Efficacy of Targeted Complement Inhibition in Experimental C3 Glomerulopathy

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ABSTRACT
C3 glomerulopathy refers to renal disorders characterized by abnormal accumulation of C3 within the kidney, commonly along the glomerular basement membrane (GBM). C3 glomerulopathy is associated with complement alternative pathway dysregulation, which includes functional defects in complement regulator factor H (FH). There is no effective treatment for C3 glomerulopathy. We investigated the efficacy of a recombinant mouse protein composed of domains from complement receptor 2 (CR2) and FH (CR2-FH) in two models of C3 glomerulopathy with either preexisting or triggered C3 deposition along the GBM. FH-deficient mice spontaneously develop renal pathology associated with abnormal C3 accumulation along the GBM and secondary plasma C3 deficiency. CR2-FH partially restored plasma C3 levels in FH-deficient mice 2 hours after intravenous injection. CR2-FH specifically targeted glomerular C3 deposits, reduced the linear C3 reactivity assessed with anti-C3 and anti-C3b/iC3b/C3c antibodies, and prevented further spontaneous accumulation of C3 fragments along the GBM. Reduction in glomerular C3d and C9/C5b-9 reactivity was observed after daily administration of CR2-FH for 1 week. In a second mouse model with combined deficiency of FH and complement factor I, CR2-FH prevented de novo C3 deposition along the GBM. These data show that CR2-FH protects the GBM from both spontaneous and triggered C3 deposition in vivo and indicate that this approach should be tested in C3 glomerulopathy.
monoclonal antibody eculizumab, showed variable efficacy in an open label study\textsuperscript{10,11} and case reports.\textsuperscript{12–19} In the FH-deficient mouse model of C3G, coexistent C5 deficiency ameliorated spontaneous glomerular inflammation but did not alter the degree of C3 staining or electron-dense changes along the glomerular basement membrane (GBM).\textsuperscript{20} The lack of a uniform response to eculizumab perhaps indicates that there are both C3- and C5-dependent mechanisms of renal injury, which may be different between patients and over time within a given patient. Arguably the most logical therapy would be that which prevented or ameliorated uncontrolled C3 regulation.

Strategies to reduce C3 activation specifically at sites of complement activation include CR2-FH,\textsuperscript{21} a fusion protein comprised of the complement regulatory domains of FH (FH\textsubscript{1–5}) linked to the C3 fragment-binding domains of complement receptor 2 (CR2\textsubscript{1–4}). Using these components, CR2-FH can interact with C3 fragments at sites of complement activation and then prevent further C3 activation. CR2-FH has demonstrated efficacy in models of AP-mediated tissue injury including paroxysmal nocturnal hemoglobinuria,\textsuperscript{22} ischemia/reperfusion injury,\textsuperscript{21,23} allergen-induced airway hyperresponsiveness,\textsuperscript{24} laser-induced choroidal neovascularization,\textsuperscript{25,26} and collagen antibody–induced arthritis.\textsuperscript{27} We investigated the efficacy of CR2-FH in two animal models of C3G, one with preexisting and one with triggered C3 deposition along the GBM. Our data indicate that CR2-FH effectively interacts with GBM-bound C3 and prevents further C3 activation within the kidney.

RESULTS

CR2-FH is Rapidly Cleared from the Circulation via the Kidney

After a single intravenous bolus injection of 40 mg/kg CR2-FH in wild-type animals, plasma CR2-FH concentration reached peak levels at 15 minutes (383.6±44.82 μg/mL), dropping rapidly by 6 hours (2.92±0.61 μg/ml, Supplemental Figure 1). AP-mediated membrane attack complex formation was inhibited at >90% at 15 minutes and 2 hours, but was restored by 6 hours. The highest concentrations of CR2-FH were detected within the kidney in wild-type and C3-deficient animals. In wild-type mice almost 90% of the CR2-FH had cleared from the kidney at 2 hours postinjection (Supplemental Figure 2), indicating rapid clearance from the circulation via the kidney.

CR2-FH Partially Restored Plasma C3 and C5 Levels in FH-Deficient (Cfh\textsuperscript{–/–}) Mice

Molar equivalent doses of CR2-FH (0.8 mg) and FH\textsubscript{1–5} (0.44 mg) were administered intravenously to Cfh\textsuperscript{–/–} mice and the animals were sacrificed 2, 24, and 48 hours postinjection. In the Cfh\textsuperscript{–/–} mice there is depletion of both C3 and C5.\textsuperscript{28,29} Both reagents were detected by western blot in plasma 2 hours after injection, but not at later time points (Figure 1, A and B). Plasma C3 levels increased (median=59.15 mg/l, range 56–59.9, n=3, Figure 1C) in CR2-FH–treated animals at the 2-hour time point compared with either PBS-injected or FH\textsubscript{1–5}-injected animals, but plasma C3 levels were comparable between the experimental groups at both 24 and 48 hours. These data were confirmed by western blot analysis (Supplemental Figure 3). Plasma C5 levels were analyzed using western blotting under nonreducing conditions (Figure 1D). In the CR2-FH group, C5 was detectable at all tested time points, but the greatest intensity was 24 hours after injection. In the FH\textsubscript{1–5}-injected animals, C5 was only present 2 hours after injection and was absent at 24 and 48 hours (Figure 1D).

CR2-FH Reduced Glomerular C3 in Cfh\textsuperscript{–/–} Mice

Cfh\textsuperscript{–/–} mice spontaneously develop C3 and C9 accumulation along the GBM.\textsuperscript{29} After a single intravenous injection of CR2-FH, there was a reduction in the linear glomerular C3 at the 2, 24, and 48 hour time points, together with the appearance of mesangial C3 staining at 24 and 48 hours (Figure 2, A and B). We confirmed that the decrease in C3 reactivity was not due to CR2-FH masking epitopes on C3 and preventing binding of the anti-C3 antibody (Supplemental Figure 4). Glomerular C3 staining did not change following administration of either FH\textsubscript{1–5} or PBS. We used two additional anti-C3 antibodies that react with either C3b/iC3b/C3c or C3d. Using the C3b/iC3b/C3c monoclonal antibody, significantly reduced linear staining was apparent in the CR2-FH group compared with the mice injected with FH\textsubscript{1–5} or PBS at all time points (Figure 3). The staining observed with the anti-C3d polyclonal antibody was linear in distribution with no mesangial reactivity. In contrast to both the anti-C3 polyclonal and anti-C3b/iC3b/C3c monoclonal antibody data, the intensity of the C3d (Supplemental Figure 5) and C9/C5b-9 staining (data not shown) did not change after CR2-FH injection and remained unchanged between all three experimental groups.

CR2-FH Colocalized with Glomerular C3 in Cfh\textsuperscript{–/–} Mice

Using an Alexa 594-conjugated polyclonal anti-mouse FH antibody, CR2-FH and not FH\textsubscript{1–5} was detectable within glomeruli, and colocalized with the linear C3 reactivity. CR2-FH did not bind along the GBM in wild-type mice (Supplemental Figure 6). The interaction of CR2-FH with the linear glomerular C3 progressively reduced in intensity following injection but was still detectable at 48 hours (Figure 2A). Glomerular CR2-FH fluorescence intensity at 2 hours (median=1001.7 arbitrary units, range 767.7–1451.2, n=3) was higher than that at 24 hours (median=384.7 arbitrary units, range 358.9–432.9, n=3) and 48 hours (median=358.7 arbitrary units, range 352.9–440.9, n=3, Supplemental Figure 7A). We did not detect an interaction between CR2-FH and mesangial C3 that developed in animals injected with CR2-FH (Figures 2A and 3A). To determine if this reflected a lack of availability of CR2-FH at the time that the mesangial C3 developed, we incubated kidney sections taken from Cfh\textsuperscript{–/–} mice 48 hours
after CR2-FH injection with CR2-FH ex vivo. The exogenous CR2-FH reacted with the C3 along the GBM ex vivo but not with the mesangial C3 (Supplemental Figure 7B), indicating that the lack of reactivity was not a consequence of CR2-FH availability.

The Reduction in Glomerular C3 Reactivity Persists after Single Injection of CR2-FH in Cfh<sup>−/−</sup> Mice

We next determined how long the reduction in linear C3 persists following a single injection of CR2-FH. Cfh<sup>−/−</sup> mice were injected with a single dose of CR2-FH and kidneys were harvested at 48 hours (n=2), 72 hours (n=4), 96 hours (n=4), and 120 hours (n=4). The C3 staining observed with the three anti-C3 antibodies at the earliest time point (48 hours) was similar in distribution pattern and intensity to that obtained at the same time point (48 hours) in the previous experiment (Figures 2A and 3A). The glomerular C3 reactivity assessed with anti-C3 polyclonal and anti-C3b/iC3b/C3c monoclonal antibodies at the 48-hour and all later time points appeared reduced compared with that observed in nontreated animals (Figure 4). No difference in the intensity of C3d staining was observed between treated and nontreated mice, even at the 120-hour time point (Figure 4). Although the staining intensity progressively reduced, CR2-FH was present in the glomeruli 120 hours after the single injection (Figure 4).

Multiple Injections of CR2-FH Ameliorated C3d and C9/C5b-9 Reactivity in Cfh<sup>−/−</sup> Mice

To determine the effects of repeated CR2-FH dosing, Cfh<sup>−/−</sup> mice received CR2-FH (0.8 mg, n=5) or PBS (n=6) daily for 7 days and were then culled at day 8, 24 hours after the last injection. Plasma C3 levels in the CR2-FH group (median=37.28 mg/L, range 33.53–42.7, n=5) were slightly higher than those in the PBS-injected group (median=32.85 mg/L, range 28.26–35.67,
Figure 2. CR2-FH colocalized with and reduced glomerular C3 in Cfh−/− mice. (A) Representative images of glomerular C3, CR2-FH, and FH(1–5) in Cfh−/− mice injected with a single dose of CR2-FH, FH(1–5), or PBS. C3 was visualized using FITC-conjugated goat anti-mouse C3 polyclonal antibody (green). Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody was used to detect either CR2-FH or FH(1–5) (red). CR2-FH (red) colocalized with C3 (green) along the GBM. No staining was observed with the Alexa 594-conjugated antibody in the PBS group.
n=6), but remained significantly lower than C3 levels in unmanipulated wild-type mice (median 278.84 mg/L, range 277.08–280.59, n=2, Figure 5A). Plasma C5 levels were comparable to those of nontreated wild-type animals, and strikingly different to arbitrary versus FH(1–5) and PBS group. Granular C3 was detected 48 hours after CR2-FH injection. Original magnification, ×40. (B) Quantitative immunofluorescence demonstrated a reduction in glomerular C3b/iC3b/C3c in the CR2-FH group compared with the FH(1–5) or PBS-injected mice at 2, 24, and 48 hours. Horizontal bars denote median values. **P<0.01 versus FH(1–5) and PBS.

Exogenous mouse and human FH restored plasma C3 levels and reduced GBM C3 deposition in Cfh−/− mice.30,31 After a single injection of 0.44 mg of FH(1–5) in Cfh−/− mice, we detected a faint band of intact C3 alpha chain in serum at 2 hours but no changes in glomerular C3. At a higher dose, a single injection of 1.6 mg FH(1–5) transiently increased plasma C3 levels at 2 hours (Supplemental Figure 9A), but did not alter glomerular C3 reactivity 2 hours (FH(1–5)); median=1149.7 arbitrary units, range 748.2–1300.3, n=4; PBS: median=1091.1 arbitrary units, range 973.9–1209.1, n=2), or at 24 hours (FH(1–5): median=1524.3 arbitrary units, range 1466.0–1582.7, n=2; PBS: median=1143.8 arbitrary units, range 1095.4–1192.1, n=2). However, following

rabbit anti-mouse FH polyclonal antibody in mice injected with either FH(1–5) or PBS. Granular C3 was detected 48 hours after CR2-FH injection. Original magnification, ×40. (B) Quantitative immunofluorescence demonstrated a reduction in glomerular C3 in the CR2-FH group compared with the FH(1–5) or PBS-injected mice at 2, 24, and 48 hours. Horizontal bars denote median values. **P<0.001 versus FH(1–5) and PBS.

FH(1–5) Reduced, but Did Not Colocalize with, Glomerular C3 in Cfh−/− Mice
Figure 4. Representative images of glomerular C3 in Cfh-/- mice injected with a single dose of CR2-FH. The reduction in glomerular C3 and C3b/iC3b/C3c intensity at 48 hours following a single injection of CR2-FH in Cfh-/- mice persisted to at least 96 hours. In contrast, no change in glomerular C3d reactivity was demonstrable (see also Supplemental Figure 5). CR2-FH reactivity, detected using the Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody, was demonstrable at all time points after injection and no reactivity was seen in nontreated in Cfh-/- mice. Original magnification, ×40.
Figure 5. Plasma and glomerular complement profile in mice injected with multiple doses of either CR2-FH or PBS. (A) Plasma C3 remained low in Cfh<sup>−/−</sup> mice despite repeated injections of CR2-FH. Horizontal bars denote median values. (B) Plasma C5 was analyzed by western blot under nonreducing conditions using 1 µl of mouse plasma. Under these conditions, C5 (band indicated by arrow) was readily detectable in mice injected with CR2-FH and unmanipulated wild-type mice, but absent in PBS-injected and unmanipulated Cfh<sup>−/−</sup> mice. (C) Representative images of glomerular C3 and C9/C5b-9. Original magnification, ×40. (D) Quantitative immunofluorescence demonstrated a reduction in glomerular C3, C3b/iC3b/C3c, C3d, and C9/C5b-9 in the CR2-FH-injected mice compared with the PBS group. Horizontal bars denote median values. *P=0.004 versus PBS group. Mann–Whitney test. AFU, arbitrary fluorescent units.
three injections of 1.6 mg of FH\(_{1-5}\) over 24 hours, there was a reduction in C3 reactivity and the appearance of mesangial C3 reactivity (Supplemental Figure 9B). Unlike CR2-FH, which was readily detectable and colocalized with glomerular C3, we were not able to detect FH\(_{1-5}\) in association with glomerular C3, suggesting that the effect was either indirect (e.g., control of C3 activation in plasma and consequent reduction of C3 deposition along the GBM), or that the interaction with glomerular C3 was very rapid (e.g., binding to C3b and rapid release following factor I [FI]-mediated cleavage of C3b to iC3b).

**CR2-FH Prevented Experimentally Triggered C3 Accumulation on the GBM**

Mice deficient in both FI and FH (Cfh\(^{-/-}\)Cfi\(^{-/-}\)) develop mesangial but not GBM accumulation of C3.\(^{32}\) Administration of a source of FI (serum from mice with a combined deficiency of C3 and FH, denoted C3\(^{-/-}\)Cfi\(^{-/-}\) serum) to these animals results in the appearance of GBM C3 deposition.\(^{32}\) We investigated whether CR2-FH could influence the development of GBM C3 by administering CR2-FH to Cfh\(^{-/-}\)Cfi\(^{-/-}\) mice (n=4) prior to injection of C3\(^{-/-}\)Cfh\(^{-/-}\) serum. As previously demonstrated,\(^{32}\) plasma C3b (alpha prime chain) was detected in control Cfh\(^{-/-}\)Cfi\(^{-/-}\) mice while, following injection of C3\(^{-/-}\)Cfh\(^{-/-}\) serum, plasma C3 alpha chain fragments were evident (Figure 6A), together with the appearance of linear staining along the GBM (Figure 6C). The appearance of the plasma C3 profile did not change with prior administration of CR2-FH at the 24-hour time point. No C5 was detected in mice injected with PBS irrespective of whether or not they received C3\(^{-/-}\)Cfh\(^{-/-}\) serum (Figure 6B). However, C5 became detectable in Cfh\(^{-/-}\)Cfi\(^{-/-}\) mice following the injection of CR2-FH and this was independent of the administration of mouse serum containing FI. As previously reported, linear glomerular C3 staining developed in Cfh\(^{-/-}\)Cfi\(^{-/-}\) mice following injection of mouse serum containing FI (Figure 6C).\(^{32}\) In marked contrast, this linear glomerular C3 staining was not seen in the animals pre-injected with CR2-FH. In these mice, there was mesangial C3 reactivity only and this was less intense than that seen in animals treated with CR2-FH or PBS that did not receive the C3\(^{-/-}\)Cfh\(^{-/-}\) serum (Figure 6D). Using the anti-FH antibody, CR2-FH was found to colocalize with the mesangial C3 in all mice injected with the reagent (Figure 6C).

In summary, a single CR2-FH injection increased plasma C5 levels in Cfh\(^{-/-}\)Cfi\(^{-/-}\) mice, colocalized with mesangial C3, and prevented the appearance of linear glomerular C3 following injection of mouse serum containing FI.

**DISCUSSION**

CR2-FH localized to C3 along the GBM in the FH-deficient mouse. The targeting domain (CR2) of CR2-FH was required for this effect because a single injection of FH\(_{1-5}\) unlike a single injection of CR2-FH, did not localize to the kidney or alter glomerular C3 reactivity. In the FH-deficient animal, CR2-FH reduced glomerular C3 reactivity after a single injection for at least 48 hours, and daily administration for 7 days resulted in reduction in glomerular C3d reactivity. Following injection, CR2-FH rapidly localized to glomerular C3 deposits along the GBM and remained detectable for up to 120 hours after a single injection. Despite a long half-life within the GBM, the agent was rapidly cleared from the circulation. It was detectable in plasma 2 hours after injection, but absent at 24 hours and later time points. Consistent with this rapid plasma clearance, plasma C3 levels rose only transiently and partially following administration. This is in contrast to the circulating half-life of FH following single administration: exogenous human FH had an estimated half-life of 6 days in human FH deficiency,\(^{33}\) was detectable in circulation 4 days after injection in FH-deficient mice,\(^{31}\) and was associated with prolonged increase in plasma C3 levels. Our data showed that CR2-FH was rapidly cleared from the plasma both in wild-type and factor H–deficient mice. So, if C3G was caused by a systemic defect in the regulation of C3 activation, we would predict that CR2-FH would not correct the systemic defect. However, based on our data, it would protect the GBM from C3 activation, which may be sufficient to ameliorate or prevent renal injury. We did not detect an interaction between CR2-FH and the mesangial C3 that appeared after administration of CR2-FH. This remains unexplained, but could reflect the nature of the mesangial C3; for example, if this was C3c or intact C3, we would not expect an interaction with CR2-FH.

CR2-FH was also able to prevent the de novo appearance of glomerular C3 in a triggered model of C3G. In this setting, the administration of a source of FI results in proteolytic cleavage of C3b and generation of C3b metabolites together with the appearance of GBM C3 reactivity.\(^{32}\) Our data show that the pre-administration of CR2-FH completely prevented the development of GBM C3 reactivity, but did not influence the metabolism of C3b. We speculate that CR2-FH interacted with C3b metabolites, preventing their association with the GBM, and/or interacted with any C3 that did associate with the GBM and prevented further amplification.

CR2-FH differentially affected plasma C3 and C5 levels in the experimental models. Potential explanations include reduction of C5 activation in fluid-phase or along surfaces within the kidney or both. C5 activation in fluid-phase is inefficient in vitro.\(^{34}\) We looked at the rate of C3 and C5 activation in human FH-depleted sera in vitro. C3 was rapidly cleaved (within 20 minutes) on addition of cations (Supplemental Figure 10). In contrast, C5 levels did not change over 24 hours as assessed by both western blotting and the ability of the test sera to reconstitute hemolytic activity to human C5-depleted sera. This indicates that C3 activation is likely to be rapid in the setting of FH deficiency in vivo, while the activation of C5 will be slower and depend on the availability of C3b. We speculate that the C5 convertase in complete FH deficiency is inefficient,\(^{35,36}\) and is therefore particularly sensitive to inhibition by exogenous regulators or the presence of properdin. Properdin deficiency differentially affected C3 and C5 levels in
FH-deficient mice, and here we could detect increases in C5 2 hours after injection of either FH1–5 or CR2-FH to FH-deficient mice. It is possible that these inhibitors transiently regulate both the C3 and C5 convertases, but the discordant changes in C3 and C5 levels derive from the different convertase efficiencies. It is also possible that C5 activation within the mesangium or along the GBM contributes to plasma C5 depletion and that this is influenced by CR2-FH and FH1–5.

Figure 6. CR2-FH prevented triggered C3 accumulation on the GBM. Mice with combined deficiency of FH and Fl (Cfh<sup>–/–</sup> Cfi<sup>–/–</sup>) were treated with CR2-FH or PBS and 1 hour later either did (+) or did not (−) receive an injection of serum deficient in both C3 and FH (as a source of Fl) to trigger GBM C3 deposition. Animals were culled 24 hours later and (A) plasma C3, (B) plasma C5, and (C,D) glomerular C3, and (C) CR2-FH assessed. (A) The C3 alpha prime chain of C3b was present in the Cfh<sup>–/–</sup> Cfi<sup>–/–</sup> mice irrespective of prior treatment with CR2-FH or PBS. C3 alpha chain fragments were detected in all mice that received C3- and FH-deficient mouse serum irrespective of pretreatment with CR2-FH or PBS. (B) C5 became detectable in animals that had received CR2-FH irrespective of subsequent injection with C3- and FH-deficient serum. (C) Representative images of glomerular C3 (green) and CR2-FH (red), original magnification ×40, and (D) quantitative glomerular C3 immunofluorescence, horizontal bars denote median values. *P<0.001, Bonferroni’s Multiple Comparison Test. NS, not significant. GBM C3 reactivity was detected in mice injected with C3- and FH-deficient serum alone, but not in those who had been pretreated with CR2-FH with consequent significant reduction in glomerular C3 staining intensity. The intensity of the mesangial C3 staining pattern seen in mice that had not received C3- and FH-deficient serum was not altered by pretreatment with CR2-FH.
However, we could not detect an interaction between FH\(_{1-5}\) and glomerular C3. Notably, glomerular C9 reactivity was not influenced by properdin deficiency in Cfh\(^{-/-}\) mice.\(^{37}\) Repeated administration of FH\(_{1-5}\) to Cfh\(^{-/-}\) mice over a 24-hour period was associated with a reduction in glomerular C3 staining. Notably, when a mutant FH protein that lacked the terminal five short consensus repeat (SCR) domains (FH\(_{1-16}\)–20) was expressed in the Cfh\(^{-/-}\) mice, abnormal GBM C3 deposition did not develop, and plasma C3 levels increased in proportion to the circulating FH\(_{1-16}\)–20 level.\(^{39}\) These data indicate that restoration of systemic C3 dysregulation by FH proteins that lack surface recognition domains is sufficient to ameliorate (exogenous FH\(_{1-5}\)) or prevent (endogenous FH\(_{1-16}\)–20) glomerular C3 accumulation in Cfh\(^{-/-}\) mice. However, the inability of FH\(_{1-5}\) to target to complement within the kidney indicated that this would not be an effective therapeutic approach in situations where C3 dysregulation is due to local activation. It would also, like FH, not be efficient in C3G associated with systemic C3 dysregulation that is driven by factors other than FH deficiency. This is important, as C3G associated with FH deficiency is extremely rare. More commonly, fluid-phase C3 dysregulation in C3G is due to the presence of C3 nephritic factors and normal levels of FH. In these settings, CR2-FH, by virtue of its ability to localize to glomerular-bound C3, would be the definitive method of specifically protecting glomerular components (GBM and/or mesangium) from the deleterious consequences of complement activation.

In summary, we have utilized two models of C3G and demonstrated that CR2-FH: (1) rapidly associates with glomerular C3 irrespective of whether it is within the mesangium or along the GBM; (2) ameliorated preexisting GBM-bound C3; and (3) prevented triggered GBM-bound C3. Our data indicate that CR2-FH could be efficacious in human C3G regardless of whether the C3 accumulation derives from systemic or renal C3 dysregulation. Ideally, this would require validation in models of C3G not driven by complete FH deficiency. Unlike C5 inhibition, from experimental data, C3 inhibition is the definitive therapeutic target in C3G and our data validate exploring the efficacy of CR2-FH in C3G.

**CONCISE METHODS**

**Generation of CR2-FH and FH\(_{1-5}\)**

The recombinant fusion protein CR2-FH, a hybrid mouse protein comprised of mouse CR2\(_{1-4}\) and mouse FH\(_{1-5}\), was generated as previously described.\(^{21}\) Subscript numbers denote SCR domains within CR2 and FH, with the first amino-terminal domain numbered one. The construct was prepared with a linker sequence between CR2 and FH encoding KEIL, a portion of the natural linker SCR\(_{4-5}\) of mouse CR2. The mouse FH\(_{1-5}\) recombinant protein was purified from the supernatant of transiently transfected EXP1293 cells by cation exchange, and complement activity evaluated using a complement alternative pathway hemolysis assay (data not shown).

**Animals and Treatment Protocols**

Mice were housed in specific pathogen-free conditions and animal procedures were performed in accordance with institutional guidelines and approved by the United Kingdom government. The generation of Cfh\(^{-/-}\),\(^{29}\) Cfh\(^{-/-}\).Cfh\(^{-/-}\), and C3\(^{-/-}\).Cfh\(^{-/-}\) mice has been described previously. Reagents were administered intravenously at the doses indicated (CR2-FH 0.8 mg; FH\(_{1-5}\) 0.44 or 1.6 mg) and samples and tissue collected at various time points. In the experiment where we administered three injections of 1.6 mg FH\(_{1-5}\), the second injection was given intraperitoneally. To trigger the C3 accumulation along the GBM in Cfh\(^{-/-}\).Cfh\(^{-/-}\) mice, we injected C3\(^{-/-}\).Cfh\(^{-/-}\) mouse serum as described previously,\(^{32}\) and mice were sacrificed 24 hours later. In these experiments the injection of C3\(^{-/-}\).Cfh\(^{-/-}\) mouse serum was preceded 1 hour earlier by intravenous injection of either 0.8 mg CR2-FH or PBS.

**CR2-FH Biodistribution**

Wild-type C57BL/6 mice received a single intravenous injection of 40 mg/kg CR2-FH. Animals were anesthetized, perfused with PBS, and sacrificed at 15 minutes, 2, 6, and 24 hours after injection. Organ homogenates were isolated and plasma collected for quantitative analysis of CR2-FH levels. CR2-FH concentrations were determined by ELISA using anti-CR2 7E9 (Hycult, The Netherlands) as capture antibody and biotinylated rabbit anti-mouse FH polyclonal antibody for detection (Alexion Pharmaceuticals, Cheshire, CT).

**Complement Inhibition Assay**

Complement activity in mouse sera was determined by its ability to lyse rabbit erythrocytes.\(^{40}\) CR2-FH at 1000, 62.5, and 3.91 nM in gelatin veronal-buffered saline (0.1% gelatin, 141 mM NaCl, 2.5 mM MgCl\(_2\), 0.15 mM CaCl\(_2\), 1.8 mM sodium barbital, 10 mM EGTA), was used as low, medium, and high lysis controls, respectively. Water and 42 mM EDTA were used as 100% lysis and negative controls, respectively. Samples were prepared in 1:2 dilutions in triplicate in gelatin veronal-buffered saline. Rabbit erythrocytes (1.5×10\(^6\)) were added to control and test samples and incubated at 37°C for 30 min. The absorbance of the supernatant was measured at 415 nm, and percent hemolysis was calculated by standard methods.\(^{41}\)

**C3 Quantification by ELISA**

Mouse blood was collected by cardiac puncture in the presence of EDTA, chilled on ice and the plasma separated. Coating antibody was a goat anti-mouse C3 polyclonal antibody (MP Biomedicals, Cedex, France) used at a dilution of 1:8000 in 0.1 M carbonate buffer, pH 9.6. Captured mouse C3 was detected using a horseradish peroxidase–conjugated goat anti-mouse C3 polyclonal antibody (MP Biomedicals) used at a dilution of 1:25,000 PBS/0.2% Tween. Plates were developed using TMB substrate (Sigma-Aldrich, Dorset, UK). The concentration of plasma C3 was estimated by reference to a calibration curve constructed using reference sera containing a known amount of mouse C3 (Serum Amyloid P mouse standard; Calbiochem, Darmstadt, Germany).

**Western Blot for CR2-FH, FH\(_{1-5}\), C3, and C5**

One microliter of EDTA plasma was used for western blot analysis. Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene
fluoride membrane. CR2-FH and FH(1-5) were detected with rabbit anti-mouse FH (Alexion Pharmaceuticals) and horseradish peroxidase–conjugated anti-rabbit immunoglobulins (DAKO). Western blotting for mouse C3 and C5 was performed as described previously.37

**Immunostaining**

Kidney samples were frozen in O.C.T. compound and 5 μm cryosections were fixed in acetone for 10 minutes. C3 was visualized using a FITC–conjugated goat anti-mouse C3 polyclonal antibody (MP Biomedicals), a rat anti-mouse C3b/iC3b/C3c monoclonal antibody (Hycult, The Netherlands), and a biotinylated goat anti-mouse C3d (R&D System, Abingdon, UK). The latter was visualized using streptavidin Alexa Fluor 488 (Life Technologies, Renfrew, UK). CR2-FH was visualized using an Alexa Fluor 594–conjugated rabbit anti-mouse FH antibody (Alexion Pharmaceuticals). Mouse C9 was visualized using a rabbit anti-rat C9 polyclonal antibody that cross-reacts with mouse C9 (a gift from Prof. B. Paul Morgan, Cardiff University, Cardiff, UK), and an Alexa Fluor 594–conjugated F(ab')2 of goat anti-rabbit IgG polyclonal antibody (Life Technologies). Quantitative immunofluorescence staining was performed as previously described.37 Ten glomeruli were assessed per section and the fluorescence intensity expressed in arbitrary units.

**Measurement of C3 and C5 Activation in FH-Depleted Sera**

FH-depleted serum (6 μl; Complement Technology, Tyler, TX) diluted in 24 μl AP buffer (5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 10 mM EGTA, 7 mM MgCl2, 0.1% (w/v) gelatin) was incubated for 0, 5, 10, 20, and 60 minutes, and 24 hours at 37°C and complement activation stopped by the addition of 10 mM EDTA. C3 activation was assessed by western blot under reducing conditions. Reaction samples containing 0.17 μl serum were subjected to SDS-PAGE and proteins were transferred onto polyvinylidene fluoride membrane. C3 was detected with horseradish peroxidase–conjugated goat anti-human C3 polyclonal antibody (MP Biomedicals). C5 was assessed by western blotting under nonreducing conditions and hemolysis assay. For western blotting, samples containing 0.25 μl serum were subjected to SDS-PAGE and proteins were transferred onto polyvinylidene fluoride membrane. C5 was detected with antibodies used for mouse C5. For the hemolysis assay, rabbit erythrocytes were washed and resuspended to 1% (v/v) in AP buffer. C5-depleted human serum (Complement Technology) was diluted in AP buffer. Samples containing FH-depleted serum were incubated for 0, 10, 60 minutes, and 24 hours and then serially diluted. Fifty microliters of each dilution was added to 50 μl of C5-depleted serum (10%), 30 μl of rabbit erythrocytes suspension (1%), and 20 μl of AP buffer. Reactions were incubated for 30 minutes at 37°C. The absorbance of the supernatant was measured at 415 nm and percent hemolysis was calculated by standard methods.41

**Statistical Analyses**

Data were analyzed using GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA). For comparison of two groups, the Mann–Whitney test was used. For comparison of three or more groups, Bonferroni’s Multiple Comparison Test was used. Statistical significance was defined as *P*<0.05.

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**DISCLOSURE**


**REFERENCES**


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Supplemental Figures 1 to 10

Efficacy of targeted complement inhibition in experimental C3 glomerulopathy

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Supplemental figure 1. CR2-FH in vivo functional activity. Levels of CR2-FH in plasma (filled squares) and inhibition of AP-mediated haemolysis by CR2-FH (open circles) were measured by ELISA and rabbit red blood cell haemolytic assay respectively. Samples were collected as indicated post-intravenous injection in wild-type mice.
Supplemental figure 2. CR2-FH biodistribution. CR2-FH concentration in kidneys (K), liver (Lv), spleen (S), lung (Lu), heart (H), brain (B) and eyes (E) of wild-type mice was measured after a single bolus injection at the indicated time points. Values are mean +/- standard error of the mean (SEM) and n = 3-4 per time point.
**Supplemental figure 3.** Assessment of plasma C3 in FH-deficient mice following either CR2-FH or FH_{1-5} by western blot analysis under reducing conditions. A single dose of CR2-FH, FH_{1-5}, or PBS was administered and plasma analysed at 2, 24 and 48 hours post-treatment. Intact C3 alpha chain was seen 2 hours following either CR2-FH or FH_{1-5}, but band intensity was greater in the CR2-FH treated animals.
Supplemental figure 4. CR2-FH does not interfere with the binding of anti-C3 antibody to glomerular C3 in vitro. Kidney sections from FH-deficient mice (n=3) were incubated with increasing concentrations of CR2-FH ex vivo. (A) Representative images of glomerular C3 and CR2-FH. Dose-dependent binding of CR2-FH to glomerular C3 was detected using an Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody (red). Glomerular C3 was detected using a FITC-conjugated goat anti-mouse C3 polyclonal antibody (green). Original magnification x40. (B) and (C) represent quantitative immunofluorescence using the FH and C3 antibody respectively. Horizontal bars denote median values. AFU - arbitrary fluorescence units.
Supplemental figure 5. Glomerular C3d staining. Representative images of glomerular C3d staining in FH-deficient mice after a single injection of CR2-FH, FH(1-5) or PBS. C3d was detected using a biotinylated goat anti-mouse C3d antibody and streptavidin-Alexa Fluor. Original magnification x40.
Supplemental figure 6. CR2-FH did not bind along the glomerular basement membrane (GBM) in wild-type mice. Representative images of glomerular C3 and CR2-FH in wild-type (n=3) and FH-deficient (n=3) mice two hours after injection of a single dose of CR2-FH. CR2-FH was detected using an Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody (red). Glomerular C3 was detected using a FITC-conjugated goat anti-mouse C3 polyclonal antibody (green). Original magnification x20.
**Supplemental figure 7.** (A) Assessment of glomerular CR2-FH binding by quantitative immunofluorescence. The highest intensity was 2 hours following a single dose of CR2-FH. Binding of CR2-FH within glomeruli was detected using an Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody. * P < 0.05 versus either 24 hour or 48 hour time point, Bonferroni multiple comparison test. Horizontal bars denote median values. AFU - arbitrary fluorescence units. (B) Representative images of glomerular staining using mouse kidney sections incubated with CR2-FH or PBS *ex vivo*. Using sections from FH-deficient mice 48 hours after a single dose of CR2-FH, the applied CR2-FH bound in a linear staining pattern, consistent with an interaction with GBM-bound C3. Using sections from untreated FH-deficient mice, the applied CR2-FH bound in a linear staining pattern, again consistent with an interaction with GBM-bound C3. Using sections from untreated wild-type mice, the applied CR2-FH co-localized to C3 along Bowman’s capsule. CR2-FH was detected using an Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody (red). Glomerular C3 was detected using a FITC-conjugated goat anti-mouse C3 polyclonal antibody (green). Original magnification x40
Supplemental figure 8. Representative images of glomerular FH staining using mouse kidney sections from FH-deficient mice treated with either single or multiple doses of CR2-FH. CR2-FH was detected using an Alexa 594-conjugated rabbit anti-mouse FH polyclonal antibody (red). Original magnification x40. Quantitative immunofluorescence demonstrated greater glomerular staining intensity in animals treated with the single compared to multiple doses indicating that CR2-FH did not accumulate within glomeruli after repeated administration. P value derived from Mann-Whitney test. Horizontal bars denote median values. AFU - arbitrary fluorescence units. For details of multiple dosing protocol see material and methods.
**Supplemental figure 9.** Plasma (A) and glomerular C3 (B) in FH-deficient mice injected with single or multiple doses of FH\(_{1-5}\). Plasma C3 levels increased significantly 2 hours after a single 1.6 mg injection of FH\(_{1-5}\). * P < 0.01 versus PBS-injected group at 2 and 24 hours and FH\(_{1-5}\)-injected group at 24 hours, Bonferroni multiple comparison test. Horizontal bars denote median values. (B) Representative images of glomerular C3 staining using sections from FH-deficient mice injected with single or multiple doses of FH\(_{1-5}\). Reduced linear C3 staining and appearance of mesangial C3 staining was evident within glomeruli from FH-deficient mice injected with multiple doses of FH\(_{1-5}\). Glomerular C3 was detected using a FITC-conjugated goat anti-mouse C3 polyclonal antibody (green). Original magnification x40. For details of multiple dosing protocol see material and methods.
Supplemental figure 10. Rate of C3 and C5 conversion in FH-depleted human sera in vitro. Human FH-depleted sera was incubated at 37 degrees centigrade in the presence of magnesium to enable spontaneous activation through the complement alternative pathway. Samples were taken at the indicated time intervals and complement activation stopped by the addition of EDTA. (A) Serum C3 activation was analysed by western blot under reducing conditions. Intact C3 alpha chain was evident only in pre-incubation sample. At 5 and 10 minutes incubation, the C3 alpha prime chain was evident together with C3 alpha chain fragments. After 10 minutes no C3 alpha prime chain was evident. (B) Serum C5 was analysed by western blot under non-reducing conditions. No reduction in C5 band intensity was evident over the time points examined. (C) Assessment of C5 haemolytic activity. Serial dilutions of FH-depleted sera, incubated for increasing times as indicated, were added to 10% C5-depleted human sera and the ability of the mixture to lyse rabbit erythrocytes was assessed. C5 remained functionally active at all tested time points with no evidence of reduction in activity with increasing incubation periods. Each point represents an average of duplicate measurements.