RGMb protects against acute kidney injury by inhibiting tubular cell necroptosis via an MLKL-dependent mechanism

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Tubular cell necrosis is a key histological feature of acute kidney injury (AKI). Necroptosis is a type of programmed necrosis, which is executed by mixed lineage kinase domain-like protein (MLKL) upon its binding to the plasma membrane. Emerging evidence indicates that necroptosis plays a critical role in the development of AKI. However, it is unclear whether renal tubular cells undergo necroptosis in vivo and how the necroptotic pathway is regulated during AKI. Repulsive guidance molecule (RGMb)-b is a member of the RGM family. Our previous study demonstrated that RGMb is highly expressed in kidney tubular epithelial cells, but its biological role in the kidney has not been well characterized. In the present study, we found that RGMb reduced membrane-associated MLKL levels and inhibited necroptosis in cultured cells. During ischemia/reperfusion injury (IRI) or oxalate nephropathy, MLKL was induced to express on the apical membrane of proximal tubular (PT) cells. Specific knockout of Rgmb in tubular cells (Rgmb cKO) increased MLKL expression at the apical membrane of PT cells and induced more tubular cell death and more severe renal dysfunction compared with wild-type mice. Treatment with the necroptosis inhibitor Necrostatin-1 or GSK-963 reduced MLKL expression on the apical membrane of PT cells and ameliorated renal function impairment after IRI in both wild-type and Rgmb cKO mice. Taken together, our results suggest that proximal tubular cell necroptosis plays an important role in AKI, and that RGMb protects against AKI by inhibiting MLKL membrane association and necroptosis in proximal tubular cells.

Significance

Necroptosis is critically involved in the development of acute kidney injury (AKI), but it has not been well demonstrated that necroptosis occurs in renal tubular epithelial cells in vivo during AKI. Now, we provide evidence that renal proximal tubular cells undergo necroptosis during ischemia/reperfusion injury or oxalate nephropathy. Repulsive guidance molecule-b protects against AKI by inhibiting mixed lineage kinase domain-like membrane association and necroptosis in proximal tubular cells.


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toxins, and is sufficient to oligomerize, bind to phosphatidylinositol lipids through a patch of positively charged amino acids, permeabilize the plasma membrane, and trigger cell death (20, 21, 23–25). However, the activation of the full-length MLKL requires phosphorylation of Thr357 and Ser358 in human MLKL (17, 23), or Ser345 and Ser347 in mouse MLKL (26, 27), to unleash the 4HBD for oligomerization and membrane recruitment of MLKL and subsequent membrane rupture (24).

Physiologically relevant necroptosis has been found in T lymphocytes (28), photoreceptors (29), microglia (30), and enteroctyes (31). Necroptosis has also been shown to contribute to the pathogenesis of ischemic injury in the brain, heart, and kidney (5, 6, 8, 32, 33), atherosclerosis (34), pancreatitis (14), inflammatory bowel diseases (35), and viral infection (36). In renal IRI, necroptosis appears to be predominant over apoptosis, and necroptosis contributes to IRI as genetic deletion of Rip3 or inhibition of RIP1 kinase by necrostatin-1 (Nec-1) each prevented renal injury (5, 6). In addition, a recent study demonstrated that acute crystal nephropathy involves RIP3-MLKL–mediated necroptosis (37). It has been well documented that tubular cells undergo necroptosis in vitro (5, 6, 37, 38). However, since the previous studies used Nec-1 administration, or global Rip3 or MLKL knockout mouse models, it is unclear whether necroptosis occurs in renal tubular cells in vivo during AKI (6, 37, 38).

Repulsive guidance molecules b (RGMb) is a member of the RGM family, which consists of RGMα, RGMb, and RGMc (hemojuvelin). The RGM proteins associate with cell membrane through a phosphatidylinositol (GPI) anchor, and have been shown to be coreceptors for BMP signaling as well as ligands for the neogenin receptor (39). RGMb, also known as Dragon, was originally identified through a genomic screen for genes regulated by the transcription factor DRG11 in embryonic dorsal root ganglion (40). The biological functions of RGMb are only beginning to emerge. Our previous study demonstrated that homozygous RGMb-deficient mice die at early postnatal ages and that RGMb inhibits IL-6 expression in macrophages through the BMP pathway (41). RGMb also promotes neurite outgrowth and peripheral nerve regeneration by modulating BMP signaling (42). In addition, RGMb binds to PD-L2, and this interaction promotes regulatory tolerance (43).

We previously showed that RGMb is highly expressed in the tubular epithelial cells of the kidney (44). Using heterozygous global Rgm knockout mice, we were able to demonstrate that RGMb induces apoptosis in the tubular cells of kidneys subjected to unilateral ureteric obstruction (UOU) (45), but this regulation was not observed in renal IRI. To further identify the specific role of RGMb in renal tubular cells during AKI, we generated renal tubular cell-specific Rgmb knockout mice. We found that RGMb protects kidneys from IRI and oxalate nephropathy by inhibiting necroptosis in proximal tubular cells. Due to its protective role, RGMb is a potential therapeutic target for renal injury.

Results

Tubule-Specific Ablation of Rgmb Aggravates AKI Induced by IR. Our previous study demonstrated that RGMb is highly expressed in renal tubular cells including proximal tubular cells (44). To examine the specific role of RGMb in renal tubules, we generated renal tubule-specific RGMb knockout mice by interbreeding floxed Rgmb mice (46) with Ksp-Cre mice (SI Appendix, Fig. S1). Ksp-Cre has been shown to efficiently ablate floxed Smad2, β-catenin, and Dicer in renal tubules including proximal tubules (47–49), although Cre recombines is expressed at lower levels in proximal tubules compared with collecting ducts and thick ascending limbs (50).

Rgmb mRNA was reduced by 65% in Rgmb cKO kidneys compared with wild-type (WT) kidneys (SI Appendix, Fig. S2A), while RGMb protein, the specificity of which was determined by the kidney sample from a global Rgmb KO (gKO) mouse, was reduced by 67% (SI Appendix, Fig. S2B). As previously demonstrated by us (44), RGMb was highly expressed in various renal tubules including proximal tubules (marked by megalin), and this expression was markedly reduced in Rgmb cKO kidneys (SI Appendix, Fig. S2 C and D).

Rgmb cKO mice appeared to be grossly normal. Rgmb cKO mice showed normal kidney sizes and structures, and normal serum creatinine levels. Therefore, RGMb is not critical for normal kidney function.

We next examined the effects of Rgmb ablation on acute tubular injury after IR. Compared with WT mice, Rgmb cKO mice exhibited more severe tubular injury, characterized by loss of brush border, tubular dilation, tubular cell depletion, and cast formation in the lumen of tubules, 24 h after IR (Fig. 1A). Overall tubular injury scores were higher in Rgmb cKO compared with WT mice (Fig. 1B). Ngal, a marker for kidney injury (51), in the kidney and urine, was increased in Rgmb cKO mice compared with WT mice (Fig. 1 C and D). Urinary chromatin protein high mobility group mobility group B1 (HMGB1), a marker of necrosis (52), was also increased in Rgmb cKO after IR (Fig. 1D). More TUNEL-positive cells were observed in Rgmb cKO than in WT kidneys (Fig. 1E). The inflammatory factors Il-6, Cxcl-1, Cxcl-2, and TNF-α was up-regulated by IR. Deletion of Rgmb further increased the expression of Il-6, Cxcl-1, and Cxcl-2, but not TNF-α (Fig. 1 F–I). Serum creatinine and blood urea nitrogen (BUN) levels were significantly higher in Rgmb cKO mice than in WT mice (Fig. 1 J and K). Taken together, it is clear that the loss of Rgmb in tubular cells aggravates IRI.

RIP3 and MLKL Expression in the Kidney Are Induced by IR. To investigate the types of cell death regulated by RGMb, we examined the expression levels of the key regulators in cell death. Cleaved caspase-3 and cleaved PARP were not altered in kidneys collected 12 and 24 h after ischemia in both WT and Rgmb cKO mice compared with the sham control. As positive control, cleaved caspase-3 and cleaved PARP were increased by UUO (SI Appendix, Fig. S3 A and B). These results suggest that the increased cell death in Rgmb cKO kidneys during IRI may not be apoptosis. Conversion of LC3-I into LC3-II did not significantly change following IR. PI62 appeared to slightly increased upon ischemia, but there were no differences between WT and Rgmb cKO mice (SI Appendix, Fig. S4). Therefore, autophagy is not a mechanism underlying RGMb's protective role.

Ferroptosis is an iron-dependent form of necrosis that occurs due to lipid peroxidation. System xuronc is known key regulators of ferroptosis, and inhibition of either Slc7a11, a subunit of the system xuronc, or GPX4 induces ferroptosis (53, 54). Interestingly, Slc7a11 and GPX4 expression was markedly increased by IR and there was no significant difference in the expression of the two genes between WT and Rgmb cKO mice (SI Appendix, Fig. S5), indicating that ferroptosis may not be a mechanism of RGMb action.

Critical for the formation of the necroosomes is a comprised caspase-8 activity (55, 56). Interestingly, cleaved caspase-8 was reduced 12 and 24 h after ischemia (Fig. 2 A and B). Rip1 mRNA in the kidney was slightly up-regulated 12 h after ischemia, and was slightly down-regulated 24 h after ischemia (Fig. 2C) in both WT and Rgmb cKO mice, with no difference between the two genotypes. Rip3 and Mlkl mRNA levels increased 12 and 24 h after ischemia (Fig. 2 D and E). Rip3 protein was barely detectable in sham-operated kidneys, but markedly increased 12 and 24 h (Fig. 2F) after ischemia. Mlkl protein did not change 12 h after ischemia, but increased 24 h after ischemia (Fig. 2G). Both Mlkl mRNA and protein levels were similar between WT and cKO kidneys 24 h after ischemia (Fig. 2 E and G). These results suggest that the necrotic pathway may be activated by IR.

IR and Deletion of Rgmb Promote Expression of MLKL in the Apical Membrane of Proximal Tubular Cells. To determine the cell types that may undergo necroptosis during IRI, we examined cellular localization of RIP3 and MLKL by immunofluorescence. RIP3 was not detectable in sham-operated kidneys, but was readily detected in proximal tubules in cortex and outer medulla 12 and 24 h after ischemia. No RIP3 expression was found in other tubules.
Similar staining pattern was obtained when a different RIP3 antibody was used (SI Appendix, Fig. S8). There was no appreciable difference in RIP3 signal between WT and Rgmb cKO mice (SI Appendix, Figs. S6 and S7).

We then examined the so-far most-terminal known end-stage effector of the necroptosis pathway MLKL. MLKL was detected only in collecting ducts (positive for AQP2) in a diffused manner in sham-operated kidneys and in injured kidneys collected 12 h after ischemia in WT and Rgmb cKO mice (SI Appendix, Fig. S9). Twenty-four hours after IR, in addition to the expression in collecting duct (SI Appendix, Fig. S10), MLKL was also found to be expressed in the apical membrane of proximal tubules in inner cortex and outer medulla 24 h after ischemia (Fig. 3A). Strikingly, MLKL apical staining in proximal tubules was much increased in Rgmb cKO kidneys compared with WT kidneys (Fig. 3A). No apparent MLKL signal was found in the proximal tubules in the outer cortex (SI Appendix, Fig. S10).

We also extracted membrane proteins from the cortex and outer medulla of injured kidneys from WT and Rgmb cKO mice and immunoblotted for MLKL. Membrane-associated MLKL levels were increased in Rgmb cKO compared with WT kidneys, whereas cytosolic MLKL was similar between the two genotypes (Fig. 3B). Since translocation of MLKL to the plasma membrane is an indicator of MLKL activation and necroptosis. Our results suggest that necroptosis is induced in proximal tubules by IR, and deletion of RGMb promotes IRI-induced necroptosis.

Ablation of Rgmb Increases Apical MLKL Expression in Proximal Tubules and Aggravates AKI Induced by Oxalate Crystals. Crystal nephropathy also involves RIP3-MLKL–mediated necroptosis (37). To confirm
RGMb's role in tubular cell necroptosis during AKI, we induced crystal nephropathy by a single injection of NaOx. Intrarenal calcium oxalate (CaOx) crystal deposition was similar between WT and Rgmb cKO mice 24 h after NaOx injection (SI Appendix, Fig. S11). Serum creatinine and BUN levels were higher in Rgmb cKO than in WT mice (Fig. 4A). Histologically, more tubular damage and higher kidney injury scores were found in Rgmb cKO than in WT mice (Fig. 4B). Urinary Ngal levels were also increased in Rgmb cKO mice compared with WT mice (Fig. 4C).

Cleaved caspase-8 was reduced in kidneys after NaOx treatment in both WT and Rgmb cKO mice (Fig. 4D). RIP3 and MLKL in the whole kidney were induced by NaOx treatment (Fig. 4E). Weak RIP3 staining was found in proximal tubular cells in both WT and Rgmb cKO kidneys injected with NaOx, with no apparent difference between the two genotypes (SI Appendix, Fig. S12). MLKL was localized to the apical membrane of proximal tubular cells in the cortex and outer medulla of injured kidneys of WT mice, and this expression was increased in Rgmb cKO mice (Fig. 4F). MLKL levels in membrane fractions were increased by NaOx treatment and further increased by Rgmb deletion (SI Appendix, Fig. S13). These results suggest that, as in the IRI model, loss of RGMb induced more MLKL membrane expression in proximal tubules and exacerbated tubular injury and renal dysfunction in the oxalate nephropathy.

**Inhibition of Necroptosis Reduces MLKL Apical Expression in Proximal Tubules and Ameliorates Tubular Injury and Renal Function Impairment After IR.** To determine the contributions of proximal tubular cell necroptosis and its regulation by RGMb to IRI, we treated WT and Rgmb cKO mice with the necroptosis inhibitor Nec-1 30 min before IR. In mice injected with the vehicle (DMSO), deletion of Rgmb increased serum creatinine levels 24 h after IR (Fig. 5A, compare bars 4 and 3). Treatment with Nec-1 reduced serum creatinine levels in both WT and Rgmb cKO mice, which were no longer different between the two genotypes (Fig. 5A, bars 4 and 5). Nec-1 also improved the tubular injury in both WT and Rgmb cKO kidneys after IR (Fig. 5B). These results confirm the previous finding that necroptosis contributes to IRI (5, 6), and also suggest that RGMb inhibits necroptosis during IRI.

Nec-1 did not significantly alter MLKL expression in the kidney (Fig. 5C). Intriguingly, Nec-1 blocked the translocation of MLKL to the apical membrane in proximal tubules in both WT and Rgmb cKO mice (Fig. 5D). Consistently, MLKL levels in membrane fractions induced by IR were reduced by Nec-1 treatment (Fig. 5E).
GSK’963, another necroptosis inhibitor structurally distinct from Nec-1 (57), also reduced the serum creatinine levels and apical MLKL signal in proximal tubules in WT and Rgmb cKO mice (SI Appendix, Fig. S14). These results further support that MLKL-mediated and RGMb-regulated necroptosis occurs in proximal tubules during IRI.

**RGMb Inhibits Necroptosis in TKPTS, HT-29, and HK-2 Cells.** To directly examine whether RGMb regulates necroptosis, we used TKPTS mouse proximal tubular cells, which have been reported to be sensitive to TNF-α/cycloheximide (CHX)/zVAD-fmk–induced cell death, a classical assay for necroptosis (5). Consistent with this previous observation, a combination of TNF-α, CHX, and zVAD-fmk (TCZ) induced more cell death than TNF-α plus CHX (TC) as determined by ATP levels (Fig. 6A). Cotreatment with Nec-1 decreased cell death induced by TCZ, thus defining the cell death induced by TCZ as RIP1-dependent necroptosis (Fig. 6A). We then transfected TKPTS cells with increasing amounts of RGMb cDNA. RGMb did not have any effects on TC-induced cell death (apoptosis), but dose-dependently inhibited TCZ-induced necroptosis (Fig. 6B). RGMb also reduced TCZ-induced release of HMGB1 into the cell culture media (Fig. 6C), while it did not affect HMGB1 mRNA expression (SI Appendix, Fig. S15B). RGMb did not alter Rip1, Rip3, and Mlkl expression after TCZ treatment (SI Appendix, Fig. S15 C–E). These results suggest that RGMb inhibits necroptosis in TKPTS cells.

We also used HT-29 colonic epithelial cells, a widely used necroptosis-sensitive cell line, to further examine RGMb’s role in necroptosis. As shown in Fig. 6D, HT-29 underwent drastic necroptosis in the presence of TNF-α, the Smae mimetic Birinapant and zVAD-fmk (TSZ). RGMb markedly inhibited TSZ-induced necroptosis while it did not affect TSZ-induced apoptosis (Fig. 6E and F). Inhibition of RGMb expression by two shRNA sequences increased TSZ-induced necroptosis, and these increases were abolished by cotransfection with Rgmb cDNA (SI Appendix, Fig. S16). These results further indicate that RGMb inhibits necroptosis in HT-29 cells.

A recent study found that crystals induce necroptosis in renal tubular cells (37). Consistent with this study, CaOx crystals induced cell death in HK-2 human proximal tubular cells, and this effect was blocked by Nec-1 (Fig. 6G). RGMb overexpression dose-dependently inhibited CaOx-induced cell death (Fig. 6H), suggesting a role of RGMb in inhibiting CaOx-induced necroptosis.

To determine whether the GPI anchor is necessary for RGMb activity, we generated chimeric Rgmb-TM, in which the GPI anchor is replaced with the transmembrane (TM) domain from platelet-derived growth factor receptor, thus RGMb is anchored to the membrane via the TM domain. The proteins of Rgmb-TM expressed at the expected molecular weights and was able to stimulate BMP signaling as WT Rgmb did (SI Appendix, Fig. S17). Interestingly, RGMb-TM also dose-dependently inhibited TSZ-induced necroptosis in HT-29 cells (Fig. 6I). These results suggest that Rgmb activity is not dependent on the presence of GPI anchor, but rather on the localization on the plasma membrane.

**RGMb Reduces Plasma Membrane-Associated MLKL.** MLKL phosphorylation, oligomerization, and plasma membrane translocation are the key steps for MLKL to execute necroptosis. We therefore examined whether RGMb regulates these modifications on MLKL. HT-29 cells transfected with Rgmb were treated with TSZ for 3 or 6 h. TSZ induced MLKL phosphorylation (Thr357) 3 h or 6 h after TSZ treatment (Fig. 7A and B). MLKL oligomerization was detected at 6 h (Fig. 7B) but not at 3 h (data not shown) after TSZ treatment. Both MLKL phosphorylation and oligomerization were inhibited by Nec-1 (Fig. 7A and B). RGMb overexpression did not alter MLKL phosphorylation and oligomerization (Fig. 7A and B).

Once oligomerized, MLKL translocates to the plasma membrane to induce cell membrane rupture and cell death. TSZ increased membrane-associated MLKL as determined by either total MLKL or phosphorylated MLKL (Fig. 7C). Membrane-associated MLKL was significantly reduced in the presence of RGMb (Fig. 7C). Immunofluorescence also showed that the ratio
of cells with TSZ-induced phospho-MLKL plasma membrane localization was reduced by RGMb overexpression (Fig. 7D). Interestingly, membrane-associated MLKL was also reduced by RGMb-TM (Fig. 7E). These results suggest that RGMb does not affect MLKL phosphorylation and oligomerization, but inhibits its membrane translocation or membrane binding.

**RGMb Does Not Regulate Ferroptosis.** Ferroptosis has been reported to contribute to acute kidney injury (7, 58, 59). We examined the effects of inhibition of system x−c by erastin on cell death in HK-2 cells. Erastin dose-dependently induced cell death (SI Appendix, Fig. S18A), which was prevented by iron chelation with desferoxamine (DFO) or by Fer-1 (SI Appendix, Fig. S18B). Thus, HK-2 cells are sensitive to erastin-induced ferroptosis. Transfection of Rgmb into HK-2 cells did not alter erastin-induced ferroptosis (SI Appendix, Fig. S18C) although RGMb protein was expressed (SI Appendix, Fig. S18D). These results suggest that RGMb does not play a role in ferroptosis.

**Discussion**

Previous studies have demonstrated that TKPTS, HK-2, and NRK-52 tubular epithelial cells, or isolated proximal tubules, undergo necroptosis in response to various treatments (5, 6, 37, 38, 60). However, whether necroptosis occurs in renal tubules in vivo is questioned by a study showing that conditional deletion of FADD or caspase-8 in renal tubules did not induce spontaneous necroptosis under basal conditions (58). In the present study, we found that RIP3 and MLKL were induced during kidney injury induced by IR or oxalate cytotoxicity, while caspase 8 cleavage was inhibited. Importantly, MLKL was induced to be expressed on the apical membrane of proximal tubules, and inhibition of necroptosis by necrostatin-1 or GSK 963 dramatically reduced this expression and ameliorated tubular injury. MLKL permeabilizes and ruptures the plasma membrane once it binds to cell membrane (20, 21, 23–25). Therefore, our results demonstrate that necroptosis occurs in proximal tubular cells at least in the mouse models of IRI and oxalate nephropathy.

In normal kidneys, RIP3 and MLKL expression was not detectable. These results may explain the failure of FADD or caspase-8 deficiency to induce spontaneous necroptosis in renal tubular cells under basal conditions (58): Necroptosis could not take place when RIP3 and MLKL are not available although the necroptosis inhibitor FADD or caspase-6 is removed. Combining this previous observation with our own results, we hypothesize...
that the necrotic pathway may not play a role in tubular cell homeostasis in kidneys under basal conditions.

We found that deletion of Rgmb in tubular cells increased the expression of MLKL on the apical membrane of proximal tubules during IRI or oxalate nephropathy. Deletion of Rgmb also exacerbated tubular injury and kidney dysfunction. Necrostatin-1 or GSK963 treatment reduced MLKL apical expression and tubular injury in both WT and Rgmb cKO mice. In vitro, we demonstrated that Rgmb inhibited necroptosis in mouse TKPTS and human HK-2 renal proximal tubular cells and in HT-29 colonic epithelial cells. Importantly, we found that Rgmb reduced membrane-associated MLKL although it did not alter MLKL expression, phosphorylation, and oligomerization. These results provide evidence that Rgmb inhibits necroptosis in proximal tubular cells by preventing MLKL membrane association. In addition, since inhibition of necroptosis by Nec-1 or GSK963 reduced tubular injury and serum creatinine to similar levels in WT and Rgmb cKO mice, necroptosis in proximal tubules induced by Rgmb deletion may play a major role in the increased IRI observed in Rgmb cKO mice.

How Rgmb reduces MLKL membrane association remains unsolved yet. Rgmb has a signal sequence at the N terminus and a GPI anchor motif at the C terminus. Rgmb is associated with cell membrane through the GPI anchor (40, 43), and the membrane-bound Rgmb functions as a coreceptor for BMP signaling and a ligand for neogenin (39, 61). In this respect, we found that membrane-bound Rgmb inhibits necroptosis no matter whether it is anchored to the plasma membrane through the GPI motif or transmembrane domains. However, our preliminary results showed that Rgmb may not act through the BMP or neogenin pathway to regulate MLKL membrane association and necroptosis. In addition, we did not observe any interaction between Rgmb and MLKL by immunoprecipitation under the necroptosis induction condition.

We previously showed that less apoptosis was observed in the collecting ducts of kidneys subjected to UUO in heterozygous global Rgmb KO mice (45) or in Ksp-cre–mediated Rgmb conditional KO mice compared with WT mice. In the IRI model, no cleaved caspase 3 was detected in kidneys after IRI, an observation consistent with a previous study (6). These results indicate that there is no or minimal tubular cell apoptosis at least during the early stage of IRI. Therefore, it is not surprising that we did not see any effects of Rgmb deletion on caspase-3 cleavage in IRI. Our results also suggest that Rgmb differentially regulates apoptosis and necroptosis, and the overall effects of Rgmb may be cell type and injury type dependent.

Ferroptosis also plays a role in AKI (7, 58, 59). Ferroptosis occurs when GPX4 is inhibited at the expression level, by small molecules such as RSL3 or by unavailability of glutathione. Inhibition of the glutamate/cysteine antiporter, system x_c by erastin or by glutamate reduces intracellular glutathione levels, thus also inducing ferroptosis (53, 54). Interestingly, a previous study demonstrated that there was a sustained decrease in glutamate in kidney tissues after IR (62). Our present study found that the expression of Slc7a11, a subunit of system x_c, and Gpx4 was markedly increased by IR. The changes in glutamate, Slc7a11, and Gpx4 do not appear to favor ferroptosis, thus the molecular mechanisms underlying ferroptosis during IRI remain to be elucidated. Nevertheless, no differences in Slc7a11 and Gpx4 expression in the kidney were found between WT and Rgmb cKO mice, and Rgmb did not alter erastin-induced ferroptosis in vitro. Therefore, Rgmb may not play a role in ferroptosis during AKI.

Taken together, our results demonstrated that Rgmb reduced membrane-associated MLKL and inhibited necroptosis in vitro. Deletion of Rgmb in renal tubular cells increased MLKL expression on the apical membrane of proximal tubules, and aggravated tubular injury and renal functional impairment in IRI or oxalate nephropathy. Inhibition of necroptosis by necrostatin-1 or GSK963 reduced MLKL apical expression in proximal tubules and improved IRI. Rgmb inhibits tubular cell necroptosis during AKI.
Materials and Methods

See SI Appendix, SI Materials and Methods for detailed descriptions.

Generation of Renal Tubule-Specific Rgmb Knockout Mice. Floxed Rgmb mice on C57BL/6 background have been described (46). Excision of the loxP-flanked region in kidney tubular epithelial cells to establish conditional kidney knockout mice (cKO) was obtained by interbreeding with Ksp-Cre mice on the C57BL/6 background.

IRI. Bilateral IRI was performed as previously described with minor modifications (63). Briefly, male Rgmb cKO (Rgmb<sup>f/f</sup>; Ksp-cre) mice and WT littermates (Rgmb<sup>f/f</sup>, Rgmb<sup>f/wt</sup>, or Rgmb<sup>wt/wt</sup>; Ksp-cre) were placed on a heat plate at a temperature of 36.5–37 °C. Right and left flank incisions were made. The renal pedicles were clamped for 40 min, followed by reperfusion. The mice were killed at 24 h after ischemia. Mice with sham operation were used as control.

Oxalate Nephropathy. Oxalate crystal kidney injury was performed as previously described (37). Male Rgmb cKO and WT littermates were injected with CaOx crystals in the absence or presence of Nec-1 for 24 h before cell viability was measured.

Fig. 6. RGMb inhibits necroptosis in TKPTS, HT-29, and HK-2 cells. (A) Necroptosis in TKPTS cells. Cells were treated with DMSO (Ctrl), TNF-α (100 ng/mL), CHX (2 μg/mL), zVAD-fmk (25 μM), and/or Nec-1 (50 μM) as indicated (TC, TCZ, or TCZN) for 24 h. Cell viability was measured using CellTiter-Glo Luminescent Cell Viability Assay. (B) RGMb inhibits TCZ-induced necroptosis but not TC-induced apoptosis in TKPTS cells. Cells were transfected with increasing amounts of Rgmb cDNA. Forty-eight hours after transfection, cells were treated with DMSO, TC (TNF-α and CHX), or TCZ (TNF-α, CHX, and zVAD-fmk) for 24 h before cell viability was measured. (C) RGMb reduces the release of HMGB1 from TKPTS cells in the presence of TCZ. Cells were transfected with or without Rgmb cDNA (300 ng/mL). Forty-eight hours after transfection, cells were treated with DMSO (lanes 1 and 2) or TCZ (lanes 3–8) for 24 h. Supernatants were collected and concentrated. Cells were lysed. Western blotting was performed for HMGB1 in supernatants and HMGB1 and RGMb in whole-cell lysates. β-actin was used as the loading control for cell lysates. (D) Necroptosis in HT-29 cells. Cells were treated with DMSO (Ctrl), TNF-α (30 ng/mL), the Smac mimic Birinapant (50 nM), zVAD-fmk (20 μM), and/or Nec-1 (50 μM) as indicated (TS, TSZ, or TSZN) for 24 h. MTT assay was performed to determine cell viability. (E) RGMb inhibits TSZ-induced necroptosis but not TS-induced apoptosis in HT-29 cells. Cells were transfected with or without Rgmb cDNA (300 ng/mL). Forty-eight hours after transfection, cells were treated with DMSO, TS, or TSZ for 24 h before the cells were subjected to MTT assay. (F) RGMb dose-dependently inhibits necroptosis in HT-29 cells. Cells were transfected with increasing amounts of Flag-Rgmb plasmid. Forty-eight hours after transfection, cells were treated with DMSO or TSZ for 8 h before the cells were subjected to MTT assay. (G) CaOx crystals induce necroptosis in HK-2 cells. Cells were incubated with 1 mg/mL CaOx crystals in the absence or presence of Nec-1 for 24 h before cell viability was measured by MTT assay. (H) RGMb inhibits CaOx-induced necroptosis in HK-2 cells. Cells were transfected with increasing amounts of Rgmb plasmid. Twenty-four hours after transfection, cells were incubated with or without CaOx crystals for 24 h before cell viability was measured. (I) RGMb-TM inhibits TSZ-induced necroptosis. Cells were transfected with increasing amounts of Flag-Rgmb-TM plasmid. Forty-eight hours after transfection, cells were treated with DMSO or TSZ for 6 h before the cells were subjected to MTT assay. n = 6 for A, B, D, F, and I; n = 3 for C; n = 10 for E and G; n = 8 for H. *P < 0.05; **P < 0.01; ***P < 0.001. In B, ** indicates the differences between the doses 150 and 300 ng/mL.
with a single dose of sodium oxalate at 100 mg/kg body weight and provided with drinking water containing 3% sodium oxalate.

Histology and Immunofluorescence. Paraffin kidney sections were used for periodic acid-Schiff staining. The degree of tubular damage including tubular dilation, tubular atrophy, and cast formation was scored by three investigators without knowing the genotypes. Cryostat kidney sections were used for immunofluorescent staining to examine RGMb, megalin, RIP3, and MLKL cellular localization. MLKL fluorescence intensity on the apical membrane of PT cells was quantified by the ImageJ software.

Cell Culture and Transfection. Human colon carcinoma HT-29 cells were originally obtained from American Type Culture Collection. Mouse proximal tubular TKPTS cells were published previously (64) and were a generous gift from Rafia Al-Lamki, University of Cambridge, Cambridge, UK. TKPTS cells were maintained in DMEM/F12 (1:1) (Invitrogen) supplemented with 7% FBS and 1% SITZ liquid medium (Sigma-Aldrich). Human proximal tubular HK-2 cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FBS.

Cells were seeded in 6-, 12-, or 96-well plates and transfected with Rgmb or 3xFlag-Rgmb expression construct using Lipofectamine 2000 (Invitrogen). Transfected cells were incubated in completed medium for 1 or 2 d before experiments.

Necroptosis Induction. Necroptosis in TKPTS cells was induced by combined treatment with TNF-α, CHX, and the pan-caspase inhibitor zVAD. Necroptosis in HT-29 cells was induced by TNF-α, the smac mimetic Birinapant (S), and zVAD. Necroptosis in HK-2 cells were induced by CaOx crystals.

Isolation of Membrane Proteins. Cytosolic and crude membrane proteins from HT-29 cells and kidney tissues were performed as previously described (24). Briefly, HT-29 cells were washed with ice-cold PBS before the cells were harvested and permeabilized in a buffer containing 0.025% digitonin. The cortical and outer medullary region was dissected and homogenized in the same buffer. Cytosolic and crude membrane fractions were separated by centrifugation at 3,000 × g for 5 min. The supernatants were taken as cytosolic samples, and the pellets were washed with PBS and then solubilized to collect membrane proteins.
Statistical Analysis. All data are represented as mean ± SEM of independent replicates. Student’s t test was applied for statistical analysis. A P value of less than or equal to 0.05 was considered statistically significant.

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