CD59 Protects Glomerular Endothelial Cells from Immune-Mediated Thrombotic Microangiopathy in Rats

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Abstract. CD59 is a cell membrane-bound complement regulatory protein on glomerular cells that inhibits C5b-9 assembly and insertion. This report describes a recently developed model of immune thrombotic microangiopathy (TMA) induced by the renal artery perfusion of anti-glomerular endothelial cell (anti-GEN) antibody. To examine the role of CD59 in protecting the GEN from immune-mediated injury, rats underwent selective renal artery perfusion with F(ab')2 fragments of anti-CD59 monoclonal antibody to block CD59 activity or control mouse IgG followed by anti-GEN antibody or control goat IgG. Neutralization of CD59 in normal rats did not result in any significant functional or histologic changes. Perfusion with anti-CD59 did not change deposition of the pathogenic anti-GEN IgG used to induce the TMA model. However, neutralization of CD59 in the TMA model resulted in more C5b-9 formation in glomeruli, accompanied by increased platelet and fibrin deposition, more severe endothelial injury, and reduced renal function compared with the animals perfused with control F(ab')2 fragments. These results demonstrate directly that CD59 serves a protective role for GEN in this TMA model of rats, and confirm that C5b-9 formation has a critical pathogenic role in the mediation of the disease. CD59 may play an important role in protecting glomerular endothelium from other complement-mediated types of injury. (J Am Soc Nephrol 9: 590–597, 1998)

Many forms of human glomerular disease are autoimmune in nature and characterized by glomerular deposition of immunoglobulin and complement (1,2). Studies of the mediation of glomerular injury have been greatly facilitated by a variety of animal models that closely mimic the structural and functional features of human glomerulonephritis. Complement activation mediates immune tissue injury through several mechanisms, including: (1) localization of circulating inflammatory cells through generation of chemotactic factors and immune adherence mechanisms and (2) direct injury to resident glomerular cells through membrane insertion of the membrane attack complex C5b-9 (2,3). The role of C5b-9 in glomerular injury has been particularly well established in the passive Heymann nephritis model of membranous nephropathy in rats (4–6). More recently, C5b-9 has also been shown to play an important role in the pathogenesis of experimental mesangio proliferative glomerulonephritis induced by antithymocyte serum, which is directed at antigens expressed on the mesangial cell membrane (ATS model) (7) and in experimental renal thrombotic microangiopathy (TMA) in rats induced by antibodies to the glomerular endothelial cell (GEN) (8).

Under normal circumstances, glomerular cells are protected from C5b-9 attack by circulating and cell-bound complement regulatory proteins (9). A principal cell-bound regulator of C5b-9 activation is CD59, an Mr 18,000 to 20,000 glycosylphosphatidylinositol-anchored protein that limits incorporation of C9 thereby preventing formation of a lytic lesion (10,11). The functional importance of CD59 in protecting cultured glomerular cells from complement attack in vitro has been demonstrated both by neutralization with antibody (12–14) and by overexpression studies (15). A role for CD59 has also been shown in vivo using a model of experimental glomerulonephritis induced by in situ formation of lectin immune complexes (16).

We hypothesized that CD59 expressed on GEN might confer protection from C5b-9-mediated injury induced by antibody binding directly to GEN antigens. We tested this hypothesis by neutralizing CD59 in a new model of TMA induced with antibody to GEN. Our results document a significant protective effect of CD59 in this model.

Materials and Methods

Induction of the TMA Model and Neutralization of CD59

The TMA model was induced by selective perfusion of the right kidney through the superior mesenteric artery with the purified IgG of
Isolation of F(ab')2 Fragments

F(ab')2 fragments of the 6D1 monoclonal antibody to rat CD59 and irrelevant mouse monoclonal IgG were isolated using an ImmunoPure IgG1 F(ab')2 preparation kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Briefly, mouse IgG was incubated in an immobilized ficin column in the presence of 1 mM cysteine to generate F(ab')2 fragments at 37°C (19), and the F(ab')2 produced was isolated using a protein A column. The eluted F(ab')2 was dialyzed against PBS, sterilized by filtration, and stored at 4°C.

Experimental Animals

Male Wistar rats weighing 200 to 300 g were purchased from Simonsen Laboratory (Gilroy, CA). All animal studies were performed in an accredited animal care facility in accord with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental Protocol

Three groups of rats were studied (group I: rats perfused with 0.15 mg of F(ab')2 fragments of 6D1 and anti-GEN [n = 8]; group II: rats perfused with 0.15 mg of F(ab')2 fragments of irrelevant mouse IgG and anti-GEN [n = 8]; and group III: to ensure that injury was not augmented by binding of anti-CD59 IgG to GEN, rats were perfused with 0.15 mg of F(ab')2 fragments of 6D1 followed by normal goat IgG [n = 3]). Ten minutes after perfusion, a survival biopsy was performed in two animals in groups I and II. Four hours after perfusion, a survival renal biopsy of the perfused kidney was performed in all groups. Rats were then housed in metabolic cages with free access to water, and urine was collected overnight. Twenty-four hours after induction of disease, a blood sample was obtained via a cardiac puncture for measurement of blood urea nitrogen (BUN), and the kidney was again biopsied for histologic analysis.

Renal Histology

Methyl Carnoy's fixed tissue was processed, and 4-μm sections were stained with various antibodies as follows. Glomerular platelet infiltration was assessed by staining with PL-1, a murine monoclonal antibody to rat platelets (generously supplied by W. W. Baker, University of Groningen, Groningen, The Netherlands) (21). GEN were stained with RECA-1, a monoclonal IgG, antibody specific for rat endothelial cells (22).

Tissue for immunofluorescence was embedded in OCT (Lab-Tek Products, Miles Laboratories, Naperville, IL) and snap-frozen in isopentane. Fibrinogen was detected by staining with FITC-conjugated goat anti-rat fibrinogen IgG (Cappel, Durham, NC) at the final concentration of 25 μg/ml. Rat C3 was detected with FITC-conjugated goat anti-rat C3 (Cappel). The presence of rat C5b-9 was determined using a biotinylated anti-rat C5b-9 monoclonal antibody 2A1 (23), followed by FITC-streptavidin.

Serum BUN and Urinary Protein Measurement

BUN was determined colorimetrically using a commercial kit for the measurement of urea nitrogen (Sigma Diagnostics, St. Louis, MO). Urine protein excretion was measured using the sulfosalicylic acid method.

Purification of Goat IgG

Goat IgG for renal artery perfusion was purified using a caprylic acid precipitation method as described previously (18). Briefly, after adjusting the pH of the plasma to 5.5 with HCl, 5.5 ml of caprylic acid solution/100 ml of plasma (Sigma, St. Louis, MO) was added dropwise with vigorous stirring. Then the solution was stirred for 1 h at room temperature and centrifuged at 14,000 rpm for 30 min at room temperature. The supernatant was collected and dialyzed against PBS. The dialyzed solution was centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was sterilized by filtration and stored at 4°C.

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Statistical Analyses
Data were reported as mean ± SD. Data were analyzed by t test with adjustments using the Bonferroni/Dunn method. In the case of histologic analysis, a total of 20 (40 for PL-1 staining) glomeruli of each animal were scored on the scale of 0 to 4 based on the intensity of the staining or the extent of tubular injury. The average scores of group I and group II were compared using Fisher’s method (n = 8 for each group).

Results
Anti-GEN IgG Deposition
All rats perfused with anti-GEN IgG exhibited diffuse glomerular staining for goat Ig along all capillary walls in a coarse, uninterrupted pattern consistent with staining GEN (Figure 1, A and B). To determine whether perfusion with anti-CD59 changed deposition of the pathogenic anti-GEN IgG, we performed immunofluorescence studies with anti-goat IgG, using biopsies 4 h after induction of disease. Quantification of immunofluorescence demonstrated that there was no difference in deposition of the pathogenic goat IgG between TMA rats perfused with anti-CD59 (group I) and those perfused with control mouse IgG (group II) (fluorescence: 379.8 ± 55.7 and 351.8 ± 47.2, respectively, P > 0.05) (Figure 1, A and B). Rats perfused with anti-CD59 and normal goat IgG did not show positive staining for goat IgG. In contrast, staining for mouse IgG, which represented binding of anti-CD59, was observed only in rats perfused with anti-CD59 (Figure 1, C and D).

Glomerular C5b-9 Formation
Using this dose of anti-GEN IgG to induce disease, segmental C5b-9 formation was observed along capillary walls in most of the glomeruli of rats perfused with anti-CD59 (group I) 4 h after induction of disease. C5b-9 staining was less often detected in TMA rats perfused with control mouse IgG (group II) (Figure 2, A and B). C5b-9 formation in glomeruli of TMA rats perfused with anti-CD59 (group I) was 0.45 ± 0.18 by semiquantification with a visual scoring system, whereas that of control TMA rats (group II) was 0.19 ± 0.12 (P < 0.05). No C5b-9 staining was seen in rats perfused with anti-CD59 and normal goat IgG.

C3 staining was not detectable in any samples obtained at 4 h after perfusion. However, there was marked and equal C3 deposition at 10 min in glomerular endothelium of TMA rats perfused with anti-CD59 (group I) and TMA rats perfused with control mouse IgG (group II) (Figure 2, C and D). The pattern

Figure 1. Deposition of anti-glomerular endothelial cell (GEN) and anti-CD59. Deposition of anti-GEN, which was observed along glomerular capillary walls, was not different between thrombotic microangiopathy (TMA) rats perfused with anti-CD59 (A; group I) and TMA rats perfused with control mouse IgG (B; group II) at 4 h. In contrast, deposition of mouse IgG was observed in glomeruli of rats perfused with anti-CD59 at 10 min (C), but not in glomeruli of rats perfused with control mouse IgG (D). Magnification, ×400.

Figure 2. C5b-9 membrane attack complex formation and C3 deposition in glomeruli. Representative glomeruli stained for C5b-9 at 4 h (A and B) and C3 at 10 min (C and D). More C5b-9 formation along glomerular capillary walls was segmentally observed 4 h after induction of disease in glomeruli of TMA rats perfused with anti-CD59 F(ab')2 fragments (A) compared with TMA rats perfused with control F(ab')2 fragments (B). In contrast, no difference in C3 deposition in glomerular endothelium was observed at 10 min between TMA rats perfused with anti-CD59 (C) and TMA rats perfused with control mouse IgG (D). Magnification, ×400.
of C3 staining was similar to that of C5b-9 with capillary wall staining in a focal and segmental distribution. There was no C5b-9 or C3 staining in rats perfused with anti-CD59 and normal goat IgG (group III).

Morphological Analysis of Glomerular and Tubulointerstitial Injury

Periodic acid-Schiff staining at day 1 demonstrated multiple thrombi in the glomerular capillary loops of TMA rats (groups I and II) (Figure 3A), whereas no thrombi were seen in control rats (group III). TMA rats perfused with anti-CD59 (group I) had more glomerular thrombi compared with those perfused with control IgG (group II) (17.2 ± 8.0% of glomeruli versus 7.8 ± 5.0%, P < 0.05). In tubulointerstitial areas, focal tubular damage such as tubular necrosis, sloughing of tubular epithelial cells, and tubular cast formation was seen in TMA rats (groups I and II) (Figure 3B), whereas no tubular damage was observed in rats perfused with anti-CD59 and control goat IgG (group III). Tubular injury was 0.50 ± 0.36 in TMA rats perfused with anti-CD59 (group I) (Figure 3C), whereas it was only 0.08 ± 0.08 in control TMA rats (group II) (Figure 3D) (P < 0.05).

Fibrin Deposition and Platelet Accumulation in Glomeruli

Fibrin deposition in glomeruli was assessed by immunofluorescence studies with samples obtained at day 1. TMA rats perfused with anti-CD59 (group I) showed more fibrin deposition compared with those perfused with control mouse IgG (group II) (Figure 4, A and B). Quantitative immunofluorescence densitometry confirmed a significant increase in fibrin deposition in glomeruli of TMA rats perfused with anti-CD59 (group I) compared with those perfused with control mouse IgG (group II) (fluorescence: 389.8 ± 220.9 and 168.2 ± 31.6, respectively, P < 0.05). Rats perfused with anti-CD59 and normal goat IgG had no fibrin deposition in glomeruli.

Platelet accumulation in glomeruli was also assessed by immunostaining with platelet-specific monoclonal antibody PL-1, using samples obtained 4 h after induction of disease. TMA rats perfused with anti-CD59 (group I) demonstrated more platelet accumulation compared with those perfused with control mouse IgG (group II) (Figure 4, C and D). Semiquantification of the immunostaining for PL-1 was performed using a visual scoring system (scale, 0 to 4). Glomerular PL-1 staining of rats perfused with anti-CD59 (group I) was 2.7 ± 0.4, whereas in control TMA rats (group II) it was 1.3 ± 0.3 (P < 0.001). No platelet accumulation was observed in rats perfused with anti-CD59 and normal goat IgG (group III).

Figure 3. Periodic acid-Schiff staining at 24 h. Multiple thrombi in the glomerular capillary loops were observed in TMA rats. Thrombus formation was more frequently seen in rats perfused with anti-CD59 F(ab')2 fragments (A) compared with rats perfused with control mouse IgG (B). Arrows indicate thrombus formation in the capillary loops. Magnification, ×400. Tubular injury was also more prominent in rats perfused with anti-CD59 F(ab')2 fragments (C) compared with rats perfused with control mouse IgG (D). Magnification, ×200.

Figure 4. Fibrin deposition and platelet accumulation in glomeruli. More fibrin deposition was observed in glomeruli of TMA rats perfused with anti-CD59 F(ab')2 fragments (A) compared with rats perfused with control F(ab')2 fragments (B). More platelet accumulation was also observed in glomeruli of rats perfused with anti-CD59 F(ab')2 fragments (C) compared with rats perfused with control F(ab')2 fragments (D). Magnification, ×400.
Staining with Endothelial Cell-Specific Antibody

To assess endothelial cell damage, we performed staining with an endothelial cell-specific monoclonal antibody, RECA-1, using samples obtained at day 1. Rats perfused with anti-CD59 and control goat IgG (group III) showed an intact staining pattern of RECA-1, whereas some of the glomeruli in TMA rats (groups I and II) showed a decrease in staining (Figure 5). Semiquantification of RECA-1 staining suggested that TMA rats perfused with anti-CD59 (group I) lost more staining pattern of RECA-1, whereas some of the glomeruli in anti-CD59 and control goat IgG (group III) showed an intact endothelial cell-specific antibody staining with an endothelial cell-specific monoclonal antibody, RECA-1, using samples with an endothelial cell-specific monoclonal antibody.

Table 1. Summary of histologic, immunohistochemical, and immunofluorescence studies

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group I Anti-CD59/</th>
<th>Group II Control/</th>
<th>Group III Anti-CD59/</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat IgG (4 h) (fluorescence units)</td>
<td>380 ± 56</td>
<td>352 ± 43</td>
<td>0</td>
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<tr>
<td>Mouse IgG (4 h) (0 to 4+)</td>
<td>++</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Rat C5b-9 (4 h) (0 to 4+)</td>
<td>0.45 ± 0.18</td>
<td>0.19 ± 0.12</td>
<td>0</td>
</tr>
<tr>
<td>Rat C3 (10 min) (0 to 4+)</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Glomerular thrombi (%)</td>
<td>17 ± 8</td>
<td>8 ± 5</td>
<td>0</td>
</tr>
<tr>
<td>Tubular injury (0 to 5+)</td>
<td>0.50 ± 0.36</td>
<td>0.08 ± 0.08</td>
<td>0</td>
</tr>
<tr>
<td>Fibrin (24 h) (fluorescence units)</td>
<td>390 ± 221</td>
<td>168 ± 32</td>
<td>0</td>
</tr>
<tr>
<td>Glomerular platelets (4 h) (0 to 4+)</td>
<td>2.7 ± 0.4</td>
<td>1.3 ± 0.3</td>
<td>0</td>
</tr>
<tr>
<td>GEN staining (24 h) (0 to 4+)</td>
<td>3.52 ± 0.23</td>
<td>3.80 ± 0.13</td>
<td>4</td>
</tr>
<tr>
<td>BUN (24 h) (mg/d)</td>
<td>59 ± 13</td>
<td>41 ± 7</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Urine protein (mg/d)</td>
<td>31 ± 10</td>
<td>27 ± 5</td>
<td>22 ± 2</td>
</tr>
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* The results of scoring of tubular injury, platelet aggregation, C5b-9 deposition, and GEN staining are shown as mean ranks. TMA, thrombotic microangiopathy; GEN, glomerular endothelial cell; BUN, blood urea nitrogen.

Effects of Neutralization of CD59

The BUN levels of anti-CD59-perfused TMA rats (group I) at day 1 were significantly higher than those of control TMA rats (group II) (58.9 ± 12.6 mg/dl versus 40.8 ± 7.4 mg/dl, P < 0.01). Rats perfused with anti-CD59 and normal goat IgG (group III) did not show an increase in BUN levels (25.8 ± 2.8 mg/dl) compared with rats perfused with only normal goat IgG (n = 2, 31.3 ± 2.8 mg/dl, P > 0.05). Urinary protein excretion was not different among the three groups of rats (group I: 30.8 ± 10.2 mg/d; group II: 27.3 ± 4.8 mg/d; group III: 22.3 ± 1.8 mg/d, P > 0.05).

Discussion

The role of complement in mediating glomerular injury has been well established in numerous studies in which antibody-induced diseases have been substantially modified or prevented by maneuvers that block or inhibit complement activation (1,2). The primary nephritogenic effect of complement activation was initially attributed to release of chemotactic factors such as C5a, leading to recruitment of inflammatory cells. However, it has subsequently become clear that insertion of C5b-9 into glomerular cell membranes is also a major mechanism of glomerular injury (1,2,5). Injury induced by C5b-9 may involve direct lysis of resident glomerular cells or may occur as a result of stimulation of cells by sublytic quantities of C5b-9 to release a variety of cytokines, growth factors, and other inflammatory mediators (15,27,28).

Subsequent to establishing the central role of the complement system in mediating glomerular injury, it has been recognized that complement activation in vivo is carefully regulated by a host of circulating and cell-bound complement regulatory proteins that act to prevent complement-mediated tissue injury (9,29). With regard to C5b-9, the principal cell-bound molecule that prevents C5b-9 attack is CD59. CD59 is an 18- to 20-kD membrane glycoprotein anchored to the membrane via glycosyl-phosphatidylinositol. CD59 was originally purified from erythrocyte membranes by several groups independently (30–33). CD59 inhibits C5b-9 formation by incorporating into the forming complex at the C5b-8 stage and blocking the uptake and insertion of C9 molecules (10,11). In

Figure 5. Glomerular endothelial staining with RECA-1. Rats perfused with anti-CD59 Fab'2 fragments (A) showed endothelial injury associated with a decrease in RECA-1 staining at 24 h that was more severe than that observed in controls treated with control Fab'2 fragments (B). Magnification, ×400.
the glomerulus of both rat and human, CD59 is expressed on the membrane of all three resident glomerular cells (12, 14, 34–37).

The functional importance of CD59 in modulating antibody complement-induced injury in cultured glomerular cells in vitro has been demonstrated by neutralization of CD59 with antibodies and by overexpression of CD59 in permanently transfected glomerular cells. Blocking of CD59 in cultured glomerular cells with neutralizing antibodies resulted in an increase in susceptibility to complement-mediated cell lysis (12–14). When rat glomerular mesangial cells were transfected with CD59, there was no change in mesangial cell phenotype, but transfected cells overexpressed CD59 and were extremely resistant to both lytic and sublytic effects of C5b-9 (15). Although less is known of the significance of CD59 but transfected cells overexpressed CD59 and were extremely resistant to both lytic and sublytic effects of C5b-9 (15). Matsuo et al. recently studied an experimental glomerulonephritis model induced by a lectin (16). They observed an increase in glomerular deposition of C9, prolonged deposition of fibrin, enhanced expression of intercellular adhesion molecule-1, increased leukocyte infiltration, cell proliferation, and hypercellularity in their lectin/antilectin model perfused with anti-CD59 to neutralize the regulatory capacity of this molecule. In the present study, we focused specifically on the GEN and examined the consequences of neutralizing CD59 with antibody before inducing TMA with antibody to rat GEN (17).

To ensure that disease severity in control animals was mild enough to permit easy detection of an increase in disease resulting from CD59 neutralization, we used a lower dose of anti-GEN than previously reported (8, 17). To ensure that prior perfusion with neutralizing anti-CD59 antibody did not cause disease by itself and thereby augment the effect of anti-GEN antibody by mechanisms independent of complement regulation, we used non-complement fixing F(ab′)2 fragments of anti-CD59 and included a control group (group III) perfused with anti-CD59 followed by control goat IgG. Anti-CD59 deposition alone induced no detectable glomerular injury. However, our findings document a significant result in both structural and functional measurements of disease severity in the animals in which CD59 was neutralized before disease induction (group I) compared with unmodified disease (group II). Compared with controls, anti-CD59-treated animals had more C5b-9 formation in glomeruli, accompanied by a significant increase in glomerular thrombosis, fibrin deposition, and platelet aggregation, as well as more severe interstitial disease and worse renal function (Table 1). More GEN damage was also indicated in anti-CD59-perfused rats using RECA-1 antibody, although we could not rule out the possibility that the endothelial cell antigens were redistributed and quickly shed in response to anti-GEN binding without associated endothelial injury (38, 39). The severe interstitial damage can be attributed, at least in part, to injury to peritubular capillaries by the pathogenic antibody, as described in our previous report (17).

CD59 inhibits the formation of C5b-9 (10, 11), whereas other complement regulatory proteins, such as decay accelerating factor, membrane cofactor protein, and Crry, reduce C3/C5 convertase activity (39–43). Therefore, CD59 likely plays a more important role in situations in which C5b-9-mediated processes are critical, whereas those regulatory proteins that work at the C3/C5 convertase level would also function in situations in which generation of local inflammatory mediators derived from the cleavage of more proximal complement components such as C5a play a major pathogenic role. The significant effect obtained by neutralization of CD59 not only demonstrates that CD59 plays an important protective role in vivo, but also suggests that C5b-9 is an important modulator of injury in our TMA model. In fact, studies using a C6-deficient strain of PVG rats indicate that almost all of the aspects of this model are mediated entirely by C5b-9 (8).

There are many possible mechanisms with which C5b-9 can mediate endothelial damage and TMA. It is well known that C5b-9 can induce cell lysis. In addition, C5b-9 inserted in the membrane of nucleated cells, which are generally more resistant to cell lysis than non-nucleated cells, can induce various effects on the function, metabolism, and morphology of the cells (2, 3, 27, 44). Furthermore, there is increasing evidence that the complement system directly changes vascular homeostasis and promotes both the adherence of blood cells to the vessel walls and the formation of fibrin through the enzymatic cascades of the coagulation system (45, 46). The mechanisms of platelet activation and fibrin deposition mediated by C5b-9 are multifactorial. C5b-9 induces secretion of multimers of endothelial von Willebrand factor (47) and stimulates endothelial prothrombinase (48) and tissue factor activity (49). Complement injury releases heparan sulfate proteoglycan from endothelium (50). Furthermore, complement induces endothelial morphologic changes, which may expose preexisting inducers of thrombosis in the underlying matrix to plasma-clotting factors and platelets (51). It was also reported that anti-CD59 antibody augmented the C5b-9-induced cellular responses that enhance the coagulation cascade, such as secretion of von Willebrand factor and expression of the catalytic surface for the prothrombinase enzyme complex, in cultured human umbilical vein endothelial cells (52). The ability of C5b-9 to upregulate expression of leukocyte adhesion molecules on the endothelial cell might also contribute to platelet localization (53). Although the precise mechanisms of the C5b-9 nephritogenic effect in this model are not established by this study, it is likely that many of the factors described above are involved and were augmented by neutralization of CD59.

In conclusion, our findings confirm earlier studies of Matsuo et al., which establish a significant protective role for CD59 in modulating immune glomerular injury in vivo, and we extend their observations by localizing the effect to the GEN in this model. Our findings strongly support the hypothesis that modulation of CD59 activity may have important benefits in multiple diseases in which anti-endothelial antibodies may be pathogenic, including vasculitis and xenograft rejection (54–56).

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