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Recombinant human IgG1 based Fc multimers, with limited FcR binding capacity, can effectively inhibit complement-mediated disease

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ABSTRACT

There is a lack of effective targeted therapies for the treatment of complement dependent diseases. We developed two recombinant Fc multimers, G207 and G211, with limited ability to interact with low/moderate affinity FcγRs, but with high avidity for C1q. These drugs effectively inhibited complement dependent cytotoxicity (CDC) *in vitro*, and prevented the deposition of C1q, C3b and MAC, on the surface of Ab-opsonized cells. Importantly, these inhibitory effects were both C1q dependent and independent. In order to determine the biologic relevance of our findings, we evaluated the clinical efficacy of these drugs in three different animal models, acute RBC hemolysis, anti-Thy-1 nephritis and passive Heymann's nephropathy (PHN), in which disease pathophysiology relies preferentially on complement activation. While G207 was protective in the anti-Thy-1 nephritis and PHN models, G211 was *protective* in all of the models tested and could effectively *treat* PHN. In the anti-Thy-1 nephritis model, G211 prevented the characteristic histologic changes associated with the disease and limited glomerular deposition of C3. Collectively, these data suggest that "complement preferential" Fc multimers offer a novel approach to the treatment of complement mediated diseases.

1. Introduction

Several lines of evidence suggest that many of the anti-inflammatory activities of intravenous immunoglobulin (IVIG) are the result of small quantities of immune aggregates within the drug preparation that, paradoxically, are targeted for elimination during drug formulation [1–4]. Based on the idea that Fc bearing immune aggregates could induce tolerance, we developed a drug candidate called GL-2045, which is a recombinant human Fc multimer designed to replace the anti-inflammatory effects of IVIG [5]. (Manuscript submitted) Preclinical studies revealed that in addition to inducing anti-inflammatory activities through active Fc receptor signaling and passive FcR blockade, GL-2045 could also inhibit complement dependent cytotoxicity (CDC) through both C1q dependent and independent mechanisms [6,7].

Surprisingly, despite its ability to sequester C1q, which should in theory drive the complement cascade through to completion with resultant formation of the membrane attack complex (MAC), GL-2045 stimulation of human serum *in vitro* induced relatively high levels of C4a, but more limited levels of C3a and virtually no C5a.

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Mechanistic studies revealed that this "phenotype" was secondary to the ability of GL-2045 – like IVIG – to potentiate factor H [8]. As a result of this attenuated activation, GL-2045 induced high levels of the "inactive" form of the complement split product C3b, called iC3b, – a potent anti-inflammatory molecule, whose relatively long half-life and ability to inhibit the adaptive immune response, likely constrains complement related injury long after elimination of the precipitating threat [9–12].

Recent progress in Fc engineering through mutagenesis and glycoform modifications have afforded opportunities to modify the affinity and/or selectivity of Fc fragments to engage with FcRs or complement, resulting in altered Ab effector functions [13,14]. However, we have found that the reported mutations and combinations of mutations that cause desired changes in affinity or selectivity in the context of an antibody [15–17] are not predictive in the context of a stradomer. In the current study, we employed GL-2045 as a platform to engineer two new recombinant, human Fc multimers, G207 and G211, which retain the inhibitory activities of GL-2045 on the complement cascade, while selectively decreasing their ability to engage the canonical FcγRs. *In vitro*, G207 and G211exhibited potent inhibition of complement activation through C1q dependent and independent mechanisms. Furthermore, these drugs showed efficacy in the prevention and/or treatment of three distinct diseases in rats whose pathophysiology is variably dependent on aberrant complement activation. These observations suggest that G207 and/or G211 might serve as novel drugs for the treatment of complement mediated human disease.

2. Materials and methods

2.1. Cells

Ramos and SUDHL4 cells lines were maintained in RPMI 1640 (Mediatech Inc., Manassas, VA) supplemented with 10% FBS (Atlanta Biologicals, GA), 1% pencillin/streptomycin, 1% Hepes and 1% Glutamax (all from Life Technologies). Human FcγR transfected CHO cells were generated and maintained in the laboratory [18]. Human RBCs were obtained from ZenBio, Inc. (Research Triangle Park, NC). Human PBMCs were prepared from buffy coats (Biospecialty, Inc. Colmar, PA) by density centrifugation.

2.2. Preparation of G001, GL-2045, G207 and G211

G001 and GL-2045 were prepared as previously described [7]. G207 and G211 were designed based on the structure of core stradomer GL-2045 [7] to have enhanced C1q binding and reduced FcγR engagement. In order to capture the spectrum of naturally occurring human IgG1 Fc polymorphisms, we made two forms of both G207 and G211, bearing either the DEL or EEM polymorphisms. These polymorphic variants are functionally indistinguishable and were used interchangeably throughout our studies [19].

Stable CHO cell pools expressing G207 and G211 were established according to the manufacturers' protocols (ExcellGene SA, Monthey, Switzerland). G207 and G211 were purified using a combination of affinity and cation exchange chromatography. Non-reduced and reduced SDS-PAGE analysis of G207 and G211 was performed as previously described [18].

2.3. ELISAs

2.3.1. C1q binding ELISA

The C1Q binding ELISA was performed as described [18].

2.3.2. Complement split products ELISAs

90 μl pooled human complement serum (Cedarlane, Burlington, NC) was incubated with 10 μl of serial diluted G001, G207, G211, and heat-aggregated IgG (HAGG) (Quidel, San diego, CA) in 96 well plate at 37 °C for 1.5 h. The reaction was stopped by addition of EDTA at final concentration of 10 mM. Treated serum samples were aliquot and stored at −80 °C for ELISA assay. C4a (BD Bioscience), C3a (eBioscience), C5a (eBioscience), and iC3b (Quidel) kits were used to measure the levels of complement split products per manufacturer's protocol. Serum sample dilutions for ELISA were: 1:50,000 for C4a, 1:10,000 for C3a, 1:100 for C5a, and 1:300 for iC3b.

2.3.3. TNF-α and IL-1RA ELISA

2×10^6/ml PBMCs were incubated with 100 μg/ml G001, G207, G211, or GL-2045 in 96 well plate at 200μl/well. Supernatants were collected at 4 h (TNF-α), and 24 h (IL-1RA) timepoints. The levels of cytokine production were measured by ELISA (TNF-α, BD Bioscience; IL-1RA, RnD systems) per manufacturer's protocol.

2.4. Surface plasma resonance

2.4.1. FcγR and FcRn binding analysis

SPR analysis of FcγR and FcRn binding were performed as previous described with the following modifications [18]. Anti-Histidine (GE Lifesciences) immobilized to 2 flow cells on a CM5 chip to 7000RU per manufacturer instructions for amine coupling. To determine dissociation constants (K_D) to FcγRI, G207 and G211 were serially diluted from 1000 nM to 1.6 nM and 100 nM to 0.16 nM, respectively, and injected for 1 min at 50 μL/min in increasing concentration without regeneration between injections. A dissociation phase of 120 s was performed between injections. At the end of each injection series, a final 6 min dissociation performed. Flow cells were fully regenerated using a 15 μL injection of Glycine pH1.5 at 50 μL/min, followed by a 60-second wash with running buffer. Each series cycle was performed in triplicate. Dissociation constants were analyzed with the single cycle titration kinetics model using the BIAevaluation software v.3.1.

Human FcRn (Sino Biologic) was diluted to 5 μg/mL in Sodium Acetate pH 4.5 and injected over an activated flow cell at 5 μL/min on a CM5 chip until an immobilization level of 1000RU was reached. After blocking all open binding sites, G207 or G211 were serially diluted in running buffer (67 mM Sodium Phosphate pH6.0, 150 mM NaCl, 0.005% Tween 20) and injected for 3 min at 25μL/minute, followed by a 4 min dissociation phase. After each injection, the chip surface was fully regenerated using running buffer at ph8.0 and then washed with running buffer pH6.0. A blank flow cell was used as a blank reference and a running buffer-only injection was subtracted from each curve. Curves were run in triplicate and dissociation constants were determined using the 1:1 Langmuir model with RI = 0 in the BIAevaluation software version 3.1.

2.4.2. C1q binding analysis

Purified human C1q (Complement Tech) was diluted to 25 μg/mL in Sodium Acetate pH 4.5 and injected over an activated flow cell on a CM5 chip at 5 μL/min until an immobilization level of 3500RU was reached. A blank flow cell was used as a reference. After blocking all open binding sites, G001, G207, G211, GL-2045, and IVIG (Atlantic Biologicals, Miami, FL) were diluted in HBSS-EP running buffer and injected for 1 min at 50 μl/minute to determine binding activity. For kinetic assays, a single cycle titration method was used. G207, G211 and GL-2045 were serially diluted in running buffer and injected in increasing concentration at 50 μl/min for 1 min, followed by a 2 min dissociation step between injections, without regeneration. The final injection of each single cycle was followed by a 6 min dissociation phase. The chip surface

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was then fully regenerated using 0.5M KCl in HBSS-EP, following by a wash with running buffer. A running buffer injection was subtracted from each curve. Dissociation constants were determined by overlaying each curve in a titration series and using the 1:1 Langmuir model in the BIA Evaluation software version 3.1.

2.5. CDC

2.5.1. Anti-CD20 mediated CDC

1×10^5 SUDHL4 or Ramos cells were pre-incubated with human serum (final concentration at 6% or 50% v/v) in the presence of serial titrations of testing compounds for 15 min at 37 °C in a 96 well plate. Anti-CD20 mAb (Rituximab, Genentech) was then added to the culture at final concentration of 10 μg/ml to induce antibody mediated CDC for additional 45 min at 37 °C. The cytotoxicity was measured by staining cells with Annexin V-PE and 7-AAD (Biolegend) and analyzed by flow cytometry. Alternatively, the assay was stopped at 15 min, or 30 min after addition of anti-CD20 mAb by adding EDTA at 10 mM for measurement of C1q, C3b and MAC deposition.

2.5.2. C1q-dependent CDC modulation

Purified C1q (10 μg/ml, Quidel) was pre-treated with testing compounds at indicated concentrations for 15 min at room temperature, then incubated with anti-CD20 mAb opsonized Ramos cells in the presence of C1q-deficient serum (6%, Quidel) at 37 °C for 45 min. Cytotoxicity was then evaluated by Annexin V/7-AAD staining.

2.5.3. C1q-independent CDC modulation

Ramos cells were first opsonized with anti-CD20 mAb (10 μg/ml) in the presence of purified C1q (10 μg/ml) for 15 min at room temperature. After washing to remove excessive mAb and C1q, Cells were then incubated with 6% C1q depleted serum that had been pre-treated with testing compounds, for an additional 45 min at 37 °C. After incubation, cells were washed and stained with Annexin V/7-AAD to assess cytotoxicity.

The percent of inhibition of CDC in both C1q dependent and independent CDC modulation was calculated as: (1-CDC with testing compounds/maximum CDC (no testing compounds)) × 100.

2.5.4. In vitro and ex vivo stradomers inhibit complement dependent hemolysis

Wistar rat serum (150μl/well) (Innovative Research) was pre-treated with testing compounds (25 μl/well) at indicated concentrations in 96 well plate for 15 min at room temperature. Alternatively, 25 μl rat serum from animals treated with G211 were added directly to 96 well plate. Human RBCs were washed and suspended in GVBS buffer (VBS with 0.1% Gelatin), then added into 96 well plate (25 μl/well) and incubated for 1 h at 37 °C. EDTA was added to terminate the hemolysis reaction. The plate was centrifuged and supernatant was collected and measured at 540 using a spectrophotometer. Positive and negative hemolysis control was set up using ACK lysing buffer (Quality Biological), and PBS, respectively. The calculation of percent of hemolysis in *in vitro* assay was: (sample OD540-negative control OD540)/(positive control OD540-negative control OD540) × 100. For the ex vivo analysis, the percent of hemolysis relative to pre-dose animal serum was calculated as: (sample OD540 − negative control OD540)/(pre-dose OD540 − negative control OD540) × 100.

2.6. Flow cytometry

2.6.1. Binding of G207 and G211 to human FcγR expressing CHO cells, human and rat PBMCs

Dylight 488 labeled G001, G207, G211, and GL-2045 were prepared in-house using Dylight 488 microscale Ab labeling kit (Thermo Scientific) per manufacturer's protocol. 10 μg/ml of each labeled compound was incubated with FcγR expressing CHO cells, or human and rat PBMCS in combination with fluorochrome labeled mAbs to human or rat immune cell markers to define specific cell populations in PBMC. The following mAbs were used: PerCP anti-human CD3 (Biolegend), APC anti-human CD19 (Biolegend), PE anti-human CD56 (BD Biosciences), PE-Cy7 anti-human CD14 (Biolegend), PE anti-rat CD3 (eBioscience), PE-eFluor710 anti-rat CD161 (eBioscience), APC anti-rat CD45RA (eBioscience), APC anti-rat CD4 (eBioscience).

2.6.2. C1q, C3b and MAC deposition on Ab-opsonized Ramos cells

The Ramos cell CDC assay was setup as described above. Cell surface deposition of C1q, C3b, and MAC were detected with FITC labeled anti-C1q, anti-C3b (Cedarlane), and anti-sc5b-9 (LifeSpan Biosciences, Seattle, WA) Abs.

To test whether G207 and G211 pull down the deposited C1q from cell surface, Ramos cells opsonized with anti-CD20 mAb and purified C1q were treated with testing compounds for 15 min at 37 °C. Cells surface C1q was detected by FITC labeled anti-C1q Ab and analyzed by FACS.

Labeled cells were acquired with an LSRII flow cytometer (BD Biosciences) and analyzed using FACSDiva (BD Biosciences) or FlowJo (Treestar) software.

2.7. Rat acute intravascular hemolysis model

Wistar rats with Jugular vein catheter were purchased from Charles River. The protocol was approved by IACUC of University of Maryland School of Medicine. Rats were sedated with ketamine-Xylazine (60 mg/kg and 7.5 mg/kg, MWI Animal health, Idaho) by IP. The first dose of drug was infused via catheter, 10 min before human RBC transfusion. Human RBCs were labeled with PKH26 Red (Sigma) per manufacturer's protocol. Two × 10^9 labeled RBCs mixed with a second dose of drug were transfused into rat circulation through catheter at a consistent rate of infusion (1 min/infusion). 200 μl rat blood samples were drawn through catheter pre-dosing and at 1, 3, 5, 10, 15, 30, and 60 min post RBC infusion. The percentage of human RBC in rat blood was assessed by flow cytometry.

2.8. Rat anti-Thy-1 glomerulonephritis model

All of the rat anti-Thy-1 nephritis studies were performed by Washington Biotechnology (Baltimore MD), Inc. Male Wistar rats were purchased from Envigo. Nephritis was induced in rats by IV injection of anti-Thy-1.1 mAb (Cedarlane) at 1 mg/kg at day 0. Testing compounds or PBS were administered by IV at day 0 (4 h before the injection of anti-Thy-1.1 Ab), 2, 4, and 6. 24 h' urine were collected on day −1, 3, 5, and 7 by housing the rats in metabolic cages for urine protein analysis. At terminal time points, animals were sacrificed by exsanguination. Both kidneys were collected and fixed in 10% formalin for subsequent embedment and immune-histological assessments.

2.9. Rat PHN model

Passive Heymann Nephritis (PHN) studies were performed by Washington Biotechnology, Inc. and Probetex Inc, (San Antonio,

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Texas). Sprague-Dawley (SD) rats were obtained from Harlan laboratories. The age and weight of rats at the initiation of treatments were 7 weeks old, between 180 and 200 grams. PHN was induced by tail vein injection of anti-FX1A serum (PTX-002S, Probetex, Inc, San Antonio, TX) on day 0. Testing compounds were administered by IV or SC, 1 or 2 times/week, for 3 weeks. The drug administration started either prior to or post injection of antisera as indicated. Urine samples were collected at four time points during the course of the study by housing the rats in metabolic cages - Pre-study (0 wk), 1 week, 2 weeks, and 3 weeks following induction of PHN.

2.10. Histology assessments

OCT-embedded sections of rat spleen and kidney were fixed for 5 min in cold acetone and washed with PBS for 15 min. After blocking with 10% FBS in PBS for 30 min, tissue sections were stained with Dylight 488 conjugated G207 and G211 for 1 h. The specific binding of G207 and G211 to rat tissues were reviewed by two pathologists and the final report reflects their consensus opinion.

Formalin fixed kidney samples were routinely embedded in paraffin. For routine microscopy, 4 micron thick sections were stained with H&E. C3 Immunohistochemistry staining was performed at Laboratory of Dermatology, University of Maryland School of medicine. The rabbit anti-rat C3 polyclonal Ab (LifeSpan Biosciences, Inc.) was used as primary antibody. Immunohistochemical staining was performed using a VECTASTAIN ABC kit (Vector Laboratories). The sections were counterstained with hematoxylin. The pathological changes were assessed in a blinded way by the pathologist.

P57 staining was performed by Probetex Inc. The rabbit anti-rat p57 polyclonal Ab (Santa Cruz Biotechnology) and ImmPRESS anti-rat IgG (peroxidase) polymer detection kit (Vector Laboratories) were used for immunohistochemical staining of p57. Podocyte injury was assessed by manually counting the number of p57 positive nuclei/glomerulus. Thirty random images at 20X objective magnification for each kidney, each field representing one glomerulus, were assessed.

2.11. Electron microscopy

Electron microscopy was performed as previously described [20].

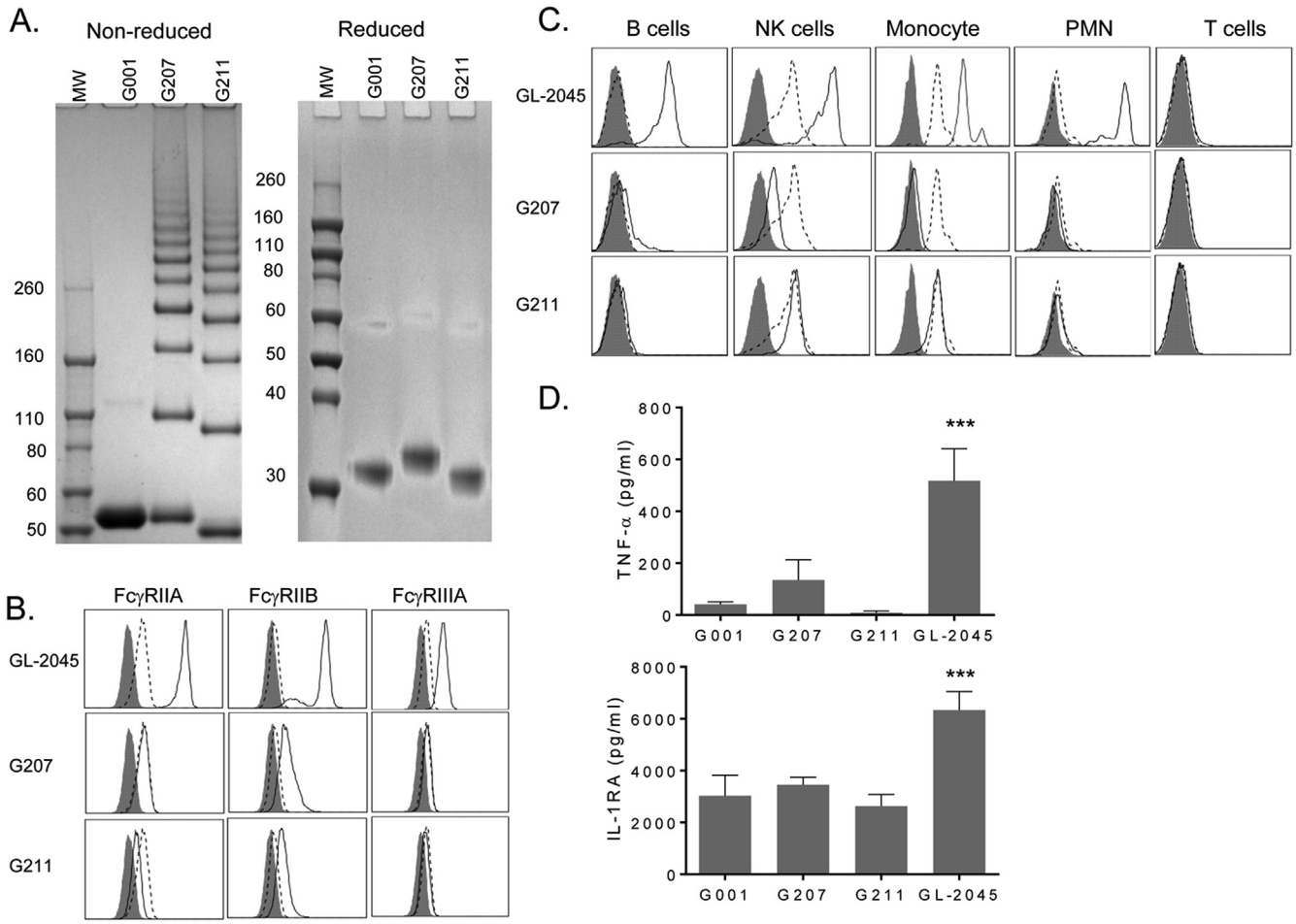

Fig. 1. G207 and G211 form Fc multimers and have limited interactions with low affinity human canonical Fcγ receptors. (A) SDS-PAGE analysis of G207 and G211 under non-reduced and reduced conditions. (B) Binding of Dylight 488 labeled G207 and G211 to low affinity CHO-human FcγR expressing cells by flow cytometry. (C) G207 and G211 do not bind to human peripheral blood Fcγ receptor expressing cells. Human whole blood from healthy donors was stained with Dylight 488 labeled G207 or G211, in combination with mAbs specific for immune cell markers. The binding of each compound to T cells ($CD3^+$), B cells ($CD3^-CD19^+$), NK cells ($CD3^-CD56^+$), Monocytes ($CD14^+$), and granulocytes (FSC vs SSC) was evaluated by flow cytometry. Filled histogram – no stain; dashed lines – G001; Solid lines – GL-2045, G207, or G211. (D) TNF-α and IL-1RA production by PBMCs stimulated with G207 and G211. PBMCs were cultured with 100 μg/ml of each compound for 24 h. The levels of cytokines in the supernatants were determined by ELISA. Data are shown as mean with SEM, summarized from four experiments using PBMCs from different donors. ***$p < 0.001$, compared to G001 control.

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2.12. Statistics

Ordinary one-way ANOVA was carried out to compare different treatments versus controls (Figs. 1D, 2E and 3E, 7E). To compare treatment effects at different dose levels, we used two-way ANOVA (Fig. 2C–F, 4A). Statistical significance of a potential dose-response effect was established by testing the hypothesis that Spearman's rank correlation coefficient, r_S, was zero. An approximate estimate (with 95% confidence limits) of the dose for 50% effect was obtained from a linear regression of the base 2 logarithm of the effect of a given drug concentration relative to the effect at zero dose. Control groups included no treatment, PBS, or G001. Only one type of control group was used in each experiment as specified in each figure. Time-dependent data from *in vivo* experiments (Figs. 5, 6 A-B and 7 A-D) were analyzed using repeated measures two-way ANOVA. The Mann-Whitney test was used to compare the number of abnormal glomeruli in G211 treated group with PBS group (Fig. 6 C). All hypothesis were tested with a two-sided level of significance of 0.05. Dunnett's adjustment procedure was applied to account for multiplicity when multiple comparisons were present. Statistical significance was indicated as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Statistical analyses were performed using Graphpad

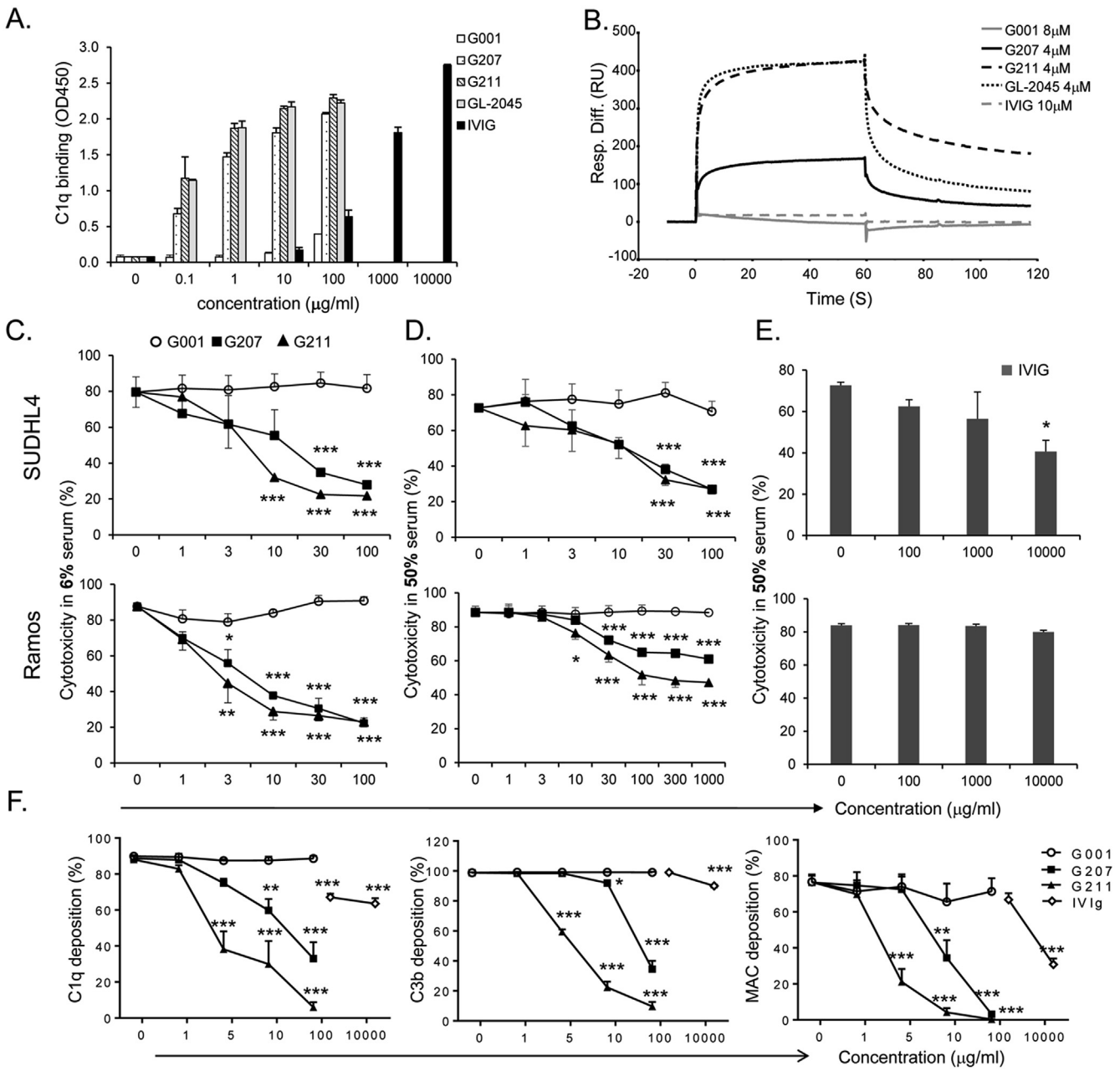

Fig. 2. G207 and G211 bind C1q and block antibody mediated CDC. (A) The binding of G207 and G211 to plate coated C1q was assessed by ELISA. (B) BiaCore plot characterizing the binding kinetics of each drug to C1q. The ability of G207, G211 and IVIG to block anti-CD20 mediated CDC of SUDHL4 or Ramos cells was tested using either 6% (C) or 50% (D, E) human serum. (F) Characterization of C1q, C3b and MAC deposition on the cell surface in the presence of defined drug concentrations. Data are shown as mean with SEM, from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, G207 vs G001, GL211 vs G001, IVIG vs no treatment control.

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softward (V6.05).

3. Results

3.1. G207 and G211 are recombinant Fc multimers, engineered to have high avidity for C1q and limited interactions with the canonical FcγRs

Building upon the GL-2045 platform [7], we generated a series of human IgG1 mutated Fc multimers and found that the published literature of antibody mutations was not predictive in identifying multimerizing stradomers with retained or enhanced Fc: C1q interactions and reduced Fc: FcγR binding. Among a large number of mutated GL-2045-derived compounds, G207 and G211 retained complement binding and CDC inhibition with greatly diminished binding to low affinity FcγRs. Analysis of G207 and G211 by SDS-PAGE revealed that both drugs are composed of sequential, highly ordered, Fc multimers (Fig. 1A). Surface plasma resonance studies, employed to characterize the ability of G207 and G211 to interact with the canonical FcγRs and the FcRn, demonstrated that both G207 and G211 bound with high *avidity* to the high *affinity* receptor, FcγRI as well as to the FcRn. In contrast, both drugs lost the ability to bind FcγRIIA, IIB, and IIIA (Supplemental Figs. 1 and 2).

Consistent with SPR analysis, neither of these drugs efficiently bound CHO cells engineered to express the FcγRIIA or FcγRIIIA. However, both G207 and G211 showed limited binding to FcγRIIB expressing cells, compared with the homodimeric Fc control, G001 (Fig. 1B). In contrast, the GL-2045 positive control, bound CHO cells expressing each of the individual FcγRs. In keeping with these findings, G207 and G211 failed to bind human PBMC at levels greater than the G001 control (Fig. 1C).

Fcγ receptor crosslinking induces cytokine production by human leukocytes [21]. In order to determine if this limited FcγR binding by G207 and G211 was functionally relevant, we tested their ability to induce secretion of TNF-α human PBMC (Fig. 1D). As anticipated, GL-2045 induced secretion of TNF-α and IL-1RA. In contrast, neither G207 nor G211 stimulated levels of TNF-α or IL-1RA release above that of G001 control, which lacks the avidity to effectively signal through the low and intermediate affinity FcγRs. Collectively, these data demonstrate that despite the fact that G207

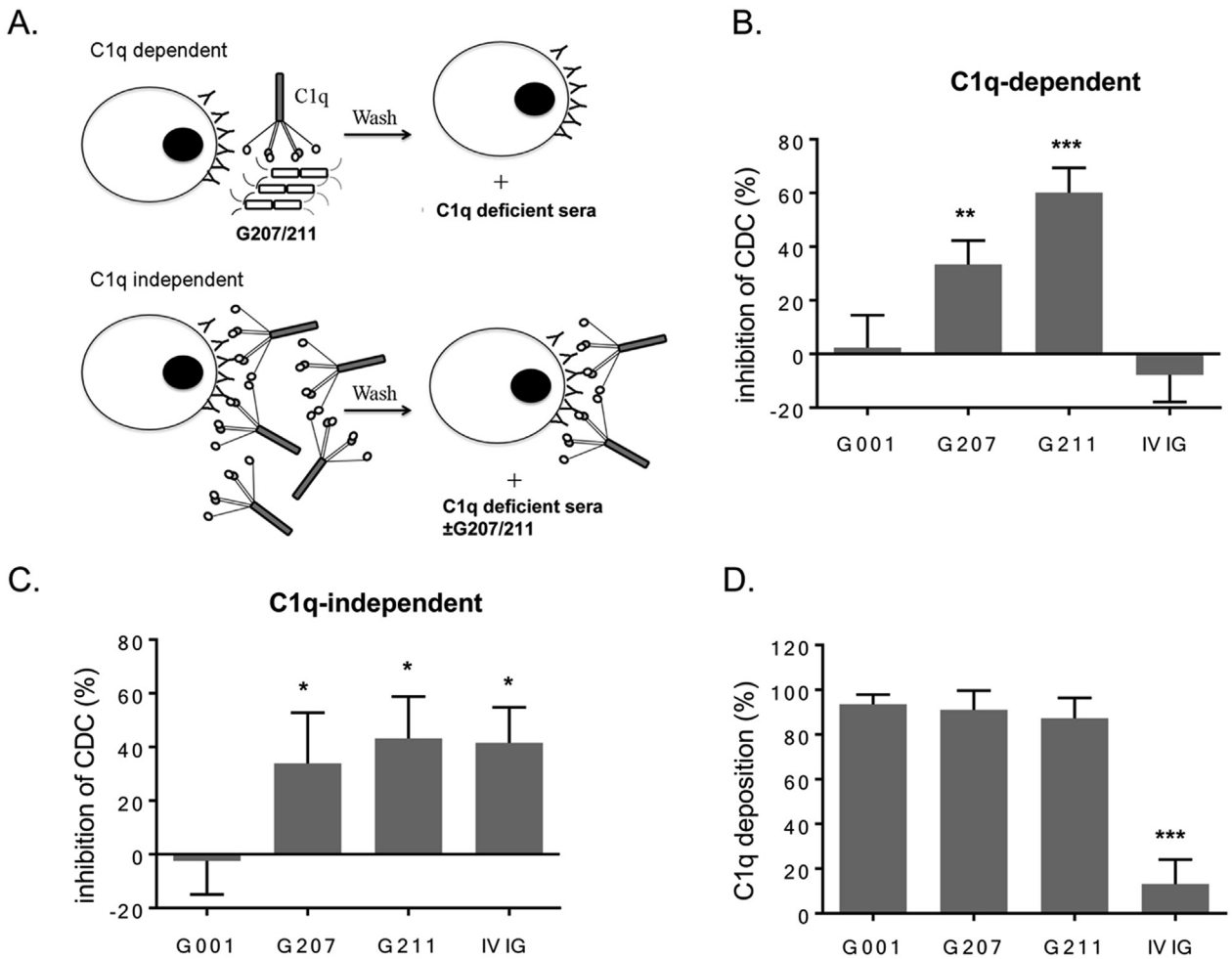

Fig. 3. G207 and G211 mediate both C1q-dependent and C1q-independent inhibition of complement activation. (A, B) Anti-CD20 opsonized Ramos cells were incubated with purified C1q that was pre-treated with G207 or G211, washed, then incubated with C1q deficient serum. (A, C) Anti-CD20 opsonized Ramos cells were treated with purified C1q, washed, then incubated with C1q deficient serum pretreated with G207 or G211. CDC activity was assessed using Annexin V/7-AAD staining kit and analyzed by flow cytometry. Data are shown as the percent inhibition of CDC relative to a no treatment control. (D) C1q deposition on Ab opsonized Ramos cells is not affected by post treatment with G207 or G211. Anti-CD20 opsonized Ramos cells were coated with purified C1q, washed, then incubated with the compounds indicated. The deposition of C1q on the cell surface was evaluated by anti-C1q mAb staining. Data are shown as mean with SEM, from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to G001 control.

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and G211 are highly ordered Fc multimers, they have limited ability to bind and interact with the low and intermediate affinity human FcγRs.

3.2. G207 and G211 avidly bind C1q and inhibit complement dependent cytotoxicity

Next, we sought to determine whether G207 and G211 retained or enhanced the ability of Fc multimers to sequester C1q and inhibit CDC. Like GL-2045, both G207 and G211 avidly bound plate coated-C1q, with a potency that was approximately 3-log orders greater than IVIG in an ELISA based assay (Fig. 2A) [7]. SPR analysis revealed different C1q binding kinetics between G207 vs G211. In comparison with G207, G211 had more than a 1-log order faster rate of association with C1q, and approximately a two times slower rate of dissociation. (Fig. 2B, Table 1, and Supplemental Fig. 3). Interestingly, IVIG showed no binding to C1q in the setting of SPR analysis, in contrast to the significant C1q binding observed at high concentrations in an ELISA based assay.

In order to determine the functional relevance of this C1q binding, we tested the ability of both drugs to inhibit CDC of two anti-CD20 opsonized malignant B cell lines, SUDHL4 (FcγRIIB$^+$) and Ramos (FcγRIIB$^-$) (Fig. 2C and D). Both G207 and G211 efficiently

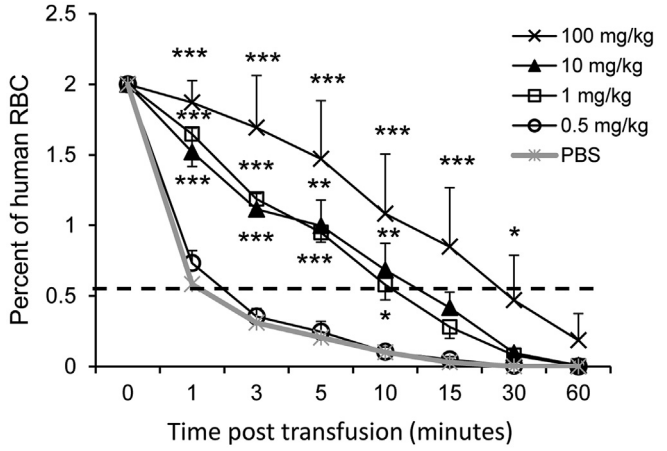

Fig. 5. G211 effectively prevents complement dependent acute intravascular hemolysis. Different doses of G211 was administered by IV 10 min before transfusion of PKH-labeled human RBC pre-mixed with the same doses of G211. Blood samples were drawn at different time points post transfusion and levels of human RBCs in the rat circulation were analyzed by flow cytometry. Data are shown as the mean with SEM of percent of human RBC, from five independent experiments. G211 treatment groups, n = 3 for each group; PBS group, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001, compared with PBS control.

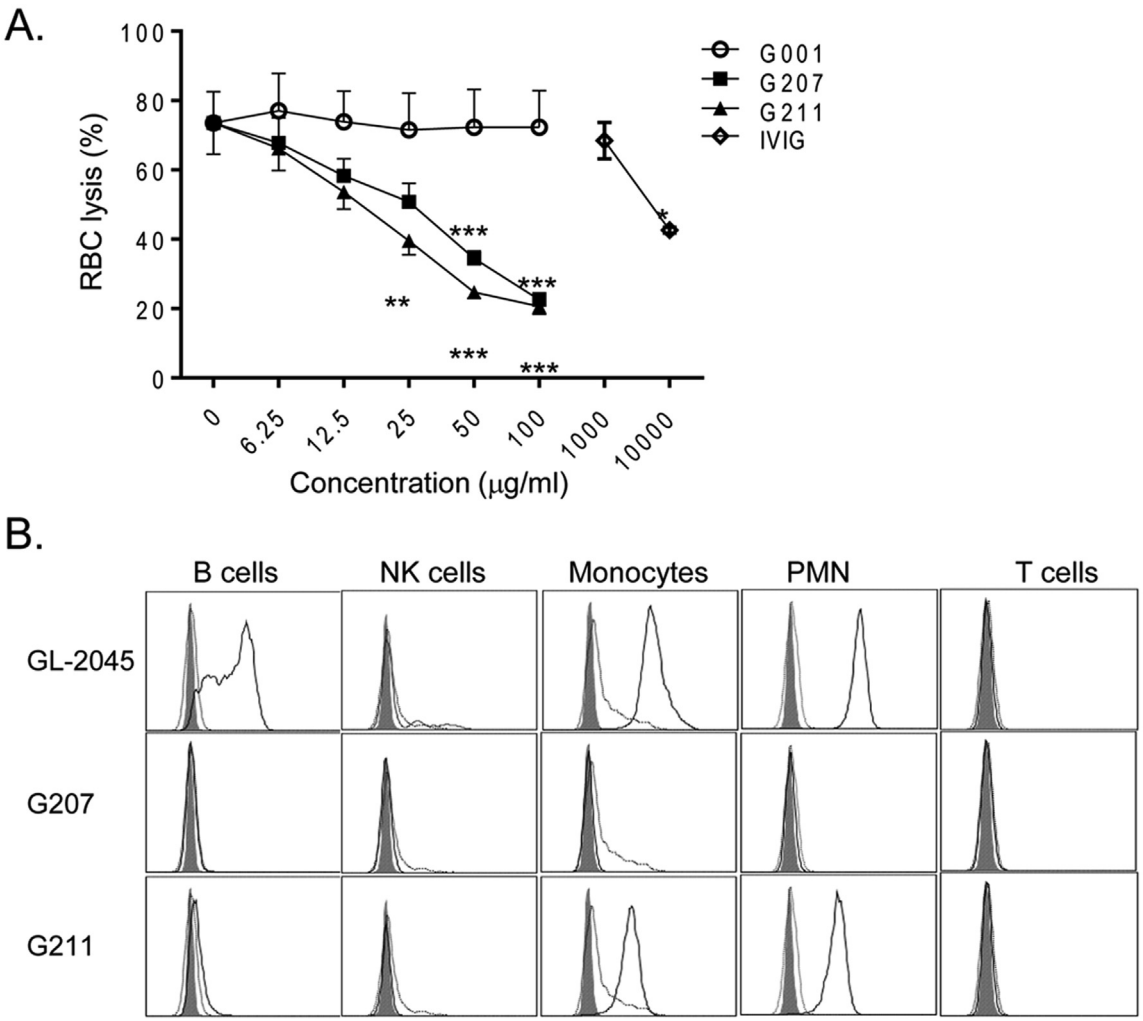

Fig. 4. G211 inhibits rat complement activation in vitro. (A) Human RBCs were incubated with 75% normal Wistar rat serum in the presence of the indicated concentrations of G207, G211 or IVIG for 1 h. RBC lysis was measured. Data are pooled from three independent experiments and shown as mean with SEM. (B) The binding of G207 and G211 to rat peripheral blood cells was assessed by flow cytometry. The markers used to define specific cell populations were: B cells (CD3- CD45R+), NK cells (CD3- CD161+), monocytes (CD3- CD4$^+$), PMN (FSC vs SSC), T cells (CD3$^+$). Filled histogram – no stain; dashed lines – G001; Solid lines – GL-2045, G207, or G211. *p < 0.05, **p < 0.01, ***p < 0.001, G207 vs G001, GL211 vs G001, IVIG vs no treatment control.

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inhibited CDC of both cell lines in 6% serum. Importantly, both drugs also protected both cells lines against CDC at the more biologically relevant serum concentration of 50%. In contrast, IVIG, at the highest concentration tested, partially protected SUDHL4 cells, but not Ramos, from CDC in 50% complement serum (Fig. 2E).

We hypothesized that G207 and G211 binding to C1q might sequester C1q away from the Ab opsonized cells and therefore inhibit CDC. In order to test this hypothesis, we assessed the deposition of C1q, C3b and the membrane attack complex (MAC) on the cell surface in a CDC assay with 6% serum (Fig. 2F). As anticipated, both drugs inhibited the deposition of C1q, C3b and MAC on cell surface in a dose-dependent manner (p-value for testing $r_S = 0$ less than 0.001 for each of C1q, C3b and MAC for both G207 and G211). However, compared with G207, G211 was more potent with estimated doses [with 95% CI] for halving the deposition of C1q (23 [18,32] μg/ml vs. 63 [50, 83] μg/ml, $p < 0.001$), C3b (27 [20, 38] μg/ml vs. 67 [59, 71] μg/ml, $p < 0.001$), and MAC (11 [9, 16] μg/ml vs. 21 [18, 24] μg/ml), $p = 0.002$), for G211 and G207 respectively. Furthermore, the maximum levels of inhibition of both G207 and G211 on C1q, C3b, and MAC deposition were greater than IVIG. Interestingly, while 10 mg/ml of IVIG exhibited potent inhibition of MAC deposition (59.8% inhibition), it was less effective in inhibiting C1q (29.3% inhibition) and C3b (9% inhibition) deposition.

In order to determine if G207 and G211 binding to C1q activates the complement cascade, we measured the levels of C4a, C3a, C5a, and iC3b in human serum exposed to escalating concentrations of each drug (Supplemental Fig. 3). Heat aggregated IVIG (HAGG), used as a positive control for naturally occurring immune aggregates, induced high levels of all complement split products tested, including C5a. In contrast, G207 and G211 induced high levels of C4a, intermediate levels of C3a, and no C5a. Similarly, both drugs induced high levels of iC3b, even at concentrations as low as 10 μg/

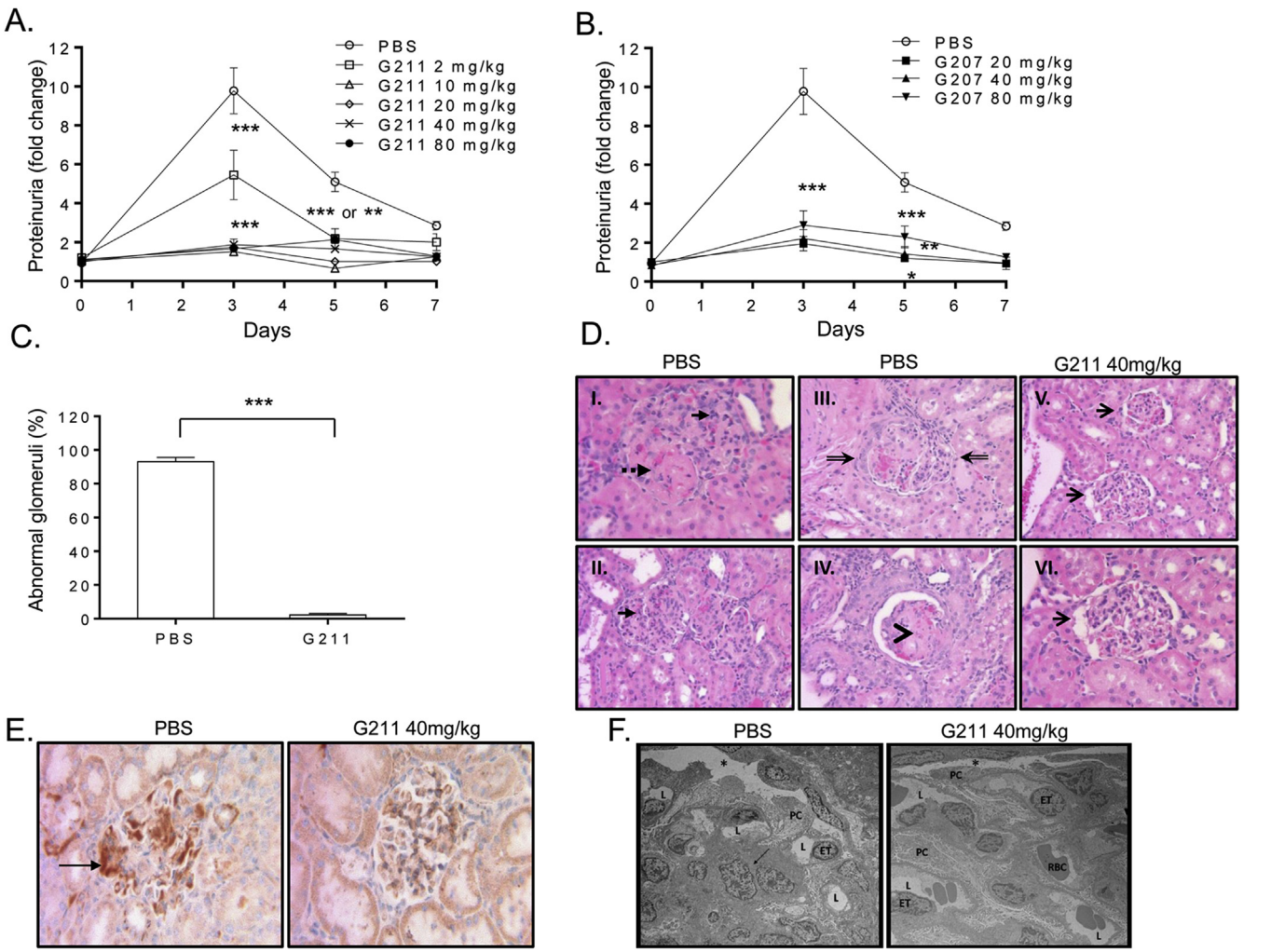

Fig. 6. G207 and G211 effectively prevents anti-Thy 1 glomerulonephritis. (A, B) Time course of the daily urinary protein excretion in anti-Thy-1 nephritis rats pre-treated with different doses of G207 or G211. The start time of anti-Thy-1 antibody injection was designated as day 0. Data are summarized from three separate experiments and are shown as the mean ± SEM of the fold change of urinary protein levels, compared to baseline (pre-*anti*-Thy-1 Ab injection), at the time points indicated. N = 24, for the PBS group; n = 8, 8, 16, 16, 8 for G211 2 mg/kg, 10 mg/kg, 20 mg/kg, 40 mg/kg, and 80 mg/kg groups respectively; n = 8 for each of G207 treatment groups. (C) Quantification of abnormal glomeruli by light microscopic analysis (n = 8 for each group), ***$p < 0.001$. (D) Light microscopic analysis of glomerular injury. Paraffin fixed kidney samples from PBS and G211 groups at day 9 were cut and stained with H&E. (I and II) In untreated diseased animals, histology revealed evidence of glomerular hypercellularity, inflammation (solid arrows) and fibrinoid necrosis (Dotted arrow). (III and IV) Additionally, these untreated kidneys had Bowman's space epithelioid crescents (double arrow) and mesangiolysis (arrowhead). (V and VI) G211 treated animals demonstrated normocellular glomeruli (open arrows) with open capillary lumina and intact Bowman's spaces. (E) C3 immunostaining of kidney sections from PBS versus G211 treated rats highlighting large glomerular capillary wall and mesangial deposits in the former. (F) Electron microscopic analysis of kidney sections from anti-Thy-1 rats treated with PBS vs G211. L: Glomerular Capillary Lumina; PC: Podocytes; ET: Endothelial Cells; RBC: Red Blood Cells within glomerular capillary lumina; Arrow: Activated Mesangial Cells; *: Bowman Space. Marked cell swelling results in pronounced narrowing of the glomerular capillary lumina in the PBS group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with PBS control.

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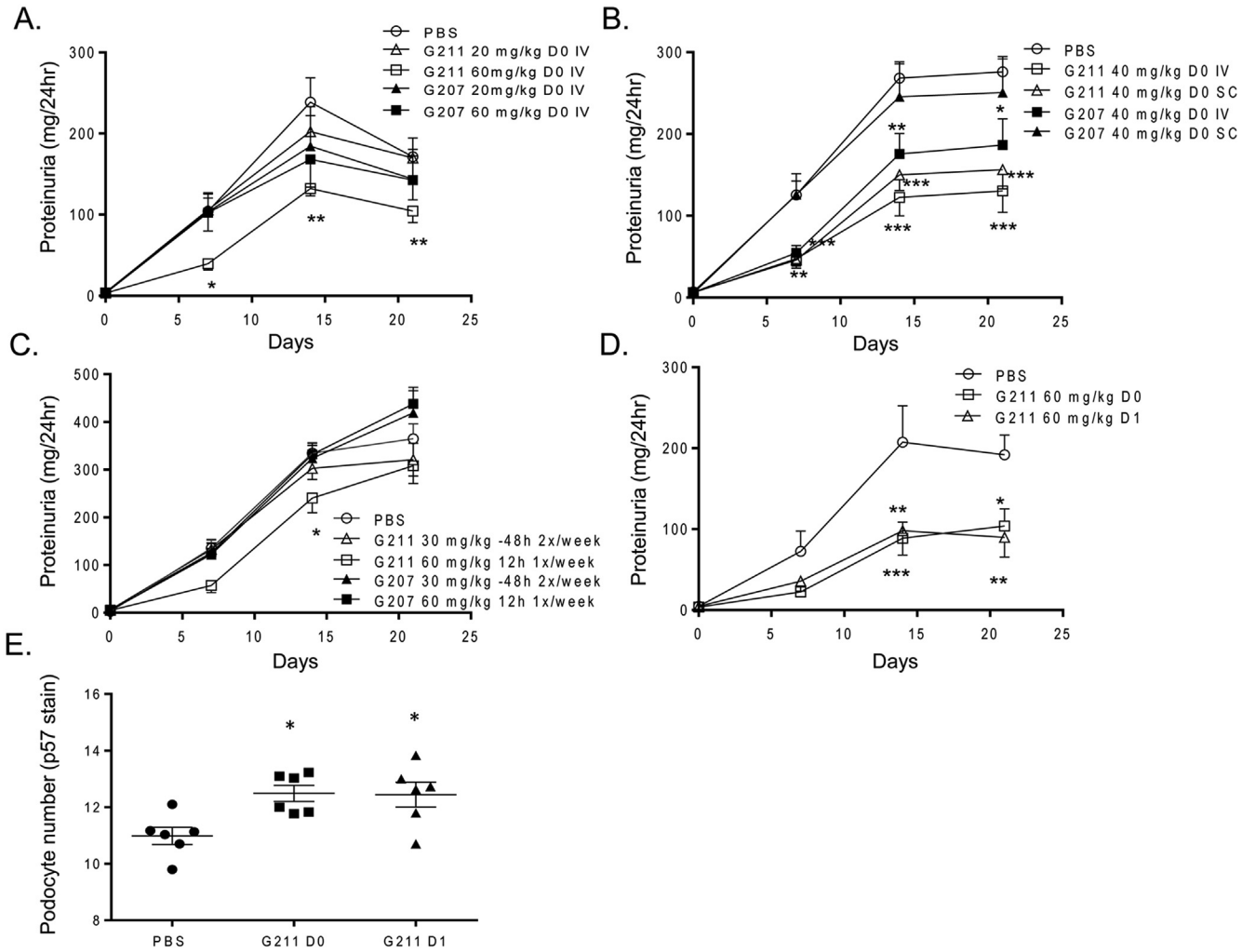

Fig. 7. G211 effectively prevents and treats passive Heymann's nephropathy. Goat Anti-Fx1A was injected at day 0 to induce PHN in rats. Treatment with G207 or G211 was initiated either before or after anti-Fx1A injection by either IV or SC administration. Daily urinary protein excretion was monitored. Data are shown as the mean with SEM of proteinuria levels at the time points indicated. (Data summarized from four independent experiments.) (A) D0 (2 h prior to anti-Fx1A injection) IV dosing of G211 at 60 mg/kg (2 doses/week) was effective in preventing proteinuria. N = 8 per group. (B) Comparison of SC vs IV dosing of G207 and G211 (2 doses/week) starting from day 0 (2 h prior to anti-Fx1A injection) in the prevention of proteinuria. N = 8 per group. (C) G207 and G211 were administered by SC at 48 h before (30 mg/kg, 2 doses/week) or 12 h after (60 mg/kg, 1 dose/week) anti-Fx1A injection. 24 h proteinuria was monitored. N = 8 per group. (D) G211 was effective in treating PHN when dosed at day 1 at 60 mg/kg (2 doses/week, SC). (E) G211 pretreatment protects against podocyte injury and death. N = 6 per group (D,and E). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to the PBS control.

ml. Collectively, these data demonstrate that G207 and G211 effectively inhibit CDC by sequestering C1q and preventing the deposition of complement split products on the surface of opsonized cells, with self-limited complement activation. Furthermore, as a byproduct of C1q engagement these drugs induce anti-inflammatory iC3b.

3.3. G207 and G211 can inhibit CDC in a C1q independent fashion

We sought to understand whether the ability of G207 and G211 to inhibit CDC was strictly dependent on their ability to bind and sequester C1q away from the surface of opsonized cells. As an experimental control, we first incubated anti-CD20 opsonized Ramos cells with C1q in the presence of defined concentrations of G001, G207, G211or IVIG (Fig. 3A and B). Cells were then washed – eliminating unbound C1q, drugs, and C1q-drug complexes from the cell surface – and subsequently incubated with C1q deficient serum. As anticipated, both G207 and G211 significantly inhibited CDC. Despite the ability to bind to plate coated-C1q as shown above (Figure 2A), 10 mg/ml of IVIG did not inhibit CDC in this setting. These data confirm that G207 and G211 sequester C1q away from the surface of opsonized cells and inhibit CDC mediated by the classical pathway.

We then tested the ability of G207 and G211 to reduce CDC in a setting where they could not competitively inhibit C1q binding to the surface of opsonized cells (Fig. 3A and C). Anti-CD20 opsonized Ramos cells were incubated with C1q in the absence of drug. Cells were washed and then exposed to C1q deficient serum bearing GL-001, GL207, G211or IVIG. Under these conditions, all of the reagents tested significantly inhibited CDC, while the G001 control lacked protective activity. These effects were not secondary to G207 or

Table 1
Binding kinetics of G207 and G211 to C1q.

Analyte	ka (1/Ms) × 10^4	kd (1/s) × 10^{-3}	KD (nM)	chi2
G207	4.9 ± 0.1	9.9 ± 0.2	204 ± 7.5	17 ± 0.4
G211	89.0 ± 9.3	4.9 ± 0.2	5.5 ± 0.7	25 ± 2.6
GL-2045	55.9 ± 2.4	8.3 ± 0.1	14.8 ± 0.7	14 ± 0.9

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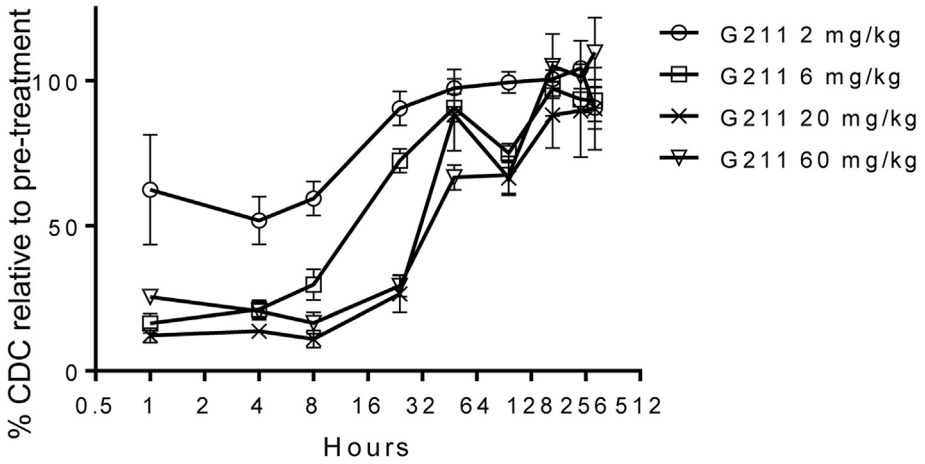

Fig. 8. ***In vivo*** **administration of G211 inhibits the CDC** ***ex vivo***. Blood samples were taken from rats at different timepoints after IV administration of G211. The levels of CDC activity in the serum samples were tested with a hemolysis assay using 50% serum concentration. Data is shown as relative percent of CDC activity to pre-treatment level. N = 4 per group.

G211 mediated elimination of C1q from the cell surface, as C1q could still be detected by anti-C1q Ab following drug exposure (Fig. 3D). Interestingly, C1q from cells treated with high concentration of IVIG became less detectable. Collectively, these data demonstrate that G207 and G211 inhibit CDC by both C1q dependent and independent effects.

3.4. G207 and G211 directly inhibit rat complement activation in vitro

Prior to testing G207 and G211 in rat models of diseases, that are complement dependent, it is necessary to assess whether these compounds directly inhibit rat complement activation *in vitro*. Due to the lack of reagents, we did not directly test the interaction of the compounds with rat C1q. *In vitro* studies, using 75% Wistar rat serum which contains anti-human RBC antibodies, revealed that both G207 and G211 inhibited rat CDC of human RBCs in a dose dependent fashion ($p < 0.001$ for both G207 and G211); (Fig. 4A). Even in this high serum concentration, small but detectable levels of inhibition were apparent at 12.5 μg/ml for both drugs. In contrast, IVIG at concentrations of 10 mg/ml, mediated less protection of human RBC than G207 and G211.

We analyzed the binding of G207 and G211 to rat blood cells (Fig. 4B). G207 did not bind any of the rat cell types tested. In contrast, G211 demonstrated positive binding to rat monocytes and PMNs. *Ex vivo* IHC studies (Supplemental Fig. 5) partially recapitulated these flow cytometric findings. In the spleen, both drugs bound a rim of cells surrounding the follicles that were morphologically consistent with mantle zone lymphocytes or macrophages. In addition, they bound intensely to a small number of intrafollicular cells, which appeared to be macrophages post − phagocytosis of necrotic debris. The ability of G207 to bind resident macrophages but not peripheral monocytes may reflect intrinsic differences between these cell populations. In contrast to this splenic staining, neither drug bound to rat kidney. It is unclear whether the binding to monocytes and PMNs is through engagement of Fc receptors, or other molecules.

3.5. G211 protects human RBC from acute intravascular hemolysis in vivo

We employed a well characterized complement dependent rat acute intravascular hemolysis model to evaluate the ability of G207 and G211 to inhibit the complement cascade in a clinically relevant setting [22]. Interestingly, despite the fact that both G207 and G211 were protective against rat CDC *in vitro*, only G211 showed protection in this *in vivo* model of acute hemolysis (Fig. 5). In this model, transfused human RBC clearance occurred within one minute of infusion without any intervention. G211 treatment significantly delayed the clearance of human RBCs from circulation. The minimal dose required for protection was at 1 mg/kg. No difference in the potency of protection was observed between 1 and 10 mg/kg doses. However, a dose of 100 mg/kg provided a significantly higher level of protection.

3.6. G207 and G211 effectively prevent anti-Thy-1 proteinuria and inhibit glomerular C3 deposition in rats

Given the importance of complement in the pathophysiology of glomeruloephritis, we evaluated the ability of G207 and G211 to prevent disease in the rat acute anti-Thy-1 mediated nephritis model. In this complement dependent model, anti-Thy-1 antibodies bind to rat mesangial cells, with resultant acute onset of proteinuria, phenotypic evidence of mesangial cell injury, and high levels of C3 and C5-9 deposition [23–25].

Three independent studies (Washington Biotechnology Inc., Baltimore, MD) were performed with the investigators blinded in which five different doses of intravenously administered G207 and G211, [2 mg/kg (1 study, 211 only), 10 mg/kg (1 study, 211 only), 20 mg/kg (2 studies, 1 study with 211 only), 40 mg/kg (2 studies), 80 mg/kg (1 study)] and placebo control were tested for their ability to prevent proteinuria (Fig. 6A and B). Both G207 and G211 significantly inhibited the development of proteinuria at doses ranging from 20-80 mg/kg. The levels of inhibition did not vary between these doses. In order to define the lower limit of efficacy, we tested lower doses of G211 and found that a dose of 10 mg/kg completely prevented proteinuria, while 2 mg/kg was only partially effective. Finally, we evaluated the ability of both drugs to treat established disease, and observed that neither drug could reduce the levels of proteinuria when administered on day 1 after administration of anti-Thy-1 Ab (Data not shown).

In order to begin to understand the nature of the observed protection, we evaluated histopathological changes of kidneys from PBS and G211 treated animals. H&E analysis revealed that while more than 90% of the glomeruli were histologically abnormal in PBS treated animals, less than 10% were abnormal in G211 treated rats

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(Fig. 6C). Glomeruli in the PBS control animals showed marked glomerular hypercellularity, inflammation, mesangiolysis, fibrinoid necrosis and crescents, all indicative of induced glomerulonephritis (Fig. 6D). Similarly, C3 immunostaining demonstrated that while diseased animals showed large, segmental and most likely sub endothelial C3 deposits, treated animals evidenced only mild, mesangial only, C3 positivity, which we postulated might be a normal finding (Fig. 6E). These findings were both reinforced and extended by electron microscopy where PBS treated animals displayed capillary occlusion, focal mesangiolysis, endocapillary hypercellularity due to proliferation of native glomerular cells and probable infiltration by monocytoid cells. In contrast, G211 treated animals showing patent peripheral capillary loops with scattered RBCs (Fig. 6F).

3.7. G211 effectively prevents and treats passive Heymann's membranous nephropathy

In order to better assess the potential clinical utility of G207 and G211 in the treatment of memranous nephropathy (MN), we also had them evaluated (Washington Biotechnology Inc., Baltimore, MD and Probetex, Inc., San Antonio, TX) by blinded investigators for efficacy in the PHN model. In this model, sheep anti-Fx1A serum are injected IV into rats to induce disease. In the first study, G211 dosed at 60 mg/kg IV, significantly inhibited the development of anti-Fx1a induced proteinuria, while, unlike in the anti-Thy-1 model, doses of 20 mg/kg were not effective (Fig. 7A). While analogous doses of G207 did not evidence a statistically significant effect, there was a trend towards efficacy at the later time points for the 60 mg/kg treatment group. Furthermore, in a second study, both G207 and G211 were efficacious in preventing disease when administered by IV at 40 mg/kg. Subcutaneous delivery of 40 mg/kg of G211 inhibited proteinuria while G207 demonstrated no clinical effect (Fig. 7B).

Next, in order to evaluate the ability of these drugs to mediate a *treatment* effect, we compared the ability of G211 to inhibit either proteinuria when administered 48 h before or 12 h after the induction of disease (Fig. 7C). Sixty mg/kg of G211 given at 12 h post disease induction significantly reduce the level of proteinuria on day 14. In a separate study (Fig. 7D), G211 (60 mg/kg, SC, 2 times/week) given at either day 0 or day 1 demonstrated equivalent levels of protection, which correlated with an increased number of podocytes (Fig. 7E).

3.8. Serum samples from rats treated with G211 have reduced ability to mediate CDC ex vivo

Finally, in order to correlate our findings with the ability of these drugs to inhibit CDC, we evaluated the ability of serum from rats treated with G211 to mediate CDC ex vivo (Fig. 8). After injection of G211, the serum hematolytic potential was significantly diminished at the one-hour post-infusion time-point, and gradually returned to normal levels between 24 and 96 h.

4. Discussion

The goal of our study was to develop a series of recombinant human IgG1 Fc multimers that could preferentially inhibit complement activity with limited ability to interact with low/intermediate affinity Fc receptors. In keeping with this goal, we developed two drugs, G207 and G211, which demonstrated restricted binding to immune cells in human whole blood, yet retained the ability of the parent drug, GL-2045, to engage with C1q and inhibit CDC by C1q dependent and independent mechanisms. Although we did not fully characterize the mechanisms by which G207 and G211 mediated C1q independent inhibition of CDC or prevented MAC generation following C1q binding, our prior studies with GL-2045 demonstrated that these effects were partially dependent on the ability to potentiate the function of Factor H [7].

We employed three pre-clinical rat models to evaluate the efficacy of G207 and G211 in mediating complement dependent activity *in vivo*. The human RBC transfusion model represents an acute complement activation model, in which human RBC are lysed and cleared from circulation within minutes of transfusion, in a complement dependent fashion [22]. The anti-Thy1 induced nephritis model is “semi-acute”, in that complement deposition and mesangial injury occur within 30 min of anti-Thy-1 antibody injection, and proteinuria resolves by day 5–7 [24]. In contrast, the PNH model, induced by passive administration of anti-Fx1A antibody, results in two distinct phases of nephropathy: 1) a heterologous phase, representing an acute nephritis within 1 week of induction, and 2) a subsequent and chronic autologous phase characterized by the production of the hosts own immune response against the exogenous sheep immunoglobulin [26–28].

G207 was effective in preventing both anti-Thy-1 and PHN nephritis. These data confirm the potential utility of complement preferential Fc multimers in modulating complement activation *in vivo*. In contrast, G211, but not G207, protected RBCs from lysis in the acute hemolysis model. While it is tempting to speculate that the ability of G211 to inhibit RBC lysis *in vivo* was secondary to its rapid and sustained interactions with C1q, we cannot completely exclude the possibility that differences in cellular binding, between the two drugs played a role in defining their functional activities. Specifically, unlike human PBMC, rat peripheral blood monocytes and PMNs preferentially bound G211 versus G207, making it difficult to separate complement dependent and independent effects for G211. This situation is further confounded by the knowledge that some investigators have demonstrated that PHN is ameliorated by antibody depletion of C6 [29], while others have shown that disease can be induced in C6 deficient rats, who develop similar levels of proteinuria as WT controls [30]. Although these concerns are partially mitigated by the fact that the anti-Thy-1 nephritis and hemolysis models employed in our study are relatively complement dependent, and that neither drug directly interacts with kidney tissue, further studies will be required to determine what, if any, role that direct drug interactions with immune cell(s) play in defining the anti-inflammatory functions of G211 in rat [22,24,31].

While we have clearly demonstrated that both G207 and G211 function similarly to GL-2045 by serving as a C1q sink, whether or not these drugs also affect the other components or pathways of complement activation need further investigation. Specifically, we found that both drugs inhibit CDC activity through both C1q dependent and C1q independent mechanisms. The self-limited complement activation, as well as increased iC3b generation, driven by both drugs, suggest that they might possess similar activity as GL-2045 in promoting C3b degradation.

To the best of our knowledge, this study is the first demonstration that recombinant Fc multimers can preferentially inhibit complement activation and mediate preventive and therapeutic effects in rodent models of complement mediated diseases. G207 and G211 differ from existing drugs targeting complement, with the possible exception of IVIG, in that in addition to inhibiting CDC they also bolster the natural anti-inflammatory activities of select complement split products, thereby facilitating an inhibitory link between the innate and adaptive immune systems and enabling a multipronged attack on complement mediated disease. An anti-C1q antibody, for example, would not be expected to be associated with initial activation of the classical complement pathway which is necessary for the generation of anti-inflammatory iC3b [32]. As such, G207 and G211 represent a new class of complement

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inhibitors that might be useful in the treatment of complement mediated human disease.

Conflict of interest statement

Dr. Strome is a Cofounder, consultant and stockholder in Gliknik Inc., a biotechnology company. He receives royalties for intellectual property, related to B7-H1 (PD-L1), licensed by the Mayo Clinic College of Medicine to third parties. He receives research support from Pfizer and Gliknik through sponsored research agreements through the University of Maryland, Baltimore. He also serves as a paid consultant to Astra Zeneca. Dr. Olsen, Mr. Mérigeon and Dr. Block are employees of Gliknik.

Authorship contribution

H.S.O., D.S.B., S.E.S, and X.Z. made substantive intellectual contributions to the experimental design and data interpretation. H.S. performed the majority of *in vitro* studies. H.S., E.S., X.Z. contributed to rat acute hemolysis studies. E.Y.M. and H.S.O. produced and characterized the proteins. E.B. performed surface plasma resonance analysis. S.K. conducted *ex vivo* complement dependent hemolysis assay. J.C.P. and C.B.D. were responsible for the assessment of IHC. S.M.B. provided statistical analysis. X.Z. and S.E.S. wrote the manuscript and share senior authorship. All authors had input into the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2017.08.004.

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