Development of C1q Affinity Chromatography for the Study of C1q-IgG Interactions

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Running Title

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Abstract

The classical complement system represents a central effector mechanism of antibodies, initiated by the binding of C1q to target bound IgG. Human C1q contains six heterotrimeric globular head groups that mediate IgG interaction, resulting in an avidity-driven binding event involving multiple IgG molecules binding a single C1q. Accordingly, surface bound IgG molecules are thought to assemble into non-covalent hexameric rings for optimal binding to the six-headed C1q. To study the C1q-Fc interaction of various antibodies and screen for altered C1q binding mutants we developed a novel HPLC based method. Employing a single chain form of C1q (scC1q) representing one C1q head group, our HPLC methodology was able to detect the interaction between scC1q and various ligands. We show that, despite a narrow window of specific binding owing to the low affinity of the monomeric C1q-IgG interaction, this approach clearly distinguished between IgG subclasses with established C1q binding properties. IgG3 displayed the strongest binding, followed by IgG1, with IgG2 and IgG4 showing the weakest binding. Fc mutants known to have increased C1q binding through oligomerisation or enhanced C1q interaction showed greatly increased column retention, and IgG glycovariants displayed a consistent trend of increasing retention upon increasing galactosylation and sialylation. Furthermore, the column retention of IgG isotypes and glycovariants matches both the cell surface recruitment of C1q and complement mediated cytotoxicity induced by each variant on an anti-CD20 antibody backbone. This methodology therefore provides a valuable tool for testing IgG antibody (glyco)variants for C1q binding, with clear relevance for therapeutic antibody development.

Key Points

* We have developed a novel HPLC method for studying C1q-Ligand interactions
* scC1q column binding correlates with biological activity of anti-CD20 IgG isotypes

Introduction

Therapeutic monoclonal antibodies (mAbs) have emerged over the last 2 decades as robust and efficacious treatment agents for many indications, including cancer and autoimmune disease(1-3). Almost all of the currently approved mAbs are of the human IgG class, and have the capacity through their Fc domain to interact with both Fc gamma receptors (FcγR) on the surface of leukocytes and C1q, a serum protein involved in the initiation of the complement cascade(4-6).

The exact mechanism of action of target depleting mAbs in vivo is not fully understood, with complement, Fc gamma receptor expressing effector cells such as NK cells and myeloid cells, and direct signalling into the tumour cell being implicated (reviewed in Sopp JCO 2018(7)). With regards complement, the data are mixed with evidence both supporting and denying its importance in antibody efficacy but components of the cascade are convincingly shown to be depleted after administration of the anti-CD20 mAb rituximab in patients with chronic lymphocytic leukaemia (CLL)(8, 9) and in vitro CLL cell killing by rituximab is reduced after anti-C5 mAb eculizumab treatment(10).

Furthermore, C1q and the complement cascade is implicated in the pathology of autoimmune diseases where autoantibodies can result in immune complex formation and complement activation on host cells and tissues causing injury, as in systemic lupus erythematosus (SLE)(11). To this end, full length porcine C1q has been used in dialysis-type columns as a more selective alternative to plasmapheresis to remove immune complexes and auto-antibodies from the plasma of SLE patients(12). Although this treatment showed some promise in several small trials with an acceptable safety profile, it has not yet been adopted for general use(13, 14).

C1q is a large protein complex consisting of 18 polypeptides- 6x C1qa, 6x C1qb and 6x C1qc encoded by the genes *C1QA, C1QB* and *C1QC* located on chromosome 1(15, 16). Each molecule of C1qa, C1qb and C1qc contains a carboxyl terminal globular domain and a collagen like domain that assemble into a triple helix structure with the carboxyl terminal globular domains forming a heterotrimeric globular head region(17). A disulphide bond between C1qa and C1qb stabilises each heterotrimer, while a second disulphide bond between adjacent C1qc molecules couples two heterotrimers together(18). The disulphide bonds and helical interactions cause C1q to adopt a large oligomeric structure with 6 heterotrimeric globular head groups and a helical coiled tail- commonly referred to as a ‘bouquet of tulips’ conformation(15, 18). It is these 6 head groups that are responsible for the ligand binding properties of C1q, while the associated proteases C1r and C1s interact with C1q to form the full C1 complex required for activation of the classical arm of the complement pathway(15, 19).

C1q binds to the upper CH2/lower hinge region of IgG, similarly to FcγRs, but with very low affinity(20-22). Accordingly, efficient recruitment of C1q by IgG requires binding to multiple IgG Fc regions immobilised to the target cell surface, whereas the naturally pentameric IgM is far more efficient at recruiting C1q(6). In keeping with this observation, IgG Fc regions have recently been shown to form ordered non-covalent hexameric rings at the target cell surface that interact with C1q(23). Disruption of this Fc-Fc interface either through mutation, or the application of a competitive binding peptide, prevents hexamerisation and thus reduces C1q binding and complement activation.

Several residues within both IgG Fc(24) and C1q(25) have been shown to impact the affinity of the C1q:IgG interaction and in particular both the presence and characteristics of the IgG Fc hinge region are critical(26). This is clearly demonstrated by the relationship between hinge size and C1q binding, with IgG3 having both the longest hinge and strongest C1q binding, whereas IgG2 and IgG4 have a short hinge and bind C1q with very low affinity (27). Furthermore, IgG1 with the hinge region removed was unable to bind to C1q(28), and various mutations to the IgG1 hinge region can increase or decrease binding(29). Although it is thought that C1q binds to the CH2 domain, the nature of the hinge may be important for orientating the CH2 domain or distancing the fab arms. In support of the latter, IgG4, unable to bind to C1q as a full length antibody due to potential Fab steric clashes(30), is able to bind as an Fc alone(31).

Unlike the binding of IgG Fc to the family of FcγRs which is of low micromolar- high nanomolar affinity (depending on the individual member), IgG Fc binding to C1q is of very low affinity- high micromolar(32). As such, it is thus difficult to study accurately by SPR or ELISA without crosslinking or aggregating the IgG(25). Furthermore, different subpopulations within a sample that exhibit differential C1q binding are not resolved by these techniques. As such we sought to develop an alternative method amenable for high throughput screening of C1q ligands using a single chain monomeric scC1q head group coupled to sepharose beads in an HPLC column. We assessed the potential of this approach for studying scC1q binding to various ligands, distinguishing altered scC1q binding of IgG mutants and glycovariants and identification of sub species within a sample that exhibit differential binding to scC1q.

Materials and Methods

Materials and Cells:

NaCl and HEPES were purchased from Merck Millipore. scC1q was produced in house on two separate occasions in HEK293 cells and purified by affinity chromatography using a His tag to give a purified product of 50 kDa. High Performance Streptavidin Sepharose and Tricorn columns were purchased from GE Healthcare. Bovine serum albumin (BSA) and plasma purified C reactive protein (Cat. No. #C4063) were purchased from Sigma Aldrich. Penicillin/streptomycin, glutamine, sodium pyruvate, Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco’s Modified Eagle Medium (DMEM) were all purchased from Life Technologies. Foetal calf serum (FCS) was purchased from Lonza. Cell lines were purchased from American Type Culture Collection (ATCC): Raji (CCL-86), Ramos (CRL-1596), SU-DHL-4 (CRL-2957) and MDA-MB-453 (HTB-131). Human C1q and anti-C1q FITC antibody were purchased from Abcam (Cat. No. #Ab 96363 and #ab4223, respectively). Serum was obtained in house from the blood of healthy donors with informed consent and stored at -80°C in glass vials.

scC1q Production and Purification:

In brief, an N-terminal gC1qC-gggsgdyka-gC1qB-meaggnikd-gC1qA-constuct was produced incorporating an AVI-tag sequence for enzymatic mono-biotinylation after the C-Terminal C1qA and a Hexa- His tag for purification. This construct was expressed using a proprietary expression vector following routine HEK293F culture. Plasmid DNA was added to FectoPro (FectoPro, PolyPlus) and transient transfection performed according to the manufacturer’s protocol. 18 hours after transfection the culture was fed with 3 g/L glucose. The cell suspension was cleared by centrifugation at 4000g after 7 days. The cleared supernatant was used for further purification using a cOmplete His-tag column (Roche, 20 ml) at 1.5 ml/minute equilibrated in 20 mM Na2HPO4 pH 7.4, 500 mM NaCl. Elution was attempted by applying a step gradient of 4%, 20%, 60% and 100% 20 mM Na2HPO4 pH 7.4, 500 mM NaCl, 500 mM imidazole. The fractions were analyzed by SDS-PAGE and Caliper.

Column Production:

scC1q was biotinylated according to manufacturer’s instructions using BirA biotin-protein ligase kit (Avidity, Aurora, Cat. No. #bulk BirA). Biotinylated scC1q was dialysed in Slide-A-Lyzer cassettes (ThermoFisher Scientific) against 150 mM NaCl, 20 mM NaH2PO4\*H2O (monohydrate) pH 7.5, and mixed dropwise with 1 ml streptavidin sepharose beads (GE Healthcare, cat. no. 17-5113-01) while shaking. scC1q bound sepharose was then packed into 4.6 x 50 mm chromatography columns (GE Healthcare, Tricorn 5/50 column, cat. no. 28-4064- 09) and used for chromatography experiments. A blank column was generated in the same manner using mock treated streptavidin sepharose beads. The columns were stored in 20 mM HEPES, pH 7.4, at 4°C.

Caliper:

35 µl sample buffer (HT Protein Express Sample Buffer) were pipetted into the wells of a microtiter plate. 5 µl (at a concentration of 1 mg/ml or comparable 5 µg) of each scC1q sample were pipetted into the well.

When finished, the plate was covered with foil to minimize evaporation. The samples (plate) were denatured at 70 °C for 10 minutes, with or without reducing agent. 70 µl water were added to each sample. The sample plate was rotated at 3000 RPM for 1 minute to eliminate bubbles and move the fluid to the bottom of the well. The samples were applied to the Caliper instrument and the result was evaluated by LabChip GX software.

SDS PAGE:

The samples were diluted in a reaction vessel with a placebo solution to a protein concentration of 1mg/ml. 20 µl (20 µg protein) diluted sample or reference standard are mixed in the reaction vessel with 20 µl of the reducing sample buffer and incubated for 5 minutes at 70 °C in a preheated heating block. 16 μl (8 μg protein) of this solution are applied to each gel pocket. 10 µL of the MW marker are applied undiluted to the gel. After installing the gel in the electrophoresis apparatus the buffer chamber has been filled with approx. 800 ml (1x) Tris-Glycine SDS Running Buffer. The run has been finished after the sample front reached the end of the gel. The gel has been stained with 60 ml SimplyBlueTM SafeStain staining solution while gently shaking for an hour.

Mass Spectrometry:

To check the integrity of the product, mass spectrometry was used. Before measurement, the samples were deglycosylated by adding Rapid PNGase F from NEB (New England Biolabs) and further processed according to the manufacturer's instructions. Final incubation of the substrate/enzyme mix was done at 50 °C for 15 minutes. The samples were prepared by an automated workstation (Biomek i7, Beckman Coulter).

The product obtained was injected and separated without pre-treatment using reversed phase UHPLC (Vanquish, Thermo Fisher Scientific). A PLRP-S column (1×50 mm; 5 µm particle diameter, 1000 Å pore size) from Agilent was used for separation. The UHPLC eluate was infused into a MaXis II ETD Q‐TOF instrument (Bruker Daltonics) operating in positive ion mode. Data was evaluated using in‐house developed software.

Size Exclusion Chromatography:

SEC was carried out using a TSKgel UP-SW3000 column (7.6 × 300 mm, 2 µm particle size; Tosoh Bioscience). An isocratic elution using 100% running buffer (200 mM KH2PO4/ K2HPO4, 250 mM KCl, pH 6.2) at a flow rate of 0.3 ml/minute was used for chromatographic separation on an UltiMate3000 HPLC system (Dionex Softron GmbH) equipped with UV detection at 280nm. 15 µg of rhC1q were injected for the chromatographic analysis (at room temperature). Relative quantification was performed by manual integration of the sample elution peaks and the areas of the relative peaks compared to indicate what proportion of the sample was in each elution peak. Realtive quantification was performed by manual integration of the sample elution peaks within the elution window to identify the purity of the protein.

Chromatographic Gradient and Eluents:

HPLC running buffers for scC1q and blank affinity columns consisted of 20 mM HEPES, pH 7.4 (Eluent A) and 20 mM HEPES, 500 mM NaCl, pH 7.4 (Eluent B). Primary scC1q affinity elution gradient used is shown in Table 1.

General scC1q Chromatography Method:

The scC1q affinity column was operated on a Shimadzu 10A HPLC system with in-line degasser. Samples were detected by measuring their absorbance at 280nm. The autosampler was maintained at 4°C and the column maintained at 25°C. Prior to injection of samples, running buffer (eluent A) was injected to equilibrate the column followed by two injections of 25 µg of antibody standard. 25-100 µg of samples were typically injected at 1 mg/ml in Eluent A from Chromacol glass sample vials (ThermoFisher Scientific), with a buffer flow rate of 0.5 ml/minute. 100% Eluent B was used to regenerate the column during each run. Chromatograms were generated and processed using Chromeleon v 7.2 SR5 (ThermoFisher Scientific).

Antibodies:

Anti-HER3 IgG1 and anti-P-Selectin IgG4 were generated in house (Roche) and used as a standard control and for column establishment. Anti-HER2, anti-CD20 and anti-IL-6R antibodies were generated in house and *in vitro* glycoengineering was performed as previously described(33). Anti-HER2 antibody was also used as a framework for Fc and RGY Fc mutations produced by Absolute Antibody. Purified human Fab and IgG1-Fc were purchased from Jackson ImmunoResearch (Cat. No. #009-000-007) and Sino Biological (Cat. No. #10702-HNAH-200), respectively. Anti-CD20 subclass variants were generated in Southampton (IgG2 and IgG4) or purchased (IgG3, InvivoGen, Cat. No. #hcd20-mab3). Serum purified IgM was purchased from Sigma Aldrich (Cat. No. #I8260).

Glycan engineering efficiency:

The degree of glycan modification was confirmed using IdeS cleavage followed by mass spectrometry analysis of the IgG Fc region, as previously described (34).

Biacore:

The binding properties of complexed anti-CD20 antibodies to single chain C1q were analyzed by surface plasmon resonance (SPR) technology using a BIAcore T200 instrument (BIAcore AB, Uppsala, Sweden). scC1q was immobilized onto a BIAcore CAP chip (GE Healthcare Bioscience, Uppsala, Sweden) via Biotin – Streptavidin. The overall level reached 12,000 Response units (RU). The assay was carried out at room temperature with PBS containing 0.05 % Tween20, pH 6.0 (GE Healthcare Bioscience) as running and dilution buffer. 500 nM of complexed or monomeric antibody samples were injected at a flow rate of 30 µl/minute at room temperature. Association time was 120 seconds, dissociation phase took 360 seconds. Regeneration of the chip surface was achieved through a short injection of 6M Guanindinium hydrochloride and 1 M NaOH.

Cell Culture:

Cell lines were maintained in complete RPMI 1640 (cRPMI) or DMEM (cDMEM) supplemented with glutamine (2 mM), sodium pyruvate (1 mM), streptomycin (100 U/ml), penicillin (100 µg/ml) and 10% FCS. Cells lines were split 3 times a week to approximately 0.5x106 cells/ml, or to approximately 20% confluency (for MDA-MB-453 cells). All cells were grown at 37°C, 5% CO2 in a humidified incubator in T25 or T75 tissue culture flasks (Sigma Aldrich).

Complement Dependent Cytotoxicity (CDC) Assay:

5x104 target cells (at 0.5x106 cells/ml) were opsonised with antibody or media (cRPMI) at room temperature for 15 minutes in a flat bottomed 96-well plate (Corning, Sigma Aldrich). Freshly thawed human serum was added to a final concentration of 30% and cells incubated at 37°C for 30 minutes in a humidified incubator at 5% CO2. The plate was then placed on ice and samples transferred to FACS tubes. 25 µl of 10 µg/ml propidium iodide (PI, Sigma Aldrich) was added and samples mixed. Data from single cells was acquired on a Calibur flow cytometer (Becton Dickinson). Serum debris was gated out and cells positive for PI were classified as dead. Data was analysed on FCS Express V3 and GraphPad Prism Version 6.05.

C1q Binding Assay:

5x104 target cells were opsonised at 1x106 cells/ml with antibody or media (cRPMI) at room temperature for 15 minutes in a flat bottomed 96-well plate. Purified human full length C1q (Abcam) was added to a final concentration of 8 µg/ml and cells incubated at 37°C for 15 minutes. Cells were transferred to FACS tubes on ice and washed 2x in 3 ml PBS with 0.1% BSA and 10 mM sodium azide for 5 minutes at 450g. Cells were stained with 1 µl per tube undiluted anti-C1q FITC (Abcam) for 30 minutes at 4°C, and then washed as above. Data from single cells was acquired on a Calibur flow cytometer (Becton Dickinson). Data was analysed on FCS Express V3 and GraphPad Prism version 6.05.

scC1q Cell Binding Assay:

1x105 target expressing cells were opsonised at 1x106 cell/ml with antibody variants or media (cRPMI) at 4°C for 30 minutes in FACS tubes and washed once in 3 ml of PBS, containing 0.1% BSA and 10 mM sodium azide, at 450g for 5 minutes. Cells were incubated with 0.1 mg/ml scC1q for 30 minutes at 4°C. 10 µl of biotin binding agents (streptavidin-FITC, streptavidin-APC, anti-biotin-PE (clone 1D4-C5) all undiluted and from Biolegend (Cat. No.’s #405202, #405207 and #409004, respectively)) were added at 4°C for 30 minutes, and cells washed once as above. Data from single cells was acquired on a Calibur flow cytometer (Becton Dickinson). Data was analysed on FCS Express V3 and GraphPad Prism version 6.05.

scC1q Binding ELISA:

A serial 1 in 2 dilution of scC1q from 100 µg/ml was immobilised onto flat bottomed 96-well Nunc MaxiSorp ELISA plates (ThermoFisher Scientific) in coating buffer (15 mM Na2CO3, 35 mM NaHCO3, both ThermoFisher Scientific) by incubating at 37°C for 2 hours. Plates were washed three times with PBS tween (0.05%) using a Skanwasher 300 plate washer (Skatron). Plates were blocked for 1 hour with PBS 1% BSA at 37°C, washed as above and 1 µg/ml antibody variants (in PBS 1% BSA) incubated in wells for 90 minutes at 37°C, and the plates washed again three times. Horseradish peroxidase (HRP) conjugated goat anti-human Fc antibody (in PBS 1% BSA, Jackson Immunoresearch, Cat. No. #109-035-098-JIR) was added for 1.5 hours at 37°C and plates were washed as above before addition of o-Phenylenediamine dihydrochloride (OPD; Sigma Aldrich, Cat. No. #P5412-100TAB) ELISA substrate. Prior to use, 40 μl of 40% H2O2 (Merck) was added to the ELISA substrate, then 100 μl of the prepared substrate was added per well. ELISA plates were incubated in the dark at room temperature for 20 minutes, until colour change had occurred, when 50 μl 2.5M H2SO4 (VWR) added. Plates were read on an Epoch microplate reader (Biotek) at 450nm. Data were analysed on Microsoft Excel and GraphPad PRISM v7.

Statistics:

Statistics were performed in GraphPad Prism version 6.05.

Results

In order to study the interaction of the globular C1q head domains with various ligands, first we produced a recombinant single chain C1q (scC1q) molecule. We used an approach similar to that of Moreau *et al.,* who linked the C1q subunits in the order found in the human genome, C1qA-C1qC-C1qB(35). Here, we linked human C1qA, C1qB and C1qC into a single chain in the order C1qA-C1qB-C1qC using short glycine-serine rich linkers at the base of the trimer(36). This construct was produced in HEK293 cells, purified by affinity chromatography and biotinylated. Analysis of the purified scC1q by caliper indicated a % pure (Figure 1A) and SDS-PAGE analysis of the scC1q product showed a main band at approximately 45kDa, with some higher bands likely to be multimers (Supplementary Figure 1A). Further analysis by size exclusion chromatography (Supplementary Figure 1B) and mass spectrometry (Supplementary Figure 1C) also confirmed mono-biotinylation of scC1q.

The biological activity of the resulting biotinylated scC1q molecule was confirmed through SPR to detect IgG Fc binding (Figure 1B). No binding was seen with monomeric IgG or F(ab’)2 alone. Binding was only detected using F(ab’)2 anti-Fab complexed IgG1 (Figure 1B, Blue line).

We further confirmed the activity of the scC1q molecule through detection of scC1q on IgG opsonised cells by means of fluorescently labelled streptavidin and an anti-biotin antibody (Supplementary Figure 1). Using two different antibodies for opsonisation we found scC1q was detectable, with mutations known to increase C1q binding giving increased scC1q signal (notably the K326W, E333S double mutant) (Supplementary Figure 1D-F and 1I). A similar trend was seen when detecting the level of anti-HER2 antibody bound to both scC1q and purified human C1q using an anti-C1q antibody, with a greater signal seen using the hexameric purified human wild type C1q (Supplementary Figure 1G-H). Finally, scC1q coated onto a plate was able to capture the K326W, E333S mutant (but not wild type or P329G mutant) anti-HER2 in an ELISA (Supplementary Figure 1J).

Based on this, we sought to produce a column suitable for scC1q affinity chromatography that may be more sensitive and allow the detection of monomeric IgG interactions with scC1q. Biotinylated scC1q was coupled to streptavidin coated sepharose beads at a density of 1 mg of biotinylated C1q per gram of sepharose streptavidin, and packed into 4.6 x 50 mm chromatography columns. Higher densities of C1q were also tested but did not prove to be stable, as their IgG1 retention time dropped off after the column was stored (Supplementary Figure 2A and 2B).

C1q binding to IgG is thought to be a largely charge based interaction, so based on this we selected a NaCl based gradient to control binding/release of ligand from the column. The gradient used is shown in Table-1. In this system, sample is injected at time zero in the absence of NaCl (0% Eluent B) to allow binding of weak affinity ligands, and then NaCl is gradually added in order to compete off the ligand based on its binding strength. The column is then regenerated by increasing the NaCl concentration to 500 mM to elute all bound material.

IgG1 (anti-HER3) eluted from the column as a retention peak during the titration of NaCl at approximately 50 mM, with a peak retention time of ~25 minutes (Figure 1C). Next, the specificity of the system was tested by injection of BSA onto the scC1q column. BSA showed no binding to the column and eluted entirely in the void peak at approximately 2 minutes (Figure 1D). The scC1q column was next assessed for its ability to distinguish between IgG1 (known to bind C1q and induce CDC) and IgG4 (which does not bind C1q or induce CDC as strongly as IgG1). Although IgG4 (anti-P-Selectin) binding to the column was detected, it was substantially less than that observed in IgG1 (~20 minutes vs 25 minutes) (Figure 1E).

In order to further confirm the specificity of the scC1q column, a blank column containing mock treated streptavidin coated sepharose was produced and the binding of IgG1 to this blank column tested under the same conditions as used above. IgG1 binding to the blank column gave rise to a similar retention profile with one major elution peak at ~22 minutes. Comparison of the elution profiles of IgG1 on the C1q column and the blank column indicate a peak retention time for the scC1q column approximately 3 minutes greater than for the blank column (Figure 1F).

Systematically increasing the NaCl concentration within the system during the antibody binding phase resulted in a progressively earlier elution peak for IgG1 injected onto the blank column (Figure 2A, left). Similarly however, increasing the NaCl concentration also reduced the retention time of IgG1 on the scC1q column (Figure 2A, right), whilst maintaining a slightly later peak retention time for each NaCl concentration tested (see Figure 2B for direct overlays for 5 nM and 25 nM NaCl).

C1q binding is mediated by the IgG Fc region. As such, Fab and Fc regions were tested independently on both the scC1q and the blank columns (Figure 2C and 2D, respectively). IgG1 Fab demonstrated apparently identical binding on both the scC1q and the blank columns, with most of the material showing no binding and a small binding peak eluting at approximately 19 minutes (Figure 2C). IgG1 Fc showed no binding on the blank column, but displayed a later elution peak on the scC1q column eluting approximately 2 minutes later than on the blank column (Figure 2D).

In order to further characterise the scC1q column we tested the effect of antibody subclass on column retention. An anti-CD20 antibody was selected and the four different human IgG subclasses (IgG1-4) were analysed on the scC1q (Figure 3A, left) and blank columns (Figure 3A, right). Anti-CD20 IgG3 showed the greatest level of binding to the scC1q column, as evidenced by the window of separation between the retention on the scC1q column and the blank column (Figure 3B, left). Anti-CD20 IgG1 showed the next highest binding, with anti-CD20 IgG4 showing the least binding (Figure 3B, right). These data were then compared to the performance of these subclasses in assays of C1q cell surface recruitment and complement dependent cytotoxicity (CDC) (Figure 3C-D). In addition to demonstrating the greatest specific binding to the scC1q column, anti-CD20 IgG3 also displayed the greatest C1q recruitment to the surface of opsonised cells (Figure 3C), and induced the strongest CDC response (Figure 3D). The pattern of results for anti-CD20 IgG1, 2 and 4 are also consistent with that seen on the scC1q binding column, with anti-CD20 IgG2 and IgG4 being the least active.

We then tested a series of different glycoforms of three different antibodies, generated by *in vitro* glycoengineering, on the scC1q column(33) (Figure 4). These glycoforms had quite similar retention profiles, although trends for increased retention were seen for all antibodies tested. Increasingly mature glycan patterns (galactose and sialic acid) resulted in prolonged retention times (Figure 4A-D). Samples with more galactose eluted slightly later on the scC1q column than samples with less or no galactosylation, and sialylated samples eluted later than galactosylated samples. This order of retention matches the levels of C1q recruitment to opsonised cells (Figure 4E), and the level of CDC induced (Figure 4F) when these glycoforms are tested on an anti-CD20 IgG1 antibody backbone. Fully galactosylated anti-CD20 IgG1 had significantly increased C1q binding recruitment compared to mock treated anti-CD20 IgG1, with sialylated anti-CD20 IgG1 having further increased C1q recruitment (Figure 4E). The same trend was seen for CDC induction, with fully galactosylated anti-CD20 IgG1 showing increased CDC over mock treated anti-CD20 IgG1, and sialylated anti-CD20 IgG1 having further increased CDC (Figure 4F).

Next, a panel of IgG1 mutants with anticipated effects on C1q binding or CDC induction were assessed (Figure 5). Three mutant antibodies showed dramatically increased binding to the scC1q column- the two double mutants E345K, K326W and K326W, E333S as well as the RGY triple mutant (E345R, E430G, S440Y) which is known to form solution state hexamers(23)(Figure 5A and 5B). These three mutants showed broadly similar binding to the wild type antibody on the blank column (Figure 5C). Interestingly, the RGY mutant gave rise to two elution peaks on the scC1q column, which match by relative size the size exclusion chromatography data indicating the hexameric peak (80% of the sample (Supplementary Figure 3)) also has high C1q binding (Figure 5D). Two of these high binding mutants (RGY and K326W, E333S) also showed increased resistance to the presence of NaCl during the antibody binding phase, as shown by the increased binding when tested at these increased NaCl concentrations (Figure 5E). The hexameric RGY peak elutes at a retention time that corresponds to 500 mM NaCl, whereas the monomeric species is more sensitive and elutes earlier, whilst retaining greater binding than seen for the wild type antibody (Figure 5D). Interestingly, the P329G mutant (known to have greatly abrogated Fc effector function) had highly similar retention profiles on both the scC1q and the blank column (Figure 5A-C).

To investigate the Fab and Fc impact on scC1q column retention time, Fc only constructs containing the RGY triple mutation, as well as various attenuating mutations were generated and tested on the scC1q column (Figure 5F, left) and the blank column (Figure 5F, right). RGY Fc showed a similar retention peak as seen for the high binding species of full length RGY anti-HER2 on the scC1q column (~82 minutes), and a slightly earlier retention peak on the blank column (~21 minutes). The effect of attenuating mutations on the RGY Fc binding to C1q is shown by the progressively earlier elution of the sample with various mutations. LALA mutations attenuate the C1q binding, whereas N297Q (aglycosylation) has a more limited effect on the C1q binding. Mutation of the P329G residue has a greater effect than the two previously described mutations. Combining the P329G mutation with the LALA mutations resulted in the earliest elution time, earlier than wild type anti-HER2. The binding of this strongly attenuated mutant was much greater than that of the IgG1 Fc, showing the effect of the avidity endowed by the presence of multiple Fc domains seen in the RGY Fc samples.

Finally, we tested two non-IgG C1q binding ligands on the scC1q column. C reactive protein (CRP) showed greater specific binding to the scC1q column versus the blank column and to a greater level than did IgG1, as exhibited by a later retention time (Figure 6A and 6B). Serum purified IgM also showed specific binding to the scC1q column and had a greater retention than IgG1 (Figure 6C and 6D).

Discussion

The broad recognition properties of C1q stem from its six heterotrimeric globular head groups. We generated a single chain format of the C1q head group (scC1q) using a method based on that which has been previously described to generate functional C1q head groups(35). As described, binding of a monomeric IgG to a single C1q head is of very low affinity (10-4 M) and therefore difficult to study by ELISA or SPR(35). The authors of this report commented that binding to IgG was not detectable in their SPR setup(35). Recently an SPR method utilising protein L to capture IgG antibodies through their light chain, thus presenting their Fc regions, has been described that facilitated detection of C1q binding to captured IgGs(37). Whilst promising, this method was unable to capture the IgG2 and IgG4 subclasses, thereby preventing the assessment of their C1q binding properties.

The weak affinity, and the functionality of our scC1q head group, was confirmed by the lack of binding to monomeric antibody by SPR measurement using immobilised scC1q, with binding only seen after the antibody was crosslinked (Figure 1B). This approach of crosslinking/aggregation is commonly used in C1q ELISAs or SPR detection methods in order to detect binding.

The biological activity of the scC1q construct was further demonstrated through its ability to bind to antibody Fc regions on the surface of opsonised cells (Supplementary Figure 1C-I). Greater recruitment of the scC1q construct was seen with antibodies carrying Fc mutations known to increase C1q binding (notably the K326W, E333S double mutant). When detecting bound C1q using an anti-C1q antibody, there appeared to be greater signal when using a wild type purified human C1q molecule compared to the scC1q molecule (Supplementary Figure 1F-G). This could be due to the greater binding avidity of the hexameric wild type C1q, which contains 6 globular Fc binding heads, compared to the single Fc binding head of the scC1q molecule. However, the scC1q molecule is demonstrably able to bind to antibody Fc regions as measured by ELISA, SPR and flow cytometry.

Biotinylated scC1q was packed into columns and tested for IgG1 binding. Initial tests showed that the NaCl based elution gradient chosen allowed for binding and elution of monomeric IgG1 (Figure 1C). We found that the optimal density of scC1q for the column was 1 mg scC1q per 1 ml sepharose column, as although increased densities initially showed a greater IgG1 retention time than the 1 mg column they were not found to be stable, as this dropped after storage (Supplementary Figure 2A and 2B). Testing of BSA on the column indicated that the binding to IgG was specific, as no BSA binding was detected (Figure 1D). Furthermore, the scC1q column showed greater binding for IgG1 than for IgG4, which matches previous data determined by other methods and the complement activating potential of these two IgG subclasses (Figure 1E). However, IgG4 still showed considerable binding to the column which was surprising.

When IgG1 was tested on the blank column, considerable binding was detected, with the retention peak eluting approximately 3 minutes earlier than on the scC1q column (Figure 1F). We hypothesised that the absence of NaCl from the buffer during the antibody binding phase was allowing for non-specific interactions between the IgG and the sepharose matrix. Increasing the NaCl concentration present within the buffer during the antibody binding phase resulted in a progressive decrease in binding to the blank column, but also to the scC1q column (Figure 2A-B). Comparison of samples tested under the same NaCl running conditions on both columns indicates that the elution of samples from the scC1q column occurs 2-3 minutes later than seen on the blank column, irrespective of the level of NaCl present (Figure 2A-B). We confirmed the run to run stability of the columns by including an IgG1 standard sample in each experiment. After an initial decrease from the first experimental run, the retention time was highly stable for both the blank column (Supplementary Figure 2C, left) and the 1 mg scC1q columns (Supplementary Figure 2C, right).

As the C1q binding region of IgG is known to be located in its Fc domain, we sought to determine whether the Fc region of IgG was responsible for the increased binding of IgG on the scC1q column over the blank column. Recombinant human IgG1 Fc showed a 2 minute shift of increased binding on the scC1q column versus the blank column (Figure 2D), whereas Fab alone showed near identical binding on the two columns (Figure 2C).

In order to further characterise the column in terms of its relevance to biology, an anti-CD20 antibody was tested on the column in each of the four IgG subclasses, as well as in cell based assays of C1q recruitment and CDC. As would be expected, anti-CD20 IgG3 showed the greatest level of C1q recruitment to the cell surface of opsonised Ramos cells, and induced the most complement dependent cytotoxicity of opsonised Ramos cells in the presence of human serum (Figure 3C and 3D). These data matched the observation that the anti-CD20 IgG3 was the strongest binder on the scC1q column, with the biggest specific window of binding over the blank column (Figure 3B, left). The relative levels of C1q recruitment and CDC induction of anti-CD20 IgG1, IgG2 and IgG4 also matched their relative binding strengths on the scC1q column (Figure 3A), giving a hierarchy of complement activation for the IgG subtypes of IgG3>IgG1>IgG2/IgG4.

IgG Fc glycosylation is known to affect multiple antibody functions, particularly antibody dependent cell mediated cytotoxicity through the fucose residue, and also complement mediated cytotoxicity(38). To see if our scC1q column could detect differences in antibody-C1q interactions due to differences in glycosylation we generated different glycovariants of three different clinical grade IgG1 antibodies. The effect of these glycovariants was characterised in terms of C1q recruitment to the cell surface and CDC induction using an anti-CD20 antibody. As reported previously, increased CDC and C1q recruitment was seen for highly galactosylated anti-CD20, but also for anti-CD20 mAb bearing increased sialylation (Figure 4E-F)(39, 40).

There are reports in the literature of sialylated IgG being anti-inflammatory and having reduced complement activating properties when compared to galactosylated antibody(40). These observations conflict with the data presented here, where sialylated anti-CD20 IgG1 has increased CDC and C1q binding over galactosylated and mock treated anti-CD20 IgG1. These differences could be explained by the methods of glycosylation manipulation applied by different groups, the purities of sialylated IgG generated (and therefore the levels of galactosylated IgG) and the methods of studying CDC, with different treatment protocols and serum sources used.

Despite this apparent conflict with some previous reports, testing these antibody glycoforms on the scC1q and blank columns appeared to recapitulate the order of complement activation from the C1q recruitment and CDC assays, albeit with only subtle differences between the glycoforms (Figure 4A-D). The order of samples was consistent across three different antibody scaffolds (anti-HER2, anti-IL-6R and anti-CD20) and repeat measurements. Most notably, addition of galactose to agalactosylated IgG caused an increase in binding, and addition of sialic acid further increased binding, but only when in an α2-6 linkage (Supplementary Figure 4). The specificity of the α2-6 linkage over the α2-3 linkage was also reported by SPR measurement of C1q binding to protein L captured IgG glycovariants(37). Although the shifts seen on the scC1q column are very subtle for all antibody backbones tested, a small increase in C1q binding for each Fc present at a target cell surface could cause a far larger increase in C1q binding to multiple Fcs through increased avidity. This may explain the larger differences between the glycoforms in CDC and C1q recruitment than on the scC1q column. For investigation of this effect glycovariants may need to be tested in combination with IgG hexamerisation engineering. Furthermore, although Fc glycans are largely buried within the core of the CH2 domains, sialic acid is believed to at least begin to protrude out of the antibody core, and thus may have some effect on Fc-Fc or Fc-C1q interactions.

We selected several Fc mutations previously reported to alter C1q binding or complement activation to test on our column and demonstrate its utility for antibody screening. Three mutants were found to have greatly increased binding to the scC1q column, with no/little increase in binding to the blank column (Figure 5A-C). Two of these mutants were also tested for their binding under higher NaCl levels, and showed a reduced sensitivity to NaCl as compared to wild type antibody (Figure 5E). Interestingly, the P329G mutant (known to have highly abrogated Fc effector function) showed no difference in binding between the scC1q column and the blank column, further suggesting that the 2-3 minutes increased binding on the C1q column is a real Fc-C1q interaction (Figure 5B and 5C, green line). There were three mutants that showed greatly increased binding to the scC1q column, with little or no change in binding to the blank column, most notably the RGY (E345R, E430G and S440Y) triple mutant reported by Diebolder *et al.* This mutant forms solution state hexamers in an approximately 80:20 ratio of hexamers to monomers, and has greatly enhanced CDC. Interestingly, 2 elution peaks were seen for the RGY mutant, likely indicating the hexameric and monomeric species as the peak areas matched the 80:20 ratio, the larger peak eluting later (Figure 5D). This highlights the potential of this method to visualise subpopulations within a sample by their affinity for C1q, which is not possible by ELISA or SPR. It is of note that the monomeric peak also showed greater binding than the wild type antibody, indicating that even in monomeric form this mutant shows increased binding to C1q.

Finally, we tested other non-IgG ligands of C1q for binding to the columns. Both IgM and C reactive protein were found to bind to the scC1q column with greater retention than IgG1 and a larger window of specificity between the retention on the blank column and the scC1q column (Figure 6). This indicates that, as reported by Moreau *et al.*, the single chain C1q is capable of binding to multiple C1q ligands and not just IgGs. Both of these ligands typically only bind to C1q after immobilisation to a surface through binding to their ligand (antigen for IgM, phosphocholine for CRP) which induces a conformational change to permit C1q binding. These molecules are not bound to ligand when being passed through the columns. However, it is possible that the buffer conditions cause IgM and CRP to adopt a conformation permissive to C1q binding, allowing them to bind to the column.

In summary we have developed an HPLC based assay that is able to detect C1q binding to multiple ligands and make biologically relevant distinctions between different IgG subtypes, mutants and glycoforms as well as being able to detect subpopulations with different C1q binding properties. This method is of use for screening various mutated ligands to detect those with altered binding for potential therapeutic use, or to probe the residues that are important for C1q interaction.

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Figure Legends

Figure 1- Establishment and initial characterisation of the scC1q column. A scC1q column was produced by encoding C1qA, C1qB and C1qC into a single chain in the order C1qA-C1qB-C1qC using short glycine-serine rich linkers at the base of the trimer. The purified material was then evaluated for purity and IgG binding capacity. A- Caliper trace of purified scC1q, representing 100% of the sample. B- monomeric IgG, F(ab’)2 or complexed IgG was flowed over scC1q immobilised to an SPR chip. The IgG association and dissociation phase is shown. C- 25 µg IgG1 was applied to the scC1q column, with the elution gradient shown. D- 50 µg bovine serum albumin was applied to the scC1q column with the elution profile shown. E- Overlaid 25 µg IgG1 (black) and 25 µg IgG4 (blue) were applied to the scC1q column, with the elution profiles shown and overlaid. F- 25 µg IgG1 was applied to the scC1q column (black) with the elution profile shown, and overlaid with the elution profile obtained from applying 25 µg IgG1 to a blank sepharose-streptavidin column (blue). C-F- All samples injected at 1 mg/ml in eluent A (20 mM HEPES).

Figure 2- Evaluation of non-specific binding for the scC1q column. A- Assessment of the impact of NaCl concentration on the binding of IgG to the scC1q and blank columns. 20 µg samples of IgG1 were applied to the scC1q column (right) and the blank column (left) under a series of different NaCl concentrations at the beginning of the run, and the elution profiles overlaid. B- Direct comparison of the overlaid elution profiles of 20 µg IgG1 applied to the scC1q column (black) with that of the blank column (blue) with 5mM (left) or 25mM (right) NaCl at the beginning of the run. C and D- Direct comparison of the overlaid retention profiles of antibody Fab (C) and Fc (D) fragments (both 25 µg samples) applied to the scC1q column (black) with the blank column (blue).

Figure 3- Comparison of the IgG binding properties of the scC1q column to cell based assays of complement activation. A- A set of IgG1-4 anti-CD20 antibodies was applied in 25 µg samples to the scC1q column (left) and the blank column (right) and the elution profiles shown and overlaid. B- Direct comparison of the overlaid elution profiles of 25µg of anti-CD20 IgG3 (left) and IgG4 (right) on the scC1q column (black) and the blank column (blue). C- Ramos cells were opsonised with anti-CD20 antibodies and incubated with full length purified human C1q. Bound C1q was detected with an anti-C1q labelled antibody. Mean + SEM of 3 independent experiments. Statistics calculated by one-way ANOVA with multiple comparisons correction; \*\* P ≤ 0.01, \*\*\*\* P ≤ 0.0001. D- Ramos cells were opsonised with anti-CD20 antibodies and incubated with 30% human serum. Dead cells were detected with propidium iodide (PI) staining. Mean + SEM of 3 independent experiments. Statistics calculated by one-way ANOVA with multiple comparisons correction; \*\*\*\* P ≤ 0.0001.

Figure 4- Investigation of the impact of antibody glycosylation on scC1q column binding. A-C- Three sets of glycoengineered IgG, anti-HER2 (A), anti-IL-6R (B) and anti-CD20 (C), were applied as 25 µg samples to the scC1q column and the elution profiles shown and overlaid. Anti-HER2 and anti-IL-6R glycovariants tested were degalctosylated (G0), fully galactosylated (G2) or fully sialylated (α2-6 linkage, S2) and anti-CD20 glycovariants tested were untreated (>85% of sample contains 0 or 1 galctose residues), fully galactosylated (G2) and highly sialylated (>98% of sample contains at least 1 sialic acid residue). D- 25 µg samples of glycoengineered anti-IL-6R antibodies were applied to the blank column and the elution profiles overlaid. E- Ramos cells were opsonised with anti-CD20 glycoengineered antibodies and incubated with full length purified human C1q. Bound C1q was detected with an anti-C1q labelled antibody. Mean + SEM of 3 independent experiments. Statistics calculated by one-way ANOVA with multiple comparisons correction; \*\* P ≤ 0.01, \*\*\* P ≤ 0.001. F- Ramos cells were opsonised with anti-CD20 glycoengineered antibodies and incubated with 30% human serum. Dead cells were detected with propidium iodide (PI) staining. Mean + SEM of 9 independent experiments. Statistics calculated by one-way ANOVA of paired data with multiple comparisons correction; \* P ≤ 0.05, \*\*\*\* P ≤ 0.0001

Figure 5- Analysis of the impact of Fc mutations on binding to the scC1q column. A- 100µg samples of anti-HER2 and mutated anti-HER2 were applied to the scC1q column and the elution profiles overlaid. B- 100µg samples of selected anti-HER2 and mutated anti-HER2 were applied to the scC1q column and the elution profiles overlaid and the time period of 20-90 minutes enlarged. C- 100µg samples of selected anti-HER2 and mutated anti-HER2 were applied to the blank column and the elution profiles overlaid. D- 25 µg of anti-HER2 RGY was applied to the scC1q column and the elution profile shown. E- 25 µg samples of anti-HER2 RGY (left) and anti-HER2 K326W, E333S (right) were applied to the scC1q column under increasing NaCl concentration at the beginning of the run. Overlaid elution profiles are shown. F- 25 µg samples of anti-HER2, anti-HER2 Fc mutants and mutated Fc fragments were applied to the scC1q column (left) and the blank column (right) and the elution profiles overlaid.

Figure 6- Evaluation of non-IgG ligands on the scC1q column. A-B- 25µg samples of C reactive protein (CRP- black) and IgG1 (blue) were applied to the scC1q column (A) and the blank column (B) and the elution profiles overlaid. C-D- 25µg samples of IgM (blue) and IgG1 (black) were applied to the scC1q column (C) and the blank column (D) and the elution profiles overlaid.