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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[1]

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Abstract

Complement is implicated in the pathogenesis of ischemia reperfusion injury (IRI). The activation pathway(s) and effectors(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. Here we used this model to dissect the pathway(s) by which complement is activated in renal IRI and to evaluate whether C3a or C5a receptor (C3aR, C5aR)-mediated inflammation or the membrane attack complex (MAC) 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 (MBL), C3aR and C5aR or immunoglobulin (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 MBL had no effect. Treatment of $DAF^{-/-}CD59^{-/-}$ mice with an anti-C5 mAb reduced renal IRI to a greater degree than 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 hr before, but not 4 or 8 hrs 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 MAC contribute to tissue injury. Further, they demonstrate a critical role of properdin and support its therapeutic targeting in renal IRI.

Introduction

Ischemia-reperfusion injury (IRI[3]) contributes significantly to morbidity and mortality in various clinical settings including acute renal failure in allograft and native kidneys (1, 2). Animal modeling studies have indicated that the complement system plays an important role in the pathogenesis of IRI, but the pathways by which complement is activated during IR and the complement effectors that are responsible for tissue injury may be organ-specific

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[3]Abbreviations used: IRI, ischemia reperfusion injury; AP, alternative pathway; DAF, decay-accelerating factor; Crry, complement receptor 1-related gene/protein y; MBL, mannose-binding lectin; CVF, cobra venom factor; WT, wild-type; BUN, blood urea nitrogen; MASP, mannose-binding lectin-associated serine protease.

and remain to be fully characterized. Studies using rodent models of skeletal muscle, intestinal and heart IRI have implicated natural antibodies and the mannose-binding lectin (MBL) pathway of complement in tissue injury (3–6). They have led to the hypothesis that ischemic assaults expose neoantigens on host tissues which are recognized by natural antibodies or lectins, and binding of these innate immune proteins to the neoantigens activates the classical or MBL pathway of complement (3–6).

The role of complement in renal IRI has also been addressed by multiple investigators using rodent models. Some studies have shown a critical role of the alternative pathway (AP) while others have implicated the MBL pathway (7–9), but mechanistic details of complement activation in renal IRI via either pathway remain to be further characterized. Regarding complement effectors, both the MAC and anaphylatoxin receptor (C5a and C3aR)-mediated signaling on neutrophils and tubular cells have been described to play a pathogenic role in renal IRI (10–15). Additionally, B cell subsets and natural antibodies have been found to influence renal IRI (16, 17). Other studies, however, have shown that renal IRI is independent of immunoglobulin and T lymphocytes (18) and inhibiting the complement system did not reduce renal IRI, suggesting a minimal role of complement in the experimental setting examined (19).

A challenge in renal IRI studies is to separate complement-mediated injury from those caused by other inflammatory pathways that may be triggered, especially when protocols involving prolonged ischemic periods are used. We previously developed a murine model of renal IRI using mice deficient in two membrane complement regulators, decay-accelerating factor (DAF) and CD59 (20). By employing a protocol of short ischemia (22 min) followed by 24 hr reperfusion, we demonstrated that wild-type (WT) mice sustained only mild renal IRI, whereas $DAF^{-/-}CD59^{-/-}$ mice incurred profound renal injury that was complement-dependent, as exacerbation of injury in the double mutant mice was prevented by complement depletion with cobra venom factor (CVF)(20). Here, we used this model of 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 alternative pathway in a properdin-dependent manner and that both C3aR and C5aR anaphylatoxin receptors and the MAC contributed to renal IRI. Further, properdin inhibition with a blocking mAb before reperfusion ameliorated renal IRI, suggesting that anti-properdin therapy may have beneficial effect in human IRI.

Materials and methods

Animals

$DAF^{-/-}CD59^{-/-}$, $fP^{-/-}$ and $fP^{flox/flox}$-lysozyme-Cre^{+} mice were generated as described previously (20–22). C57BL/6, 129J and Balb/c wild-type (WT) and MBL-$A^{-/-}C^{-/-}$ mice ($MBL^{-/-}$) were purchased from The Jackson Laboratory. The sources of $C3^{-/-}$, $C4^{-/-}$, $fB^{-/-}$, $C3aR^{-/-}$ and $C5aR^{-/-}$ mice were described previously (23, 24). $Ig^{-/-}$ (JHT) mice (25) were kindly provided by Dr R. Eisenberg (University of Pennsylvania, Philadelphia, PA). All mutant mice except $fB^{-/-}$, $fP^{-/-}$ and $fP^{flox/flox}$-lysozyme-Cre^{+} mice were on the C57BL/6 background. $DAF^{-/-}CD59^{-/-}$ mice were crossed with the relevant mutant mouse strains to generate $DAF^{-/-}CD59^{-/-}$ $C3^{-/-}$, $DAF^{-/-}CD59^{-/-}$ $C4^{-/-}$, $DAF^{-/-}CD59^{-/-}$ $MBL^{-/-}$, $DAF^{-/-}CD59^{-/-}C3aR^{-/-}$, $DAF^{-/-}CD59^{-/-}$ $C5aR^{-/-}$ and $DAF^{-/-}CD59^{-/-}$ $Ig^{-/-}$ mice, for which $DAF^{-/-}CD59^{-/-}$ mice on C57BL/6 background were used as controls. To generate $DAF^{-/-}CD59^{-/-}$ $fB^{-/-}$ and $DAF^{-/-}CD59^{-/-}$ $fP^{-/-}$ mice, $DAF^{-/-}CD59^{-/-}$ mice were crossed with $fB^{-/-}$ and $fP^{-/-}$ mice on a 129/C57BL/6 mixed background. For the study of these mice, littermate $DAF^{-/-}CD59^{-/-}$ mice with 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

weeks 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 ischemia reperfusion injury induction

Renal ischemia/reperfusion 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 hrs. Mice were sacrificed and kidneys were harvested for histologic analysis. Serum samples were collected before ischemia and 24 hr after reperfusion. In some experiments, $DAF^{-/-}CD59^{-/-}$ mice were treated intraperitoneally with an anti-C5 mAb (BB5.1) (26) or a control mAb (MOPC-31C, ATCC, cat# CCL-130) 24 hrs before ischemia at a dosage of 1mg/mouse as previously described (27). In other experiments, mice were treated intraperitoneally with the mouse anti-mouse properdin mAb 14E1 at a dosage of 2 mg/mouse 24 hrs before ischemia or at 4, 8 hrs after reperfusion. Blood urea nitrogen (BUN) levels were measured using urea nitrogen reagents (Sigma-Aldrich) by following the manufacturer's instructions.

Histopathology, immunohistochemistry, and immunofluorescence

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 were evaluated blindly and scored semi-quantitatively as described before (20). Cryostat sections (4 μm) of frozen kidneys were stained for neutrophils, complement factors C3, C9 (MAC) and properdin as descried before (20, 28). Briefly, slides were dried and fixed in methanol/acetone. 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-rat C9 Ab (for MAC, kindly provided by Dr. P. Morgan, University of Wales College of Medicine, Cardiff, U.K.) which cross-reacts with mouse C9. For staining complement factor C3 and properdin, a biotin-labeled goat anti-mouse C3 (29) and a rabbit anti-mouse properdin (22) antibody was used, respectively. Neutrophils were counted by examining 5–10 viewing fields (400 x) randomly selected from the outer medulla and corticomedullary junction on each slide. The percentage of tubules with peritubular capillary staining of C3 or C9 in 10 randomly selected viewing fields was graded as follows. Grade 0, negative; Grade 1, <25%; Grade 2, <50%; Grade 3, <75%; Grade 4, >75%.

Expression of recombinant mouse properdin

The mouse properdin full-length cDNA (nucleotide 71 to 1465, NM_008823.3) was amplified by RT-PCR using liver RNA and the following two primers: mprop-S (upstream), 5′-CCCGGGGCCACCATGCCTGCTGAAATGCAA-3′; mprop-AS (downstream), 5′-AAGCTTTTAGGGTTTCTTCTCTTCTGGGTC-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 carboxyl terminus of the protein. First, PCR amplification was performed using mprop-S (upstream) and R1 (downstream) as primers, 5′-atgatgatgatgatgCTTATCGTCATCGTC*GGGTTTCTTCTCTTCTGGGTC TTTGCAGAC*-3′ (lower case: 5xHis 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′-CGATCTTTATTAAGATCCACTatgatgatgatgatgatgatgCTTATCGTCATCGTC-3′ (lower case: 8xHis tag sequence; underlined: enterokinase cleavage site sequence). The final PCR product was cloned into the pCR2.1 vector and then sub-cloned at EcoRI site into the

pCAGGS expression vector (kindly provided by Dr. J. Miyazaki, *Osaka University,* Japan). This construct was transfected into HEK cells (ATCC, Rockville, MD) together with a pGK-Hyg vector. After selection on hygromycin for 2 weeks, stable HEK cell lines expressing recombinant mouse properdin were established and were confirmed to secret high levels of the protein by Western blot analysis (described below). Recombinant properdin was purified by Ni^{2+}-chelating chromatography (Qiagen) from cell culture supernatants of stable HEK cells (collected 48 hours after switching to serum free Dulbecco's modified Eagles medium, GIBCO, 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 PVDF membranes, which were then probed with rabbit anti-mouse properdin IgG (2 μg/ml)(22) for 1 hr, followed by HRP-conjugated goat anti-rabbit IgG (1:4000 dilution; Bio-Rad) and the ECL chemiluminescent detection system (Amersham Pharmacia).

Generation of anti-mouse properdin monoclonal antibodies

To generate anti-mouse properdin mAbs, a C57BL/6 line of fP-deficient mice derived from $fP^{flox/flox}$ mice (22) were used. Fully backcrossed (>9 generations) C57BL/6 $fP^{flox/flox}$ mice were crossed with EIIa-Cre mice which express the Cre recombinase in germline (31). Cre-negative progeny with a mutant properdin gene allele (indicative 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 complete Freund's adjuvant. Boosting immunization (100ug/mouse each time) was performed three times at 2-week intervals with incomplete Freund's adjuvant. Properdin antibody titers in the immunized mouse sera were determined by ELISA. Prior to hybridoma production, mice were injected daily with recombinant properdin for three consecutive days (50μg/mouse in PBS, i.p). Splenocytes were harvested and fused with P3-X63-Ag8.653 myeloma cells (ATCC cat CRL-1580). After selection and cloning of hybridomas, single clones were grown in culture flasks in Hypoxanthine-Thymidine (HT) medium (Sigma) containing Hybridoma-SFM (GIBCO) supplemented with FBS (Hyclone), gentamicin (GIBCO) and penicillin/streptomycin (GIBCO). mAbs were purified from serum-free culture supernatant by Protein G affinity chromatography using the Äkta FPLC system (Amersham Pharmacia, Uppsala, Sweden).

ELISA

To measure serum anti-properdin antibody titer in immunized mice, Maxisorp plates (Nunc, Roskilde, Denmark) were coated for 1hr at 37 °C with 50 μl of recombinant properdin (5 μg/ml). Wells were washed three times in washing buffer (PBS/0.05% Tween 20) and then treated with 200 μl of 1%BSA/PBS for 1hr at 25°C. The plate were then incubated with mouse serum (50 μl/well, diluted at1:62.5 to 1:32000) in 1% BSA/PBS, for 60 min at 25 °C. Wells were washed three times with washing buffer and incubated for 60 min at 25 °C with 50 μl of HRP-anti mouse IgG (Sigma, 1:4000) in 1%BSA/PBS. Wells were washed six times in washing buffer and incubated at 25 °C for 10 min with 100 μl of TMB peroxidase substrate (BD Pharmingen). The reaction was stopped with 50 μl of 2N H_2SO_4, and the OD of the samples was measured at 450 nm.

To screen hybridoma cells for desired antibody and to confirm reactivity of purified mAbs, Maxisorp plates (Nunc) were coated for 1hr at 37 °C with 50 μl of recombinant properdin (5

μg/ml). Wells were washed three times in washing buffer and then treated with 200 μl of 3%BSA/PBS for 1hr at 25°C. The plate were then incubated with the culture supernatants from hybridoma cells (50 ul/well, diluted at1:3 or 1:10) or 10-fold serially diluted purified mAbs (50 μl/well, diluted at 10 to 10^{-6} μg/ml) in 3%BSA/PBS, for 60 min at 25 °C. Plates were then processed as described above.

To measure serum fP levels, Maxisorp plates (Nunc) were coated for 1hr at 37 °C with anti-properdin mAb 14E1 (50 μl/well, 2 μg/ml). Wells were washed three times in washing buffer (PBS/0.05% Tween 20) and then treated with 1% BSA/PBS (200 μl/well) for 1hr at 25°C. After adding 50 μl/well of standard (recombinant mouse properdin) or serum samples, both serially diluted in 1%BSA/PBS, the plates were incubated for 60 min at 25 °C. Wells were washed three times with washing buffer and then incubated for 60 min at 25 °C after adding biotinylated anti-properdin mAb 5A6-7 (50 μl/well, 2 μg/ml) in 1%BSA/PBS. Biotinylation of mAb 5A6-7 was performed using EZ-link Sulfo-NHS-Biotin (Thermo Scientific, Rockford, IL) by following the manufacturer's instructions. Wells were washed three times in washing buffer and incubated for 60 min at 25 °C with streptavidin conjugated HRP (100 μl/well, BD Pharmingen) diluted 1/1000. Plates were then processed as described above.

In vivo inhibition of properdin activity in mice by mAb 14E1

C57BL/6 WT mice weighing 20–22 g were injected intraperitoneally or intravenously with 0.4 or 1.2 mg mAb 14E1. Serum samples were collected at different time points and their LPS-dependent alternative pathway complement activity were determined.

Assay of serum alternative pathway complement activity

Serum alternative pathway complement activity was determined as previously described (21). Briefly, ELISA plates were coated with LPS at 4°C overnight. After washing and blocking with BSA (50 μl/well, 1% in PBS buffer), diluted mouse serum (50 μl/well, 1:10 in Mg^{++}-EGTA GVB^{++} buffer, Sigma) was incubated on plates at 37°C for 1 hr. The plates were washed in washing buffer and plate-bound activated C3 fragments were detected using HRP-conjugated goat anti-mouse C3 antibody (50 μl/well, 1:4,000 dilution in 1% in PBS buffer, MP Biomedicals, Solon, OH).

Results

We previously demonstrated that $DAF^{-/-}CD59^{-/-}$ 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 $DAF^{-/-}CD59^{-/-}$ 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 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 hrs of reperfusion led to more severe renal IRI in $DAF^{-/-}CD59^{-/-}$ mice than in WT mice (Fig 1 and table 1). In WT mice, BUN level increased from 24.2±0.2 mg/dl before IR to 68.8±8.8 mg/dl after IR, while 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 level (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 as compared with $DAF^{-/-}CD59^{-/-}$ mice (Fig 1B and Table 1). Thus, exacerbation of renal IRI in $DAF^{-/-}CD59^{-/-}$ mice was confirmed to be critically dependent on C3.

Natural antibodies and the lectin pathway of complement have been implicated in other rodent models of IRI (3–6). To determine if they might also be involved in renal IRI of $DAF^{-/-}CD59^{-/-}$ mice, we crossed $DAF^{-/-}CD59^{-/-}$ mice with $Ig^{-/-}$, $C4^{-/-}$ and MBL-A/MBL-C double knockout ($MBL^{-/-}$) mice. We found that deficiency of antibodies, C4 or MBL did not significantly affect the susceptibility of $DAF^{-/-}CD59^{-/-}$ mice to renal IRI (Fig 2 and table 1). 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 peritubular capillaries among the different groups (Fig 2B and table 1). Together, they 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 antibodies and neither the classical nor the MBL pathway was involved.

In light of the above findings, we next examined the role of the alternative pathway (AP) of complement in this process by crossing $DAF^{-/-}CD59^{-/-}$ mice with mice deficient in complement factor B (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 (Fig 1, 3 and Table 1). 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 factor properdin ($fP^{-/-}$). 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 has suggested that fP may also bind to susceptible surfaces to initiate AP complement activation (34, 35). Comparison of $DAF^{-/-}$ $CD59^{-/-}$ $fP^{-/-}$ mice and their fP-sufficient littermates revealed that fP played a major role in renal IRI, as BUN, tubular injury, neutrophil infiltration and microvascular complement deposition in $DAF^{-/-}CD59^{-/-}$ $fP^{-/-}$ mice were all significantly reduced (Fig 3A, B and Table 1). Notably, we detected fP staining in peritubular capillaries of IRI-challenged $DAF^{-/-}CD59^{-/-}$ $fP^{+/+}$ mice but not $DAF^{-/-}CD59^{-/-}$ $fP^{-/-}$ mice littermates (Fig 3C).

Activation of the AP complement generates as effectors the anaphylatoxins C3a and C5a, as well as the membrane attack complex (MAC). The anaphylatoxins function through binding to their cognate receptors, C3aR and C5aR, 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 if 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 and Table 1). However, the magnitude of BUN reduction by either C3aR or C5aR deficiency was less than when the C3 or fB gene was deleted (Fig 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 if MAC-mediated cellular injury also played a role in the pathogenesis of renal IRI, we used an anti-C5 mAb which 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 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 if 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, and one of which, 14E1, was found to be function-blocking in a LPS-dependent AP complement activation assay (Fig 5B, C). 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 days, 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 fP$^{flox/flox}$-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 15–20 μg/ml, and that fP$^{flox/flox}$-lysozyme-Cre^{+} mice had less than 5% of WT mouse serum fP level (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 that of WT mice (Fig 5F).

We next tested if 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 intraperitoneal injection of mAb 14E1 at 24 hrs before ischemia or at 4 or 8 hrs 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). On the other hand, blocking fP at 4 hr after reperfusion was marginally effective and the protection was completely lost if the mAb was given at 8 hrs post reperfusion (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 but should be attempted prophylactically, e.g. in the setting of renal transplantation.

Discussion

In this study, we have 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 where natural antibodies and the classical or the MBL pathway were implicated (3–6), we found that complement was activated via the alternative pathway 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, non-muscle myosin heavy chain II was identified as a self antigen that became exposed on host tissues after ischemia and binding of this protein by a natural IgM antibody (CM-22) triggered MBL pathway complement activation (4, 37). Others reported natural antibodies to be pathogenic in intestinal IRI by recognizing annexin IV as a neoantigen on ischemic tissues (38). In addition, deficiency of MBL or MASP-2 was found to be protective in mouse and rat models of myocardial, intestinal and renal IRI (6, 9, 39, 40). In the present study, we confirmed that exacerbation of renal IRI in DAF$^{-/-}$CD59$^{-/-}$ mice was complement-dependent as C3 deficiency rescued the phenotype. However, we found that deficiency of Ig, C4 or MBL had no effect on renal IRI. Our data thus exclude any involvement of the classical pathway or MBL in the complement-mediated renal IRI of DAF$^{-/-}$CD59$^{-/-}$ mice. Why MBL deficiency ameliorated renal IRI in other studies (9) but not in the DAF$^{-/-}$CD59$^{-/-}$ mouse model of this study is unknown. It is possible that the discrepancy is related to differences in the experimental protocols used (e.g. wild-type vs DAF$^{-/-}$CD59$^{-/-}$ mice, length of ischemia). It should be noted, however, that although MBL and C4 are not involved in the renal IRI of DAF$^{-/-}$CD59$^{-/-}$ mice, we cannot yet rule out the possibility that

a ‘by-pass’ 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 in $DAF^{-/-}CD59^{-/-}$ mice of MASP-2, the key activation enzyme of the lectin pathway.

In contrast to the non-involvement of antibodies, MBL and the classical pathway, we found that renal IRI in $DAF^{-/-}CD59^{-/-}$ mice was completely dependent on the alternative pathway, as fB deficiency prevented exacerbation of renal IRI. Furthermore, we showed that properdin, a positive regulator of the alternative pathway, played a major pathogenic role in this process. Whether the alternative pathway 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 alternative pathway 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). These events together would render the ischemic tissues susceptible to spontaneous alternative pathway 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 has been shown that bilateral renal ischemia and reperfusion 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 ischemia and reperfusion (11). These observations are in agreement with our finding that C3a receptor deficiency was partially protective for renal IRI in $DAF^{-/-}CD59^{-/-}$ mice. Likewise, we found that C5aR receptor deficiency also ameliorated renal IRI, suggesting that C5a may have contributed to neutrophil infiltration into the outer medulla of the kidney as we previously described (20). That C5a-mediated inflammation contributed to renal IRI in $DAF^{-/-}CD59^{-/-}$ mice is also consistent with findings from other renal IRI studies where C5aR function was inhibited by a small molecule antagonist or siRNA (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 by 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 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 alternative pathway 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 a fP-deficient mouse with recombinant mouse fP 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 15–20 μg/ml in WT mice of different genetic background. 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 linage 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 alternative pathway complement, in renal IRI is imparted during the initial phase (< 4 hours) of reperfusion after ischemia.

In summary, by using $DAF^{-/-}CD59^{-/-}$ mice with an increased sensitivity to complement injury, we have dissected the complement activation pathways and pathogenic effectors in renal IRI. Our genetic and pharmacological data identify the alternative pathway, properdin, C3aR, C5aR and the MAC as components responsible for renal IRI and exclude the participation of natural antibodies, C4 and MBL. The finding of a beneficial effect of properdin targeting in renal IRI adds to a growing list of alternative pathway complement-mediated pathologies whereby anti-properdin therapy may be effective (22, 44), even though recent studies have also shown settings where properdin inhibition may not be desirable (28, 45).

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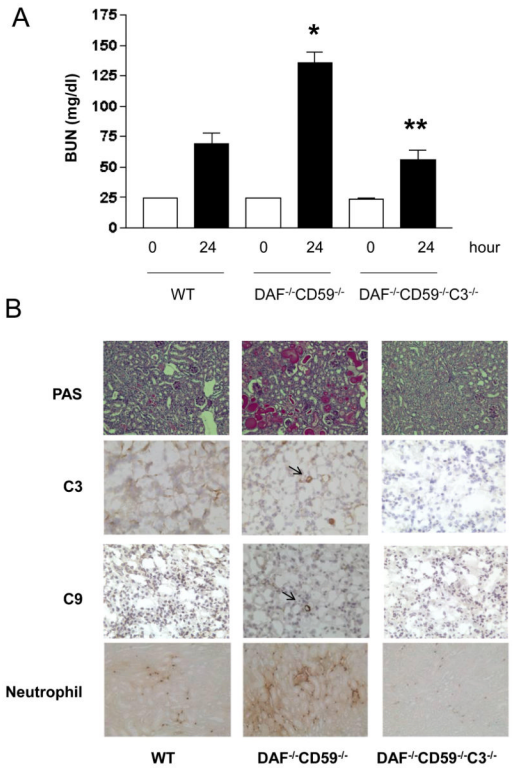


Figure 1. Deficiency of C3 ameliorates renal IRI in DAF$^{-/-}$CD59$^{-/-}$ mice

Renal pedicles were clamped bilaterally for 22 min. Blood was collected before renal pedicle clamping (0 h, □) and 24 h after reperfusion (24 h, ■) 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. *, $p < 0.0001$ vs WT mice; **, $p < 0.0001$ vs DAF$^{-/-}$CD59$^{-/-}$ mice, Student t test. (**B**) Representative images of PAS, C3, C9 and neutrophil staining in kidney sections of WT, DAF$^{-/-}$CD59$^{-/-}$ and DAF$^{-/-}$CD59$^{-/-}$ C3$^{-/-}$ mice at 24h after renal I/R, showing severe tubular injury (cast formation), deposition of C3 and C9 in peritubular capillaries 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.

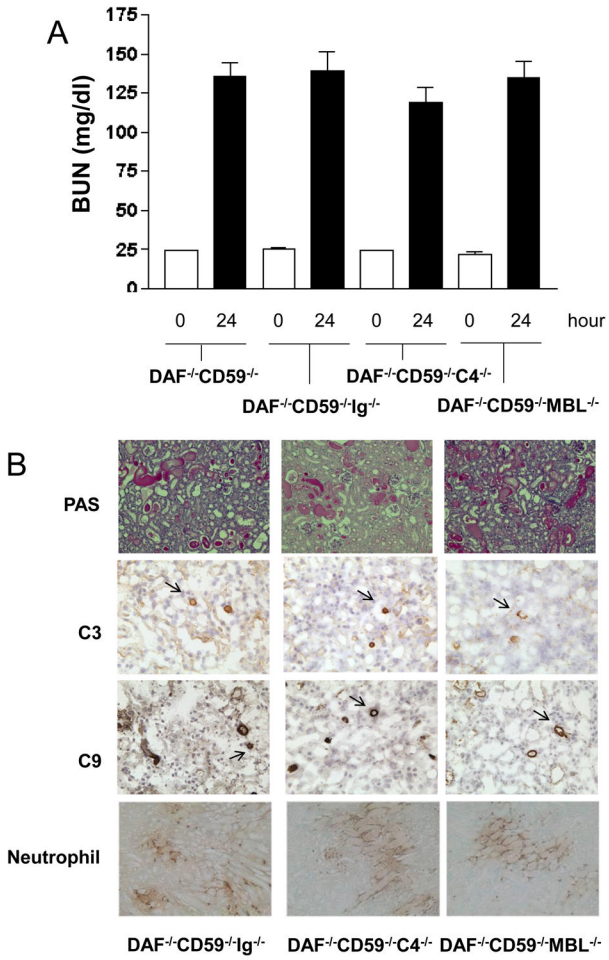


Figure 2. Deficiency of antibodies, C4 or MBL does not affect renal IRI in DAF$^{-/-}$ CD59$^{-/-}$ mice

Blood was collected before renal pedicle clamping (0 h, □) and 24 h after reperfusion (24 h, ■) for BUN determinations. (**A**) Serum levels of BUN in DAF$^{-/-}$CD59$^{-/-}$(n=37), DAF$^{-/-}$CD59$^{-/-}$ Ig$^{-/-}$ (n=25), DAF$^{-/-}$CD59$^{-/-}$ C4$^{-/-}$ (n=37) and DAF$^{-/-}$CD59$^{-/-}$ MBL$^{-/-}$(n=15) mice. (**B**) Representative images of PAS, C3, C9 and neutrophil staining in kidney sections of DAF$^{-/-}$CD59$^{-/-}$ Ig$^{-/-}$, DAF$^{-/-}$CD59$^{-/-}$ C4$^{-/-}$ and DAF$^{-/-}$CD59$^{-/-}$ MBL$^{-/-}$ mice at 24h after renal I/R, showing no effect on tubular injury, deposition of C3 and C9 in peritubular capillaries and neutrophil infiltration by Ig, C4 or MBL deficiency. Original magnification: PAS, ×200; C3, ×400; C9, ×400; neutrophil, ×200. BUN data of DAF$^{-/-}$CD59$^{-/-}$ mice in panel A are the same as in Figure 1 and are presented here for comparison.

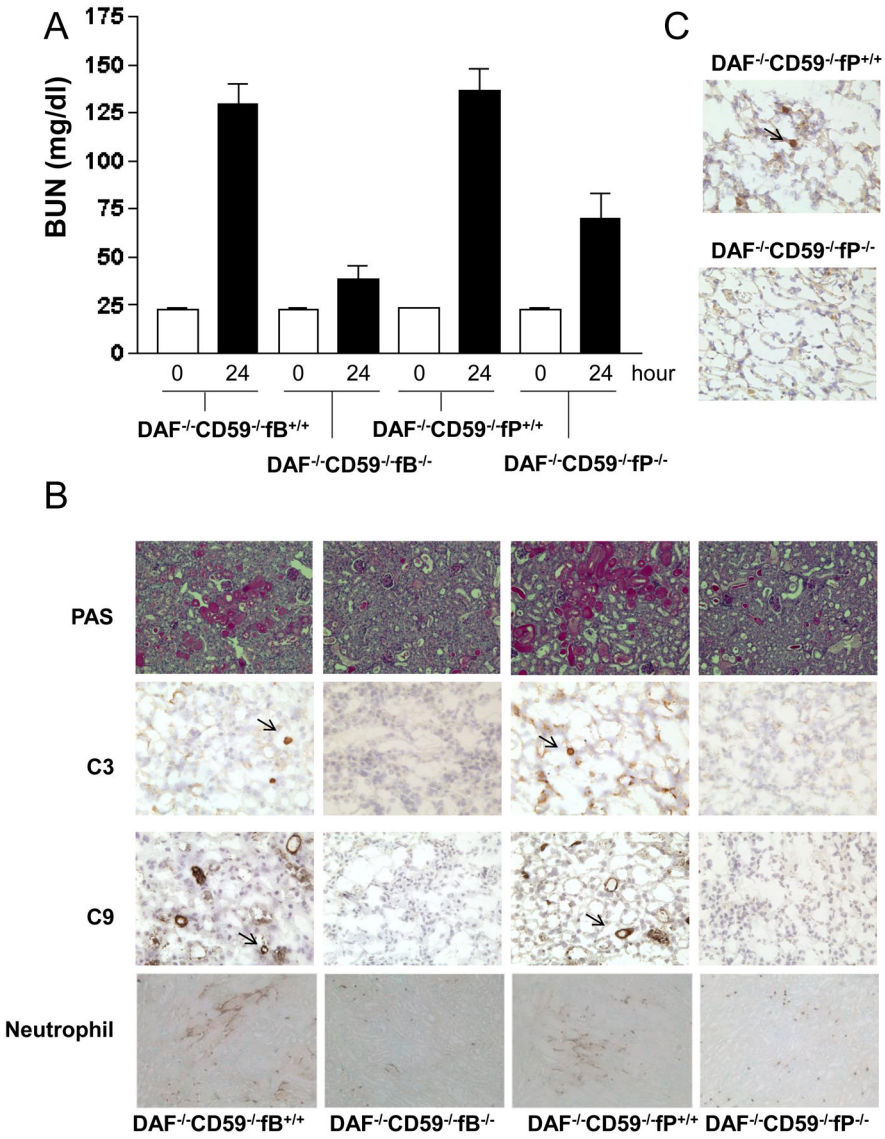
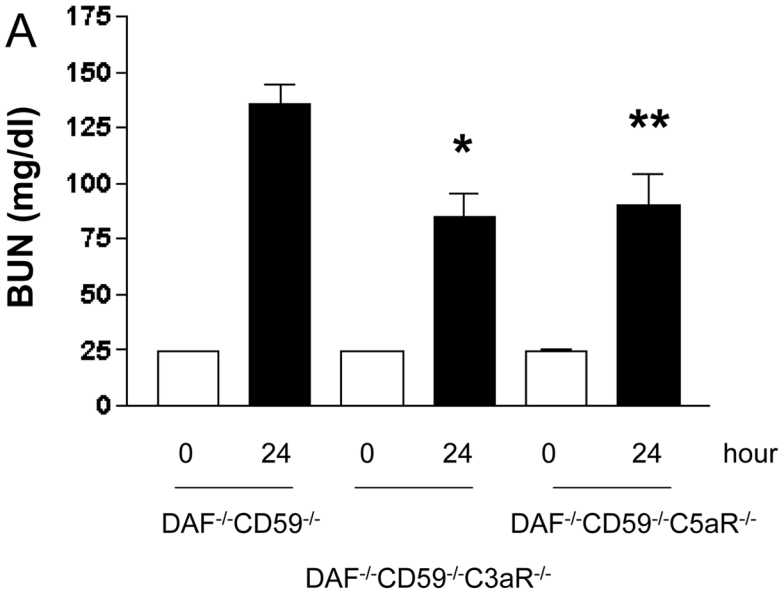


Figure 3. Deficiency of factor B or properdin ameliorates renal IRI in DAF$^{-/-}$CD59$^{-/-}$ mice

Blood was collected before renal pedicle clamping (0 h, □) and 24 h after reperfusion (24 h, ■) 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., * $p < 0.0001$ vs DAF$^{-/-}$CD59$^{-/-}$fB$^{+/+}$ mice; ** $p < 0.001$ vs DAF$^{-/-}$CD59$^{-/-}$ fP$^{+/+}$ mice. Student t test. (**B**) Representative images of PAS, C3, C9 and neutrophil staining in kidney sections of DAF$^{-/-}$CD59$^{-/-}$ fB$^{+/+}$, DAF$^{-/-}$CD59$^{-/-}$ fB$^{-/-}$, DAF$^{-/-}$CD59$^{-/-}$ fP$^{+/+}$ and DAF$^{-/-}$CD59$^{-/-}$ fP$^{-/-}$ mice at 24h after renal I/R, showing a significant reduction of tubular injury, deposition of C3 and C9 in peritubular capillaries 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 at 24h after renal I/R, showing deposition of properdin in peritubular capillaries in DAF$^{-/-}$CD59$^{-/-}$ fP$^{+/+}$ but not in DAF$^{-/-}$CD59$^{-/-}$ fP$^{-/-}$ mice. Original magnification: PAS, ×200; C3, ×400; C9, ×400; neutrophil, ×200; properdin, ×400.



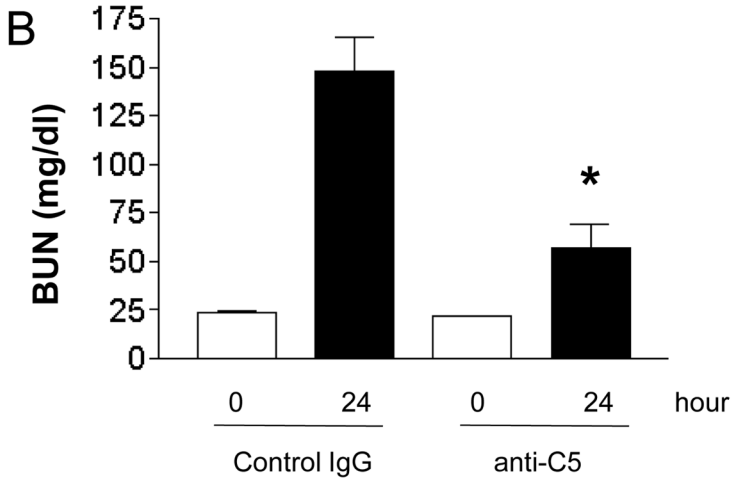


Figure 4. Both anaphylatoxin receptors C3aR and C5aR and the membrane attack complex contribute to renal IRI in DAF$^{-/-}$CD59$^{-/-}$ mice

Blood was collected before renal pedicle clamping (0 h, □) and 24 h after reperfusion (24 h, ■) for BUN determinations. (**A**) Serum levels of BUN at 24hr after renal I/R in DAF$^{-/-}$CD59$^{-/-}$C3aR$^{-/-}$(n=20) and DAF$^{-/-}$CD59$^{-/-}$ C5aR$^{-/-}$ mice (n=33) were significantly lower than that of DAF$^{-/-}$CD59$^{-/-}$ mice (n=37). DAF$^{-/-}$CD59$^{-/-}$ mouse data are the same as in Figure 1 and are presented here for comparison. Data shown are mean ± SEM. *, $p < 0.001$, **, $p < 0.01$ vs DAF$^{-/-}$CD59$^{-/-}$ mice, Student t test. (**B**) Serum levels of BUN at 24hr after renal I/R in DAF$^{-/-}$CD59$^{-/-}$ mice treated with an anti-C5 mAb (BB5.1, n=13) or a control mAb (MOPC, n=7) 24 hours before ischemia surgery. Data shown are mean ± SEM. *, $p < 0.001$ vs control IgG, Student t test.

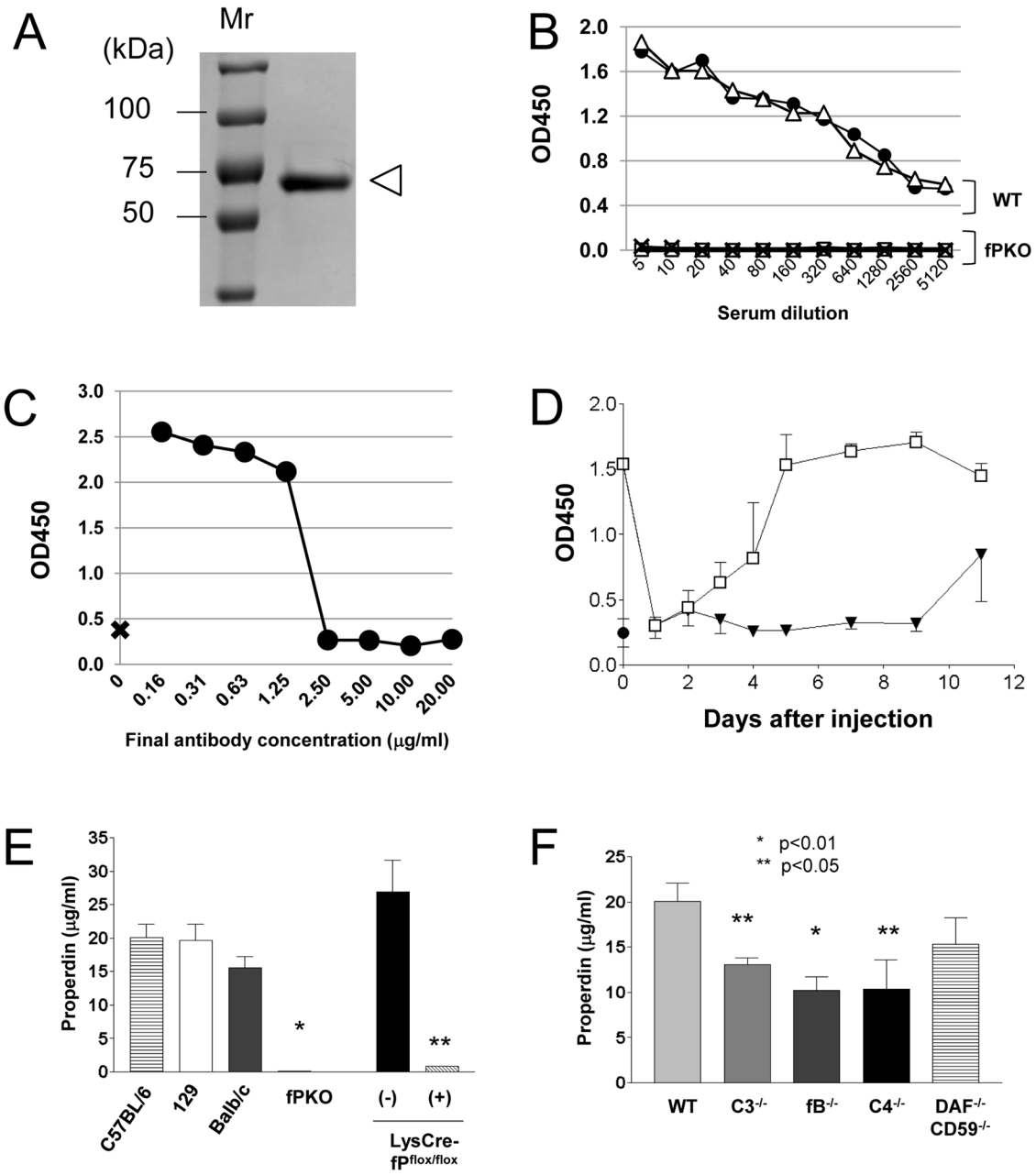


Figure 5. Generation and characterization of monoclonal antibodies against mouse properdin

(**A**) SDS-PAGE analysis of purified recombinant mouse properdin. Mr: molecular weight markers (**B**) Reactivity of mAbs with mouse serum properdin by sandwich ELISA. Two mAbs, 14E1 and 5A6-7, were used as the capture and detection antibody, respectively, in the sandwich ELISA assay. Properdin was detected in WT mouse sera (n=2) but not in the sera of a line of fP knockout mice (n=2) derived from $fP^{flox/flox}$ mice after Cre-mediated germline deletion (fPKO). (**C**) At 2.5 μg/ml or above, mAb 14E1 (●) blocked mouse properdin activity in a LPS-induced alternative pathway complement activation assay using WT mouse serum (1:10 dilution). $fP^{-/-}$ mouse serum of the same dilution and without 14E1 mAb added (×) was used as a control. (**D**) *In vivo* effect of mAb 14E1. WT mice (n=3 per group) were given a single intraperitoneal injection of 0.4 mg (□) or 1.2 mg (▼) of the

antibody. Serum samples were collected at the indicated time points and assayed (at 1:10 dilution) for LPS-induced alternative pathway complement activity as in panel **C**. Similarly diluted $fP^{-/-}$ mouse serum (●) was used as a control. (**E**) Serum levels of properdin in WT mice of different genetic background, and in mice with myeloid lineage cell-specific deletion of properdin ($LysCre^{+}$-$fP^{flox/flox}$) and their Cre-negative littermates. Sera from fPKO mice were used as a negative control. C57BL/6 (n=5), 129J (n=4), Balb/c (n=4), fPKO(n=4), Lyso-Cre^{+}-$fP^{flox/flox}$ (n=6) and Lyso-Cre^{-}-$fP^{flox/flox}$ (n=5). Values are mean ± SEM; * P< 0.0001 vs C57BL/6; ** P< 0.001 vs Lyso-Cre^{-}-$fP^{flox/flox}$, Student t test. (**F**) Serum properdin levels in C57BL/6 (n=5, same as in panel **E**), $C3^{-/-}$ (n=6), $fB^{-/-}$ (n=4), $C4^{-/-}$ (n=5) and $DAF^{-/-}CD59^{-/-}$ (n=5) mice, showing that the level was significantly lower in $C3^{-/-}$, $fB^{-/-}$ and $C4^{-/-}$ mice but not in $DAF^{-/-}CD59^{-/-}$ mice. Values are mean ± SEM; * P< 0.01 and ** P< 0.05 vs WT, Student t test.

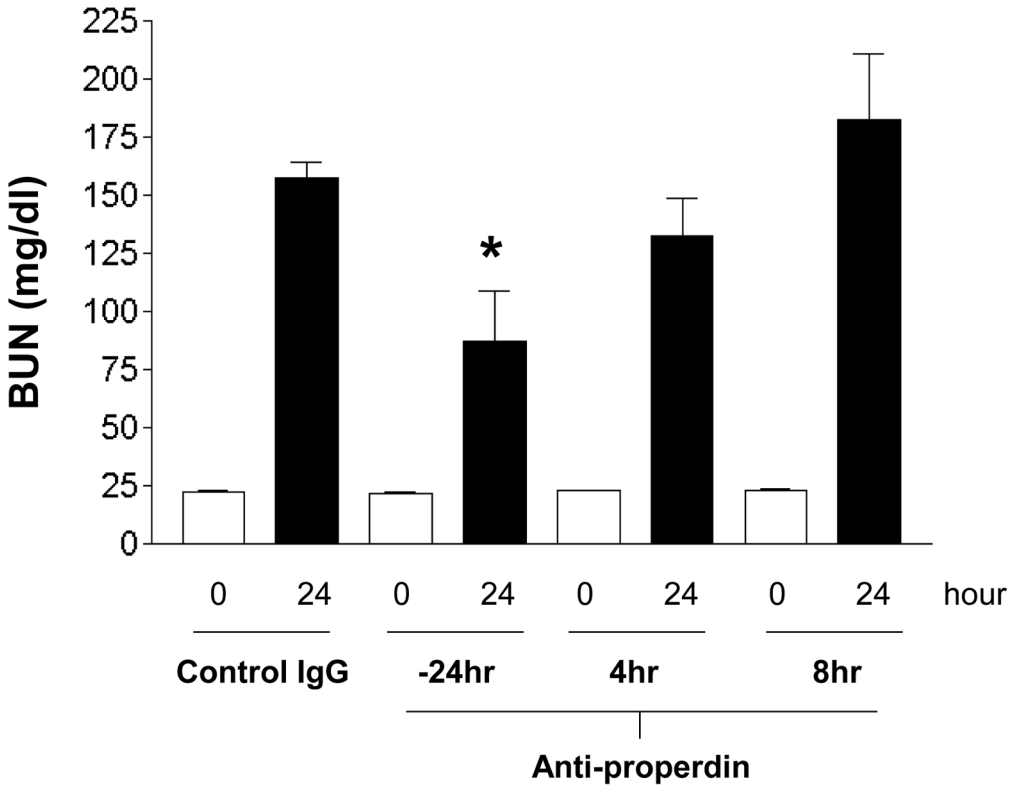


Figure 6. Systemic inhibition of properdin with mAb before but not after IR ameliorates renal IRI in DAF$^{-/-}$CD59$^{-/-}$ mice

Mice were treated with a control IgG (MOPC, n=10) at 24hr before renal I/R surgery, or with a mouse anti-mouse properdin mAb (14E1) at 24 hours before I/R surgery (n=7), 4 hour (n=4) or 8 hours (n=5) after reperfusion. Blood was collected before renal pedicle clamping (0 h, □) and 24 h after reperfusion (24 h, ■) for BUN determinations. Data shown are mean ± SEM. *, $p < 0.05$ vs control IgG, Student t test.

Table 1

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 viewing field. Tubular damage and C3, C9 staining are scored semi-quantitatively as described in Materials and Methods.

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 *
$DAF^{-/-}CD59^{-/-}$	3.2±0.6	96.5±13.4	1.4±0.2	1.3±0.2
$C3^{-/-}DAF^{-/-}CD59^{-/-}$	0.4±0.4 *	33.2±11.0 *	0±0 *	0.4±0.2 *
$Ig^{-/-}DAF^{-/-}CD59^{-/-}$	3.2±0.7	95.7±18.9	1.3±0.5	1.3±0.2
$C4^{-/-}DAF^{-/-}CD59^{-/-}$	3.6±0.5	90.7±20.2	1.3±0.3	1.1±0.3
$MBL^{-/-}DAF^{-/-}CD59^{-/-}$	3.1±1.0	95.0±15.9	1.1±0.1	1.1±0.1
$fB^{+/+}DAF^{-/-}CD59^{-/-}$	3.3±0.7	96.8±15.3	1.2±0.2	1.3±0.2
$fB^{-/-}DAF^{-/-}CD59^{-/-}$	0.5±0.6 **	19.2±4.9 **	0.1±0.1 **	0.4±0.2 **
$fP^{+/+}DAF^{-/-}CD59^{-/-}$	2.6±0.9	101.0±15.3	1.0±0.2	1.3±0.3
$fP^{-/-}DAF^{-/-}CD59^{-/-}$	1.1±1.2 ***	58.0±11.6 ***	0.4±0.1 ***	0.6±0.2 ***
$C5aR^{-/-}DAF^{-/-}CD59^{-/-}$	1.9±1.0 *	46.1±10.9 *	0.6±0.4 *	0.7±0.2 *
$C3aR^{-/-}DAF^{-/-}CD59^{-/-}$	0.9±1.0 *	49.7±11.9 *	0.3±0.3 *	0.7±0.2 *

Values shown are mean ± SEM.

* $p < 0.05$ vs $DAF^{-/-}CD59^{-/-}$ mice;

** $p < 0.05$ vs $fB^{+/+}DAF^{-/-}CD59^{-/-}$ mice;

*** $p < 0.05$ vs $fP^{+/+}DAF^{-/-}CD59^{-/-}$ mice.