CD59a Deficiency Exacerbates Accelerated Nephrotoxic Nephritis in Mice

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Abstract. CD59 is a complement regulatory protein that inhibits the terminal part of the complement system, the membrane attack complex (MAC), a mediator of renal injury. Mice deficient in the Cd59a gene (mCd59a−/−) were used to investigate the role of CD59 in experimentally induced accelerated nephrotoxic nephritis, a model of immune complex–mediated glomerulonephritis. After accelerated nephrotoxic nephritis was induced by administration of sheep nephrotoxic globulin, mCd59a−/− mice and strain-matched controls on two genetic backgrounds, 129/Sv × C57BL/6 and 129/Sv, were examined. For both, mCd59a−/− mice developed significantly greater glomerular cellularity than wild-type (WT) mice at day 5 after administration. At day 10 post-administration, mCd59a−/− mice exhibited more glomerular thrombosis than WT mice (thrombosis score, 1.8 [range, 1.4 to 4.0] versus 0.8 [range, 0.2 to 1.5] quadrants thrombosed per glomerulus, respectively; P = 0.0006). In the majority of experiments, mCd59a−/− mice also had significantly more proteinuria than controls; however, there was no difference in serum creatinine or albumin. Quantitative immunofluorescence of kidney sections revealed significantly more C9 (as a marker of MAC) deposition within glomeruli of mCd59a−/− mice than WT controls (P < 0.001). There was no difference in deposition of C3 and sheep IgG between the two experimental groups. The lack of CD59a, by allowing unregulated MAC deposition, exacerbates the renal injury in this model of immune complex–mediated glomerulonephritis.

Many forms of human glomerulopathy are mediated by Ig deposition and complement activation within the glomerulus. Complement activation mediates tissue injury via a number of mechanisms, including the generation of the chemotactic anaphylatoxins C3a and C5a and the insertion of the membrane attack complex (MAC), C5b-9, into cells. The kidney is normally protected from complement-mediated damage by circulating and membrane-bound regulatory proteins. The latter act at two levels within the complement cascade: at the levels of the C3 convertase enzyme and MAC. Decay-accelerating factor (DAF; CD55), membrane cofactor protein (CD46), and, in rodents, Crry, act at the C3 convertase level, whereas CD59 is the only membrane-bound factor that prevents the formation of C5b-9 (1). CD59 is an 18 to 20 kD glycosyl-phosphatidylinositol (GPI)–anchored protein that is ubiquitously expressed (2,3). Deficiency of this protein in humans is associated with the acquired hemolytic disorder paroxysmal nocturnal hemoglobinuria (PNH). In PNH, a mutation in a hematopoietic stem cell gives rise to a clone of blood cells that lack glycolipid-anchored molecules and are highly susceptible to complement-mediated lysis in vitro and in vivo. PNH is an acquired disorder; however, in a single isolated case of hereditary CD59 deficiency, a PNH-like pathology was described (4,5). In contrast to humans, mice have two CD59 genes encoded on chromosome 2: mCd59a and mCd59b. Both genes seem to be widely expressed; however, mCd59b is expressed at higher levels in the testes, whereas mCd59a predominates in kidney, brain, and liver (6).

Mice with a targeted deletion of the mCd59b gene have recently been reported to develop a severe hemolytic anemia and progressive male infertility, whereas mice lacking the mCd59a gene developed a mild, spontaneous, intravascular hemolysis (7,8). Although these observations in gene-targeted mice indicate that CD59b may play an important role in protecting erythrocytes and sperm cells against complement-mediated damage, it is likely that the relative contribution of these molecules varies depending on the organ or tissue analyzed as well as on the mechanisms and/or level of complement activation (8,9). Importantly it has been demonstrated that the absence of either isoform of CD59 does not alter the tissue expression of the other isoform (7,10).

In addition to lysis, complement-mediated damage by MAC can occur by the insertion of sublytic quantities of C5b-9 into cell membranes. It has been demonstrated that sublethal MAC can mediate proliferative, proinflammatory and profibrotic effects in vitro in glomerular cells (11–23). MAC has also been shown to be involved in the pathogenesis of a number of glomerular diseases in vivo, including membranous nephropa-
thy (24,25) and immune complex nephritis (26,27). Although the significance of CD59 in protecting human glomerular cells from MAC-mediated damage has been previously demonstrated in vitro both by neutralization experiments with antibody (28–30) and by overexpression studies (31), the importance of CD59 activity in vivo is not established. Previous studies in rats have shown that neutralization of CD59 by an anti-CD59 monoclonal antibody or F(ab')2 fragment caused a marked exacerbation of the phenotype in a model of immune complex–mediated nephritis, suggesting that CD59 does play a key role in vivo in this model. However, the possibility that binding of the anti-CD59 antibody or F(ab')2 fragment to renal cells per se caused glomerular injury could not be entirely excluded (32,33).

Accelerated nephrotic nephritis (ANTN) in mice is an immune complex–mediated glomerular disease characterized by an early glomerular hypercellularity followed by proteinuria, crescent formation, and thrombosis. Among the several factors that have been implicated in the pathogenesis of this disease, the roles of Fcγ receptors (34) and complement (35–37) have been most comprehensively investigated. Although the involvement of Fcγ receptors in immune complex–mediated nephritis is well documented, the role of complement in this model is still controversial. For example, deficiency of C3 or C1q may affect the development of nephritis, but the effects are influenced by the genetic background of the mice and by the experimental conditions applied (35).

Nevertheless, there is evidence of complement-dependent inflammatory injury initiated by nephrotoxic serum, but the extent to which MAC mediates this damage has not been fully elucidated. In this study, we used mCd59a−/− deficient mice (mCd59a−/−) and demonstrated that mCd59a deficiency exacerbated the complement-mediated inflammatory damage induced by a sheep anti-mouse glomerular basement membrane globulin preparation. In particular, mCd59a−/− mice developed more severe glomerular histologic features accompanied by more proteinuria than matched WT controls, suggesting that CD59a plays an important protective role in vivo in autoimmune tissue injury.

Materials and Methods

Animals

mCd59a−/− were generated as described previously (8). Age-, strain-, and gender-matched wild-type (WT) mice were used in all experiments. Mice were studied on two genetic backgrounds: pure 129/Sv and a hybrid (129/Sv x C57BL/6)F2. Mice were kept in a specific-pathogen-free environment, and experiments were performed according to institutional guidelines.

Preparation of Sheep Nephrotoxic Globulin

Preparation of mouse glomeruli was performed as described previously (35). A sheep was repeatedly immunized with this preparation according to institutional guidelines. Total glomerular cell counts were quantified by multiplying the concentration by the volume collected.

Histologic Analysis

Kidneys were snap-frozen in isopentane and stored at −70°C. Frozen sections were cut at a thickness of 5 μm. An observer without knowledge of the sample identity performed all quantitative immunofluorescence analyses. Mouse anti-sheep IgG (FITC conjugated; Sigma), rabbit anti-mouse IgG (FITC conjugated; Southern Biotechnology Associates, Birmingham, AL) and goat anti-mouse C3 (FITC conjugated; ICN, Basingstoke, UK) antibodies were used for direct immunofluorescence studies. Indirect immunofluorescence was performed with a rabbit anti-rat C9 (cross-reactive with mouse C9 (32)) and a FITC-conjugated mouse anti-rabbit IgG secondary (Sigma). All incubations were performed for 1 h at room temperature, and all antibodies were used at a 1/50 dilution in PBS except for rabbit anti-mouse IgG (1/200). For the anti-C9 antibody, a negative control (PBS) was used in each experiment to ensure there was no binding of the secondary antibody to the kidney section.

In quantitative immunofluorescence studies, to exclude artifacts due to variable decay of the fluorochrome, all sections from one experiment were stained and analyzed at the same time. Sections were
examined at ×400 magnification with an Olympus BX4 fluorescence microscope (Olympus Optical, London, UK). A Photonic Science Color Coolview digital camera (Photonic Science, East Sussex, UK) was attached to the microscope, and, with Image-Pro Plus software (Media Cybernetics, Silver Spring, MD), images were captured for analysis. For each section, 20 glomeruli were examined and the mean fluorescence intensity recorded, with results expressed in arbitrary fluorescence units (AFU).

**Immunohistochemistry**

Sections were fixed in paraformaldehyde-lysine-periodate for 4 h, then transferred to 7% sucrose overnight before snap freezing in isopentane and storing at −70°C. For macrophage staining, a primary monochonal rat anti-mouse CD68 antibody (Serotec, Oxford, UK) was used. Sections were blocked with a 1% solution of hydrogen peroxide in 50% methanol. A mouse anti-rat secondary antibody and a rat peroxidase anti-peroxidase tertiary antibody (both purchased from Jackson Immunoresearch, Cambridge, UK) were then applied. The sections were developed with diaminobenzidine (Sigma), counterstained with hematoxylin (Sigma), and then dehydrated through graded alcohols.

**Statistical Analyses**

All values described in the text and figures are expressed as median and range for n observations. Statistical analysis was carried out by GraphPad Prism 3.02 (GraphPad Software, San Diego, CA). Data were analyzed by the Mann-Whitney U test for all comparisons, and P < 0.05 was considered to be significant.

**Results**

**mCd59a-Deficient Mice Are More Susceptible to ANTN**

To determine whether the lack of CD59 may affect different stages in the development of renal injury induced by NTG, we studied mice at 5 and 10 d after injection of NTG. WT, strain-matched male mice killed at 5 d after administration of NTG developed a mild disease associated with slight increase in glomerular cellularity. However, the glomeruli in the mCd59a−/− mice at this time appeared enlarged and displayed more marked glomerular hypercellularity (Figure 1, a and b) (median total cell counts 36.8; range: 34.8 to 40.1; n = 10 for mCd59a−/− versus 30.0; 24.2 to 34.5; n = 9 for WT, P = 0.002, Figure 1c). This increase in cellularity was in part because of an excess of macrophages in the glomeruli of mCd59a−/− mice, although this did not reach statistical significance (13.9 macrophages per 20 glomeruli; 0.0 to 49.0 in mCd59a−/− mice versus 1.4; 0.0 to 8.0 in controls P = 0.11).

There was, however, no neutrophil influx or evidence of apoptotic cells on light microscopy in either mCd59a−/− or WT animals. Mice lacking the mCd59a gene have been shown to develop normally, and no morphologic differences were apparent in kidneys from the mCd59a−/− mice when compared with strain-matched controls (data not shown).

In both groups, the baseline glomerular cellularity ranged from 22.4 to 24.0 cells per glomerulus. To exclude the possibility that the increased cellularity in the mCd59a−/− animals was due to greater deposition of NTG, we quantified glomerular sheep IgG deposition. No difference in sheep IgG deposition between mCd59a−/− animals and controls was detected (77.6 AFU; 52.3 to 85.3; n = 10 versus 66.3; 60.1 to 82.2; n = 9, respectively; Figure 2).

In addition, deposition of mouse IgG was also similar in mCd59a−/− deficient mice and controls (83.1 AFU; 27.4 to 110.3; n = 10 versus 69.8; 30.2 to 124.3; n = 9, respectively; Figure 2), demonstrating no differences between the two experimental groups in the immune response to NTG administration. Quantitative immunofluorescence for C9, as a marker of MAC deposition, was then performed to determine if lack of CD59, in vivo, allowed increased C5b-9 deposition to occur. As predicted, there was significantly more C9 deposition in mCd59a−/− deficient mice than controls (41.1 AFU; 25.1 to 66.5; n = 10 versus 27.0; 18.1 to 40.8; n = 9, respectively, P = 0.012) but no difference in C3 deposition between the experimental groups (39.2 AFU; 19.5 to 54.2; n = 10 for mCd59a−/− versus 39.9; 18.6 to 73.9; n = 9 for WT; Figure 2), confirming the key role for CD59 in preventing MAC deposition. Although on day 5 after administration of NTG the renal histology showed evidence of a more severe glomerular hypercellularity in the mCd59a−/− than WT mice, at this early stage in the disease, there were no significant differences between mCd59a−/− and controls in 24 h albuminuria (816 µg/24 h; 388 to 1386; versus 512; 26 to 910, respectively).
Figure 2. Immunofluorescence staining of sheep IgG, mouse IgG, C3, and C9 deposition in glomeruli of wild-type (WT) and mCd59a−/− mice. In both experimental groups, sheep IgG and mouse IgG were predominantly distributed along the glomerular capillary loops, and deposition of C3 was also observed in the mesangium. No quantitative differences between mCd59a−/− and WT animals in these parameters were detected. In contrast, there was significantly greater glomerular C9 deposition in the glomeruli of mCd59a−/− animals than matched controls. Data are representative of three separate experiments performed with similar numbers of mice on the pure 129/Sv genetic background, with comparable statistical significance reached. Equivalent results were also obtained in three experiments that used similar numbers of mice on the hybrid genetic background (129/Sv × C57BL/6)F2.
respectively), serum creatinine (43.0 μmol/L; 35.0 to 56.0; versus 46.0; 33.0 to 68.0), or serum albumin (23.0 μmol/L; 21.0 to 26.0; versus 21.5; 10.0 to 25.0).

ANTN is characterized by proteinuria, glomerular thrombosis, and tubulointerstitial injury after the initial hypercellularity stage. In view of our initial findings of excess glomerular cellularity in mCd59a−/− animals, we decided to investigate whether mCd59a deficiency would also exacerbate the subsequent thrombotic phase of the disease. We therefore studied the disease at 10 d after administration of NTG. Glomerular thrombosis, as evidenced by PAS-positive material previously shown to be fibrin (38), was worse in mCd59a−/− mice than in matched WT animals (Figure 3). Quantification of this glomerular thrombosis revealed significantly greater thrombosis counts in the mCd59a−/− animals than controls (median quadrants thrombosed per glomerulus, 1.8; range, 1.4 to 4.0; n = 9 versus 0.8; range, 0.2 to 1.5; n = 10, respectively, \( P = 0.0006 \); Figure 4a).

As seen in the early phases of the renal disease, there was significantly more C9 deposition in mCd59a-deficient animals than controls (46.0 AFU; 30.0 to 64.5; n = 9 versus 24.9; 18.9 to 32.5; n = 10, respectively, \( P = 0.0002 \), Figure 4b), but no difference in sheep IgG (73.8 AFU; 31.5 to 93.0; versus 84.2; 53.3 to 104.1), mouse IgG (77.1 AFU; 49.6 to 111.7; versus 74.2; 54.6 to 94.3), or C3 (32.9 AFU; 22.4 to 53.8; versus 36.9; 9.8 to 52.3) staining. Consistent with the more severe phenotype observed, and in contrast to earlier time points in the nephritis, mCd59a−/− mice killed at this later stage developed significantly more albuminuria than controls (4.7 mg/24 h; 2.8 to 6.1; n = 9 versus 2.4; 0.3 to 3.1; n = 10, respectively, \( P = 0.0006 \), Figure 4c). However, no differences in serum creatinine (41.0 μmol/L; 34.0 to 44.0; for mCd59a−/− mice versus 41.0; 31.0 to 58.0; for controls) or serum albumin (22.5 μmol/L; 9.0 to 29.0; for mCd59a−/− mice versus 23.0; 12.0 to 27.0; for WT) was detected between the two groups.
Addition of LPS to NTG Exacerbates Renal Damage in mCd59a−/− Mice

Previous work has demonstrated that the level of LPS contained within each batch of nephrotoxic preparation can affect the development of tissue damage in nephrotoxic nephritis (39,40). LPS is known to activate the alternate pathway of the complement system (41), and this may contribute to the exacerbation of the renal disease in this experimental model. For this reason, we measured the amount of LPS present in our NTG. The amount of LPS in the NTG was below the limit of detection of the LPS assay kit (<0.1 EU/ml). In view of this result, we wished to explore whether the addition of a small quantity of LPS would further increase the susceptibility of mCd59a−/− mice to ANTN. For these experiments, we used only mice on a pure 129/Sv genetic background to avoid any confounding factors related to the varying LPS sensitivity of mice from different genetic backgrounds.

To exclude an effect of LPS alone on the kidney, we injected 100 ng LPS in the absence of NTG. LPS alone did not cause proteinuria, and renal histology was normal in both mCd59a−/− and control 129/Sv mice whether or not the animals had been preimmunized with sheep IgG (data not shown). The addition of 100 ng LPS to the NTG, however, did increase the severity of the nephrotoxic nephritis, because mice became clinically unwell earlier than 10 d after administration of NTG and therefore were killed at day 8. In these circumstances of increased tissue injury, mCd59a−/− animals again developed greater glomerular thrombosis (thrombosis counts 2.6; 1.0 to 4.0; n = 14 for mCd59a−/− mice versus 0.5; 0.0 to 2.2; n = 12 for WT, P = 0.001; Figure 5a) and albuminuria (15.2 mg/24 h; 9.9 to 25.0; versus 6.5; 0.0 to 10.0; P = 0.0003; Figure 5b) but no difference in serum creatinine or albumin (data not shown) than WT animals. As observed in the previous experiments in mCd59a−/− mice, there was a significantly greater C9 deposition (59.3 AFU; 47.3 to 73.1; for CD59a−/− versus 42.80; 8.9 to 46.9; for controls, P < 0.0001; Figure 5c) that was not accompanied by increased glomerular C3, mouse IgG, or sheep IgG (data not shown) when compared with WT mice.

Discussion

In this report, we have demonstrated that mice lacking the mCd59a gene were more susceptible to ANTN than matched controls. They developed greater glomerular cellularity early in the disease process and more severe glomerular thrombosis and proteinuria at later time points. The excess C9 deposition that we observed reflects the presence of a greater quantity of MAC and most likely represents the mechanism whereby the absence of CD59a caused greater tissue injury.

ANTN is a type of immune complex glomerulonephritis mediated by, among other factors, Fcγ receptors and complement. Although it has been convincingly demonstrated that Fcγ receptors play a critical role in the pathogenesis of this form of renal disease (34), data on the involvement of the complement system in mediating tissue damage are less clear (35,37). However, there is evidence for a protective role of the complement regulators DAF and Crry in this experimental model of nephritis. Crry acts at the C3 convertase level but is only present in rodents and not in primates. It is the major regulator of C3 activation in rodents in which complement activation at the maternal-fetus interface is fatal to the devel-
opposing embryo of Crry-knockout mice (42,43). Transgenic mice overexpressing Crry are protected from immune complex–mediated glomerular disease, suggesting that complement activation at this level is important in the pathogenesis of the renal injury (43). DAF is a GPI-bound complement regulatory protein that also inhibits the C3 convertase enzymes. Mice with a targeted deletion of the GPI-DAF (Daf1) gene, when injured by subnephritogenic doses of anti–glomerular basement membrane antibody in the ANTN model, developed glomerular disease that was absent in controls (36). Overall, these studies demonstrated that regulation of C3 activation is important in preventing renal damage but did not clarify whether tissue injury is mediated by the anaphylatoxins C3a and C5a or via lytic or sublytic MAC insertion. CD59 acts by preventing the formation of MAC on the cell surface and has been previously shown to protect glomerular cells from complement-mediated injury in vitro. In addition, in vivo studies that used a neutralizing antibody to CD59 in rat models of thrombotic microangiopathy (33) and immune complex–mediated nephritis (32) have suggested a protective role for this molecule. However, these experiments did not exclude the possibility that binding of the anti-CD59 antibody or F(ab')2 fragment to renal cells per se caused glomerular injury.

Here we have demonstrated for the first time that deficiency of the mCd59a gene can exacerbate glomerular disease in a model of immune complex–mediated renal injury in mice. In mCd59a−/− mice, we have detected an increase in MAC deposition, which is in keeping with the in vitro data that CD59 has a specific role in inhibiting the formation of C5b-9 on the cell surface. We did not observe any difference in C3 deposition between mCd59a-deficient animals and matched controls, confirming that CD59a, unlike DAF or Crry, does not regulate C3 activation.

ANTN is characterized by an early increase in glomerular cellularity followed by progressive glomerular thrombosis and interstitial injury. We studied both phases of this disease to elucidate the role of MAC and CD59 at each stage. We have shown that at the hypercellular stage of the disease, mCd59a deficiency exacerbates the early increase in cell number. There are several lines of evidence that may explain the role of MAC in causing this excess cellularity. First, MAC has been shown to cause proliferation of glomerular mesangial, endothelial, and epithelial cells in vitro and therefore may be directly involved in inducing a increase in intrinsic cell number (17,21,44,45). Additionally, there are data indicating that MAC can induce the synthesis and/or secretion of a number of proinflammatory cytokines such as TNF-α and IL-1 (22,44), adhesion molecules such as ICAM-1 and E selectin (23), and chemokines such as IL-8 (46) and that these effects may play a role in the infiltration of cells to the glomerulus. We have also demonstrated that later in the progression of ANTN, mCd59a deficiency, by allowing unregulated MAC deposition, exacerbates glomerular thrombosis. One possible mechanism for this increase in thrombosis is that MAC is able to expose the prothrombinase or factor Va binding sites on platelets, which can cause platelet aggregation and thrombosis (47).

The genetic background of mice may modulate the development of renal damage in nephrotoxic nephritis. For example, a study that used cobra venom factor to induce complement depletion suggested that complement played a role in mediating tissue damage in BALB/c, but not in C57BL/6, mice (48).

Our initial experiments using heterogeneous F2 intercrossed mice demonstrated significantly worse disease in CD59a−/− mice compared with matched controls. These results were replicated with mice on a pure 129/Sv genetic background, which allowed us to bypass any potential problems generated by genetic polymorphisms between different strains.

Antibody-mediated glomerulonephritis in humans may be exacerbated by infection, and this effect may be mediated by LPS. The LPS content of nephrotoxic preparations has previously been shown to play an important role in the pathogenesis of heterologous nephrotoxic nephritis in rodents (39,40); however, it has not been reported in ANTN. LPS activates the endothelium and the alternate pathway of complement, and thus we hypothesized that the addition of a small dose of LPS, shown to be insufficient to cause renal damage or a significant systemic inflammatory response, to our NTG may slightly exacerbate tissue injury and make it more dependent on complement activation. As expected, we found that the addition of LPS to the NTG in male 129/Sv mice increased both the severity of glomerular thrombosis and the degree of proteinuria in this model. In this context, the mCd59a−/− animals were also more susceptible to renal injury than controls.

In summary, our data demonstrate that CD59a, by preventing deposition of the MAC of complement, reduces the severity of glomerular injury in an experimental model of immune complex–mediated glomerulonephritis in mice.

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References


