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Blocking Properdin, the Alternative Pathway, and Anaphylatoxin Receptors Ameliorates Renal Ischemia-Reperfusion Injury in Decay-Accelerating Factor and CD59 Double-Knockout Mice

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Complement is implicated in the pathogenesis of ischemia-reperfusion injury (IRI). The activation pathway(s) and effector(s) of complement in IRI may be organ specific and remain to be fully characterized. We previously developed a renal IRI model in decay-accelerating factor (DAF) and CD59 double-knockout (DAF−/−CD59−/−) mice. In this study, we used this model to dissect the pathway(s) by which complement is activated in renal IRI and to evaluate whether C3aR- or C5aR-mediated inflammation or the membrane attack complex was pathogenic. We crossed DAF−/−CD59−/− mice with mice deficient in various complement components or receptors including C3, C4, factor B (FB), factor properdin (FP), mannose-binding lectin, C3aR, C5aR, or Ig and assessed renal IRI in the resulting mutant strains. We found that deletion of C3, FB, FP, C3aR, or C5aR significantly ameliorated renal IRI in DAF−/−CD59−/− mice, whereas deficiency of C4, Ig, or mannose-binding lectin had no effect. Treatment of DAF−/−CD59−/− mice with an anti-C5 mAb reduced renal IRI to a greater degree than did C5aR deficiency. We also generated and tested a function-blocking anti-mouse FP mAb and showed it to ameliorate renal IRI when given to DAF−/−CD59−/− mice 24 h before, but not 4 or 8 h after, ischemia/reperfusion. These results suggest that complement is activated via the alternative pathway during the early phase of reperfusion, and both anaphylatoxin-mediated inflammation and the membrane attack complex contribute to tissue injury. Further, they demonstrate a critical role for properdin and support its therapeutic targeting in renal IRI. The Journal of Immunology, 2013, 190: 3552–3559.
heightened complement sensitivity to dissect the activation pathway(s) and effector(s) of complement in renal IRI. We found that classical and MBL pathways were not involved in this model of renal IRI. Rather, complement was activated via the AP in a pro-
dermin-dependent manner, and both C3aR and C5aR anaphyla-
toxin receptors and the MAC contributed to renal IRI. Further, pro-
dermin inhibition with a blocking mAb before reperfusion ameliorated renal IRI, suggesting that antiproperdin therapy may have beneficial effects in human IRI.

Materials and Methods

Animals

DAF-2/C59+/+, IP-2−, and IPflx/flox-lysosome-Cre+ mice were generated as described previously (20-22). C57BL/6, 129, and BALB/c WT and MBL-A-/- C-/- mice (MBL-/-) were purchased from The Jackson Laboratory. The sources of C3−/−, C4−/−, IB−/−, C3aR−/−, and C5aR−/− mice were described previously (23, 24). Ig−/− (JIT) mice (25) were kindly provided by Dr. R. Eisenberg (University of Pennsylvania). All mutant mice, with the exception of IB−/−, IP−/−, and IPflx/flox-lysosome-
Cre+ mice, were on the C57BL/6 background. DAF-2/C59+/− mice were crossed with the relevant mutant mouse strains to generate DAF-/−/− C59−/−, C3−/−, C4−/−, C5−/−, C9−/−, C3aR−/−, C5aR−/−, and IP−/− /− C59−/− mice, for which DAF-2/C59+/− mice on the C57BL/6 background were used as controls. To generate DAF-/−/− CD59−/−, IB−/−, and DAF-/−/− CD59−/− IP−/− mice, DAF-/−/− C59−/− mice were crossed with IB−/− or IP−/− mice on a 129/C57BL/6 mixed background. For the study of these mice, littermate DAF-/−/− CD59−/− mice with the 129/ C57BL/6 background were used as controls. Male mice weighing 25–35 g were used in all renal IRI experiments, and mice aged 7–12 wk were used for serum properdin assays. Mice were housed in a specific pathogen-
free facility and were confirmed to be negative for common murine viral pathogens by routine sera analysis. Experiments were conducted by following established guidelines for animal care, and all protocols were approved by the appropriate institutional committees.

Renal IRI induction

Renal IR in mice was performed as described previously (20). In short, both renal pedicles were clamped for 22 min using microaneurysm clamps, followed by reperfusion for 24 h. Mice were sacrificed, and kidneys were harvested for histologic analysis. Serum samples were collected before ischemia and 24 h after reperfusion. In some experiments, DAF−/− C59+ mice were treated i.p. with an anti-C5 mAb (BB5.1) (26) or a control mAb (27). In other experiments, mice were treated i.p. with an anti-C5 mAb (BB5.1) (26) or a control mAb (27). In other experiments, mice were treated i.p. with an anti-C5 mAb (BB5.1) (26) or a control mAb (27).

Histopathology and immunohistochemistry

Kidneys were fixed in methyl Carnoy’s solution overnight and processed for paraffin embedding. Sections were then stained with periodic acid–Schiff (PAS). Tubular injury was evaluated blinded and scored semiquan-
titatively, as described previously (20). Cryostat sections (4 μm) of fixed kidneys were stained by immunohistochemistry for neutrophils, complement factors C3 and C9 (MAC), and properdin, as described previously (20, 28). Briefly, slides were dried and fixed in methanol/acetic. They were then treated with a rabbit anti-mouse lactoferrin Ab (for neutrophils; kindly provided by Dr. C. Teng, National Institute of Environmental Health Sciences, Research Triangle Park, NC) or a rabbit anti-
neutrophil Ab (for MAC; kindly provided by Dr. P. Morgan, University of Wales College of Sciences, Research Triangle Park, NC) or a rabbit anti-
C3a receptor Ab (for C3aR; kindly provided by Dr. J. Miyazaki, Osaka University, Osaka, Japan). This construct was transfected into HEK cells

Expression of recombinant mouse properdin

The mouse properdin full-length cDNA (nt 71–1465, NM_008823.3) was amplified by RT-PCR using liver RNA and the following two primers: mprop-S (upstream), 5′-CCCCGGGCCACCATGCTGTGAATGCAA-3′ and mprop-AS (downstream), 5′-AACCTTTAGGGTTTCTCTTCTTTGTCGTCT-3′. This cDNA was then used as a template in subsequent PCR reactions to incorporate an eight-histidine (8XHis) tag and an enterokinase cleavage site at the C terminus of the protein. First, PCR amplification was performed using mprop-S (upstream) and R1 (downstream) as primers, 5′-agtagtagatagatgTTATCGCATCTGCGGGTTTCTCTTCTTCTTGTCGTCT-3′ (lower case: 8XHis tag sequence; underlined: enterokinase cleavage site sequence (30); italicized: nucleotide sequence of the 3′ coding region of properdin). The PCR product from the above reaction was used as a template in a further round of PCR using mprop-S (upstream) and R2 (downstream) as primers, 5′-GATCTTTAATAGCCACATagtagtagatagatgTTATCGCATCTGCGGGTTTCTCTTCTTCTTGTCGTCT-3′ (lower case: 8XHis tag sequence; underlined: enterokinase cleavage site sequence). The final PCR product was cloned into the pCR2.1 vector and then subcloned at the EcoRI site into the pCAGGS expression vector (kindly provided by Dr. J. Miyazaki, Osaka University, Osaka, Japan). This construct was transfected into HEK cells

FIGURE 1. Deficiency in C3 ameliorates renal IRI in DAF−/−/CD59−/− mice. Renal pedicles were clamped bilaterally for 22 min. Blood was collected before renal pedicle clamping (0 h) and 24 h after reperfusion for BUN determinations. (A) Serum levels of BUN in WT (n = 29), DAF−/−/CD59−/− (n = 37), and DAF−/−/CD59−/− C3−/− (n = 20) mice. Data shown are mean ± SEM. (B) Representative images of PAS, C3, C9, and neutrophil staining in kidney sections of WT, DAF−/−/CD59−/−, and DAF−/−/CD59−/− C3−/− mice at 24 h after renal IR, showing severe tubular injury (cast formation), deposition of C3 and C9 in peritubular capillaries (arrows), and abundant neutrophil infiltration in DAF−/−/CD59−/− mice but not in WT and DAF−/−/CD59−/− C3−/− mice. Original magnification: PAS, ×200; C3, ×400; C9, ×400; neutrophil, ×200. *p < 0.0001, versus WT mice. **p < 0.0001, versus DAF−/−/CD59−/− mice, Student t test.
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(American Type Culture Collection) together with a pGK-Hyg vector. After selection on hygromycin for 2 wk, stable HEK cell lines expressing recombinant mouse properdin were established and were confirmed to secrete high levels of the protein by Western blot analysis (described below). Recombinant properdin was purified by Ni+-chelating chromatography (Qiagen, Valencia, CA) from cell culture supernatants of stable HEK cells (collected 48 h after switching to serum-free DMEM; Life Technologies, Grand Island, NY).

**SDS-PAGE and Western blot analysis**

The purity of the recombinant mouse properdin was assessed by Coomassie staining of proteins resolved on 8% Tris-glycine gels under reducing conditions. To screen for stable properdin-expressing HEK cell lines, serum-free cell culture media were subjected to electrophoresis on 8% Tris-glycine gels under reducing conditions. Separated proteins on gels were transferred to polyvinylidene difluoride membranes, which were then probed with rabbit anti-mouse properdin IgG (2 μg/ml) (22) for 1 h, followed by HRP-conjugated goat anti-rabbit IgG (1:4000 dilution; Bio-Rad) and the ECL chemiluminescent detection system (Amersham Pharmacia, Uppsala, Sweden).

**Generation of anti-mouse properdin mAbs**

To generate anti-mouse properdin mAbs, a C57BL/6 line of Fp-deficient mice derived from Fp<sup>−/−</sup> mice (22) was used. Fully backcrossed (>9 generations) C57BL/6 Fp<sub>loss/loc</sub> mice were crossed with Elia-Cre mice, which express the Cre recombinase in germline (31). Cre− progeny with a mutant properdin gene allele (inducible of germline deletion of the Fp gene) were then intercrossed to generate homozygous properdin-mutant mice. Mice were immunized with 100 μg of recombinant properdin emulsified in CFA. Boosting immunization (100 μg/mouse each time) was performed three times at 2-wk intervals with FpA. Properdin Ab titers in the immunized mouse sera were determined by ELISA. Prior to hybridoma production, mice were injected i.p. daily with recombinant properdin for three consecutive days (50 μg/mouse in PBS). Splenocytes were harvested and fused with P3-X63-Ag8.653 myeloma cells (ATCC cat. #CRL-1580).

To measure serum Fp levels, Maxisorp plates (Nunc) were coated for 1 h at 37˚C with anti-properdin mAb 14E1 (50 μl/well, 1% PBS buffer; MP Biomedicals, Solon, OH). We previously demonstrated that DAF<sup>−/−</sup>/CD59<sup>−/−</sup> mice were highly susceptible to renal IRI compared with WT controls and that complement depletion with CVF markedly reduced this susceptibility (20). Exacerbated renal IRI in DAF<sup>−/−</sup>/CD59<sup>−/−</sup> mice was correlated with microvascular injury and C3/C9 deposition, increased neutrophil infiltration into the outer medulla, and higher tubular injury scores (20). To confirm the complement-dependent nature of this model as suggested by the CVF experiment, we

| Table I. Histological evaluation of kidney sections |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mice           | Tubular Damage  | Neutrophil Infiltration | C3 Staining | C9 Staining |
| WT             | 0.3 ± 0.4*      | 16.0 ± 8.8*        | 0.3 ± 0.3*    | 0.3 ± 0.2*     |
| DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 3.2 ± 0.6      | 96.5 ± 13.4       | 1.4 ± 0.2      | 1.3 ± 0.2       |
| C3<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 0.4 ± 0.4*      | 33.2 ± 11.0*       | 0 ± 0*         | 0.4 ± 0.2*     |
| Ig<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 3.2 ± 0.7      | 95.7 ± 18.9       | 1.3 ± 0.5      | 1.3 ± 0.2       |
| C4<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 3.6 ± 0.5      | 90.7 ± 20.2       | 1.3 ± 0.3      | 1.1 ± 0.3       |
| MBL<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 3.1 ± 1.0      | 95.0 ± 15.9       | 1.1 ± 0.1      | 1.1 ± 0.1       |
| Ig<sup>−/−</sup>/MBL<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 3.3 ± 0.7      | 96.5 ± 15.3       | 1.3 ± 0.2      | 1.3 ± 0.2       |
| Fb<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 0.5 ± 0.6**     | 19.2 ± 4.9**      | 0.1 ± 0.1**    | 0.4 ± 0.2**     |
| Fb<sup>−/−</sup>/MBL<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 2.6 ± 0.9      | 101.0 ± 15.3      | 1.0 ± 0.2      | 1.3 ± 0.3       |
| Fb<sup>−/−</sup>/C5<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 1.1 ± 1.2***    | 58.0 ± 11.6***    | 0.4 ± 0.1***   | 0.6 ± 0.2***    |
| C5A<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 1.9 ± 1.0*      | 46.1 ± 10.9*      | 0.6 ± 0.4*     | 0.7 ± 0.2*      |
| C5A<sup>−/−</sup>/MBL<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> | 0.9 ± 1.0*      | 49.7 ± 11.9*      | 0.3 ± 0.3*     | 0.7 ± 0.2*      |

Seven mice from each group were randomly selected for histological measures of tubular damage, neutrophil infiltration, and deposition of C3 and C9 in peritubular capillaries. Neutrophil values are numbers per field of view. Tubular damage and C3 and C9 staining were scored semiquantitatively, as described in the Materials and Methods. Data are mean ± SEM.

*<i>p < 0.05</i>, versus DAF<sup>−/−</sup>/CD59<sup>−/−</sup> mice; **<i>p < 0.05</i>, versus Ig<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> mice; ***<i>p < 0.05</i>, versus Fb<sup>−/−</sup>/DAF<sup>−/−</sup>/CD59<sup>−/−</sup> mice.
crossed DAF−/−CD59−/− mice with C3−/− mice to generate DAF−/−CD59−/−C3−/− mice and assessed renal IRI. As we observed before, 22 min of bilateral renal ischemia followed by 24 h of reperfusion led to more severe renal IRI in DAF−/−CD59−/− mice than in WT mice (Fig. 1, Table I). In WT mice, the BUN level increased from 24.2 ± 0.2 mg/dl before IR to 68.8 ± 8.8 mg/dl after IR, whereas it increased from 24.4 ± 0.5 to 135.6 ± 8.4 mg/dl in DAF−/−CD59−/− mice (Fig. 1A). Importantly, we found that C3 deficiency in DAF−/−CD59−/− mice reduced BUN to WT mouse levels (56 ± 7.5 mg/dl) (Fig. 1A). Corroborating the BUN data, renal histology revealed significantly milder tubular injury, lower numbers of neutrophils in the outer medulla, and less C3 and C9 deposition in peritubular capillaries of DAF−/−CD59−/−C3−/− mice compared with DAF−/−CD59−/− mice (Fig. 1B, Table I). Thus, exacerbation of renal IRI in DAF−/−CD59−/− mice was confirmed to be critically dependent on C3.

Natural Abs and the lectin pathway of complement have been implicated in other rodent models of IRI (3–6). To determine whether they might also be involved in renal IRI of DAF−/−CD59−/− mice, we crossed DAF−/−CD59−/− mice with Ig−/−, C4−/−, or MBL-A/MBL-C double-knockout (MBL−/−) mice. We found that deficiency of Abs, C4, or MBL did not significantly affect the susceptibility of DAF−/−CD59−/− mice to renal IRI (Fig. 2, Table I). Thus, after renal IR challenge, DAF−/−CD59−/− Ig−/−, DAF−/−CD59−/−C4−/−, and DAF−/−CD59−/−MBL−/− mice had BUN levels that were not significantly different from each other or from DAF−/−CD59−/− mice (Fig. 2A). This result was further corroborated by histological evaluations of tubular injury, neutrophil infiltration, and complement staining in peri-tubular capillaries among the different groups (Fig. 2B, Table I). Together, the data suggested that, unlike skeletal muscle, intestinal, and myocardial IRI models (3–6), complement-dependent renal IRI in DAF−/−CD59−/− mice was not mediated by natural Abs and neither the classical nor the MBL pathway was involved.

In light of the above findings, we next examined the role of the AP of complement in this process by crossing DAF−/−CD59−/− mice with mice deficient in complement fB. Examination of DAF−/−CD59−/−fB−/− mice showed that renal IRI was markedly reduced, with BUN and tubular injury scores essentially reverting to WT mouse levels (Figs. 1, 3, Table I). This result indicated that exacerbated renal IRI in DAF−/−CD59−/− mice was mediated by the AP complement. To further dissect AP complement activation under this setting, we crossed DAF−/−CD59−/− mice with mice deficient in IP (IP−/−). Properdin is the only known positive regulator of complement, and it promotes AP complement activation by stabilizing the C3 convertase C3bBb (32, 33). Recent evidence suggested that IP may also bind to susceptible surfaces to initiate AP complement activation (34, 35). Comparison of DAF−/−CD59−/− IP−/− mice and their IP-sufficient littermates revealed that IP played a major role in renal IRI, because BUN, tubular injury, neutrophil infiltration, and microvascular complement deposition in DAF−/−CD59−/− IP−/− mice were significantly reduced (Fig. 3A, 3B, Table I). Notably, we detected IP staining in peritubular capillaries of IRI-challenged DAF−/−CD59−/− IP−/− mice but not in DAF−/−CD59−/− IP+/− mice littermates (Fig. 3C).

Activation of the AP complement generates as effectors the anaphylatoxins C3a and C5a, as well as the MAC. The anaphylatoxins function through binding to their cognate receptors, C3aR and C5aR on neutrophils and macrophages, and play a major role in complement-mediated inflammatory injury (36). To characterize the complement effectors responsible for exacerbated renal IRI in DAF−/−CD59−/− mice, we crossed DAF−/−CD59−/− mice with C3aR−/− and C5aR−/− mice to determine whether a C3aR- or C5aR-mediated inflammatory response was involved. We found that, compared with DAF−/−CD59−/− mice, renal IRI in DAF−/−CD59−/−C3aR−/− and DAF−/−CD59−/−C5aR−/− mice was significantly ameliorated, as assessed by BUN and histological parameters (Fig. 4A, Table I). However, the magnitude of BUN reduction by either C3aR or C5aR deficiency was less than when the C3 or fB gene was deleted (Figs. 1, 3), suggesting that both C3aR- and C5aR-mediated inflammation contributed to renal IRI, and deletion of either effector pathway was not sufficient to completely rescue the DAF−/−CD59−/− mouse phenotype. To further investigate whether MAC-mediated cellular injury also played a role in the pathogenesis of renal IRI, we used an anti-C5 mAb that blocks C5 cleavage and prevents the generation of C5a, as well as the MAC (26). This experiment demonstrated that blocking C5 in DAF−/−CD59−/− mice was more effective than...
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Deficiency in fB or fP ameliorates renal IRI in DAF−/−CD59−/− mice. Blood was collected before renal pedicle clamping (0 h) and 24 h after reperfusion for BUN determinations. (A) Serum levels of BUN in DAF−/−CD59−/− fB+/+ (n = 9), DAF−/−CD59−/− fP+/+ (n = 14), and fB- or fP-sufficient littermate controls (n = 10 and n = 11, respectively). Data shown are mean ± SEM. (B) Representative images of PAS, C3, and CD59 staining in kidney sections of DAF−/−CD59−/− fB+/+, DAF−/−CD59−/− fB−/−, DAF−/−CD59−/− fP+/+, and DAF−/−CD59−/− fP−/− mice at 24 h after renal IR, showing a significant reduction of tubular injury, deposition of C3 and CD9 in peritubular capillaries (arrows), and neutrophil infiltration in DAF−/−CD59−/− fB+/+ and DAF−/−CD59−/− fP−/− mice. (C) Representative images of properdin staining in kidney sections of DAF−/−CD59−/− fP+/+ and DAF−/−CD59−/− fP−/− mice. Original magnification: PAS, ×200; C3, ×400; CD59, ×400; neutrophil, ×200; properdin, ×400. Data shown are mean ± SEM. *p < 0.0001 versus DAF−/−CD59−/− fP+/* mice, **p < 0.001 versus DAF−/−CD59−/− fP−/− mice. Student t test.

was C5aR gene deletion in reducing renal IRI (Fig. 4B, BUN for DAF−/−CD59−/− C5aR−/− mice, 85.2 ± 10.2 mg/dl; BUN for DAF−/−CD59−/− mice treated with anti-C5 mAb, 57.1 ± 12.1 mg/dl), suggesting that the MAC was also likely to have contributed to renal IRI.

The finding that properdin gene deletion ameliorated renal IRI in DAF−/−CD59−/− mice prompted us to investigate whether properdin might be amenable to therapeutic targeting in the setting of renal IRI. To test this concept, we expressed and purified recombinant mouse fP in HEK293 cells (Fig. 5A) and used it to immunize a line of fP−/− mice obtained by germline deletion of the floxed fP gene (22). By this approach, we produced several high-affinity mouse anti-mouse fP mAbs, one of which (14E1) was found to be function blocking in an LPS-dependent AP complement-activation assay (Fig. 5B, 5C). When tested in mice, 14E1 effectively blocked AP complement activity in vivo. For example, injection of 0.4 and 1.2 mg of 14E1 to WT mice blocked LPS-dependent AP complement activity for 2 and 9 d, respectively (Fig. 5D). By sandwich ELISA assays using these mAbs, we surveyed serum fP levels in WT mice of different genetic background and in mutant mice lacking various complement components, as well as in fPflox/flox−lysozyme–Cre+ mice with tissue-specific fP gene deletion in myeloid lineage cells (22). We found that serum fP levels in C57BL/6J, 129J, and BALB/c mice ranged between 15 and 20 ng/ml and that serum levels of fPflox/flox−lysozyme–Cre+ mice were <5% WT mouse levels (Fig. 5E), suggesting that myeloid lineage cells are the major source of serum fP. Of interest, we found serum properdin levels in C3−/−, fB−/−, and C4−/− mice to be significantly lower than in WT mice (Fig. 5F).

We next tested whether administration of a function-blocking mAb against fP to DAF−/−CD59−/− mice before or after renal IR challenge would be effective at preventing renal injury. DAF−/−CD59−/− mice were treated by i.p. injection of mAb 14E1 at 24 h before ischemia or at 4 or 8 h after reperfusion. As shown in Fig. 6, we found that systemic blockade of fP before ischemia significantly ameliorated renal IRI, and this treatment appeared to be as effective as fP gene deletion (Fig. 3). In contrast, blocking fP at 4 h after reperfusion was marginally effective, and the protection was completely lost if the mAb was given at 8 h postreperfusion (Fig. 6). These results suggested that fP played
a major role in AP complement activation in the initial phase of reperfusion, and therapeutic targeting of circulating properdin to reduce renal IRI is feasible and effective when carried out prophylactically (e.g., in the setting of renal transplantation).

**Discussion**

In this study, we used the DAF-/-CD59-/- mouse model to dissect the pathways and effectors of complement activation in renal IRI. In contrast to several other rodent IRI models in which natural Abs and the classical or MBL pathway were implicated (3–6), we found that complement was activated via the AP in renal IRI of DAF-/-CD59-/- mice and that properdin played a major pathogenic role in this process. In previous studies of murine models of intestinal and skeletal muscle IRI, nonmuscle myosin H chain II was identified as a self-Ag that became exposed on host tissues after ischemia, and binding of this protein by a natural IgM Ab (CM-22) triggered MBL pathway complement activation (4, 37). Other investigators reported natural Abs to be pathogenic in intestinal IRI by recognizing annexin IV as a neoantigen on ischemic tissues (38). In addition, deficiency of MBL or mannose-binding lectin-associated serine protease 2 was found to be protective in mouse and rat models of myocardial, intestinal, and renal IRI (6, 9, 39, 40). In the current study, we confirmed that exacerbation of renal IRI in DAF-/-CD59-/- mice was complement dependent, because C3 deficiency rescued the phenotype. However, we found that deficiency of Ig, C4, or MBL had no effect on renal IRI. Thus, our data exclude any involvement of the classical pathway or MBL in the complement-mediated renal IRI (6, 9, 39, 40). In the current study, we confirmed that exacerbation of renal IRI in DAF-/-CD59-/- mice was complement dependent, because C3 deficiency rescued the phenotype. However, we found that deficiency of Ig, C4, or MBL had no effect on renal IRI. Thus, our data exclude any involvement of the classical pathway or MBL in the complement-mediated renal IRI of DAF-/-CD59-/- mice. It is unknown why MBL deficiency ameliorated renal IRI in other studies (9) but not in the DAF-/-CD59-/- mouse model in this study. It is possible that the discrepancy is related to differences in the experimental protocols used (e.g., WT versus DAF-/-CD59-/- mice, length of ischemia). However, it should be noted that, although MBL and C4 are...
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FIGURE 6. Systemic inhibition of properdin with mAb before, but not after, IR ameliorates renal IRI in DAF−/− CD59−/− mice. Mice were treated with a control IgG (MOPC, n = 10) at 24 h before renal IR surgery or with a mouse anti-mouse properdin mAb (14E1) at 24 h before IR surgery (n = 7) and at 4 h (n = 4) and 8 h (n = 5) after reperfusion. Blood was collected before renal pedicle clamping (0 h) and 24 h after reperfusion for BUN determinations. Data shown are mean ± SEM. *p < 0.05, versus control IgG. Student t test.

not involved in the renal IRI of DAF−/− CD59−/− mice, we cannot rule out the possibility that a “bypass” lectin pathway (i.e., direct C3 cleavage) (40, 41) mediated by ficolins may have played a role. Such a scenario could be tested in future experiments by blocking the function of mannose-binding lectin-associated serine protease 2, the key activation enzyme of the lectin pathway, in DAF−/− CD59−/− mice.

In contrast to the noninvolvement of Abs, MBL, and the classical pathway, we found that renal IRI in DAF−/− CD59−/− mice was completely dependent on the AP because fB deficiency prevented exacerbation of renal IRI. Furthermore, we showed that properdin, a positive regulator of the AP, played a major pathogenic role in this process. Whether the AP was the primary and sole pathway activated or functioned as an amplification loop secondary to other triggers, such as the ficolins, remains to be determined. It is certainly possible that the AP was activated independently. Ischemic challenge may impair the function of the remaining membrane complement regulator Crry in DAF−/− CD59−/− mice (42), and ischemia-induced plasma membrane remodeling on endothelial cells may reduce their affinity for the fluid-phase complement regulator factor H (43). Together, these events would render the ischemic tissues susceptible to spontaneous AP complement attack, which was likely to be facilitated by properdin as a positive regulator.

Data from the literature suggest that C3a–C3aR interaction may play a role in renal IRI. It was shown that bilateral renal IR in mice resulted in a significant increase in systemic levels of C3a, and C3a was required for the induction of MIP-2 and KC, potent neutrophil chemotaxins, after renal IR (11). These observations are in agreement with our finding that C3aR deficiency was partially protective for renal IRI in DAF−/− CD59−/− mice. Likewise, we found that C5aR deficiency also ameliorated renal IRI, suggesting that C5a may have contributed to neutrophil infiltration into the outer medulla of the kidney, as we described previously (20). That C5aR-mediated inflammation contributed to renal IRI in DAF−/− CD59−/− mice is also consistent with findings from other renal IRI studies in which C5aR function was inhibited by a small molecule antagonist or small interfering RNA (13, 14). Of interest, a recent study using mice deficient in C3aR and/or C5aR also showed that both C3aR- and C5aR-mediated pathways contributed to renal IRI (12). Because C5 activation generates C5a, as well as C5b, which initiates the formation of the lytic MAC on target cells, we also evaluated the involvement of the MAC in renal IRI of DAF−/− CD59−/− mice using a C5-blocking mAb. We found that administration of anti-C5 mAb to DAF−/− CD59−/− mice reduced renal IRI to a greater degree than did C5aR gene deletion, implying that the MAC had also contributed to renal IRI. This conclusion is also supported by our earlier observation that renal IRI was more severe in DAF−/− CD59−/− mice than in DAF−/− mice (20), presumably reflecting increased MAC formation in the absence of CD59 as a MAC inhibitor.

As the only known positive regulator of the complement system, properdin facilitates AP complement activation either by stabilizing the C3 convertase C3bBb or by binding to susceptible surfaces to serve as a platform for de novo C3bBb assembly (32, 33, 35). Given that genetic deficiency of properdin was protective and that both C3 and terminal complement activation effectors were pathogenic in renal IRI, we tested the concept of therapeutic inhibition of properdin in renal IRI. We successfully generated function-blocking mAbs against mouse properdin by immunizing an IP-deficient mouse with recombinant mouse IP protein. Using a newly developed ELISA assay, we showed that systemic properdin is mainly produced by myeloid lineage cells, and its serum levels ranged between 15 and 20 μg/ml in WT mice of different genetic backgrounds. Interestingly, serum properdin levels in C3−/−, fB−/−, and C4−/− mice were significantly lower than that of WT mice, implying that either the number of myeloid lineage cells or their properdin-secreting activity was affected in these mutant mice. We found that systemic inhibition of properdin by mAb before reperfusion significantly reduced renal IRI in DAF−/− CD59−/− mice. The effect of mAb inhibition on reducing renal IRI was similar to that of properdin gene deletion, suggesting that the pathogenic role of properdin in this disease setting is fully amenable to pharmacological targeting. By manipulating the timing of properdin inhibition, we further showed that the pathogenic role of properdin and, therefore, the AP complement in renal IRI, is imparted during the initial phase (<4 h) of reperfusion after ischemia.

In summary, by using DAF−/− CD59−/− mice with an increased sensitivity to complement injury, we dissected the complement activation pathways and pathogenic effectors in renal IRI. Our genetic and pharmacological data identify the AP, properdin, C3aR, C5aR, and the MAC as components responsible for renal IRI and exclude the participation of natural Abs, C4, and MBL. The finding of a beneficial effect of properdin targeting in renal IRI adds to a growing list of AP complement–mediated pathologies for which antiproperdin therapy may be effective (22, 44), although recent studies also showed settings in which properdin inhibition may not be desirable (28, 45).

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Disclosures
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References

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