Two independent pathways of regulated necrosis mediate ischemia–reperfusion injury

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Regulated necrosis (RN) may result from cyclophilin (CypD)-mediated mitochondrial permeability transition (MPT) and receptor-interacting protein kinase (RIPK)1-mediated necroptosis, but it is currently unclear whether there is one common pathway in which CypD and RIPK1 act in or whether separate RN pathways exist. Here, we demonstrate that necroptosis in ischemia–reperfusion injury (IRI) in mice occurs as primary organ damage, independent of the immune system, and that mice deficient for RIPK3, the essential downstream partner of RIPK1 in necroptosis, are protected from IRI. Protection of RIPK3-knockout mice was significantly stronger than of CypD-deficient mice. Mechanistically, in vivo analysis of cisplatin-induced acute kidney injury and hyperacute TNF shock models in mice suggested the distinctness of CypD-mediated MPT from RIPK1/RIPK3-mediated necroptosis. We, therefore, generated CypD-RIPK3 double-deficient mice that are viable and fertile without an overt phenotype and that survived prolonged IRI, which was lethal to each single knockout. Combined application of the RIPK1 inhibitor necrostatin-1 and the MPT inhibitor sanglifehrin (Sf)A and necroptosis by Nec-1 con

Unlike apoptosis, necrosis was considered to be an accidental form of cell death until genetically determined, regulated processes that mediate necrotic cellular demise were identified in vivo (1, 2). Histologically, the majority of cellular damage in renal and other forms of ischemia–reperfusion injury (IRI) is caused by necrosis (3, 4). With the recognition of regulated necrosis (RN), the intriguing opportunity to pharmacologically interfere with these pathways has emerged. RN can be mediated by mitochondrial permeability transition (MPT), a process that critically depends on the protein cyclophilin (CypD) (5,6,7,8). The MPT has been successfully modi

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(Fig. S1 A–C). To avoid the presence of collateral vessels, which are known to influence the readout systems in myocardial ischemia and stroke models, we focused on a renal model of IRI that is lethal to wild-type (wt) mice after ~72 h following reperfusion to investigate the CypD-RIPK3 double-knockout (dko) mice in comparison with wt, RIPK3-ko, and CypD-ko mice (Fig. 1A). Whereas CypD-deficient mice and RIPK3-ko mice exhibited significantly prolonged survival in this model ($P < 0.01$ and $< 0.001$, respectively), but died after no longer than 120 h, all investigated CypD-RIPK3-dko mice survived long term ($P < 0.001$ in comparison with all other groups). In line with this finding, markers for the loss of kidney function (elevated serum concentrations of creatinine and urea) were significantly reduced 48 h after reperfusion in CypD-ko mice and RIPK3-ko mice, but the strongest protection was again seen in the CypD-RIPK3-dko mice (Fig. 1 D and E). Histological analysis revealed significant reduction of kidney damage in all ko and dko mice, with the strongest protection again noted for the CypD-RIPK3-dko mice (Fig. 1 B and C). However, significantly increased organ damage was still observed in the CypD-RIPK3-dko mice compared with sham-treated mice (Fig. 1 B and C).

To investigate the role of necroptosis in the kidney in more detail, we examined the expression levels of regulators of cell death in renal lysates from IRI-treated mice. Expression of the survival parameter p38 (34) and the RIPK1 regulator SHARPIN revealed a role of this system in the time-course of IRI (Fig. S2). Cleaved caspase-3 was not detectable in kidney lysates within the ischemic period and over the first 96 h after reperfusion (Fig. S2), indicating that cell death was not apoptotic. In addition, changes in expression of RIPK1 in the renal tubules 48 h after reperfusion were analyzed by immunohistochemistry, demonstrating subcellular changes in the expression profile (Fig. S3 A and B). In whole-kidney lysates, RIPK1 expression appeared to slightly increase upon ischemia in both wt and RIPK3-deficient mice (Fig. 2A) but was slightly down-regulated between 2–8 h after the beginning of reperfusion. We failed to clearly detect RIPK3 expression in the renal lysates, most probably because of overall low expression levels in the kidney. To further understand the role of necroptosis in the kidney, we assessed the in vivo model of cisplatin-induced acute kidney injury (CP-AKI) (35), which we found to be attenuated by Nec-1 (Fig. S3 C–E). In line with our previous suggestion that death receptor-mediated apoptosis is of minor pathophysiological importance in kidney IRI (11), and to further investigate the in vivo relevance of necroptosis beyond the use of Nec-1, we compared RIPK3- to caspase-8–RIPK3 double-deficient mice in a standard renal IRI model. As expected from Fig. 1A, RIPK3-ko mice were protected from IRI, but no additional protection was recognized for the caspase-8–RIPK3-dko mice (Fig. 2 B–E). This result is in line with the absence of cleaved caspase-3 in the time course of IRI (Fig. S2D). It was suggested that the protective effect of CypD is most significant in a mild model of IRI (36). Therefore, to precisely define the relative contribution of RN mediated by CypD and RIPK3 to the overall damage in IRI, we reduced the duration of the ischemic phase and directly compared wt mice, CypD-ko mice and RIPK3-ko mice. Clearly, the level of protection from mild kidney IRI was significantly stronger in RIPK3-ko mice compared with CypD-ko mice (Fig. 2 F–I).

Having delineated the in vivo role of necroptosis in IRI, we next aimed to pharmacologically inhibit necroptosis by using a previously described inhibitor of the kinase activity of RIPK1, Nec-1 (10, 26). However, Nec-1 is also known to inhibit indolamin-2,3-dioxygenase (IDO), which means that it can exert effects on the immune system that are independent of the inhibition of the kinase activity of RIPK1 (28, 37). To overcome the IDO inhibition-mediated effects of Nec-1 on the immune cells, we used freshly isolated renal tubules and immunodeficient SCID-Beige mice. In renal proximal tubular cells, we found that application of tumor necrosis factor (TNF), TNF-related weak inducer of apoptosis (TWEAK), and IFN-$\gamma$, referred to as the TTI model, resulted in caspase-independent cell death (CICD), as reported previously (38), which we here identified as necroptosis by the addition of Nec-1 (Fig. S4 A–D). Consistent with previous concepts of necroptosis (1, 39), addition of the pan-caspase inhibitor zVAD-fmk (zVAD) shifted apoptotic cell death to necroptosis (Fig. S4 B and D). We applied the TTI model in murine freshly isolated wt tubules and performed TUNEL staining, a nonspecific marker that detects double-strand breaks that are produced during apoptosis but can also arise from the action of nucleases during necrotic death (40) and detected TUNEL-positive cells after combined application of TTI and zVAD. Addition of Nec-1 reduced the number of TUNEL-positive cells to the level of control tubules, thereby identifying this CICD as necroptosis (Fig. 3 A and B; higher magnification is provided in Fig. S5). In immunodeficient SCID-Beige mice, we investigated kidney IRI in the presence and absence of Nec-1. Analysis of histopathological staining of representative kidney sections revealed significant protection upon the use of Nec-1 (Fig. 3 C and D) and prevention of the associated increase in serum creatinine and serum urea levels (Fig. 3 E and F). We then isolated renal tubules from RIPK3-deficient
mice that showed ex vivo protection from hypoxia–reoxygenation, as evaluated by lactate dehydrogenase (LDH) release and propidium iodide (PI) positivity (Fig. 3 G and H). From these data, we conclude that kidney cells readily undergo necroptosis and the prevention of necroptosis by Nec-1 is independent of the immune system.

To confirm that CypD-mediated MPT and RIPK3-mediated RN display two distinct RN pathways, we provide four further lines of evidence. First, whereas Nec-1 protected L929 cells from death in a standard model of TNF/zVAD-induced necroptosis, the MPT inhibitor SfA did not exhibit any protective effects (Fig. S6 A), and, conversely, Nec-1 did not protect Jurkat cells in standard model of MPT pore opening, whereas SfA did (Fig. S6 B). Second, as demonstrated above, in the model of cisplatin-induced AKI, RIPK3-ko mice, despite a significant protection compared with wt mice, did not reach the level of protection seen in CypD-ko mice (Fig. S7 A). Third, in cisplatin-induced AKI, whereas RIPK3-ko mice survived significantly longer in the presence of zVAD or on caspase-8-deficient background (Fig. S7B), any combination of zVAD and Nec-1 or the combined CypD-RIPK3 double-deficient background did not prolong survival of CypD-ko mice (Fig. S7C). Fourth, RIPK3-deficient, but not CypD-deficient, mice were protected from hyperacute TNF-induced shock (41, 42) (Fig. S8). These lines of evidence led us to conclude that CypD-mediated RN and necroptosis are two distinct pathways of RN. To pharmacologically interfere with both of these pathways, we first confirmed the pathway specificity of Nec-1 for necroptosis and SfA for CypD-mediated RN by applying Nec-1 in RIPK3-deficient mice and SfA in CypD-deficient mice, and no significant differences in organ damage were recognized (Fig. S9). As demonstrate in Fig. 4 A and B, no additional protection compared with the one described in Fig. 1 D and E for the single-ko mice was detected when inhibitors were applied correspondingly. In contrast, when we applied combination therapy of Nec-1 and SfA 15 min before ischemia in the severe kidney IRI model into wt mice, a significant survival benefit was detected compared with mice treated with Nec-1 or SfA alone (Fig. 4C). The superior therapeutic effect of the Nec-1/SfA combination was also detected by a marked reduction in serum creatinine and serum urea concentrations (Fig. 4 F and G) and upon histological evaluation of kidneys harvested 48 h after reperfusion (Fig. 4 D and E). Even though the protection from kidney damage upon long ischemia afforded by combined application of Nec-1 and SfA before reperfusion was substantial, it was not complete suggesting the involvement of other pathways of regulated necrosis.

Discussion

The parallel existence of two pathways that induce regulated necrosis in the same organ following the same ischemic stimulus demonstrates the complexity in the pathophysiology of IRI. Whereas intracellular changes in pH and lipid peroxidation may favor necrosis as a result of MPT (1, 6), necroptosis is triggered by stimulation of death receptors and controlled by regulatory inhibitors, most prominently by the polyubiquitination of RIPK1, which is mediated by cellular inhibitor of apoptosis (43) and the linear ubiquitin chain assembly complex (LUBAC) (44). Independently of these considerations, concomitant application of Nec-1 and SfA prevents both of these pathways from inducing...
cell death upon ischemia–reperfusion and, thereby, appears to provide a previously unreached level of protection from IRI. The strong additive benefit of CypD- and RIPK3-ko in the present studies raises the interesting question of whether mice deficient in more than two RN pathways would be further protected. Because all injury is not prevented in the CypD-RIPK3 dko and the absence of the RN pathways is well tolerated under physiological conditions, future work along these lines is both feasible and potentially highly informative.

The importance of necroptosis suggested by the earlier work with Nec-1, and conclusively shown in the present studies with the mutant mouse model, raises the question as to what triggers necroptosis in tubular cells. TNF receptor 1/2 double-deficient mice are not protected from renal IRI, and interference with TNF does not protect mice from IRI (45). However, other members of the TNF superfamily have recently been implicated in the induction of necroptosis [e.g., TNF related apoptosis inducing ligand receptor (TRAIL-R) (46), Fas, or TWEAK] and are just as likely to be involved as members of the Toll-like receptors, some of which may trigger the intracellular RHIM-containing protein TRIF (TIR-domain-containing adapter-inducing interferon-β) (16). Our data (Fig. 3A) implicate a role for the TWEAK/FN14 system, at least in isolated renal tubules.

The absence of an overt phenotype in the CypD/RIPK3-dko mice contrasts with the early lethality and tumor susceptibility usually seen in mutant mice lacking major central pathways of apoptosis (16, 47, 48) and indicates the predominant involvement of RN mechanisms in severe stress and injury settings as opposed to the physiologic role of apoptosis during development, adult tissue remodeling, and immune system regulation. A similar distinction holds true for at least two of the other RN pathways, pyroptosis (31) and parthanatos (49), because caspase-11–deficient mice that do not undergo pyroptosis and PARP1–deficient mice that do not undergo parthanatos are also viable and fertile. An increasing number of RN pathways have recently been described, including MPT, necroptosis, pyroptosis, ferroptosis (30, 50), heat stroke-associated cell death (33), direct lysosomal membrane permeabilization (51), etc., but the complex regulation and interconnectivity among these RN pathways is not understood today.
Targeting RN pathways depends on the availability of effective pharmacological agents, and net effects in complex disease settings are not fully predictable from expected targets. In this regard, two groups have recently reported that Nec-1 can accelerate time to death in the TNF-shock model (11, 28, 29). In view of the strong role played by these RN pathways, their pharmacologic modification merits further testing, particularly in transplant models that involve isolated ischemia to the organ at predictable time points and in the absence of systemic pathophysiology. Sterile tissue damage from ischemic injury that produces delayed graft function is known to play a major role in both primary nonfunction of theraft and as trigger for CDAMPs (13). Attempts to prevent IRI, however, face the problem that interference with RN pathways needs to be quick within the reperfusion phase. For necroptosis, we demonstrated that applying Nec-1 fifteen minutes following reperfusion results in loss of major parts of the protective effect, which is completely absent when Nec-1 is applied 30 min after reperfusion (11), but to the best of our knowledge, the precise therapeutic window has not been investigated for other RN pathways. From a therapeutic perspective, this is disappointing because RN inhibitors might be applied too late to achieve benefits for stroke or myocardial infarction. However, the ideal application arises when solid organs be anticipated, as in solid organ transplantation or, regarding the kidney, in cardiac surgery. In these cases, protective interference with necroptosis and MPT might indeed be beneficial.

In conclusion, the present studies emphasize the importance of immunogenic cell death (52) attributable to regulated necrosis during IRI, the particular role therein of the MPT and necroptosis as independent pathways, and the additive benefit of targeting both. Further understanding of these processes and the additional pathways of regulated necrosis promises new therapeutic possibilities. In addition, IRI is known to significantly deteriorate the outcome after stroke and myocardial infarction (52, 53). Only if we precisely unravel the kinetics of the presumably narrow therapeutic window, RN-inhibiting combinatorial therapies might also provide protection in other tissues damaged by IRI.

**Materials and Methods**

See SI Material and Methods for detailed descriptions.

**Reagents and Mice.** Nec-1 was purchased from Sigma-Aldrich. SFA was provided by Novartis Pharma. RIPK3-deficient mice were obtained from V. Dixit (Genentech, La Jolla, CA) (54). CypD-deficient mice and SCID-Beige mice were purchased from The Jackson Laboratory, and C57BL/6 mice were from Charles River. Genotypes were confirmed by tail-snip PCR as described previously. All in vivo experiments were performed according to the Protection of Animals Act after approval of the German local authorities or the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals after approval from the University of Michigan IACUC. In all experiments, mice were carefully matched for age, sex, weight, and genetic background.

**Mouse Models of IRI.** Induction of kidney IRI was performed as described previously (11). Briefly, we performed a midline abdominal incision and a bilateral renal pedicle clamping for the indicated time using microaneurysm clamps (Aesculab). Throughout the surgical procedure, the body temperature was maintained between 37 and 38 °C by continuous monitoring using a temperature-controlled self-regulated heating system (Fine Science Tools). After removal of the clamps, reperfusion of the kidneys was assisted by tail-snip PCR as described previously. All in vivo experiments were performed according to the Protection of Animals Act after approval of the German local authorities or the Institutional Animal Care and Use Committee (IACUC) of the University of Michigan and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals after approval from the University of Michigan IACUC. In all experiments, mice were carefully matched for age, sex, weight, and genetic background.

**Statistical Analysis.** For all experiments, differences of data sets were considered statistically significant when P values were lower than 0.05, if not otherwise specified. Statistical comparisons were performed using the two-tailed Student t test. Asterisks are used in the figures to specify statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001).
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