Development and characterisation of novel anti-C5 monoclonal antibodies capable of inhibiting complement in multiple species.

Running title; Cross-species C5-blocking monoclonal antibodies.

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Abbreviations: C5, complement component 5; C5b6, stable complex of C5b and C6; C6, complement component 6; CP, classical pathway; AP, alternative pathway; mAb, monoclonal antibody; MAC, membrane attack complex; NHS, normal human serum; NMS, normal mouse serum; NRS, normal rat serum; NRbS, normal rabbit serum; ELISA, enzyme linked immunosorbent assay; WB, western blot; RT, room temperature; HBS, HEPES buffered saline, BSA, bovine serum albumin; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence; Abs, absorbance; ShEA, antibody-sensitized sheep erythrocytes; EAMG, experimental autoimmune myasthenia gravis; NE, neutrophil elastase; CI, cocktail of protease inhibitors.
Abstract
Over the last decade there has been an explosion in complement therapies; one third of the drugs in the clinic or in development target C5 protein. Eculizumab, a monoclonal antibody (mAb) that binds C5 and blocks its cleavage by the convertase, is the current gold standard treatment for atypical haemolytic uremic syndrome (aHUS) and paroxysmal nocturnal haemoglobinuria (PNH) and in clinical trials for many other diseases. Here we describe a panel of novel anti-C5 mAb, including mAb that, like Eculizumab, are efficient inhibitors of complement but, unlike Eculizumab, inhibit across species, including human, rat, rabbit and guinea pig. Several inhibitory anti-C5 mAb were identified and characterised for C5 binding and lytic inhibitory capacity in comparison to current therapeutic anti-C5 mAb; three clones, 4G2, 7D4 and 10B6, were selected and further characterised for ligand specificity and affinity and cross-species inhibitory activity. The mAb 10B6 was human-specific while mAb 4G2 and 7D4 efficiently inhibited lysis by human, rabbit and rat serum, and weakly inhibited guinea pig complement; 7D4 also weakly inhibited mouse complement in vitro. The rat C5-cross-reactive mAb 4G2, when administered intraperitoneally in a rat model of myasthenia gravis, effectively blocked the disease and protected muscle endplates from destruction. To our knowledge this is the first report of an anti-C5 function blocking mAb that permits preclinical studies in rats.
Introduction;
The complement system is a central component of innate immunity responsible for recognition and killing of bacteria and promotion of phagocytosis through opsonisation. The system evolved to protect from pathogens; however, recent reports have highlighted other homeostatic roles of complement, including as a driver of inflammation, modulator of adaptive immunity and metabolism and architect of neural development. Dysregulation of complement leads to pathology, important in a large number of inflammatory and degenerative diseases. Genome wide association studies (GWAS) have provided supporting evidence that complement is involved in many common diseases. Complement can be the main cause of the disease, for example in paroxysmal nocturnal haemoglobinuria (PNH) and atypical haemolytic uremic syndrome (aHUS), or contribute to the pathology by mediating tissue injury and inflammation; in either event, it offers a good therapeutic target.

Over the last decade there has been an explosion in complement drug development. Eculizumab, an anti-C5 monoclonal antibody, was a landmark in this work, approved by Food and Drug Administration (FDA) in 2007 for use in PNH, four years later for aHUS and recently for myasthenia gravis (MG). Eculizumab rapidly became the standard treatment for these two ultra-rare complement driven diseases (PNH, aHUS). A host of new agents are in development, targeting the cascade at different stages. A recent review listed 34 drugs in the pipeline, many targeting C3 and C5 convertases and the terminal pathway. C5 occupies a central position in the complement cascade; the C5 convertase cleaves C5 into C5a and C5b, the latter essential for assembly of the membrane attack complex (MAC); inhibition of C5 is thus an attractive therapeutic target and the focus of several drugs in development. Eculizumab prevents cleavage of C5 by the C5 convertase, blocking generation of the two most inflammatory products of the complement cascade; C5a anaphylatoxin and MAC. The risk of blocking C5 is relatively low; the sole iatrogenic impact of treatment with Eculizumab is an increased risk of Neisserial infections, resolved by vaccination prior to treatment and prophylactic use of antibiotics.
Despite abundant interest in complement drugs, only three are currently FDA approved; Eculizumab and a recently approved variant engineered for extended half-life, Ravalizumab (ALXN1210; https://ascopost.com/News/59600), and serum-derived C1-Inhibitor marketed as Cinryze or Berinert. Eculizumab has changed outlook in patients with PNH and aHUS, is now in the clinic for generalised MG, and is in clinical trials for many other diseases. Although effective in these rare diseases, the cost of treatment and high dosage are huge disadvantages for extending use to more common diseases. For example, annual cost of Eculizumab treatment of a PNH patient is £340,000 in the UK, and the antibody must be administered bi-weekly by intravenous infusion (900mg/dose). There are many agents in development that target C5, for example, SKY59 (RO7112689) an anti-C5 mAb now in phase II clinical trials. This mAb incorporates a pH dependent recycling technology that increases half-life and decreases dose required for efficient C5 inhibition. The modified form of Eculizumab, ALXN1210, uses a similar pH switch technology to increase recycling efficiency and drug half-life. Unlike Eculizumab, RO7112689 also binds C5b6, the intermediate product of MAC formation, and thus inhibits reactive lysis. RO7112689 and Eculizumab were used in this study for comparison with the novel in-house mAbs.

We describe novel anti-C5 mAbs that, like Eculizumab, are efficient inhibitors of complement, but unlike Eculizumab, inhibit efficiently across species, including human, rabbit, rat (clones: 4G2 and 7D4) and weakly guinea pig and mouse (7D4). The species cross-reactivity of these mAbs makes them powerful tools for proof-of-concept animal studies. To our knowledge, this is the first report of cross-species blocking anti-C5 mAb. One of these mAb was shown to efficiently inhibit complement in vivo in rats and to prevent disease in a rat model of MG (passive transfer experimental autoimmune MG; EAMG). Surface plasmon resonance (SPR) analysis of selected mAb demonstrated strong and stable binding to both human and rat C5, making these antibodies very strong candidates for tool therapeutics.
MATERIALS AND METHODS

All chemicals, except where otherwise stated, were obtained from either Fisher Scientific UK (Loughborough, UK) or Sigma Aldrich (Gillingham, UK) and were of analytical grade. All tissue culture reagents and plastics were from Invitrogen Life Technologies (Paisley, UK). Sheep and guinea pig erythrocytes in Alsever’s solution were from TCS Biosciences (Claydon, UK). Eculizumab was kindly donated by Prof David Kanavagh (Newcastle University, UK), and RO7112689 by Roche Diagnostics (Basel, Switzerland).

Human and animal sera were prepared in-house from freshly collected blood. For human, rabbit, rat and guinea pig, blood was clotted at room temperature for one hour, and then placed on ice for two hours for clot retraction prior to centrifugation and harvesting of serum. For mouse, blood was placed on ice immediately after harvest and clotted for two hours on ice prior to serum harvest. Sera were stored in aliquots at -80°C and not subjected to freeze-thaw cycles.

Generation of anti-C5 mAb

Mouse mAbs to C5 were generated by immunization of C6-deficient mice (bred in-house) with C5b6 using standard schedules. C5b6 was used as immunogen to increase the likelihood of obtaining function-blocking mAbs. C6 deficient mice were derived from a spontaneous C6 deficient mouse, back-crossed 8 generations onto C57Bl/6. C5b6 was prepared in house by incubating C5 and C6 with a fluid phase convertase comprising cobra venom factor and activated factor B (CVFBB); the complex was then purified by gel filtration. Immunised mice were screened for antibody responses by ELISA, mice with the highest titre response selected and re-boosted prior to sacrifice and harvesting of spleens. Plasma cells were harvested, fused with SP2 myeloma and aliquoted to 96-well plates. Positive hybridomas were selected by direct ELISA on immobilised C5b6 and by haemolysis assay for blocking activity as described below. C5b6-positive complement inhibitory mAb-secreting clones were sub-cloned by limiting dilution to monoclonality. Mouse mAbs were isotyped using IsoStrips (Roche, # 11493027001). Over multiple fusions, approximately 864,000 hybridoma clones were
screened, 139 antibodies selected from ELISA, 12 confirmed to be inhibitory, and three of these, 4G2, 7D4 and 10B6, chosen for full characterisation.

**Haemolytic Assays**

The inhibitory activity of mAbs in human and animal sera was investigated by classical pathway (CP; CH50) haemolysis assay using antibody-sensitised sheep erythrocytes (ShEA) or alternative pathway (AP; AH50) assays utilising rabbit erythrocytes (RabE) animal blood was from TCS Bioscience and anti-ShE antiserum (#ORLC25, Siemens Amboceptor) was from Cruinn Diagnostics (Dublin, UK). ShEA were suspended in HBS containing Ca\(^{2+}\) and Mg\(^{2+}\) at 2% (vol:vol), RabE in HBS containing 5mM EGTA and 3mM MgCl\(_2\). For measurement of CP activity in male mouse serum, ShEA were additionally incubated with mouse anti-rabbit IgG at 25µg/ml (Invitrogen, Paisley, UK. #3123) for 30 minutes at 37°C prior to washing into HBS. A serial dilution series of each test mAb (100 – 0µg/ml; 50 µl/well) was prepared in HBS and aliquoted in duplicate into a 96-well round-bottomed plate at 50 µl/well, then serum and 2% ShEA (50 µl/well of each) added. Serum dilutions for each species were selected in preliminary experiments to give near-complete haemolysis in the CP assay in the absence of test mAb: normal human serum; NHS, 2.5%; normal male mouse serum; NMS, 25% (using the double-sensitised cells as described above), normal rat serum; NRS, 2.5%; normal guinea pig serum; NGpS, 2.5%; normal rabbit serum; NRbS, 25%. Plates were incubated at 37°C for 30 minutes, centrifuged and haemoglobin in the supernatant measured by absorbance at 405nm. Percentage lysis was calculated according to: % Lysis =Absorbance (Abs) sample-Abs background)/(Abs max -Abs background)*100%. GraphPad Prism was used for data analysis. Hybridoma supernatants were screened for blocking mAbs using the same assay but with neat tissue culture supernatant in place of the purified mAb. C5a generation in CP haemolysis assay supernatants was measured using an ELISA kit (Hycult Biotech, # HK349-02).

**Characterisation of novel mAb by ELISA**

Direct ELISA and sandwich assay were used to test whether the new mAbs bound C5, C6 or C5b6. Sandwich ELISA were used to confirm C5 binders in order to eliminate
issues around denaturation and demonstrate separate epitopes for test mAbs to RO7112689 used as capture. Standard curves were generated using in-house proteins; C5, C5b6 and C6 purified as described previously described\textsuperscript{20,23}.

In the direct ELISA, Maxisorp (Nunc, Loughborough, UK) 96-well plates were coated with C5, C5b6 or C6 (0.5µg/ml in bicarbonate buffer pH 9.6) at 4°C overnight; wells were blocked (1 hour at 37°C with 2% BSA (bovine serum albumin) in PBS (phosphate buffered saline)), washed in PBS containing 0.05% Tween 20 (PBS-T). Dilutions of purified mAbs, 1000-0ng/ml (stock concentrations of all proteins used established using the BCA assay) in 0.2% BSA-PBS, were added in triplicate to wells coated with each of the three antigens and incubated for 1 hour at 37°C. Wells were washed with PBS-T then incubated (1 hour, 37°C) with secondary antibody (donkey anti-mouse-HRP; Jackson ImmunoResearch, Ely, UK) for 1 hour at 37°C.

In the sandwich ELISA, Maxisorp plates were coated with RO7112689 or Eculizumab (1µg/ml in bicarbonate buffer pH 9.6) at 4°C overnight; wells were blocked (1 hour at 37°C with 2% BSA-PBS) and washed in PBS-T. Standard curves of in-house purified proteins; C5, C5b6 and C6 diluted in 0.2% BSA-PBS, were added in triplicate and incubated for 1 hour at 37°C. Wells were washed with PBS-T then incubated (1 hour, 37°C) with in-house mAb anti-C5 (1 in 1000 dilution in PBS-T), followed after washing with HRP-labelled anti-mouse IgG as above.

After washing, plates were developed using o-Phenylenediamine dihydrochloride (OPD, SIGMAFAST\textsuperscript{TM}, Sigma-Aldrich) and absorbance (492nm) was measured. GraphPad Prism was used for data analysis. Assays detection limits, working ranges and assay performance were determined as described.\textsuperscript{24}

**Characterisation of mAbs by western blot.**

C5, C5b6 and C6 (in house; 1µg) were placed in separate wells and resolved on 4-20% SDS-PAGE gels (Biorad, #4561093) under reducing and non-reducing conditions, then electrophoretically transferred onto 0.45µm nitrocellulose membrane (GE Healthcare). After transfer, non-specific sites on the membrane were blocked with 3% BSA in PBS-T. After washing in PBS-T, membrane strips were incubated overnight at 4°C with individual test mAbs (clone 4G2, 7D4, 10B6; each at 1µg/ml in 3% BSA PBS-T) or
controls RO7112689 (1µg/ml) or polyclonal (goat) anti-human C5 (CompTech; A220; 2µg/ml. After washing, bound test mAb were detected by incubation with donkey anti-mouse IgG-HRP (Jackson ImmunoResearch, 715-035-150), RO7112689 with donkey anti-human IgG-HRP (Jackson ImmunoResearch, 709-036-149) and polyclonal anti-C5 with rabbit anti-goat IgG HRP conjugate (Jackson ImmunoResearch, 305-035-045) at 1:5000 in PBS-T. After washing, the blot was developed with enhanced chemiluminescence (ECL, GE Healthcare) and visualised by autoradiography.

**Testing impact of mAbs on atypical cleavage of C5 by neutrophil elastase.**
C5 was mixed with test mAb at 5x molar excess in HBS, then incubated for 15 minutes at RT. Neutrophil elastase (NE, Athens Research, Athens, Georgia; # 16-14-051200) was added at 420nM final concentration, incubated 30 minutes at 37°C in a shaking water bath, and the reaction stopped by addition of 61nM protease cocktail inhibitors (CI, Sigma Aldrich, #P8340). C5 cleavage was determined by measuring generation of C5a, detected by WB and by ELISA (Hycult Biotech, # HK349-02). For WB, samples were diluted 1 in 2 in HBS, separated and transferred as above. Blots were probed with anti-C5a mAb (Hycult Biotech, #HM2079), detected using donkey anti-mouse IgG-HRP (Jackson ImmunoResearch, 715-035-150). Positive controls included intact C5 (in house), C5a (Comptech, Texas, USA), NE and CI diluted in HBS.

**SPR analysis to determine test mAbs binding affinity to human and rat C5.**
The human C5 mAb binding analyses were carried out on a Biacore S200, while rat C5 mAb binding was tested using a T200 instrument (GE Healthcare, Amersham, UK); in both, a Mouse Antibody Capture kit (GE Healthcare, # BR-1008-38) was used to immobilise capture antibody on a CM5 sensor chip (GE Healthcare, #29-1496-03) at RU = 500 (for human C5 testing) or 400 (for Rat C5 analysis). Test mAb were flowed to saturate the surface, then C5 proteins (human or rat) in HEPES-buffered saline (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.005% surfactant P20) flowed over the immobilised mAb. For kinetic analysis the flow rate was maintained at 30 µl/min, and data were collected at 25°C. Data from a reference cell were subtracted to control for bulk refractive index changes. The Rmax was kept low and the flow rate high to eliminate
mass transfer. All reagents used were of high purity, polished by size exclusion chromatography immediately before use to ensure removal of any aggregates. Data were evaluated using the software appropriate to the instrument (Biacore Evaluation Software).

**Passive Transfer Experimental Autoimmune MG (EAMG) in rats.**

To test in vivo effects and therapeutic efficacy of test mAb, Wistar Han IGS rats (100 – 150g) were obtained from Charles River Laboratories (Edinburgh, UK) and allowed to acclimatize for one week prior to disease induction by intraperitoneal administration of anti-Acetylcholine receptor (AChR) mAb35 at 1 mg/kg in PBS as described previously.\(^\text{25}\) MAb35 binds the main immunogenic region of AChR, activating complement and damaging the neuromuscular junction endplates, causing severe muscle weakness. Animals were assessed hourly post-disease initiation as described previously.\(^\text{25,26}\) mAb35-injected rats were split into two groups: Group one (n = 4) was treated with test mAb at 40 mg/kg dose (selected dose determined in a pilot study) subcutaneously (sc) at time zero, with a second sc dosage of 13 mg/ kg at 24 hours. The second group (n = 3) received an irrelevant isotype control antibody at the same times, routes and doses. All animals were euthanised at 40 hours post-induction, blood taken for serum assays, soleus muscles harvested and frozen in OCT mounting medium for sectioning as described previously.\(^\text{25,26}\) Sections were fixed in ice cold acetone for 15 min at -80°C and then blocked for 30 minutes in 10% horse serum 2% BSA. After washing in PBS, sections were stained overnight at 4°C with primary antibodies, C3/30 anti-C3b/iC3b mAb (in house) at 10µg/ml, and rabbit anti-rat C9/MAC polyclonal IgG (in house) at 50µg/ml, both in the block buffer. Anti-C3b/iC3b sections were washed and incubated for 15 min at RT with amplifier antibody goat anti-mouse (Vector Labs, Peterborough, UK, VectaFluor DyLight 488, # DK-2488). After washing, secondary antibody, horse anti-goat IgG – Alexa Fluor 488 (DyLight 488, # DK-2488) for C3b/ iC3b or goat anti-rabbit-FITC (Oxford Biomedical Research, #45002) for anti-C9/MAC added as appropriate, together with alpha-bungarotoxin-TRITC (Btx) (labels AChR; Boitum, # 00012) at 0.5% and Hoechst stain 1:10 000 (ThermoFisher, # 62249), then incubated 40 minutes at RT in dark. Sections were washed in PBS and mounted in VectorShield
Vibrance (Vector labs, #H-1700-2) before analysis using an Apotome fluorescent microscope (Zeiss Apotome Axio Observer microscope). Ten fields were captured from comparable regions of muscle in each sample at the same exposure and magnification (x 40) and the number of BuTx-reactive endplates in each section was measured using density slicing in an image analysis system (ImageJ). For co-localisation of complement activation products, sections were additionally imaged on a Zeiss confocal microscope (Zeiss LSM800 confocal laser scanning microscope).

RESULTS

Cross-species complement inhibition by mAbs in haemolysis assays.

Hybridoma clones were initially selected for further characterisation based upon the capacity of clone supernatants to cause inhibition of CP haemolysis of ShEA by human serum; three of the selected monoclonal clones; 4G2 (IgG2b, K), 7D4 (IgG2b, K), 10B6 (IgG1, K), were expanded for further characterisation, secreted mAbs purified and further tested in haemolysis assays. As expected, each of the selected mAbs efficiently inhibited NHS-induced CP haemolysis, with clone 10B6 showing equivalent dose-response performance to the benchmark mAbs RO7112689 and Eculizumab (Fig. 1A). When the impact of the mAbs on CP haemolysis induced by other species sera was tested, mAb 4G2 and 7D4 efficiently inhibited haemolysis mediated by either rat or rabbit serum (Fig. 1B, C) and weakly inhibited haemolysis by guinea pig and mouse serum (Fig. 1D, E). In contrast, neither the new mAb 10B6 nor Eculizumab inhibited any of these non-human sera, while RO7112689 inhibited mouse, guinea pig and rabbit but not rat serum induced haemolysis. The calculated 50% complement inhibitory doses of all mAbs are shown (Fig. 1F); Guinea pig serum is not included in this summary as levels of inhibition did not reach 50% for any of the mAbs. The novel mAbs 10B6, 4G2 and 7D4 also showed efficient inhibition of AP lysis in human serum (Fig. 1G), while 4G2 and 7D4 efficiently inhibited rat AP (Fig. 1H). C5a release was inhibited by the novel mAbs at the same levels as MAC generation (66nM) in the human CP assay (Fig. 1I).

Binding of mAbs to C5 and C5b6
The direct ELISA showed that all the selected new mAbs recognised C5 and C5b6 and none recognised C6 (Fig. 2A – C). In a sandwich ELISA with RO7112689 (anti-C5β) as capture and 4G2 mAb as detection, C5 and C5b6, but not C6, were detected demonstrating that mAb 4G2 and RO7112689 antibodies recognised different epitopes on C5 and C5b in the C5b6 complex (Fig. 2D).

To confirm which chains of C5 the new mAbs bound we performed western blotting on purified C5. The blots demonstrated that mAb 4G2 and 7D4 bound the α-chain in C5; of note, while 7D4 recognised both residual α-chain and the α’-chain in C5b6, 4G2 only bound residual intact α-chain (Fig. 3A, B). 10B6 recognised the β-chain of C5 (Fig. 3C); RO712689 also recognised the β-chain (Fig. 3D), while Eculizumab is known to bind the macroglobulin 7 domain in the α-chain.26 Polyclonal anti-C5 recognised both C5 α- and β-chains (not shown).

To test whether the novel anti-C5 mAb recognised epitopes distinct from the commercial mAb, sandwich ELISA were developed utilising RO7112689 or Eculizumab as the capture mAb. With RO7112689 as capture, both the C5α-specific mAb (7D4 and 4G2) bound strongly but the C5β-specific 10B6 did not bind (Fig. 2E). With Eculizumab (anti-C5α) capture, the β-chain specific mAb 10B6 bound most strongly while the anti-C5α mAb bound weakly (4G2) or not at all (7D4), suggesting that they competed for similar epitopes (Fig 2F).

SPR analysis on immobilised antibody with human C5 flowed over (Fig. 4 A–C) showed binding between human C5 and each of the novel mAb. The mAb 10B6 and 7D4, showed very strong binding to human C5 in SPR analyses (KD = 4.105 x 10^{-10}, 1.264 x 10^{-9} respectively) with negligible off rates, suggesting that 10B6 and 7D4, respectively targeting the β and α chains of C5, might be promising candidates for therapeutics. Binding of human C5 to 4G2 was relatively weak (KD = 1.389 x 10^{-8}; Fig. 4A); in contrast, binding of rat C5 to 4G2 was strong and stable (KD = 4.872 x 10^{-9}). The relatively slow off rate of rat C5 from mAb 4G2 suggested that this mAb offered promise for use in vivo in rats because 4G2 and C5 will form a stable complex, delivering prolonged inhibition. Technical issues precluded the analysis by SPR of 7D4 binding to rat C5.
**Induction of EAMG in rats and effect of mAb 4G2 on clinical disease and pathology**

Rats given mAb35 at 1mg/kg intraperitoneally and an irrelevant isotype control mAb subcutaneously began to lose weight and show signs of hind limb weakness (Fig. 5A). Clinical symptoms, comprising limp tails, piloerection, hind limb weakness and reduced grip strength, were detectable in isotype control treated animals by 18 hours post-induction, and all exhibited severe disease with hind limb weakness and/or partial paralysis, reaching clinical score 4 on a standardised scale (0, no disease; 1, reduced grip strength in front legs (can grip cage lid but cannot lift) and floppy tail; 2, loss of grip in front legs; 3, loss of grip and hind limb weakness and wasting; 4, loss of grip and hind limb paralysis; 5, moribund) by endpoint. In contrast, animals given mAb 4G2 subcutaneously at the time of disease induction continued to gain weight over the time course of the experiment and did not develop detectable weakness or other clinical manifestations for the duration of the experiment (Fig. 5B). Animals were sacrificed by a Schedule 1 method when weight loss was equal to or exceeded 20% of original bodyweight, or when clinical score reached 4. CP haemolytic activity in serum was essentially absent at 2 hours post-induction in 4G2-treated animals but at later time points, residual haemolytic activity of approximately 35% of controls was detected (Fig. 5C), likely due to the high sensitivity of the haemolytic assay for residual C5. As expected, serum from the untreated control animals retained full haemolytic activity across the time course.

Soleus muscle were harvested at sacrifice (40 hours), sections (10µm) were prepared, fixed in acetone and endplates identified by staining AChR with α-Bungarotoxin-TRITC. Receptor numbers were quantified across 10 different representative fields in an automated imaging system and analysed using ImageJ software. The number of endplates in isotype control animals was significantly decreased compared to mAb 4G2 treated animals (~2-fold less; Fig. 5D); endplate numbers in the 4G2 group were not significantly different to numbers in naïve animals (data not shown). Residual endplates were frequently fragmented in isotype control animals, while most endplates in 4G2-treated animals displayed the typical continuous, linear character. The α-Bungarotoxin fluorescence intensity was significantly higher in the 4G2-treated animals (P = 0.002,
Fig. 5E). Whereas C3 fragment staining intensity was similar between the groups, C9/MAC staining was reduced more than 3-fold in the 4G2-treated group (P<0.0001; Fig. 5F, G). Confocal analysis and co-localisation of C3b/iC3b and C9/MAC deposition at the endplate demonstrated strong and specific deposition of C3b/iC3b and MAC at the endplate in isotype control animals at 40 hours (Fig.5H), whereas in 4G2-treated animals, C3b/iC3b was deposited to a similar degree at endplates but C9/MAC deposition was weak or absent (Fig. 5I).

**mAb 4G2 but not mAb 7D4 and 10B6 block atypical cleavage of C5 by neutrophil elastase.**

To test whether any of the new mAb inhibited atypical cleavage of C5, C5 was incubated with NE with or without an excess of each of the mAbs; C5a generation, detected by western blot, was used as an index of C5 cleavage. Although C5 cleavage products were present in all cases, complete inhibition of NE-mediated C5a generation was observed when mAb 4G2 was used, while 10B6 and 7D4 caused a partial inhibition of C5a generation compared to the no-mAb control (Fig. 6A). When tested in this assay, RO7112689 completely inhibited C5a generation while Eculizumab had no effect on C5a production; an irrelevant non-blocking anti-C5 (3D3) also did not inhibit C5a generation (Fig. 6A). Densitometric analysis of the C5a band showed a ~95% inhibition of C5a generation by 4G2 and RO7112689, 40% by 10B6 and 20% by 7D4 compared to cleavage in absence of antibody (Fig. 6B). These data were confirmed by ELISA; when compared to the amount of C5a generated by NE in the absence of antibody (670ng/ml in the assay; 100%), the amount of C5a generated was reduced to ~4% by inclusion of 4G2 or RO7112689; 73% by 7D4; 59% by 10B6; Eculizumab did not inhibit C5 cleavage in this assay (Fig. 6 C).

**DISCUSSION**

Twelve years ago, the first complement inhibitor, Eculizumab was approved for use in therapy of PNH. The discovery of Eculizumab and its dramatic success in this ultra-rare disease catalysed Pharma interest in anti-complement drugs, resulting in many new agents targeting different complement pathways being developed.4,14,16 Eculizumab is
an effective terminal pathway blocker; however, for several years its use has been restricted to only two ultra-rare diseases, PNH and aHUS, and latterly approved for MG treatment. In recent years, pathological roles of complement in more common conditions like myasthenia gravis, age related macular degeneration, and neurodegeneration have been demonstrated, building a case for the broader use of anti-complement drugs in the clinic.\textsuperscript{29-31} Cost and convenience are limiting factors in broadening anti-complement drug use; current cost and dosing schedules for Eculizumab are incompatible with use in treatment of common, chronic diseases. It is therefore imperative that new agents and approaches are brought to bear on C5 inhibition; increasing drug half-life (and hence reducing dose) has been a first approach both with the Eculizumab-derived ALXN1210 and the competitor mAb RO7112689, but other approaches, including small molecule inhibitors of C5,\textsuperscript{32} are in train. A second limiting factor is that the currently available agents are human-specific, restricting proof-of-concept of efficacy of C5 inhibition in rodent disease models. The mAb BB5.1 has been widely used in mouse models,\textsuperscript{33,34} and a single report described a blocking anti-C5 mAb that was not further utilised,\textsuperscript{35} apart from these, no anti-C5 mAb inhibiting in other animal species have been described.

Here we immunised C6-deficient mice with C5b6 in order to develop functionally optimal mAb, selecting from the outset for function-blocking activity. Of 864,000 hybridoma clones screened, 139 were ELISA-positive, 12 of these were inhibitory, and three inhibitory clones were selected for full characterisation. All mAb selected based on blocking activity bound native C5 and C5b6 (the immunogen), but not C6, in ELISA. Three of these human C5 blocking mAb were selected for further characterisation; testing for inhibition in other species showed that two of the mAb, 7D4 and 4G2 (both against C5α chain), inhibited C5 in rat, rabbit, guinea pig and mouse sera, whereas 10B6 (against C5β chain) was human-specific. RO7112689 bound the C5β chain, as reported previously,\textsuperscript{20} and was a weak inhibitor of guinea pig and mouse C5 (Fig. 3), while Eculizumab bound C5α and is human-specific.\textsuperscript{27} The critical residue in the Eculizumab binding site that dictates human specificity is Trp917, replaced by Ser in other species, including non-human primates, rat, mouse, rabbit, guinea pig, sheep, pig and horse as reported,\textsuperscript{27,28} and from our data base search
The fact that the two blocking anti-C5α mAb 7D4 and 4G2 work across species suggests that they bind an epitope distinct from that in C5α bound by Eculizumab; this is supported by the demonstration that these mAb bind C5b6 (the immunogen) in the direct ELISA while Eculizumab does not. The mAb 4G2 detected C5 captured on Eculizumab in a sandwich assay, confirming that the epitopes are distinct; however, 7D4 did not bind in this assay, indicating that its epitope overlaps that of Eculizumab. When RO7112689 (C5β-specific) was used as capture in the assay, both the C5α-binding mAb detected the bound C5 as anticipated; in contrast, the C5β-specific mAb 10B6 gave no signal, demonstrating that its epitope was masked by the capture mAb. Of note, RO7112689, like 10B6, also binds C5b6. These data indicate that the epitopes for these two mAb are overlapping; the fact that RO7112689 inhibits across species while 10B6 is human-specific suggests that the epitopes are non-identical. It is clear from the above findings, and from published work comparing tick-derived C5 inhibitors with Eculizumab, that there are numerous sites on C5 where binding of an inhibitor can effectively block function. This supports the suggestion that these diverse inhibitors act as conformation locks, preventing a structural “priming” event that is necessary for cleavage of C5 by the convertase.

The mAb were also tested for their capacity to block the atypical cleavage of C5 by neutrophil elastase (NE), a process that has been implicated in the generation of C5a and MAC at inflammatory sites such as in the rheumatoid joint and cystic fibrosis lung; mAb that inhibited this cleavage might therefore have additional value as therapeutics. The mAb 4G2 strongly inhibited NE-mediated C5 cleavage and C5a generation in vitro, whereas the other two novel mAbs weakly inhibited atypical C5 cleavage (Fig. 6). Eculizumab did not inhibit C5 cleavage by NE but RO7112689 effectively blocked generation of C5a by NE (Fig. 6C).

SPR analysis (Biacore) showed very strong and stable binding of 7D4 and 10B6 to human C5 with dissociation rates and calculated affinities comparable to RO7112689 (Fig. 4). In contrast, 4G2 binding to human C5 was much weaker – but this mAb bound rat C5 much more strongly than human (KD 4.872 x 10^-9 for rat and 1.389 x 10^-8 for human) and with a slow off-rate; these binding data support the functional assays where
4G2 was a more potent inhibitor of rat C5 than human (Fig. 1). The 4G2 mAb was tested \textit{in vivo} as a prophylactic therapy in a model of MG, previously demonstrated to be dependent on MAC assembly in man and rodents and suppressed by terminal pathway deficiencies or inhibition.\textsuperscript{24,26} While control animals developed severe muscle weakness and weight loss, mAb 4G2 treated rats were protected from disease and weight loss (Fig. 5 A, B). Endplates in controls were reduced in number, fragmented and richly decorated with C3b/iC3b and C9/MAC, whereas in 4G2 treated animals endplates were preserved in number and integrity; although endplates were strongly C3b/iC3b-positive in these animals, C9/MAC deposition was markedly reduced or absent (Fig. 6). Although mAb 4G2 proved to be an efficient blocker of EAMG, assays of serum haemolytic activity showed \~35\% residual lysis after the 2 hour time-point in 4G2 treated animals. We have previously demonstrated that as little as 20 ng/ml C5 added back to undiluted C5-depleted serum is sufficient to cause measurable lysis and 50\% lysis in the assay was attained at 2µg/ml C5 added back.\textsuperscript{20} These findings illustrate the exquisite sensitivity of the haemolytic assay and show that it is not a good predictor of efficacy of C5 inhibition in disease. We have not yet tested this mAb for amelioration of established disease in the EAMG model, difficult given the acute nature of the model, or in more chronic disease models; such studies are planned in the future.

The novel mAbs described here are efficient blockers of human C5, with binding sites and other properties distinct from existing anti-C5 mAb. All three mAb bind C5b6, used as the immunogen, and block reactive lysis, a property absent from Eculizumab that may provide a therapeutic advantage. Notably, two of the mAb inhibit C5 in common laboratory animal species, raising the prospect of their use as tools in proof-of-concept studies, illustrated here using EAMG as an example. One of the new mAb was a strong inhibitor of elastase-mediated C5 cleavage, raising the possibility of testing the impact of blocking atypical C5 cleavage in inflammatory diseases where neutrophil activation predominates in rodents or man.

\textbf{ACKNOWLEDGEMENTS}

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CONFLICT OF INTEREST
BPM has provided advice on complement to Roche and is a consultant to GlaxoSmithKline; all fees are paid to Cardiff University. Other authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

AUTHOR CONTRIBUTIONS
WMZ performed all the laboratory analyses and wrote the first draft of the manuscript; PRT provided critical expertise on animal immunisation and antibody clone screening; BPM conceived and planned the study and oversaw the data handling and manuscript preparation. All authors contributed to and have approved the final manuscript.
REFERENCES


FIGURE LEGENDS

**Figure 1.** Functional assays to determine whether mAb 4G2, 7D4 and 10B6 inhibit complement in different species. A-E. Classical pathway haemolysis (CH50). Sera tested were human (A), rat (B), rabbit (C), guinea pig (D) and mouse (E). Commercial mAb RO7112689 and Eculizumab were used as comparators. F. Calculation of 50% inhibitory dose showed that human C5 inhibition by mAb 10B6 was equivalent to the two comparator mAbs, RO7112689 and Eculizumab, and that 4G2 and 7D4 strongly inhibited rat C5. G,H. Alternate pathway (AP) haemolysis (AH50) assay utilising human (G) and rat (H) serum; all tested mAb inhibited AP in human serum, while 4G2 and 7D4 inhibited rat AP. I. Inhibition of C5α generation by the novel mAbs in a classical pathway assay with human serum; all three mAb efficiently inhibited C5α generation in a dose-dependent manner. All experiments were repeated three times with the same results. The error bars are standard errors of triplicates. The dashed lines correspond to the comparator mAbs.

**Figure 2.** Direct and sandwich ELISA for characterisation of new mAb. In direct ELISA, plates were coated with human C5 (A), C5b6 (B) or C6 (C). All new mAbs detected C5 and C5b6 complex, but not C6. In a sandwich ELISA with RO7112689 as the capture and in house 4G2 mAb as detection (D), both C5 and C5b6, but not C6 were detected showing that mAb 4G2 and RO7112689 antibodies recognised different epitopes in C5. Sandwich ELISA comparing RO7112689 (E; anti-C5β) and Eculizumab (F; anti-C5α) as capture mAb showed that both the C5α-specific mAb (7D4 and 4G2) bound strongly to RO7112689-captured C5 but the C5β-specific 10B6 did not bind (E); the β-chain specific mAb 10B6 bound most strongly to Eculizumab-captured C5 while the anti-C5α mAb bound weakly (4G2) or not at all (7D4), suggesting that they competed for similar epitopes (F). All experiments were repeated three times with the same results. The error bars are standard errors of triplicates.

**Figure 3.** Western blot (WB) using novel mAbs (4G2, 7D4, 10B6) in comparison to anti-C5 mAb RO7112689. Proteins; human C5, C5b6 and C6 (1µg) were resolved on 4-20%
PAGE gels under non-reducing (NR) and reducing (R; 5% β-mercaptoethanol) conditions. Blots were probed with mAb 4G2 (A), 7D4 (B), 10B6 (C), RO7112689 (D). All mAbs detected intact C5 and C5b but not C6 in the C5b6 complex. mAb 4G2 and 7D4 bound the C5α chain, while mAb 10B6 and RO7112689 bound the C5β chain. All mAbs except 4G2 detected C5b6 in reducing condition. In house produced C5b6 probed with RO7112689 showed trace contaminating human antibody bands from the immunoaffinity purification process detected by the anti-human IgG secondary. Results are representative of multiple analyses.

**Figure 4.** Binding kinetics of the novel mAbs to human and rat C5. Each mAb (4G2, 7D4, 10B6) was immobilised on the mouse IgG capture sensor chip (GE Healthcare) at approximately 60 RU (insets in each). C5 was flowed in HBS at 66 to 8nM for human and 33 to 4nM for rat C5 and interactions with the immobilised mAbs analysed. Sensorgrams were collected and KDs calculated using the Langmuir 1:1 binding model with RI values set to zero. Representative sensorgrams are shown with raw data in black lines and fitted data in grey (n = 3). A, mAb 4G2 binding human C5; B, mAb 7D4 binding human C5; C, mAb 10B6 binding human C5; D, mAb 4G2 binding rat C5; E, summary table of KDs from the aggregate studies.

**Figure 5.** Therapeutic effect of mAb 4G2 in EAMG. EAMG was induced in rats and weight loss (A) and clinical score (B) were monitored. Control animals (EAMG) rapidly developed weight loss and muscle weakness, reaching clinical scores of four or five and all were sacrificed at 40 hours. The mAb 4G2 treated animals were protected from disease and weight loss; all were sacrificed at 40 hours. Serum lytic activity was measured at multiple time points (0, 2, 18, 24 and 40 hours) in each animal (C). Results are means of determinations from four 4G2-treated and three isotype control treated EAMG animals and vertical bars represent SD. Soleus muscles were harvested at sacrifice and snap frozen in OCT. Sections (10µm) were stained for AChR with TRITC-conjugated α-BuTX and visualised by Zeiss Apotome microscope; AChR-positive endplates were counted in ten fields from each animal using ImageJ software (D). Mean fluorescence intensity of BuTx staining in the...
sections (mean pixel intensity values from ImageJ) was significantly higher in 4G2 treated animals compared to controls (E; P = 0.002). Mean fluorescence intensity of C3b/iC3b was not significantly (NS) different between the two groups (F), while C9/MAC staining was markedly reduced in 4G2 treated animals (G; P<0.0001). Statistical significance was obtained by t-test and p<0.05 was considered as significant. Tissue sections from isotype control (H) or 4G2-treated (I) animals were double stained for AChR together with anti-C3b/iC3b (top panel) or C9/MAC (bottom panel) and imaged on a Zeiss confocal microscope. The scale bar is shown in the first plate; all images were captured at identical magnification.

Figure 6. Impact of anti-C5 mAbs on cleavage of C5 by neutrophil elastase. (A) Western blot of atypical cleavage of C5 by neutrophil elastase (NE). C5 was mixed with each mAb at 5 x molar excess in HBS, NE was added at 420nM, incubated and reaction stopped by addition of protease inhibitors. Samples (1µg) were resolved on 4-20% SDS-PAGE gels under reducing (R) conditions and processed for WB to detect intact or cleaved C5 and C5a using an anti-C5a mAb that also detects native C5α chain (Hycult; mAb 2942). C5α (115 kDa), C5a (10.4 kDa), Cl; proteases cocktail inhibitors, Ecul.; Eculizumab, RO; RO7112689, 3D3; irrelevant non-blocking anti-C5 mAb. Note that numerous unidentified C5 cleavage products are present in all NE-treated samples. (B) Densitometry analysis of the C5a band using ImageJ (expressed as % relative to the C5a generation in the absence of antibody; 100%) confirmed that mAb 4G2 and commercial mAb RO7112689 efficiently inhibited generation of C5a. (C) measurement of C5a generation by ELISA confirmed that mAbs 4G2 and RO7112689 inhibited C5a generation while other mAb inhibited weakly. ELISA results are presented as percentage relative to C5a generation in the absence of any mAb (set as 100%; measured as 670 ng/ml in the ELISA). Results are representative of three independent experiments. The error bars are standard errors of triplicates.
Figure 1

A) **CH50 Human**

B) **CH50 Rat**

C) **CH50 Rabbit**

D) **CH50 Guinea Pig**

E) **CH50 Mouse (male)**

F) **Antibody**

<table>
<thead>
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<th>Antibody</th>
<th>50% Complement inhibitory dosage (ng/ml)</th>
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</tr>
<tr>
<td>4G2</td>
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<td>7D4</td>
<td>2187.8</td>
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<tr>
<td>10B6</td>
<td>841.4</td>
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<tr>
<td>RO7112689</td>
<td>668.3</td>
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<tr>
<td>Eculizumab</td>
<td>695.0</td>
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G) **AH50 Human**

- Lysis (%) vs. log10 antibody dose (µg/ml)
- 4G2, 7D4, 10B6, RO., Ecul.

H) **AH50 Rat**

- Lysis (%) vs. log10 antibody dose (µg/ml)
- 4G2, 7D4, 10B6, RO., Ecul.

I) **C5a measurement in CH50 supernatant**

- C5a generation (%) vs. Log10 antibody dose (µg/ml)
- 4G2, 7D4, 10B6
Figure 2

A) Binding of C5 in direct ELISA

B) Binding of C5b6 in direct ELISA

C) Binding of C6 in direct ELISA

D) Binding of C5, C5b6, C6 in sandwich ELISA (RO7112689 capture, mAb 4G2 detection)

E) Binding of C5 in sandwich ELISA (RO7112689 capture)

F) Binding of C5 in sandwich ELISA (Eculizumab capture)
Figure 3

A) Non-reduced

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Reduced

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D) Non-reduced

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- C5α
- C5α'
- C5β
Figure 4

A)

[Graph showing time vs. response for 4G2 + human C5 with concentrations of 66, 33, 17, and 8 nM.

B)

[Graph showing time vs. response for 7D4 + human C5 with concentrations of 66 nM, 33 nM, 17 nM, and 8 nM.]
<table>
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<tr>
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<th>$ka$ (1/Ms)</th>
<th>$kd$ (1/s)</th>
<th>$KD$ (M)</th>
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<td>4G2</td>
<td>2.463E5</td>
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<tr>
<td>Human C5</td>
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<td>4G2</td>
<td>8.998E+5</td>
<td>0.01250</td>
<td>1.389E-8</td>
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<td>7D4</td>
<td>2.801E+5</td>
<td>3.539E-4</td>
<td>1.264E-9</td>
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<tr>
<td>10B6</td>
<td>1.652E+5</td>
<td>6.783E-5</td>
<td>4.105E-10</td>
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Figure 5

A) Weight loss

- mAb 4G2 treated (n=4)
- EAMG Control (n=3)

B) Clinical score

- mAb 4G2 treated (n=4)
- EAMG Control (n=3)

Clinical Score:
1). Reduced grip strength in front legs and floppy tail
2). Loss of grip in front legs
3). Loss of grip and hind limb weakness and wasting
4). Loss of grip and hind limb paralysis
5). Moribund

C) Haemolysis assay

- mAb 4G2 treated
- EAMG Control
Mean number of AChR per field (10 fields per animal)

**D)**

<table>
<thead>
<tr>
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<th>EAMG Control (40 hours)</th>
<th>mAb 4G2 treated (40 hours)</th>
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<tr>
<td>Mean ± SD</td>
<td>2.9 ± 0.87</td>
<td>1.54 ± 0.65</td>
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**E)**

<table>
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<th>EAMG Control (40 hours)</th>
<th>mAb 4G2 treated (40 hours)</th>
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<tr>
<td>α-BuTX mean intensity</td>
<td>2.25 ± 0.16</td>
<td>3.04 ± 0.29</td>
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**F)**

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<th>EAMG Control (40 hours)</th>
<th>mAb 4G2 treated (40 hours)</th>
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<tr>
<td>C3b/ iC3b mean intensity</td>
<td>11.03 ± 1.26</td>
<td>11.47 ± 1.25</td>
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**G)**

<table>
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<tr>
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<th>EAMG Control (40 hours)</th>
<th>mAb 4G2 treated (40 hours)</th>
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<tr>
<td>C9/ MAC mean intensity</td>
<td>7.5 ± 1.05</td>
<td>2.4 ± 0.26</td>
</tr>
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**P < 0.0001**

**P = 0.002**

**NS**
EAMG Controls (40 hours)

mAb 4G2 treated (40 hours)
Figure 6

A)

B)

<table>
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<tr>
<th>Sample</th>
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<tr>
<td>C5 cleaved by NE + mAb 4G2</td>
<td>5.6</td>
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<tr>
<td>C5 cleaved by NE + mAb 7D4</td>
<td>79.9</td>
</tr>
<tr>
<td>C5 cleaved by NE + mAb 10B6</td>
<td>60.2</td>
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<tr>
<td>C5 cleaved by NE + RO7112689</td>
<td>4.8</td>
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<tr>
<td>C5 cleaved by NE + Eculizumab</td>
<td>98.3</td>
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<tr>
<td>C5 cleaved by NE</td>
<td>100.0</td>
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C)

C5a measurement in NE C5 cleavage assay