Nonapoptotic cell death in acute kidney injury and transplantation

Andreas Linkermann[1]

[1]Clinic for Nephrology and Hypertension and Georges-Köhler-Haus for Biomedical Research and Transplantation, Christian-Albrechts-University, Kiel, Germany

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.

Kidney International (2016) **89,** 46–57; http://dx.doi.org/10.1016/j.kint.2015.10.008

KEYWORDS: apoptosis; ferroptosis; mitochondrial permeability transition; MLKL; programmed cell death; regulated cell death; RIP1; RIPK1; RIP3; RIPK3

Correspondence: *Andreas Linkermann, Clinic for Nephrology and Hypertension and Georges-Köhler-Haus for Biomedical Research and Transplantation, Christian-Albrechts-University, Kiel, Germany. E-mail: andreas.linkermann@uksh.de*

Received 1 June 2015; revised 21 July 2015; accepted 28 July 2015

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.[4] 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.[8,10] 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 phylogenic 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,[21] 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 caspases, such as vaccinia virus, cytomegalovirus, cowpox virus, and so on.[11,26,27] In these viral infections, RIPK3 is no longer inhibited by caspase-8, and the effect is spontaneous initiation of necroptosis[11,28] 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.[16] 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 central to this form of killing. Only 1 of the 3 known phosphorylation sites, Ser 345 in the MLKL activation loop, 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 necrosome, 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 necrosome formation (Figure 1). Three major physiological patterns result in necrosome 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.[33,52] 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 RIPK3[34,37] and thereby enables viruses to pull the necrotic trigger in a necrosome-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 [TNFR1]), 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

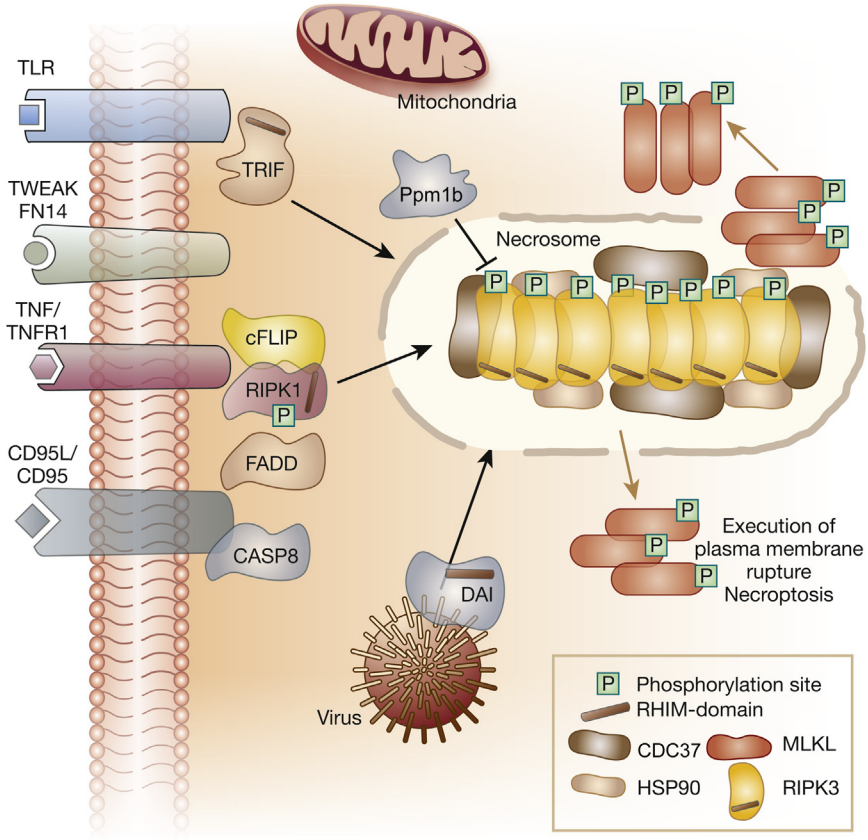

Figure 1 | The pathway of necroptosis. The necrosome, a supramolecular structure with an amyloid-like backbone of phosphorylated receptor-interacting protein kinase 3 (RIPK3) multimers, is stabilized by the chaperone heat shock protein 90 and a co-chaperone CDC37. In several circumstances, proteins like Fas-associated protein with death (FADD) and others are associated with the necrosome. Only proteins that are associated with the necrosome and are required for necrosome function are indicated here for reasons of simplicity. Phosphorylation of RIPK3 is balanced by protein phosphatase 1b (Ppm1b). The necrosome is engaged by interaction of its Rip homotypic interacting motif (RHIM) domain with the RHIM domain of other proteins. Only 3 more proteins in the entire human genome contain a typical RHIM domain: RIPK1, DNA-dependent activator of interferon, and TIR-domain–containing adapter-inducing interferon (TRIF) (see text for details). All these integrate necroptotic signals from death receptors (tumor necrosis factor receptor [TNFR] and CD95, both of which are regulated by FN14), viruses or Toll-like receptors (TLRs), respectively. In death receptor ligation-mediated regulated necrosis, best studied *in vitro*, inhibition of caspase-8 activation is required for necroptosis, but in murine or human kidney epithelial cells, this does not appear to be the case, presumably because of very low basic caspase-8 activation. The execution of necroptosis critically relies on phosphorylation of pseudokinase mixed lineage kinase domain-like (MLKL), which undergoes a conformational change and oligomerizes *in vitro* to expose a 4-helical bundle domain, which is required and sufficient for induction of necroptosis. The 4-helical bundle domain binds to phosphoinositides in any of the cellular membranes and by unknown means leads to influx of extracellular fluid into the cell, causing the process of oncosis and ultimately plasma membrane rupture. The release of intracellular content of the epithelial cell is thought to drive a necroinflammatory autoamplification circuit referred to as "necroinflammation." CASP8, caspase 8; cFLIP, cellular FLICE-like inhibitory protein; HSP-90, heat shock protein 90; RIPK1, receptor-interacting protein kinase 1; TNF, tumor necrosis factor; TWEAK, tumor necrosis factor (TNF)-like weak inducer of apoptosis.

death is tightly controlled by Fas-associated protein with death domain (FADD),[53] cellular inhibitor of apoptosis proteins 1 and 2, and transforming growth factor beta–activated kinase,[54,55] and the polyubiquitination status of RIPK1 (see further on).[14,56] 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.[17,36,54–59] 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 antiapoptotic 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.[15] Therefore, a tightly regulated complex of cellular FLICE-like inhibitory protein, FADD, caspase-8, and RIPK1 prevents spontaneous necroptosis.[60,61] 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),[62–64] 2 mechanistically clearly separate means to regulated RIPK1 polyubiquitination.[65] Other linkages also contribute to the stability of this complex. Similar polyubiquitination patterns may also be found in RIPK3.[66] 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.[36,67] IL-33 may signal through the ST2 receptor to stabilize a regulatory T-cell response, as shown for the gut.[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,[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.[69] In addition, however, IL-1α is actively produced during necroptosis.[70–72] It is currently unclear and under debate how IL-1α in necroptosis contributes to the necrotic damage *in vivo*,[67,72] and what consequences this may have for renal models of inflammatory injury.[73]

In vivo *relevance of necroptosis.* RIPK3-deficient mice *per se* do not exhibit a dramatic phenotype, apart from slight failure to gain weight over months.[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,[4,74] ischemia-reperfusion injury (IRI) in the kidney[3,75–81] and the heart,[82–89] were protected from cerulean-induced pancreatitis,[17,18,90] although this was strongly debated in other reports.[4,80] Protection has also been observed in doxorubicin-induced lethality,[91] TNF-induced shock models,[92–95] and models of sepsis.[96] Conversely, RIPK3 deficiency exquisitely promotes sensitivity to vaccinia virus infections.[16] In addition, these mice are significantly protected in ethanol-induced liver injury,[97] nonalcoholic steatohepatitis,[98] acetaminophen-induced hepatic toxicity,[99] a model of Gauchers disease,[100] and inhibited hepatocarcinogenesis.[101] RIPK3 additionally partially mediates the skin phenotype in SHARPIN-deficient mice, a detrimental skin disorder.[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.[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.[83] It has been published that MLKL-ko mice are protected from pancreatitis[90] (see earlier discussion of "putative" RIPK3-ko–mediated protection in this model) and cisplatin-induced AKI[74] and may be protected from renal IRI and in the model of TNF shock (Linkermann *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 *in vivo* but also affects ferroptosis.[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.

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.[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 *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.

Mitochondrial permeability transition-mediated RN and parthanatos

Poly(ADP-ribose) and mitochondrial permeability transition-mediated RN signaling. Activation of poly(ADP-ribose) polymerase-1 (PARP-1) leads to reversible protein modification by poly(ADP-ribosyl)ation, 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.[106] Although poly(ADP-ribosyl)ation represents a common means of posttranslational protein modification, uncontrolled PARP-1 activation (so-called PARP-1 overactivation[107]) leads to caspase-independent, nonapoptotic cell death with a necrotic phenotype,[106] classically induced by the compound N-methyl-N'-nitro-N-nitrosoguanidine.[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.[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.[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.[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.[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.

In vivo *relevance of MPT-RN and parthanatos.* Three independent laboratories have described protective effects for CypD deficiency in murine renal IRI,[81,114,115] in line with 1 report that used a CypD small, interfering RNA in rats.[116] CypD-ko mice are also protected from cisplatin-induced AKI.[117] Consistent with these results, inhibitors of MPT, such as sanglifehrin A (SfA) and CsA protect wild-type animals from renal IRI.[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[118] and stroke.[118,119] Similarly, PARP-1–mediated cell death has been demonstrated to contribute to IRI after renal, cerebral, and myocardial ischemia.[120–129] In contrast to necroptosis, inhibition of MPT-RN also protects from cerulein-induced pancreatitis.[130]

Human relevance of MPT-RN. One clinical trial has been performed by Piot *et al.*,[113] 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.[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 compounds, it cannot be excluded that the frequent superiority in clinical trials is partially the result of inhibition of immunogenic cell death (see further on).

Ferroptosis

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.[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.[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.[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).[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.[137] Glutathione peroxidase 4 (GPX4) requires GSH to be active in its function of antagonizing lipoxygenase activity and lipid peroxidation (phospholipids and cardiolipin).[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[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.[139]

SLC7A11, as referred to earlier, is a critical subunit of system Xc- that, by its expression, regulates and inhibits the activation of ferroptosis.[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 $p53^{3KR}$, and wild-type p53 are therefore capable of inhibiting SLC7A11 expression and thereby promote ferroptosis.[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.[142–144] Additionally, interference with p53 by small, interfering RNA[145] 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 adjacently dying cells that need to be removed quickly after the damage has occurred and therefore prevent "necroinflammation" (see further on).[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.[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.[147]

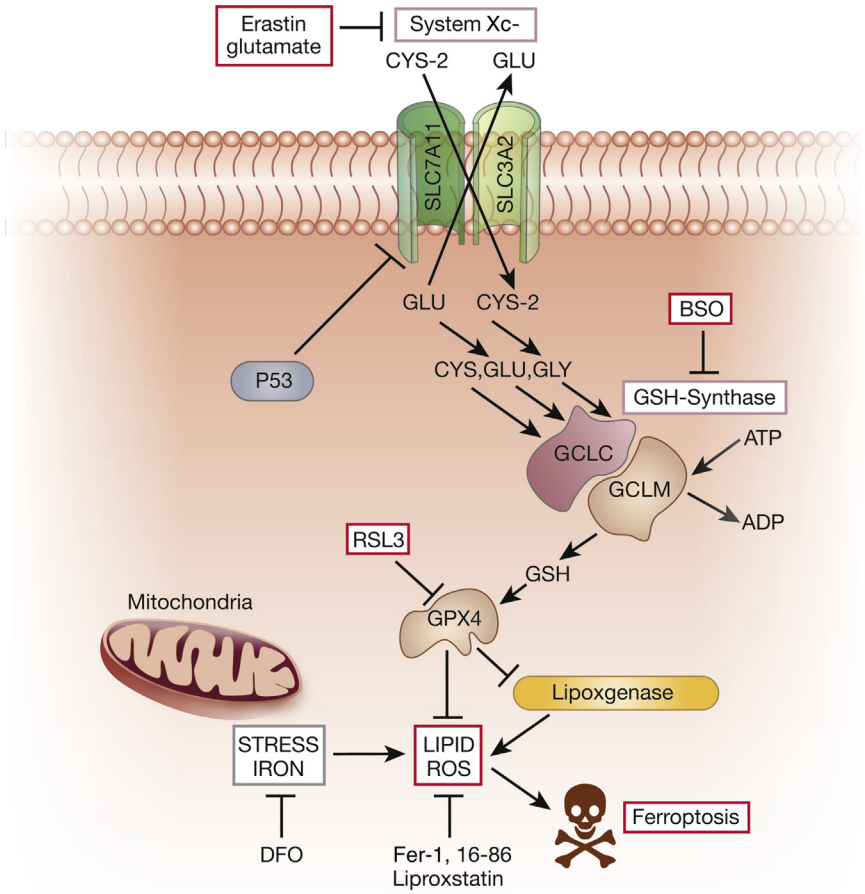

Figure 2 | Control of glutathione peroxidase 4 (GPX4) and the signaling pathway ferroptosis. The glutamate/cystine antiporter system Xc in the plasma membrane consists of the 2 subunits SLC3A2 and SLC7A11, the expression of the latter of which is controlled by p53. The import of cystine into the cell 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.

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/IRI[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/6J 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

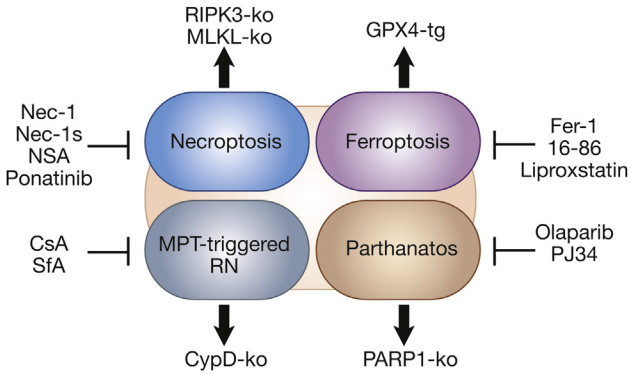

Figure 3 | The matrix to determine the relative contribution of several pathways of regulated necrosis (RN) to the overall organ damage. Several pathways of RN have been shown to be clearly separated (like necroptosis and mitochondrial permeability transition-induced regulated necrosis [MPT-RN] (Linkermann *et al.*[4]) and necroptosis and ferroptosis (Dixon *et al.*[134]). 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, parthanatos, 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 S (SfA), olaparib, and 16-86 for the prevention of necroptosis, MPT-RN, parthanatos, 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, cyclophylin 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 knockout; PARP1-ko, poly(ADP-ribose) polymerase-1 triple knockout; RIPK3-ko, receptor-interacting protein kinase 3 knockout.

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.

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 (necrosome)	Necrostatin-1[76,89,93] Necrostatin-1s[94] Necrosulfonamide (NSA)[47]
Ferroptosis	Cellular necrosis caused by a specific oxylipidomics signature	Glutathione peroxidase 4 (GPX4)	Ferrostatins[4] Liproxstatin-1[104]
MPT-RN	Cellular necrosis caused by MPT	Cyclophilin D (CypD)	Cyclosporine (CsA)[112] Sanglifehrin A[80]
Parthanatos	Cellular necrosis caused by PARP-1 hyperactivation	PARP-1	Olaparib (and others)[105,106]

MLKL, mixed lineage kinase domain-like; MPT, mitochondrial permeability transition; MPT-RN, mitochondrial-permeability transition-mediated regulated necrosis; q-VD, N-(2-Quinolyl)-L-valyl-L-aspartyl-(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,[75,79,80] rat kidneys,[75] hearts[85] of mice and pigs,[155] in renal transplantation,[75] and several other models of non-IRI diseases such as cisplatin-induced AKI[74,79,156] and others.[74,117] 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,[30,58,59] 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

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,[158] 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 death[161–163] and "necroinflammation"[24] 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.[39] 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.

DISCLOSURE

AL declares having received research grants from Pfizer, Novartis, Fresenius Medical Care, and the Else Kröner-Fresenius Stiftung. Further, this research was supported by Genentech, GlaxoSmith Kline, and Apogenix (material transfer agreements). In addition, the author declares to having received honoraria, travel grants, or both, from Astellas, Otsuka, Genentech, Alexion, and Tekmira.

ACKNOWLEDGMENTS

The work in the Linkermann group is funded by the German Research Foundation (DFG) in the Cluster of Excellence, Inflammation at interfaces (EXC 306), Kiel, Germany, and by the companies mentioned in the Disclosures section. The author thanks Doug Green, John Silke, Peter Vandenabeele, Henning Walczak, Lorenzo Galluzzi, Alexei Degterev, Mathieu Bertrand, Bill Kaiser, and Tom Vanden Berghe for continuous discussions on cell death.

REFERENCES

1. Ferrell N, Sandoval RM, Bian A, et al. Shear stress is normalized in glomerular capillaries following (5/6) nephrectomy. *Am J Physiol Renal Physiol*. 2015;308:F588–F593.
2. Hall AM, Rhodes GJ, Sandoval RM, et al. In vivo multiphoton imaging of mitochondrial structure and function during acute kidney injury. *Kidney Int*. 2013;83:72–83.
3. Linkermann A, Hackl MJ, Kunzendorf U, et al. Necroptosis in immunity and ischemia-reperfusion injury. *Am J Transplant*. 2013;13:2797–2804.
4. Linkermann A, Skouta R, Himmerkus N, et al. Synchronized renal tubular cell death involves ferroptosis. *Proc Natl Acad Sci U S A*. 2014;111:16836–16841.
5. Miura M, Zhu H, Rotello R, et al. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the C. elegans cell death gene ced-3. *Cell*. 1993;75:653–660.
6. Vaux DL, Haecker G, Strasser A. An evolutionary perspective on apoptosis. *Cell*. 1994;76:777–779.
7. Boldin MP, Varfolomeev EE, Pancer Z, et al. A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. *J Biol Chem*. 1995;270:7795–7798.
8. Oberst A, Dillon CP, Weinlich R, et al. Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. *Nature*. 2011;471:363–367.
9. Oberst A, Green DR. It cuts both ways: reconciling the dual roles of caspase 8 in cell death and survival. *Nat Rev Mol Cell Biol*. 2011;12:757–763.
10. Kaiser WJ, Upton JW, Long AB, et al. RIP3 mediates the embryonic lethality of caspase-8-deficient mice. *Nature*. 2011;471:368–372.
11. Kaiser WJ, Upton JW, Mocarski ES. Viral modulation of programmed necrosis. *Curr Opin Virol*. 2013;3:296–306.
12. Kang TB, Yang SH, Toth B, et al. Caspase-8 blocks kinase RIPK3-mediated activation of the NLRP3 inflammasome. *Immunity*. 2013;38:27–40.
13. Christofferson DE, Yuan J. Necroptosis as an alternative form of programmed cell death. *Curr Opin Cell Biol*. 2010;22:263–268.
14. Declercq W, Vanden Berghe T, Vandenabeele P. RIP kinases at the crossroads of cell death and survival. *Cell*. 2009;138:229–232.
15. Dillon CP, Oberst A, Weinlich R, et al. Survival function of the FADD-CASPASE-8-cFLIP(L) complex. *Cell Rep*. 2012;1:401–407.
16. Cho YS, Challa S, Moquin D, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell*. 2009;137:1112–1123.
17. He S, Wang L, Miao L, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. *Cell*. 2009;137:1100–1111.
18. Zhang DW, Shao J, Lin J, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science*. 2009;325:332–336.
19. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell*. 1994;76:969–976.
20. 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;24:1464–1469.
21. Krammer PH, Arnold R, Lavrik IN. Life and death in peripheral T cells. *Nat Rev Immunol*. 2007;7:532–542.
22. Watson ML, Rao JK, Gilkeson GS, et al. Genetic analysis of MRL-lpr mice: relationship of the Fas apoptosis gene to disease manifestations and renal disease-modifying loci. *J Exp Med*. 1992;176:1645–1656.
23. Ortiz A, Lorz C, Egido J. The Fas ligand/Fas system in renal injury. *Nephrol Dial Transplant*. 1999;14:1831–1834.
24. Linkermann A, Stockwell BR, Krautwald S, Anders HJ. Regulated cell death and inflammation: an auto-amplification loop causes organ failure. *Nat Rev Immunol*. 2014;14:759–767.
25. Pasparakis M, Vandenabeele P. Necroptosis and its role in inflammation. *Nature*. 2015;517:311–320.
26. Krautwald S, Ziegler E, Tiede K, et al. Transduction of the TAT-FLIP fusion protein results in transient resistance to Fas-induced apoptosis in vivo. *J Biol Chem*. 2004;279:44005–44011.
27. Krautwald S, Ziegler E, Rolver L, et al. Effective blockage of both the extrinsic and intrinsic pathways of apoptosis in mice by TAT-crmA. *J Biol Chem*. 2010;285:19997–20005.
28. Tait SW, Green DR. Caspase-independent cell death: leaving the set without the final cut. *Oncogene*. 2008;27:6452–6461.
29. Chan FK, Luz NF, Moriwaki K. Programmed necrosis in the cross talk of cell death and inflammation. *Annu Rev Immunol*. 2015;33:79–106.
30. Zhou W, Yuan J. Necroptosis in health and diseases. *Semin Cell Dev Biol*. 2014;35:14–23.
31. Ofengeim D, Yuan J. Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. *Nat Rev Mol Cell Biol*. 2013;14:727–736.
32. Linkermann A, Green DR. *Necroptosis. N Engl J Med*. 2014;370:455–465.
33. Kaiser WJ, Sridharan H, Huang C, et al. Toll-like receptor 3-mediated necrosis via TRIF, RIP3 and MLKL. *J Biol Chem*. 2013;288:31268–31279.
34. Kaiser WJ, Upton JW, Mocarski ES. Receptor-interacting protein homotypic interaction motif-dependent control of NF-kappa B activation via the DNA-dependent activator of IFN regulatory factors. *J Immunol*. 2008;181:6427–6434.
35. Bleriot C, Dupuis T, Jouvion G, et al. Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection. *Immunity*. 2015;42:145–158.

36. Rickard JA, O'Donnell JA, Evans JM, et al. RIPK1 regulates RIPK3-MLKL-driven systemic inflammation and emergency hematopoiesis. *Cell*. 2014;157:1175–1188.
37. Rebsamen M, Heinz LX, Meylan E, et al. DAI/ZBP1 recruits RIP1 and RIP3 through RIP homotypic interaction motifs to activate NF-kappaB. *EMBO Rep*. 2009;10:916–922.
38. Challa S, Chan FK. Going up in flames: necrotic cell injury and inflammatory diseases. *Cell Mol Life Sci*. 2010;67:3241–3253.
39. Debout A, Foucher Y, Trebern-Launay K, et al. Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney Int*. 2015;87:343–349.
40. Galluzzi L, Bravo-San Pedro JM, Vitale I, et al. Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. *Cell Death Differ*. 2014;22:58–73.
41. Linkermann A, Chen G, Dong G, et al. Regulated cell death in AKI. *J Am Soc Nephrol*. 2014;25:2689–2701.
42. Vanden Berghe T, Linkermann A, Jouan-Lanhouet S, et al. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. *Nat Rev Mol Cell Biol*. 2014;15:135–147.
43. Rodriguez DA, Weinlich R, Brown S, et al. Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis [e-pub ahead of print]. *Cell Death Differ*. doi: 10.1038/cdd.2015.70. Accessed May 29, 2015.
44. 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;111:15072–15077.
45. 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;457:369–377.
46. Murphy JM, Czabotar PE, Hildebrand JM, et al. The pseudokinase MLKL mediates necroptosis via a molecular switch mechanism. *Immunity*. 2013;39:443–453.
47. Sun L, Wang H, Wang Z, et al. Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell*. 2012;148:213–227.
48. Zhao J, Jitkaew S, Cai Z, et al. Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. *Proc Natl Acad Sci U S A*. 2012;109:5322–5327.
49. Li D, Xu T, Cao Y, et al. A cytosolic heat shock protein 90 and cochaperone CDC37 complex is required for RIP3 activation during necroptosis. *Proc Natl Acad Sci U S A*. 2015;112:5017–5022.
50. Li J, McQuade T, Siemer AB, et al. The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis. *Cell*. 2012;150:339–350.
51. Chen W, Wu J, Li L, et al. Ppm1b negatively regulates necroptosis through dephosphorylating Rip3. *Nat Cell Biol*. 2015;17:434–444.
52. Kaiser WJ, Offermann MK. Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. *J Immunol*. 2005;174:4942–4952.
53. Vanden Berghe T, Van LG, Saelens X, et al. Differential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD. *J Biol Chem*. 2004;279:7925–7933.
54. Dondelinger Y, Aguileta MA, Goossens V, et al. RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. *Cell Death Differ*. 2013;20:1381–1392.
55. Vanlangenakker N, Vanden Berghe T, Bogaert P, et al. cIAP1 and TAK1 protect cells from TNF-induced necrosis by preventing RIP1/RIP3-dependent reactive oxygen species production. *Cell Death Differ*. 2011;18:656–665.
56. Festjens N, Vanden Berghe T, Cornelis S, Vandenabeele P. RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Differ*. 2007;14:400–410.
57. Roderick JE, Hermance N, Zelic M, et al. Hematopoietic RIPK1 deficiency results in bone marrow failure caused by apoptosis and RIPK3-mediated necroptosis. *Proc Natl Acad Sci U S A*. 2014;111:14436–14441.
58. Kaiser WJ, Daley-Bauer LP, Thapa RJ, et al. RIP1 suppresses innate immune necrotic as well as apoptotic cell death during mammalian parturition. *Proc Natl Acad Sci U S A*. 2014;111:7753–7758.
59. Dillon CP, Weinlich R, Rodriguez DA, et al. RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. *Cell*. 2014;127:1189–1102.
60. Weinlich R, Dillon CP, Green DR. Ripped to death. *Trends Cell Biol*. 2011;21:630–637.
61. Weinlich R, Oberst A, Dillon CP, et al. Protective roles for caspase-8 and cFLIP in adult homeostasis. *Cell Rep*. 2013;5:340–348.
62. Gerlach B, Cordier SM, Schmukle AC, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature*. 2011;471:591–596.
63. Keusekotten K, Elliott PR, Glockner L, et al. OTULIN antagonizes LUBAC signaling by specifically hydrolyzing Met1-linked polyubiquitin. *Cell*. 2013;153:1312–1326.
64. Mevissen TE, Hospenthal MK, Geurink PP, et al. OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis. *Cell*. 2013;154:169–184.
65. de Almagro MC, Goncharov T, Newton K, Vucic D. Cellular IAP proteins and LUBAC differentially regulate necrosome-associated RIP1 ubiquitination. *Cell Death Dis*. 2015;6:e1800.
66. Onizawa M, Oshima S, Schulze-Topphoff U, et al. The ubiquitin-modifying enzyme A20 restricts ubiquitination of the kinase RIPK3 and protects cells from necroptosis. *Nat Immunol*. 2015;16:618–627.
67. Silke J, Rickard JA, Gerlic M. The diverse role of RIP kinases in necroptosis and inflammation. *Nat Immunol*. 2015;16:689–697.
68. Schiering C, Krausgruber T, Chomka A, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature*. 2014;513:564–568.
69. Kearney CJ, Cullen SP, Tynan GA, et al. Necroptosis suppresses inflammation via termination of TNF- or LPS-induced cytokine and chemokine production. *Cell Death Differ*. 2015;22:1313–1327.
70. England H, Summersgill HR, Edye ME, et al. Release of interleukin-1alpha or interleukin-1beta depends on mechanism of cell death. *J Biol Chem*. 2014;289:15942–15950.
71. Schmidt SV, Seibert S, Walch-Ruckheim B, et al. RIPK3 expression in cervical cancer cells is required for PolyIC-induced necroptosis, IL-1alpha release, and efficient paracrine dendritic cell activation. *Oncotarget*. 2015;6:8635–8647.
72. Lukens JR, Vogel P, Johnson GR, et al. RIP1-driven autoinflammation targets IL-1alpha independently of inflammasomes and RIP3. *Nature*. 2013;498:224–227.
73. Kurts C, Panzer U, Anders HJ, Rees AJ. The immune system and kidney disease: basic concepts and clinical implications. *Nat Rev Immunol*. 2013;13:738–753.
74. Xu Y, Ma H, Shao J, et al. A role for tubular necroptosis in cisplatin-induced AKI. *J Am Soc Nephrol*. 2015;26:2647–2658.
75. Zhao H, Ning J, Lemaire A, et al. Necroptosis and parthanatos are involved in remote lung injury after receiving ischemic renal allografts in rats. *Kidney Int*. 2015;87:738–748.
76. Tristao VR, Goncalves PF, Dalboni MA, et al. Nec-1 protects against nonapoptotic cell death in cisplatin-induced kidney injury. *Ren Fail*. 2012;34:373–377.
77. Timsit MO, Kleinclauss F. Ischemia-reperfusion in the renal allograft: new clues in a cold-case. *Prog Urol*. 2014;24(suppl 1):S1–S3.
78. Menke J, Sollinger D, Schamberger B, et al. The effect of ischemia/reperfusion on the kidney graft. *Curr Opin Organ Transplant*. 2014;19:395–400.
79. 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;81:751–761.
80. 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;110:12024–12029.
81. Jouan-Lanhouet S, Riquet F, Duprez L, et al. Necroptosis, in vivo detection in experimental disease models. *Semin Cell Dev Biol*. 2014;35:2–13.
82. Smith CC, Davidson SM, Lim SY, et al. Necrostatin: a potentially novel cardioprotective agent? *Cardiovasc Drugs Ther*. 2007;21:227–233.
83. Pavlosky A, Lau A, Su Y, et al. RIPK3-mediated necroptosis regulates cardiac allograft rejection. *Am J Transplant*. 2014;14:1778–1790.
84. Oerlemans MI, Liu J, Arslan F, et al. Inhibition of RIP1-dependent necrosis prevents adverse cardiac remodeling after myocardial ischemia-reperfusion in vivo. *Basic Res Cardiol*. 2012;107:270.
85. Luedde M, Lutz M, Carter N, et al. RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction. *Cardiovasc Res*. 2014;103:206–216.

86. Kung G, Konstantinidis K, Kitsis RN. Programmed necrosis, not apoptosis, in the heart. *Circ Res*. 2011;108:1017–1036.
87. Koshinuma S, Miyamae M, Kaneda K, et al. Combination of necroptosis and apoptosis inhibition enhances cardioprotection against myocardial ischemia-reperfusion injury. *J Anesth*. 2014;28:235–241.
88. Dmitriev YV, Minasian SM, Demchenko EA, Galagudza MM. Study of cardioprotective effects of necroptosis inhibitors on isolated rat heart subjected to global ischemia-reperfusion. *Bull Exp Biol Med*. 2013;155:245–248.
89. Degterev A, Zhou W, Maki JL, Yuan J. Assays for necroptosis and activity of RIP kinases. *Methods Enzymol*. 2014;545:1–33.
90. Wu J, Huang Z, Ren J, et al. Mlkl knockout mice demonstrate the indispensable role of Mlkl in necroptosis. *Cell Res*. 2013;23:994–1006.
91. Hakroush S, Cebulla A, Schaldecker T, et al. Extensive podocyte loss triggers a rapid parietal epithelial cell response. *J Am Soc Nephrol*. 2014;25:927–938.
92. Najjar M, Suebsuwong C, Ray SS, et al. Structure guided design of potent and selective ponatinib-based hybrid inhibitors for RIPK1. *Cell Rep*. 2015;24:1850–1860.
93. Linkermann A, Brasen JH, De ZF, et al. Dichotomy between RIP1- and RIP3-mediated necroptosis in tumor necrosis factor alpha-induced shock. *Mol Med*. 2012;18:577–586.
94. 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.
95. Duprez L, Takahashi N, Van HF, et al. RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. *Immunity*. 2011;35:908–918.
96. Sharma A, Matsuo S, Yang WL, et al. Receptor-interacting protein kinase 3 deficiency inhibits immune cell infiltration and attenuates organ injury in sepsis. *Crit Care*. 2014;18:R142.
97. Ramachandran A, McGill MR, Xie Y, et al. The receptor interacting protein kinase 3 is a critical early mediator of acetaminophen-induced hepatocyte necrosis in mice. *Hepatology*. 2013;58:2099–2108.
98. Gautheron J, Vucur M, Reisinger F, et al. A positive feedback loop between RIP3 and JNK controls non-alcoholic steatohepatitis. *EMBO Mol Med*. 2014;6:1062–1074.
99. Li JX, Feng JM, Wang Y, et al. The B-Raf(V600E) inhibitor dabrafenib selectively inhibits RIP3 and alleviates acetaminophen-induced liver injury. *Cell Death Dis*. 2014;5:e1278.
100. Vitner EB, Salomon R, Farfel-Becker T, et al. RIPK3 as a potential therapeutic target for Gaucher's disease. *Nat Med*. 2014;20:204–208.
101. Vucur M, Reisinger F, Gautheron J, et al. RIP3 inhibits inflammatory hepatocarcinogenesis but promotes cholestasis by controlling caspase-8- and JNK-dependent compensatory cell proliferation. *Cell Rep*. 2013;4:776–790.
102. Rickard JA, Anderton H, Etemadi N, et al. TNFR1-dependent cell death drives inflammation in Sharpin-deficient mice. *Elife*. 2014;3.
103. Lau A, Wang S, Jiang J, et al. RIPK3-mediated necroptosis promotes donor kidney inflammatory injury and reduces allograft survival. *Am J Transplant*. 2013;13:2805–2818.
104. 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;16:1180–1191.
105. 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;54:133–146.
106. Fatokun AA, Dawson VL, Dawson TM. Parthanatos: mitochondrial-linked mechanisms and therapeutic opportunities. *Br J Pharmacol*. 2014;171:2000–2016.
107. Andrabi SA, Umanah GK, Chang C, et al. Poly(ADP-ribose) polymerase-dependent energy depletion occurs through inhibition of glycolysis. *Proc Natl Acad Sci U S A*. 2014;111:10209–10214.
108. Sosna J, Voigt S, Mathieu S, et al. TNF-induced necroptosis and PARP-1-mediated necrosis represent distinct routes to programmed necrotic cell death. *Cell Mol Life Sci*. 2014;71:3313–3348.
109. Yu SW, Wang H, Poitras MF, et al. Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science*. 2002;297:259–263.
110. Alano CC, Ying W, Swanson RA. Poly(ADP-ribose) polymerase-1-mediated cell death in astrocytes requires NAD+ depletion and mitochondrial permeability transition. *J Biol Chem*. 279:18895–18902.
111. Galluzzi L, Kepp O, Krautwald S, et al. Molecular mechanisms of regulated necrosis. *Semin Cell Dev Biol*. 2014;35:24–32.
112. Koh DW, Dawson TM, Dawson VL. Mediation of cell death by poly(ADP-ribose) polymerase-1. *Pharmacol Res*. 2005;52:5–14.
113. Piot C, Croisille P, Staat P, et al. Effect of cyclosporine on reperfusion injury in acute myocardial infarction. *N Engl J Med*. 2008;359:473–481.
114. Devalaraja-Narashimha K, Diener AM, Padanilam BJ. Cyclophilin D gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol*. 2009;297:F749–F759.
115. Park JS, Pasupulati R, Feldkamp T, et al. Cyclophilin D and the mitochondrial permeability transition in kidney proximal tubules after hypoxic and ischemic injury. *Am J Physiol Renal Physiol*. 2011;301:F134–F150.
116. Hu W, Chen Z, Ye Z, et al. Knockdown of cyclophilin D gene by RNAi protects rat from ischemia/reperfusion-induced renal injury. *Kidney Blood Press Res*. 2010;33:193–199.
117. 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;24:1545–1557.
118. Lim SY, Hausenloy DJ, Arjun S, et al. Mitochondrial cyclophilin-D as a potential therapeutic target for post-myocardial infarction heart failure. *J Cell Mol Med*. 2011;15:2443–2451.
119. Vaseva AV, Marchenko ND, Ji K, et al. p53 opens the mitochondrial permeability transition pore to trigger necrosis. *Cell*. 2012;149:1536–1548.
120. Devalaraja-Narashimha K, Singaravelu K, Padanilam BJ. Poly(ADP-ribose) polymerase-mediated cell injury in acute renal failure. *Pharmacol Res*. 2005;52:44–59.
121. Devalaraja-Narashimha K, Padanilam BJ. PARP-1 inhibits glycolysis in ischemic kidneys. *J Am Soc Nephrol*. 2009;20:95–103.
122. Endres M, Wang ZQ, Namura S, et al. Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab*. 1997;17:1143–1151.
123. Gibson BA, Kraus WL. New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. *Nat Rev Mol Cell Biol*. 2012;13:411–424.
124. Ha HC, Snyder SH. Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion. *Proc Natl Acad Sci U S A*. 1999;96:13978–13982.
125. Kim J, Padanilam BJ. Loss of poly(ADP-ribose) polymerase 1 attenuates renal fibrosis and inflammation during unilateral ureteral obstruction. *Am J Physiol Renal Physiol*. 2011;301:F450–F459.
126. Kim J, Long KE, Tang K, Padanilam BJ. Poly(ADP-ribose) polymerase 1 activation is required for cisplatin nephrotoxicity. *Kidney Int*. 2012;82:193–203.
127. McCullough LD, Zeng Z, Blizzard KK, et al. Ischemic nitric oxide and poly (ADP-ribose) polymerase-1 in cerebral ischemia: male toxicity, female protection. *J Cereb Blood Flow Metab*. 2005;25:502–512.
128. Szabo G, Bahrle S, Stumpf N, et al. Poly(ADP-ribose) polymerase inhibition reduces reperfusion injury after heart transplantation. *Circ Res*. 2002;90:100–106.
129. 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:F387–F398.
130. Karch J, Kanisicak O, Brody MJ, et al. Necroptosis interfaces with MOMP and the MPTP in Mediating cell death. *PLoS One*. 2015;10:e0130520.
131. Cung TT, Morel O, Cayla C, et al. Cyclosporine before PCI in patients with acute myocardial infarction. *N Engl J Med*. 2015;373:1021–1031.
132. Sogabe K, Roeser NF, Venkatachalam MA, Weinberg JM. Differential cytoprotection by glycine against oxidant damage to proximal tubule cells. *Kidney Int*. 1996;50:845–854.
133. Dixon SJ, Stockwell BR. The role of iron and reactive oxygen species in cell death. *Nat Chem Biol*. 2014;10:9–17.
134. Dixon SJ, Lemberg KM, Lamprecht MR, et al. Ferroptosis: an iron-dependent form of nonapoptotic cell death. *Cell*. 2012;149:1060–1072.
135. Yagoda N, von Rechenberg M, Zaganjor E, et al. RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. *Nature*. 2007;447:864–868.
136. Boutter J, Huang Y, Marovca B, et al. Image-based RNA interference screening reveals an individual dependence of acute lymphoblastic leukemia on stromal cysteine support. *Oncotarget*. 2014;5:11501–11512.

137. Zhang H, Forman HJ. Glutathione synthesis and its role in redox signaling. *Semin Cell Dev Biol*. 2012;23:722–728.
138. Seiler A, Schneider M, Forster H, et al. Glutathione peroxidase 4 senses and translates oxidative stress into 12/15-lipoxygenase dependent- and AIF-mediated cell death. *Cell Metab*. 2008;8:237–248.
139. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014;515:431–435.
140. Jiang L, Kon N, Li T, et al. Ferroptosis as a p53-mediated activity during tumour suppression. *Nature*. 2015;520:57–62.
141. Bieging KT, Attardi LD. Cancer: a piece of the p53 puzzle. *Nature*. 2015;520:37–38.
142. Zhou L, Fu P, Huang XR, et al. Activation of p53 promotes renal injury in acute aristolochic acid nephropathy. *J Am Soc Nephrol*. 2010;21:31–41.
143. Ying Y, Kim J, Westphal SN, et al. Targeted deletion of p53 in the proximal tubule prevents ischemic renal injury. *J Am Soc Nephrol*. 2014;25:2707–2716.
144. Zhang D, Liu Y, Wei Q, et al. Tubular p53 regulates multiple genes to mediate AKI. *J Am Soc Nephrol*. 2014;25:2278–2289.
145. Molitoris BA, Dagher PC, Sandoval RM, et al. siRNA targeted to p53 attenuates ischemic and cisplatin-induced acute kidney injury. *J Am Soc Nephrol*. 2009;20:1754–1764.
146. Sutton TA, Hato T, Mai E, et al. p53 is renoprotective after ischemic kidney injury by reducing inflammation. *J Am Soc Nephrol*. 2013;24:113–124.
147. 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;10:1604–1609.
148. Koberlin MS, Snijder B, Heinz LX, et al. A conserved circular network of coregulated lipids modulates innate immune responses. *Cell*. 2015;162:170–183.
149. Saifudeen Z, Dipp S, Stefkova J, et al. p53 regulates metanephric development. *J Am Soc Nephrol*. 2009;20:2328–2337.
150. Guo JK, Marlier A, Shi H, et al. Increased tubular proliferation as an adaptive response to glomerular albuminuria. *J Am Soc Nephrol*. 2012;23:429–437.
151. Mason S, Hader C, Marlier A, et al. Met activation is required for early cytoprotection after ischemic kidney injury. *J Am Soc Nephrol*. 2014;25:329–337.
152. Huen SC, Cantley LG. Macrophage-mediated injury and repair after ischemic kidney injury. *Pediatr Nephrol*. 2015;30:199–209.
153. Huen SC, Huynh L, Marlier A, et al. GM-CSF Promotes macrophage alternative activation after renal ischemia/reperfusion injury. *J Am Soc Nephrol*. 2015;26:1334–1345.
154. Pelletier S, Gingras S, Green DR. Mouse genome engineering via CRISPR-Cas9 for study of immune function. *Immunity*. 2015;42:18–27.
155. Koudstaal S, Oerlemans MI, Van der Spoel TI, et al. Necrostatin-1 alleviates reperfusion injury following acute myocardial infarction in pigs. *Eur J Clin Invest*. 2015;45:150–159.
156. Linkermann A, Himmerkus N, Rolver L, et al. Renal tubular Fas ligand mediates fratricide in cisplatin-induced acute kidney failure. *Kidney Int*. 2011;79:169–178.
157. Fauster A, Rebsamen M, Huber KV, et al. A cellular screen identifies ponatinib and pazopanib as inhibitors of necroptosis. *Cell Death Dis*. 2015;6:e1767.
158. Jouan-Lanhouet S, Arshad MI, Piquet-Pellorce C, et al. TRAIL induces necroptosis involving RIPK1/RIPK3-dependent PARP-1 activation. *Cell Death Differ*. 2012;19:2003–2014.
159. Skouta R, Dixon SJ, Wang J, et al. Ferrostatins inhibit oxidative lipid damage and cell death in diverse disease models. *J Am Chem Soc*. 2014;136:4551–4556.
160. Vanden Berghe T, Linkermann A. Take my breath away: necrosis in kidney transplants kills the lungs! *Kidney Int*. 2015;87:680–682.
161. Kepp O, Semeraro M, Bravo-San Pedro JM, et al. eIF2alpha phosphorylation as a biomarker of immunogenic cell death. *Semin Cancer Biol*. 2015;33:86–92.
162. Kepp O, Senovilla L, Kroemer G. Immunogenic cell death inducers as anticancer agents. *Oncotarget*. 2014;5:5190–5191.
163. Kepp O, Senovilla L, Vitale I, et al. Consensus guidelines for the detection of immunogenic cell death. *Oncoimmunology*. 2014;3:e955691.