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Characterization of novel anti-factor H antibodies reveals the role of factor H in protecting human erythrocytes from complement

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Abstract

Complement factor H (FH) is a crucial inhibitor of the alternative pathway of complement activation on human cell surfaces. FH has distinct domains which are involved in the interaction with human cells and complement C3 activation fragments. We generated and characterized twenty novel anti-FH monoclonal antibodies (mAbs) to further study the function of FH on the erythrocyte cell surface. With the use of recombinant FH fragments, we mapped the epitope location of the anti-FH mAbs. The mAbs could be divided in nine groups, each binding to a different epitope spread along the different functional regions of FH. This allowed us to determine the contribution of each targeted domain to complement regulation by FH. We demonstrate that blocking either of the two binding regions of FH resulted in complement activation on both sheep and human erythrocytes, revealing that FH plays a pivotal role in preventing alternative pathway-mediated complement activation on healthy human erythrocytes.

Introduction

One of the most important regulators of the alternative pathway (AP) of the complement system is the plasma protein factor H (FH). This 155 kDa glycoprotein consists entirely of twenty complement control protein domains (CCP, also known as short consensus repeat or SCR domains) and is abundantly present in human plasma with a reported average concentration ranging from 233 μg/mL to 320 μg/mL[1–3]. FH is the main member of the FH protein family, which comprises FH, the splice variant of FH (FH-like 1, comprising domains 1-7 of FH), and six individually encoded FH-related proteins (FHR-1, FHR-2, FHR-3, FHR-4A, FHR-4B and FHR-5)[4]. The function of the FHR proteins is still poorly understood and currently they are hypothesized to act as antagonists of FH. FH specifically inhibits the AP via three modes of regulation. It competes with factor B (FB) for binding to the spontaneously formed C3(H_2O) or the enzymatically generated C3b, thereby preventing the formation of the AP C3-convertases C3(H_2O)Bb and C3bBb. FH accelerates the decay of formed AP C3-convertases, known as decay accelerating activity, and FH bound to C3b serves as a co-factor for factor I (FI), which cleaves C3b into inactive C3b (iC3b)[5]. Regulation of the AP by FH occurs both in fluid phase and on cell surfaces following C3b deposition. However, on cell surfaces, FH regulation requires not only C3b binding but also interactions with polyanion residues like sialic acid, heparan sulfate and glycosaminoglycan chains. These residues are mainly present on human cells and thereby direct FH regulation towards human cells and allowing AP-mediated complement activation on pathogens.

Over the years the different activities of FH have been mapped to distinct regions comprising multiple domains. The first four domains of FH were identified to be necessary and sufficient for the regulatory activities of FH[6–8]. These domains include C3b binding activity sufficient to bind C3b in fluid phase[6,7]. However, experiments with FH constructs lacking specific domains at different sites of the FH molecule clearly revealed the requirement of additional binding sites for regulation by FH on surfaces. Two main regions have been identified to contain these surface-binding sites. One site is located at the C-terminus of FH and comprises domains 19 and 20[9]. These domains contain a second C3b binding region next to the C3b binding in domain 1-4 and are crucial for FH regulation on surfaces[10]. In addition, binding affinity for polyanions has also been reported for domain 20[11,12]. The second main surface binding region of FH comprises domains 6 to 8, with a crucial role for domain 7, and seems to be mainly involved in polyanion binding, although a low C3b-binding capacity has been reported as well[13–15]. Furthermore, while the FHR proteins lack domains similar to the regulatory domains 1-4, all FHR proteins have domains that are similar or even completely identical to the domains comprising the binding regions of FH.

While the domains of the two binding regions do not seem to play a role in fluid phase

regulation by FH, their importance in complement regulation on a cell surface have been indicated by the association of certain diseases with genetic variations in the exons of *CFH* encoding domains 6-8 and 19-20. For instance, the tyrosine-to-histidine substitution of amino acid position 402 (FH_{402H}) is located in domain 7 and is associated with one of the most common causes for blindness in the western world: age-related macular degeneration (AMD)[16–18]. FH_{402H} binds less well to the Bruch's membrane of human macular tissue resulting in decreased local complement regulation[19]. In contrast, mutations located in domain 20 are commonly associated with atypical hemolytic uremic syndrome (aHUS, reviewed in Rodriguez de Cordoba *et al.* 2014[20]). The distinct localization of FH variants in either of the two binding regions associated with different diseases, indicates surface specificity of the two surface binding regions. Clark *et al.* (2013) demonstrated that the two polyanion-binding sites in domains 7 and 20 may indeed confer such tissue-specificity to FH[19]. Depending on the surface, the two binding regions vary in their contribution to FH binding[21]. The binding of FH and recognition of different tissues through this "heparan sulfate code" has mainly been investigated *in vitro* on endothelial cells and *ex vivo* in tissues like the macula and renal glomeruli, whereas little is known on the binding characteristics of FH to erythrocytes.

Erythrocytes, like all human cells, express membrane-bound complement regulators on their membrane. Their dependence on FH-mediated complement regulation has been poorly investigated although there are some indications on the crucial role of FH in protecting erythrocytes. For instance, erythrocytes of paroxysmal nocturnal hemoglobinuria (PNH) patients, in which a sub-population of erythrocytes lack expression of the GPI-anchored complement regulators CD55 (DAF) and CD59, are dependent on FH for protection against complement-mediated lysis and their survival *in vivo*[22]. In addition, normal human erythrocytes exposed to serum of aHUS patients with decreased FH functionality undergo eryptosis due to complement activation, which in turn could be rescued by the addition of functional FH[23]. The protection of erythrocytes by FH could be partially inhibited by the addition of recombinant FH fragments consisting of domains 19 and 20, indicating that at least these two domains play a role in FH-mediated complement protection on the erythrocytes[22]. We set out to further investigate the role of the different binding domains of FH in protecting erythrocytes against complement. For this we used monoclonal antibodies (mAbs) that were generated in mice and of which the epitope locations were mapped using recombinant human FH fragments. Effects of the mAbs on FH functionality were tested in a hemolytic assay using sheep erythrocytes as a model for human erythrocytes, while C3b deposition as a result of FH inhibition with mAbs was assessed on human erythrocytes. Our results clearly show that FH-mediated protection of erythrocytes occurs via both domains 6-8 and domains 19-20 and that FH plays a crucial role in protecting erythrocytes from complement under normal conditions.

Materials and methods

Samples

All human-derived samples were obtained from anonymous, healthy volunteers after informed, written consent in accordance with Dutch regulations and this study was approved by the Sanquin Ethical Advisory Board in accordance with the Declaration of Helsinki. Serum was obtained by collecting blood, allowing it to clot for 1 hour at room temperature and collecting the supernatant after centrifugation at 3,000 rpm for 10 minutes. Serum samples were stored in small aliquots at -80°C until use to avoid repetitive freeze/thawing. To obtain human erythrocytes, peripheral blood was drawn from healthy volunteers in 10 mM EDTA tubes. Human erythrocytes were pelleted by centrifugation at 2,500 rpm for 10 minutes. After removing plasma and peripheral blood mononuclear cells, human erythrocytes were washed 3 times with PBS, re-suspended to a 50% cell suspension in saline-adenine-glucose-mannitol (SAGM) solution (Fresenius Kabi, Zeist, the Netherlands) and stored at 4°C for a maximum of one week. Normal healthy human serum pool comprises serum from 400 healthy donors.

Proteins and reagents

Human plasma-derived factor H (FH), factor I (FI), C3b, iC3b and C3d were purchased from Complement Technology (Tyler, TX, USA). Rat anti-mouse kappa (RM-19) monoclonal antibody (mAb), mouse anti-human C3.9 mAb, mouse anti-human C4.1 mAb, mouse anti-human IL-6.8 mAb (IgG1 isotype control) and mouse anti-human CRP mAb (IgG2a isotype control) were provided by Sanquin Reagents (Amsterdam, the Netherlands). Mouse mAb against human FHR-1 (Cat. #MAB4247), human FHR-2 (Cat. #MAB5484), human FHR-4 (Cat. #MAB5980) and human FHR-5 (Cat. #MAB3845) were purchased from R&D systems (Minneapolis, MN, USA). Polyclonal rabbit anti-human FHR-5 antibodies were purchased from Abnova (Cat. #H00081494, Taipei, Taiwan). Polyclonal rabbit anti-human FHR-3 antibodies were generated in house as part of another study[24]. High Performance ELISA buffer (HPE) was provided by Sanquin. Polyclonal rabbit anti-human FH was obtained from Sanquin, polyclonal goat anti-human FH was purchased from Quidel (Quidel Corporation, San Diego, CA, USA). Proteins were biotinylated according to the manufacturer's instructions using EZ-Link Sulfo-NHS-LC-Biotin, No-Weigh Format (Thermo Scientific, Waltham, MA, USA), when indicated.

Immunization and hybridoma generation

Anti-FH mAbs were generated by immunizing BALB/c mice via intraperitoneal injection with 25 μg human FH in montanide as adjuvant at four week intervals. Three days after the fourth booster immunization, spleen cells were fused with the mouse myeloma cell line SP2/0. The presence of FH specific antibodies in the supernatants of the hybridomas was tested by ELISA. In short, microtiterplates were coated with RM-19 mAb to capture mouse kappa

immunoglobulins. Specificity of the antibodies was determined by addition of biotinylated FH (FH-bt), diluted in HPE. The assay was developed with streptavidin conjugated with horse radish peroxidase (strep-HRP, Sanquin) and 100 µg/mL 3,5,3',5'-tetramethylbenzidine (TMB) in 0.1 M sodium acetate containing 0.003% (v/v) H_2O_2, pH 5.5. Substrate conversion was stopped by addition of 100 µL H_2SO_4. Absorbance was measured at 450 nm and corrected for the absorbance at 540 nm with a Synergy 2 Multi-Mode plate reader (BioTek Instruments, Winooski, VT, USA). Positive wells were made monoclonal by multiple limiting dilution steps. Isotypes of mAbs were determined by ELISA or with the use of the Mouse Mab Isotyping Kit (Hycult Biotech, Uden, the Netherlands).

Competition ELISA

Microtiterplates were coated with 100 µL of 2 µg/mL anti-FH mAbs, in 0.1 M carbonate buffer, pH 9.6, by incubating overnight (O/N) at room temperature. Binding of FH-bt in the presence or absence of anti-FH mAbs was determined as described previously.

Epitope mapping mAbs

Recombinant human FH fragments composed of multiple domains (domains 1-4, 1-7, 4-6, 6-8, 8-15, 12-13, 15-18, 15-19, 18-20 or 19-20) were produced as described before[15,25]. Anti-FH mAbs (5 µg) were mixed with 25 µg of RM-19 coupled to 1 mg CNBr-activated sepharose (GE Healthcare, Freiburg, Germany). The FH fragments were labelled with ^{125}I and 100 µL (40,000 c/min) was added to each sample followed by incubation O/N. The assay was performed in PBS with 0.1% (w/v) Tween-20 and 0.1% (w/v) BSA (PTB). Samples were washed 5 times with PBS with 0.1% (w/v) Tween-20 (PT) and counted for 30 seconds. Sepharose-bound radioactivity was measured and compared to total input (40,000 c/min, set to 100%).

Depletion of FH from human serum

FH was depleted from normal human serum using anti-FH.16. Serum was run over a 3 mL column containing anti-FH.16 coupled to CNBr-activated Sepharose 4B (GE Healthcare, 25 mg mAb per 1 gram sepharose coupled, according to the manufacturer's instructions). Flow through was collected and used for IP as described above. Depletion of FH was confirmed using an in house developed FH ELISA.

Immunoprecipitation

Immunoprecipitation (IP) was performed by incubating 200 µL FH-depleted serum with 500 µL of 5 mg/mL CNBr-activated sepharose (GE Healthcare) to which RM-19 was coupled (25 mg mAb per 1 gram sepharose coupled, according to the manufacturer's instructions), and 50 µL of 100 µg/mL mAb as indicated, diluted in PT supplemented with 0.1% (w/v) BSA and 10 mM EDTA. IP with polyclonal anti-FHR-3 was performed using ProtG sepharose (GE Healthcare). Following overnight incubation at 4°C, while rotating, the sepharose was washed

three times with 1 mL PT and two times with 1 mL PBS. Precipitated proteins were eluted by addition of 75 µL 1x NuPAGE Sample buffer solution (Invitrogen, Carlsbad, CA, USA) and incubation at 70°C for 10 minutes. After spinning down the sepharose, SDS-PAGE under non-reducing conditions was performed using Novex NuPAGE 10% Bis-Tris gels followed by Western Blot onto nitrocellulose membranes (Novex iBlot Gel Transfer kit, Invitrogen). Membranes were blocked with 1% (v/v) Western Blocking Reagent (WBR, Roche, Basel, Switzerland) in PBS for >30 minutes and incubated with 1 µg/mL biotinylated polyclonal rabbit anti-FHR-5, anti-FHR-2, polyclonal rabbit anti-FHR-3, anti-FHR-4 or polyclonal rabbit anti-FHR-5, respectively, in PBS with 0.5% (v/v) WBR, O/N. After washing three times with PT, membranes were incubated with 0.1% (v/v) Strep-HRP in PBS with 0.5% (v/v) WBR. After 1 hour, the membranes were washed three times with PT followed by two washes with PBS. Western Blots were developed with the Pierce ECL 2 Western Blotting substrate kit (Thermo Scientific) according to the manufacturer's instructions and analyzed using the ChemiDoc™ MP System and Image Lab Software, version 5.0 (BioRad, Hercules, USA).

FH co-factor activity assay

FH co-factor activity was determined by a fluid phase assay as described previously with some adjustments[26]. Fluid phase C3b degradation by FI was achieved by incubating 200 ng FH with 3 µg C3b and 90 ng FI, in a total reaction volume of 20 µL, at 37°C for 30 minutes. The reaction was stopped by adding 30 µL of stop buffer (12.5 µL 4x NuPAGE LDS Sample Buffer, 5 µL 10x NuPAGE Reducing Agent, both from Invitrogen, Life Technologies, Carlsbad, USA, and 12.5 µL H_2O) and heating the samples at 70°C for 10 minutes. To assess FH co-factor activity in the presence of anti-FH mAbs, FH was incubated with a four-fold excess of anti-FH mAbs (800 ng) in a total volume of 12.5 µL for 15 minutes at room temperature prior to the addition of FI and C3b. Degradation products of C3b were separated by SDS-PAGE under reducing conditions and visualized with PageBlue Protein Staining Solution (Thermo Scientific). FI-mediated degradation of C3b was determined by calculating the ratio between the intact C3b α'-chain at 114 kDa and the β-chain at 75 kDa using the BioRad ChemiDoc MS System and Image Lab Software, version 5.0 (BioRad).

ELISA for C3 and C4 activation

C3 and C4 activation in serum were measured by assessing the amount of the activation products C3b/bi/c (abbreviated further as C3b/c) and C4b/c, respectively. For the quantification of these activation products ELISA's were performed as described previously[27]. Briefly, anti-C3.9 mAb (recognizing a neo-epitope on activated C3) or anti-C4.1 (recognizing a neo-epitope on activated C4) were used as catching antibodies. Biotinylated polyclonal sheep anti-human-C3b/c or sheep anti-human-C4b/c was used for detection. Aged human serum, containing a known amount of C3b/c and C4b/c, was used as standard.

Hemolytic assay

FH functionality was measured with the use of a hemolytic assay as described previously with some adjustments[28,29]. Sheep erythrocytes were obtained from Håtunalab (Håtunaholm, Sweden). Sheep erythrocytes were diluted in veronal buffer (3 mM barbital, 1.8 mM sodium barbital, 145 mM NaCl, pH 7.4) supplemented with 5.8% (w/v) sucrose (VBS) to a final concentration of $2.1*10^8$ cells/mL. Normal human serum pool was diluted to 20% (v/v) in VB containing 0.05% (w/v) gelatin, 10 mM $MgCl_2$, 20 mM EGTA (VBG-AP) with the indicated anti-FH mAbs at appropriate concentrations. The assay was performed by mixing 50 µL of serum sample with 50 µL sheep erythrocyte suspension to reach a final concentration of 10% (v/v) pool serum with $1.05*10^8$ cells/mL in 5 mM $MgCl_2$, 10 mM EGTA followed by incubation at 37°C for 75 minutes while shaking at 200 rpm. The lysis was stopped by addition of 100 µL ice-cold VB containing 20 mM EDTA followed by centrifugation in a pre-chilled centrifuge (7°C) at 1,800 rpm for 2.5 minutes. The absorbance of the supernatants was measured at 412 nm on a Synergy 2 Microplate Reader (BioTek). The hemolysis of each sample was expressed as percentage compared to the 100% lysis control (sheep erythrocytes incubated in H_2O with 0.6% (w/v) Saponin). As negative control, sheep erythrocytes were incubated with serum diluted in VB supplemented with 10 mM EDTA to prevent complement activation.

Flow cytometry analysis of C3 deposition on human erythrocytes

Stored human erythrocytes were washed with PBS prior to use. Subsequently, cell suspensions of 0.16% human erythrocytes were incubated with ABO-matched normal human serum pre-incubated with the indicated mAbs and VB supplemented with 0.05% (w/v) gelatin, 10 mM $CaCl_2$ and 2 mM $MgCl_2$ (VBG++) at 37°C. After 1 hour, human erythrocytes were washed with FACS buffer (Millipore, Billerica, MA, USA). C3 deposition was stained with FITC-conjugated anti-C3.19 mAb (Sanquin). To control for possible classical pathway activation C4 deposition was stained with APC-conjugated anti-C4.10 mAb (Sanquin). After staining human erythrocytes were washed with FACS buffer and complement deposition was determined using a LSR Fortessa flow cytometer (BD Biosciences, Franklin Lakes, USA) and analyzed using FlowJo software, v1.0 (Treestar, Ashland, USA).

Statistical analysis

Analysis and statistical tests were performed using GraphPad Prism, version 6.04 (GraphPad Software, La Jolla, CA, USA).

Results

Anti-FH mAbs characteristics

We obtained 21 mAbs against human FH, numbered following the order of identification.

One mAb (anti-FH.01) appeared to be an IgA antibody and was not pursued any further. The remaining twenty mAbs were either of IgG1 or IgG2a isotype (**Table 1**). All antibodies were able to capture soluble plasma derived human FH. A competition ELISA was used to identify whether the mAbs competed for FH binding with each other (**Fig. 1a**). Competition would indicate close proximity of, or even shared, epitopes. We identified five groups of mAbs competing with each other for FH binding. Four mAbs, anti-FH.02, anti-FH.07, anti-FH.16 and anti-FH.19, did not compete with any of the other mAbs, thereby forming a group on their own.

Table 1 Characteristics of anti-FH mAbs

Group	Designation	Isotype	Epitope location (domain)	Cross-reactivity
	Anti-FH.01	IgA	-	-
I				
	Anti-FH.04	IgG1	20	-
	Anti-FH.10[a]	IgG1	20	FHR-1, FHR-2
	Anti-FH.14	IgG1	20	-
	Anti-FH.17	IgG1	20	-
	Anti-FH.21	IgG1	20	-
II				
	Anti-FH.03[a]	IgG1	Unknown[b]	FHL-1
	Anti-FH.05	IgG1	Unknown	-
	Anti-FH.06	IgG1	Unknown	-
III				
	Anti-FH.12	IgG1	5	-
	Anti-FH.13	IgG2a	5	-
	Anti-FH.15[a]	IgG1	5	FHL-1
IV				
	Anti-FH.08	IgG2a	1-4	-
	Anti-FH.11[a]	IgG2a	1-4	FHL-1
	Anti-FH.20	IgG2a	1-4	-
V				
	Anti-FH.09[a]	IgG1	6	FHL-1, FHR-3, FHR-4A
	Anti-FH.18	IgG2a	6	-
VI				
	Anti-FH.02[a]	IgG1	20	FHR-1, FHR-2
VII				
	Anti-FH.07[a]	IgG1	18	FHR-1, FHR-2
VIII				
	Anti-FH.16[a]	IgG1	16-17	None
IX				
	Anti-FH.19[a]	IgG1	20	FHR-1, FHR-2

-, not tested.
[a]Selected representative of each group for functional testing. [b]The mAbs of group II did not react to any of the FH fragments in the epitope mapping assays. However, due to cross-reactivity of anti-FH.03 with FHL-1, the epitope location must reside within domains 1 to 7 of FH.

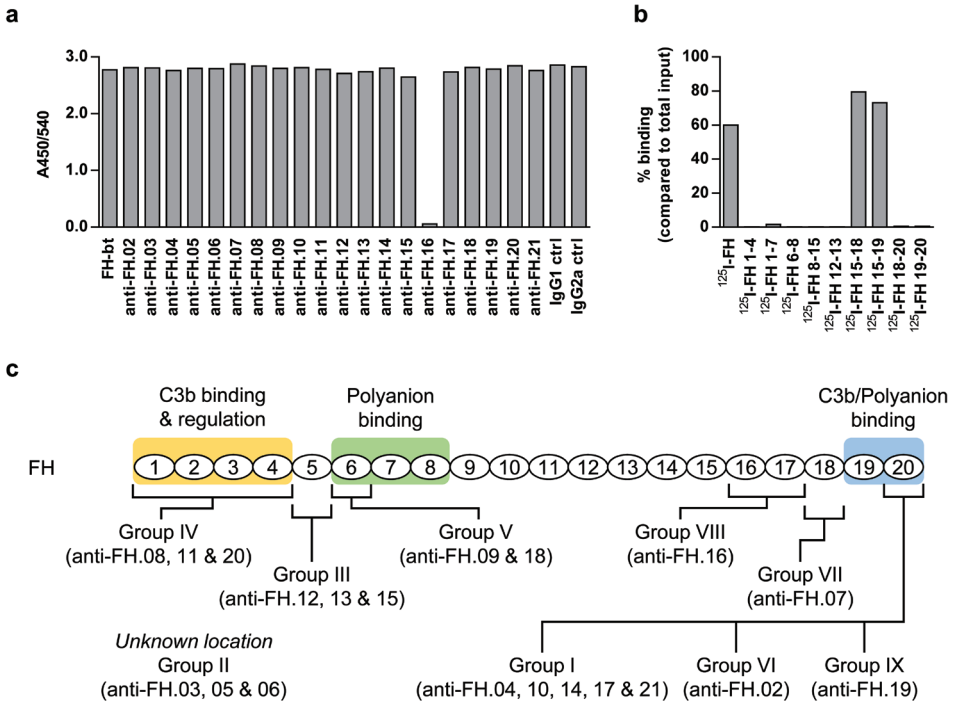

Figure 1 Characterisation of the anti-FH mAbs. (**a**) Representative of a competition assay with anti-FH.16. Anti-FH.16 was coated and binding of FH-bt in the presence or absence of the indicated mAbs was determined by ELISA to determine the competition between anti-FH mAbs. (**b**) Representative of epitope mapping of anti-FH.16 using ^{125}I-labelled FH fragments. Binding is expressed as percentage of total input (40,000 c/min). (**c**) Schematic overview of the binding location of the anti-FH mAbs.

In order to map the binding site of each mAb, we tested the reactivity towards a panel of radiolabelled recombinant human FH fragments consisting of various domains. Binding of the mAbs was tested in a radioimmunoassay (**Fig. 1b**). We also performed an ELISA-based competition assay using unlabelled FH fragments and FH-bt. However, the results did not differ from the experiments with the ^{125}I-labelled FH fragments (data not shown). Group I mAbs (anti-FH.04, anti-FH.10, anti-FH.14, anti-FH.17 and anti-FH.21) were directed towards domain 20, group III mAbs (anti-FH.12, anti-FH.13 and anti-FH.15) bind domain 5, group IV mAbs (anti-FH.08, anti-FH.11 and anti-FH.20) bind domains 1-4, group V (anti-FH.09 and anti-FH.18) binds domain 6, group VI (anti-FH.02) binds domain 20, group VII (anti-FH.07) binds domain 18, group VIII (anti-FH.16) bind domains 16-17 and group IX (anti-FH.19) binds domain 20 (**Table 1** and **Fig. 1c**). The epitope location of the mAbs in group II (anti-FH.03, anti-FH.05 and anti-FH.06) could not be identified with the use of the recombinant FH fragments. Of note, mAbs from group I, group VI and group IX were all

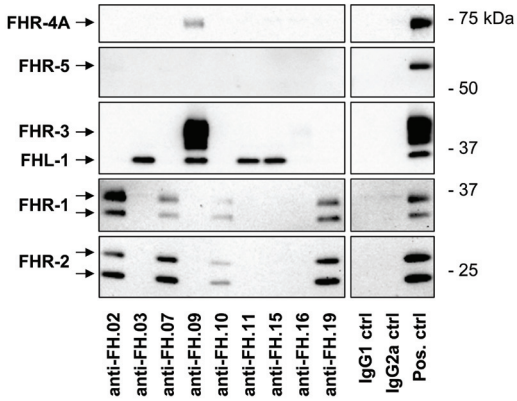

Figure 2 Cross-reactivity of anti-FH mAbs against FH-related proteins. Immunoprecipitation of other members of the FH protein family by the indicated anti-FH mAbs from FH-depleted serum. Blots were developed with biotinylated polyclonal anti-FHR-5, anti-FHR-2, polyclonal anti-FHR-3, anti-FHR-4 and polyclonal anti-FHR-5, respectively. The positive controls (Pos. ctrl) on each respective blot are immunoprecipitations performed with either commercially available monoclonal anti-FHR-1, anti-FHR-2, anti-FHR-4 or anti-FHR-5, or in house developed polyclonal anti-FHR-3.

identified to be directed against domain 20, but apparently to non-overlapping epitopes as these mAbs did not compete for binding of FH. One representative mAb of each group was selected for further characterization.

To test possible cross-reactivity to the splice variant of FH, FH-like 1 (FHL-1), and the FH-related proteins (FHRs), immunoprecipitation was performed. Because FH is a highly abundant plasma protein we first depleted FH from normal human serum to allow binding of FHL-1 and FHR proteins to the anti-FH mAbs. For the depletion of FH, we used anti-FH.16 which binds to an epitope in domains 16/17 of FH, which are not present in any of the other members of the FH protein family. Anti-FH.02, anti-FH.07, anti-FH.10 and anti-FH.19 precipitated FHR-1 (37/43 kDa) and FHR-2 (24/29 kDa) from FH-depleted serum (**Fig. 2**). FHL-1 (42 kDa), detected by the biotinylated polyclonal anti-FHR-3 antibodies on the FHR-3 blot, was immunoprecipitated by anti-FH.03, anti-FH.09, anti-FH.11 and anti-FH.15. Anti-FH.09 also immunoprecipitated FHR-3 (36-50 kDa) and FHR-4A (86 kDa). No band corresponding with FHR-4B could be detected by Western blot. None of the mAbs immunoprecipitated FHR-5 (62 kDa).

Fluid phase regulation of FH is only blocked by mAbs directed against domain 1-4

To determine the effect of the anti-FH mAbs on the co-factor activity of FH, we performed a co-factor activity assay using purified C3b, FH and FI (**Fig. 3a**). FH-mediated cleavage of C3b by FI was visualized using SDS-PAGE under reducing conditions, followed by staining with PageBlue, and quantification by calculating the ratio between the intensities of the 114 kDa α'-chain and the 75 kDa β-chain (**Fig. 3b**). The ratio between the α'-chain and the β-chain of C3b incubated with only FI was used as reference and set to 1.0. Incubation of FI with C3b in the presence of FH resulted in a ratio of 0.12, reflecting degradation of the C3b α'-chain. Only

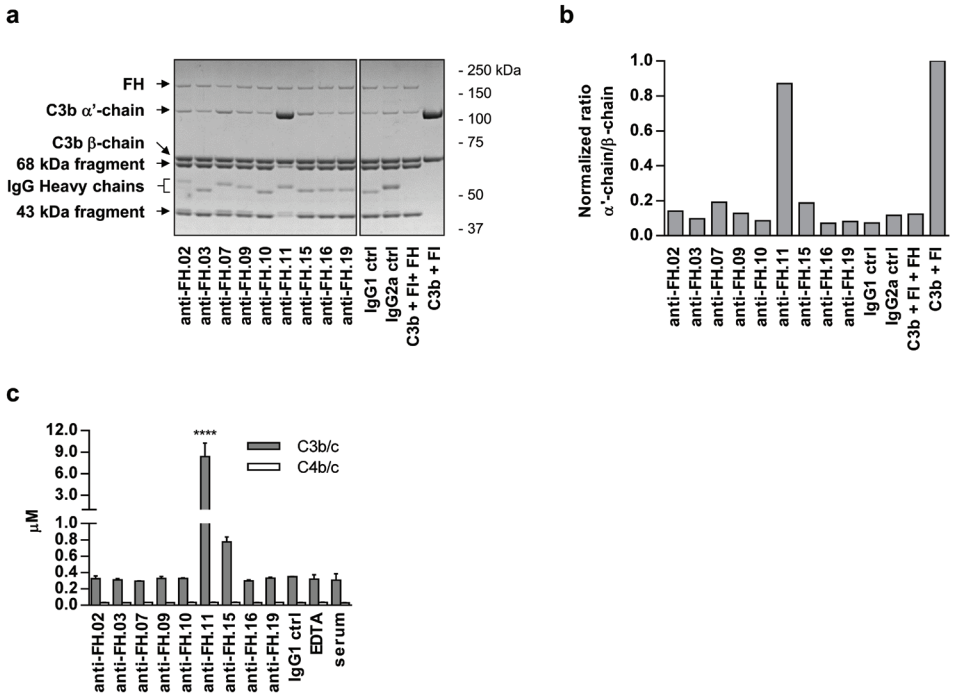

Figure 3 Anti-FH.11 mAb blocks FH functionality in fluid-phase. (**a**) Fluid-phase co-factor activity of FH in the presence of indicated anti-FH mAbs, visualized on a SDS-PAGE run under reducing conditions and stained with PAGEblue. Representative of multiple experiments. (**b**) Quantification of α'-chain degradation by FI in the presence of FH and anti-FH mAbs, as performed in (**a**). The ratio in band intensity between the α'-chain and the β-chain of C3b in the presence of anti-FH mAbs, was normalized against the ratio in the absence of FH (set to 1.0). (**c**) C3b/c levels, reflecting complement activation, and C4b/c levels, reflecting classical and lectin pathway activation, determined by ELISA, in serum incubated at RT with the indicated mAbs or EDTA. Bars represent the mean of two independent experiments with error bars indicating the SD. ****, $p < 0.0001$; two-way ANOVA with Dunnett's multiple comparison post-test, compared to serum.

the addition of anti-FH.11, which binds domains 1-4, resulted in almost complete abrogation of the α'-chain degradation by FI, resulting in a ratio of 0.87.

We also measured C3b/c levels in normal human serum pool after incubation with the anti-FH mAbs, as described before[27]. C3b/c is an activation product of C3 and increased levels reflect complement activation. As expected, incubation of serum with anti-FH.11 resulted in a clear increase in C3b/c levels ($p < 0.0001$). None of the other mAbs induced C3 activation (**Fig. 3c**, grey bars). Anti-FH.11 is the only tested mAb of the IgG2a isotype. This mouse isotype is known to activate the classical pathway, which possibly accounts for the increased C3b/c levels. To determine whether the classical pathway was activated, the activation product

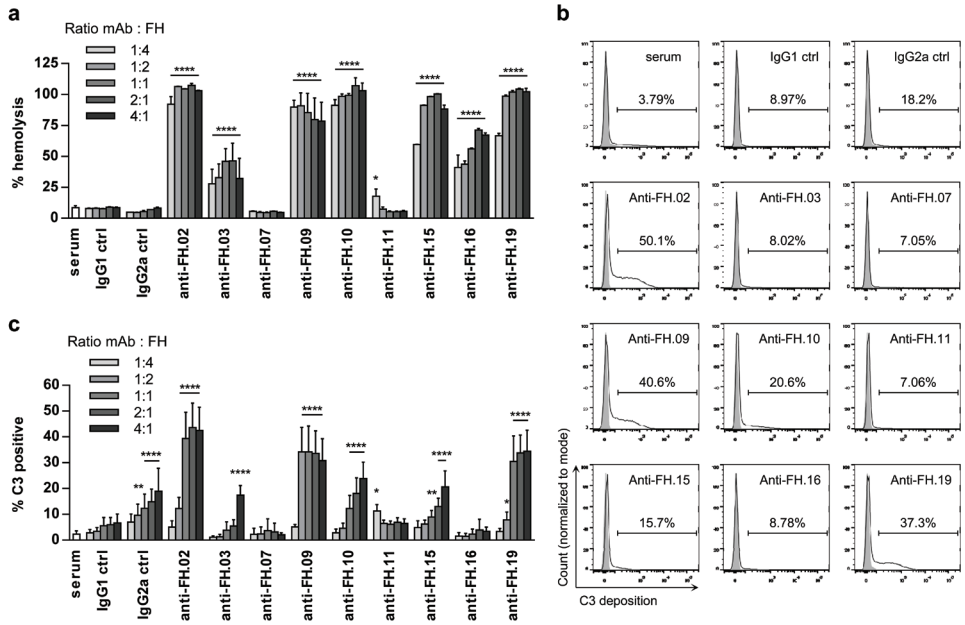

Figure 4 Anti-FH mAbs inhibit FH function on erythrocytes. (**a**) Sheep erythrocytes were incubated with 10% (v/v) normal human serum with a titration of the indicated anti-FH mAbs. Hemolysis was determined by measuring the supernatants at 412 nm and expressed as percentage of the 100% lysis control. Bars represent the mean of two independent experiments with error bars indicating the SD. (**b**) C3 deposition on human erythrocytes was determined by flow cytometry after incubation with 25% (v/v) serum with the addition of a titration of the indicated mAb. Representative results of erythrocytes from one experiment with the addition of a two-times molar excess of the indicated mAbs are shown. Grey histograms indicate the erythrocytes stained for C3 without pre-incubation with serum, black lines indicate the erythrocytes stained for C3 after incubation with serum. (**c**) Results from the flow cytometry analysis of C3 deposition on human erythrocytes as performed in (**b**). Bars represent the mean percentage of C3 positive human erythrocytes from three individual healthy donors with error bars indicating the SD. In (**a**) and (**c**); *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$; two-way ANOVA with Dunnett's multiple comparison post-test, compared to serum.

of C4, C4b/c, was measured as well (**Fig. 3c**, white bars). However, incubation of serum with none of the anti-FH mAbs resulted in increased C4b/c levels as compared to serum without anti-FH mAbs. This indicated that the measured C3b/c resulting from incubating with anti-FH.11 is AP mediated and due to the inhibition of FH. Together, these results demonstrate that blocking domain 1-4 of FH with anti-FH.11 inhibits fluid-phase FH regulation and results in complete C3 consumption in serum.

Anti-FH mAbs binding domains 5, 6 or 20 inhibit FH cell surface regulation on erythrocytes

The effect of the anti-FH mAbs on FH-mediated complement regulation at cell surfaces was

first determined with the use of a sheep erythrocyte hemolytic assay as described before[28]. Addition of anti-FH.02 (domain 20), anti-FH.09 (domain 6), anti-FH.10 (domain 20), anti-FH.15 (domain 5) and anti-FH.19 (domain 20) resulted in almost complete hemolysis of the sheep erythrocytes, while addition of anti-FH.03 and anti-FH.16 resulted in intermediate hemolysis (**Fig. 4a,** $p < 0.0001$ for all mAbs at all concentrations used). Anti-FH.02, anti-FH.09 and anti-FH.10 appeared to have the strongest inhibiting activity compared to the other mAbs as even at a four-fold lower molar concentration compared to the FH levels in the serum pool used in this assay, up to 90% hemolysis was observed. Addition of anti-FH.07 or anti-FH.11 did not result in relevant hemolysis.

Next, we determined whether FH function on human erythrocytes was also affected by the anti-FH mAbs. As human erythrocytes do not lyse due to the presence of other human complement regulators on their cell surface, we measured C3 deposition by FACS (**Fig. 4b** and **4c**). Incubation of human erythrocytes with 25% (v/v) serum did result in negligible C3 deposition, possibly reflecting low level activation of the AP. However, after incubation with anti-FH.02, anti-FH.09, anti-FH.10, anti-FH.15 or anti-FH.20, C3 deposition was markedly increased on human erythrocytes in a dose dependent manner. The most C3 deposition was observed after blocking FH domain 20 with anti-FH.02, which resulted in about 40% of the human erythrocytes containing C3 deposition at equimolar concentrations ($p < 0.0001$). Both of the other anti-FH mAbs that bind to an epitope in domain 20 (anti-FH.10 and anti-FH.19) increased C3 deposition on human erythrocytes ($p < 0.0001$ at equimolar concentrations). Corresponding with the lysis of sheep erythrocytes, addition of anti-FH.09 or anti-FH.15, binding domain 6 and 5, respectively, resulted in C3 deposition on human erythrocytes ($p < 0.0001$ and $p < 0.01$, respectively, at equimolar concentrations). In addition, anti-FH.03 also increased C3 deposition but only at a 4-fold molar excess compared to FH ($p < 0.0001$). Anti-FH.07, anti-FH.11 and anti-FH.16 did not induce C3 deposition on human erythrocytes.

Remarkably, two populations of human erythrocytes were observed after inhibiting FH (**Fig. 4b**). A major group of human erythrocytes remained negative for C3 deposition even at the highest concentration of inhibiting anti-FH mAbs, while a complete shift of the population, with all human erythrocytes becoming positive for C3 deposition was expected. We observed no correlation with either the size of the human erythrocytes (which reflects age), nor the expression of complement regulators DAF or CR1 with the deposition of C3 (data not shown). In addition, C4 deposition was measured to exclude activation of the CP or LP. However, none of the anti-FH mAbs induced C4 deposition (data not shown), which indicates that the increased C3 deposition measured resulted solely from activation of the AP due to inhibition of FH.

Discussion

We successfully generated and characterized 21 novel anti-FH mAbs. Based on competition assays these mAbs could be categorised into nine groups. Three groups (I, VI and IX, comprising seven mAbs), bind FH at non-overlapping epitopes in domain 20, while all other groups bind to other domains. We did not succeed in mapping the epitope location of the mAbs in group II. The representative mAb of this group, anti-FH.03, could also precipitate FHL-1 from human serum, indicating that the epitope must reside within the first seven domains of FH. It is possible that the mAbs in group II are directed against the N-terminus of FH. This site differs in the recombinant FH fragments used for the epitope mapping, thereby explaining the lack of binding of the recombinant FH fragments to the mAbs of group II[15]. Lack of *N*-linked glycans on the FH fragments does not seems to be involved, as the only *N*-linked glycosylation site predicted in domains 1-7 of FH is reported to be unused in plasma-derived human FH[30].

With the use of nine representative mAbs (one of each group), we demonstrated that most of the anti-FH mAbs cross-react with one or more of the other members of the FH protein family. For most mAbs this correlated well with the epitope mapping, as all mAbs binding domain 20 cross-reacted with FHR-1 and FHR-2, both having domains highly similar to domain 20. The mAbs directed against one of the first seven domains (anti-FH.09, anti-FH.11 and anti-FH.15) of FH all cross-reacted with FHL-1. Anti-FH.07, which binds to an epitope in domain 18, did precipitate FHR-2 although it lacks a domain similar to domain 18 of FH. This is explained by hetero-dimerization of FHR-1 with FHR-2, which was described before[31]. Domain 3 of FHR-1 is almost completely identical to domain 18 of FH and is consequently immunoprecipitated by anti-FH.07, which through hetero-dimerization results in co-immunoprecipitation of FHR-2. Therefore, we also cannot exclude that all other mAbs that precipitate FHR-2 and FHR-1 only cross-react with one of the two proteins, co-precipitating the other due to the dimerization. Only one anti-FH mAb, anti-FH.16, was found to be mono-specific for FH, which is in line with its specificity for an epitope within domain 16-17 of FH. These domains are not present in the other FH protein family members. Therefore, this mAb is suitable for assays to specifically distinguish FH from any of the other related proteins of the FH protein family, including the FH splice variant FHL-1.

Of the representative anti-FH mAbs, only one affected the fluid phase regulatory function of FH. Anti-FH.11 binds to an epitope in domains 1-4, which are also the domains known to be involved in the regulation of C3b. Indeed, anti-FH.11 inhibited the fluid phase co-factor activity resulting in fluid phase consumption of C3. While anti-FH.11 is inhibiting the function of FH, addition of anti-FH.11 did not result in increased complement-mediated hemolysis, nor increased C3b deposition. All C3 seems to be effectively consumed in fluid

phase upon FH inhibition by anti-FH.11, leaving no C3 left to be deposited and initiate complement activation on the cell surface.

All three mAbs directed against domain 20 (anti-FH.02, anti-FH.10 and anti-FH.19) inhibited FH function on sheep and human erythrocytes, emphasizing the importance of this domain. Next to domain 20, domain 6 was also involved. Blocking this domain with mAb anti-FH.09 resulted in increased hemolysis and C3 deposition. This demonstrated that both binding regions are involved in inhibiting complement activation on the erythrocyte cell surface by FH. Blocking domain 5 with anti-FH.15 also inhibited FH on the erythrocyte cell surface but we believe that this effect is due to steric hindrance. No specific function has been described for domain 5 besides forming a physical link between the regulatory domains 1-4 with the binding region in domains 6-8. It is possible that binding of an antibody to domain 5 hinders the function of either of these regions. Indeed, we measured a small, although not statistically significant, increase in serum C3b/c levels after addition of anti-FH.15, indicating that blocking domain 5 might affect the fluid phase regulation by FH. However, we did not find an effect of this antibody on the co-factor activity of FH, making it more likely that anti-FH.15 sterically hinders domain 6-8.

Despite that human erythrocytes express multiple other complement inhibitors on their surface, we were able to induce a pronounced C3 deposition by only inhibiting FH. Apparently, the other surface regulators are unable to completely control the initial activation and deposition of C3b via the AP at least on a subset of erythrocytes. Note that these erythrocytes were drawn from healthy donors, thus indicating that FH plays a crucial role in the protection of healthy erythrocytes from AP-mediated complement activation. The other complement regulators were able to prevent further activation of the complement cascade, as no hemolysis occurred. In addition, a distinct human erythrocyte population was observed upon FH inhibition that remained negative for C3 deposition. We could not find any correlation between the age of the human erythrocytes or the expression of other complement regulators and C3 deposition, and are currently looking further into this phenomenon. Possibly the C3-positive human erythrocytes expose a trigger capable of activating the AP.

In conclusion, we have generated and characterized twenty anti-FH mAbs which we used to demonstrate the important role of both binding regions of FH in the protection of erythrocytes against complement-mediated damage.

Author Contributions

RBP, MCB and AJFT performed the experiments. CQS provided critical materials and reagent

and helped to interpret the data. RBP, TWK and DW designed the study and wrote the manuscript. All contributing authors provided critical review of the manuscript and agreed to its final form.

Conflict of interest disclosure

RBP, MCB, TWK, and DW are inventors of a patent application (PCT/NL2015/050584) describing the therapeutic use of potentiating anti-FH antibodies. AJFT and CQS declare no relevant competing financial interests.

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References

1. Esparza-Gordillo, J. *et al.* Genetic and environmental factors influencing the human factor H plasma levels. *Immunogenetics* **56,** 77–82 (2004).
2. Hakobyan, S. *et al.* Measurement of factor H variants in plasma using variant-specific monoclonal antibodies: application to assessing risk of age-related macular degeneration. *Investig. Ophthalmol. Vis. Sci.* **49,** 1983–1990 (2008).
3. Sofat, R. *et al.* Distribution and determinants of circulating complement factor H concentration determined by a high-throughput immunonephelometric assay. *J. Immunol. Methods* **390,** 63–73 (2013).
4. Józsi, M. & Zipfel, P. F. Factor H family proteins and human diseases. *Trends Immunol.* **29,** 380–387 (2008).
5. Walport, M. J. Complement; first of two parts. *N. Engl. J. Med.* **344,** 1058–1066 (2001).
6. Gordon, D. L., Kaufman, R. M., Blackmore, T. K., Kwong, J. & Lublin, D. M. Identification of complement regulatory domains in hHuman factor H. *J. Immunol.* **155,** 348–356 (1995).
7. Kühn, S., Skerka, C. & Zipfel, P. F. Mapping the complement regulatory domains in the human factor H-like protein 1 and in factor H. *J. Immunol.* **155,** 5663–5670 (1995).
8. Kühn, S. & Zipfel, P. F. Mapping of the domains required for decay acceleration activity of the human factor H-like protein 1 and factor H. *Eur. J. Immunol.* **26,** 2383–2387 (1996).
9. Jokiranta, T. S., Hellwage, J., Koistinen, V., Zipfel, P. F. & Meri, S. Each of the three binding sites on complement factor H interacts with a distinct site on C3b. *J. Biol. Chem.* **275,** 27657–27662 (2000).
10. Józsi, M., Oppermann, M., Lambris, J. D. & Zipfel, P. F. The C-terminus of complement factor H is essential for host cell protection. *Mol. Immunol.* **44,** 2697–2706 (2007).
11. Blackmore, T. K., Sadlon, T. A., Ward, H. M., Lublin, D. M. & Gordon, D. L. Identification of a heparin binding domain in the seventh short consensus repeat of complement factor H. *J. Immunol.* **157,** 5422–5427 (1996).
12. Jokiranta, T. S. *et al.* Binding of complement factor H to endothelial cells is mediated by the carboxy-terminal glycosaminoglycan binding site. *Am. J. Pathol.* **167,** 1173–1181 (2005).
13. Blackmore, T. K. *et al.* Identification of the second heparin-binding domain in human complement factor H. *J. Immunol.* **160,** 3342–3348 (1998).
14. Prosser, B. E. *et al.* Structural basis for complement factor H linked age-related macular degeneration. *J. Exp. Med.* **204,** 2277–2283 (2007).
15. Schmidt, C. Q. *et al.* A new map of glycosaminoglycan and C3b binding sites on factor H. *J. Immunol.* **181,** 2610–2619 (2008).
16. Klein, R. J. *et al.* Complement factor H polymorphism in age-related macular degeneration. *Science* **308,** 385–389 (2005).
17. Haines, J. L. *et al.* Complement factor H variant increases the risk of age-related macular degeneration. *Science* **308,** 419–421 (2005).
18. Edwards, A. O. *et al.* Complement factor H polymorphism and age-related macular degeneration. *Science* **308,** 421–424 (2005).
19. Clark, S. J. *et al.* Tissue-specific host recognition by complement factor H is mediated by differential activities of its glycosaminoglycan-binding regions. *J. Immunol.* **190,** 2049–2057 (2013).
20. Rodríguez de Córdoba, S., Tortajada, A., Harris, C. L. & Morgan, B. P. Complement dysregulation and disease: From genes and proteins to diagnostics and drugs. *Immunobiology* **217,** 1034–1046 (2012).
21. Loeven, M. A. *et al.* The role of heparan sulfate as determining pathogenic factor in complement factor H-associated diseases. *Mol. Immunol.* **63,** 203–208 (2015).
22. Ferreira, V. P. & Pangburn, M. K. Factor H-mediated cell surface protection from complement is critical for the survival of PNH erythrocytes. *Blood* **110,** 2190–2192 (2007).
23. Lang, P. A. *et al.* Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J. Mol. Med.* **84,** 378–388 (2006).
24. Pouw, R. B. *et al.* Complement factor H-related protein 3 serum levels are low compared to factor H and mainly determined by gene copy number variation in CFHR3. *PLoS ONE* **11,** e0152164 (2016).
25. Morgan, H. P. *et al.* Structural basis for engagement by complement factor H of C3b on a self surface. *Nat. Struct. Mol. Biol.* **18,** 463–471 (2011).
26. Pechtl, I. C., Kavanagh, D., McIntosh, N., Harris, C. L. & Barlow, P. N. Disease-associated N-terminal complement factor H mutations perturb cofactor and decay-accelerating activities. *J. Biol. Chem.* **286,** 11082–11090 (2011).
27. Wolbink, G. J. *et al.* Application of a monoclonal antibody against a neoepitope on activated C4 in an

ELISA for the quantification of complement activation via the classical pathway. *J. Immunol. Methods* **163,** 67–76 (1993).
28. Sánchez-Corral, P., González-Rubio, C., Rodríguez de Córdoba, S. & López-Trascasa, M. Functional analysis in serum from atypical Hemolytic Uremic Syndrome patients reveals impaired protection of host cells associated with mutations in factor H. *Mol. Immunol.* **41,** 81–84 (2004).
29. Wouters, D., Brouwer, M. C., Daha, M. R. & Hack, C. E. Studies on the haemolytic activity of circulating C1q-C3/C4 complexes. *Mol. Immunol.* **45,** 1893–1899 (2008).
30. Fenaille, F. *et al.* Site-specific N-glycan characterization of human complement factor H. *Glycobiology* **17,** 932–944 (2007).
31. Goicoechea De Jorge, E. *et al.* Dimerization of complement factor H-related proteins modulates complement activation in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 4685–4690 (2013).