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Tissue-specific deletion of Crry from mouse proximal tubular epithelial cells increases susceptibility to renal ischemia–reperfusion injury

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The murine cell surface protein Crry (complement receptor 1–related protein/gene y) is a key complement regulator with similar activities to human membrane cofactor protein (MCP) and decay-accelerating factor. MCP has a critical role in preventing complement-mediated tissue injury and its mutation has been implicated in several human kidney diseases. The study of Crry in mice has relevance to understanding MCP activity in human diseases; however, such efforts have been hampered by the embryonic lethality phenotype of Crry gene knockout. Here we used a conditional gene-targeting approach and deleted Crry from the mouse proximal tubular epithelial cells where Crry is prominently expressed. Absence of Crry from proximal tubular epithelial cells resulted in spontaneous C3 deposition on the basolateral surface but no apparent renal disease in unchallenged mice. However, mice deficient in Crry on proximal tubular epithelial cells developed exacerbated renal injury when subjected to renal ischemia–reperfusion, showing increased blood urea nitrogen levels, higher tubular injury scores, more tubular epithelial cell apoptosis, and inflammatory infiltrates. Renal ischemia–reperfusion injury in the Crry conditional knockout mice was prevented by blocking C3 and C5 activation using an anti-properdin or anti-C5 monoclonal antibody (mAb), respectively. Thus, Crry has a critical role in protecting proximal tubular epithelial cells during ischemia–reperfusion challenge. Our results highlight the latent risk for inflammatory kidney injury associated with defects in membrane complement regulators.

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The complement system is a finely tuned innate immune system that, although having a critical role in host defense, has the potential to cause significant tissue injury if not properly controlled.[1,2] The kidney appears to be especially vulnerable to complement attack, and clinical studies have long revealed the importance of complement as an inflammatory pathway in the pathogenesis of various human kidney diseases.[3–5] More recently, several rare and genetically predisposed kidney pathologies including C3 glomerulopathy and atypical hemolytic uremic syndrome have been linked to dysregulation of complement, often arising from defects in complement regulators such as factor H (fH) and membrane cofactor protein (MCP, CD46).[6] Complement regulator insufficiency can be caused by mutations in the genes encoding these proteins or by autoantibodies against them. In addition to C3 glomerulopathy and atypical hemolytic uremic syndrome, complement has also been implicated in the outcomes of kidney transplantation and contributes to tubular injury during renal ischemia–reperfusion.[3,7–10] Thus, understanding how complement is regulated in the various compartments of the kidney has relevance to the prevention and treatment of rare, as well as common, kidney pathologies.

Apart from MCP and fH, host cells are also protected by other membrane complement regulators including decay-accelerating factor (DAF) and CD59. Although all these regulators are expressed in the human kidney, MCP appears to be the only membrane regulator of C3 activation present in abundance on human renal tubular epithelial cells.[3,11,12] MCP inhibits complement activation by acting as a cofactor for factor I–mediated cleavage of C4b and C3b to prevent the formation of both classical and alternative pathway (AP) C3 convertases.[13] Although an MCP gene exists in mice and rats, its expression in these rodent species is rather limited, being detected primarily in the testis.[14–17] Crry (complement receptor 1–related protein/gene y) is a rodent transmembrane protein with both DAF and MCP activities. As Crry has MCP activity and is expressed in many mouse and rat tissues where MCP is absent,[14,18] the study of Crry bears relevance to understanding human MCP in health and disease. However, investigation of the physiological role of Crry *in vivo* has been

hampered by the fact that global gene knockout of the Crry gene in mice is embryonically lethal.[19] Nevertheless, previous studies have provided evidence that Crry has a critical role in protecting the mouse kidney from AP complement attack.[20–24]

To circumvent the embryonic lethality phenotype of global *Crry* gene deficiency and directly assess the physiological role of Crry on proximal tubular epithelial cells (PTECs), we used conditional gene targeting and selectively deleted Crry expression in mouse PTECs. We found that Crry deficiency from PTECs resulted in local C3 deposition on the cells and the presence of activated C3 fragments in plasma. Although no constitutive renal injury was observed, the mutant mice developed exacerbated tubular injury when subjected to renal ischemia–reperfusion (IR) stress. Importantly, blocking AP complement activation or terminal complement pathway by using an anti-properdin or anti-C5 monoclonal antibody (mAb), respectively, prevented renal IR injury, suggesting that IR injury in the mutant mice was caused by increased sensitivity to AP complement attack. Our results provide direct evidence for an important role of Crry in protecting PTECs from AP complement injury during IR stress and suggest that defects in membrane complement regulators can be a latent risk factor for complement-mediated kidney pathologies.

RESULTS

PTEC-specific Crry gene deletion in mice

We screened homozygous Crry-floxed mice ($Crry^{flox/flox}$) with or without Cre transgene expression by polymerase chain reaction (PCR) analysis of tail DNA. Bands for wild-type (WT) or floxed *Crry* alleles were observed at ~970 and 1100 bp, respectively, whereas expression of the Cre transgene was indicated by a 500-bp PCR product (Figure 1a). Previous characterization of the phosphoenolpyruvate carboxykinase (PEPCK)-Cre transgenic mouse established that Cre expression is restricted to the proximal tubules in the renal cortex and medulla and a subset of periportal hepatocytes.[25] Cre-mediated deletion of exon 5 from the floxed *Crry* allele could be indicated by the presence of a 350-bp band. As shown in Figure 1b, mutant *Crry* allele was detected in the kidney, but not in the spleen, of PEPCK-Cre^{+}-$Crry^{flox/flox}$ mice, consistent with expected Cre expression driven by the PEPCK gene promoter.[25] To confirm *Crry* gene deletion at the protein level, we first produced, purified, and validated a rabbit anti-Crry short consensus repeat 3/4 (SCR3/4) antibody. Figure 1c and d shows that on western blot this antibody reacted strongly with the recombinant Crry SCR3/4 and detected endogenous Crry protein in the kidney of WT but not in $Crry^{-/-}C3^{-/-}$ mice. Using immunofluorescence, the same antibody detected abundant Crry expression in the glomeruli and basolateral surfaces of PTECs of WT but not $Crry^{-/-}C3^{-/-}$ mice (Figure 1e), consistent with previously reported Crry expression pattern in the mouse kidney.[22,26,27] We next used immunofluorescence to compare Crry expression in the kidneys of Cre^{-}- and Cre^{+}-$Crry^{flox/flox}$ mice by costaining with megalin, a specific marker for PTECs.[28,29] Although megalin and glomerular Crry expression were unchanged, the basolateral expression of Crry on PTECs normally observed in Cre (−) mice was markedly reduced, and in some areas was almost absent, in Cre (+) mice (Figure 1f and Supplementary Figure S1 online). These results confirmed Crry deletion from PTECs of the mutant mice.

Spontaneous complement activation on PTECs of Crry mutant mice

Global *Crry* gene knockout is embryonically lethal[19] and $Crry^{-/-}$ mice rescued by maternal complement inhibition displayed spontaneous plasma complement activation and consumption after birth.[30,31] To assess the impact of Crry deletion from PTECs on complement activation, we measured circulating levels of intact and activated C3 using enzyme-linked immunosorbent assay (ELISA) and examined C3 deposition on PTECs. We found that deletion of Crry from PTECs had no discernible effect on plasma intact C3 levels but slightly increased activated C3 fragments in circulation (data not shown). Furthermore, immunofluorescence staining of C3 showed spontaneous C3 deposition on the basolateral surface of PTECs in Cre^{+}-$Crry^{flox/flox}$ mice where Crry had been deleted (Figure 2), suggesting that there was local complement activation on these cells, and some activated C3 fragments may have been released into the systemic circulation. Despite the clear evidence of increased complement activation on PTECs, however, we found no functional or histological signs of kidney injury in Cre^{+}-$Crry^{flox/flox}$ mice up to 24 months of age (Figure 3 and data not shown). The *Crry* mutant mice had normal blood urea nitrogen (BUN) levels and showed no proteinuria (Figure 3a and b). Periodic acid–Schiff (PAS) staining revealed normal kidney architecture with prominent brush borders in the proximal tubules in both the cortex and outer medulla (Figure 3c).

Deficiency of Crry in PTECs increases sensitivity to renal IRI

We next subjected Cre^{+}-$Crry^{flox/flox}$ mice and their Cre^{-} littermates to renal IR challenge to determine whether Crry deficiency from PTECs may render the mutant mice more susceptible to renal injury. Compared with the 0-h time point, BUN levels at 24 h after reperfusion increased both in Cre^{-}-$Crry^{flox/flox}$ (0 h: 24.50 ± 0.80 mg/dl vs. 24 h: 45.19 ± 5.31 mg/dl, $P = 0.0014$) and Cre^{+}-$Crry^{flox/flox}$ mice (0 h: 24.29 ± 0.60 mg/dl vs. 24 h: 75.93 ± 12.28 mg/dl, $P = 0.0007$). Notably, at 24 h after reperfusion, BUN levels in Cre^{+}-$Crry^{flox/flox}$ mice were significantly higher than that in Cre^{-}-$Crry^{flox/flox}$ mice ($P < 0.05$; Figure 4a). Increased kidney injury in Cre^{+}-$Crry^{flox/flox}$ mice was corroborated by histological findings. At 1-h post reperfusion, kidney tubular injury was not seen in Cre^{-}-$Crry^{flox/flox}$ mice; however, cast formation was observed in some tubular lumen in the cortex and medulla of Cre^{+}-$Crry^{flox/flox}$ mice (Figure 4b). The difference between Cre^{-} and Cre^{+} mice became more pronounced at 24 h after reperfusion. Compared with

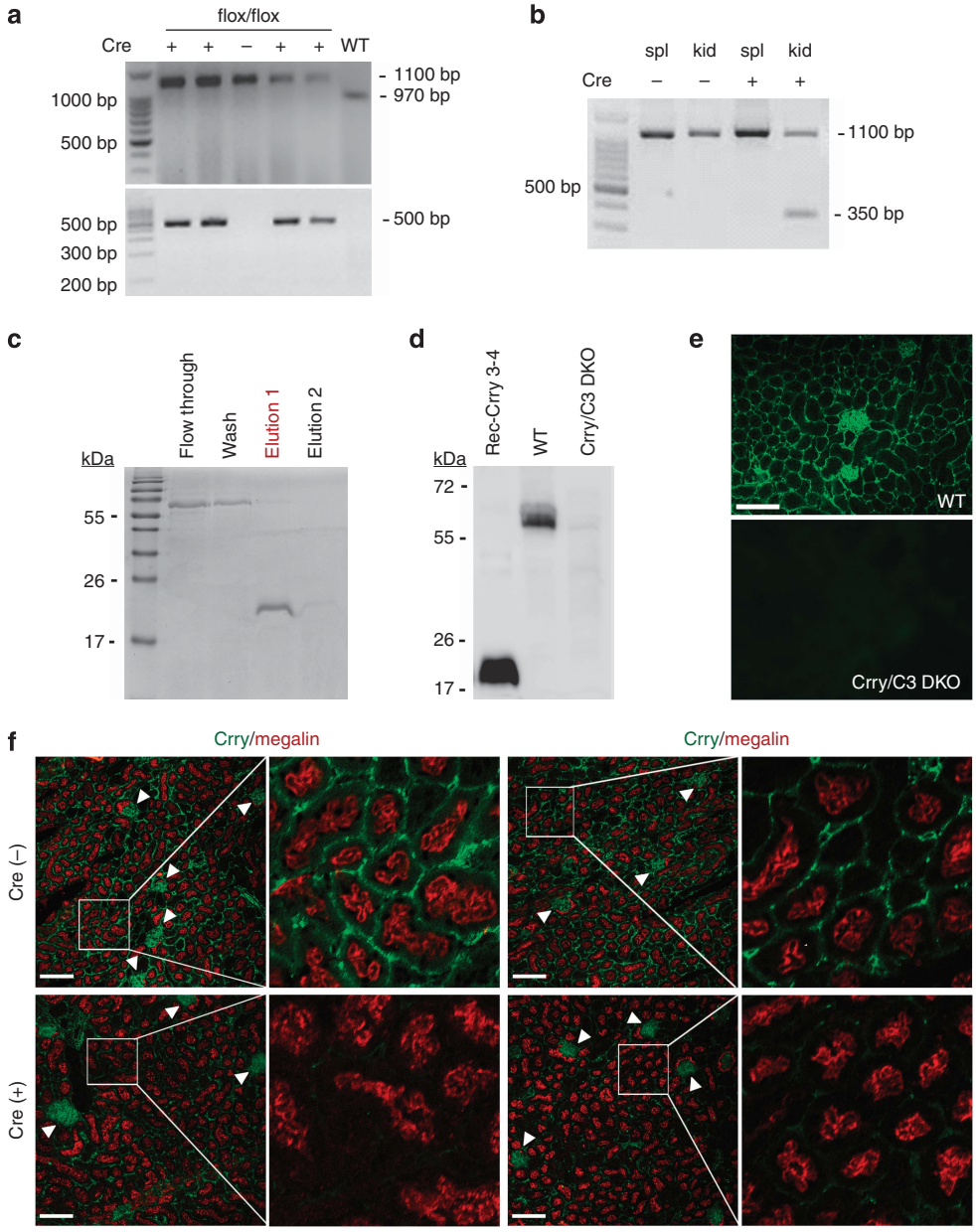

Figure 1 | Generation and confirmation of a PTEC-specific Crry (complement receptor 1–related protein/gene y) knockout mouse. (**a**) Polymerase chain reaction (PCR) genotyping of wild-type (WT) and floxed Crry gene alleles using primers flanking exon 5 of the *Crry* gene. WT Crry alleles produced a 970-bp product, whereas the floxed allele produced an 1100-bp product (upper panel). Mice positive for the Cre transgene (+) showed a PCR product of ~500 bp (lower panel). (**b**) PCR confirmed the specific deletion of the Crry allele in Cre+ kidney tissue (kid) by the presence of a 350-bp band that was not observed in spleen tissue (spl) from the same mouse. (**c**) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis shows the expression and purification of a His-tagged recombinant Crry short consensus repeat 3/4 (SCR3/4) protein (in 'elution 1' fraction) on the Ni^{+} column. (**d**) Western blot showing that a rabbit anti-Crry (SCR3-4) antibody recognized recombinant Crry (SCR3-4) and endogenous Crry protein in the kidney homogenate from a WT mouse. As expected, no signal was detectable in the kidney homogenate of a $Crry^{-/-}C3^{-/-}$ mouse (Crry/C3 DKO), validating the specificity of the antibody. (**e**) Immunofluorescence staining was also used to confirm the specificity of rabbit anti-Crry (SCR3-4) antibody in tissues. Positive staining was observed in WT but not in $Crry^{-/-}C3^{-/-}$ mouse kidneys. Original magnification ×200, scale bar = 50 μm. (**f**) Costaining of megalin (red color) and Crry (green) in Cre^{-} (upper row) and Cre^{+} (lower row) mouse kidneys shows specificity of Crry deletion to PTECs. Megalin is expressed on the apical surface of PTECs, and no difference in its expression was observed between Cre^{-} and Cre^{+} mice. Crry is expressed in both the glomeruli and TECs of Cre^{-} mice. No difference in glomerular Crry expression (arrowhead) was observed between Cre^{-} and Cre^{+} mice. In contrast, Crry expression on PTECs of Cre^{+} mice was markedly reduced, and it was nearly absent in some areas. Representative images from two different mice are shown for each genotype (Cre^{+} and Cre^{-}). Original magnification ×200 for each panel on the left (scale bars = 50 μm); box indicates the section of tissue shown in the corresponding right panel at ×5 greater magnification. DKO, double knockout; IRI, ischemia-reperfusion injury; PTECs, proximal tubular epithelial cells.

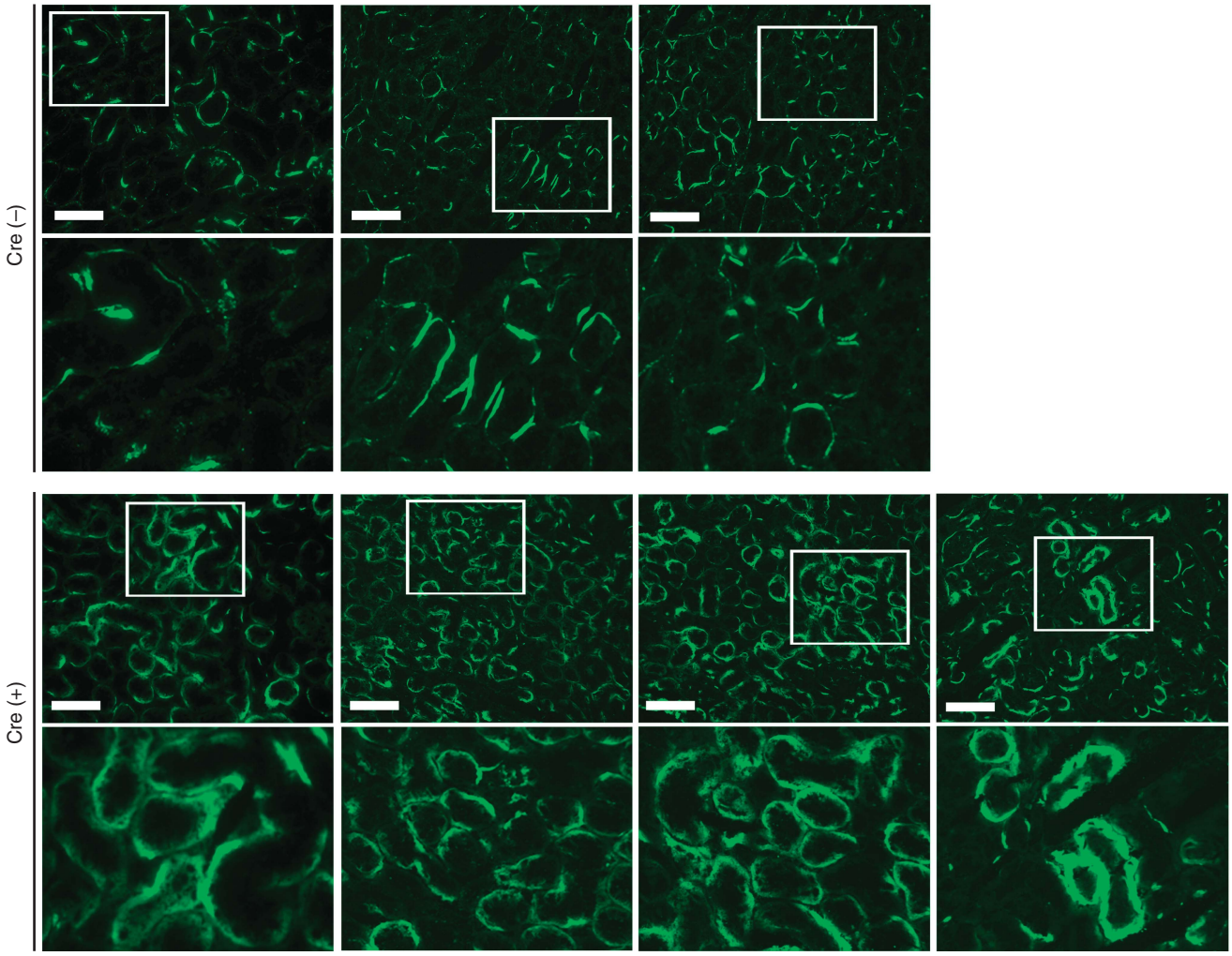

Figure 2 | Effect of Crry (complement receptor 1–related protein/gene y) deletion from PTECs on local complement activation. Representative immunofluorescence pictures showing that Crry deletion from PTECs caused increased C3 deposition on the basolateral surface. In the Cre^- mouse kidney, segmental linear staining of C3 was detectable along the basement membrane, possibly reflecting local C3 synthesis.[47] In the Cre^+ mouse kidney, thicker and ribbon-like C3 staining was seen on basolateral portions of the tubular cells. Images from three Cre^- and four Cre^+ mice are shown. Original magnification for top panels, ×200. Boxed areas are shown underneath at ×2 greater magnification. All scale bars indicate 100 μm length. PTECs, proximal tubular epithelial cells.

Cre^--$Crry^{flox/flox}$ mice, Cre^+-$Crry^{flox/flox}$ mice had markedly more severe tubular injury with pronounced tubular necrosis, brush border disruption, cast formation, tubular dilatation, and inflammatory cell infiltration (Figure 4b and Supplementary Figure S2 online). The significant increase in tubular injury in the Cre^+-$Crry^{flox/flox}$ group was also confirmed by a semiquantitative pathology grading (4.36 ± 0.28 vs. 2.27 ± 0.26, $P<0.001$; Figure 4c). It is interesting that IR-induced renal injury in Cre^+-$Crry^{flox/flox}$ mice was not correlated with more tubular C3 deposition at either 1 or 24 h (Figure 5). In fact, tubular C3 staining in Cre^+-$Crry^{flox/flox}$ mice at 24 h after IR was significantly decreased compared with that detected at 1 or 0 h (before IR challenge; Figure 5). Nevertheless, it is apparent by visual scoring or quantitative image analysis of fluorescence intensity (data not shown) that at all time points there was more tubular C3 deposition in PEPCK-Cre^+-$Crry^{flox/flox}$ mice than in Cre^- mice (Figure 5).

Exacerbated renal IRI in PTEC-specific Crry knockout mice correlates with increased mononuclear phagocyte infiltration and PTEC apoptosis

To assess and compare inflammatory infiltrate into the kidney after IR challenge, we stained mononuclear phagocytes using immunofluorescence with an F4/80 antibody. IR challenge caused a noticeable but only modest increase in F4/80 + mononuclear phagocyte infiltration in the kidneys of Cre^--$Crry^{flox/flox}$ mice at 24 h (0 h: 12.20 ± 0.8602, 1 h: 12.60 ± 1.435, 24 h: 18.40 ± 1.208; Figure 6a). In comparison, infiltration of F4/80 + mononuclear phagocytes at 24 h after IR treatment was much more striking in Cre^+-$Crry^{flox/flox}$ mice (0 h: 12.60 ± 1.502, 1 h: 16 ± 0.707, 24 h: 50.8 ± 2.289;

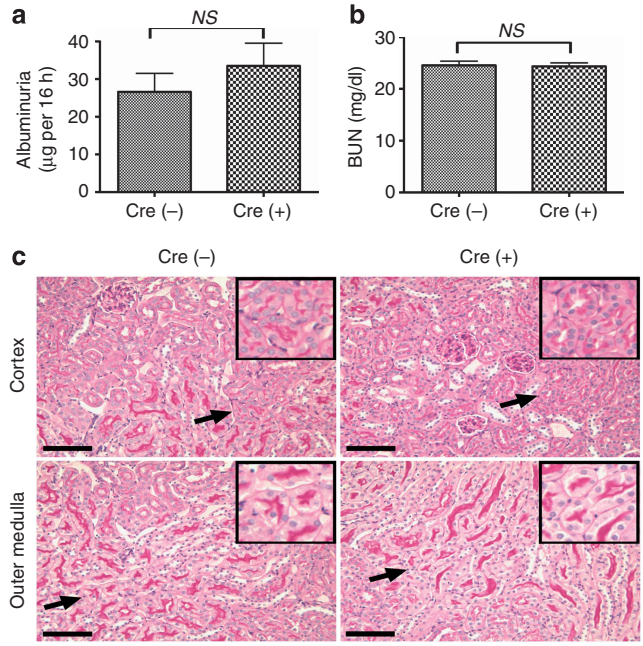

Figure 3 | Crry (complement receptor 1–related protein/gene y) deletion from PTECs does not cause spontaneous renal injury. Compared with Cre^- mice, no abnormal albuminuria (**a**) or elevated blood urea nitrogen (BUN) (**b**) was detected in Cre^+ mice (NS: no significance, $n = 8$ mice per group, ~3 months of age). In both Cre^- and Cre^+ mice, periodic acid–Schiff (PAS) staining showed normal kidney architecture with prominent brush border in the proximal tubules of the cortex and outer medulla (**c**). Original magnification, ×200. Inset panels show ×2 higher magnification of the area indicated by the arrow. Pictures in **c** are representative of eight kidneys in each genotype examined. All scale bars = 50 μm.

Figure 6a). Increased infiltration of mononuclear phagocytes in the kidneys of Cre^+-$Crry^{flox/flox}$ mice was unlikely caused by any intrinsic phenotype differences in these cells, as Crry expression on bone marrow cells of Cre^- and Cre^+ mice was the same (data not shown).

Apoptosis is also a common feature of inflammatory organ damage. Therefore, we next determined the degree of apoptosis in the kidneys of IR-challenged Cre^- and Cre^+ mice using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In both groups of mice, we observed increased numbers of apoptotic tubular cells at 24 h after IR compared with naive mice (TUNEL-positive cells in Cre^- mice: 6.4 ± 0.8124 at 0 h vs. 17.40 ± 2.293 at 24 h, $P < 0.05$; Cre^+ mice: 8.200 ± 1.281 at 0 h vs. 66.00 ± 4.301 at 24 h, $P < 0.001$). The degree of apoptosis at 24-h post reperfusion was significantly greater in Cre^+-$Crry^{flox/flox}$ mice compared with Cre^--$Crry^{flox/flox}$ mice ($P < 0.001$; Figure 6b), indicating that Cre^+ mice had greater sensitivity to IR-induced tissue damage.

IR-induced renal injury in PEPCK-Cre^+-$Crry^{flox/flox}$ mice is ameliorated by blocking alternative pathway or terminal pathway of complement activation

To confirm that exacerbated renal IR injury in PTEC-specific Crry knockout mice was caused by increased complement activation, we blocked the alternative pathway or terminal pathway of complement activation with an anti-properdin or anti-C5 mAb, respectively. Figure 7a shows that pretreatment with either anti-properdin or anti-C5 mAb significantly reduced BUN levels in Cre^+-$Crry^{flox/flox}$ mice ($P < 0.001$ for both groups, compared with control mAb). The reduction in BUN in the anti-complement mAb–treated mice correlated with significant improvement in renal pathology. In control mAb–treated Cre^+-$Crry^{flox/flox}$ mice, both the cortex and medulla architecture was distorted and the tubules were filled with hyaline casts and sloughed cells/necrotic debris with inflammatory cells detected prominently in the tubular interstitium (Figure 7b and Supplementary Figure S3 online). On the other hand, mice pretreated with either anti-C5 mAb or anti-properdin mAb had little necrosis or cast formation in the cortex and only focal necrotic and cast areas in the medulla (Figure 7b and Supplementary Figure S3 online). Semiquantitative scoring of tubular damage confirmed the protective effect of anti-C5 and anti-properdin mAbs (Figure 7c). As expected, anti-properdin but not anti-C5 mAb significantly reduced the intensity of tubular C3 staining (Figure 8a and b). Furthermore, compared with control mAb–treated mice, mononuclear phagocyte infiltration in the kidney at 24 h was significantly reduced in both anti-C5 and anti-properdin mAb–pretreated mice (control mAb: 53.60 ± 1.965; anti-C5: 22.00 ± 2.168; anti-properdin: 19.60 ± 2.249; $P < 0.001$ vs. control mAb; Figure 8c and d).

DISCUSSION

In this study, we have used a conditional gene-targeting approach to evaluate the physiological role of Crry on PTECs of the kidney. Crry is a key membrane complement regulator in rodent species, and its study bears relevance to understanding the role of human MCP in health and disease. It is the only membrane-bound C3 regulator expressed on PTECs within the mouse kidney.[3,11,27] Previous studies have shown that Crry is localized to the basolateral aspect of PTECs and loss of polarity in Crry expression on these cells preceded AP complement activation in a renal IR injury model.[27] Inhibition of Crry in cultured primary murine PTECs also rendered these cells susceptible to AP complement attack upon exposure to mouse serum.[22] Similar sensitivity to AP complement activation was observed when PTECs from Crry and factor B double-knockout mice were treated with normal mouse serum.[22] In other studies, antibody-mediated neutralization of Crry in rat kidneys allowed C3 activation in the tubules and perivascular capillaries and transient glomerular C3 staining.[32] $Crry^{-/-}/C3^{-/-}$ mouse kidneys developed remarkable inflammatory cell infiltration, tubular damage, and interstitial fibrosis when transplanted into syngeneic WT mice, a phenomenon that was not observed in transplanted wild-type kidneys.[20]

Despite these earlier lines of evidence of Crry function in the kidney, a direct evaluation of the *in vivo* role of Crry on tubular epithelial cells has not been possible until now owing to the embryonic lethality phenotype of global

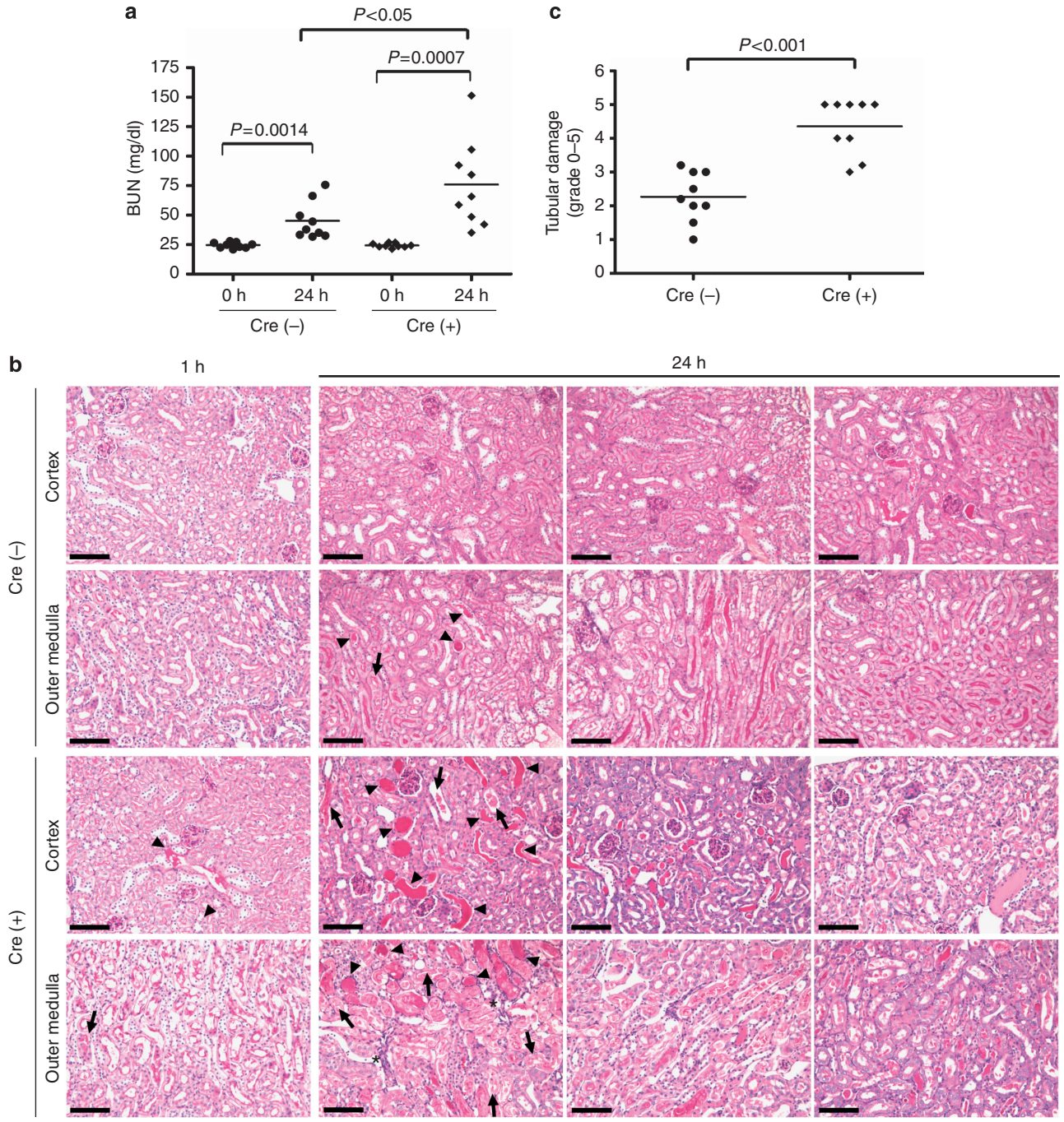

Figure 4 | Crry (complement receptor 1-related protein/gene y) deletion from PTECs renders mice more susceptible to renal IRI. (**a**) Although blood urea nitrogen (BUN) was higher in both Cre^- and Cre^+ mice 24-h post IR challenge compared with 0 h, BUN in Cre^+ mice at 24 h was significantly higher than that in Cre^- mice ($P<0.05$, $n=9$ mice per group). (**b**) Representative histology of kidneys harvested at 1 and 24 h after renal IR challenge. Periodic acid–Schiff (PAS) staining of Cre^+ mice showed more severe tubular injury than Cre^- mice both in the cortex and the outer medulla. Arrows and arrowheads indicate tubules with necrotic debris and cast formation, respectively, and inflammatory infiltrate is denoted with asterisks. Images from one mouse at 1 h and three mice at 24 h per genotype are shown to illustrate the range of injury severity. Original magnification, ×200. Scale bars = 100 μm. (**c**) Semiquantitative analysis confirmed that tubular injury in Cre^+ mice was significantly more severe than in Cre^- mice ($P<0.001$, $n=9$ mice per group). Mice were ~3 months of age with body weight of 25–30 g. IRI, ischemia–reperfusion injury; PTECs, proximal tubular epithelial cells.

Crry deletion.[19] In two recent studies, live $Crry^{-/-}$ mice were obtained by inhibiting the complement system during gestation.[30,31] However, such mice were observed to have systemic complement activation and depletion, thus also preventing analysis of Crry function *in vivo* in the context of normal complement activity.[30,31] In the present study, we circumvented these limitations by using the Cre/loxP system to selectively inactivate the *Crry* gene in renal PTECs.

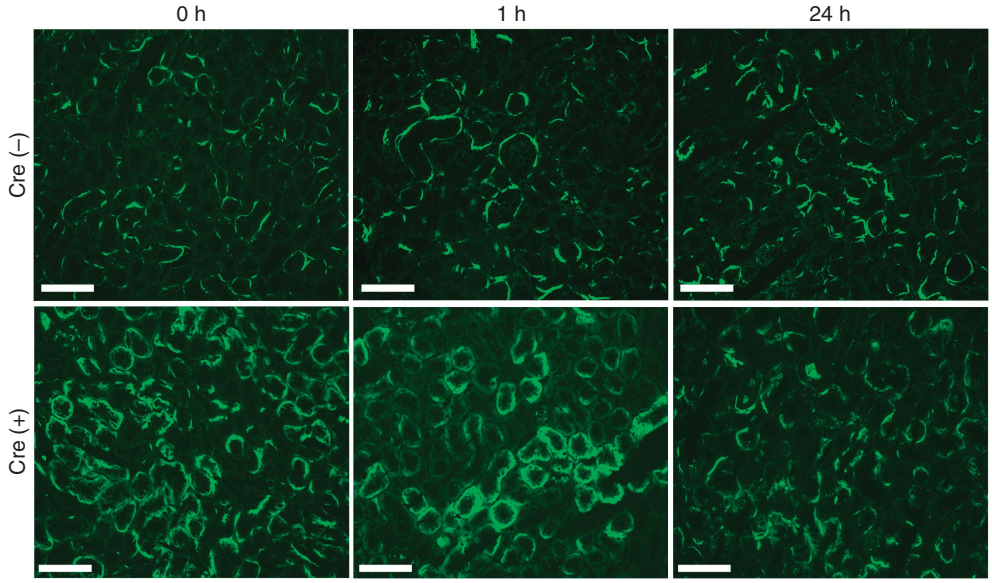

Figure 5 | Tubular C3 deposition in Cre$^-$ and Cre$^+$ mice after renal IRI challenge. Tubular C3 deposition was more prominent in Cre$^+$ mice than in Cre$^-$ mice at all time points examined. However, there was no significant difference between 0 and 1 h or 24 h after IR in Cre$^-$ mice. There was also no difference between 0 and 1 h in Cre$^+$ mice, but tubular C3 deposition at 24 h after IR was significantly reduced in Cre$^+$ mice compared with that at 0 or 1 h. Original magnification, ×200. Scale bars = 100 μm. IRI, ischemia–reperfusion injury.

We confirmed *Crry* gene deletion in PTECs and established that Cre$^+$ mutant mice had normal plasma C3, indicating that no systemic complement depletion and secondary complement insufficiency has occurred. The latter finding was consistent with data from two other tissue-specific *Crry* gene knockout studies. Using the same Crry$^{flox/flox}$ mouse strain crossed to Cre-transgenic mice, we found in earlier experiments that Crry deletion from mouse T cells or platelets did not affect systemic complement levels and activity.[33,34]

Using immunofluorescence staining, we detected significant C3 deposition on PTECs of naive Cre$^+$ mice. This suggested that lack of Crry on these cells rendered them susceptible to spontaneous complement attack. Although the degree of complement activation on PTECs did not significantly affect plasma levels of intact C3, we did observe increased C3b fragments in the plasma, thus providing an independent marker of spontaneous complement activation in the mutant mice. Previous studies of Crry$^{-/-}$ mice similarly showed the presence of activated C3b fragments in the circulation,[30,31] presumably reflecting an excess of activated C3 fragments from uncontrolled surface convertase activity that did not immediately link covalently to cell surface hydroxyl or amine groups. Surprisingly, despite clear signs of complement activation on PTECs, we did not detect any appreciable renal injury in either young or aged Cre$^+$-Crry$^{flox/flox}$ mice by functional (BUN) or histopathological evaluation. However, when Cre$^-$ and Cre$^+$ mice were subjected to IR challenge, we found that Cre$^+$ mice incurred significantly more renal injury than Cre$^-$ mice. We noted considerable spread in the BUN levels in challenged Cre$^+$-Crry$^{flox/flox}$ mice, which may reflect variations in Crry deletion efficiency in different animals. Of interest, increased sensitivity to renal IRI in Cre$^+$ mice was not correlated with more intense tubular C3 staining at 1 or 24 h after reperfusion. In fact, tubular C3 staining at 24 h after reperfusion in the kidneys of Cre$^+$ mice was significantly reduced when compared with that in naive mice without IR challenge, potentially a consequence of cellular apoptosis and necrosis observed by this time point, which might have limited local complement availability. A similar finding of reduced tubular C3 staining at 24 h after reperfusion was observed previously in the DAF$^{-/-}$CD59$^{-/-}$ mouse model of renal IRI.[35] Thus, depending on the disease model and time course, C3 deposition on tissues may not always be a meaningful marker of complement injury.

Although not directly correlated with tubular C3 deposition, exacerbated renal IRI in Cre$^+$-Crry$^{flox/flox}$ mice was clearly mediated by complement activation, as anti-properdin or anti-C5 mAb ameliorated renal injury. IR challenge may augment complement activation, particularly the terminal pathway, on tubular cells by impairing the activity of factor H (fH). In addition to Crry, previous studies have suggested that the fluid-phase complement regulator fH also has a role in protecting tubular cells of the kidney.[22,36] By interacting with surface-deposited C3b and host cell-specific glycosaminoglycans, fH can act as a potent inhibitor of AP complement activation on host cells. Tubular epithelial cells of the kidney are known to express glycosaminoglycans,[37] which together with spontaneously deposited C3b may attract fH binding to PTECs in Cre$^+$-Crry$^{flox/flox}$ mice. Prevention by fH of excessive AP and terminal pathway

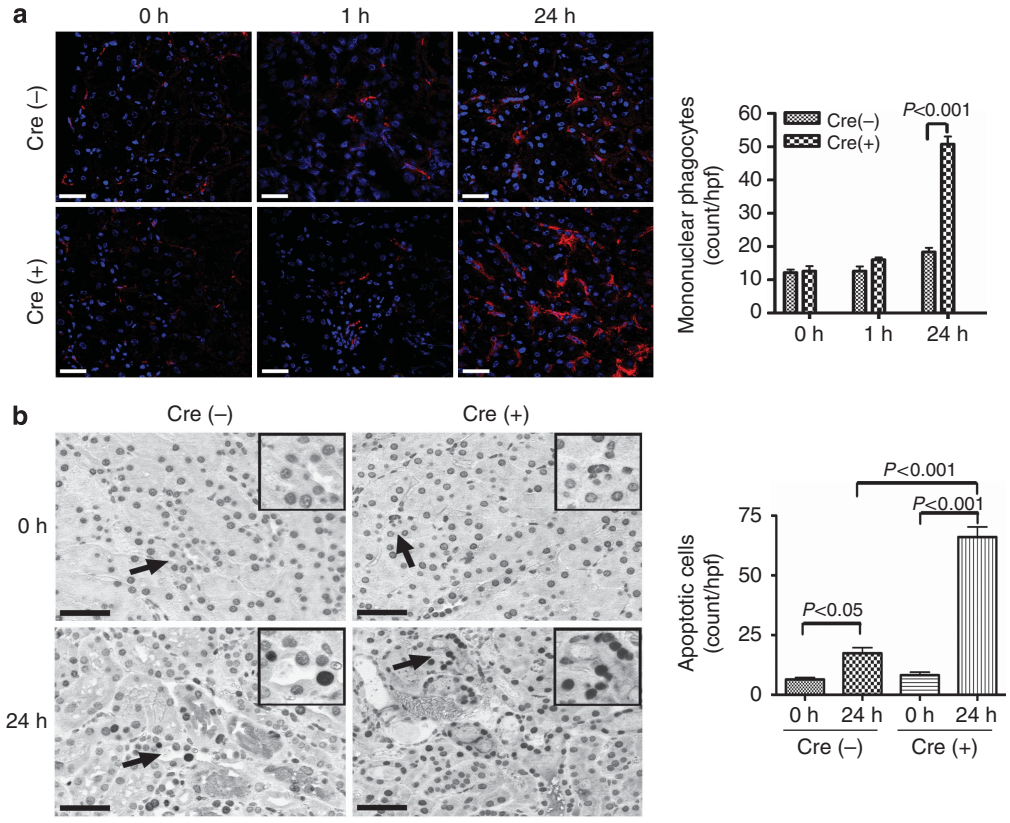

Figure 6 | Deficiency of Crry (complement receptor 1–related protein/gene y) from PTECs exacerbates IR-induced inflammation and tubular apoptosis. (**a**) Representative tissue sections from kidneys harvested before (0 h) and 1 or 24 h after IR were stained for mononuclear phagocyte infiltration with an F4/80 antibody (red). Few F4/80 + cells were observed per high-power field (hpf) in the Cre^- mouse kidneys and they showed only modest increase after IR. Although there was no significant difference between 0 and 1 h in Cre^+ mice, the number of mononuclear phagocytes in the medulla of Cre^+ mice had markedly increased by 24-h post IR ($P<0.001$, $n=5$ mice per group). Scale bars = 20 μm. (**b**) Apoptotic cells in the outer medulla of the kidney were identified using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and counted. Representative tissue sections from Cre^- and Cre^+ mice before IR (0 h) and 24 h after IR challenge are shown. Original magnification ×400. Insets show ×2 higher magnification of the areas indicated by arrows. All scale bars = 25 μm. The number of TUNEL-positive cells had significantly increased by 24 h in both in Cre^- and Cre^+ mice compared with 0 h ($P<0.05$ and $P<0.001$, respectively); however, there were still significantly more apoptotic cells in the Cre^+ mouse kidney than in the Cre^- mouse kidney ($P<0.001$, $n=5$ mice per group). IR, ischemia–reperfusion; PTECs, proximal tubular epithelial cells.

complement activation on PTECs would explain the lack of F4/80 + mononuclear phagocyte infiltration and associated renal injury in naive Cre^+ mice despite an appreciable degree of AP complement activation on Crry-deficient PTECs. During IR challenge, tubular cell membrane remodeling may lead to the loss or reduction of glycosaminoglycans and other fH ligands, and this may decrease fH binding and protection, resulting in unchecked complement-dependent renal injury.

The finding that anti-properdin and anti-C5 mAbs are effective in ameliorating renal IRI in Crry mutant mice is consistent with our previous data on IR-challenged DAF/CD59 knockout mice.[38] It, however, contrasted with the finding from recent C3 glomerulopathy models in which properdin deficiency was shown to exacerbate kidney injury.[39,40] This difference may relate to differential requirement of properdin in AP complement activation on certain host cells (for example, endothelial and renal tubular cells) vs. other cellular structures (for example, the GBM) or in the fluid phase.[39,41] Thus, although anti-properdin therapy may be ineffective or counterproductive in disorders involving fluid-phase complement activation, it is expected to benefit other cell surface–driven AP complement–mediated pathologies. Our data here showed that one such setting where prophylactic anti-properdin or anti-C5 therapy can be considered could be transplantation-related IRI.

In summary, this study has provided direct evidence for a role of Crry in controlling spontaneous AP complement activation on renal tubular epithelial cells. It also demonstrates that a defect in AP complement control on target cells may not always produce a spontaneous pathological phenotype but may instead pose an increased risk for injury in settings where complement regulation may be further compromised. This relates to the partial penetrance of complement gene defects observed in human patients. Not all individuals carrying fH or MCP mutations develop complement-mediated disorders, and those who do often

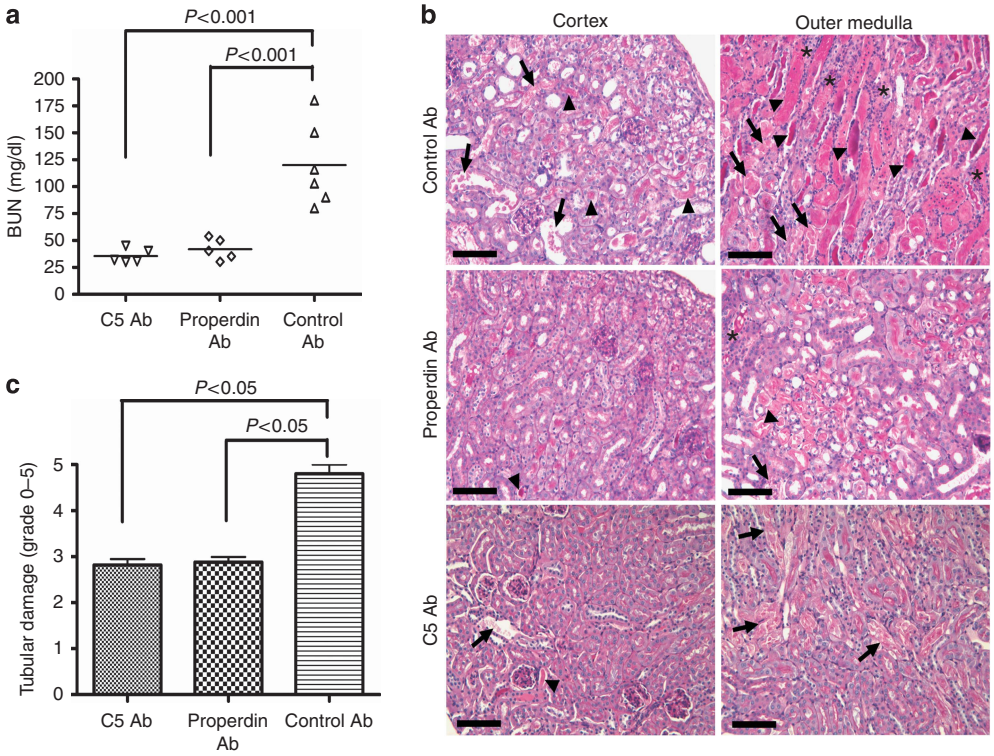

Figure 7 | Complement inhibition ameliorates renal IRI in PTEC-specific Crry (complement receptor 1-related protein/gene y) knockout mice. (**a**) Compared with control monoclonal antibody (mAb) (Control Ab), pretreatment of Cre$^+$-Crry$^{flox/flox}$ mice (2 h before IR) with anti-properdin mAb (Properdin Ab) or anti-C5 mAb (C5 Ab) significantly reduced blood urea nitrogen (BUN) levels 24-h post IR injury ($P<0.001$, $n=5$–6 mice per group). (**b**) Representative periodic acid–Schiff (PAS)-stained kidney sections from mAb pretreated mice. In mice pretreated with the control mAb, the cortex and medulla architecture was greatly distorted and tubules were filled with hyaline casts (arrowhead) and sloughed cells/necrotic debris (arrow), and inflammatory infiltrate was prominent within the tubular interstitium (asterisk). In mice pretreated with either anti-C5 mAb or anti-properdin mAb, there was little necrosis or cast formation in the cortex, and only focal necrotic areas and casts were visible in the medulla. Original magnification ×200. Scale bars = 100 μm. (**c**) Semiquantitative scoring of tubular injury showed significant ($P<0.05$, $n=5$–6 mice per group) protection from renal IRI by C5 mAb or properdin mAb pretreatment compared with pretreatment with the control mAb. IRI, ischemia-reperfusion injury; PTEC, proximal tubular epithelial cell.

have their disease onset or flare-ups triggered by potentially complement-activating events such as infection, surgery, or trauma.[42,43] In addition, our results showing that renal IRI in Cre$^+$-Crry$^{flox/flox}$ mice was correlated with F4/80+ mononuclear phagocyte infiltration and PTEC apoptosis provide mechanistic insight on the pathogenesis of this condition. Finally, the finding that renal IRI was ameliorated by anti-properdin and anti-C5 mAbs suggests that these treatments may benefit human patients in settings of complement-mediated IRI.

MATERIALS AND METHODS

Generation of PTEC-specific Crry knockout mice

To generate PTEC-specific Crry knockout (KO) mice, Crry$^{flox/+}$ mice on a mixed 129/C57BL6 background[33] were crossed with PEPCK-Cre$^+$ transgenic mice (kindly provided by Dr Volker Haase, Vanderbilt University). PEPCK-Cre$^+$ transgenic mice were generated by using a modified portion of the PEPCK gene promoter to selectively drive Cre expression in PTECs, although Cre expression was also noted in the medulla and a subset of hepatocytes.[25] The resulting PEPCK-Cre$^+$-Crry$^{flox/+}$ mice were then intercrossed to obtain PEPCK-Cre$^+$-Crry$^{flox/flox}$ and Crry$^{flox/flox}$ mice as breeders to produce experimental mice. In all experiments, Cre-negative littermates were used as controls for PEPCK-Cre$^+$-Crry$^{flox/flox}$ mice. Mouse genotyping was performed using PCR of tail and/or kidney DNAs. For the WT, floxed, and deleted *Crry* allele, fragments of 970, 1100, and 350 bp, respectively, were expected with the use of the following pair of primers: TX-1 (5′-CAGAGTAATCTACAGTT TCACC-3′) and E5S2 (5′-GTTCACTGTATTCCCTCATCCAGA-3′). PEPCK-Cre transgenic mice were identified using a pair of Cre-specific primers, 5′-ATTCTCCCACCGTCAGTACG-3′ and 5′-CGTTTTCTGAGCATACCTGGA-3′. Experiments were conducted by following the established guidelines for animal care, and all protocols were approved by the appropriate institutional committees.

Generation and purification of rabbit anti-mouse Crry SCR3/4 antibody

The targeting strategy for *Crry* was to delete exon 5, which encodes SCRs 3 and 4 critical for Crry function.[19,44,45] As a small amount of mutant, nonfunctional Crry protein was still made in the *Crry* gene targeted mice,[33] we prepared a SCR3/4-specific rabbit polyclonal antibody to distinguish WT and mutant Crry proteins. We expressed

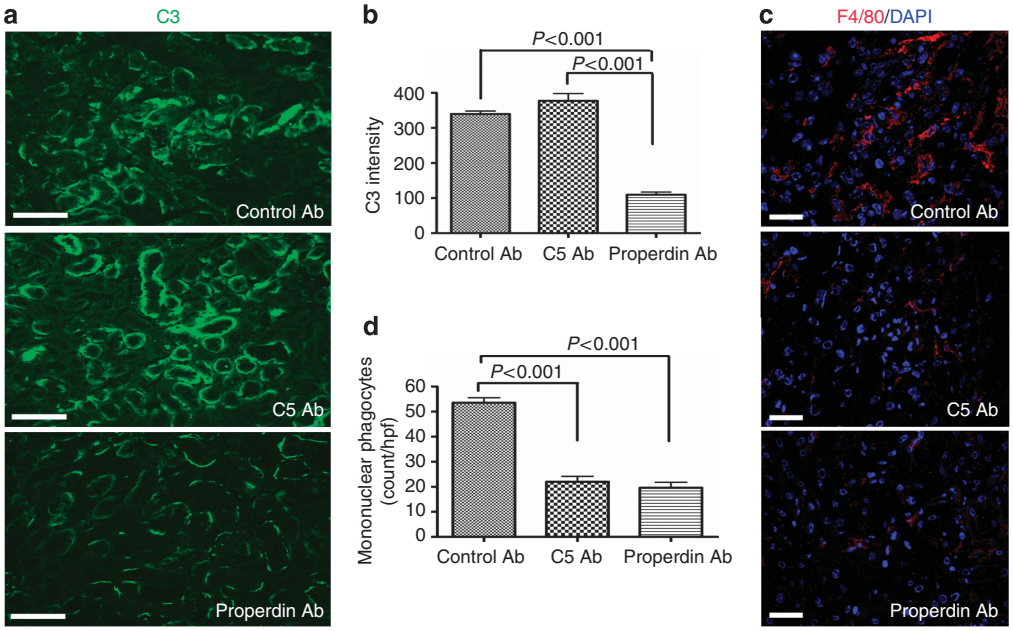

Figure 8 | Effects of anti-properdin and anti-C5 monoclonal antibodies (mAbs) on tubular C3 deposition and mononuclear phagocyte infiltration in response to ischemia–reperfusion (IR) challenge in Crry (complement receptor 1–related protein/gene y) mutant mice. (**a**) Compared with control mAb treatment, pretreatment of Cre^{+}-$Crry^{flox/flox}$ mice with anti-properdin mAb significantly reduced tubular C3 staining at 24 h after IR challenge, whereas anti-C5 mAb-treated kidneys were unaffected. Original magnification, ×200. Scale bars = 100 μm. (**b**) Quantitative image analysis of C3 deposition confirmed tubular C3 staining intensity to be significantly reduced in anti-properdin mAb–treated mice compared with control or anti-C5 mAb treatment groups ($P < 0.001$, $n = 3$ mice per group). (**c**) Immunofluorescence staining showed that mononuclear phagocyte infiltration in the kidney of control mAb–treated Cre^{+}-$Crry^{flox/flox}$ mice at 24 h after IR was largely prevented by anti-properdin mAb or anti-C5 mAb (red = F4/80, blue = 4′,6-diamidino-2-phenylindole (DAPI)). (**d**) Quantification of mononuclear phagocyte infiltration. Compared with control mAb–treated mice, the number of mononuclear phagocytes per high-power field (hpf) in the kidneys of anti-C5 or anti-properdin mAb-treated mice was significantly ($P < 0.001$, $n = 5$ mice per group) reduced. Original magnification ×400. Scale bars = 20 μm.

and purified a 6xHis-tagged mouse Crry SCR3/4 fragment using the pCAGGS vector and HEK293 cells, as described previously.[34] This recombinant Crry protein was then used as an antigen to produce a polyclonal antibody in rabbits by Cocalico Biologicals (Reamstown, PA, USA). Total IgGs were purified from rabbit serum by Protein A affinity column, and Crry SCR3/4-specific IgGs were purified on a second affinity column constructed by conjugating recombinant Crry SCR3/4 protein onto CNBr-Sepharose beads (Amersham, Arlington Heights, IL). The specificity of this antibody was confirmed using western blot and immunostaining experiments.

C3 ELISA assays

ELISA assays to measure intact and activated C3 levels in the mouse plasma were performed as described previously.[39]

Functional evaluation of renal injury

Urinary albumin was quantified using a mouse albumin ELISA kit according to the manufacturer's instructions (Bethyl Laboratories, Montgomery, TX). Urine samples were collected in metabolic cages for 16 h, and volumes were recorded. Total urinary albumin was determined by multiplying albumin concentration (determined by ELISA) by total urine volume of 16 h. BUN levels were measured using urea nitrogen reagents (Sigma-Aldrich, St Louis, MO), as described previously.[35]

Induction of renal ischemia–reperfusion injury

Renal ischemia–reperfusion was performed as described previously.[35] In brief, both renal pedicles were clamped for 25 min using micro-aneurysm clamps, followed by reperfusion for 1 or 24 h. Mice were killed at the end of the reperfusion experiment and kidneys were harvested for histological analysis. Blood samples were collected before and after IR surgery.

Renal pathology grading

Kidneys were fixed in 10% formalin-phosphate-buffered saline before processing and paraffin embedding. Samples were cut in 4-μm sections and stained with PAS reagent. As described previously,[35] tubular injury was scored by estimating the percentage of tubules in the cortex and in the outer medulla that showed epithelial necrosis or had necrotic debris or cast as follows: 0, none; 1+, <10%; 2+, 10–25%; 3+, 26–45%; 4+, 46–75%; 5+, >75%. Ten viewing fields, randomly selected from the cortex and outer medulla on each slide section, were examined at ×200 magnification.

Immunofluorescence for C3 and F4/80

Cryostat sections (4 μm) of kidneys snap frozen in optimal cutting temperature compound (OCT) were fixed with ice-cold acetone for 10 min and blocked with 10% goat serum to decrease background staining. Fluorescein isothiocyanate-conjugated goat anti-mouse C3 Ab (4.0 mg/ml, MP Biomedicals, Santa Ana, CA) was used directly at a 1:500 dilution. Under ×200 magnification, 10 viewing fields

from sections of each animal were photographed. Areas of positive staining were highlighted and the fluorescence intensity of C3 was determined using the plugin 'Measure particles' of the ImageJ software (NIH, Bethesda, MD). F4/80-positive mononuclear phagocytes were visualized by staining with a rat anti-mouse F4/80 Ab (1.0 mg/ml, AbD seroTEC, Raleigh, NC) used at a 1:50 dilution followed by Alexa Fluor 555-goat anti-rat IgG (2.0 mg/ml, Invitrogen, Grand Island, NY) used at a 1:2000 dilution. Under ×400 magnification, F4/80+ cells were counted by examining 10 viewing fields randomly selected from the outer medulla on each slide.

TUNEL assay for apoptosis

Deparaffinized and rehydrated tissue sections were prepared according to the standard protocols. Slides were incubated with proteinase K for 20 min at room temperature. TUNEL labeling was carried out using an *in situ* cell death detection kit (Roche, Indianapolis, IN) according to the manufacturer's instructions, and color was developed using the diaminobenzidine substrate kit (Vector, Burlingame, CA, USA). The number of apoptotic cells in the outer medulla was counted from 10 different fields at ×400 magnification for each sample and were averaged.

Administration of anti-properdin and anti-C5 mAbs

Mice were pretreated with neutralizing monoclonal anti-C5 antibody BB5.1, anti-properdin antibody 14E1,[38] or isotype control mouse IgG1, MOPC-31C (all at a dose of 1 mg/mouse) by intraperitoneal injection 2 h before the induction of ischemia. Selection of mAb dosages was based on previously published studies.[38,46]

Statistical analysis

Statistical comparisons were performed using the GraphPad Prism 4.0 software (La Jolla, CA). All data are reported as the mean ± s.e.m. The difference between two groups was calculated using unpaired *t*-test for normally distributed data. For data with nonparametric distributions, Mann–Whitney test was applied for two groups. For multiple group comparisons, one-way analysis of variance with a Tukey's test was used. A *P*-value of less than 0.05 was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Additional images of Cre-mediated Crry deletion in PTECs.
Figure S2. Histology of kidneys from IR-challenged Cre^+ and Cre^- mice.
Figure S3. Kidney histology of mAb-treated Cre^+ mice at 24 h after IR challenge.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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