TWEAK and RIPK1 mediate a second wave of cell death during AKI

Diego Martin-Sanchez[a], Miguel Fontecha-Barriuso[a], Susana Carrasco[a], Maria Dolores Sanchez-Niño[a], Anne von Mässenhausen[b], Andreas Linkermann[b], Pablo Cannata-Ortiz[c], Marta Ruiz-Ortega[a,d], Jesus Egido[e,f], Alberto Ortiz[a,e,1,2], and Ana Belen Sanz[a,1,2]

[a]Red de Investigacion Renal (REDINREN), Research Institute–Fundación Jiménez Díaz, Autonomous University of Madrid (UAM), 28040 Madrid, Spain; [b]Department of Internal Medicine III, Division of Nephrology, University Hospital Carl Gustav Carus at the Technische Universität Dresden, 01307 Dresden, Germany; [c]Department of Pathology, Research Institute–Fundación Jiménez Díaz, School of Medicine, UAM, 28040 Madrid, Spain; [d]School of Medicine, UAM, 28029 Madrid, Spain; [e]Instituto Reina Sofía de Investigación Nefrológica, School of Medicine, UAM, 28040 Madrid, Spain; and [f]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Research Institute-Fundación Jiménez Díaz, UAM, 28040 Madrid, Spain

Edited by Xiaodong Wang, National Institute of Biological Sciences, Beijing, China, and approved March 1, 2018 (received for review September 27, 2017)

Acute kidney injury (AKI) is characterized by necrotic tubular cell death and inflammation. The TWEAK/Fn14 axis is a mediator of renal injury. Diverse pathways of regulated necrosis have recently been reported to contribute to AKI, but there are ongoing discussions on the timing or molecular regulators involved. We have now explored the cell death pathways induced by TWEAK/Fn14 activation and their relevance during AKI. In cultured tubular cells, the inflammatory cytokine TWEAK induces apoptosis in a proinflammatory environment. The default inhibitor of necroptosis [necrostatin-1 (Nec-1)] was protective, while caspase inhibition switched cell death to necroptosis. Additionally, folic acid-induced AKI in mice resulted in increased expression of Fn14 and necroptosis mediators, such as receptor-interacting protein kinase 1 (RIPK1), RIPK3, and mixed lineage domain-like protein (MLKL). Targeting necroptosis with Nec-1 or by genetic RIPK3 deficiency and genetic Fn14 ablation failed to be protective at early time points (48 h). However, a persistently high cell death rate and kidney dysfunction (72–96 h) were dependent on an intact TWEAK/Fn14 axis driving necroptosis. This was prevented by Nec-1, or MLKL, or RIPK3 deficiency and by Nec-1 stable (Nec-1s) administered before or after induction of AKI. These data suggest that initial kidney damage and cell death are amplified through recruitment of inflammation-dependent necroptosis, opening a therapeutic window to treat AKI once it is established. This may be relevant for clinical AKI, since using current diagnostic criteria, severe injury had already led to loss of renal function at diagnosis.

AKI | cell death | RIPK1 | TWEAK | Fn14

Acute kidney injury (AKI) results in an acute and usually transient decrease in renal function. AKI is associated with long-term mortality and a higher risk of chronic kidney disease (CKD) progressing to end-stage renal disease (ESRD) (1, 2). Furthermore, no satisfactory treatment attenuates AKI or accelerates recovery.

During AKI, tubular cell death is followed by tubular dedifferentiation, proliferation, and regeneration and is accompanied by inflammation (3). Different pathways of regulated necrosis (e.g., necroptosis, ferroptosis) may contribute to tubular cell loss, as observed in ischemia/reperfusion injury or folic acid-induced (FA)-AKI (4, 5). Necroptosis is the best characterized form of regulated necrosis and relies on phosphorylation of receptor-interacting protein 3 (RIPK3) by RIPK1, and subsequent RIPK3-mediated phosphorylation of the pseudokinase mixed lineage kinase domain-like protein (MLKL) (6–8). Necrostatin-1 (Nec-1) inhibits necroptosis by maintaining the RIPK1 kinase inactive, thereby indirectly preventing necrosome formation. There is interventional evidence in vivo that regulated necrosis may mediate the initial wave of tubular cell death, dependent on the initial insult (4, 5). Cells dying through necrosis release intracellular organelles and inflammatory damage-associated molecular patterns (DAMPs) that potentiate the inflammatory response, recruiting inflammatory cells (necroinflammation) (9). Recruitment of inflammatory mediators may lead to a second wave of cell death, not directly related to the original insult, but to cell death induced by inflammatory cytokines and infiltrating immune cells. We recently observed that a single preemptive dose of the ferroptosis inhibitor ferrostatin-1 prevented tubular cell death and also reduced the expression of proinflammatory molecules in FA-AKI, thus attenuating renal dysfunction at 48 h (5). By contrast, interference with necroptosis was not protective at this early time point (5). However, necroptosis may represent an important pathway in nephrotoxic AKI induced by cisplatin, since Nec-1 was protective when assessed at 48 h or 72 h (4, 10, 11). Therefore, multiple cell death pathways are involved in AKI but the precise molecular mechanisms and their specific contribution to different AKI stages require further clarification to optimize the therapeutic approach.

TWEAK is a TNF superfamily cytokine that activates the receptor Fn14 and promotes kidney injury (12). In both FA-AKI and ischemia-reperfusion AKI, functional interventional studies have disclosed a key role of TWEAK (13–15). However, much remains to be understood of the molecular mechanisms of nephroprotection resulting from TWEAK neutralization. In tubular renal cells, TWEAK, in combination with proinflammatory cytokines TNFα and IFNγ, induces apoptosis but caspase inhibition did not prevent TWEAK-induced cell death (16).

We have now explored the relative role of necroptosis in a second wave of injury in toxic FA-AKI as well as the contribution of the TWEAK to this process. We found that Fn14-deficient mice

Significance

Acute kidney injury (AKI) has a mortality of 50%. There is no satisfactory therapy and the incidence is increasing. The etiology of AKI is heterogeneous, and this has therapeutic implications. Here we show that necroptosis plays a role in a second wave of tubular cell death in experimental toxic AKI. This second wave of death is triggered by TWEAK activation of the Fn14 receptor and contributes to persistence of injury. We previously observed that the initial wave of cell death was ferroptosis dependent and necroptosis independent. The identification of a pathway contributing to AKI persistence may facilitate the design of therapies, as exemplified by the protection afforded by RIPK1 inhibitors when administered after AKI had been induced.

Author contributions: D.M.-S., A.O., and A.B.S. designed research; D.M.-S., M.F.-B., S.C., M.D.S.-N., and A.B.S. performed research; P.C.-O. performed analysis of histological renal injury; A.v.M. and A.L. contributed new reagents/analytic tools; M.R.-O., J.E., and A.O. provided financial support for the study; D.M.-S., M.F.-B., P.C.-O., M.R.-O., J.E., A.O., and A.B.S. analyzed data; and A.L., A.O., and A.B.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]A.O. and A.B.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: AOrtiz@fjd.es or ASanz@fjd.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1716578115/-/DCSupplemental.

Published online March 27, 2018.

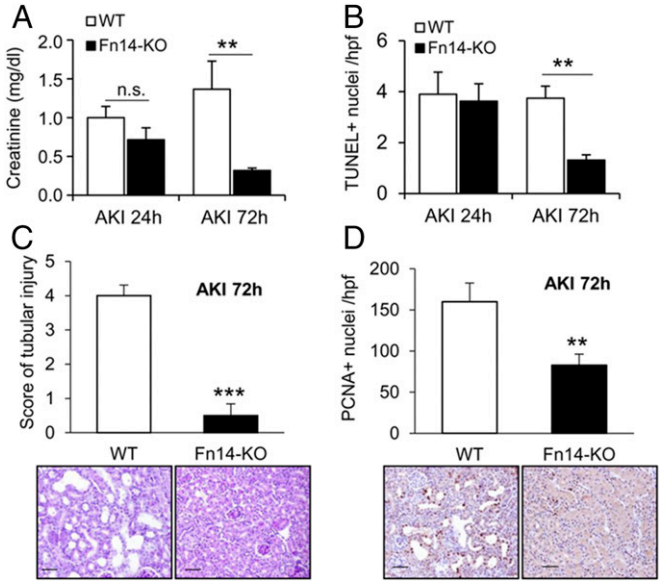

Fig. 1. Fn14 deficiency preserves renal function and reduces cell death in folic acid-induced AKI. AKI was induced by a folic acid overdose in WT and Fn14-KO mice. Mice were killed at 24 and 72 h. (*A*) Renal function assessed by plasma creatinine. (*B*) Cell death was assessed by TUNEL staining. (*C*) Histological injury score. Representative images of PAS staining and quantification according to a tubular damage score. Original magnification, 200×. (Scale bars: 50 μm.) (*D*) Immunohistochemistry of PCNA and quantification. Original magnification, 200×. Data are expressed as mean ± SEM of n = 7 mice per group. $^{**}P < 0.01$; $^{***}P < 0.001$. n.s., nonsignificant.

showed improved renal function and reduced cell death when assessed 72–96 h following induction of AKI, but not at earlier time points. Moreover, TWEAK/TNF/IFNγ (TTI)-induced apoptosis switched to necroptosis in the presence of caspase inhibition, and both apoptosis and necroptosis are prevented by Nec-1 in cultured cells. Finally, we observed that necroptosis, likely a consequence of TWEAK/Fn14 activation, triggers a second wave of injury in AKI that leads to prolonged kidney dysfunction.

Results

Fn14 Targeting Reduces Cell Death in AKI. Recently, ferroptosis was reported to play a key role in initial insult leading to FA-AKI, as assessed at 48 h, not only by promoting cell death, but also by up-regulating inflammatory proteins such as Fn14 (5). Since Fn14 blockade prevents TWEAK-induced cell death in cultured tubular cells (16), but the impact of genetic Fn14 deficiency over cell death in AKI had not been previously assessed (12), we used Fn14 knockout mice (Fn14-KO) to test the role of Fn14 in FA-AKI. Fn14 deficiency resulted in improved renal function at 72 h, as assessed by plasma blood urea nitrogen (BUN) and creatinine levels, while it was not protective at 24 h (Fig. 1*A* and Fig. S1). Moreover, in Fn14-deficient mice, cell death assessed by TUNEL was reduced at 72 h, but not at 24 h (Fig. 1*B*). The reduced cell death in Fn14-KO mice correlated with reduced tubular injury and renal cell proliferation (Fig. 1 *C* and *D*). This result indicates that the TWEAK/Fn14 axis may mediate a second wave of cell death during AKI, likely a consequence of the first wave of cell death triggering inflammation. This hypothesis is supported by our previous report on the inhibition of ferroptosis that prevented both cell death and Fn14 up-regulation (5). We next explored the molecules involved in cell death induced by Fn14 activation.

RIPK1 Kinase Activity Mediates TWEAK-Induced Apoptosis. First, we addressed the mechanisms of cell death induced by TWEAK in cultured tubular cells. Previously it was reported that TWEAK in combination with the proinflammatory cytokines TNFα and IFNγ (TTI) promoted apoptosis in cultured tubular cells, but inhibition of caspases with the general pan-caspase inhibitor zVAD (TTI/zVAD) switched the cell death type to necroptosis (16). Indeed, TTI/zVAD induced necroptosis, because it was prevented by the RIPK1 inhibitor Nec-1 (4) (Fig. 2*A*). However, caspase inhibition was achieved by adding a therapeutic agent and may not be occurring in vivo, so we focused on the molecules involved in tubular cell death induced by the cytokine mixture. Surprisingly, Nec-1 also prevented cell death induced by cytokine mixture alone. In fact, Nec-1 prevented the presence of hypodiploid cells and pyknotic nuclei (Fig. 2*C* and Fig. S2*A*). However, the specificity of Nec-1 has been questioned, since it also inhibits other types of cell death, such as ferroptosis (17). Thus, we tested Nec-1 stable (Nec-1s), a more specific derivative of Nec-1, to rule out nonspecific effects of Nec-1. Indeed, Nec-1s also prevented cell death induced by TTI alone (Fig. 2*B*). Moreover, in clonogenic assays, cells treated with TTI alone formed colonies for a long time, but this was not observed when apoptosis was inhibited by a caspase inhibitor (TTI/zVAD). Nec-1 also reverted the decreased clonogenic survival induced by the combination of cytokines and caspase inhibition (Fig. S2*B*). These results suggest that the kinase activity of RIPK1 is necessary to promote TTI-induced cell death, despite its morphological and functional features consistent with apoptosis.

To clarify whether Nec-1 acts specifically over RIPK1, RIPK1 was targeted with a specific siRNA. RIPK1-deficient cells lost the protection afforded by Nec-1 against TTI-induced cell death (Fig. 2*D*), indicating that in cultured tubular cells, Nec-1 is acting specifically by inhibiting the kinase activity of RIPK1.

RIPK3 and MLKL in TTI/zVAD-Induced Necroptosis. Since in our experimental conditions Nec-1 inhibits both apoptosis and necroptosis, we explored whether TTI/zVAD-induced cell death is mediated by necroptosis. First, we studied MLKL activation by assessing phospho-MLKL (p-MLKL) by Western blot. p-MLKL was not observed in TTI-stimulated cells, while following TTI/zVAD stimulation, p-MLKL was already observed at 3 h and increased at 24 h (Fig. 3*A*). Moreover, RIPK3 knockdown by a specific siRNA prevented MLKL phosphorylation, confirming that RIPK3 mediates MLKL activation (Fig. 3*B* and Fig. S3*A*). Then, we confirmed the contribution of both RIPK3 and MLKL to TTI/zVAD-induced cell death by targeting them with specific

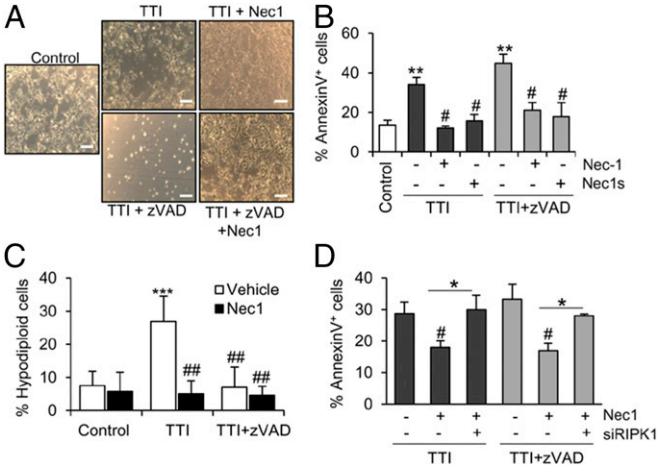

Fig. 2. Nec-1 and Nec-1s prevent cell death induced by TWEAK/TNFα/IFNγ (TTI) in cultured tubular cells. Cultured tubular cells were pretreated with zVAD and/or Nec-1 or Nec-1s for 1 h and subsequently stimulated with TTI for 24 h. (*A*) Representative contrast phase microscopy photographs of tubular cells (original magnification, 200×) are depicted. (Scale bars: 200 μM.) (*B*) Percentage of annexin V positive tubular cells. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. vehicle. (*C*) Percentage of hypodiploid cells. $^{***}P < 0.001$ vs. control; $^{\#\#}P < 0.01$ vs. TTI alone. (*D*) Effect of RIPK1 targeting over protection provided by Nec-1. Percentage of annexin V positive cells at 24 h. $^{*}P < 0.05$; $^{\#}P < 0.05$ vs. TTI or TTI/zVAD alone. Data are expressed as mean ± SEM of three independent experiments.

CELL BIOLOGY

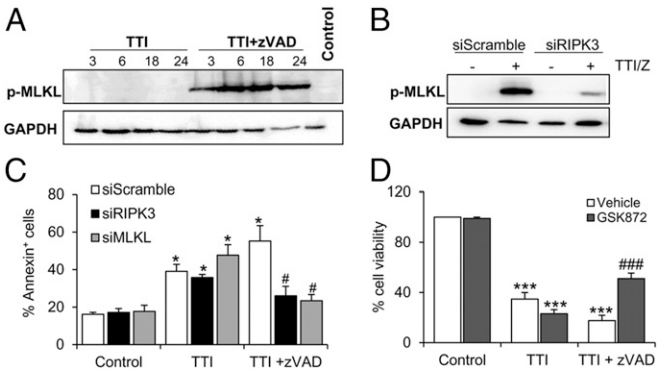

Fig. 3. RIPK3 and MLKL play a key role in TTI/zVAD-induced cell death but not in TTI-induced apoptosis in MCT cells. (*A*) p-MLKL protein levels in cultured mouse tubular cells following exposure to TTI or TTI/zVAD. (*B*) p-MLKL protein levels in tubular cells transfected with a RIPK3 siRNA and exposed to TTI/zVAD for 6 h. (*C*) Percentage of annexin V positive tubular cells transfected with RIPK3, MLKL, or control (Scramble) siRNA. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. TTI/zVAD with siScramble. (*D*) Cell viability assessed by the MTT assay in tubular cells treated with the RIPK3 inhibitor GSK872 (1 μM). $^{***}P < 0.001$ vs. control; $^{\#\#\#}P < 0.001$ vs. TTI/zVAD alone. (*C* and *D*) Data are expressed as mean ± SEM of three independent experiments.

siRNA. Targeting RIPK3 or MLKL prevented cell detachment, reduced the percentage of annexin V positive cells, and prevented the loss of mitochondrial membrane potential (MMP) induced by TTI/zVAD, while there was no protection from TTI (Fig. 3*C* and Fig. S3 *B* and *C*). Furthermore, a chemical inhibitor of RIPK3, GSK872, prevented cell death induced by TTI/zVAD, but did not protect from TTI alone (Fig. 3*D* and Fig. S3*D*).

Finally, we explored the role of RIPK1 on MLKL phosphorylation. Nec-1 completely prevented p-MLKL induced by TTI/zVAD, indicating activity of the necroptosis-signaling pathway in the presence of caspase inhibitors (Fig. 4*A*). However, RIPK1 knockdown did not prevent cell death induced by TTI or TTI/zVAD (Fig. 4 *B* and *C*). In line with this observation, RIPK1 knockdown partially prevented TTI/zVAD-induced p-MLKL, and promoted p-MLKL in TTI-stimulated cells (Fig. 4*D*). This result suggests that RIPK1 may have a dual role; its kinase activity is necessary to induce cell death, but a scaffolding function of RIPK1 is required to prevent cell death. This is consistent with prior reports in which the kinase activity of RIPK1 promoted cell death, but the presence of the RIPK1 protein prevented necrosome formation (18, 19). To test the role of RIPK3 in RIPK1-KO cells, these cells were pretreated with GSK872, observing that RIPK3 inhibition prevented cell death and p-MLKL in presence of both TTI and TTI + zVAD, suggesting that RIPK3 can induce cell death in the absence of RIPK1 (Fig. 4 *E* and *F*).

In summary, Nec-1 prevented cell death induced by either TTI or TTI/zVAD and this protective action required RIPK1. However, functional evidence of necroptosis was observed only in death induced by TTI/zVAD, and necroptosis pathway inhibition only protected from cell death induced by TTI/zVAD, but not from cell death induced by TTI.

Necrostatin-1 Prevents Caspase Activation and Loss of MMP Induced by TTI. Therefore, we explored whether Nec-1 protection of TTI-induced cell death resulted from the prevention of caspase activation. TTI promoted caspase-8 and caspase-3 cleavage. As expected, zVAD inhibited caspase-8 and caspase-3 cleavage, but did not efficiently block the initial step in caspase-8 activation, consistent with its lack of effect on p43 generation (20, 21) (Fig. 5*A*). Additionally, Nec-1 prevented caspase-8 and caspase-3 processing, indicating that RIPK1 could trigger TTI-induced apoptosis acting upstream of caspase-8 activation (Fig. 5*A*). Confocal microscopy of cleaved caspase-3 and quantification of caspase-3 activity confirmed this result (Fig. 5*B* and Fig. S4*A*). Moreover, Nec-1 also prevented loss of MMP in the presence of either TTI or TTI/zVAD (Fig. 5*C*). In addition, RIPK1-deficient cells lost the protection afforded by Nec-1 against TTI-induced caspase activation (Fig. 5*D*), indicating that in cultured tubular cells, the kinase activity of RIPK1 could mediate caspase activation. However, down-regulation of whole RIPK1 protein did not protect from TTI-induced caspase activation (Fig. 5*D*), consistent with the dual role of RIPK1 over cell death activation.

Caspase-8 Inhibition Mediates the Transition from Apoptosis to Necroptosis in TTI-Stimulated Cells. Since the pan-caspase inhibitor zVAD switched TTI-induced cell death from apoptosis to necroptosis, we explored the effect of the specific caspase-8 and -3 inhibitors IETD-fmk (IETD) and DEVD-fmk (DEVD) (22, 23). Inhibition of caspase 8 is sufficient to promote necroptosis, since IETD increased TTI-induced cell death in a dose-dependent manner (Fig. S4 *B* and *D*). In line with this observation, IETD increased p-MLKL in TTI-stimulated cells (Fig. S4*E*). However, the caspase-3 inhibitor DEVD did not increase the rate of cell death (Fig. S4 *C* and *D*) and had a weak effect on MLKL phosphorylation (Fig. S4*E*).

Fn14 Deficiency Prevents Features of Apoptosis and Necroptosis. Next, we addressed whether Fn14 deficiency prevented apoptosis, necroptosis, or both in vivo. The expression of necroptosis-related RIPK3, RIPK1, and MLKL proteins was increased in AKI (5) (Fig. S5). Fn14 deficiency results in decreased levels of these proteins (Fig. 6 *A*–*C*), and in less accumulation of MLKL in the cellular membrane (Fig. 6*D*) at 72 h following induction of AKI compared with WT mice. Cleaved poly(ADP-ribose) polymerase (PARP), identified by an antibody specific for the 25-kDa fragment, and

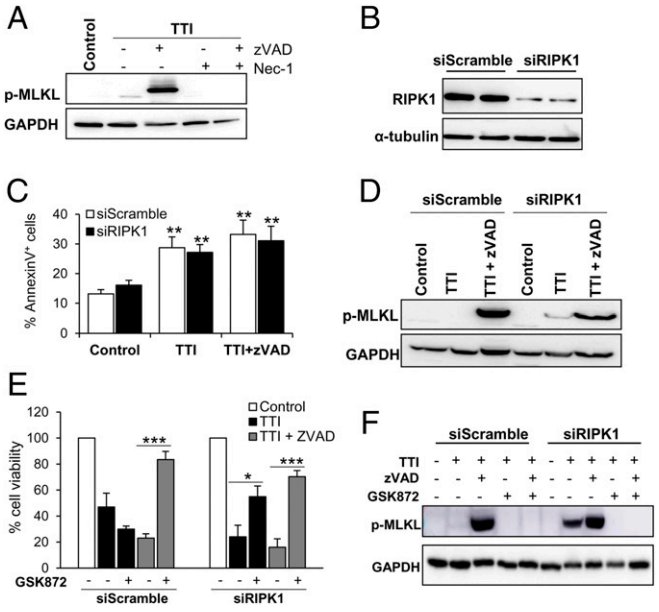

Fig. 4. Role of RIPK1 in TTI/zVAD-induced necroptosis in cultured tubular cells. (*A*) p-MLKL protein levels in tubular cells exposed to TTI with zVAD and Nec-1 for 6 h. (*B*) MCT cells were transfected with specific siRNA against RIPK1. After 24 h, the expression of RIPK1 was checked by Western blot. (*C*) Percentage of annexin V positive tubular cells transfected with RIPK1 or control siRNA after 24 h of treatment. $^{**}P < 0.01$ vs. control. (*D*) p-MLKL protein in tubular cells transfected with a RIPK1 or control siRNA after 6 h of treatment. (*E*) Pretreatment with a RIPK3 inhibitor (GSK872) in MCT cells transfected with RIPK1 or control siRNA and treated with TTI or TTI + zVAD. Cell viability was assessed by MTT. $^{***}P < 0.001$; $^{*}P < 0.05$. (*F*) p-MLKL is prevented with GSK872 pretreatment in MCT cells transfected with RIPK1 or control siRNA and treated with TTI or TTI + zVAD. (*A*, *B*, *D*, and *F*) Representative Western blots of three independent experiments are shown. (*C* and *E*) Mean ± SEM of three independent experiments.

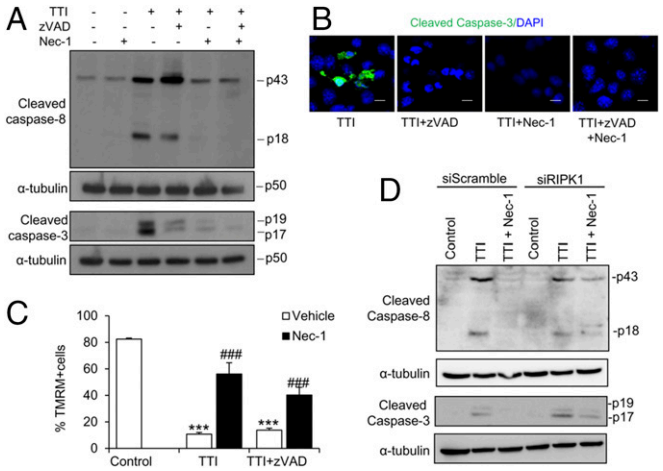

Fig. 5. Nec-1 prevents caspase activation in cultured tubular cells. (*A*) Cleaved caspase-8 and cleaved caspase-3 proteins in tubular cells exposed to TTI or TTI + zVAD for 6 h following pretreatment with Nec-1. (*B*) Cleaved caspase-3 staining, detected by immunofluorescence. Cleaved caspase 3 is shown in green and DAPI, in blue. Original magnification, 630×. (Scale bars: 15 μm.) (*C*) MMP in tubular cells at 24 h of treatment. Graph shows TMRM staining. Data are expressed as mean ± SEM of three independent experiments. ***$P < 0.001$ vs. control; ###$P < 0.001$ vs. TTI or TTI/zVAD vehicle. (*D*) Cleaved caspase-8 and cleaved caspase-3 proteins in tubular cells transfected with a RIPK1 or control siRNA and exposed to TTI for 6 h. (*A* and *D*) Representative Western blots of three independent experiments are demonstrated.

cleaved caspase 3, assessed by immunohistochemistry, were also significantly reduced in Fn14-KO mice (Fig. 6 *E* and *F*). These results suggest that the TWEAK/Fn14 axis may mediate a second wave of cell death during AKI, and that both features of necroptosis and apoptosis were prevented by Fn14 deficiency.

Nec-1 Prevented the Second Wave of Cell Death During FA-AKI. Finally, we tested whether Nec-1 protection of cytokine-stimulated tubular cells has a therapeutic relevance in vivo. Recently, it was reported that pretreatment with a single dose of Nec-1 did not prevent renal injury in FA-AKI at 48 h (5). However, the effect of longer Nec-1 treatment was not explored. Now we observed that daily i.p. administration of Nec-1 reduces creatinine and urea (Fig. 7*A* and Fig. S6*A*), and the expression of tubular cell injury marker NGAL (Fig. S6*B*) in FA-AKI when assessed at the longer time period of 96 h. Moreover, Nec-1 also reduced cell death assessed by TUNEL staining and compensatory proliferation assessed by proliferating cell nuclear antigen (PCNA) staining (Fig. 7 *B* and *C*). These results suggest that Nec-1 prevented a second wave of tubular injury during FA-AKI. Then, we explored the cell death pathway targeted by Nec-1 in vivo. There was a trend toward lower cleaved PARP levels in the kidney from mice treated with Nec-1 than in controls (Fig. 7*D*). To execute necroptosis, MLKL translocates to the plasma membrane. Indeed, MLKL located to cellular membranes in AKI kidneys, and this was partially reduced by Nec-1, while cytosolic levels remained unchanged (Fig. 7 *E* and *F*) (24). These results suggest that Nec-1 may reduce both apoptosis and necroptosis in vivo. In addition, we compared the effect of RIPK1 inhibition before and after AKI induction. In this model we used Nec-1s, a more specific inhibitor of RIP1 kinase than Nec-1, which protected at similar levels, corroborating the role of RIPK1 in AKI. Moreover, similar protection was observed with both approaches: Nec-1s administration before or after AKI induction (Fig. 8 and Fig. S6 *C* and *D*). This result is in line with a role for RIPK1 in the second wave of injury.

In addition, to test the role of necroptosis in the second wave of injury during FA-AKI in a more specific way, we studied WT, RIPK3 (RIPK3-KO), and MLKL-deficient mice (MLKL-KO) at 96 h. In accordance with the cell culture observations, RIPK3-KO and MLKL-KO mice had attenuated renal dysfunction and lower cell death rates at 96 h after AKI than WT mice (Fig. 9 *A*–*D* and Fig. S7). Caspase activation was assessed as cleaved PARP by Western blot. As expected, RIPK3 deficiency did not prevent caspase activation and PARP cleavage (Fig. 9*E*), suggesting lack of impact on apoptosis. However, it prevented MLKL translocation to the membrane of tubular cells (Fig. 9*F*), suggesting a role of RIPK3 in necroptosis during the second wave of cell death in AKI.

Discussion

The main finding of this study is that a second wave of injury during AKI depends on the inflammatory response, requiring the TWEAK/Fn14 axis and RIPK1 activation. The Fn14/TWEAK axis or RIPK1 could be therapeutic targets for AKI, once AKI has been established. Indeed, our vitro studies support these data, since TWEAK-induced tubular cell death is mediated by both apoptosis or necroptosis, depending on the microenvironment.

AKI is histologically characterized by tubular cell death and inflammation, but the exact mechanisms of and molecular contributors to cell death are unclear. Recently, regulated necrosis pathways such as necroptosis and ferroptosis were proposed to have a key role in AKI. Intervention studies demonstrated a role of necroptosis in ischemia–reperfusion kidney injury, cisplatin nephrotoxicity, contrast media- and rhabdomyolysis-induced AKI at 48 or 24 h (25). However, necroptosis may not be the main tubular cell death pathway in ischemia–reperfusion injury, where the beneficial effect of necroptosis inhibitors may be due to endothelial protection (3). In this regard, a previous report showed that ferroptosis was an important cell death pathway following the initial insult in toxic experimental AKI, while interference with apoptosis (zVAD) or necroptosis (Nec-1, RIPK3-KO, or MLKL-KO) did not prevent early renal injury, assessed at 48 h, despite up-regulation of RIPK3 and MLKL protein in early AKI (5). This suggested that induction of AKI might sensitize the kidney to necroptosis, but necroptosis is not itself involved in the initial wave of damage in FA-AKI.

Ferroptosis could be a driver for other pathways of cell death such as apoptosis or necroptosis, since it promotes inflammatory responses such as up-regulation of inflammatory cytokines that themselves are known to promote cell death (26). Specifically, Fn14 expression is up-regulated during AKI and this is at least partially mediated by ferroptosis (5). Previously, the role of the TWEAK/Fn14 axis in renal injury was demonstrated in different animal models of renal injury (13–15), but the specific effect of

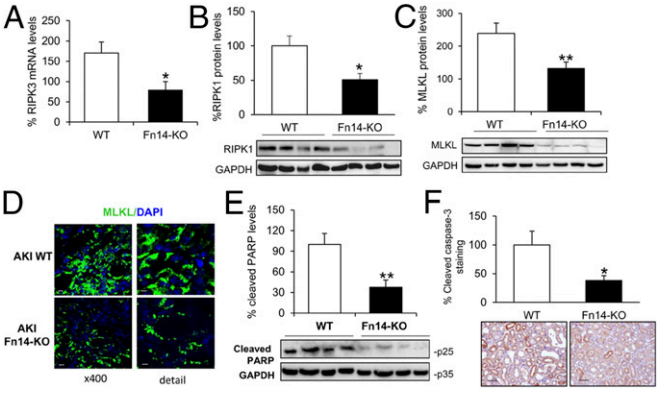

Fig. 6. Fn14 regulates the expression of necroptosis proteins during AKI at 72 h. (*A*–*C*) RIPK3 mRNA levels and RIPK1 and MLKL protein levels at 72 h of AKI. (*D*) MLKL immunofluorescence in AKI at 72 h. Representative images are shown. Magnification, 400× (scale bars: 10 μm); detail, 800× (scale bars: 5 μm). (*E*) Cleaved PARP protein levels in AKI at 72 h. Quantification and representative Western blots are shown. (*F*) Cleaved caspase-3 staining and quantification. Original magnification, 200×. (Scale bars: 50 μm.) (*A*–*C*, *E*, and *F*) Data are expressed as mean ± SEM of seven mice per group. *$P <$ 0.05 vs. WT mice; **$P < 0.01$ vs. WT mice.

CELL BIOLOGY

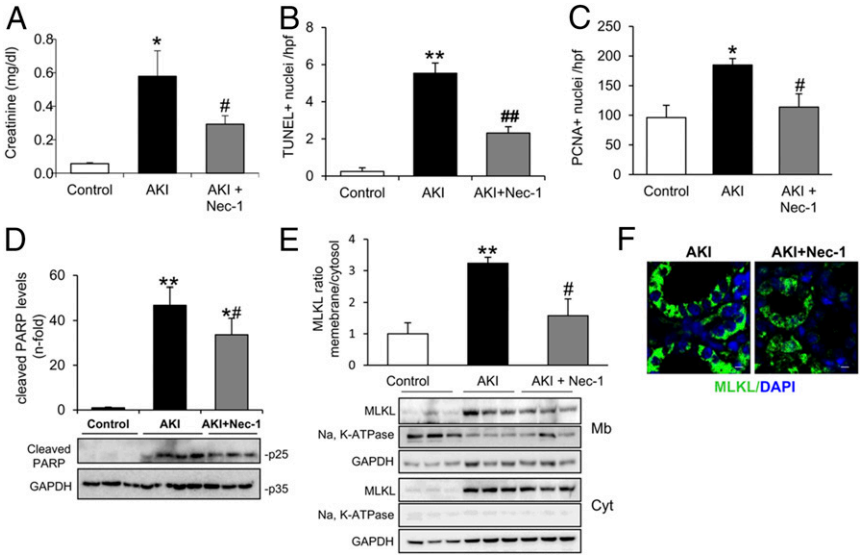

Fig. 7. Nec-1 functionally prevents folic acid-induced AKI at 96 h in mice. (*A*) Renal function assessed by plasma creatinine levels. (*B*) Cell death assessed by TUNEL. (*C*) Cell proliferation assessed by PCNA staining. (*D*) Quantification and representative Western blot of cleaved PARP. (*E*) Western blot analysis demonstrating that MLKL locates at the membrane during AKI and this is partially prevented with Nec-1. (*F*) MLKL membrane accumulation is reduced with Nec-1 treatment. Representative images of confocal microscopy. Magnification, 800×. (Scale bars: 5 μm.) (*A*–*E*) Data expressed as mean ± SEM of $n = 10$ mice per group. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. AKI; $^{\#\#}P < 0.01$ vs. AKI.

TWEAK/Fn14 or the consequences of genetic targeting of Fn14 over cell death pathways was not explored. In this line, Fn14-KO mice had better preserved renal function and reduced cell death levels 72 h after injury, but not at 24 h, suggesting that the beneficial effect relates to late events in the course of injury and not to early events, that is, to direct actions of the insult. Moreover, we showed that Fn14 deficiency reduces the levels of the necroptotic proteins RIPK1, MLKL, and RIPK3, active caspase 3, and cleaved PARP, which is a target of caspase 3, suggesting that both pathways, apoptosis and necroptosis, could be activated by TWEAK at later stages of AKI. Further supporting this sequence of events, our in vitro studies showed that TWEAK, in the presence of proinflammatory cytokines, activates apoptosis, but the form of cell death switches to necroptosis in the presence of caspase inhibitors, and both, apoptosis and necroptosis, depend on Fn14 activation (16). Specifically, caspase-8 inhibition activates MLKL- and RIPK3-dependent necroptosis. However, both apoptosis and necroptosis induced by an inflammatory milieu are inhibited by Nec-1, independently of caspase inhibitors. Furthermore, Nec-1 improved renal function and reduced cell death at later time points during AKI, whereas, as was the case for Fn14 targeting, it was not protective in early AKI. This may be due to ferroptosis predominance at this early time point. Moreover, we have compared the effect of RIPK1 inhibition before and after AKI induction, detecting similar levels of protection with both approaches. These results support a model in which the role of RIPK1 is limited to the second wave of injury. However, further studies are required to characterize the precise pathway targeted by Nec-1, although our data support the concept of Nec-1 to inhibit both apoptosis and necroptosis.

Nec-1 is an inhibitor of the kinase activity of RIPK1, but it also stabilizes the scaffolding function of inactive RIPK1, thereby inhibiting necroptosis by two different mechanisms (27). However, our results show that Nec-1 also prevents caspase activation and TTI-induced apoptosis, suggesting that the kinase activity of RIPK1 could activate apoptosis. This is in accordance with previous reports where RIPK1 mediated caspase activation and apoptosis in immortalized fibroblasts treated with TNF and in the absence of IKK complex (28, 29). Therefore, the protection offered by Nec-1 in our model of AKI and in other experimental

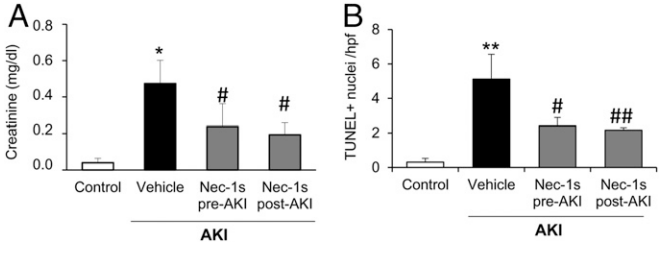

Fig. 8. Nec-1s prevents features of AKI at 96 h. Nec-1s was administered either before or 6 h after induction of folic acid-AKI and then daily until 96 h. (*A*) Renal function assessed by plasma creatinine levels. (*B*) Cell death assessed by TUNEL. Data are expressed as mean ± SEM of $n = 5$ mice per group. $^{*}P < 0.05$ vs. control; $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. AKI; $^{\#\#}P < 0.01$ vs. AKI.

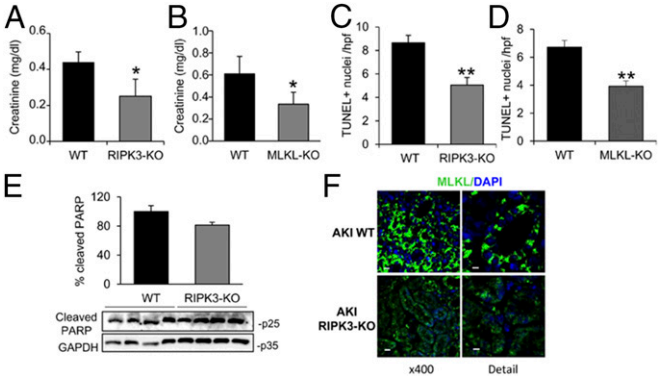

Fig. 9. RIPK3 and MLKL deficiency prevents features of AKI at 96 h. (*A* and *B*) Renal function assessed by plasma creatinine levels. (*C* and *D*) Cell death assessed by TUNEL staining. (*E*) Cleaved PARP protein levels in AKI at 72 h. Quantification and representative Western blots are shown. (*F*) Representative images of confocal microscopy of MLKL localization. Magnification, 400× (scale bars: 10 μm); detail, 800× (scale bars: 5 μm). Data are expressed as mean ± SEM of $n = 5$–10 mice per group. $^{*}P < 0.05$; $^{**}P < 0.01$.

models may reflect its ability to inhibit both apoptosis and necroptosis. The kinase activity of RIPK1 is an upstream activator of necroptosis. However, the RIPK1 protein devoid of kinase activity appears to function as a cell death inhibitor (18, 19). In this regard, RIPK1$^{-/-}$ mice die perinatally, while mice that lack RIPK1, RIPK3, and caspase 8 survive, suggesting that RIPK1 controls both apoptosis and necroptosis at birth (30, 31). In line with this observation, genetic silencing of RIPK1 demonstrated the specificity of Nec-1 RIPK1: Nec-1 did not protect in the absence of RIPK1. However, RIPK1 knockdown did not prevent cell death, and it promotes a RIPK3-dependent cell death. Two hypotheses could explain this result: (*i*) RIPK1 devoid of kinase activity limits TTI-induced cell death, or (*ii*) RIPK3/MLKL may lead to RIPK1 kinase-independent necroptosis. Further studies are needed to discern these pathways in tubular cells.

AKI is characterized by an inflammatory response that amplifies kidney injury. RIPK3 may promote inflammation in early AKI, independent of cell death (5). We now observed that RIPK3-KO mice were protected at later time points in AKI and this could be a consequence of the reduced inflammation observed early in the course of the disease, of necroptosis inhibition at later time points, or a combination of both. It will be interesting to elucidate the functionally relevant targets of RIPK3 in AKI and their role in the mechanisms of the proinflammatory effect of RIPK3 independently from regulated necrosis.

In conclusion, these data demonstrate that a Nec-1 sensitive cell death pathway, presumably driven by an inflammatory response involving TWEAK/Fn14 to an initial wave of cell death, appears to be responsible for amplification of the tubular cell death response and for persistence of AKI (Figs. S6 and S7). While inhibiting ferroptosis is highly effective in preventing AKI when therapy is started before AKI occurs, there are few clinical situations in which this is possible, such as prevention of AKI following kidney transplantation or heart surgery. However, an agent that protects from the second, inflammation-dependent wave of cell death, after AKI has been induced, may be applied to a wider range of clinical situations, since current diagnostic criteria for AKI assume that kidney injury severe enough to decrease kidney function has already occurred by the time a diagnosis is made and therapeutic decisions can be reached.

Materials and Methods

Animal Model. All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the animal ethics committee of IIS-FJD. Folic acid nephropathy is a classical model of AKI (13) that has been reported in humans (32). Female 12- to 14-wk-old C57BL/6 wild-type (WT) mice or Fn14-KO (Biogen) (7 mice per experimental group) received a single i.p. injection of folic acid (Sigma) 250 mg/kg in 0.3 mol/l sodium bicarbonate, or vehicle, and were killed 24 and 72 h later. In a second experiment, WT mice (10 mice per group) received an i.p. injection of 1.65 mg/kg Nec-1 (Santa Cruz Biotechnology) or DMSO (vehicle) 30 min before folic acid injection and every 24 h until being killed at 96 h. Dose was based on previous reports (4). Additionally, RIPK3-KO mice (kindly provided by Kim Newton and Vishva Dixit, Genentech, San Francisco) (33), MLKL-KO (provided by John Silke, The Walter & Eliza Hall Institute of Medical Research, Parkville, Australia and James Murphy, The University of Melbourne, Parkville, Australia to A.L.) or WT mice, on the C57B1/6 background, received an i.p. folic acid injection, and were killed 96 h later (5–10 mice per experimental group). Finally, a WT group received an i.p. injection of 1.65 mg/kg Nec-1s (Santa Cruz Biotechnology) or DMSO (vehicle) 30 min before folic acid injection or 6 h after folic acid injection, and every 24 h until being killed at 96 h (5 mice per experimental group).

Cells. MCT cells are a proximal tubular epithelial cell line.

Statistics. Statistical analysis was performed using SPSS 11.0 statistical software. Results are expressed as mean ± SEM. Significance at the $P < 0.05$ level was assessed by nonparametric Mann–Whitney *U* test for two groups and analysis of ANOVA for three or more group.

ACKNOWLEDGMENTS. This work was supported by Fondo de Investigación Sanitaria Grants PI15/00298, CP12/03262, CP14/00133, PI17/01495, PI14/00386, PI16/02057, PI16/01900, Research Institute Carlos III (ISCIII)–Networks for Cooperative Research in Health, Red de Investigación Renal Grant RD016/0009, Fondo Europeo de Desarrollo Regional, Sociedad Española de Nefrología, Fundación Renal Iñigo Álvarez de Toledo, Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, ISCIII Miguel Servet (A.B.S. and M.D.S.-N.), Fundación Conchita Rabago (D.M.-S.), Consejería de Educación, Juventud y Deporte Comunidad de Madrid/FSE (M.F.-B.), and the Heisenberg Professorship Program of the German Research Foundation (A.L.).

1. Siew ED, et al. (2015) Predictors of recurrent AKI. *J Am Soc Nephrol* 27:1190–1200.
2. Bellomo R, Kellum JA, Ronco C (2012) Acute kidney injury. *Lancet* 380:756–766.
3. Linkermann A, Stockwell BR, Krautwald S, Anders HJ (2014) Regulated cell death and inflammation: An auto-amplification loop causes organ failure. *Nat Rev Immunol* 14: 759–767.
4. Linkermann A, et al. (2013) Two independent pathways of regulated necrosis mediate ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 110:12024–12029.
5. Martin-Sanchez D, et al. (2017) Ferroptosis, but not necroptosis, is important in nephrotoxic folic acid-induced AKI. *J Am Soc Nephrol* 28:218–229.
6. Cho YS, et al. (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* 137:1112–1123.
7. Wu XN, et al. (2014) Distinct roles of RIP1-RIP3 hetero- and RIP3-RIP3 homo-interaction in mediating necroptosis. *Cell Death Differ* 21:1709–1720.
8. Sun L, et al. (2012) Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. *Cell* 148:213–227.
9. Mulay SR, Linkermann A, Anders HJ (2015) Necroinflammation in kidney disease. *J Am Soc Nephrol* 27:27–39.
10. Xu Y, et al. (2015) A role for tubular necroptosis in cisplatin-induced AKI. *J Am Soc Nephrol* 26:2647–2658.
11. Tristão VR, et al. (2016) Synergistic effect of apoptosis and necroptosis inhibitors in cisplatin-induced nephrotoxicity. *Apoptosis* 21:51–59.
12. Sanz AB, et al. (2014) TWEAK and the progression of renal disease: Clinical translation. *Nephrol Dial Transplant* 29(Suppl 1):i54–i62.
13. Sanz AB, et al. (2008) The cytokine TWEAK modulates renal tubulointerstitial inflammation. *J Am Soc Nephrol* 19:695–703.
14. Hotta K, et al. (2011) Direct targeting of fibroblast growth factor-inducible 14 protein protects against renal ischemia reperfusion injury. *Kidney Int* 79:179–188.
15. Xia Y, et al. (2014) Deficiency of fibroblast growth factor-inducible 14 (Fn14) preserves the filtration barrier and ameliorates lupus nephritis. *J Am Soc Nephrol* 26: 1053–1070.
16. Justo P, et al. (2006) Cytokine cooperation in renal tubular cell injury: The role of TWEAK. *Kidney Int* 70:1750–1758.
17. Friedmann Angeli JP, et al. (2014) Inactivation of the ferroptosis regulator Gpx4 triggers acute renal failure in mice. *Nat Cell Biol* 16:1180–1191.
18. Orozco S, et al. (2014) RIPK1 both positively and negatively regulates RIPK3 oligomerization and necroptosis. *Cell Death Differ* 21:1511–1521.
19. Dannappel M, et al. (2014) RIPK1 maintains epithelial homeostasis by inhibiting apoptosis and necroptosis. *Nature* 513:90–94.
20. Biton S, Ashkenazi A (2011) NEMO and RIP1 control cell fate in response to extensive DNA damage via TNF-α feedforward signaling. *Cell* 145:92–103.
21. Guo X, et al. (2016) TAK1 regulates caspase 8 activation and necroptotic signaling via multiple cell death checkpoints. *Cell Death Dis* 7:e2381.
22. Lorz C, et al. (2004) Paracetamol-induced renal tubular injury: A role for ER stress. *J Am Soc Nephrol* 15:380–389.
23. Justo P, Lorz C, Sanz A, Egido J, Ortiz A (2003) Intracellular mechanisms of cyclosporin A-induced tubular cell apoptosis. *J Am Soc Nephrol* 14:3072–3080.
24. Günther C, et al. (2016) The pseudokinase MLKL mediates programmed hepatocellular necrosis independently of RIPK3 during hepatitis. *J Clin Invest* 126:4346–4360.
25. Martin-Sanchez D, et al. (2018) Targeting of regulated necrosis in kidney disease. *Nefrologia* 38:125–135.
26. Sanchez-Niño MD, et al. (2010) TNF superfamily: A growing saga of kidney injury modulators. *Mediators Inflamm* 2010:182958.
27. Linkermann A, Green DR (2014) Necroptosis. *N Engl J Med* 370:455–465.
28. Dondelinger Y, et al. (2013) RIPK3 contributes to TNFR1-mediated RIPK1 kinase-dependent apoptosis in conditions of cIAP1/2 depletion or TAK1 kinase inhibition. *Cell Death Differ* 20:1381–1392.
29. Dondelinger Y, et al. (2015) NF-κB-independent role of IKKα/IKKβ in preventing RIPK1 kinase-dependent apoptotic and necroptotic cell death during TNF signaling. *Mol Cell* 60:63–76.
30. Dillon CP, et al. (2014) RIPK1 blocks early postnatal lethality mediated by caspase-8 and RIPK3. *Cell* 157:1189–1202.
31. Kondylis V, Kumari S, Vlantis K, Pasparakis M (2017) The interplay of IKK, NF-κB and RIPK1 signaling in the regulation of cell death, tissue homeostasis and inflammation. *Immunol Rev* 277:113–127.
32. Metz-Kurschel U, et al. (1990) Folate nephropathy occurring during cytotoxic chemotherapy with high-dose folinic acid and 5-fluorouracil. *Ren Fail* 12:93–97.
33. Newton K, Sun X, Dixit VM (2004) 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* 24:1464–1469.