Selectivity of C3-opsonin targeted complement inhibitors: a distinct advantage in the protection of erythrocytes from paroxysmal nocturnal hemoglobinuria patients

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Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by complement-mediated cell lysis due to deficiency of GPI-anchored complement regulators. Blockage of the lytic pathway by eculizumab is the only available therapy for PNH patients and shows remarkable benefits, but regularly yields PNH erythrocytes opsonized with fragments of complement protein C3, rendering such erythrocytes prone to extravascular hemolysis. This effect is associated with insufficient responsiveness seen in a subgroup of PNH patients. Novel C3-opsonin targeted complement inhibitors act earlier in the cascade, at the level of activated C3 and are engineered from parts of the natural complement regulator Factor H (FH) or complement receptor 2 (CR2). This inhibitor class comprises three variants of "miniFH" and the clinically developed "FH-CR2" fusion-protein (TT30). We show that the approach of FH-CR2 to target C3-opsonins was more efficient in preventing complement activation induced by foreign surfaces, whereas the miniFH variants were substantially more active in controlling complement on PNH erythrocytes. Subtle differences were noted in the ability of each version of miniFH to protect human PNH cells. Importantly, miniFH and FH-CR2 interfered only minimally with complement-mediated serum killing of bacteria when compared to untargeted inhibition of all complement pathways by eculizumab. Thus, the molecular design of each C3-opsonin targeted complement inhibitor determines its potency in respect to the nature of the activator/surface providing potential functionality in PNH.

Introduction

In absence of strict regulation the triggering of any of the three complement activation pathways, the lectin (LP), the classical (CP) or the alternative pathway (AP) can lead to the initiation of the lytic, terminal pathway (TP) (Supplemental Figure 1A). Due to the deficiency in producing glycosylphosphatidylinositol (GPI) anchors, PNH cells lack the two important membrane-based complement regulators CD55 (Davitz et al., 1986; Nicholson-Weller et al., 1983; Pangburn et al., 1983) and CD59 (Holguin et al., 1990, 1989) leaving PNH erythrocytes susceptible to complement-mediated lysis (reviewed in (Parker, 2007)). CD55 is a potent regulator of all convertases throughout the proximal complement cascade (Sun et al., 1999; Telen and Green, 1989), while CD59 specifically inhibits the formation of the membrane attack complex (MAC) (Davies et al., 1989), which introduces lytic pores into cell membranes. The CP is typically initiated by the sensing of immune complexes, but can, similarly to the LP, also be launched by recognition of pathogen or danger patterns. In contrast the AP is continuously and indiscriminately activated at low level (which is also called "tick-over" activation) posing a constant threat to vulnerable cells (reviewed in (Ricklin et al., 2010)). Yet the AP is not merely one of the three complement activation pathways, but functions as a positive feedback loop to all initiation pathways and amplifies the C3 activation product C3b regardless of the underlying activation pathway that produced the initial C3b molecules (reviewed in (Lachmann, 2009)).

Selectivity within the AP is achieved by providing healthy host cells with a set of regulators that tightly control AP amplification on such surfaces. On PNH cells, complement receptor 1 (CR1) is the only remaining membrane anchored AP regulator on the cell surface. The number of CR1 molecules on erythrocytes differs considerably and in Caucasians correlate with a restriction fragment length polymorphism (Xiang et al., 1999). Low numbers of CR1 molecules on PNH erythrocytes correlate with reduced complement control and higher numbers of C3-opsonins being fixed to PNH erythrocytes, which predisposes those erythrocytes for clearance by

the reticuloendothelial system, a phenomenon termed "extravascular hemolysis" (Rondelli et al., 2014). The reduced set of surface-bound regulators puts more emphasis for protecting PNH erythrocytes from complement on the soluble AP regulator Factor H (FH). Owing to its ability to recognize sialic acid moieties located on erythrocytes, FH was shown to be a crucial contributor to the protection of PNH erythrocytes (Ferreira and Pangburn, 2007). While FH and CR1 appear to provide sufficient protection of PNH erythrocytes from AP tick-over activation under steady state conditions, after initiation of the complement cascade by a stimulus (e.g. infection or surgery) (Peffault de Latour et al., 2015) the two remaining complement regulators are overwhelmed and fail to sufficiently control bystander AP activation on the PNH surface (Ezzell et al., 1991; Ferreira and Pangburn, 2007; Rondelli et al., 2014). This situation of relative underregulation of complement renders PNH cells, but not healthy host tissue, susceptible to lysis by the AP.

Therapy with eculizumab (Soliris, Alexion Pharmaceuticals), a humanized monoclonal antibody directed against terminal complement component C5, inhibits MAC formation and thus intravascular lysis (Rother et al., 2007) (Supplemental Figure 1B). Despite the clear benefits of complete blockage of complement TP for PNH patients (Brodsky et al., 2008; Hillmen et al., 2006; Kelly et al., 2011), the high treatment cost, insufficient response in some patients and increased risk for meningococcal infections need to be considered. Whereas the infection risk may be reduced through use of a vaccination panel (Verhave et al., 2014), residual anemia remains an issue for a substantial fraction of patients. Under eculizumab treatment, C5 inhibition prevents MAC formation and intravascular lysis, but due to an under-controlled proximal AP (absence of CD55), AP-triggered C3-opsonization (covalent attachment of C3 activation fragments) of PNH erythrocytes has been observed (Höchsmann et al., 2012; Risitano et al., 2009). C3 opsonization of PNH erythrocytes is a finding seen only in eculizumab treated patients (it is assumed that, prior to therapeutic C5-inhibition, PNH erythrocytes fixing C3-opsonins would lyse immediately - being unavailable for detection). C3-opsonised PNH erythrocytes suffer from reduced circulation half-life due to clearance via extravascular hemolysis through phagocytosis by tissue macrophages in spleen and liver (Risitano et al., 2009). Whereas C3b and its degradation product iC3b are the primary phagocytic opsonins, the accumulation of the endstage opsonin C3dg on circulating PNH erythrocytes in patients receiving anti-C5 therapy may further facilitate clearance via interaction with complement receptor 3 on phagocytes, as a recent in vitro study shows (Lin et al., 2015). Importantly, the level of "extravascular hemolysis" correlates negatively with the hematologic response to eculizumab in a substantial fraction of patients (Hill et al., 2010; Risitano et al., 2011, 2009).

To prevent opsonization and extravascular hemolysis of PNH cells, novel complement inhibitors were developed that block at the point of C3 activation; some of these inhibitors were shown to efficiently inhibit both intravascular hemolysis and opsonization in preclinical PNH models (Risitano et al., 2014, 2012; Schmidt et al., 2013). With the potential safety profile in mind a new class of bioengineered inhibitors was constructed to specifically block only the PNH implicated AP of complement activation keeping the CP and LP intact for immunological surveillance (Supplemental Fig. 1C). Apart from regulating complement at the level of C3b (i.e. activated C3) by utilizing N-terminal domains of the natural regulator FH (Schmidt et al., 2007) these inhibitors possess various strategies to also bind efficiently to C3b's degradation products iC3b and C3dg, which occur frequently on sites of ongoing complement turnover on vulnerable host tissue (e.g. PNH cells that have at least a residual set of complement regulators) (Risitano et al., 2012; Schmidt et al., 2013). By targeting AP-specific inhibitors directly to vulnerable cells,

local AP-inhibitory capacity is markedly increased while systemic AP activity is less affected, thus apparently preventing increased susceptibility to infection (Atkinson et al., 2005). The in vitro and ex vivo studies presented here simultaneously compare the ability of four different AP-specific complement inhibitors to selectively target to C3-opsonized (C3b, iC3b, C3dg) surfaces and protect PNH erythrocytes. The regulators under investigation are: FH-CR2, an analog of the inhibitor TT30 (Fridkis-Hareli et al., 2011) and three inhibitors that underwent different levels of engineering and are denominated miniFH versions 1, 2 and 3 (Fig. 1A). In terms of regulating the AP (via C3b) FH-CR2 and miniFH proteins all utilize N-terminal portions of the natural regulator FH (Schmidt et al., 2007). In regards to targeting to C3-inactivation products, FH-CR2 relies on an N-terminal portion of the complement receptor 2 (CR2), while the miniFH approach utilizes C-terminal portions of FH. The simultaneous evaluation of all four C3-opsonin targeted inhibitors on various non-native and native surfaces and the comparison to eculizumab in a bactericidal serum killing assay identifies the targeting mechanisms with highest selectivity and efficiency for human erythrocytes in vitro, revealing important considerations for the engineering of complement targeted inhibitors.

Material and Methods

Human erythrocytes

PNH erythrocytes were collected by venipuncture from a PNH patient and used immediately in the AP lysis assay. This project was conducted under the ethic approval 279/09 granted from the ethics commission of Ulm University.

Proteins

FH was purchased from CompTech (Tyler, USA). MiniFH-1 (Schmidt et al., 2013), miniFH-2 (Hebecker et al., 2013) and miniFH-3 (Nichols et al., 2015) were obtained as published. FH-CR2 was generated by PCR amplification of CCP1-4 of CR2 from plasmid BillNeo-hCR2-E-E (gift from Prof Mike Holers, Denver, USA) and CCPs1-5 from an in-house vector containing the complete FH cDNA (NP_000177.2); the PCR products were cloned into pDR2EF1α-nMCS vector (Charreau et al., 1994; Yanagawa et al., 2003). The construct was sequence verified and transfected into Chinese Hamster Ovary (CHO) cells using jetPEI (Polyplus, VWR, Leicestershire, UK) following standard protocols. FH-CR2 was purified from transfected CHO cell culture supernatants using nickel-affinity chromatography (GE Healthcare) followed by an OX-24 affinity-column and size exclusion chromatography. Eculizumab was obtained from remnants left in the infusion pipe. The concentration of eculizumab was determined by measuring the OD280nm employing a standard extinction coefficient for IgG antibodies of 210,000 M^{-1} cm^{-1}.

ELISA

ELISAs measuring the complement inhibition activities of the various inhibitors were based on standard methods and performed as described (Schmidt et al., 2013). In brief, 50 µl of a LPS solution (50 µg/ml) from Salmonella typhimurium (Sigma-Aldrich) were coated on 96-well plates in PBS (pH 7.4) for 2 h at RT or overnight at 4°C followed by washing (PBST: PBS containing 0.05% Tween 20) and blocking (1% BSA in PBS). In a 96-well plate analytes in PBS (30 µl) were mixed with 30 µl of 50% serum containing 10 mM MgEGTA to block CP/LP activation. The final serum content was 25%. The mix was incubated

for 1.5 h at 37 °C prior to washing (2xPBST) and detection with exposure to goat anti-human C3 (HRP conjugate from MP Biomedicals) at a 1:1000 dilution in 1% BSA/PBS. Further washing (3xPBST) was followed by detection with a solution of ABTS (0.5 mg/ml; Roche) and 0.03% H_2O_2 in 0.1 M sodium citrate buffer at pH 4.3. Absorbance was read at 405 nm. EDTA at a final concentration of 5 mM and PBS were used as negative and positive controls, respectively.

PNH hemolysis assay

Patient blood was collected in EDTA. Erythrocytes were washed with PBS. ABO-matched serum was obtained from healthy volunteers (ethic approval 155/12, Ulm University) or German Red Cross BloodTransfusion Service Baden-Württemberg-Hessen and shock-frozen and stored at -80 ºC prior to use.
For each assay, 2 µl of washed and packed PNH erythrocytes were incubated with a mix of 8 µl of inhibitors or controls in PBS and 30 µl of HCl-acidified serum (pH 6.6–6.9) with addition of $MgCl_2$ to 1.5 mM. The hematocrit approximated 8%, the final serum content was 75%. Lowering the pH triggers brisk activation of the AP (Ezzell et al., 1991). Samples were incubated for 24 h at 37°C. Hemolysis of PNH II and III erythrocytes was measured by flow cytometry with an phycoerythrin-coupled anti-CD59 antibody (Clone OV9A2; e-Bioscience) similarly as described before (Höchsmann et al., 2011; Schmidt et al., 2013). The level of hemolysis of PNH II and III erythrocytes at the different inhibitor concentration points was normalized to the lysis of PNH II and III erythrocytes observed in acidified serum alone (i.e. in absence of any spiked inhibitors or controls).

Rabbit erythrocyte hemolysis assay

30 µl of a serum/MgEGTA mix (with serum being derived from three healthy donors) were mixed with 4 µl of inhibitors or controls in PBS and 6 µl of a rabbit erythrocyte (CompTech or TCS) suspension in PBS/Mg-EGTA. The final serum content was 75%. This mixture was incubated for 30 min at 37 °C. Reactions were stopped with 120 µl ice-cold PBS/EDTA (5 mM). Hemolysis was determined by reading the OD405 of 100µl supernatant.

Bacterial killing assays

XL1-Blue (Stratagene) E. coli containing the pPICZαB plasmid, which confers resistance to Zeocin, were grown in LB-medium until an OD600 of 0.6 was reached, were washed in PBS and then incubated for 1 h in NHS spiked with PBS (as control) or complement inhibitors dissolved in PBS at the indicated final concentration. Colony forming units were evaluated by colony counting after plating 10 to 10000-fold serial dilutions on selective LB (low salt) medium containing Zeocin at a final concentration of 25 µg/ml.

Blood polymorphism genotyping and erythroid CR1 levels

CR1 levels on erythrocytes vary between individuals. In Caucasians a restriction fragment length polymorphism in an intron of the CR1 gene has been identified to correlates with the number of CR1 molecules on erythrocytes (Wilson et al., 1986). Subsequently, a single nucleotide polymorphism (SNP) within exon 22 (A3650G) in the CR1 gene was linked to high CR1 expression (H, high allele) or low CR1 expression (L, low allele) (Xiang et al., 1999). Homozygous HH individuals have higher erythrocyte surface levels of CR1 (>1000), homozygous LL individuals have <200 molecules per erythrocyte, and HL individuals having intermediate CR1 surface levels (a comprehensive table that lists CR1 SNPs is found in

(Schmidt et al., 2015)). To test the CR1 phenotype of the PNH erythrocytes 200 µl of whole blood from the PNH patient or a healthy control were used to extract DNA using the QIAamp DNA Blood Mini Kit (Qiagen). Samples were genotyped for the SNP (A/G) in the CR1 gene at nucleotide 3650 in exon 22, which was analysed by restriction enzyme digest by RsaI, as described in (Tham et al., 2010; Xiang et al., 1999). In brief, a 682 base pair spanning sequence within exon 22 was amplified via PCR using the cloned Pfu® DNA Polymerase (Agilent Technologies) according to the suppliers protocol and the forward primer 5'-ttcacattggatagccagagc-3' and the reverse primer 5'-ccagaggttaatctccctgga-3'. The restriction digest with

RsaI (NewEnglandBiolabs) was performed according to the supplier's specifications and analysed by agarose gel electrophoresis using SYBR® safe DNA stain (Thermo fisher scientific) for visualisation of the DNA bands. The A3650 SNP corresponds to the HH phenotype and yields restriction fragments of 520 and 162 base pair length while the G3650 SNP produces 458, 162, 62 base pair fragments. Erythrocytes from both donors were also submitted to analysis by flow cytometry to analyse the surface expression of CR1. Cells were stained with a mouse anti-human CD35 antibody (clone E11; AbD Serotec) labelled with R-phycoerythrin for 30 min at room temperature in the dark or an isotype control prior to analysis by a FACSVerse™ flow cytometer (BD Biosciences).

Results

Due to unavailability of the clinically developed TT30 we expressed an analog FH-CR2 fusion protein, which shares 99.6% sequence identity with TT30 (Figure 1A). All proteins enrolled in this study were analyzed by SDS-PAGE under reducing and non-reducing conditions demonstrating comparable high quality of the preparations (Figure 1B).

Inhibition of AP activation induced by LPS

Using LPS to induce specifically activation of the AP, we evaluated the potency of FH and the C3-opsonin targeted inhibitors to block AP activity. LPS coated to micro-titer plates was exposed to serum spiked (separately) with various amounts of either the natural regulator FH or one of the engineered inhibitors. Of note, serum already contains FH (~300 to 500 µg/ml (Hakobyan et al., 2008; Sofat et al., 2013) equating to ~2-3 µM), so the effective FH concentration in this assay, with a final serum proportion of 25%, is about 0.6 µM higher than the amount of externally added FH. Addition of purified FH to a final concentration of 2 µM completely abolished AP-activation by LPS, which was measured by detecting (or failing to detect) the C3-activation products C3b and iC3b (Figure 2A). The estimated IC50 of added FH is 173 nM (Table I). With an apparent IC50 of 14 nM, FH-CR2 exhibited the highest potency for controlling AP-mediated C3b-deposition followed by miniFH-3, miniFH-1 and miniFH-2 (estimated IC50 of 23 nM, 33 nM and 71 nM, respectively). Thus FH-CR2, miniFH-3, miniFH-1 and miniFH-2 are about 12-, 8-, 5- and 2-fold more active than FH in inhibiting LPS-induced complement activation. The inhibitory potential of FH and miniFH-1 observed here compares well to the observations in a previous study in the same LPS-induced AP activation assay (Schmidt et al., 2013). With its apparent IC50 of 14 nM, FH-CR2 showed higher absolute inhibitory activity in blocking LPS-driven AP-activation than was originally reported for TT30 (40 nM) (Fridkis-Hareli et al., 2011), when TT30 has been assayed in the Wieslab complement system AP ELISA assay. This difference in absolute numbers most likely originates from the different serum concentration and detection method used in the Wieslab assay. This observation underlines the importance of comparing different complement inhibitors simultaneously in the

same assay in order to establish a conclusive ranking of inhibitory potency.

MiniFH is most active in protecting PNH cells from complement AP-mediated lysis

To probe the complement inhibitory potency of the C3-opsonin targeted inhibitors in a more physiological setting, we tested the inhibitor set in a clinically relevant assay of PNH erythrocyte lysis in acidified serum (Risitano et al., 2012; Schmidt et al., 2013) with a final serum content of 75%. Acidification of serum results in brisk activation (enhanced tick-over activation of C3) of the AP simulating a complement activation trigger (Ezzell et al., 1991). Similarly to the LPS assay, here the acidified serum was spiked with the C3-opsonin targeted inhibitors prior to incubation with patient-derived PNH erythrocytes. Blood SNP genotyping revealed that the PNH erythrocytes were of the heterozygous (high/low) genotype for the CR1 expression number polymorphism, which represents the most commonly observed CR1 phenotype, and thus areexpected to have an intermediate number of CR1 molecules (200-1000) on their surface. A direct comparison of CR1 surface expression by flow cytometry between the erythrocytes of a healthy donor with a heterozygous high/low CR1 expression phenotype and the PNH patient revealed a similar CR1 expression pattern, thereby confirming that the PNH cells supplied for this assay exhibit a common CR1 phenotype (Supplemental Figure 2).

In the PNH erythrocyte lysis assay miniFH-1 exhibited the highest inhibitory potency with an estimated IC50 of 78 nM, followed by miniFH-3 and miniFH-2 with IC50 values of 112 nM and 120 nM, respectively (Figure 2B, Table I). FH-CR2 showed an estimated IC50 of 385 nM and was substantially less active than any of the miniFH versions. However, FH-CR2 is still about twice as active as externally added FH with an estimated IC50 of 887 nM. MiniFH-1 and TT30 have already been evaluated in the lysis assay of PNH cells in acidified serum and the values obtained here for miniFH-1 and FH-CR2 (from one PNH patient) fit excellently with the previously published activities when these two proteins have been tested on PNH erythrocytes from two or 20 different PNH patients (respectively) (Risitano et al., 2012; Schmidt et al., 2013). The good agreement validates the PNH lysis assay employed here and importantly confirms that FH-CR2, which is nearly identical to TT30, shares the same functional activity in protecting PNH erythrocytes. In analogy to the inhibition of LPS-induced AP activation, any targeting of the N terminal FH regulatory domains to C3-opsonins (as seen in the engineered inhibitors) potentiates the regulatory efficiency of FH in protecting patient-derived PNH cells. Of note, the relative complement-inhibitory ranking observed for PNH cells differs markedly from the ranking observed for LPS-induced complement activation. In protecting PNH cells from AP mediated lysis, miniFH-1 is at least 10-fold more active than FH, followed by miniFH versions 2 and 3, which exhibit about an 8-fold activity gain over FH, with FH-CR2 being at least twice as active as FH. Thus, the miniFH-1 targeting approach is significantly more efficient (~0.2 µM) in the protection of host erythrocytes (full inhibition of lysis, Fig 2B) than the FH-CR2 approach (~0.8 µM).

Equal activity of miniFH and FH-CR2 in protecting rabbit erythrocytes from complement AP mediated

lysis

Next we set out to test if the increased efficiency of miniFH analogs over FH-CR2 derives from a general higher complement regulatory activity or instead from a higher selectivity for host erythrocytes. We therefore tested the AP regulatory activities of miniFH-1 and FH-CR2 on

foreign rabbit erythrocytes, which are well known activators of the AP. In this established standard assay (here with final serum content of 75%) miniFH-1 and FH-CR2 exhibited similar activity, requiring ~1 µM to block lysis (Figure 3, Table I). Since the complement regulatory functions for both inhibitors is mediated by the N-terminal portion of FH, and both are targeted to C3-degradation products (though different mechanisms) the increased efficiency of miniFH proteins on human PNH cells appears to be due to the increased selectivity for host surface polyanions, which are recognized by the C-terminal FH domain 20, rather than an intrinsic increase in AP regulatory potency.

C3-opsonin targeted inhibitors with AP specificity maintain bactericidal serum activity contrary

to eculizumab

Extending this idea of specificity for vulnerable host cell surfaces, we evaluated the effect that the AP specific, C3-opsonin targeted inhibitors exert on complement mediated bactericidal serum activity. Therefore we compared the impact on serum killing of E. coli cells (final serum content of 75%) between untargeted inhibition of the TP by eculizumab with the more selective inhibition by miniFH-1 and FH-CR2 (Figure 4). Under a final concentration of 0.3 µM eculizumab in this assay, which closely corresponds to the C5 plasma concentration, bacterial survival was comparable to heat-inactivated serum. The final eculizumab concentration of 0.3 µM in this assay may be compared to therapeutic serum levels of the drug typically measured in patients of 0.2-1.0 µM (0.035 – 150 µg/ml) (Hillmen et al., 2004). Contrary to eculizumab, both targeted inhibitors showed substantially smaller effects on serum bactericidal activity with a similar dose-response behavior in this assay. Although not reaching statistical significance, differences between the CFU values in the miniFH-1 and FH-CR2 groups were observed, particularly when taking the effective concentration for the protection of PNH cells into consideration. A final miniFH concentration of 0.2 µM, which was fully protective in the PNH assay (Figure 2B), barely interfered with bactericidal serum activity. In contrast, an FH-CR2 concentration of 0.8 µM, which was required to fully protect PNH erythrocytes, yielded 100-fold more surviving bacteria than miniFH-1 at its efficient concentration, which emphasizes the very high regulatory selectivity of the miniFH approach for host polyanion-bearing surfaces.

Discussion

The therapeutic utilization of FH's regulatory function can be an attractive strategy in reestablishing the delicate balance between complement activation and regulation in clinical conditions that are primarily AP-mediated (Holers et al., 2013). Although the clinical experience with long-term therapeutic inhibition of complement is still limited, selectively inhibiting AP activation on self-cells is expected to better preserve the immune surveillance functions of the complement cascade. Novel inhibitors that maintain the selectivity of FH but are more potent regulators hold great promise for further preclinical and clinical development.

All C3-opsonin targeted inhibitors are more active than FH

Combining of the N-terminal regulatory domains of FH (CCPs 1-4 or 1-5) with a portion that targets the C3b-inactivation products iC3b or C3dg, as seen in all of the C3-opsonin targeted inhibitors, clearly potentiates the inhibitory function towards the AP when compared to FH

(Figure 2). It is remarkable that this effect can either be achieved by CR2 domains 1-4, which bind to the thioester domain (TED) in iC3b and C3dg with a KD of 0.5 μM (van den Elsen and Isenman, 2011) or by the C-terminal domains 19-20 in FH, which appear to be cryptic in fulllength FH (Oppermann et al., 2006; Schmidt et al., 2013), but when uncoupled from FH bind to iC3b and C3dg with an affinity of ~5 μM (Morgan et al., 2011; Schmidt et al., 2013, 2008). Recently, the concept of a cryptic C-terminus was also confirmed experimentally (Herbert et al., 2015). Hence, different means of targeting complement regulators to sites of ongoing complement turnover (presence of iC3b and C3dg opsonins) appears to generally boost regulatory capacity, which may be due to the local increase of inhibitor concentration on those sites. Remarkably, CR2 domains 1-4 exhibit a 10-fold higher affinity for the C3-inactivation products iC3b/C3dg than FH domains 19-20 do (Morgan et al., 2011; Schmidt et al., 2008; van den Elsen and Isenman, 2011), but this higher affinity for these late-stage opsonins did not translate into higher AP inhibitory activity of FH-CR2 on foreign surfaces like rabbit erythrocyte (Figure 3). It can be anticipated that due to faster iC3b/C3dg dissociation rates, which are generally associated with a higher KD, miniFH molecules transit faster to surface patches that experience a fresh insult of C3b-deposition than FH-CR2 may do, thus compensating for its lower affinity to those sites of complement turnover.

Molecular design of engineered inhibitors correlates with specificity for self-surfaces

The FH-CR2 fusion protein approach (targeting of iC3b/C3dg via CR2; reviewed in (Holers et al., 2013)) showed the highest inhibitory activity towards AP activation induced by LPS (Figure 2A) and is at least tenfold more active than FH in this assay. In regards to preventing lysis of PNH cells the miniFH approach of targeting complement regulation to iC3b/C3dg-sites (via an isolated FH C-terminus) was found to be most active (Figure 2B). The activity gain of FH-CR2 over FH in the PNH lysis assay is only twofold when compared to the tenfold higher activity in the LPS assay. Therefore, on the human PNH erythrocyte surfaces, the FH protection mechanism appears to have gained advantageous features in comparison to the activity ranking of FH-CR2 and FH in the LPS-based assay. A probable explanation is that FH contains recognition sites for host specific polyanionic markers that are absent in FH-CR2. The reason for the higher activity of the miniFH proteins over FH-CR2, on host erythrocytes, likely also originates from the host-cell recognition properties of FH domains 19-20 (Blaum et al., 2015; Ferreira et al., 2006; Ferreira and Pangburn, 2007; Meri and Pangburn, 1990; Oppermann et al., 2006; Schmidt et al., 2013). Apart from binding to the thioester domain in C3b, iC3b and C3dg, FH domains 19-20 also adhere to host specific sialic acid molecules on human tissue, which increases the avidity for C3 opsonins present on host surfaces. In comparison to FH the miniFH versions benefit from higher availability of the FH domains 19-20, which enables targeting to C3b inactivation products; in full-length FH these recognition domains reside in a cryptic conformation. The observed activity gain of miniFH-1 over the other miniFH versions most likely results from the optimized peptide linker (Schmidt et al., 2013), which allows miniFH-1 to bind simultaneously and optimally with all of its domains (FH domains 1-4 and 19-20) to the C3-activation product C3b and the host polyanion cell surface markers. The miniFH-2 molecule is a direct fusion of the FH domains 1-4 with domains 19-20, whereas miniFH-3 extents these key functional parts of FH by FH domain 5 and 18. As a result miniFH versions -2 and -3 are probably unable to bind simultaneously to one C3b molecule and thus are predicted to have a different avidity behavior for C3b covered surfaces than miniFH-1 has. Nonetheless, all miniFH versions benefit from the avidity that was introduced by directly connecting the N-terminal FH domains with domains 19-

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20 thus releasing these domains from the natural cryptic C-terminal orientation in FH. Highselectivity for host tissue specific polyanions is generally expected to result in a superior safety profile since pathogens are commonly void of these surface markers. Indeed, miniFH effectively inhibits PNH cell lysis without significantly modulating serum bactericidal properties. Contrary to this, complete blockage of the TP by eculizumab abolished bactericidal serum activity, as anticipated. Of note though, our bactericidal serum assay solely relies on bacterial killing through the terminal MAC, which cannot form under eculizumab, whereas in vivo C3-opsonisation of bacteria (still possible under eculizumab) constitutes another anti-bacterial action of complement and enhances bacterial clearance by phagocytes. Nonetheless, in the light of the Neisseria infections observed under eculizumab, our finding of miniFH's selective AP complement inhibition on PNH erythrocytes is very relevant for future preclinical development.

C3-opsonin targeting and clinical outlook

Our in vitro comparison shows that, contrary to eculizumab, the C3-opsonin targeted, APspecific inhibitors act more selectively on host cells and largely maintain bactericidal serum activity. The C3-opsonin targeting approach introduces a bias for protecting host surfaces since the objectives of the targeting, i.e. the late stage C3-opsonins iC3b and C3dg (and specific polyanions), more readily occur on host surfaces which are (better) equipped with the regulator machinery to process C3b to late stage opsonins. Another reason for the marginal interference with bactericidal serum activity of miniFH and FH-CR2 may lie in their AP selectivity. Antibodydependent and independent CP activation on E. coli has been demonstrated (Wang et al., 2012) providing a rationale why AP-specific inhibition interferes to a much lesser extent with the bactericidal serum activity. MiniFH-1 and FH-CR2 show similar dose-dependent interference with lytic serum activities. However, when the inhibitor concentrations that are needed to fully protect the PNH cells from lysis are factored in, a notable difference between the two inhibitors is apparent. At the effective concentration in the PNH assay of 0.2 µM, miniFH-1 maintains a higher (but not significantly different) bactericidal activity in serum than observed for FH-CR2 at 0.8 µM. The direct comparison of the different targeting approaches employed in FH-CR2 or miniFH shows that the latter approach overall is more selective for PNH erythrocytes. This implies that in a potential future clinical application miniFH may mitigate the current safety concerns in respect to eculizumab predisposing to Neisseria infections. In addition, targeted inhibition may favorably influence dose requirements. While application of FH from external sources (Brandstätter et al., 2012; Fakhouri et al., 2010; Schmidt et al., 2011) to patients should restore complement regulation, it is evident that a significant quantity of the natural regulator would have to be administered to achieve sufficient control over an under-regulated AP. The lysis assay of PNH cells in acidified serum herein was performed at a serum concentration of 75%, thus resulting in a final FH concentration in the assay of about 1.5- 2.2 µM. Addition of at least the same amount of external FH (to achieve a final concentration of externally added FH at 2 µM) (Figure 2B) was necessary to protect the majority of PNH cells from lysis. Our in vitro comparison of C3-opsonin targeted inhibitors, demonstrates that only one tenth (= 0.2 µM) of that concentration of externally added FH would have to be achieved if PNH patients were to be treated with the most active fusion protein in the PNH assay, miniFH-1. The C3-opsonin targetedinhibitors TT30 and miniFH-1 have already demonstrated that those biopharmaceutical candidates efficiently prevent C3 opsonization of PNH erythrocytes, which is an effect observed under eculizumab therapy and appears to correlate negatively with the hematologic response to eculizumab in a substantial subgroup of PNH patients (Risitano et al., 2011, 2009). In the direct comparison in this clinically relevant ex vivo models, miniFH-1 is at least 5-fold more active than

FH-CR2 in protecting PNH cells from lysis, which correlates well with the previously estimated 10-fold increase in activity when two separate studies using the two inhibitors were compared (Risitano et al., 2012; Schmidt et al., 2013). This clear and substantial activity gain of the miniFH versions (and especially miniFH-1) over TT30 in protecting PNH cells should warrant future clinical investigation of this potent class of FH-based and engineered proteins after successful completion of ongoing preclinical animal studies. In addition to the subgroup of PNH patients that suffer from extravascular hemolysis second to C3-opsonization (Hill et al., 2010; Risitano et al., 2009), patients with genetic variants in C5 have been reported that also suffer from a poor response to eculizumab (Nishimura et al., 2014). Our findings suggest that the C3-opsonin targeted inhibitors may introduce significant clinical benefit to PNH patients, particularly to those who currently suffer from suboptimal or poor response to eculizumab.

Conflict-of-interest

C.Q.S, D.R. and J.D.L. are inventors of a patent application that describes the use of miniFH for therapeutic applications. J.D.L. is the founder of Amyndas Biopharmaceuticals, which develops complement therapeutics. B.H., C.Q.S. and H.S. received honoraria for speaking at symposia organized by Alexion Pharmaceuticals. H.S. and B.H. served on an advisory committee for and received research funding from Alexion Pharmaceuticals. E.M.N is now working in the C3 DPU, Immunoinflammation TA of GSK. The other authors have disclosed no conflicts of interest.

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Table 1. Summary of estimated IC$_{50}$ values for complement AP inhibition

SPIKED INHIBITORS	IC$_{50}$ values for different activator surfaces[a]		
	LPS (ELISA plate)	PNH erythrocytes	Rabbit erythrocytes
FH	173 *(R^2=0.96, CI=130 to 230)*	887 *(R^2=0.91, CI=687 to 1146)*	-
FH-CR2	14 *(R^2=0.92, CI=10 to 22)*	385 *(R^2=0.91, CI=314 to 473)*	650 *R$_2$=0.91 CI=441 to 959*
miniFH-1	33 *(R^2=0.98, CI= 28 to 39)*	78 *(R^2=0.90, CI=62 to 98)*	567 *R$_2$=0.94 CI=457 to 703*
miniFH-2	71 *(R^2=0.92, CI=52 to 98)*	120 *(R^2=0.95, CI=101 to 142)*	-
miniFH-3	23 *(R^2=0.97, CI=19 to 29)*	112 *(R^2=0.83, CI=85 to 142)*	-

[a] Values are in nM.

Estimated IC$_{50}$ values were obtained by fitting the obtained curves to a sigmoidal dose-response using GraphPad Prism software; the goodness of the fit is indicated by R^2 followed by the 95% confidence interval (CI)
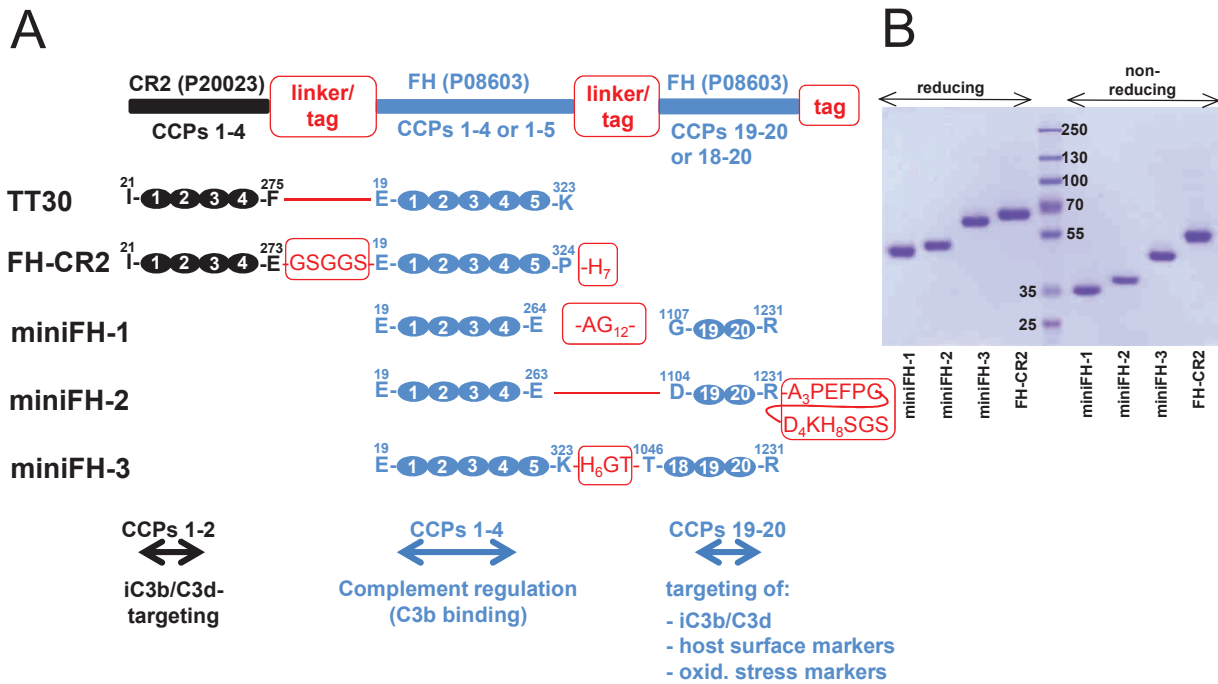
Figure 1. SCHMIDT *et al* 2015

Figure 1. C3-opsonin targeted complement inhibitors: design and production. (A) Schematic representation of the molecular composition of inhibitors enrolled in this study. Numbering based on encoded protein sequences including signal sequence according to UniProt (accession number in parenthesis). Each oval represents a CCP domain (domain numbers are indicated). Native N- and C-terminal residues are denoted in one letter code; non-native linker sequences and purification tags are indicated by box. Key functional properties of CCP domains are highlighted at the bottom. In terms of regulating the AP (via C3b) FH-CR2 and miniFH proteins all utilize N-terminal portions of the natural regulator Factor H (FH). In regards to targeting to C3-inactivation products, FH-CR2 relies on an N-terminal portion of the complement receptor 2 (CR2), while the miniFH approach utilizes C-terminal portions of FH, which are cryptic and not fully available in natural FH. MiniFH-1 is polymorphic for isoleucine at position 62. **(B)** SDS-PAGE gel analysis of C3-targeted inhibitors produced for this study under reducing and non-reducing conditions (2µg each lane).
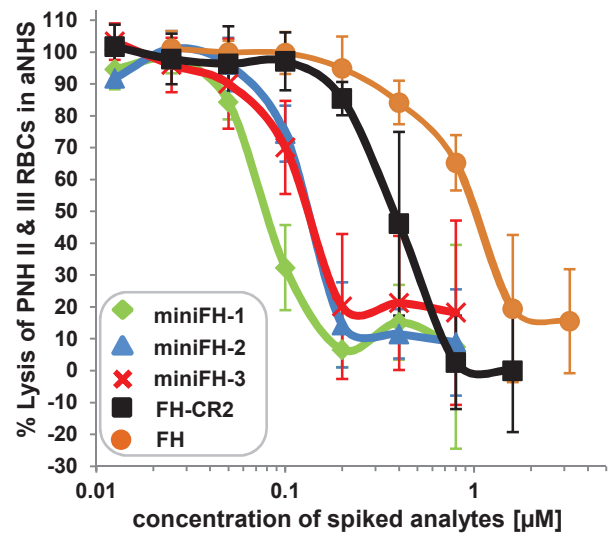
Figure 2. SCHMIDT *et al* 2015
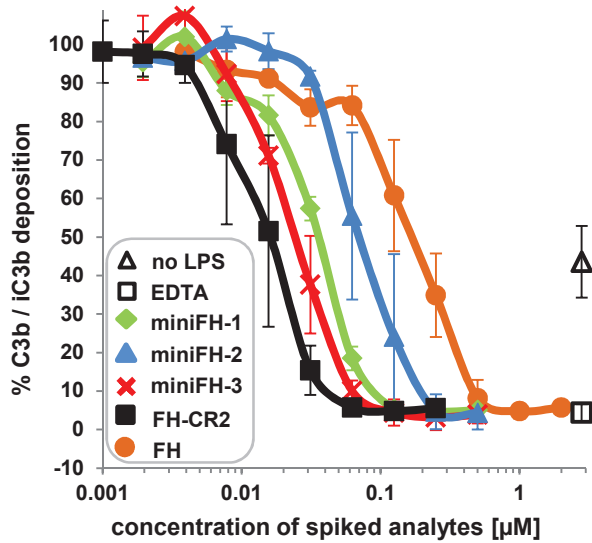
Figure 2. Regulatory activity of C3-opsonin targeted complement inhibitors on LPS and PNH erythrocytes (A) Inhibition of LPS-driven AP-activation. Serum spiked with C3-opsonin targeted inhibitors or FH was exposed to LPS coated on an ELISA plate (25% final serum conc.). Deposition of C3b/iC3b was detected as a measure for complement activation (Average of 2 independent assays with SD is shown). **(B)** Protection of PNH cells from complement AP mediated lysis. PNH erythrocytes were incubated for 24h in acidified human serum spiked with complement inhibitors (75% final serum conc.). The lysis of PNH II & III erythrocytes was measured by flow cytometry and normalized to lysis of PNH II & III erythrocytes observed in acidified serum in absence of spiked inhibitors (Average of 4 independent assays with SD).

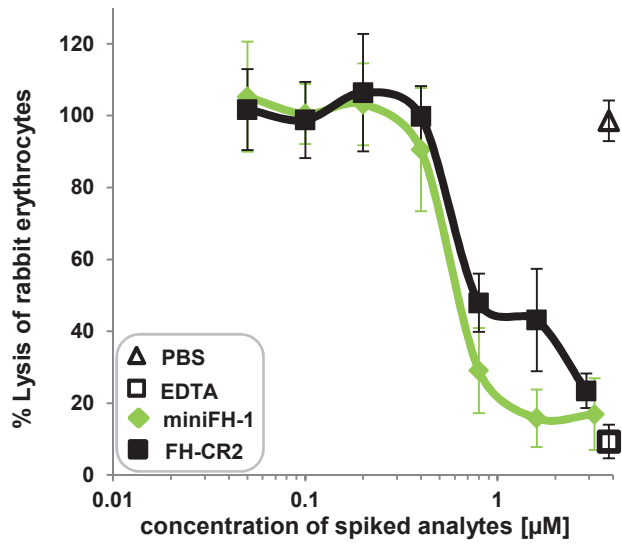# Figure 3. SCHMIDT *et al* 2015

Figure 3. Protection of rabbit erythrocytes from complement AP mediated lysis. Rabbit erythrocytes were incubated for 30 min in serum (from healthy donors) spiked with complement inhibitors (75% final serum conc.). The lysis of rabbit erythrocytes was measured by hemoglobin release and normalized to lysis observed in water (Average of 3 or 2 independent assays with SD is shown for miniFH-1 and FH-CR2, respectively).
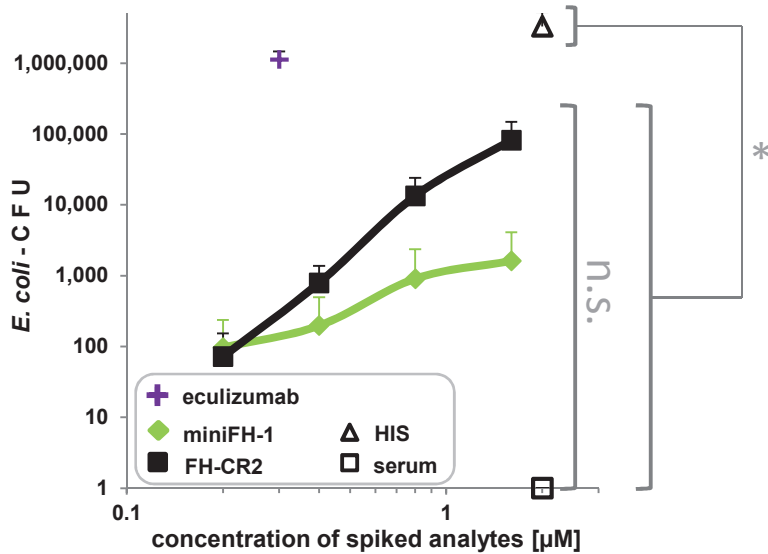
Figure 4. SCHMIDT *et al* 2015

Figure 4. Complement mediated killing of *E. coli* in NHS. **(A)** *E. coli* cells containing a selective marker were incubated for 1h in NHS spiked with complement inhibitors or PBS. Colony forming units (CFU) were evaluated by colony counting after plating the reactions on selective medium. Eculizumab was used at 0.3 µM, which nearly corresponds to the known human C5 plasma conc. of 0.38µM (HIS: heat inactivated serum; average of 3 assays with SD is shown, except for eculizumab which was assayed only twice). Selected points were analyzed for significant changes in CFU (non-parametric one-way analysis of variance with a significance level of 0.05; GraphPad Prism). The number of CFU of the serum control and the two selected concertation points of the targeted inhibitors that fully protected PNH erythrocyte hemolysis (0.2 µM miniFH-1; 0.8 µM FH-CR2) were significantly smaller (*) than the number of CFU for heat-inactivated serum. No statistical significant (n.s.) change was noted between the serum control and the concentration points of miniFH-1 and FH-CR2.

Supplemental Figure 1 SCHMIDT *et al* 2015

A

B

C
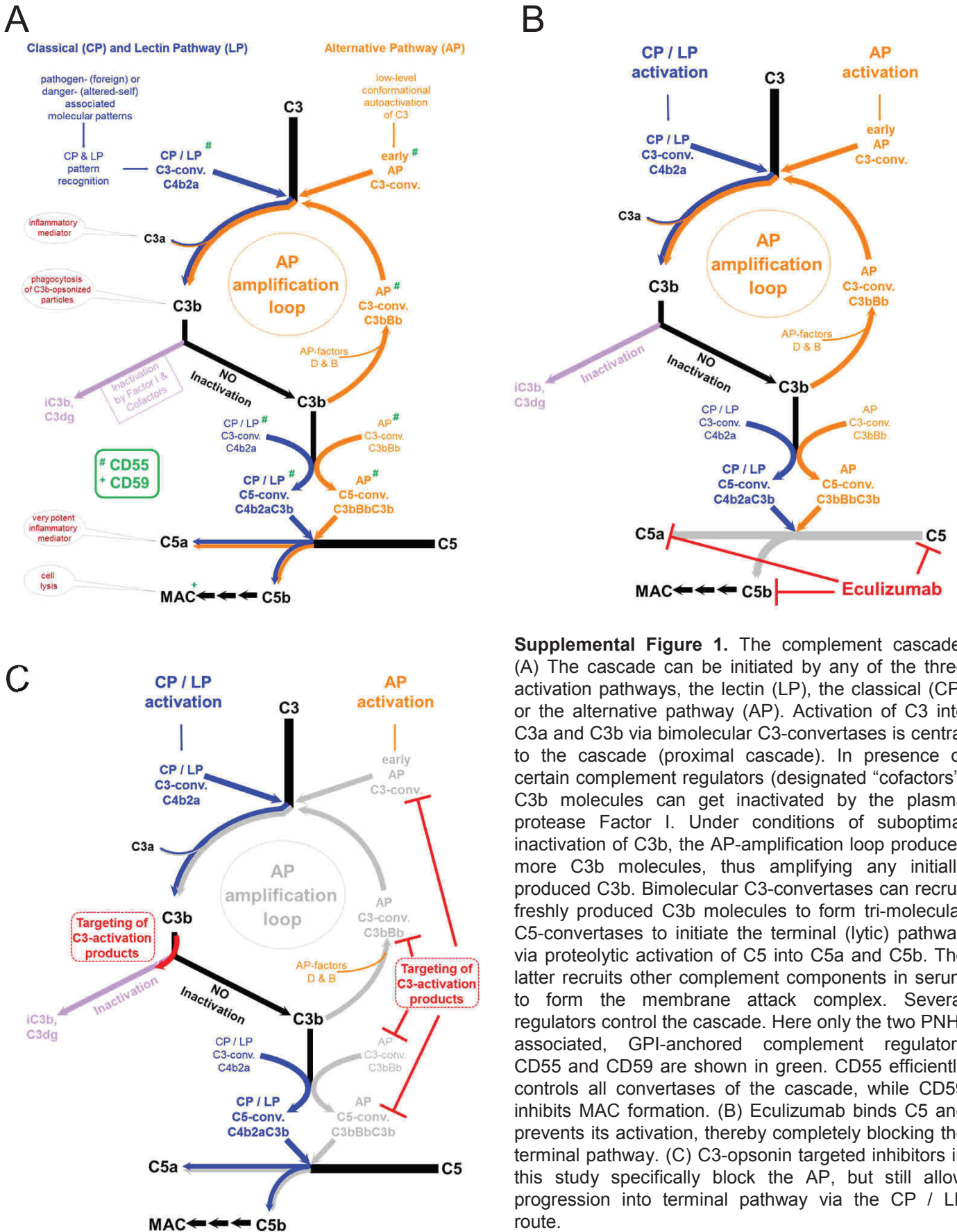
Supplemental Figure 1. The complement cascade. (A) The cascade can be initiated by any of the three activation pathways, the lectin (LP), the classical (CP) or the alternative pathway (AP). Activation of C3 into C3a and C3b via bimolecular C3-convertases is central to the cascade (proximal cascade). In presence of certain complement regulators (designated "cofactors") C3b molecules can get inactivated by the plasma protease Factor I. Under conditions of suboptimal inactivation of C3b, the AP-amplification loop produces more C3b molecules, thus amplifying any initially produced C3b. Bimolecular C3-convertases can recruit freshly produced C3b molecules to form tri-molecular C5-convertases to initiate the terminal (lytic) pathway via proteolytic activation of C5 into C5a and C5b. The latter recruits other complement components in serum to form the membrane attack complex. Several regulators control the cascade. Here only the two PNH-associated, GPI-anchored complement regulators CD55 and CD59 are shown in green. CD55 efficiently controls all convertases of the cascade, while CD59 inhibits MAC formation. (B) Eculizumab binds C5 and prevents its activation, thereby completely blocking the terminal pathway. (C) C3-opsonin targeted inhibitors in this study specifically block the AP, but still allow progression into terminal pathway via the CP / LP route.

Supplemental Figure 2 SCHMIDT *et al* 2015
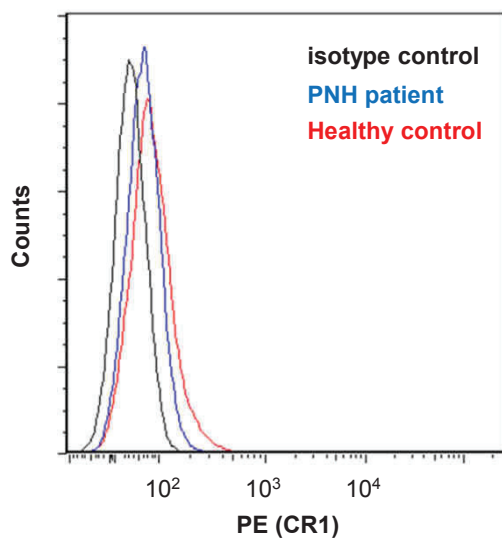
Supplemental Figure 2. Determination of CR1 (CD35) on erythrocyte surfaces. Erythrocytes of a healthy control subject and the PNH patient were stained with an anti-human CD35 antibody (clone E11; AbD Serotec) marked with RPE dyes. Both subjects were of heterozygous phenotype (high/low) in regards to the CR1 expression polymorphism.