REGULATED NECROSIS IN KIDNEY ISCHEMIA-REPERFUSION INJURY

Aspasia Pefanis, MBBS[1,2], Francesco L Ierino, MBBS[2,3], James M Murphy, PhD[4,5], Peter J Cowan, PhD[1,2]

1. Immunology Research Centre, St Vincent's Hospital Melbourne, Fitzroy, Australia
2. Department of Medicine, University of Melbourne, Melbourne, Australia
3. Department of Nephrology, St Vincent's Hospital Melbourne, Fitzroy, Australia
4. The Walter and Eliza Hall Institute of Medical Research, Parkville, Australia
5. Department of Medical Biology, The University of Melbourne, Parkville, Australia

Corresponding author:

Professor Peter J Cowan

Immunology Research Centre, St Vincent's Hospital Melbourne

P.O. Box 2900, Fitzroy, Victoria 3065, Australia

Telephone number: +613 9231 3144; Fax number: +613 9231 3151

Email address: peter.cowan@svha.org.au

Sources of support

JMM acknowledges the National Health and Medical Research Council of Australia (NHMRC) for fellowship (1105754), grants (1124735, 1124737), and infrastructure (IRIISS 9000433) support; and the Victorian Government Operational Infrastructure Support scheme. PJC acknowledges the NHMRC for grant support (447706, 1083006).

Running head

Regulated necrosis in kidney IRI

ABSTRACT

Ischemia-reperfusion injury (IRI) is the outcome of an inflammatory process that is triggered when an organ undergoes a transient reduction or cessation of blood flow, followed by re-establishment of perfusion. In the clinical setting, IRI contributes to significant acute kidney injury, patient morbidity and mortality and adverse outcomes in transplantation. Tubular cell death by necrosis and apoptosis is a central feature of renal IRI. Recent research has challenged traditional views of cell death by identifying new pathways in which cells die in a regulated manner but with the morphological features of necrosis. This 'regulated necrosis' (RN) takes several forms, with necroptosis and ferroptosis being the best described. The precise mechanisms and relationships between the RN pathways in renal IRI are currently the subject of active research. The common end point of RN is cell membrane rupture, resulting in the release of cytosolic components with subsequent inflammation and activation of the immune system. We review the evidence and mechanisms of RN in the kidney following renal IRI, and discuss the use of small molecule inhibitors and genetically modified mice to better understand this process and guide potentially novel therapeutic interventions.

Keywords

ischemia-reperfusion injury, apoptosis, necrosis, necroptosis, ferroptosis, acute kidney injury, kidney transplantation.

INTRODUCTION

Ischemia-reperfusion injury (IRI) can be precipitated by multiple clinical scenarios including periods of significant hypotension, myocardial infarction, stroke, or following organ transplantation. IR sets off a cascade of deleterious cellular responses in the affected organ, resulting in cell injury and eventual cell death via necrosis or apoptosis.[1] Indeed, the presence of necrotic tubular cells is a hallmark of renal IRI.[2] Although necrosis is traditionally viewed as a non-specific and unregulated process, several regulated pathways have now been described, with increasing recognition of their potential contribution to several pathophysiological conditions. This review will focus on the pathways leading to cell death following renal IRI, with an emphasis on regulated necrosis (RN).

RENAL ISCHEMIA-REPERFUSION INJURY (IRI)

Renal ischemia-reperfusion injury is observed widely across the spectrum of clinical practice, from embolic or thrombotic events, all causes of circulatory "shock" and hypotension, surgical settings such as cardiac by-pass surgery, the obligatory renal injury associated with organ procurement, and a range of other kidney insults. This injury results in a rapid decline in kidney function and increased patient morbidity and mortality. The short- and long-term outlook of the patient varies depending on the reversibility and recovery of the injury. Acute kidney injury may predispose to chronic kidney disease and end-stage kidney disease leading to dialysis.

The kidneys are highly susceptible to IRI. The mismatch between oxygen supply and demand causes a reduction in oxidative metabolism, with progressive injury and death of tubular epithelial cells.[1] This leads to loss of the kidney's homeostatic control of metabolic waste

products, with impaired water and electrolyte balance and associated morbidity and mortality.

Renal cell injury and death

Cellular damage occurs during both the ischemic insult and subsequent reperfusion of the kidney.[1,3] Ischemic renal tissue switches metabolism from aerobic to anaerobic and intracellular ATP levels fall precipitously, with the resultant acidosis worsened by an increase in lactate-dependent ATP production.[4] Inhibition of membrane-bound Na^+/K^+ ATPase activity[5] increases intracellular sodium and water, causing cellular oedema.[4] Reperfusion restores aerobic metabolism and normalizes pH in the kidney, but concomitantly generates reactive oxygen species (ROS) that damage functional cellular components and induce cell death.[3,6] Epithelial cell injury associated with renal IR is most apparent in the proximal tubules.[1] The appearance of casts and the presence of tubular cells in the urine is an indicator of tubular cell damage and death by necrosis and/or apoptosis.[2]

Inflammation and immune activation

During renal IR, injured and dying cells release endogenous molecules termed Damage-Associated Molecular Patterns (DAMPs) and secrete proinflammatory and chemotactic cytokines.[7,8] DAMPs include heat shock proteins, high-mobility group box 1 (HMGB1) and deoxyribonucleic acid (DNA) fragments. These molecules activate cell surface receptors (e.g. Toll-like receptors (TLR) 2 and 4 and the receptor for advanced glycation end products (RAGE)), triggering inflammatory and cytotoxic responses. The release of proinflammatory cytokines such as interleukin (IL)-1α and tumour necrosis factor alpha (TNFα) stimulates

migration and activation of dendritic cells, which in turn activate T cells and macrophages.[9-11]

Microvascular dysfunction and fibrosis

Total renal blood flow can be impaired by up to 50% following IR.[12] This phenomenon is caused by several factors including endothelial damage by inflammatory mediators and recruited leukocytes[13], compression of capillaries by interstitial oedema and congestion,[14] and an imbalance between vasodilation and vasoconstriction,[15] Endothelial cells in peritubular capillaries have limited regenerative capacity[14] with ongoing microvascular damage leading to permanent rarefaction.[16] Chronic kidney hypoperfusion causes persistent regional hypoxia, which is a fibrogenic stimulus for tubular epithelial cells, interstitial fibroblasts and renal microvascular endothelial cells.[17] Experimental renal transplant models have shown a link between IRI and progressive interstitial fibrosis.[18]

IRI in renal transplantation: clinical consequences and therapeutic approaches

IRI is detrimental to kidney transplants, with consequences including delayed graft function,[3] acute and chronic rejection,[19-21] and fibrosis. Delayed graft function is influenced by both cold and warm ischemia.[21-24] IRI is thought to enhance the immunogenicity of the graft by facilitating antigen presentation by dendritic cells to naïve T cells, leading to T cell-mediated rejection.[25] Clinically, the severity of IRI has been associated with increased frequency of acute rejection episodes.[26] Animal and human studies have been used to investigate a wide range of protective therapies including machine perfusion, ischemic pre- and post-conditioning, cell therapy, and various pharmacological agents.[23]. However, none of these interventions have yet been translated to routine clinical practice. The discovery of

RN has provided further insight into the mechanisms of IRI and new pathways that may be amenable to pharmacological intervention.

REGULATED NECROSIS (RN)

Traditionally, scientists have maintained a dichotomous view on how cells die: apoptosis is a tightly regulated ATP-dependent process of programmed cell death, whereas necrosis is a passive process by which cells die following an overwhelming chemical or physical insult. Apoptotic cells are characterized by nuclear and cytoplasmic condensation, DNA fragmentation, cell membrane blebbing with the presence of apoptotic bodies,[27] and externalization of phosphatidylserine (PS), which is a signal for macrophages to engulf and remove the cells without mounting an inflammatory response.[28,29] Necrotic cells, on the other hand, exhibit cytoplasmic and mitochondrial swelling, with loss of plasma membrane integrity resulting in the release of proinflammatory intracellular contents.[30]

The past decade has seen a wealth of research expanding the traditional view of necrosis. New evidence has emerged for highly regulated non-apoptotic cell death with the morphological features of necrosis, called regulated necrosis (RN). RN takes several forms: necroptosis, ferroptosis, pyroptosis, mitochondrial permeability transition (MPT)-driven necrosis, and parthanatos. In contrast to apoptosis, RN leads to cell membrane rupture and release of DAMPs, resulting in inflammation and immune activation. Necroptosis and ferroptosis are the most extensively studied forms of RN occurring in the kidney and will be the main focus of this review.

Necroptosis

TNFα has long been known to induce cell death.[31] but this was first observed to occur in a controlled fashion in cells pre-treated with a pan-caspase inhibitor to block apoptosis.[32,33] A subsequent study using Fas ligand as the stimulus identified the kinase receptor-interacting protein (RIP, later known as RIPK1) as a key component of caspase-8-independent non-apoptotic cell death.[34] The term necroptosis was coined to describe this regulated form of necrosis.[35] Necroptosis has been reported to contribute to various disease states including sepsis, colitis, pancreatitis and stroke, although its relative importance in these conditions remains somewhat controversial.[36] Necroptosis is also involved in defence against cancer and viral infections.[37,38] Necroptosis is believed to play a role in several models of renal injury, including IRI (discussed in more detail below), cisplatin-induced acute kidney injury (AKI),[39,40] contrast-induced nephropathy,[41] and folic acid-induced AKI.[42]

The necroptotic signaling pathway

Necroptosis is dependent on the cytoplasmic molecules RIPK3 (receptor-interacting protein kinase 3) and MLKL (mixed lineage kinase domain-like protein).[43,44] The best studied trigger of necroptotic cell death is TNFα, however necroptosis can also be initiated by other members of the TNFα death ligand family such as Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL), as well as interferons, TLR signalling, and viral infections via the adaptor DAI/ZBP1.[45] For this review, we will focus on the prototypic TNFα-initiated necroptotic pathway (Figure 1).

When TNF binds to its receptor TNFR1, the receptor-associated **Complex I** assembles (Figure 1a). This complex consists of TNFR1, TNFR1-associated death domain (TRADD), TNFR-associated factor 2 (TRAF2), RIPK1, cellular inhibitor of apoptosis protein 1 (cIAP1), cIAP2

and the linear ubiquitin chain assembly complex (LUBAC).[46] Cylindromatosis (CYLD) is a deubiquitinating enzyme that contributes to the destabilization of complex I.[47] Complex I provides the platform for a series of reactions which determine whether a cell will survive or enter the apoptotic or necroptotic pathways. Activation of NF-κB triggers a *cell survival* signal involving ubiquitination of RIPK1 by cIAP1 and cIAP2, which stabilizes Complex I and enables recruitment of factors such as transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) in complex with TAK1-binding protein 2 (TAB2). Ubiquitination of RIPK1 by LUBAC recruits the inhibitor of NF-κB kinase (IKK) complex, which phosphorylates RIPK1 and sustains a pro-survival mode by igniting the canonical NF-κB pathway.[48]

Complex I-related *cell death* occurs by one of three separate routes. Destabilization of Complex I results in the formation of **Complex IIa** (Figure 1a), which contains TRADD, RIPK1, caspase-8 and FAS-associated death domain (FADD), leading to apoptosis via caspase activation.[49] The formation of **Complex IIb** (Figure 1a) is promoted by depletion of cIAPs,[50] TAK1 or NF-κB essential modulator (NEMO).[51] Complex IIb consists of RIPK1, RIPK3, FADD and caspase-8 which favours a RIPK1 kinase activity-dependent apoptosis.

Complex IIc (Figure 1a) (the 'necrosome') is formed under conditions where there is sufficient expression of RIPK1 and MLKL,[52,53] and caspase-8 activity is suppressed by viral or chemical inhibitors or reduced activity of $FLIP_L$ (FLICE-like inhibitory protein long isoform). Heteromeric pro-caspase-8:$FLIP_L$ negatively controls necroptosis by cleaving RIPK1, RIPK3 and CYLD.[54] RIPK1 and RIPK3 associate in the necrosome via their RIP homotypic interaction motif (RHIM) domains and undergo a series of auto- and trans-phosphorylation events. Activated RIPK3 phosphorylates the pseudokinase domain of MLKL,[55-57] which triggers MLKL

killing of mouse cells[55] and likely promotes activated MLKL oligomer formation or dissociation from the necrosome to kill human cells.[58]

MLKL, the executioner protein in necroptosis, has two functional domains, an N-terminal four-helix bundle (4HB) and a C-terminal pseudokinase domain (PsKD) that contains the RIPK3 phosphorylation site. These domains are bridged by two 'brace' helices,[55] which function by communicating stimulatory phosphorylation events in the PsKD to the killer 4HB and in assembly of MLKL complexes that mediate cell killing.[59] Recent research implicates inositol phosphate (IP) kinases as essential mediators of necroptosis. Binding of a higher-order IP has been proposed to promote an active conformation of MLKL by mediating the structural rearrangement of the brace helices to relieve a 'plug'[60,61] that blocks the killing function of the 4HB.[62] This results in MLKL oligomerization and translocation to the cell membrane, followed by membrane permeabilization and cell death,[63] although the exact mechanism remains contentious. The primary role of MLKL appears to be in necroptosis, but recent evidence suggests that it has an additional role in endosomal function.[64]

Regulation of necroptosis downstream of MLKL activation

In vitro studies have shown that activation of MLKL results in rapid exposure of phosphatidylserine (PS) prior to cell membrane rupture.[65,66] The fate of the cell after PS exposure depends on the endosomal sorting complexes required for transport (ESCRT) III. ESCRT-III is made up of cytosolic proteins that enable membrane remodelling with resultant membrane bending/budding away from the cytoplasm. Indeed, small bubbles are seen at the plasma membrane following oligomerization of MLKL.[65] ESCRT-III is required for cell viability[67] and prevents or delays loss of plasma membrane integrity and subsequent cell

death caused by active MLKL.[65] This delay may allow expression of cytokines and chemokines prior to the demise of the cell, as well as antigenic cross-priming of $CD8^+$ T cells.[65] It has been argued that active MLKL is not a 'point of no return' for cell survival,[65] although whether this is true across species requires further investigation. It has been hypothesised that cells can be resuscitated in the presence of ESCRT-III if further MLKL activation is blocked.[65]

Repulsive guidance molecule-b (RGMb) works at an earlier stage than ESCRT-III by inhibiting MLKL binding to the plasma membrane,[68] by a mechanism that remains unknown. Conditional deletion of RGMb in renal tubular epithelial cells in mice worsened renal injury induced by IR or oxalate crystals *in vivo*.[68]

Inflammation during necroptosis

In vitro data suggest that necroptosis takes at least 3 hrs from induction until cell membrane rupture,[53,69] although the *in vivo* kinetics may vary depending on the nature and strength of the stimulus and the abundance of MLKL and RIPK3. Necroptosis promotes inflammation in a variety of ways (Figure 1b): by DAMP and cytokine release, DAMP-independent activation of the inflammasome by MLKL or RIPK3, and activation of RIPK1 driving cytokine secretion.[70]

DAMPs released during necroptosis include proinflammatory intracellular components with predominantly non-immunological functions (e.g. nucleic acids, histones, HMGB1 and ATP).[71] Adding to the inflammatory milieu are cytokines from the IL-1 family, which includes IL-33, a 'necroptotic DAMP' that contributes to both innate and adaptive immune

responses.[72] DAMPs are detected by pattern recognition receptors that subsequently activate inflammasomes that are formed in response to cellular stress or microbial ligands.

Both RIPK3[73] and MLKL[70] can activate the NLRP3 inflammasome independently of DAMP release. Inflammasomes engage and activate pro-caspase-1,[70] resulting in maturation of IL-1β and IL-18.[74] Caspase-8 can have both a stimulatory and inhibitory role in inflammasome assembly; it directly processes IL-1β and/or activates caspase-1 within NLRP3, but also limits RIPK3/MLKL-dependent NLRP3 assembly and caspase-1 activation.[75]

RIPK1 can also induce proinflammatory cytokines independently of RIPK3 and MLKL by functioning as a scaffold during TNFα-driven NF-κB activation to promote cytokine production.[76] In addition, RIPK1 kinase activity has been shown to be crucial for the increase in circulating IL-1α levels and the development of spontaneous inflammatory disease in mice,[77] for the autocrine production of TNFα from TNFα-treated cells,[78] and for the sustained activation of ERK, FOS and NF-κB following TLR4 activation.[79]

Many of the inflammatory cytokines released during necroptosis directly promote further cell death, which in turn promotes inflammation creating an auto-amplification loop.[80] If this self-amplifying circuit ('necroinflammation') is not restrained, the ongoing DAMP release can trigger systemic inflammation resulting in fibrosis, tissue loss and organ failure. It has been proposed that the participation of necroptosis in certain types of AKI may predominantly be in this second wave of cell death induced by inflammatory cytokines released in response to the original insult. Inhibiting necroptosis in folic acid-induced AKI was not protective at 48 hrs[81] despite the upregulation of RIPK3 and MLKL expression in this

early phase, suggesting that induction of AKI might sensitize the kidney to necroptosis, but that necroptosis itself is not involved in the initial wave of damage in this model.[42] Inhibiting necroptosis resulted in protection at 96 hrs but not earlier, supporting the second wave hypothesis.[42] This second wave of injury during AKI depends on the inflammatory response and requires RIPK1 activation.[42]

Necroptosis pathway knockout mice

RIPK3 KO mice do not have major developmental defects and are fertile with normal NF-κB activation and apoptosis.[82] However, they do have a subtle phenotype, including defects in the Circle of Willis[36] and impaired platelet function.[83] MLKL KO mice are anatomically normal, viable and fertile.[55,84] In contrast, RIPK1 KO mice do not survive beyond 1-2 days after birth, dying of systemic inflammation.[85,86] This lethal phenotype is rescued by co-deletion of caspase-8 and either RIPK3 or MLKL,[86-88] demonstrating the interplay between apoptosis and necroptosis.[55,89]

MLKL KO mice but not RIPK3 KO mice showed protection in some models of inflammation.[36] Conversely, RIPK3 KO mice but not MLKL KO mice showed protection from tubular injury in a sepsis model of renal disease.[90] This discrepancy may be due to pleiotropic actions of RIPK3 beyond necroptosis. An interesting hypothesis is that RIPK3 serves an additional role as an apoptosis inhibitor,[89] and may undergo auto-activation when overexpressed to alleviate the requirements of RIPK1 for initiation of necroptosis.[91] RIPK3 was reported to induce oxidative stress and mitochondrial dysfunction involving upregulation of NADPH oxidase-4 (NOX4) and inhibition of mitochondrial complex I and III, promoting kidney tubular injury.[90] RIPK3 KO mice have been shown to be resistant to cerulean-induced

necrotic pancreatitis[92] and vaccinia virus-induced hepatic necrosis.[93] In a mouse transplant model, mice receiving RIPK3 KO kidneys survived longer with improved renal function compared to controls.[94]

Inhibitors of necroptosis

Small molecule inhibitors of various components of the necroptotic pathway have been reviewed recently.[43,95] GSK2982772, a RIPK1 inhibitor, is currently being tested in subjects with active ulcerative colitis in a phase 2 double randomized intervention trial (NCT02903966). Necroptosis inhibitors that have been tested in models of renal IRI are listed in Table 1 and discussed in further detail later in this review. It is also conceivable that molecules with broader modes of action, such as statins, may influence necroptosis. The precise mechanism by which MLKL permeabilizes membranes is debated, and why permeabilization does not proceed immediately upon membrane localization is unclear. This raises the prospect that additional factors, such as membrane composition, might influence susceptibility to MLKL permeabilization. It is therefore possible that statins, compounds that affect lipid organization and assembly into rafts, may influence the propensity for MLKL-mediated membrane permeabilization, although this remains to be examined formally.

Ferroptosis

The ferroptotic pathway

The role of iron in renal disease has been apparent for decades following the observation that iron chelators inhibit renal tubular cell death. It is now known that this iron-dependent pathway, termed ferroptosis, is a form of regulated necrosis characterized by lipid peroxidation (Figure 2).[96] Unlike apoptosis and necroptosis, there are no known receptors

that trigger ferroptosis, rather it is initiated intracellularly by the failure of glutathione-dependent antioxidant defences. The cysteine-glutamate antiporter system (X_c) allows the intracellular entry of cysteine which is required for the generation of glutathione (GSH) by glutathione synthase.[97] GSH is crucial for the function of glutathione peroxidase 4 (GPX4), an enzyme that inhibits lipoxygenase and prevents the accumulation of lipid ROS.[98] Either the depletion of GSH or the absence of GPX4 results in cardiolipin oxidation and subsequent phospholipid peroxidation, and this unique lipid peroxidation signature leads to the loss of plasma membrane integrity by an unknown mechanism.[99] The glutathione-GPX4 axis is the sole cellular system responsible for the efficient repair of oxidized phospholipids, and *in vivo* studies in mice have demonstrated that regulation of ferroptosis is a key function of GPX4.[100] The lipid metabolic enzyme acyl-CoA synthetase long-chain family member 4 (ACSL4) sensitizes cells to ferroptosis by enriching membranes for particular oxidation-sensitive fatty acids,[101-104] and the expression level of ACSL4 appears to be a useful marker of ferroptosis.[101]

Inhibitors of ferroptosis

Ferroptosis inhibitors have been reviewed elsewhere,[95,105] and only those inhibitors relevant to renal IRI are described in Table 1 and later. Much of the *in vivo* data should be interpreted with caution due to the lability of inhibitors such as ferrostatin-1.[96] Like necroptosis, ferroptosis may also conceivably be affected by agents such as statins that impact lipid-ROS metabolism.

Other forms of RN

Pyroptosis is a highly inflammatory caspase-dependent cell death modality that is mainly described in monocytes.[106] Various inflammasomes activate caspase-1, which cleaves gasdermin D to release an N-terminal fragment which forms plasma membrane pores,[107] resulting in osmotic swelling and cell membrane rupture with rapid release of cytosolic contents. Caspase-1 also mediates the secretion of IL-1β and IL-18, perpetuating inflammation.[106,108] There is some evidence to suggest that pyroptosis is induced in tubular epithelial cells during renal IRI, although the precise signalling pathways are yet to be elucidated.[109,110]

Parthanatos has mainly been studied in the context of neuronal damage and neurodegeneration. The hallmark of parthanatos is over activation of poly(ADP-ribose) polymerase-1 (PARP-1) by a wide array of stimuli including ROS.[111] This results in the synthesis of poly(ADP-ribose), which causes translocation of apoptosis-inducing factor from mitochondria to the nucleus where it mediates caspase-independent cell death.[112,113] Parthanatos can be blocked genetically by deleting PARP-1 or pharmacologically by using PARP-1 inhibitors. PARP1-deficient mice are protected in stroke and renal IRI,[114] and pilot clinical trials testing PARP-1 inhibitors in myocardial infarction[115] and stroke are currently underway (NCT01983358).

MPT-triggered RN is dependent on Cyclophilin D (CypD) and the opening of the mitochondrial permeability transition pore (MPTP). While the exact mechanism of MPT-triggered RN is unknown, it has been implicated in various pathologies including cardiac and renal IRI,[116] muscular dystrophy,[117] and diabetes.[118] Ions and various metabolic intermediates such as ROS allow for the assembly of the MPTP.[119] Pharmacological

inhibition using immunophilin-binding ligands such as cyclosporin A or Sanglifehrin A reduces IRI in the kidney,[40] brain,[120] and heart,[121] although some of the protective effects may be due to calcineurin inhibition by the cyclosporine A-cyclophilin complex.

RN IN RENAL IRI

The involvement of RN pathways in renal IRI has been investigated using mouse models in which the renal pedicles are bilaterally clamped for various periods of time, and tubular injury, renal function and mortality are assessed at selected times post-reperfusion.[36,40,101,122-124] Comparison between studies is complicated by several variables, including the degree of injury, which is influenced by ischemia time (usually between 27 and 43 min) and even by the type of anesthesia: isoflurane, for example, is protective.[125] In the case of knockout mouse studies, the use of commercially purchased WT controls rather than the more appropriate littermates may also be a confounding factor,[126] although this is not always explicitly stated. Despite these limitations, mouse models have provided valuable insights into the role and interplay of the different RN pathways in renal IRI.

The most extensive evidence linking RN to renal IRI has been gathered for necroptosis and ferroptosis (discussed below). The contribution of the other RN pathways is less well established. A role for pyroptosis was suggested by the upregulation of pyroptosis-related proteins in renal tissue in a rat model of renal IRI, with *in vitro* studies indicating that endoplasmic reticulum stress is a trigger for activation of the pyroptotic pathway in renal tubular epithelial cells.[127] PARP activation contributes to renal proximal tubular cell injury in ischemic kidneys by augmentation of the inflammatory cascade, suggesting the involvement of parthanatos in renal IRI.[123,124] This is supported by the finding that pharmacological

inhibition or gene knockout of PARP-1 improved renal function in a model of renal IRI.[128] Similarly, inhibition or knockout of CypD (an essential component of the MPTP) protected mice from renal IRI by reducing necrotic cell death, implicating MPT-RN.[40,116] Combinatorial studies using RIPK3 KO and CypD KO mice and various inhibitors suggest that necroptosis and MPT-RN may operate independently in renal IRI.[40]

Necroptosis in renal IRI

In vitro studies hint at a cell-specific sensitivity to necroptosis within the kidney, with tubular cells and glomerular endothelial cells more susceptible than mesangial cells and podocytes.[129] Increased expression of RIPK1, RIPK3 and MLKL may indicate a cell's predisposition to necroptosis.[130] RIPK1 and RIPK3 have been detected in lysates of whole mouse kidney and isolated mouse proximal tubules by Western blot analysis,[129] and MLKL expression was increased post-reperfusion.[101] Activation of RIPK1, RIPK3 and MLKL can be detected by changes in their phosphorylation status or membrane accumulation using immunoblotting or immunohistochemistry,[131,132] but the latter method in particular suffers from a lack of suitable antibodies. Furthermore, phosphorylated MLKL denotes the activation of necroptosis within a cell, but does not indicate that the cell has undergone necroptotic death *per se*. The study of necroptosis in renal IRI has therefore relied heavily on the use of small molecule inhibitors and knockout mice (Table 1).[130]

Necroptosis inhibitor studies

The RIPK1 inhibitor Necrostatin-1 (Nec-1) protected rat and human tubular cells in an *in vitro* model using TNFα stimulation and ATP depletion to mimic renal ischemic injury.[133,134] Nec-1 was tested in a mouse model of renal IRI (30 min ischemia).[129] Nec-1 treatment at

either 15 min pre-ischemia or 15 min post-reperfusion significantly reduced renal damage and preserved renal function as measured by serum creatinine and urea at 48 hrs.[129] Furthermore, Nec-1 given at both the pre and post time points significantly reduced mortality in a more severe model (40 min ischemia).[129] However, Nec-1 is not specific for necroptosis as it also inhibits indoleamine 2,3-dioxygenase (IDO), which influences innate and adaptive immune function.[95,135,136] Nec-1 has also been shown to function as a ferrostatin[100] and is relatively toxic.[136] The newer generation inhibitor Nec-1s lacks IDO-inhibitory activity and is more stable and less toxic than Nec-1.[137] Nec-1s locks RIPK1 in an inactive conformation,[138] preventing its phosphorylation and ubiquitination within the necrosome.[139] Nec-1s treatment protected against TNFα-induced systemic inflammatory response syndrome (SIRS)[140] and against folic-acid-induced AKI,[42] but its effect in renal IRI has not yet been reported.

Another RIPK1 inhibitor, the chemotherapeutic drug Sorafenib tosylate, also protected against SIRS and was protective in the same model of renal IRI (30 min ischemia) used to test Nec-1.[140] However, while low-dose Sorafenib (10 mg/kg) given 15 min pre-ischemia reduced renal damage and preserved renal function at 48 hrs, a higher dose (100 mg/kg) actually worsened renal injury.[140] Whether this was due to an effect on RIPK1 and/or another of the kinases known to be inhibited by Sorafenib (including RIPK3[140]) remains unknown. Further complicating the interpretation of Sorafenib's effects is the finding that it had no effect on necroptosis in mouse fibroblasts *in vitro*.[131]

The RIPK3 inhibitors GSK843, GSK872 and the human-specific GSK840 inhibit TNFα-induced necroptosis *in vitro*, but they are untested in renal IRI and their therapeutic potential may

be limited by a concentration-dependent induction of apoptosis via the recruitment of RIPK1 and activation of caspase-8.[141]

Inhibition of MLKL currently offers the most specific pharmacological means to block necroptotic cell death. Necrosulfonamide (NSA) prevents necroptosis *in vitro*[57] but is uninformative in murine models because it is specific for human MLKL. 'Compound 1' (also known as GW806742X or SYN1215) targets the pseudokinase domain of murine MLKL and protects mouse fibroblasts from necroptosis *in vitro*,[131] although the extent to which protection is attributable to MLKL engagement or inhibition of the upstream RIPK1 remains unclear. The effect of Compound 1 in renal IRI is yet to be determined.

A potential limitation to the testing of therapeutic necroptosis inhibitors in mouse models of renal IRI is that despite the fact that both RIPK3 and MLKL share >60% amino acid identity between mice and humans,[142] there appear to be species-specific differences in their mechanism of action.[53,58,59,142] This may necessitate testing in chimeric mice in which the human RIPK3 and/or MLKL genes are knocked in to replace their mouse homologs.

Necroptosis knockout mouse studies

Studies with KO mice support a role for necroptosis in renal IRI (Table 1), although whether RIPK3 also mediates MLKL-independent injury in this setting remains an open question. Linkermann et al. reported a modest survival advantage for RIPK3 KO mice in a "lethal-to-WT" renal IRI model (43 min ischemia).[40] RIPK3 KO and MLKL KO mice also showed a modest improvement in 48-hr renal function compared to WT controls in the same group's "standard" model (30 min ischemia).[122] Newton et al. used a 30 min renal ischemia model to

demonstrate markedly lower mortality of RIPK3 KO mice compared to WT mice.[36] RIP1 KD (kinase-dead) mice were also protected in this model.[36] However, despite a 4-day prolongation in median survival, overall survival of MLKL KO mice was not significantly different to that of WT mice.[36] Muller et al. reported protection of MLKL KO mice in a 35 min ischemia model at 48 and 72 hrs after reperfusion, but not at 24 hrs.[101]

Do necroptosis pathway components have cell death-independent effects in renal IRI?

Several mouse studies suggest that manipulation of RIPK1, RIPK3 or MLKL may have unexpected effects on microvascular function in the kidney. The RIPK1 inhibitor Nec-1 affected the dilation of peritubular capillaries in a model of contrast-induced AKI.[41] Peritubular capillary diameter was increased in untreated RIPK3 KO mice,[143] and renal blood flow was increased in both these mice[143] and untreated MLKL KO mice.[122] Since impairment of blood flow to the kidney is a feature of renal IRI,[12] these cell death-independent effects should be taken into consideration when studying necroptosis in models of renal IRI.

Evidence for necroptosis in clinical renal IRI

Immunohistological detection of phosphorylated MLKL (pMLKL) is a logical method to assess necroptosis in human kidneys affected by IRI, but this approach suffers from a lack of properly validated antibodies. With this caveat, human kidney biopsies taken immediately post-transplant showed some endothelial staining for pMLKL, although interestingly not all positive cells showed features of necrosis.[65] Another study by the same group found that 10-15% of necrotic tubules were pMLKL-positive.[122] It remains unclear whether the presence of pMLKL-negative necrotic cells was due to a technical issue or was an indication of the involvement of other pathways of RN. A different group failed to detect pMLKL in

human kidney biopsies taken 7 days post-transplant, although this may have reflected the unsuitability of available antibodies and/or the later timing of the biopsies.[101]

Ferroptosis in renal IRI

Linkermann et al. reported that treatment of WT mice 15 min pre-ischemia with ferrostatin-1 or its more stable derivative 16-86 was protective in a model of severe IRI (40 min ischemia).[143] Tissue injury, serum creatinine and urea were all reduced at 48 hrs.[143] These results indicate a role for ferroptosis in the pathogenesis of renal IRI in this model, although the apparent efficacy of ferrostatin-1 in particular was surprising given its *in vivo* instability.[96] Additional combined pre-treatment with the RIPK1 inhibitor Nec-1 and the MPT-RN inhibitor Sanglifehrin A in an "ultrasevere" model (50 min ischemia) potentiated the protection mediated by 16-86, suggesting the involvement of multiple RN pathways in renal IRI.[143] This is consistent with an earlier study with RIPK3 KO and CypD KO mice that implicated both necroptosis and MPT-RN.[40]

Muller et al. identified ACSL4 as a mediator of ferroptosis and proposed that increased ACSL4 expression may be a useful marker of the process.[101] Analysis of biopsies from a patient with acute tubular injury 7 days post-transplant revealed strong staining for ACSL4 in some renal tubules.[101] *In vitro* analysis of cells deficient in MLKL or ACSL4 suggested that resistance to necroptosis sensitized cells to ferroptosis, and vice versa.[101] Consistent with this, MLKL KO mice subjected to renal IRI (35 min ischemia) exhibited earlier upregulation of renal ACSL4 expression than WT mice, indicating more rapid activation of ferroptosis in the absence of necroptosis. As mentioned earlier, MLKL KO mice showed a modest improvement in renal function at 48 and 72 hrs post-reperfusion; treatment with 16-86 at

15 min pre-ischemia and every 3 hrs for 24 hrs thereafter returned the kinetics of ACSL4 expression to the WT pattern, but did not result in a further improvement in the outcome at 72 hrs.[101] The authors suggested several possible reasons for this apparent discrepancy, including insufficient duration of treatment and/or *in vivo* instability of 16-86.[101]

CONCLUSIONS

Renal IRI is a very complex process, and deciphering any role of the various pathways of RN is complicated by a range of factors: a lack of truly specific, physiologically stable inhibitors, validated detection antibodies, and informative/reliable biomarkers; the pleiotropic functions of several RN effector molecules; variation in mouse models, and species-specific differences in some aspects of RN; and incomplete understanding of the intricate inter-relationships between different RN pathways. Nevertheless, the evidence that RN plays a role in the pathophysiology of renal IRI continues to grow.

It is interesting to speculate on how this knowledge might ultimately be used in the clinical treatment of renal IRI. Meaningful protection may require targeting of multiple RN pathways, since inhibition of one pathway may simply sensitize to another.[101] Furthermore, the *in vivo* fate of a cell in which RN is initiated but blocked from following its full course remains unclear. There are also potential drawbacks. In the case of renal transplantation, inhibiting necroptosis in an immunosuppressed recipient may increase susceptibility to infection, considering the putative role of necroptosis in defence against bacterial and viral pathogens.[93,144-146] However, this may be a short-term risk because RN blockade is likely to be required only during the peri-transplant period.

The past decade has seen significant evolution in our understanding of how cells die. RN has been shown to play an important role in renal IRI, albeit only in animal models to date. The accelerating research into the mechanisms and interactions of cell death pathways and the development of improved inhibitors may one day impact on clinical nephrology and transplantation practice.

Disclosure

JMM contributes to a project with Anaxis Pharma developing small molecule inhibitors of necroptosis.

FIGURE LEGENDS

Figure 1. The prototypic TNFα-initiated necroptosis pathway. a) Alternative signalling pathways triggered by TNFα. Ligation of TNFR1 results in the generation of Complex I, which can act as a platform for the assembly of Complexes IIa or IIb (leading to apoptosis), or of the 'necrosome', Complex IIc (leading to necroptosis). b) The pro-inflammatory process of necroptosis. The necrosome, formed under conditions where caspase-8 activity is suppressed, recruits and phosphorylates MLKL, inducing a conformational change that exposes the death effector domain of MLKL. Oligomerization of phospho-MLKL, which appears to require binding of a higher-order inositol phosphate (IP), is followed by its translocation to the plasma membrane and induction of cell lysis, resulting in the release of proinflammatory DAMPs. Phospho-MLKL also activates the NLRP3 inflammasome, causing secretion of IL-1β and IL-18. Repulsive guidance molecule-b (RGMb) inhibits necroptosis in renal tubular cells by interfering with MLKL binding to the plasma membrane. Endosomal sorting complexes required for transport (ESCRT) III can delay or prevent necroptotic cell death by enabling membrane remodelling. Small molecule inhibitors of the key components of necroptosis are shown.

Figure 2. The ferroptosis pathway. Various stressors including intracellular free iron promote the generation of lipid peroxides, which mediate loss of plasma membrane integrity by an unknown mechanism. GPX4 detoxifies lipid peroxides and is a key regulator of ferroptosis. Acyl-CoA synthetase long-chain family member 4 (ACSL4) sensitizes cells to ferroptosis by enriching membranes for particular fatty acids. Small molecule inhibitors of

ferroptosis are shown. Abbreviations: PUFA, polyunsaturated fatty acids; PL, phospholipid; PE, phosphatidyl ethanolamine; ROS, reactive oxygen species.

REFERENCES

1. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest.* 2011;121(11):4210-4221.
2. Brady HR, Singer GG. Acute renal failure. *Lancet.* 1995;346(8989):1533-1540.
3. Perico N, Cattaneo D, Sayegh MH, Remuzzi G. Delayed graft function in kidney transplantation. *The Lancet.* 2004;364(9447):1814-1827.
4. Kosieradzki M, Rowinski W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant Proc.* 2008;40(10):3279-3288.
5. Kako K, Kato M, Matsuoka T, Mustapha A. Depression of membrane-bound Na+-K+-ATPase activity induced by free radicals and by ischemia of kidney. *The Americal Physiological Society.* 1988:330-337.
6. Castaneda MP, Swiatecka-Urban A, Mitsnefes MM, et al. Activation of mitochondrial apoptotic pathways in human renal allografts after ischemiareperfusion injury. *Transplantation.* 2003;76(1):50-54.
7. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007;81(1):1-5.
8. Bianchi ME, Manfredi AA. High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev.* 2007;220:35-46.
9. Li L, Okusa MD. Blocking the immune response in ischemic acute kidney injury: the role of adenosine 2A agonists. *Nat Clin Pract Nephrol.* 2006;2(8):432-444.
10. Chadha R, Heidt S, Jones ND, Wood KJ. Th17: contributors to allograft rejection and a barrier to the induction of transplantation tolerance? *Transplantation.* 2011;91(9):939-945.
11. Tesmer LA, Lundy SK, Sarkar S, Fox DA. Th17 cells in human disease. *Immunol Rev.* 2008;223:87-113.
12. Molitoris BA, Sutton TA. Endothelial injury and dysfunction: role in the extension phase of acute renal failure. *Kidney Int.* 2004;66(2):496-499.
13. Zhao H, Alam A, Soo AP, George AJT, Ma D. Ischemia-Reperfusion Injury Reduces Long Term Renal Graft Survival: Mechanism and Beyond. *EBioMedicine.* 2018;28:31-42.
14. Basile DP, Donohoe D, Roethe K, Osborn JL. Renal ischemic injury results in permanent damage to peritubular capillaries and influence long-term function. *Am J Physiol Renal Physiol.* 2001;281(5):887-899.
15. Legrand M, Mik EG, Johannes T, Payen D, Ince C. Renal hypoxia and dysoxia after reperfusion of the ischemic kidney. *Mol Med.* 2008;14(7-8):502-516.
16. Basile DP, Friedrich JL, Spahic J, et al. Impaired endothelial proliferation and mesenchymal transition contribute to vascular rarefaction following acute kidney injury. *Am J Physiol Renal Physiol.* 2011;300(3):F721-733.
17. Norman JT, Clark IM, Garcia PL. Hypoxia promotes fibrogenesis in human renal fibroblasts. *Kidney Int.* 2000;58(6):2351-2366.
18. Gueler F, Gwinner W, Schwarz A, Haller H. Long-term effects of acute ischemia and reperfusion injury. *Kidney Int.* 2004;66(2):523-527.
19. Fletcher JT, Nankivell BJ, Alexander SI. Chronic allograft nephropathy. *Pediatr Nephrol.* 2009;24(8):1465-1471.
20. van der Vliet JA, Warle MC. The need to reduce cold ischemia time in kidney transplantation. *Curr Opin Organ Transplant.* 2013;18(2):174-178.

21. Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. *Nephrology Dialysis Transplantation.* 2014;29(8):1612-1612.
22. Yarlagadda SG, Coca SG, Formica RN, Jr., Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrol Dial Transplant.* 2009;24(3):1039-1047.
23. Saat TC, van den Akker EK, Ijzermans JN, Dor FJ, de Bruin RW. Improving the outcome of kidney transplantation by ameliorating renal ischemia reperfusion injury: lost in translation? *J Transl Med.* 2016;14:20.
24. Ortiz F, Paavonen T, Tornroth T, et al. Predictors of renal allograft histologic damage progression. *J Am Soc Nephrol.* 2005;16(3):817-824.
25. Denecke C, Tullius SG. Innate and adaptive immune responses subsequent to ischemia-reperfusion injury in the kidney. *Progrès en Urologie.* 2014;24:S13-S19.
26. Barba J, Zudaire JJ, Robles JE, et al. Is there a safe cold ischemia time interval for the renal graft? *Actas Urol Esp.* 2011;35(8):475-480.
27. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell.* 2004;116(2):205-219.
28. Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA. Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity.* 2008;29(1):21-32.
29. Asano K, Miwa M, Miwa K, et al. Masking of phosphatidylserine inhibits apoptotic cell engulfment and induces autoantibody production in mice. *J Exp Med.* 2004;200(4):459-467.
30. Krysko DV, Vanden Berghe T, D'Herde K, Vandenabeele P. Apoptosis and necrosis: detection, discrimination and phagocytosis. *Methods.* 2008;44(3):205-221.
31. Carswell EA, Old LL, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci U S A.* 1975;72(9):3666-3670.
32. 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(9):1477-1485.
33. Vercammen D, Brouckaert G, Denecker G, et al. Dual Signaling of the Fas Receptor: Initiation of Both Apoptotic and Necrotic Cell Death Pathways. *The Journal of Experimental Medicine.* 1998;188(5):919-930.
34. 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. *Nature Immunology.* 2000;1(6):489-495.
35. 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(2):112-119.
36. Newton K, Dugger DL, Maltzman A, et al. RIPK3 deficiency or catalytically inactive RIPK1 provides greater benefit than MLKL deficiency in mouse models of inflammation and tissue injury. *Cell Death Differ.* 2016;23(9):1565-1576.
37. Krysko O, Aaes TL, Kagan VE, et al. Necroptotic cell death in anti-cancer therapy. *Immunol Rev.* 2017;280(1):207-219.
38. Jorgensen I, Rayamajhi M, Miao EA. Programmed cell death as a defence against infection. *Nat Rev Immunol.* 2017;17(3):151-164.

39. Tristao VR, Goncalves PF, Dalboni MA, Batista MC, Durao Mde S, Jr., Monte JC. Nec-1 protects against nonapoptotic cell death in cisplatin-induced kidney injury. *Ren Fail.* 2012;34(3):373-377.
40. Linkermann A, Brasen JH, Darding M, et al. Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proc Natl Acad Sci U S A.* 2013;110(29):12024-12029.
41. Linkermann A, Heller JO, Prokai A, et al. The RIP1-kinase inhibitor necrostatin-1 prevents osmotic nephrosis and contrast-induced AKI in mice. *J Am Soc Nephrol.* 2013;24(10):1545-1557.
42. Martin-Sanchez D, Fontecha-Barriuso M, Carrasco S, et al. TWEAK and RIPK1 mediate a second wave of cell death during AKI. *Proc Natl Acad Sci U S A.* 2018;115(16):4182-4187.
43. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature.* 2015;517(7534):311-320.
44. Linkermann A, Green DR. Necroptosis. *N Engl J Med.* 2014;370(5):455-465.
45. Galluzzi L, Kepp O, Chan FK, Kroemer G. Necroptosis: Mechanisms and Relevance to Disease. *Annu Rev Pathol.* 2017;12:103-130.
46. Conrad M, Angeli JPF, Vandenabeele P, Stockwell BR. Regulated necrosis: disease relevance and therapeutic opportunities. *Nature Reviews.* 2016.
47. Orozco S, Oberst A. RIPK3 in cell death and inflammation: the good, the bad, and the ugly. *Immunol Rev.* 2017;277(1):102-112.
48. Dondelinger Y, Jouan-Lanhouet S, Divert T, et al. NF-kappaB-Independent Role of IKKalpha/IKKbeta in Preventing RIPK1 Kinase-Dependent Apoptotic and Necroptotic Cell Death during TNF Signaling. *Mol Cell.* 2015;60(1):63-76.
49. Micheau O, Tschopp J. Induction of TNF Receptor I-Mediated Apoptosis via Two Sequential Signaling Complexes. *Cell.* 2003;114(2):181-190.
50. Wang L, Du F, Wang X. TNF-alpha induces two distinct caspase-8 activation pathways. *Cell.* 2008;133(4):693-703.
51. Legarda-Addison D, Hase H, O'Donnell MA, Ting AT. NEMO/IKKgamma regulates an early NF-kappaB-independent cell-death checkpoint during TNF signaling. *Cell Death Differ.* 2009;16(9):1279-1288.
52. Cook WD, Moujalled DM, Ralph TJ, et al. RIPK1- and RIPK3-induced cell death mode is determined by target availability. *Cell Death Differ.* 2014;21(10):1600-1612.
53. Tanzer MC, Tripaydonis A, Webb AI, et al. Necroptosis signalling is tuned by phosphorylation of MLKL residues outside the pseudokinase domain activation loop. *Biochem J.* 2015;471(2):255-265.
54. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature.* 2011;471(7338):363-367.
55. Murphy JM, Czabotar PE, Hildebrand JM, et al. The Pseudokinase MLKL Mediates Necroptosis via a Molecular Switch Mechanism. *Immunity.* 2013;39(3):443-453.
56. Murphy JM, Lucet IS, Hildebrand JM, et al. Insights into the evolution of divergent nucleotide-binding mechanisms among pseudokinases revealed by crystal structures of human and mouse MLKL. *Biochem J.* 2014;457(3):369-377.
57. Sun L, Wang H, Wang Z, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell.* 2012;148(1-2):213-227.

58. Petrie EJ, Sandow JJ, Jacobsen AV, et al. Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis. *Nat Commun.* 2018;9(1):2422.
59. Davies KA, Tanzer MC, Griffin MDW, et al. The brace helices of MLKL mediate interdomain communication and oligomerisation to regulate cell death by necroptosis. *Cell Death Differ.* 2018.
60. Su L, Quade B, Wang H, Sun L, Wang X, Rizo J. A plug release mechanism for membrane permeation by MLKL. *Structure.* 2014;22(10):1489-1500.
61. Quarato G, Guy CS, Grace CR, et al. Sequential Engagement of Distinct MLKL Phosphatidylinositol-Binding Sites Executes Necroptosis. *Mol Cell.* 2016;61(4):589-601.
62. Dovey CM, Diep J, Clarke BP, et al. MLKL Requires the Inositol Phosphate Code to Execute Necroptosis. *Mol Cell.* 2018;70(5):936-948 e937.
63. Petrie EJ, Hildebrand JM, Murphy JM. Insane in the membrane: a structural perspective of MLKL function in necroptosis. *Immunol Cell Biol.* 2017;95(2):152-159.
64. Yoon S, Kovalenko A, Bogdanov K, Wallach D. MLKL, the Protein that Mediates Necroptosis, Also Regulates Endosomal Trafficking and Extracellular Vesicle Generation. *Immunity.* 2017;47(1):51-65 e57.
65. Gong YN, Guy C, Olauson H, et al. ESCRT-III Acts Downstream of MLKL to Regulate Necroptotic Cell Death and Its Consequences. *Cell.* 2017;169(2):286-300 e216.
66. Zargarian S, Shlomovitz I, Erlich Z, et al. Phosphatidylserine externalization, "necroptotic bodies" release, and phagocytosis during necroptosis. *PLoS Biol.* 2017;15(6):e2002711.
67. Christ L, Raiborg C, Wenzel EM, Campsteijn C, Stenmark H. Cellular Functions and Molecular Mechanisms of the ESCRT Membrane-Scission Machinery. *Trends Biochem Sci.* 2017;42(1):42-56.
68. Liu W, Chen B, Wang Y, et al. RGMb protects against acute kidney injury by inhibiting tubular cell necroptosis via an MLKL-dependent mechanism. *Proc Natl Acad Sci U S A.* 2018;115(7):E1475-E1484.
69. Tonnus W, Linkermann A. The in vivo evidence for regulated necrosis. *Immunol Rev.* 2017;277(1):128-149.
70. Conos SA, Chen KW, De Nardo D, et al. Active MLKL triggers the NLRP3 inflammasome in a cell-intrinsic manner. *Proc Natl Acad Sci U S A.* 2017;114(6):E961-E969.
71. Krysko DV, Garg AD, Kaczmarek A, Krysko O, Agostinis P, Vandenabeele P. Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer.* 2012;12(12):860-875.
72. Liew FY, Girard JP, Turnquist HR. Interleukin-33 in health and disease. *Nat Rev Immunol.* 2016;16(11):676-689.
73. Lawlor KE, Khan N, Mildenhall A, et al. RIPK3 promotes cell death and NLRP3 inflammasome activation in the absence of MLKL. *Nat Commun.* 2015;6:6282.
74. Weinlich R, Oberst A, Beere HM, Green DR. Necroptosis in development, inflammation and disease. *Nat Rev Mol Cell Biol.* 2017;18(2):127-136.
75. Kang TB, Yang SH, Toth B, Kovalenko A, Wallach D. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity.* 2013;38(1):27-40.

76. Kondylis V, Kumari S, Vlantis K, Pasparakis M. The interplay of IKK, NF-kappaB and RIPK1 signaling in the regulation of cell death, tissue homeostasis and inflammation. *Immunol Rev.* 2017;277(1):113-127.
77. Berger SB, Kasparcova V, Hoffman S, et al. Cutting Edge: RIP1 kinase activity is dispensable for normal development but is a key regulator of inflammation in SHARPIN-deficient mice. *J Immunol.* 2014;192(12):5476-5480.
78. Christofferson DE, Li Y, Hitomi J, et al. A novel role for RIP1 kinase in mediating TNFalpha production. *Cell Death Dis.* 2012;3:e320.
79. Najjar M, Saleh D, Zelic M, et al. RIPK1 and RIPK3 Kinases Promote Cell-Death-Independent Inflammation by Toll-like Receptor 4. *Immunity.* 2016;45(1):46-59.
80. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol.* 2014;14(11):759-767.
81. Martin-Sanchez D, Ruiz-Andres O, Poveda J, et al. Ferroptosis, but Not Necroptosis, Is Important in Nephrotoxic Folic Acid-Induced AKI. *J Am Soc Nephrol.* 2017;28(1):218-229.
82. Newton K, Sun X, Dixit VM. Kinase RIP3 is dispensable for normal NF-kappa Bs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol.* 2004;24(4):1464-1469.
83. Zhang Y, Zhang J, Yan R, et al. Receptor-interacting protein kinase 3 promotes platelet activation and thrombosis. *Proc Natl Acad Sci U S A.* 2017;114(11):2964-2969.
84. Wu J, Huang Z, Ren J, et al. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res.* 2013;23(8):994-1006.
85. 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(3):297-303.
86. Rickard JA, O'Donnell JA, Evans JM, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell.* 2014;157(5):1175-1188.
87. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature.* 2011;471(7338):368-372.
88. Green DR, Oberst A, Dillon CP, Weinlich R, Salvesen GS. RIPK-dependent necrosis and its regulation by caspases: a mystery in five acts. *Mol Cell.* 2011;44(1):9-16.
89. Newton K, Dugger DL, Wickliffe KE, et al. Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. *Science.* 2014;343(6177):1357-1360.
90. Sureshbabu A, Patino E, Ma KC, et al. RIPK3 promotes sepsis-induced acute kidney injury via mitochondrial dysfunction. *JCI Insight.* 2018;3(11).
91. Moujalled DM, Cook WD, Okamoto T, et al. TNF can activate RIPK3 and cause programmed necrosis in the absence of RIPK1. *Cell Death Dis.* 2013;4:e465.
92. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell.* 2009;137(6):1100-1111.
93. Cho Y, Challa S, Moquin D, et al. Phosphorylation-Driven Assembly of the RIP1-RIP3 Complex Regulates Programmed Necrosis and Virus-Induced Inflammation. *Cell.* 2009;137(6):1112-1123.
94. Lau A, Wang S, Jiang J, et al. RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *Am J Transplant.* 2013;13(11):2805-2818.

95. Degterev A, Linkermann A. Generation of small molecules to interfere with regulated necrosis. *Cell Mol Life Sci.* 2016;73(11-12):2251-2267.
96. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell.* 2012;149(5):1060-1072.
97. Conrad M, Sato H. The oxidative stress-inducible cystine/glutamate antiporter, system x (c) (-) : cystine supplier and beyond. *Amino Acids.* 2012;42(1):231-246.
98. Yang WS, SriRamaratnam R, Welsch ME, et al. Regulation of ferroptotic cancer cell death by GPX4. *Cell.* 2014;156(1-2):317-331.
99. Linkermann A. Nonapoptotic cell death in acute kidney injury and transplantation. *Kidney Int.* 2016;89(1):46-57.
100. Friedmann Angeli JP, Schneider M, Proneth B, et al. Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol.* 2014;16(12):1180-1191.
101. Muller T, Dewitz C, Schmitz J, et al. Necroptosis and ferroptosis are alternative cell death pathways that operate in acute kidney failure. *Cell Mol Life Sci.* 2017;74(19):3631-3645.
102. Dixon SJ, Winter GE, Musavi LS, et al. Human Haploid Cell Genetics Reveals Roles for Lipid Metabolism Genes in Nonapoptotic Cell Death. *ACS Chem Biol.* 2015;10(7):1604-1609.
103. Doll S, Proneth B, Tyurina YY, et al. ACSL4 dictates ferroptosis sensitivity by shaping cellular lipid composition. *Nat Chem Biol.* 2017;13(1):91-98.
104. Kagan VE, Mao G, Qu F, et al. Oxidized arachidonic and adrenic PEs navigate cells to ferroptosis. *Nat Chem Biol.* 2017;13(1):81-90.
105. Li Y, Qian L, Yuan J. Small molecule probes for cellular death machines. *Curr Opin Chem Biol.* 2017;39:74-82.
106. Fink SL, Cookson BT. Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. *Cellular Microbiology.* 2006;8(11):1812-1825.
107. Liu X, Zhang Z, Ruan J, et al. Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature.* 2016;535(7610):153-158.
108. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, Walczak H, Vandenabeele P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol.* 2014;15(2):135-147.
109. Wu H, Huang T, Ying L, et al. MiR-155 is Involved in Renal Ischemia-Reperfusion Injury via Direct Targeting of FoxO3a and Regulating Renal Tubular Cell Pyroptosis. *Cell Physiol Biochem.* 2016;40(6):1692-1705.
110. Yang J-R, Yao F-H, Zhang J-G, et al. Ischemia-reperfusion induces renal tubule pyroptosis via the CHOP-caspase-11 pathway. *American Journal of Physiology-Renal Physiology.* 2014;306(1):F75-F84.
111. Virag L, Robaszkiewicz A, Rodriguez-Vargas JM, Oliver FJ. Poly(ADP-ribose) signaling in cell death. *Mol Aspects Med.* 2013;34(6):1153-1167.
112. David KK, Andrabi SA, Dawson TM, Dawson VL. Parthanatos, a messenger of death. *Front Biosci.* 2009;14:1116-1128.
113. Andrabi SA, Kim NS, Yu SW, et al. Poly(ADP-ribose) (PAR) polymer is a death signal. *Proc Natl Acad Sci U S A.* 2006;103(48):18308-18313.

114. del Moral RM, Gomez-Morales M, Hernandez-Cortes P, et al. PARP inhibition attenuates histopathological lesion in ischemia/reperfusion renal mouse model after cold prolonged ischemia. *ScientificWorldJournal.* 2013;2013:486574.
115. Morrow DA, Brickman CM, Murphy SA, et al. A randomized, placebo-controlled trial to evaluate the tolerability, safety, pharmacokinetics, and pharmacodynamics of a potent inhibitor of poly(ADP-ribose) polymerase (INO-1001) in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention: results of the TIMI 37 trial. *J Thromb Thrombolysis.* 2009;27(4):359-364.
116. Devalaraja-Narashimha K, Diener AM, Padanilam BJ. Cyclophilin D gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol.* 2009;297(3):F749-759.
117. Millay DP, Sargent MA, Osinska H, et al. Genetic and pharmacologic inhibition of mitochondrial-dependent necrosis attenuates muscular dystrophy. *Nat Med.* 2008;14(4):442-447.
118. Fujimoto K, Chen Y, Polonsky KS, Dorn GW, 2nd. Targeting cyclophilin D and the mitochondrial permeability transition enhances beta-cell survival and prevents diabetes in Pdx1 deficiency. *Proc Natl Acad Sci U S A.* 2010;107(22):10214-10219.
119. Javadov S, Kuznetsov A. Mitochondrial permeability transition and cell death: the role of cyclophilin d. *Front Physiol.* 2013;4:76.
120. Nighoghossian N, Berthezene Y, Mechtouff L, et al. Cyclosporine in acute ischemic stroke. *Neurology.* 2015;84(22):2216-2223.
121. Keogh A. Calcineurin inhibitors in heart transplantation. *J Heart Lung Transplant.* 2004;23(5 Suppl):S202-206.
122. von Massenhausen A, Tonnus W, Himmerkus N, et al. Phenytoin inhibits necroptosis. *Cell Death Dis.* 2018;9(3):359.
123. Zheng J, Devalaraja-Narashimha K, Singaravelu K, Padanilam BJ. Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol.* 2005;288(2):F387-398.
124. Devalaraja-Narashimha K, Padanilam BJ. PARP-1 inhibits glycolysis in ischemic kidneys. *J Am Soc Nephrol.* 2009;20(1):95-103.
125. Kim M, Ham A, Kim JY, Brown KM, D'Agati VD, Lee HT. The volatile anesthetic isoflurane induces ecto-5'-nucleotidase (CD73) to protect against renal ischemia and reperfusion injury. *Kidney Int.* 2013;84(1):90-103.
126. Holmdahl R, Malissen B. The need for littermate controls. *Eur J Immunol.* 2012;42(1):45-47.
127. Yang JR, Yao FH, Zhang JG, et al. Ischemia-reperfusion induces renal tubule pyroptosis via the CHOP-caspase-11 pathway. *Am J Physiol Renal Physiol.* 2014;306(1):F75-84.
128. Devalaraja-Narashimha K, Singaravelu K, Padanilam BJ. Poly(ADP-ribose) polymerase-mediated cell injury in acute renal failure. *Pharmacol Res.* 2005;52(1):44-59.
129. Linkermann A, Brasen JH, Himmerkus N, et al. Rip1 (receptor-interacting protein kinase 1) mediates necroptosis and contributes to renal ischemia/reperfusion injury. *Kidney Int.* 2012;81(8):751-761.
130. Jouan-Lanhouet S, Riquet F, Duprez L, Vanden Berghe T, Takahashi N, Vandenabeele P. Necroptosis, in vivo detection in experimental disease models. *Seminars in Cell & Developmental Biology.* 2014;35:2-13.

131. Hildebrand JM, Tanzer MC, Lucet IS, et al. Activation of the pseudokinase MLKL unleashes the four-helix bundle domain to induce membrane localization and necroptotic cell death. *Proc Natl Acad Sci U S A.* 2014;111(42):15072-15077.
132. Wang H, Sun L, Su L, et al. Mixed lineage kinase domain-like protein MLKL causes necrotic membrane disruption upon phosphorylation by RIP3. *Mol Cell.* 2014;54(1):133-146.
133. Zhang L, Jiang F, Chen Y, et al. Necrostatin-1 attenuates ischemia injury induced cell death in rat tubular cell line NRK-52E through decreased Drp1 expression. *Int J Mol Sci.* 2013;14(12):24742-24754.
134. Liang X, Chen Y, Zhang L, et al. Necroptosis, a novel form of caspase-independent cell death, contributes to renal epithelial cell damage in an ATP-depleted renal ischemia model. *Mol Med Rep.* 2014;10(2):719-724.
135. Prendergast GC, Chang MY, Mandik-Nayak L, Metz R, Muller AJ. Indoleamine 2,3-dioxygenase as a modifier of pathogenic inflammation in cancer and other inflammation-associated diseases. *Curr Med Chem.* 2011;18(15):2257-2262.
136. 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(2):185-187.
137. 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.
138. Xie T, Peng W, Liu Y, et al. Structural basis of RIP1 inhibition by necrostatins. *Structure.* 2013;21(3):493-499.
139. de Almagro MC, Goncharov T, Izrael-Tomasevic A, et al. Coordinated ubiquitination and phosphorylation of RIP1 regulates necroptotic cell death. *Cell Death Differ.* 2017;24(1):26-37.
140. Martens S, Jeong M, Tonnus W, et al. Sorafenib tosylate inhibits directly necrosome complex formation and protects in mouse models of inflammation and tissue injury. *Cell Death Dis.* 2017;8(6):e2904.
141. Mandal P, Berger SB, Pillay S, et al. RIP3 induces apoptosis independent of pronecrotic kinase activity. *Mol Cell.* 2014;56(4):481-495.
142. Tanzer MC, Matti I, Hildebrand JM, et al. Evolutionary divergence of the necroptosis effector MLKL. *Cell Death Differ.* 2016;23(7):1185-1197.
143. Linkermann A, Skouta R, Himmerkus N, et al. Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A.* 2014;111(47):16836-16841.
144. Pearson JS, Giogha C, Muhlen S, et al. EspL is a bacterial cysteine protease effector that cleaves RHIM proteins to block necroptosis and inflammation. *Nat Microbiol.* 2017;2:16258.
145. Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. *Curr Opin Virol.* 2013;3(3):296-306.
146. Nailwal H, Chan FK. Necroptosis in anti-viral inflammation. *Cell Death Differ.* 2018.

Table 1. Evidence for necroptosis and ferroptosis in mouse models of renal IRI

Pathway	Target	Intervention	Ischemia time	Outcome	Reference
Necroptosis	RIPK1	RIPK1 KD[a]	30 min	↓ mortality	36
		Necrostatin-1 (Nec-1)[b]	30 min	↓ injury at 48 hrs	130
			40 min	↓ mortality	130
			40 min	↓ injury at 24 hrs	68
		Sorafenib[c]	30 min	↓ injury at 48 hrs (low dose) ↑ injury at 48 hrs (high dose)	141
	RIPK3	RIPK3 KO	30 min	↓ injury at 48 hrs	123
			30 min	↓ mortality	36
			43 min	↓ mortality	40
	MLKL	MLKL KO	30 min	↓ mortality (not significant)	36
			35 min	↓ injury at 48 & 72 hrs (not at 24 hrs)	102
	RGMb	RGMb KO[d]	40 min	↑ injury at 24 hrs	68
Ferroptosis	ROS	Ferrostatin-1	40 min	↓ injury at 48 hrs	83
		16-86	40 min	↓ injury at 48 hrs	83

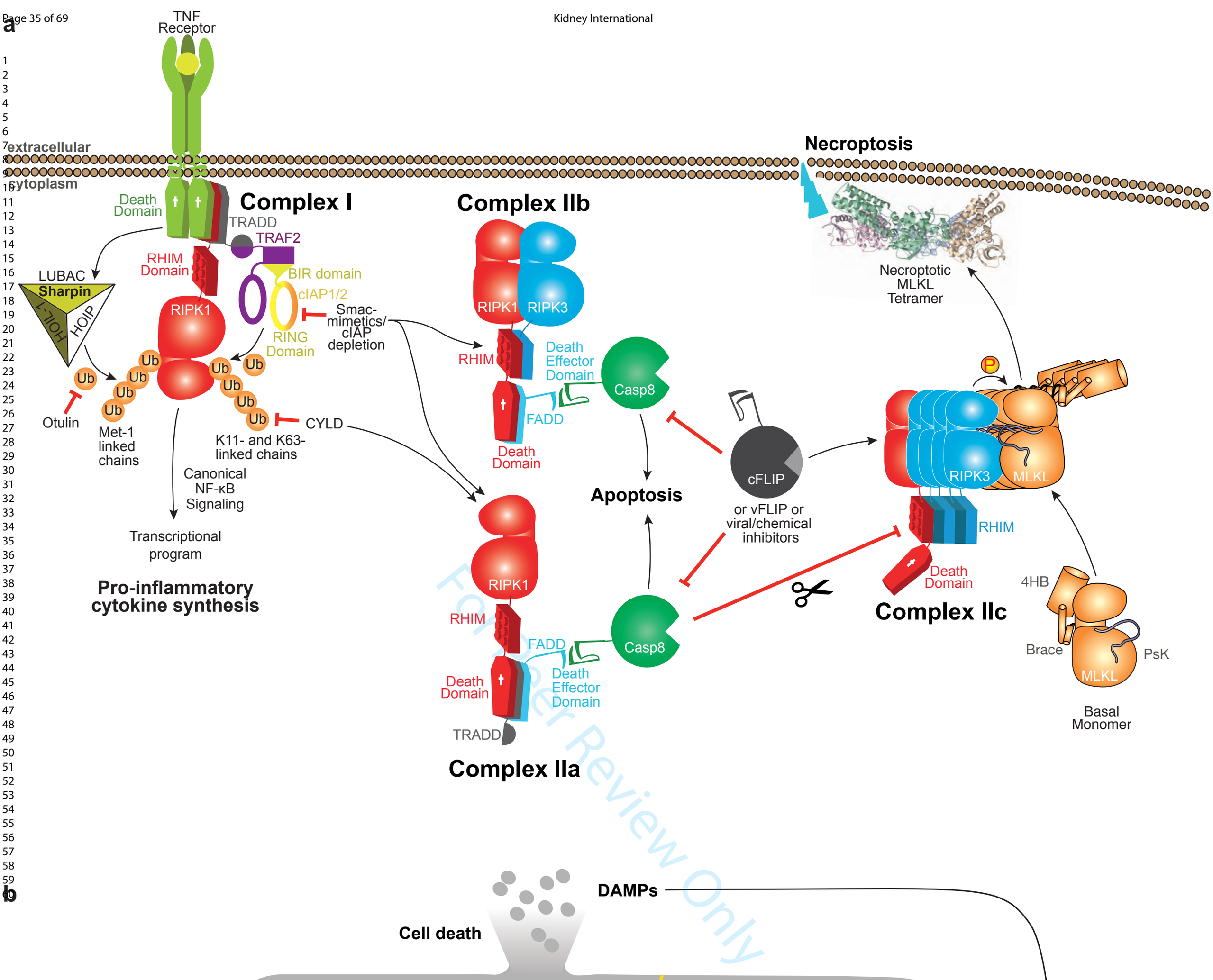
[a] RIPK1 kinase-dead.

[b] Also inhibits IDO and has ferrostatin activity.

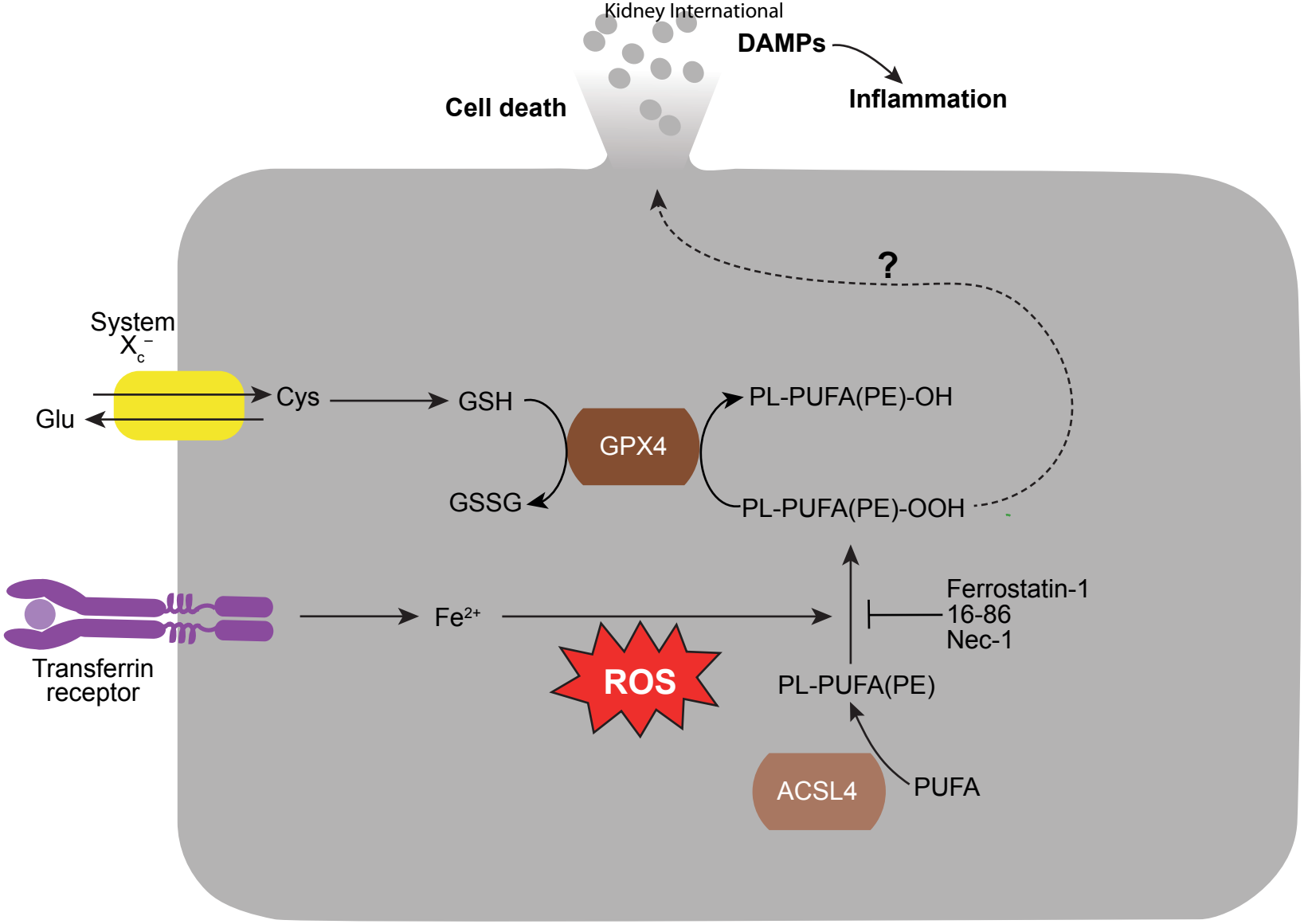
[c] Also inhibits other kinases including RIPK3.

[d] Conditional KO of RGMb in renal tubular cells.

a



b


DAMPs
Inflammation
Cell death
?
System
X_c^-
Glu
Cys
GSH
GSSG
GPX4
PL-PUFA(PE)-OH
PL-PUFA(PE)-OOH
Transferrin
receptor
Fe^{2+}
ROS
Ferrostatin-1
16-86
Nec-1
PL-PUFA(PE)
ACSL4
PUFA