situations, simply by traveling the blood stream. As a result, the lung is the predominant organ of remote injury. Remote organ injury is not limited to kidney necrosis, but may occur after any significant necrotic cell death–associated injury, as in trauma, fulminant hepatitis, transplantation of other solid organs, and sepsis. This concept might provide a possible explanation for acute respiratory distress syndrome, symptomatic transitory psychiatric syndrome, and other common clinical observations after trauma, surgery, and sepsis that are accompanied by large-scale necrosis within several organs of the organism. It remains to be seen whether or not this distinct injury might benefit from antinecrotic therapy.

**Concepts of immunogenic cell death and necroinflammation**

In contrast to apoptosis, which is generally considered an antiinflammatory means of metabolic cellular turnover in physiological conditions, RN is immunogenic. The concepts of immunogenic cell death46–57 and “necroinflammation” describe this phenomenon and thereby provide a hypothesis for inflammation after primary necrotic cell death that may be beneficial for induction in cancers and harmful during ischemic damage or sepsis. Necroinflammation additionally considers that different pathways of RN induce each other, consistent with the observation of synchronized necrosis.4,24 In this scenario, an autoamplification loop of immunogenicity and RN fuels itself, resulting in the exacerbation of local organ injury beyond the margins of initiating organs into the systemic vasculature and resulting in multiple organ injury. In that sense, pharmacologic inhibition of RN, if directly associated with inflammation, might functionally be considered an immunosuppressant at an upstream site of the injury cascade.

**CONCLUSION**

RN inhibitors (RIPK1 inhibitors) have entered clinical trials. Despite current euphoria in the field, these first-in-class therapeutic agents will face several obstacles. How safe will it be to inhibit nonapoptotic cell death pathways in patients, especially in those receiving immunosuppressive medication? In fact, we are also inhibiting backup mechanisms that represent our collective viral defense that could determine our transplant patient’s survival in some scenarios. However, optimists will argue that inhibiting RN will permit the use of fewer immunosuppressive drugs as evidenced by living kidney donors. Are these only the healthiest patients or does the interruption of necroinflammation prevent memory B cells from being primed? It is conceivable that standard immunosuppression, at least, will not interfere with the priming of such cells but only with their proliferation. When standard immunosuppression is tapered after several months in most protocols, antibody-mediated rejection provides a major problem that might well be prevented by inhibiting RN (as occurs in our living donors). In conclusion, IRI is
likely to be a major factor that contributes to graft loss, even in the long term, and is not restricted to only short-term effects such as delayed graft function. This concept is strongly supported by a recent study that clearly highlights how each additional hour of cold ischemic time increases the risk of graft failure and, more importantly, of mortality after renal transplantation. Given the increasing numbers of expanded-criteria donors, prevention of RN represents a promising strategy to improve the rate of solid organ transplantation.

The progression of synchronized necrosis, as demonstrated to occur in renal tubules, progresses within hours. This provides an idea about the therapeutic window for RN inhibitors. Importantly, synchronized necrosis is likely to happen in other functional units on infarction, like the brain and the contracting heart. Although these conditions may as well be therapeutic targets for RN inhibition, clinical trials for such disorders will require additional information on the pharmacodynamics and pharmacokinetics. In the future, data obtained from trials on AKI and kidney transplantation will contribute to this pharmacologic understanding and are expected to ultimately pave the way to novel therapeutics for such widespread diseases.

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