Prevention of Fatal C3 Glomerulopathy by Recombinant Complement Receptor of the Ig Superfamily

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ABSTRACT

Background C3 glomerulopathy (C3G) is a life-threatening kidney disease caused by dysregulation of the alternative pathway of complement (AP) activation. No approved specific therapy is available for C3G, although an anti-C5 mAb has been used off-label in some patients with C3G, with mixed results. Thus, there is an unmet medical need to develop other inhibitors of complement for C3G.

Methods We used a murine model of lethal C3G to test the potential efficacy of an Fc fusion protein of complement receptor of the Ig superfamily (CRIg-Fc) in the treatment of C3G. CRIg-Fc binds C3b and inhibits C3 and C5 convertases of the AP. Mice with mutations in the factor H and properdin genes (FHm/mP2/2) develop early-onset C3G, with AP consumption, high proteinuria, and lethal crescentic GN.

Results Treatment of FHm/mP2/2 mice with CRIg-Fc, but not a control IgG, inhibited AP activation and diminished the consumption of plasma C3, factor B, and C5. CRIg-Fc–treated FHm/mP2/2 mice also had significantly improved survival and reduced proteinuria, hematuria, BUN, glomerular C3 fragment, C9 and fibrin deposition, and GN pathology scores.

Conclusions Therapeutics developed on the basis of the mechanism of action of soluble CRIg may be effective for the treatment of C3G and should be explored clinically.


C3 glomerulopathy (C3G) is a rare kidney disease that is defined mechanistically by dysregulation of the alternative pathway of complement (AP) activation. C3G can be caused by defects in fluid phase or cell surface complement regulators.1–5 In addition, autoimmune forms of C3G attributable to autoantibodies against complement regulators or C3 convertase are also well documented.6 Pathologically, C3G is characterized by plasma C3 consumption and prominent C3 fragment depositions in the mesangium and capillary walls, with typically no or trace Ig presence in the glomeruli. Two subcategories of C3G are distinguished histologically by electron microscopy: dense deposit disease (DDD) and C3 glomerulonephritis (C3GN).7 A pathognomonic feature of DDD is dense deposit in the lamina densa of the glomerular basement membrane (GBM), whereas in C3GN, the pattern of electron-dense deposit is more variable and can present as mesangial, subendothelial, intramembranous, or subepithelial.8,9 The complement effectors responsible for C3G pathogenesis may not be identical across the clinical spectrum of the disease, but both human and mouse model studies have clearly shown the importance of C5-mediated terminal complement pathway in C3G.
pathogenesis,10–17 and the blood level of soluble C5b-9 (sC5b-9) has been used as a biomarker for disease activity and predicted response of anti-C5 therapy.11,14,16,18,19

Prognosis of C3G, particularly DDD, is poor and approximately 50% patients progress to end stage renal failure within 10 years of diagnosis.1,20 Currently there is no specific therapy for C3G and the disease is managed primarily by empirical and supportive treatments, including plasma infusion/exchange, immunosuppression, and BP control.21,22 These conventional treatments lack consistent efficacy and do not usually achieve long-term satisfactory clinical outcome. Renal transplantation in C3G is also not a reliable option because disease recurrence in the transplanted kidneys is common.1 More recently, eculizumab, an anticomplement C5 mAb, has been tested for C3G in isolated cases and in a small, open-label, clinical trial. Although some patients responded positively to anti-C5 treatment, others showed no improvement.12,14,16,23 Blocking C5 will inhibit terminal complement activation but is not expected to have an effect on plasma C3 activation and glomerular C3 fragment deposition. Whether and to what degree these processes contribute to C3G pathogenesis is not yet fully understood, but given the underlying mechanism of C3G being dysregulation of the AP, there is a strong rationale and need to explore other complement inhibitors targeting the proximal steps of AP that can block both C3 and C5 activation in C3G.

In this study, we have tested a soluble Fc fusion protein of the extracellular domain of complement receptor of the Ig superfamily (CR1g-Fc) in a murine model of lethal C3G. CR1g-Fc was previously shown to be a potent inhibitor of AP activation.24–26 It works by binding to and interfering with, the substrate binding function of C3b, thereby inhibiting the formation of both C3 and C5 convertases.27,28 CR1g-Fc is a specific AP inhibitor because it does not interfere with C5 binding to the classic pathway C5 convertase C3bC4bC2a.27 We previously created a factor H (FH) and properdin (P) gene double-mutant mouse (FH<sup>−/−</sup>P<sup>−/−</sup>) that developed lethal C3G with features of human disease. FH<sup>−/−</sup>P<sup>−/−</sup> mice displayed dense deposits in the GBM, had low plasma C3, factor B (FB), and C5 levels, and developed early-onset proteinuria.5 All FH<sup>−/−</sup>P<sup>−/−</sup> mice died from rapidly progressive GN by 8–12 weeks.5 We show here that treatment of FH<sup>−/−</sup>P<sup>−/−</sup> mice with CR1g-Fc, but not an isotype control mAb, markedly ameliorated C3G when assessed by multiple disease parameters including survival, renal pathology, and renal function. Our results suggest that soluble CR1g may inform a promising therapeutic strategy for human C3G and should be explored clinically.

**METHODS**

**In Vitro Complement Activation Assays**

Mouse CR1g-Fc (murine IgG1 Fc) and an isotype control murine α-HIV gp120-IgG1 antibody were produced as described previously.25 LPS-dependent AP activity assay of wild-type (WT) mouse sera with or without CR1g-Fc was performed as described.29 Classical pathway complement activity, in the presence or absence of CR1g, was measured using an ovalbumin (OVA)/anti-OVA-based ELISA method and FB knockout mouse serum as described.29

**In Vivo Kinetics of CR1g-Fc Activity**

WT C57BL/6 mice were purchased from The Jackson Laboratory. FH<sup>m/m</sup> mice were produced as described.5 Mice were intraperitoneally injected with 50 mg/kg or 1 mg per mouse (corresponding to 40–50 mg/kg) of CR1g-Fc, as specified. Blood was collected before and at various time points post-treatment for LPS-dependent AP activity assay or plasma C3 detection.5,29

**Treatment of FH<sup>−/−</sup>P<sup>−/−</sup> Mice**

FH<sup>−/−</sup>P<sup>−/−</sup> mice were generated as described.5 Beginning at 4 weeks of age, they were treated every 4 days (50 mg/kg, administered intraperitoneally) with either CR1g-Fc or the control IgG (n=10 per group). Treatment lasted for 10 weeks unless the mice became moribund and had to be euthanized. Only male mice were used in the treatment experiment.

**Survival Curve**

Survival curve was analyzed using the GraphPad Prism program (La Jolla, CA) as described previously.30

**Measurement of Renal Function**

Blood and urine samples were collected every 2 weeks during treatment. Urine samples were collected using metabolic cages for 16 hours and volumes recorded. Blood urea nitrogen (BUN), serum creatinine, proteinuria, leukocyturia, and hematuria were assessed as described.5,10,31

**Immunofluorescence Staining and Histology**

Immunofluorescence staining of C3, C9, and fibrin in the mouse kidney, preparation of paraffin sections and hematoxylin and eosin and periodic acid–Schiff staining of kidney sections were performed as described previously.5,10,32 In some experiments, glomerular C3 deposition was also stained with a FITC-conjugated mouse anti-mouse C3b/iC3b/C3d mAb (used at 1 μg/ml; Cederlane Labs) or a biotinylated polyclonal goat anti-mouse C3d antibody (catalog #BAF2655, used at 1 mg/ml; R&D Systems) with streptavidin–FITC (catalog #554060, used at 1:100 dilution; BD Pharamingen) as a detecting reagent. Renal pathology was graded in a blind fashion. The following characteristics were recorded: glomerular hypercellularity and glomerular crescent formation. The severity of each change in a given mouse was graded as a percentage of glomeruli showing the respective changes (0–100%). Approximately 50 glomeruli were examined for each sample. Electron microscopy analysis of kidney sections was performed at the Electron Microscopy Resource Laboratory of University of Pennsylvania, as described previously.5,10

**Western Blotting of C3, FB, and C5**

Western blotting of mouse plasma C3, FB, and C5 was performed as described.5,10

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**RAPID COMMUNICATION**

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Detection of Anti CRIg-Fc Antibody Response

To detect antibody response against CRIg-Fc, ELISA plates were coated with 2 μg/ml purified CRIg-Fc in PBS for 1 hour at 37°C. Plates were then blocked with 1% BSA-PBS for 1 hour at room temperature and washed three times with PBS-Tween 20, followed by addition of serially diluted mouse serum in 1% BSA-PBS, starting at 1:100. After washing, plates were incubated with a light-chain-specific, horseradish peroxidase-conjugated goat anti-mouse IgG (catalog #115–005–174, used at 2 μg/ml; Jackson Immuno-research) for 1 hour at room temperature. After washing, plates were developed using Ultra TMB ELISA substrate (Thermo Fisher Scientific).

Statistical Analyses

Statistical comparisons were performed using GraphPad Prism 4.0 software. Data are presented as mean±SD. The difference between two groups was calculated using the two-tailed t test for normally distributed data. For multiple group comparison, one-way ANOVA with a Tukey test was used. P value <0.05 was considered statistically significant.

RESULT AND DISCUSSION

To confirm the activity of CRIg-Fc as an AP-specific inhibitor, we performed

![Graphs and images representing experimental data](image_url)

**Figure 1.** CRIg-Fc inhibits AP activity in normal and mutant mice and prevents fatal C3G disease. (A) At 40 μg/ml or higher, CRIg-Fc effectively inhibited AP activity in 50% WT mouse serum. Data from two independent experiments (one with duplicate and the other triplicate assays) were pooled and plotted together. Results are presented as mean (SD). (B) CRIg-Fc inhibited AP activity in vivo in WT mice. Data from four WT mice (M01, M02, M03, and M04) were plotted individually. Mice were injected with 50 mg/kg CRIg-Fc and LPS-dependent AP activity was measured using 10% serum, in duplicate assays (average is shown). AP activity at day 2 and day 4 was normalized to that of the pretreatment sample (pre). (C) Western blotting shows that CRIg-Fc treatment inhibited C3 consumption and markedly elevated plasma C3 levels in two FHm/m mice (M1 and M2). Western blot band shown denotes intact C3 α-chain. A single dose of 1 mg CRIg-Fc was administered to each mouse. Blood samples were collected immediately before and at 6 hours and 1, 2, 3, and 4 days after CRIg-Fc injection. (D–I). Treatment of FHm/mPm/m mice with control IgG had no effect on survival (100% mortality) and C3G disease development as assessed by proteinuria, hematuria, leukocyturia, BUN, and serum creatinine. In contrast, treatment with CRIg-Fc significantly improved survival (80%) and multiple disease parameters. FHm/mPm/m mice received treatments (n=10 mice per group) starting at 4 weeks of age and lasting for 10 weeks (50 mg/kg, administered intraperitoneally). Data shown in (E–I) represent mean (SD). ***P<0.001, log-rank test. ctrl, control; conc, concentration.
LPS-dependent AP and OVA/anti-OVA immune complex-mediated classical pathway complement activation assays using ELISA. Figure 1A shows that at 40 mg/ml CRIg-Fc completely suppressed AP activity in 50% mouse serum diluted in EGTA-containing gelatin-veronal buffered saline. Using OVA/anti-OVA immune complex and FB knockout mouse serum, we confirmed previous findings that CRIg does not inhibit classical pathway complement activity both in normal mice and mice suffering from AP dysregulation.

To test the therapeutic potential of CRIg-Fc, we next tested CRIg-Fc in FHm/m mice who develop a more aggressive and lethal form of C3G. FHm/m mice were randomized into either CRIg-Fc or isotype control IgG treatment group (50 mg/kg, intraperitoneal injection every fourth day, n=10 mice per group). Treatment was initiated at 4 weeks of age and lasted for 10 weeks. Figure 1D shows that FHm/m mice treated with control IgG all died or became moribund within 8 weeks of initiating treatment, whereas eight out of ten (80%) CRIg-Fc–treated mice survived the entire 10-week treatment period. Furthermore, CRIg-Fc but not control IgG treatment reversed and/or maintained at baseline levels several C3G disease markers, including proteinuria, hematuria, leukocyturia, and BUN (Figure 1, D–I). Renal pathology was performed on eight out ten and seven out of ten mice in the CRIg-Fc and control IgG group, respectively. These mice represented all surviving mice in the CRIg-Fc group at week 10 of treatment and those that became moribund in the control IgG group that had to be terminated before week 10. We were not able to collect tissues from the remainder of experimental mice (two in the CRIg-Fc group and three in the control IgG group) as they died suddenly. As shown in Figure 2 and Supplemental Table 1, moribund mice in the control IgG group developed glomerular hypercellularity, diffuse cellular and fibrocellular glomerular crescents, and segmental...
fibrinoid necrosis. Interstitial fibrosis, tubular protein, and erythrocyte casts were also present. In contrast, CRlg-Fc–treated mice showed essentially normal kidney histology, with no glomerular crescent seen and hypercellularity in only 2–5% of the glomeruli (Figure 2, Supplemental Table 1). On electron microscopy, glomeruli of control IgG–treated mice had extensive foot process effacement, irregular GBM thickening, and intramembranous dense deposit, whereas CRlg-Fc–treated mice had normal GBM architecture with intact podocyte foot processes (Figure 2).

Results of immunofluorescence staining of C3, C9, and fibrin/fibrinogen in kidney sections of CRlg-Fc–treated mice compared with that of control IgG–treated mice. Compared with control IgG–treated group, CRlg-Fc treatment significantly decreased glomerular C3 and fibrin/fibrinogen deposition in FHm/mP−/− mice. Representative fluorescence images from a control IgG– and a CRlg-Fc–treated mouse are shown in the top panels and quantitative analysis of C3, C9 and fibrin/fibrinogen staining is provided in the scatter plots underneath the immunofluorescence images. Each dot in the scatter plot represents a single mouse. Fluorescence intensity of C3, C9, and fibrin/fibrinogen was calculated using ImageJ and expressed in arbitrary units. Arbitrary unit was calibrated separately for each antibody staining group. We analyzed and scored 10–15 glomeruli in three different viewing fields of each kidney section. Horizontal bars through scatter plots represent average values (n=7 mice in control IgG group and n=8 mice in CRlg-Fc group). Bars, 100 μm. * P<0.05; *** P<0.001 (t test).

Figure 3. Reduction of immunofluorescence staining of C3, C9, and fibrin/fibrinogen in kidney sections of CRlg-Fc–treated mice compared with that of control IgG–treated mice. Compared with control IgG–treated group, CRlg-Fc treatment significantly decreased glomerular C3 and fibrin/fibrinogen deposition in FHm/mP−/− mice. Representative fluorescence images from a control IgG– and a CRlg-Fc–treated mouse are shown in the top panels and quantitative analysis of C3, C9 and fibrin/fibrinogen staining is provided in the scatter plots underneath the immunofluorescence images. Each dot in the scatter plot represents a single mouse. Fluorescence intensity of C3, C9, and fibrin/fibrinogen was calculated using ImageJ and expressed in arbitrary units. Arbitrary unit was calibrated separately for each antibody staining group. We analyzed and scored 10–15 glomeruli in three different viewing fields of each kidney section. Horizontal bars through scatter plots represent average values (n=7 mice in control IgG group and n=8 mice in CRlg-Fc group). Bars, 100 μm. * P<0.05; *** P<0.001 (t test).
and C3 than on FB. This differential effect is likely related to the mechanism of action of CRIg-Fc, which inhibits C3 and C5 cleavage by the respective C3 and C5 convertases but does not directly inhibit the cleavage of FB by factor D once C3bB is formed. The moderate increase in plasma FB likely reflected a secondary effect of C3 inhibition, leading to less C3b and C3bB production. It is also interesting that CRIg-Fc treatment produced a more dramatic inhibitory effect on glomerular C9 deposition than C3 deposition (Figure 3). This observation is consistent with the known property of CRIg-Fc, which has a higher affinity for, and is a better inhibitor of, the C5 convertase than the C3 convertase.

C3G is a serious kidney disease in need of a specific and effective treatment. Several proximal complement inhibitors are currently under development for C3G, but their efficacy in vivo remains to be tested. Our data here using the robust FH<sup>−/−</sup> mouse model have provided strong proof of concept that therapeutics on the basis of the mechanism of action of CRIg-Fc could represent a new and effective therapy for C3G. The treatment regimen of CRIg-Fc used in this study (50 mg/kg, every 4 days) is similar to what is required to inhibit mouse C5 by an mAb. If translatable to humans and by analogy of anti-C5 mAb therapy in humans, the pharmacology of CRIg-Fc may be superior to inhibitors targeting C3 or factor D, considering the abundance or fast turnover rate of the latter two complement proteins. Of note, by ELISA assay we detected low titers of antibody response to CRIg-Fc in the treated mice (Supplemental Figure 3). However, the observed efficacy of CRIg-Fc therapy in C3G mice suggested that such immune response likely had a limited effect on the pharmacology of CRIg-Fc.

On the other hand, as a macrophage phagocytic receptor, CRIg plays a critical role in removing complement-opsonized bacteria in circulation, and whether the use of CRIg-Fc as a therapeutic drug creates an unacceptable or manageable infection risk needs to be carefully considered. In this context, complement inhibitors that share a similar mechanism of action to CRIg-Fc but do not block cellular functions of CRIg, e.g., C3b-specific mAbs as reported in the literature, may be another class of promising drugs that could be explored clinically for the treatment of C3G.

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W.-C.S., X.W., T.M., D.G., and G.X. designed experiments and interpreted data. X.W., T.M., D.G., Y.U., and Y.W. performed experiments. M.P. performed pathological analysis and interpreted data. M.V.L.C. and K.J.K. provided testing reagents. X.W., T.M., D.G., Y.W., and W.-C.S. prepared figures. W.-C.S. and X.W. wrote the manuscript and all coauthors critically commented and/or edited the manuscript.

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DISCLOSURES

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