Unrestricted C3 Activation Occurs in Crry-Deficient Kidneys and Rapidly Leads to Chronic Renal Failure

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Deficiency of the C3 convertase regulator Crry is embryonic lethal in mice unless C3 also is absent. For evaluation of the effect of local kidney Crry deficiency in the setting of an intact complement system, Crry<sup>−/−</sup> C3<sup>−/−</sup> mouse kidneys were transplanted into syngeneic C57BL/6 wild-type mice. These Crry-deficient kidneys developed marked inflammatory cell infiltration, tubular damage, and interstitial fibrosis, whereas similar changes were absent in control transplanted kidneys. Strong C3 deposition in the vessels and tubules that correlated significantly with measures of disease supported that complement activation was pathogenic in this model. Microarray studies showed upregulation of a number of chemokine and extracellular matrix genes, which were validated for CCL2 and CXCL10 mRNA and collagen III protein. The functional significance of these pathophysiologic findings was evaluated by removing both native kidneys, so the transplanted kidney alone provided renal function. Within 21 d of transplantation, seven of eight Crry-deficient kidneys in complement-sufficient wild-type hosts failed, compared with two of 13 controls (P = 0.001), with final blood urea nitrogen levels of 133.9 ± 33.0 and 55.6 ± 8.3 mg/dl, respectively (P = 0.015). These data show that mouse Crry is a critical complement regulator in the kidney. When absent, unrestricted complement activation occurs and quickly leads to marked inflammation and progressive renal failure, with features relevant to human diseases with underlying defects in complement regulation, such as hemolytic uremic syndrome.


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ISSN: 1046-6673/1803-0811

Received October 30, 2006. Accepted December 6, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

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In humans, complement activation by antibodies that are directed against foreign antigens (e.g., streptococcal exotoxin B [5]) or self-molecules (e.g., native DNA) has the clear potential to be pathogenic in organs such as the kidney. Even in diseases that traditionally are not considered immune in nature, complement activation may underlie disease pathogenesis. For instance, reperfusion injury that occurs in myocardial infarction can be reduced by inhibiting activation of C1 (6), C3 (7), or C5 (8). There also are a growing number of disease states in which complement activation occurs because of inherited or acquired abnormalities in one or more of the complement regulators; these include paroxysmal nocturnal hemoglobinuria (9), age-related macular degeneration (10), membranoproliferative glomerulonephritis (11,12), atypical hemolytic uremic syndrome (aHUS) (13), and acute tubular necrosis (14). The last three have predominant effects in the kidney, which can be attributed to certain distinct features of this organ, such as its high plasma flow, unique vasculature, and the daily production of approximately 170 L of largely protein-free glomerular ultrafiltrate.

The functional relevance of Crry is evident in Crry gene-targeted mice in which the Crry deficiency is embryonic lethal (15). This can be attributed to unrestricted complement activation and concommitant placental inflammation, because it can be averted by coexisting C3 deficiency in Crry<sup>−/−</sup> C3<sup>−/−</sup> double knockout (DKO) mice. Because of the resultant C3 deficiency in these DKO mice, the role for Crry to protect an organ such as the kidney from C3 activation has not been determined. To address this, we transplanted DKO mouse kidneys into syngene-
Mice

Crry<sup>+/−</sup> and C<sup>3</sup><sup>+/−</sup> mice, provided by Dr. Hector Molina (Washington University School of Medicine, St. Louis, MO) (15,16) were crossed with WT C57BL/6 mice at least eight generations. C<sup>3</sup><sup>+/−</sup> mice were intercrossed to generate C<sup>3</sup><sup>−/−</sup> mice and with Crry<sup>+/−</sup> mice to produce C<sup>3</sup><sup>+/−</sup>-Crry<sup>−/−</sup> mice, which then were intercrossed to generate Crry<sup>−/−</sup>-C<sup>3</sup><sup>−/−</sup> mice. Genotyping for Crry and C3 was performed using PCR-based approaches (15,16) on purified tail DNA (DNasey Tissue kit; Qiagen, Valencia, CA). Absence of Crry and C3 proteins in respective KO mice also was confirmed by Western blotting of lysed erythrocyte proteins and sera, respectively.

The genomic markers that were polymorphic between 129X1/SvJ and C57BL/6J strains and used to genotype KO mice were D1Mit159.1, D1Mit111.1, D1Mit206.1, D17Mit10.1, and D17Mit180.1 (PRISM Mouse Mapping Set; Applied Biosystems, Foster City, CA). The first three in mouse chromosome 1 are 25.0, 14.3, and 10.8 cM centromeric from Crry; the last two in mouse chromosome 17 are 9.8 and 4.9 cM centromeric from C3 and separate C3 from the mouse MHC complex. Labeled PCR-amplified products were analyzed on 3730XL Gene Analyzer using Gene Mapper software (Applied Biosystems). DNA samples from WT 129 and C57BL/6 mice were used in each reaction to confirm the expected sizes of the strain-specific PCR products.

Mouse Experimental Protocols

All experimental procedures performed here were approved by the University of Chicago Animal Care and Use Committee. Male mice between 10 to 12 wk of age were used either as kidney donor or recipient. Donor mice were anesthetized, and the donor left kidney was removed with artery, vein, and ureter attached and preserved in cold saline on ice. The right kidney from the donor was removed as an additional control for these experiments. The recipient then was anesthetized, and the left kidney was excised. Renal transplantation was performed with end-to-side anastomoses of the donor renal vein, artery, and ureter to the recipient inferior vena cava, aorta, and bladder, respectively (17). Total cold ischemic time of the donor kidney ranged between 45 and 60 min.

In the first set of studies, DKO kidneys were transplanted into WT recipients (n = 14). Controls included WT (n = 5) and C<sup>3</sup><sup>−/−</sup> kidneys (n = 5) transplanted into WT hosts and DKO kidneys transplanted into DKO hosts (n = 4). In these experiments, one recipient kidney was left in place to provide renal function, thereby ensuring that the host would survive for detailed assessment of the transplanted kidney. Groups were divided equally (±1) for study 1, 4, and 7 d after transplantation.

In subsequent experiments, WT hosts with DKO kidneys (n = 8) and control transplants (n = 13) underwent nephrectomy of the right native kidney 7 d after transplantation. These mice relied solely on the transplanted kidney to provide renal function. Blood urea nitrogen (BUN) concentrations were determined with a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA). Urinary albumin concentrations were measured with a mouse albumin ELISA kit (Bethyl Laboratories, Montgomery, TX) and normalized to urinary creatinine measured colorimetrically (Stanbio Laboratory, Boerne, TX) as described previously (18).

Skin transplantation experiments were performed by grafting full-thickness tail skin (0.5 × 0.5 cm) onto the flank of recipients, which was secured with bondage. Skin grafts were assessed daily up to 9 d. The following groups of C57BL/6 mice were studied: WT to WT (n = 3), C<sup>3</sup><sup>−/−</sup> to WT (n = 2), WT to C<sup>3</sup><sup>−/−</sup> (n = 1), DKO to WT (n = 2), and DKO to DKO (n = 2). As a control for rejection of allogeneic skin, BALB/c mouse skin also was grafted onto WT C57BL/6 mice.

Measurements from Tissue

Four-micrometer sections of formalin-fixed, paraffin-embedded kidney tissues were stained with periodic acid-Schiff and examined by a renal pathologist (M.H.) in a blinded manner. For each slide, the severity of TI nephritis was graded from 0 to 4 in 0.5 increments as described previously (19). Using this protocol, semiquantitative grading of proliferative glomerulonephritis, glomerulosclerosis, and arteritis also was done.

For immunofluorescence microscopy, 4-μm cryostat sections were fixed in ether-ethanol and stained with FITC–anti-mouse C3 (Cappel Pharmaceuticals, Aurora, OH) or with biotin-conjugated antibodies to human collagen III (reactive with mouse collagen III but not other mouse collagens; SouthernBiotech, Birmingham, AL), followed by FITC-streptavidin (ICN Biomedical, Aurora, OH). The staining intensity of C3 or collagen III in the TI was scored semiquantitatively from 0 to 3 by an observer who was blinded to the origin of the specimens as described previously by others (20). Macrophages were stained using FITC-conjugated rat F4/80 mAb (Serotec, Oxford, UK) and counted in at least 20 high-power fields (×400) of the cortical TI in a blinded manner as described previously (21).

For immunohistochemistry, 4% paraformaldehyde-fixed kidney sections were heated in a microwave oven for 10 min in citrate buffer (pH 6.0) to ensure optimal antigen accessibility. Sections were blocked with 0.3% H<sub>2</sub>O<sub>2</sub> and Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) followed by 10% normal mouse serum. The slides then were incubated with rat anti-mouse neutrophil (Serotec) or rat anti-mouse B220 mAb (eBioscience, San Diego, CA), followed by mouse anti-rat IgG (BD Pharmingen, San Diego, CA). Neutrophils and B cells were quantified in kidneys as for macrophages.

For detection of apoptotic cells in tissue sections, the TACS XL-Blue in situ apoptosis detection kit (Trevenig, Gaithersburg, MD) was used according to the manufacturer’s instruction and as described previously (21). Apoptotic cells, which stained intensively blue, were counted in a blinded manner from at least 20 glomeruli or 20 high-power fields (×400) of TI per specimen.

Microarray Experiments

DKO kidneys that were transplanted into WT mice (n = 4) and DKO mice (n = 3) were studied 4 d after transplantation. Renal cortical RNA was purified using Trizol reagent (Invitrogen, Carlsbad, CA) and processed and hybridized with Mouse Genome 430 2.0 Arrays (Affymetrix, Santa Clara, CA) as described previously (22). From previous studies with unmanipulated WT kidneys, the underlying transcriptional state of a normal kidney also was used for comparison purposes. Robust Multichip Analysis software (23) was used to adjust array background levels and normalize interarray expression levels. Mean expression values for each gene in experimental and control groups were calculated, and differences were detected using a two-tailed t test with
corrections for multiple comparisons. Gene ontology relationships and their significance were determined using the “Gene Ontology” function in GeneSpring software (v. 6.0; Agilent Technologies, Santa Clara, CA).

**Quantitative Reverse Transcriptase–PCR**
cDNA was produced from renal cortical RNA using a commercial kit (Superscript III; Invitrogen). Quantitative reverse transcriptase–PCR was performed with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) using an Applied Biosystems 7300 Real Time PCR System. Expression data were normalized to 18S RNA measured contemporaneously from the same samples. The primers used were as follows: CXCL10, left 5’-ggtgcctctctctcctc-3’ and right 5’-ataacccctggagcttgg-3’; CCL2, left 5’-cccaatgagtaggctggaga-3’ and right 5’-gctgaagaccttagggcaga-3’; and 18S, left 5’-atggccgttcttagttggtg-3’ and right 5’-cgtgagctcagctgtag-3’.

**Statistical Analyses**
All data are expressed as means ± SEM and were analyzed using Minitab software (State College, PA). For comparisons between two groups when measured at a single time, two-sample t and Kruskal-Wallis tests were used for parametric and nonparametric data, respectively. For comparisons between two groups when measurements were obtained over time, ANOVA followed by Tukey pair-wise comparisons was used. χ² testing was used to analyze the incidences between two groups. Potential correlations among variables were examined by Pearson product moment correlation coefficient.

**Results**

**Acute C3 Activation in Crry-Deficient Kidneys Leads to Progressive TI Inflammation**
Pathologic effects that occurred acutely in Crry-deficient kidneys in complement-sufficient hosts were evaluated by transplantation of kidneys from Crry<sup>−/−</sup> C3<sup>−/−</sup> DKO mice into WT hosts (n = 14). In these sets of experiments, one of the recipient kidneys was removed at the time of transplantation and the second recipient kidney was left in place to ensure that all of the mice would survive this study. Controls included transplantation of WT (n = 5) or C3<sup>−/−</sup> kidneys (n = 5) into WT hosts and DKO kidneys into DKO hosts (n = 4). In all experiments, DKO and C3<sup>−/−</sup> mice were backcrossed at least eight generations onto C57BL/6. Microsatellites that were polymorphic between C57BL/6 and 129 strains were exclusively of C57BL/6 origin, including those that were 4.9 and 10.8 cM centromeric from C3 and Crry, respectively.

Strong C3 deposition was found in the vessels and tubules in the TI of Crry-deficient kidneys as early as day 1 and progressed during the next 6 d (Figure 1, D through F), indicative of spontaneous complement activation in the Crry-deficient TI. In the control groups, such TI C3 deposition was completely absent (Figure 1, A through C), even in the C3-deficient kidneys that were transplanted into WT controls (data not shown). Discontinuous linear C3 staining of the tubular basement membranes is normal in WT mice (18,24). Although such C3 staining along the tubular basement membranes was absent in all C3-deficient kidneys, it became apparent within 1 d after transplantation into WT hosts. This staining clearly was distinguishable from the C3 staining that extended to the basolateral aspects of tubular cells observed here (e.g., compare Figure 1, C and F). C3 staining scores in DKO kidneys in WT hosts were 1.0 ± 0.2 on day 1, 1.4 ± 0.2 on day 4, and 1.8 ± 0.2 on day 7 and absent in all control kidneys at all time points (P < 0.01). Notably, there was no C3 deposition in glomeruli in any of the transplanted kidneys.

At the time of transplantation, the contralateral kidneys from WT and DKO donors histologically were normal (Figure 2, A and E). Mild inflammation occurred in control transplanted kidneys was marked C3 staining that extends into the basolateral portions of the tubular cells. Magnification, ×200.
kidneys and tended to resolve over time (Figure 2, B through D) and likely is a consequence of the short period of cold ischemia (<1 h) and subsequent reperfusion. In contrast, Crry-deficient kidneys in WT hosts had marked progressive TI inflammation (Figure 2, F through H). At this time, the scores that reflected the severity of TI nephritis were 1.7 ± 0.4 compared with 0.5 ± 0.3 in the control groups (P < 0.05). In all of the transplanted kidneys irrespective of donor or recipient Crry and C3 status, there were little to no acute or chronic pathologic changes in glomeruli.

The nature of the cellular inflammation in Crry-deficient kidneys was determined next. There were few neutrophils and B cells in control kidneys up to 7 d after transplantation (Figure 3, A and D). In contrast, there was marked infiltration of neutrophils and B cells in Crry-deficient kidneys in WT hosts (Figure 3, B and E), which was cumulative over time (Figure 3, C and F). Similar findings were observed for macrophages (Figure 3G). Consistent with the histologic data, there were very few infiltrating cells in the glomeruli from all groups (Figure 3, A and D, showing glomeruli that contained one neutrophil and no B cells, respectively).

Apoptosis is another relevant feature of organ damage, which can occur as a direct result of complement activation and/or be initiated by infiltrating inflammatory cells (24–30). In control transplanted kidneys, there were few apoptotic cells at all times studied (Figure 4A), whereas in Crry-deficient kidneys in WT recipients, apoptosis above controls was evident by day 4 after transplantation, which increased further by day 7 (Figure 4, B and C). Consistent with the previous histologic data, the majority of apoptotic cells were in the interstitium/vasculature (Figure 4B, arrowheads) or tubules (Figure 4B, arrows), whereas little apoptosis was evident in glomeruli from any of the groups (Figure 4A).

**Increased Expression of Chemokines and Extracellular Matrix Proteins in Crry-Deficient Kidneys**

For these experiments, we focused on transcriptional events that were brought about by acute complement activation in Crry-deficient kidneys. To limit any confounding effects of reperfusion as well as to generate comparisons among alike kidneys, we performed initial microarray experiments 4 d after transplantation of DKO kidneys into WT (n = 4) and DKO (n = 3) hosts. Given that deficiency of Crry resulted in spontaneous complement activation, followed by inflammatory cell recruitment and TI fibrosis, here we focused on chemokine and extracellular matrix genes that had a statistically significant increase of at least two-fold. By these criteria, 11 chemokine ligand (Table 1) and three collagen genes (Table 2) were upregulated in Crry-deficient kidneys. These genes were expressed at low to undetectable levels in normal kidneys, which also was true of the control transplanted kidneys. Hence, one result of acute complement activation was the transcriptional activation of CCL/CXCL and interstitial collagen (I/III) genes by intrinsic and/or infiltrating cells in the TI.

To expand on these transcriptional data from kidneys that were obtained 4 d after transplantation, we performed quantitative reverse transcriptase–PCR using kidneys from all groups and time points for representative genes from each group. CCL2 and CXCL10 were examined as highly expressed CCL and CXCL genes in these studies, as well as in human and experimental HUS (31,32). Both were upregulated from days 1

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**Figure 2.** Acute tubulointerstitial nephritis develops in Crry-deficient kidneys. Shown is representative histopathology in WT kidneys before (A) and after transplantation into WT hosts (B through D) and in Crry−/−/C3−/− DKO kidneys before (E) and after transplantation into WT hosts (F through H) 1 d (B and F), 4 d (C and G), and 7 d (D and H) after transplantation. Control WT kidneys have largely normal tubulointerstitium (TI) and glomeruli, whereas Crry-deficient kidneys in complement-sufficient WT hosts exhibit marked abnormalities of the TI, including tubular dilation and cellular sloughing and inflammatory infiltrates. Magnification, ×200.
through 7 in Crry-deficient kidneys in a WT host relative to the control group kidneys, with CXCL10 peaking at day 4 (Figure 5A) and CCL2 rising over time (Figure 5B). In unmanipulated DKO kidneys, expression of both genes was low (CXCL10/18S = 0.017 ± 0.04 and CCL2/18S = 0.07 ± 0.02; n = 5), with expression 1 d after transplantation into WT mice, resulting in ≥40-fold increases in respective mRNA levels. Collagen III protein also was assessed by immunofluorescence staining. Immediately after transplantation, there was little TI collagen III in either group (Figure 6, A and D), which is typical for normal kidneys. There was no change in control transplanted kidneys (Figure 6, B and C), whereas in Crry-deficient kidneys, there was progressive collagen III accumulation in the TI (Figure 6, E and F), which was statistically significant on day 7 (Figure 6G).

**Correlations among Variables**

There were strong correlations among some of the quantitative measures that accumulated from the Crry-deficient kidneys that were transplanted into WT hosts. Not unexpected, the extent of TI infiltration with each of the three inflammatory cells and TI nephritis scores were significantly related to each other ($r^2 = 0.79$ to 0.94; $P \leq 0.001$). The relevance of complement activation to downstream inflammation and scarring is

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**Figure 3.** Progressive infiltration of Crry-deficient kidneys with neutrophils, B cells, and macrophages. Shown is representative histochemistry for neutrophils (A and B) and B cells (D and E) 7 d after transplantation of WT (A and D) or Crry$^{-/-}$C3$^{-/-}$ DKO kidneys (B and E) into WT hosts. Quantification of neutrophils (C), B cells (F), and macrophages (G) in both transplant groups over time are shown. $^aP < 0.05$, $^bP < 0.01$ versus the control group at the same time point. Magnification, ×400.

**Figure 4.** Progressive apoptosis in Crry-deficient kidneys. Shown is representative histochemistry for apoptotic cells 7 d after transplantation of a WT (A) or Crry$^{-/-}$C3$^{-/-}$ DKO kidney (B) into WT hosts. The arrows and arrowheads depict apoptotic cells in the tubules and interstitium, respectively. A glomerulus that lacked apoptotic cells is shown in A, as typically was true of both groups. Quantitative data for apoptotic cells over time also are shown (C). $^aP < 0.01$ versus the control group at the same time point. Magnification, ×400.
supported by the significant correlation of C3 staining with TI infiltration by inflammatory cells (Table 3) and collagen III staining scores ($r^2 = 0.72, P < 0.005$). As could be anticipated on the basis of its known function, CCL2 mRNA expression significantly correlated with macrophage numbers, as well as with scores for the extent of TI nephritis and collagen III deposition ($r^2 \geq 0.75, P < 0.003$ for each). TI apoptotic cellular numbers also were related to neutrophil and macrophage numbers, illustrating a link between inflammation and programmed cell death (30).

**C3 Activation in Crry-Deficient Kidneys Leads to Organ Failure**

In this set of experiments, the functional significance of complement activation in Crry-deficient DKO kidneys in WT hosts was assessed. To do this, we performed nephrectomy of the first host kidney at the time of transplantation as before, whereas the second host kidney was removed 7 d later. Therefore, during the subsequent 14 d, renal function on which the animal would survive was provided solely by the transplanted kidney. Of the 13 kidneys in the control group, only two failed as determined by BUN levels $\geq 60$ mg/dl, whereas renal failure occurred in seven of eight Crry-deficient kidneys ($P = 0.007$).

**Table 1. Chemokine genes upregulated in Crry-deficient kidneys 4 d after transplantation into WT recipients**

<table>
<thead>
<tr>
<th>GenBank Accession No.</th>
<th>Gene Name</th>
<th>Fold Change (DKO versus Control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_021274</td>
<td>CXCL10</td>
<td>11.3</td>
<td>0.002</td>
</tr>
<tr>
<td>NM_008599</td>
<td>CXCL9</td>
<td>8.0</td>
<td>0.000</td>
</tr>
<tr>
<td>NM_008176</td>
<td>CXCL1</td>
<td>6.7</td>
<td>0.000</td>
</tr>
<tr>
<td>NM_009140</td>
<td>CXCL2</td>
<td>5.8</td>
<td>0.001</td>
</tr>
<tr>
<td>NM_013653</td>
<td>CCL5</td>
<td>3.9</td>
<td>0.016</td>
</tr>
<tr>
<td>AF065933</td>
<td>CCL2</td>
<td>2.9</td>
<td>0.019</td>
</tr>
<tr>
<td>BC019961</td>
<td>CXCL16</td>
<td>2.7</td>
<td>0.020</td>
</tr>
<tr>
<td>NM_019494</td>
<td>CXCL11</td>
<td>2.6</td>
<td>0.008</td>
</tr>
<tr>
<td>NM_009141</td>
<td>CXCL5</td>
<td>2.5</td>
<td>0.021</td>
</tr>
<tr>
<td>AF128196</td>
<td>CCL9</td>
<td>2.4</td>
<td>0.003</td>
</tr>
<tr>
<td>BC002073</td>
<td>CCL6</td>
<td>2.0</td>
<td>0.043</td>
</tr>
</tbody>
</table>

*Microarray experiments were performed with four double knockout (DKO) kidneys and three wild-type (WT) kidneys 4 d after transplantation into WT hosts. Shown are chemokine genes that had a statistically significant increase of twofold or greater in DKO kidneys compared with WT kidneys.

**Table 2. Extracellular matrix genes upregulated in Crry-deficient kidneys 4 d after transplantation into WT recipients**

<table>
<thead>
<tr>
<th>GenBank Accession No.</th>
<th>Gene Name</th>
<th>Fold Change (DKO versus Control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>U08020</td>
<td>Procollagen, type I, $\alpha 1$</td>
<td>4.3</td>
<td>0.005</td>
</tr>
<tr>
<td>AW550625</td>
<td>Procollagen, type III, $\alpha 1$</td>
<td>4.0</td>
<td>0.008</td>
</tr>
<tr>
<td>BF227507</td>
<td>Procollagen, type I, $\alpha 2$</td>
<td>3.4</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*Microarray experiments were performed with four DKO kidneys and three WT kidneys 4 d after transplantation into WT hosts. Shown are extracellular matrix genes that had a statistically significant increase of twofold or greater in DKO kidneys compared with WT kidneys.

**Figure 5. Upregulation of CXCL10 and CCL2 mRNA in Crry-deficient kidneys.** Shown are relative mRNA expressions of CXCL10 (A) and CCL2 (B) over time in DKO and WT kidneys that were transplanted into WT hosts. *$P < 0.05$ versus the control group at the same time point.
which led to death of five of the animals between 9 and 13 d after transplantation. Final BUN levels were 55.6 ± 8.3 and 133.9 ± 33.0 mg/dl in control and Crry-deficient groups, respectively (P = 0.015; Figure 7). In contrast to the abnormal excretory renal function in Crry-deficient kidneys, albuminuria measured at multiple time points in both groups was not elevated (data not shown). Although deficiency of Crry profoundly affected the TI, glomeruli were spared, as shown by histopathologic and functional measurements.

Crry-Deficiency Does not Affect Skin Transplants

The studies using renal transplantation showed that the potent complement regulator Crry is necessary in the TI of kidneys. In contrast, skin seems to rely more on DAF to prevent C3 activation (33). To examine this in the context of these studies, we performed skin transplants between Crry/C3-deficient DKO and Crry/C3-sufficient WT mice to evaluate whether skin would suffer a similar fate as kidneys. In contrast to the renal failure that occurred in Crry-deficient DKO kidneys, Crry-

**Table 3. Correlations among various measurements in Crry-deficient kidneys transplanted into WT hosts**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Interstitial Nephritis</th>
<th>TI Neutrophils</th>
<th>TI Macrophages</th>
<th>TI B Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3c</td>
<td>0.582 (0.029)</td>
<td>0.760 (0.002)</td>
<td>0.616 (0.019)</td>
<td>0.767 (0.001)</td>
</tr>
<tr>
<td>Apoptosisc</td>
<td>0.764 (0.001)</td>
<td>0.733 (0.003)</td>
<td>0.711 (0.004)</td>
<td>0.701 (0.005)</td>
</tr>
<tr>
<td>CCL2d</td>
<td>0.763 (0.001)</td>
<td>0.710 (0.004)</td>
<td>0.745 (0.002)</td>
<td>0.507 (0.064)</td>
</tr>
<tr>
<td>Collagen IIIc</td>
<td>0.823 (0.000)</td>
<td>0.906 (0.000)</td>
<td>0.844 (0.000)</td>
<td>0.835 (0.000)</td>
</tr>
</tbody>
</table>

*The first value is the correlation coefficient, and the value in parentheses is the P value. TI, tubulointerstitium.

bVariable assessed by histology.

Variables assessed by immunohistology.

Relative mRNA levels assessed by quantitative reverse transcriptase–PCR.
deficient skin grafts all survived in WT mice, as was true of all skin grafts among the various C57BL/6 strains (WT, DKO, and C3−/−) that were used in the kidney transplant studies. As expected, skin grafts between C57BL/6 and Balb/c mice were rejected quickly. Therefore, although Crry is essential in the kidney, it is dispensable in skin.

Discussion

Crry is a potent inhibitor of C3 activation. Compelling evidence for its importance is seen in Crry-deficient fetuses in which unrestricted alternative pathway C3 activation leads to fatal inflammation (15,16). Because of the concurrent C3 deficiency that is required to surmount this embryonic lethality, the absence of Crry is inconsequential after birth in these in Crry−/−C3−/− DKO mice. As such, to determine the functional role of Crry as a complement regulator in the adult mouse kidney, we relied on transplantation of DKO Crry−/−C3−/− kidneys into complement-sufficient WT hosts. On restoration of blood flow to Crry-deficient kidneys, the intact complement system from the host rapidly became activated on unprotected vascular and tubular cells in the renal TI. From our data accumulated over time, this C3 activation was followed by CCL and CXCL chemokine mRNA production; neutrophil, monocyte/macrophage, and B cell infiltration; apoptosis; and production of matrix proteins such as the interstitial collagens I and III. These events in the kidney were followed quickly by morphologic and functional features of chronic kidney failure. Thus, Crry is required in the normal mouse renal TI, where it prevents spontaneous complement activation and a cascade of events that lead quickly to inflammation and the ultimate loss of functional tissue and development of fibrosis.

Given that plasma C3 largely originates from the liver (34), our strategy of kidney transplantation allowed us to generate a model in which a Crry-deficient kidney was present in a complement-sufficient environment, which does have certain limitations. Of necessity, the transplanted kidneys also had targeted KO of the C3 gene, which has relevance given the capacity of the kidney to contribute substantially to systemic C3 pools as well as C3 for local use (35,36). This local production of C3 seems to be particularly important in allograft rejection (37) and ischemia-reperfusion injury (38). The latter is relevant to our studies in which there was a brief period of cold ischemia, in that DKO kidneys that lack intrinsic renal C3 production would be relatively protected from reperfusion injury (38), yet it is clear that the abundant C3 that was derived from the WT recipient plasma became activated acutely in the DKO kidney, leading to downstream pathologic events. Despite the absence of intrinsic renal C3 production, with its effects on reperfusion injury, whether our results could be attributable to some degree of amplified plasma complement activation from reperfusion events cannot be excluded. Potential strategies to evaluate this include temporary systemic or local complement inhibition during the transient period of reperfusion injury or the use of conditional KO mice. For instance, the use of a Cre/loxP recombination strategy in which Cre recombinase expression is directed by slgl2 or γGT promoters has allowed the targeting of gene KO with relative specificity to the proximal tubular brush border (39,40). Although, in this study we determined the role of Crry in every cell within the kidney, our data support that complement activation occurred primarily on the vasculature of the TI and the basolateral aspects of renal tubules for which a gene targeting approach has not been produced to our knowledge.

Whereas the complement regulators DAF and MCP are widely distributed in humans, they have a more restricted distribution in rodents (41–44). Instead, Crry seems to replace DAF and/or MCP in many sites, including the erythrocyte and kidney (3,45–47). At least one exception to this is skin, in which DAF is a necessary complement regulator (33), whereas Crry seems dispensable on the basis of our studies with skin transplantation.

Until now, the relevance of Crry to protect the kidney from complement attack under normal (20) and disease conditions (48–50) has been defined largely through the use of function-inhibiting antibodies. When Crry in the normal rat kidney was functionally inhibited by specific antibodies that were delivered locally, animals developed renal tubular injury that was characterized by cast formation, dilation of tubular lumens, and mononuclear cell infiltration (20). Of relevance is that these changes from partial and transitory Crry inhibition were prominent early after antibody administration but had undergone resolution by 7 d; furthermore, at no time was there any apparent affect on renal function (20). Additional insights into the importance of Crry as a complement regulator in renal tubules have come from studies by Thurman et al. in warm ischemic-reperfusion injury in mouse kidneys (14,51,52). In this model, ischemia resulted in transient loss of basolateral tubular Crry. With reperfusion, spontaneous alternative pathway complement activation that could be blocked with an inhibitory anti-

Figure 7. Local Crry deficiency leads to functional kidney failure. Shown are final blood urea nitrogen (BUN) concentrations from WT hosts with WT kidneys (Control) or Crry−/−C3−/− (DKO) kidneys. Each circle is from an individual animal. ○, animals that died before d21. *P = 0.015 versus control.
factor B mAb occurred. The histopathologic events that occurred early in this model have considerable similarities to those shown here, as well as acute kidney injury in humans (14), yet in ischemia-reperfusion, Crry was regenerated quickly by the tubules, and the kidneys recovered (51). In contrast, Crry was absent throughout the course in our studies. Rather than resolution, the Crry-deficient kidneys developed progressive and irreversible TI damage and, eventually, renal failure.

Consistent with these previous studies in which Crry was inhibited or lost, our data show that absence of Crry leads to TI damage, whereas glomeruli seem to be spared, even in pathologic processes that are severe enough to cause renal failure. This can be explained by Crry’s being a key complement regulator in mouse endothelial and tubular epithelial cells (51), whereas other complement regulators in addition to Crry, such as DAF and CD59, are protective in rodent glomeruli (41,53,54). Furthermore, factor H potently protects the glomerular capillary wall from complement activation (55,56). Presumably, these complement regulators compensate for lack of Crry in the glomerulus. It is interesting that marked complement activation in the glomerulus, such as can occur when factor H is absent, leads to substantial inflammation and scarring in glomeruli, yet renal excretory failure is a late manifestation in only a minority of animals (55–58). This fits with the widely held view that the extent of TI pathology predicts kidney outcome much better than does glomerular pathology, even when disease is initiated in glomeruli (59,60).

It has become increasingly apparent that acquired or inherited abnormalities in complement regulators can underlie human disease states, such as paroxysmal nocturnal hemoglobinuria and aHUS (13). Overall, our findings in Crry-deficient kidneys seem to have the greatest relevance to aHUS that occurs in individuals with MCP mutations. For example, more than 20 mutations and a spectrum of functional defects have been described in MCP that result in aHUS (61–63). End-stage renal failure results in many cases, for which renal transplantation is curative in 90% of cases; this contrasts to the much lower success in individuals with factor H or I defects, in whom disease recurs in the allograft (62). Thus, MCP is a necessary local complement regulator in the human kidney, as also is true for its functional analogue, Crry, in the mouse kidney.

The progressive influx of neutrophils into the TI of Crry-deficient kidneys was striking, with approximately 10-fold greater numbers present by day 7 after transplantation than on day 1. A similar increase over time was apparent for B cells and macrophages, although of lesser magnitude and achieving statistical significance only for macrophages. In the setting of marked complement activation, it seems likely that the generation of C3a and C5a, each with significant chemotactic activities, contributed to this inflammation. Complement activation products are capable of inducing chemokine release (64,65), and tubular cells can be a major source of chemokine production in vivo (66) and in vitro (66,67). Although the renal pathologic features in Crry-deficient mice have dissimilarities from HUS, it is notable that marked upregulation of CXC and CC chemokines has been observed in human and experimental HUS (31,32), including CXCL1, 5, 9, and 10 and CCL2 and 5, as we observed here. Taken together, our data support that complement activation in Crry-deficient kidneys directly and/or indirectly led to chemokine production, which contributed to the ensuing inflammatory reaction in the TI.

Apoptosis has contrasting roles in the development of renal diseases. Although it is necessary to clear damaged and inflammatory cells (68), it also can cause death of needed cells, thereby contributing to disease rather than aiding in its resolution. For instance, a critical role for apoptosis in the progression of inflammation and tubular cell injury is shown by the findings that inhibition of apoptosis ameliorated inflammation and tubular damage after ischemia-reperfusion injury (26,69). The involvement of apoptosis in complement-dependent renal diseases was supported by our studies in experimental lupus nephritis in which inhibition of C3aR and C5aR signaling led to a significant reduction in apoptosis and overall disease activity (21,70). Although traditionally considered to be a noninflammatory process, there are situations in which apoptosis and organ inflammation are linked, such as in the renal TI in endotoxemic acute kidney injury (30). In our study, apoptosis was induced by spontaneous complement activation in Crry-deficient kidneys, which strongly correlated with disease parameters, such as the extent of interstitial nephritis, inflammatory cell infiltration, and collagen III accumulation, supporting that inflammation and fibrosis were related to apoptosis in this model.

Because of the kidney’s uniquely high blood flow, its cells are exposed continuously to the plasma pool of complement proteins. Without active complement regulation in the TI vessels and tubules, such as by Crry in mice, spontaneous activation of C3 from the plasma rapidly ensues. This quickly leads to inflammatory cell recruitment from generated complement anaphylatoxins and chemokines, cell death through apoptosis, a fibrogenic response, and subsequent organ failure. Thus, Crry is essential to the mouse kidney to maintain its normal structure and function.

Acknowledgments

This work was supported by National Institutes of Health grant R01 DK041873.

We are grateful to Dr. Hector Molina for generating and supplying the mice that were used in these studies.

Disclosures

None.

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