FUNCTION AND DYSFUNCTION OF COMPLEMENT FACTOR H

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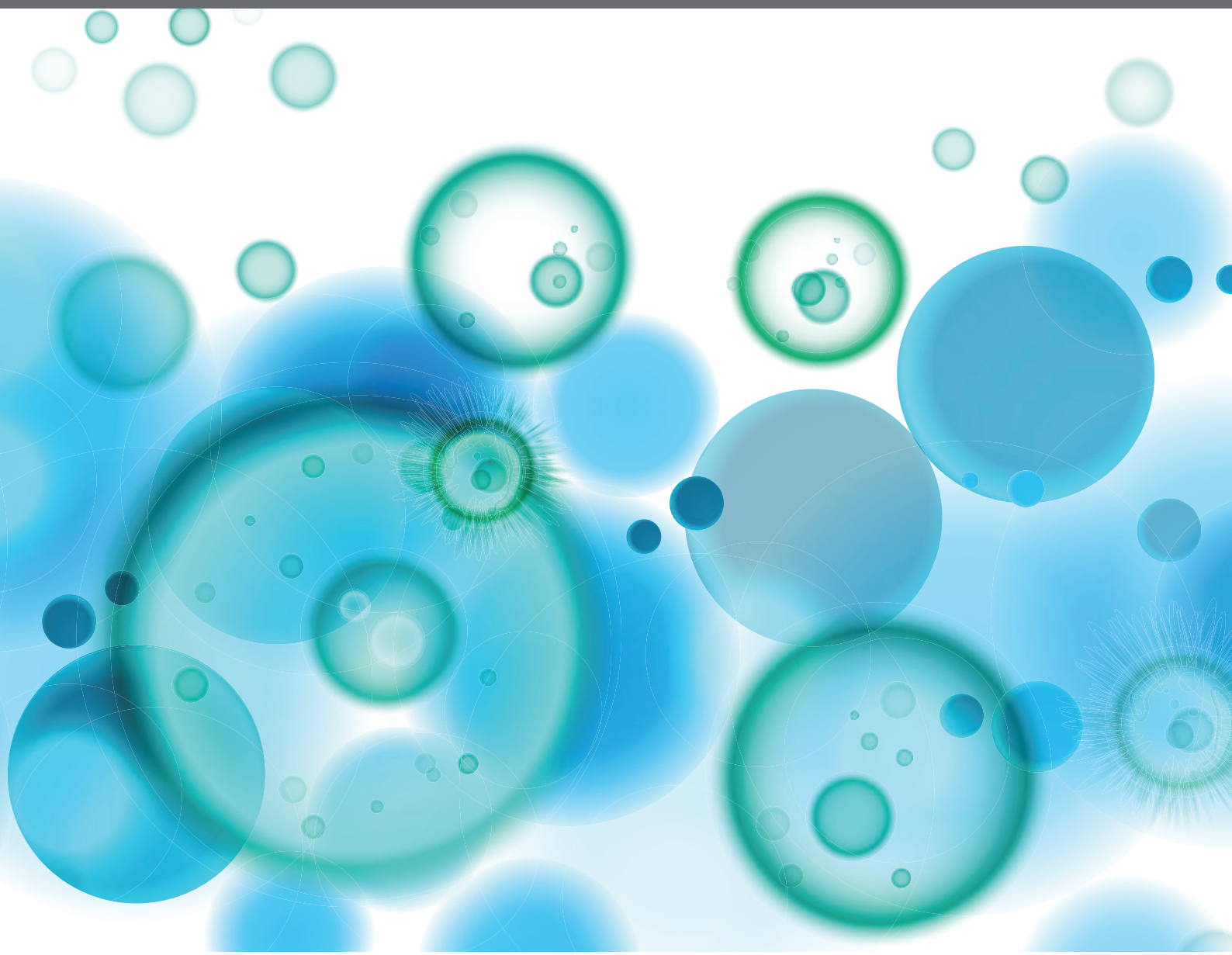

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FUNCTION AND DYSFUNCTION OF COMPLEMENT FACTOR H

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Table of Contents

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EDITORIAL
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Editorial: Function and Dysfunction of Complement Factor H

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Editorial on the Research Topic

Function and Dysfunction of Complement Factor H

The complement system responds very quickly to danger due to the eternal vigilance of its alternative pathway (AP). In the AP, an always-on positive-feedback C3b-amplification loop (**Figure 1**) is maintained at "tick-over" level on autologous surfaces by protective membrane-bound and soluble regulators. Conversely, the unprotected surfaces of invading microbes rapidly become opsonised by AP-generated C3b, tagging them for clearance, triggering the cytolytic terminal pathway and releasing anaphylatoxins C3a and C5a. Pathogenic microbes, however, often resist attack by complement, thus avoiding elimination.

Complement factor H (FH, encoded by *CFH*) is a soluble AP suppressor operating on autologous surfaces by recognising self-surfaces, directly *via* specific glycosaminoglycans and sialic acid or indirectly e.g. *via* C-reactive protein (CRP), and in fluid-phase. FH also aids non-inflammatory clearance of damaged cells and cell debris. Further "non-canonical" FH functions are mediated *via* binding sites, notably for complement receptor (CR3), malondialdehyde (MDA)-modified proteins and apolipoprotein E (apoE), distributed along its 20 CCP modules (CCPs) also called short consensus repeats (**Figure 1**). FH-like 1 (FHL-1) is an AP-regulating smaller splice variant that lacks self-*versus*-nonself discrimination. Six FH-related proteins (FHRs-1-3, 4A and 4B, and 5) complete the family (**Figure 1**). Encoded by a gene cluster situated *3'* of *CFH* (*CFHR3*;*CFHR1*;*CFHR4*;*CFHR2*;*CFHR5*), these products of genomic duplication events may antagonise FH.

In *CFH* and *CFHRs*, single-nucleotide polymorphisms, copy-number variations, and exon duplications, deletions and rearrangements are commonplace. These can alter risks of atypical haemolytic uraemic syndrome (aHUS), C3 glomerulopathy (C3G) or age-related macular degeneration (AMD) amongst many other diseases. In a fast-moving field, the 12 papers in this Research Topic take stock of efforts to understand how these proteins work, and sometimes fail.

The interplay between FH-family members is unpicked in Poppelaars et al. and Mannes et al. The C-terminal CCPs of FHRs resemble, and may compete with, the self-surface-recognising and C3b-binding CCPs 19-20 of FH. But CCPs equivalent to the C3b-binding CCPs 1-4 of FH, critical for AP regulation, are absent from FHRs. N-terminal CCPs in FHRs-1, 2 and 5 stabilise homo-/hetero-dimerisation (**Figure 1**). Poppelaars et al. discuss discrepancies in reported functions and serum-levels of FH-family proteins. Conversely, FHL-1 (reviewed by Mannes et al.) possesses the CCPs 1-4 of FH along with its CCPs 5-7 that augment their function, but lacks FH's C-terminal CCPs, accounting for its lack of self/non-self discrimination. Being smaller, FHL-1 reaches locations poorly accessible to FH, *e.g.* by crossing Bruch's membrane in the eye. These two reviews highlight

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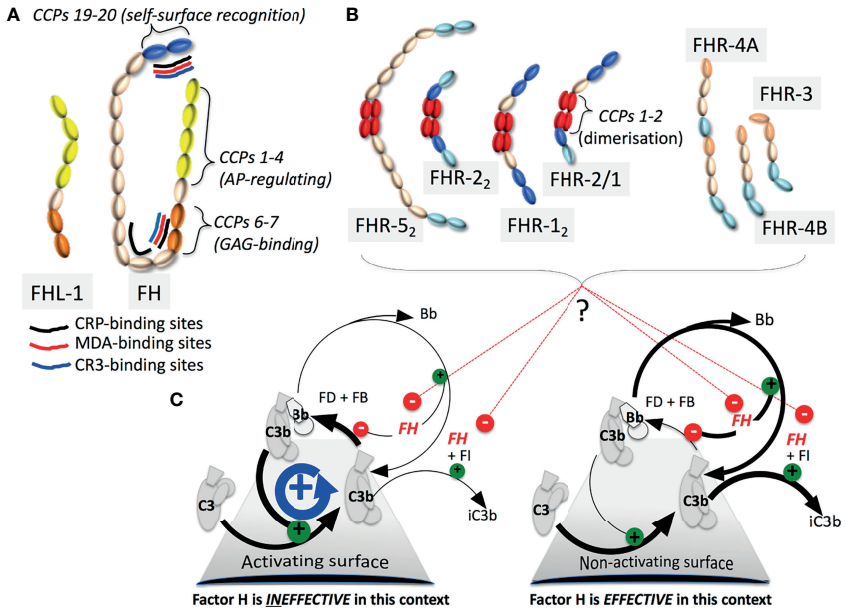

FIGURE 1 | *The FH family and C3b amplification*: **(A)** FHL-1 and FH contain seven and 20 CCPs (SCRs) respectively. Functional sites on FH are indicated. **(B)** The CCPs of the FHRs are colour-coded to highlight similarity with CCPs in FH. Dimerisation-mediating CCPs 1-2, are unique to FHRs-1, 2 and 5. Of potential heterodimers, only FHR-2/1 is detectable. **(C)** In a positive-feedback cycle factor B (FB) binds nascent C3b (that can covalently attach to virtually any surface) whereupon factor D (FD) cleaves FB to yield C3b.Bb, a "C3 convertase", that cleaves C3 into C3a and C3b. C3b also enters other complement pathways, promoting C5a release and cytolysis. FH destabilises C3b.Bb, competes with FB for binding to C3b, and is a cofactor for factor I (FI) that cleaves C3b to the CR3-ligand iC3b. Some FHRs likely antagonize FH.

challenges with working on FHRs and FHL-1 including shortages of specific antibodies and good animal models.

Proposed mechanisms for FH invoke simultaneous engagement of CCPs 1-4 and CCPs 19-20 of FH with the same C3b molecule, facilitated by 14 connecting CCPs (**Figure 1**). Dunne et al. identify a low-affinity dimerization site in CCPs 17-18, potentially explaining disease-linked mutations therein. Dimerisation could help FH molecules gather at sites requiring robust AP suppression. The role of FH's sialylated N-glycans is incompletely understood. Delgado et al. describe desialylation of FH by bacterial neuraminidase in cases of *Streptococcus pneumoniae*-precipitated aHUS (*Sp*-aHUS). *In-vitro* enzymatic desialylation rendered FH less effective at preventing complement-mediated haemolysis, suggesting a pathological mechanism underlying *Sp*-aHUS. Rare genetic variations of the *CFH*/*CFHR* cluster, observed in many patients in this study, might also contribute to disease.

Screening for genetic variants, routine in aHUS management, is informed by knowledge of the consequences of specific mutations. Wong et al. describe a workflow for characterising "variants of unknown significance" identified in aHUS, C3G and AMD patients, within the AP-regulating CCPs 1-4 of FH. Of six new SNPs investigated, Q81P emerged as a dysfunctional, potentially causative, mutation in aHUS. Less impacted variants (*e.g.* D130N in this study) might contribute to AMD, a slower progressing disease. This brings to a useful total of 16 the number of biochemically characterised FH N-terminal variants.

Zhang et al. found FH autoantibodies (FH-AAs) in about 1-in-9 and 1-in-30 members of North American aHUS and C3G cohorts, respectively. Consistent with patterns of genetic variation, most FH-AAs in aHUS bind to and compromise CCPs 19-20, while C3G-associated FH-AAs often bind to and compromise N-terminal CCPs. Of aHUS patients with FH-AAs, ~75% were Δ*CFHR3*;*CFHR1* (*cf* ~3.5% of controls and in the C3G cohort). One hypothesis suggests C-terminal CCPs of FHR-1 (98% identity with FH CCPs 19-20) induce tolerance to a cryptic epitope in FH's C-terminal region that becomes exposed during infection.

Autoantibodies binding CCPs 19-20 of FH and inhibiting C3b binding were also found in four of 45 cases of neuromyelitis optica spectrum disorder (Uzonyi et al.) a rare inflammatory disease of the CNS, also associated with other autoantibodies, that responds to therapeutic complement suppression. These FH-AAs cross-reacted with FHR-1, but none of these individuals is Δ*CFHR3*;*CFHR1*, or had kidney disease. Studies on larger cohorts will determine how the FH-AAs are generated and contribute to underlying pathophysiology.

By definition, successful human pathogens evade the AP. Hijacking by pathogens of FH for self-protection is achieved by many organisms; Moore et al. list >30 such bacterial species, plus fungi, protozoa, helminths and viruses. Pathogens utilise diverse molecules to anchor FH including, but not limited to, glycans and proteins that mimic host equivalents. FH-binding proteins of pathogens are often, although not invariably, important

virulence factors with expression levels depending on the abundance of FH in the milieu. Antimicrobial strategies targeting FH capture include uses of sialic acid analogues, small molecules to lock-in non-FH binding conformations of FH-anchoring proteins, and – as illustrated by Laabei et al. - engineered FH-like molecules that displace host FH. In this case a chimeric protein, fusing the *Moraxella catarrhalis*-binding CCPs (6-7) of FH to IgG Fc, outcompeted FH for binding to its receptor on bacteria whereupon it activated classical complement *via* its Fc, promoting bacterial elimination.

Beyond its canonical roles in complement, surface-bound FH binds and influences the behaviour of neutrophils and monocytes. Research described in Kárpáti et al. extends this property to surface-immobilised FHL-1, FHR-1 and FHR-5, detailing how these proteins may affect extravasation and pathogen-killing by variously modulating adherence and migration, as well as production of IL-8, IL-1β, TNFα, and anti-inflammatory IL-10. A key challenge is to confirm the identity of the cell-surface FH/FHR receptor(s); CR3 is the lead candidate. In related work, Kozma et al. explored use of engineered FH to supress the cytokine storm potentially triggered by C3a/C5a binding to receptors on peripheral blood mononuclear cells. In cells cultured with autologous serum and subjected to artificial immune activation, a "mini"- FH, comprising CCPs 1-4 linked to CCPs 19-20, mildly suppressed IL-6 production and stimulated IL-10 production. Also of growing interest is how FH deters formation of lipid-rich deposits (LRD), and suppresses their inflammatory potential. As argued by Meri and Haapasalo, LRDs characterise not only AMD (drusen) and some forms of C3G (dense deposits) but also occur as plaques in Alzheimer's disease and atherosclerosis. Noting that FH binds lipid-free and high-density lipoprotein-associated ApoE, oxidized lipids and bisretinoids in drusen, and malondialdehyde-conjugates (by-products of lipid peroxidation), these authors suggest potential involvement of aberrantly functioning FH in the pathophysiology underlying all these conditions.

Together, the articles in this Research Topic emphasise the pivotal importance of FH. The viability and health of our cells and tissues depend strongly on FH-mediated protection. The multitude of pathogenic microbes that exploit FH for their survival in human tissues attests to its role as the "master regulator" of the complement system. The growing list of diseases linked to variants or mutations in the *CFH* and *CFHR*s cluster underlines the need to develop better tools and animal models to aid in research, and supports establishment of units, within hospital laboratory settings, specialising in complement diagnostics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Antibacterial Fusion Proteins Enhance *Moraxella catarrhalis* Killing

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Moraxella catarrhalis is a human-specific commensal of the respiratory tract and an opportunistic pathogen. It is one of the leading cause of otitis media in children and of acute exacerbations in patients with chronic obstructive pulmonary disease, resulting in significant morbidity and economic burden. Vaccines and new immunotherapeutic strategies to treat this emerging pathogen are needed. Complement is a key component of innate immunity that mediates the detection, response, and subsequent elimination of invading pathogens. Many pathogens including *M. catarrhalis* have evolved complement evasion mechanisms, which include the binding of human complement inhibitors such as C4b-binding protein (C4BP) and Factor H (FH). Inhibiting C4BP and FH acquisition by *M. catarrhalis* may provide a novel therapeutic avenue to treat infections. To achieve this, we created two chimeric proteins that combined the Moraxella-binding domains of C4BP and FH fused to human immunoglobulin Fcs: C4BP domains 1 and 2 and FH domains 6 and 7 fused to IgM and IgG Fc, respectively. As expected, FH6-7/IgG displaced FH from the bacterial surface while simultaneously activating complement via Fc-C1q interactions, together increasing pathogen elimination. C4BP1-2/IgM also increased serum killing of the bacteria through enhanced complement deposition, but did not displace C4BP from the surface of *M. catarrhalis*. These Fc fusion proteins could act as anti-infective immunotherapies. Many microbes bind the complement inhibitors C4BP and FH through the same domains as *M. catarrhalis*, therefore these Fc fusion proteins may be promising candidates as adjunctive therapy against many different drug-resistant pathogens.

Keywords: pathogen, *Moraxella catarrhalis*, fusion proteins, complement, antibacterial

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INTRODUCTION

Moraxella catarrhalis is a Gram-negative diplococcus which commonly colonizes the nasopharyngeal cavity of humans asymptomatically (1). However, in recent years, a greater appreciation of the virulent nature of this bacterium has emerged and *M. catarrhalis* is now considered an opportunistic pathogen (1, 2). *M. catarrhalis* is the third most common cause of

Abbreviations: AF, Alexa Fluor; BHI, brain-heart infusion; BSA, bovine serum albumin; C4BP, C4-binding protein; CCP, complement control protein; CFSE, carboxyfluorescein succinimidyl ester; CFU, colony forming unit; CHO, Chinese hamster ovary; FH, factor H; gMFI, geometric mean fluorescence intensity; GVB, gelatin veronal buffer; Ig, immunoglobulin; MAC, membrane attack complex; NHS, normal human serum; OD, optical density; Usp, ubiquitous surface protein; WT, wild-type.

acute sinusitis and otitis media in children and is responsible for a variety of lower respiratory tract infections in immunocompetent hosts and patients with chronic lung disease (2, 3). Rarely, *M. catarrhalis* can cause bacteraemia and pneumonia in immunocompromised individuals (4).

Humans have evolved a variety of mechanisms to evade infections caused by a multitude of microbial pathogens. The complement system represents an effective arm of innate immunity involved in detecting, labeling and eradicating potential microbial threats. Bacterial activation of complement is mediated by specific recognition molecules, which bind to conserved structures on the bacterial surface, initiating an enzymatic cascade resulting in the formation of the C3 convertases and cleavage of C3, the central protein of the complement cascade (5, 6). A strong activator of the classical pathway is the recognition subunit of the C1 complex, C1q (7). C1q interacts with high avidity with the Fc region of clustered immunoglobulin (Ig)Gs or multivalent IgM molecules and in conjunction with serine proteases, C1r and C1s, initiates proteolytic events resulting in C3 convertase formation (7). C3 can be cleaved into multiple fragments with opsonic properties and when deposited on the microbial surface, can interact with complement receptors expressed on the surface of professional phagocytes culminating in uptake and destruction of the pathogen (8). Further activation and processing of C3 forms C5 convertases, that cleave C5 into C5a, a potent chemoattractant, and C5b, an essential building block of the membrane attack complex (MAC). Interaction of C5b with complement proteins C6 through C9 results in formation and insertion of MAC leading to a reduction in membrane potential and bacterial lysis of Gram-negative bacteria (5, 6).

To prevent complement destruction of host cells, a suite of soluble and cell surface regulators maintain complement homeostasis (9). Two soluble proteins, Factor H (FH) and C4b-binding protein (C4BP) are pivotal for preventing unwanted complement activation, both exerting their influence at the level of C3 convertase inhibition (9–11). FH is the major soluble inhibitor of the alternative pathway (AP), binds to C3b via complement control protein (CCP) domains 1–4 and accelerates the decay of the alternative C3 convertase while also acting as a cofactor for the serine protease, factor I mediated inactivation of C3b (9, 10). C4BP is the major inhibitor of the classical and lectin pathways, interacting with and limiting the function of complement protein C4b (9, 11). In similar fashion to FH, C4BP acts as a cofactor for both FI proteolysis of cell-bound and soluble C4b, disrupting formation of the classical C3 convertase (9, 11) and fluid phase C3b inhibiting AP activity (12). Furthermore, C4BP can accelerate the decay of formed classical pathway C3 convertase (11).

The success of any disease-causing organism depends on its ability to resist host immunity (13–15). As FH and C4BP are soluble proteins, a wide variety of pathogens have evolved mechanisms to bind and recruit these proteins to their surface, thus disrupting complement deposition (13, 16). Most bacteria recruit FH through CCP domains 6–7 and 18–20, thereby permitting FH domains 1–4 to inhibit complement (10, 15). Pathogen binding of C4BP is generally associated with CCP1-3, which is also responsible for C4b and C3b binding (11, 15). However, C4BP is a multimeric protein with seven identical alpha chains, which permits its simultaneous binding to different ligands while maintaining complement inhibitory activity (11, 15).

Infections are primarily curtailed by antibiotics or vaccines. In recent years, antibiotic resistance has become a major health problem globally, with the proliferation of multidrug-resistant bacteria (17). Development of new antibiotics and vaccines does not appear to meet the current medical demand, therefore new antimicrobial approaches are urgently needed. Chimeric proteins that fuse pathogen binding domains of either FH (CCPs 6–7) of the Fc region of human IgG (FH6–7/IgG) or C4BP (CCPs 1–2) fused to the constant domains CH2, CH3, and CH4 of IgM (C4BP1–2/IgM) resulting in a hexavalent chimeric protein have been developed as alternative strategies to control infection (18, 19). The net result is displacement of complement inhibitors from the bacterial surface with simultaneous complement activation via Fc-C1q interaction, which increases pathogen elimination. This approach has shown promise in enhancing killing of Non-typeable *Haemophilus influenzae* (NTHi) (20), *Neisseria meningitidis* (18), *Neisseria gonorrhoeae* (19, 21), and *Streptococcus pyogenes* (22). In this study we tested the bactericidal activity of both fusion proteins against *M. catarrhalis*.

MATERIALS AND METHODS

Bacteria and Cell Line Culture Conditions

Bacteria used in this study were *M. catarrhalis* strain RH4 (23), isogenic mutants of RH4 devoid of UspA1 (Δ*uspA1*), UspA2 (Δ*uspA2*), and both UspA1 and UspA2 (Δ*uspA1*Δ*uspA2*) (24), and clinical isolates KR473, KR478, KR479, KR485, KR502, KR507, KR508, KR530, KR533, KR488, KR504, KR506, KR531, KR539, KR540, KR477, KR480, KR482, KR510, KR515, and KR527 (25). All strains of *M. catarrhalis* were routinely cultured on chocolate blood agar and grown overnight at 37°C with 5% CO_2. Prior to experiments, bacteria were sub-cultured from overnight plates and streaked onto new chocolate blood agar plates and grown for 6–8 h. Bacteria were scraped from plates and resuspended into freezing medium [25% brain-heart infusion (BHI)/glycerol], and subsequently aliquoted and stored at −80°C until use.

Staphylococcus aureus strain USA300 JE2 were grown in tryptic soy broth at 37°C with shaking (200 rpm). Overnight cultures were normalized to an $OD_{600\ nm}$ = 0.1 in fresh medium and grown under the same conditions until an $OD_{600\ nm}$ = 0.3–0.4, corresponding to mid-exponential phase of growth. Bacteria were centrifuged and washed once in sterile PBS and normalized to an $OD_{600\ nm}$ = 1 which equates to 1–2 × 10^8 CFU/ml.

FreeStyle Chinese hamster ovary (CHO) S suspension cells (Life Technologies), grown in FreeStyle CHO expression medium supplemented with 8 mM L-glutamine were used for the expression of FH6-7/IgG fusion protein as described (22). Cell cultures were grown in 250 ml Erlenmeyer flasks at 37°C with 8% CO_2 with shaking (130 rpm). Adherent CHO cells grown

in serum-free OptiMEM Glutamax medium were used for the expression of C4BP1-2/IgM as described (19). For expression and collection of C4BP1-2/IgM fusion protein, adherent cells were washed twice in PBS and grown in Opti-MEM for 48 h. Following 48 h, cells were washed and grown in CHO expression medium for 24 h and this procedure repeated for 15 days.

Purification of Fusion Proteins

Fusion protein FH6-7/IgG were expressed in CHO cells and purified on protein A/G columns as described previously (22). Bound proteins were eluted using 0.1M glycine, pH = 2.7. Protein eluate was dialyzed three time in PBS at 4°C and protein concentrations calculated on aBio photomoter. The C4BP1-2/IgM fusion protein was purified using a HiTrap Normal human serum (NHS)-activated Sepharose 5 ml column coupled with antibody MK104, which recognizes the CCP-1 domain of C4BP as described previously (19).

Binding of Fusion Proteins to *M. catarrhalis*

Moraxella catarrhalis glycerol stocks were thawed at 37°C for 30 min and washed once in PBS for 5 min at 5000 × *g*. Bacteria were normalized to $OD_{600\ nm}$ of 1, stained with carboxyfluorescein succinimidyl ester (CFSE, 10 μM) for 30 min at 37°C and washed once in PBS, for gating in flow cytometry assays. Bacterial concentrations were adjusted to an $OD_{600\ nm}$ of 0.5 in PBS and thereafter 50 μl was mixed with 50 μl of increasing concentrations of fusion proteins for 30 min at 37°C in a covered V-bottomed 96 well plate. Bacteria were centrifuged for 5 min at 5000 × *g* at room temperature (RT) and washed once in PBS. To detect FH6-7/IgG binding to *M. catarrhalis*, bacteria were stained with Alexa Fluor (AF) – 488 goat anti-human IgG (1:1000; Invitrogen) in 1% (w/v) BSA/PBS for 30 min at RT. To detect C4BP1-2/IgM binding, bacteria were stained with polyclonal rabbit anti-human IgM (1:1000; Dako) for 30 min at RT. Bacteria were centrifuged and washed once in 1% (w/v) BSA/PBS and for C4BP1-2/IgM detection, followed by a secondary antibody staining (AF-647 goat anti-rabbit, 1:1000; Invitrogen) for 30 min in the dark. For both fusion proteins, following Ab staining, bacteria were centrifuged and washed once in 1% (w/v) BSA/PBS, resuspended in 150 μl PBS and deposited fusion proteins were assessed using a CytoFLEX flow cytometer. Bacteria incubated without fusion protein was used as a negative control. CFSE-stained and non-stained bacteria were used for gating purposes and a minimum of 20,000 events were examined. To assess the binding of C4BP-IgM in the presence of serum, the fusion protein was labeled with AF 488 using AF 488 microscale labeling kit (A10235, Molecular Probes).

Serum Bactericidal Assays

Bacteria were prepared as described in bacteria-fusion protein binding section, with the omission of the CFSE staining. NHS was prepared from freshly drawn blood with informed consent from at least eight healthy volunteers as described (26) and in accordance with the recommendations of the ethical committee at Lund University (Permit 2017/582) and the Declaration of Helsinki (27). Bacteria were normalized to $OD_{600\ nm}$ of 0.05. Fifty microliters of bacteria were incubated in the presence or absence of 40 μl of fusion protein (final concentration 50 μg/ml) for 30 min at 37°C in GVB^{++} buffer [5 mM veronal buffer pH 7.3, 0.1% (w/v) gelatin, 140 mM NaCl, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$]. After 30 min, 10 μl of pooled NHS was added and incubated with bacteria for a further 30 min at 37°C. Alternatively, bacteria and fusion protein in GVB^{++} buffer (0–100 μg/ml) in addition to serum were added simultaneously and incubated for 30 min at 37°C. For calculation of bacteria survival, samples of bacteria were removed at time 0 and time 30 min following incubation at 37°C, serially diluted in PBS and spread onto chocolate agar plates for colony enumeration after growth overnight at 37°C with 5% CO_2. As a control heat-inactivated serum was used following treatment at 56°C for 30 min.

Binding Competition Experiments

Bacteria were thawed, washed once in PBS and normalized to an $OD_{600\ nm}$ of 0.5. Two experimental protocols were utilized: (1) Bacteria were pre-incubated with varying concentrations of either FH6–7/IgG or C4BP1–2/IgM in GVB^{++} buffer for 30 min at 37°C. Following incubation, NHS treated with the complement C5 inhibitor OmCI (10 μg/ml; 0.625 μM) (expression vector obtained from Swedish Orphan Biovitrum) (28) on ice for 30 min was added to a final concentration of 10% and incubated at 37°C for 30 min; (2) Bacteria, FH6-7/IgG fusion protein (at a final concentration of 0, 50, or 100 μg/ml) and OmCI-treated NHS (final concentration of 10%) were added simultaneously and incubated at 37°C for 30 min. Following incubation, bacteria were centrifuged and washed once in 1% (w/v) BSA/PBS. To determine serum FH binding, biotinylated Ab specific to CCP domain 5 of FH (MRC OX-24-biotin; 1:500) was used for 30 min at RT. Bacteria were washed once in 1% (w/v) BSA/PBS and stained with AF647-streptavidin (1:1000; Invitrogen) in the dark for 30 min at RT. To determine serum C4BP binding, Ab specific to CCP domain 4 of the alpha chains of C4BP (MK67; 1:1000) (29) was used for 30 min at RT. Bacteria were washed once in 1% (w/v) BSA/PBS and stained with AF-647 goat anti-mouse (1:1000; Invitrogen). Bacteria were centrifuged and washed once in 1% (w/v) BSA/PBS, resuspended in 150 μl PBS and serum FH or C4BP binding were assessed using a CytoFLEX flow cytometer.

Complement Deposition Assays

Bacteria were prepared as described in the binding assay section. For detection of complement components C3d and iC3b, bacteria, fusion protein, and OmCI-treated NHS (final concentration of 5%) were incubated for 30 min at 37°C. Bacteria were centrifuged and washed once in PBS and stained with either a monoclonal murine anti-human C3d Ab (1:1000, Quidel, A207) or a monoclonal murine anti-human iC3b Ab (1:1000, Quidel, A209) in 1% (w/v) BSA/PBS for 30 min at RT. For detection of MAC, NHS at a final concentration of 10% was used and bacteria incubated for 20 min at 37°C. Following washing, bacteria were stained with monoclonal mouse anti-human C9 neoantigen (1:1000, Hycult, aE11) for 30 min at RT. Bacteria were centrifuged and washed once in PBS followed by staining with AF-647 goat

anti-mouse, 1:1000; Invitrogen) for 30 min in the dark at RT. Bacteria were centrifuged and washed once in PBS, resuspended in 150 μl of PBS and deposited complement components detected using a CytoFLEX flow cytometer (Beckman Coulter).

Whole Blood Survival and Phagocytosis Assay

Moraxella catarrhalis were prepared as described in the binding assay section. *S. aureus* were grown as described on the bacteria section. Bacteria were normalized to $OD_{600\ nm}$ of 1, stained with CFSE (10 μM) for 30 min at 37°C and washed once in PBS.

Human blood was taken from healthy volunteers and treated with lepirudin (Refludan 50 μg/ml; Celgene). Five hundred microliters of blood were incubated for 30 min with 1 μM OmCI, 10 μM cytochalasin D (Sigma-Aldrich) or both, on an end-over-end shaker at 37°C and 5% CO_2. Approximately 5×10^5 CFU of CFSE-stained bacteria were added to blood, and blood suspensions were incubated on a shaker at 37°C and 5% CO_2.

To assess the survival of bacteria in blood, at specific time points a sample of the suspension was collected, serially diluted in PBS and plated onto chocolate agar plates (*M. catarrhalis*) or blood agar plates (*S. aureus*). Agar plates were incubated overnight at 37°C and 5% CO_2 and CFU/mL determined following enumeration of surviving bacteria.

To assess the phagocytosis of bacteria, at specific time points a sample of the suspension was collected, red blood cells lysis was performed by addition of water followed by addition of 10× PBS to restore osmotic pressure. Cells were centrifuged at 300 *g* for 5 min and fixed by incubation in 4% paraformaldehyde in PBS for 15 min at RT. Cells were stained with anti-CD14 APC antibody (Beckman Coulter) for 30 min, washed once in PBS and resuspended in PBS for analysis using a CytoFLEX flow cytometer.

Statistical Analysis

To examine differences between experimental groups, a one-way or two-way ANOVA was used (GraphPad Prism v7.0); a *p*-value was <0.05 was considered statistically significant. The *p*-values shown in figure legends represent the *post hoc* tests.

RESULTS

Fusion Proteins Bind to *M. catarrhalis*

The fusion protein FH6–7/IgG was created by combining the CCP6 and CCP7 domains of FH with the Fc region of human IgG (**Figure 1A**). C4BP1–2/IgM was made by fusing the CCP1 and CCP2 domains of C4BP together with the constant domains CH2, CH3, and CH4 of human IgM (**Figure 1B**). C4BP1–2/IgM forms multimers similarly to IgM, hexamers (dodecamers of C4BP CCPs 1–2; ∼80%) or pentamers (decamers of C4BP CCPs 1–2; ∼20%) (19).

To understand whether fusion proteins (FH6–7/IgG and C4BP1–2/IgM) could be used as alternative treatment strategies, we first explored whether these proteins could bind the well-characterized *M. catarrhalis* strain RH4. *M. catarrhalis* is known to bind both soluble complement inhibitors, FH and C4BP (24, 30). Fusion proteins incubated with bacteria over a range of concentrations confirmed that *M. catarrhalis* interacts with FH6–7/IgG (**Figure 1C**) and C4BP1–2/IgM (**Figure 1D**) in a dose-dependent fashion.

The ubiquitous surface proteins (Usp) A1 and A2 are high-molecular weight proteins that are abundantly expressed at the surface of *M. catarrhalis* (31). UspA2 plays an important role in immunity evasion as it can bind the complement inhibitors C4BP (24), vitronectin (32) and plasminogen (33). To test the possibility that the fusion proteins bind *M. catarrhalis* through UspA1 and/or UspA2, we used mutants lacking UspA1 and/or UspA2. The binding of FH6–7/IgG was reduced in the single mutant Δ*uspA2* and the double mutant Δ*uspA1*Δ*uspA2*, but not in Δ*uspA1*, compared to the wild-type (WT) strain (**Figure 1E**). A similar observation was made when assessing the binding of whole FH (**Figure 1G**), suggesting that the fusion protein FH6–7/IgG, like FH, binds partly through UspA2. The binding of C4BP1–2/IgM was unchanged in Δ*uspA1* compared to WT *M. catarrhalis*, but almost completely abrogated in Δ*uspA2* and Δ*uspA1*Δ*uspA2* (**Figure 1F**), similar to the binding of C4BP from NHS (**Figure 1H**). Therefore, the fusion protein C4BP1–2/IgM binds *M. catarrhalis* mainly through UspA2.

FH6–7/IgG but Not C4BP1–2/IgM Prevents Binding and Outcompetes Respective Soluble Complement Inhibitor

We investigated the interplay between fusion proteins and the ability of *M. catarrhalis* to recruit FH and C4BP to the bacterial surface. Firstly, we pre-incubated *M. catarrhalis* strain RH4 with increasing concentrations of FH6–7/IgG or C4BP1–2/IgM followed by the addition of OmCI-treated serum as a source of FH and C4BP. After incubation we examined the amount of FH or C4BP binding with antibodies specific to CCP domains present in serum-derived FH and C4BP, but not in the respective Fc fusion proteins. Preincubation of RH4 with FH6–7/IgG with concentrations as low as 1.5 μg/ml resulted in a significant reduction in FH binding from serum with an inverse correlation between FH6–7/IgG and FH binding continuing in a dose-dependent fashion (**Figure 2A**). Next, we assessed whether FH6–7/IgG could outcompete serum FH in binding to *M. catarrhalis* when added concurrently with NHS. Under these experimental settings, FH6–7/IgG also outcompeted FH binding from serum (**Figure 2B**). In contrast, no difference was observed in serum C4BP binding when bacteria were pre-incubated with increasing concentrations of C4BP1–2/IgM (**Figure 2C**). These data show that FH6–7/IgG can successfully displace FH at the surface of *M. catarrhalis*, while C4BP1–2/IgM does not out-compete serum C4BP, suggesting that C4BP binds the bacteria with a higher affinity than the fusion protein. Based on the observation that C4BP-IgM does not outcompete C4BP for binding to the bacteria, we tested whether C4BP-IgM could bind bacteria in the presence of serum (OmCI-treated). We found that the binding of C4BP-IgM is decreased when increasing concentrations of serum are added at the same time (**Figure 2D**), but there seems to be still

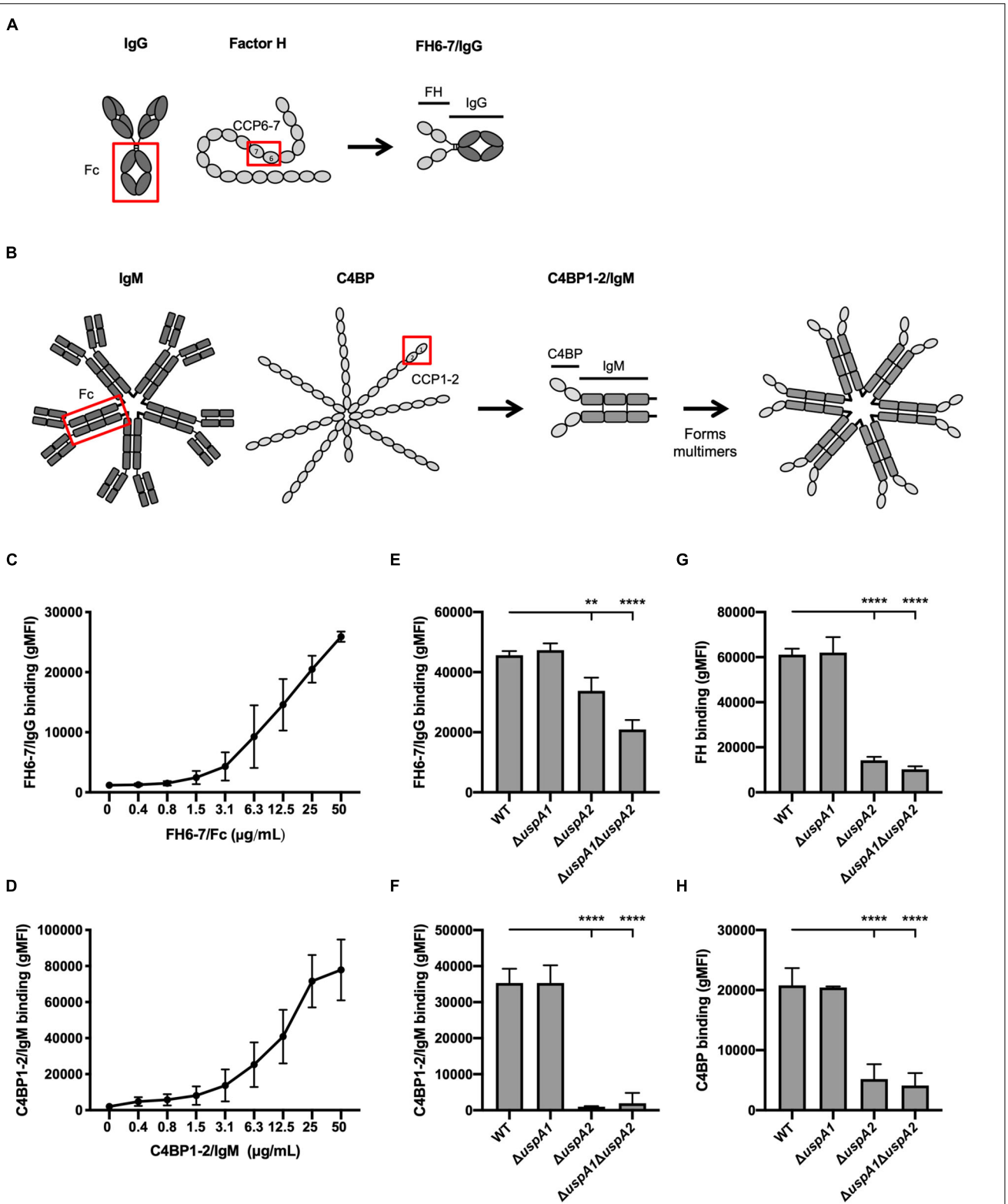

FIGURE 1 | FH 6-7/IgG and C4BP1-2/IgM bind to *M. catarrhalis*. **(A,B)** Schematic representation of fusion proteins FH6-7/IgG and C4BP1-2/IgM, respectively. **(C)** Binding of FH6-7/IgG and **(D)** C4BP1-2/IgM to *M. catarrhalis* RH4 [wild-type (WT) reference strain] assessed by flow cytometry. **(E–H)** Binding of RH4 WT and isogenic mutants lacking *uspA1* and/or *uspA2* to **(E)** FH6-7/IgG, **(F)** C4BP1-2/IgM, **(G)** factor H (FH), and **(F)** C4b-binding protein (C4BP). Mean (±SD) from three independent experiments are shown. Statistical significance of differences was calculated using one-way ANOVA with Dunnett's post-test **(E–H)**; $^{**}p < 0.01$ and $^{****}p < 0.0001$.

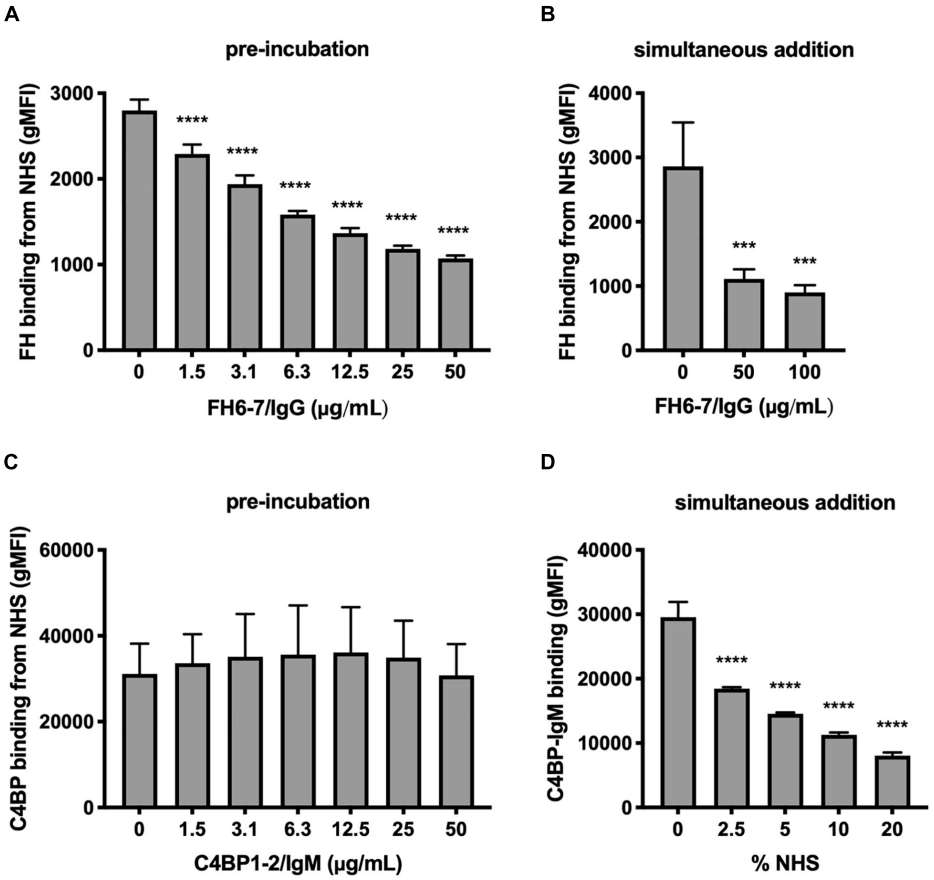

FIGURE 2 | FH6-7/IgG competes out serum FH, but C4BP1-2/IgM does not compete out serum C4BP. **(A)** *M. catarrhalis* were pre-incubated in increasing amounts of FH6-7/IgG, followed by the addition of OmCI-treated serum (C5-inhibitor). The binding of FH from NHS was assessed by flow cytometry using antibodies specific to domains present in serum derived FH but absent on the fusion proteins. **(B)** FH6-7/IgG and NHS were added simultaneously to the bacteria and binding of FH from NHS was assessed. **(C)** *M. catarrhalis* pre-incubated in increasing amounts of C4BP1-2/IgM, followed by OmCI-treated serum were tested for the binding of C4BP from NHS by flow cytometry using antibodies specific to domains present in serum derived C4BP but absent on the fusion proteins. **(D)** Alexa Fluor 488-labeled C4BP-IgM was added simultaneously with differing concentrations of OmCI-treated serum to bacteria to assess the binding of the fusion protein. Mean (±SD) from three independent experiments are shown. Statistical significance of differences was calculated using one-way ANOVA with Dunnett's post-test; $^{***}p < 0.001$ and $^{****}p < 0.0001$.

a significant binding in the presence of 10% NHS which is the amount used in serum bactericidal assays.

Serum Bactericidal Activity Is Enhanced by Fusion Proteins

Complement activation results in phagocytic clearance of bacteria as well as direct bacterial killing through the formation of lytic MAC. Gram-positive bacteria are not sensitive to MAC-mediated killing, but it plays a critical role against Gram-negative bacteria such as *M. catarrhalis* (34). In order to assess the respective roles of phagocytosis and MAC-mediated killing in clearance of *M. catarrhalis*, we employed a whole blood infection model, using human blood pre-treated with complement C5 inhibitor OmCI, phagocytosis inhibitor cytochalasin D or both. We observed that *M. catarrhalis* survival in whole blood is increased when complement is inhibited but not significantly changed when phagocytosis was inhibited (**Figure 3A**). However, phagocytosis of *M. catarrhalis* by granulocytes and monocytes remained unchanged by OmCI compared to untreated blood, while cytochalasin D inhibited phagocytosis as expected (**Figure 3B**). It is worth noting that this method does not distinguish between live and dead bacteria being taken up by phagocytes. This confirms that phagocytosis does not play a major role in *M. catarrhalis* killing in whole blood as it does not correlate with bacteria survival numbers: bacteria survive much better in OmCI-treated blood than untreated blood, however phagocytosis is unchanged. To confirm that our model works, and to highlight the difference between Gram-negative *M. catarrhalis* and a Gram-positive bacteria, we performed the same experiments in parallel with *S. aureus*. As expected, inhibition of phagocytosis increased the survival of *S. aureus*, while complement inhibition at the level of C5 did not (**Figure 3C**). Phagocytosis of *S. aureus* was similar to *M. catarrhalis*, as it was unchanged by OmCI but inhibited by cytochalasin D (**Figure 3D**). Altogether, this experiment confirms

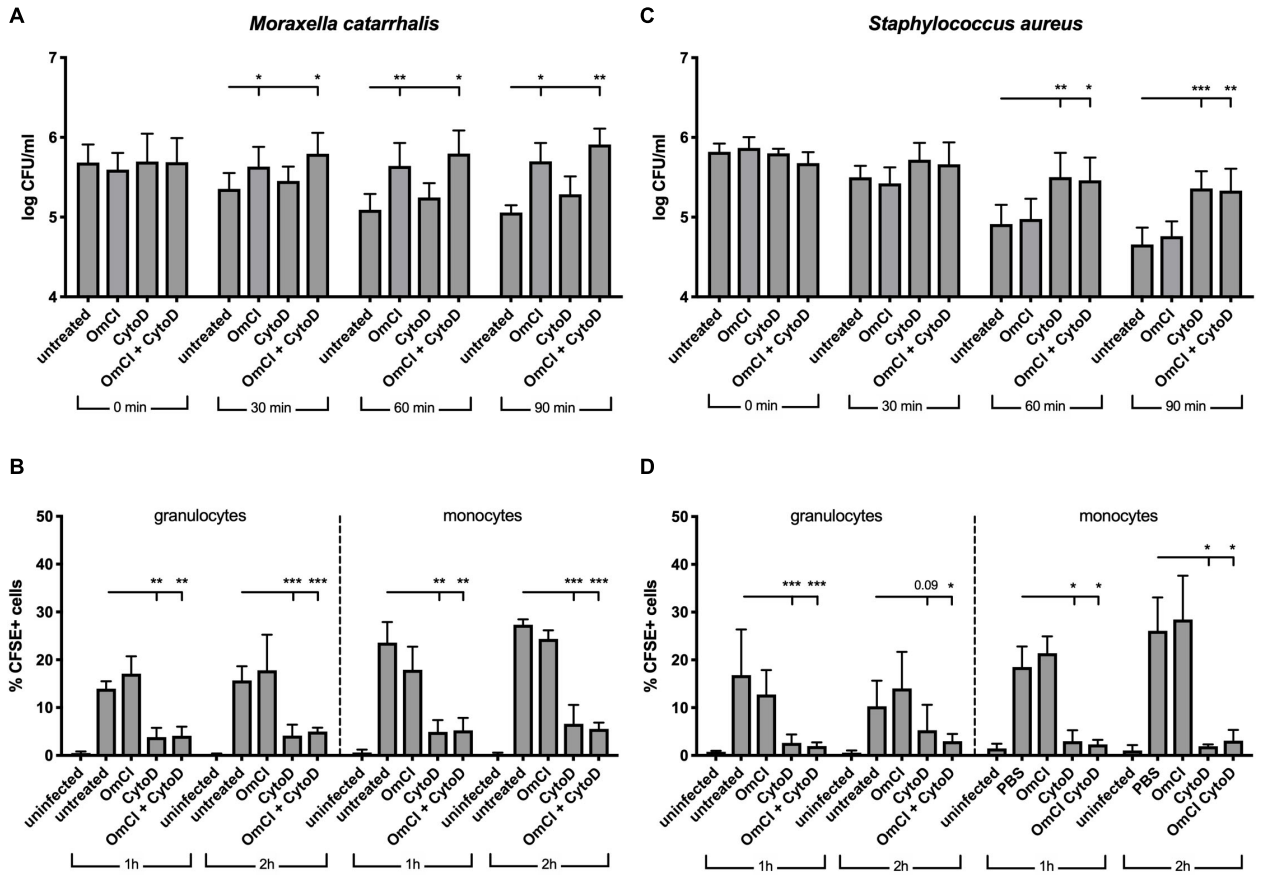

FIGURE 3 | In a whole blood model, *M. catarrhalis* is killed through MAC-mediated killing. CFSE-labeled bacteria were added to whole blood previously incubated with 1 μM OmCI, 10 μM cytochalasin D or both. **(A,B)** *M. catarrhalis* clinical isolate #473 was picked as more serum resistant than most clinical isolates and the reference strain RH4 **(C,D)** *Staphylococcus aureus* JE2 was used as an example of MAC-resistant bacteria **(A,C)** Survival of bacteria overtime was assessed by collecting a sample at t = 0, 30, 60, and 90 min post-infection, and plating bacteria on agar plates. The number of bacteria is represented as log of CFU/ml. **(B,D)** Phagocytosis was assessed by detection of CFSE+ cells among granulocytes and monocytes from whole blood. At 1 and 2 h post-infection, a sample was collected, red blood cells were lysed, cells were fixed and stained with anti-CD14, and cells were analyzed using flow cytometry. The percentage of CFSE+ cells was measured in granulocytes and monocytes (gated using Side Scatter and CD14 staining). Mean (±SD) from four **(A)** or three **(B–D)** independent experiments are shown. Statistical significance of differences was calculated using two-way ANOVA with Dunnett's post-test; *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

in an environment containing both complement and phagocytes, *M. catarrhalis* is killed through MAC-mediated lysis. Based on this, the effect of the fusion proteins in killing *M. catarrhalis* will be evaluated in serum alone.

Recruitment of FH and or C4BP to the bacterial surface interferes with complement deposition and contributes to serum resistance. Therefore, we tested the ability of FH6–7/IgG and C4BP1–2/IgM to augment serum killing. Both fusion proteins, when pre-incubated with *M. catarrhalis* at a final fusion protein concentration of 50 μg/ml, resulted in a significant reduction in bacterial survival (**Figure 4A**). C4BP1–2/IgM did not displace serum C4BP (**Figure 2C**), but still bound bacteria in the presence of serum (**Figure 2D**), and the hexavalent structure of the C4BP1–2/IgM fusion protein would most likely be a potent activator of the classical pathway of complement, explaining the reduced bacterial survival compared to no protein serum control. Regarding the enhanced FH6–7/IgG-dependent serum killing, both displacement of FH from the bacterial surface and Fc-mediated activation of the classical pathway were likely to have contributed to enhanced killing.

When fusion proteins were added with bacteria and serum simultaneously we observed a statistically significant decrease in survival with FH6–7/IgG and C4BP1–2/IgM (**Figures 4A,B**). However, the reduction is less apparent with C4BP1–2/IgM compared to FH6–7/IgG, likely because the C4BP1–2/IgM fusion protein does not prevent serum C4BP binding on the bacterial surface. Finally, we confirm that FH6–7/IgG enhances serum killing of strain RH4 in a dose-dependent fashion that requires active complement (**Figure 4C**).

FH6–7/IgG Enhances C3b and MAC Deposition

In order to understand how FH6–7/IgG increased serum sensitivity of *M. catarrhalis* we examined the deposition of complement components iC3b, C3d, and MAC in the presence

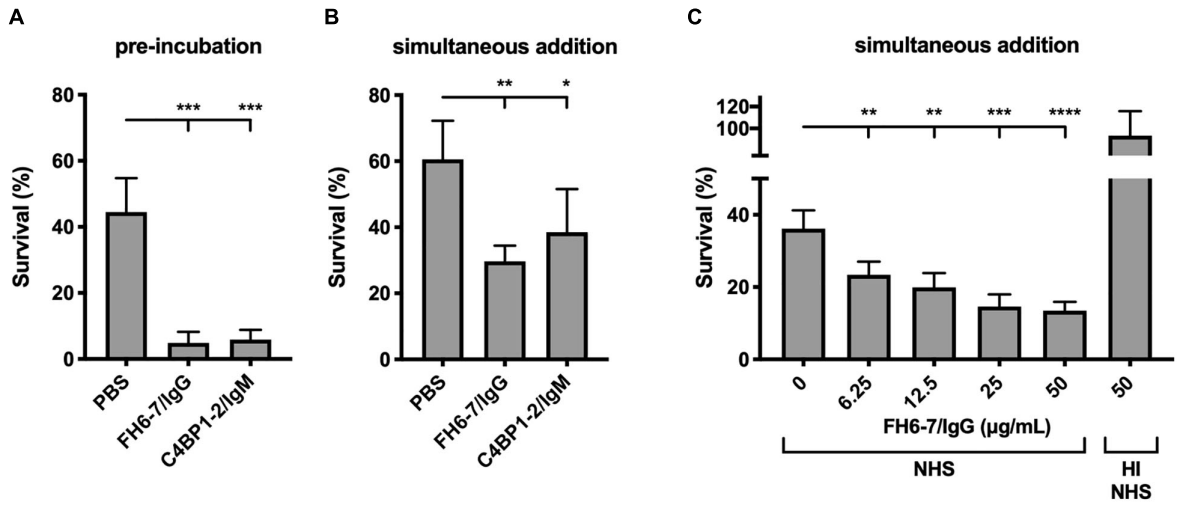

FIGURE 4 | FH6-7/IgG and C4BP1-2/IgM increase serum killing of *M. catarrhalis*. **(A)** Bacteria were pre-incubated with PBS (control), 50 μg/ml of FH6-7/IgG or 50 μg/ml of C4BP1-2/IgM for 30 min before addition of 10% NHS. Bacteria were counted at $t = 0$ and $t = 30$ min post-addition of serum and survival percentage was calculated. **(B)** Fusion proteins and serum were added simultaneously to the bacteria, and bacteria survival was calculated. **(C)** Increasing amounts of FH6-7/IgG were added simultaneously with NHS to bacteria and survival was calculated. Heat-inactivated NHS was used as a negative control of bacteria killing. Mean (±SD) from three **(A,C)** or four **(B)** independent experiments are shown. Statistical significance of differences was calculated using one-way ANOVA with Dunnett's post-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; and $^{****}p < 0.0001$.

and absence of fusion protein using flow cytometry. We choose to investigate both iC3b and C3d as markers of C3b deposition due to our previous experience with non-specific binding and anomalies with Ab detection of cleaved complement components. In accordance with the serum bactericidal data, the presence of FH6–7/IgG resulted in a significant increase in iC3b, C3d, and MAC deposition compared to no FH6–7/IgG control (**Figures 5A–C**). In contrast, no significant differences in complement deposition were observed when C4BP1–2/IgM was simultaneously added with serum, likely because this assay is not sensitive enough to detect a smaller increase in complement deposition (**Figures 5D–F**). However, an increase in complement deposition is observed when bacteria were pre-treated with C4BP1–2/IgM, in accordance with the increase in serum killing (**Figures 5G–I**).

FH6–7/IgG Binds a Broad Panel of *M. catarrhalis* Clinical Isolates Resulting in Enhanced Serum Killing

To ascertain whether FH6–7/IgG could be used as a novel immunotherapeutic we investigated the activity of this fusion protein against a panel of genetically diverse clinical isolates of *M. catarrhalis*. These isolates represent a broad diversity of virulent *M. catarrhalis* strains, and several UspA types: UspA2H (like RH4), UspA2 NTER2A or NTER2B. Both fusion proteins bind RH4 through the adhesin UspA2 (**Figures 1E,F**), therefore it is of interest to know whether different UspA2 types can also bind FH6–7/IgG and C4BP1–2/IgM. All *M. catarrhalis* clinical isolates bound FH6–7/IgG, similarly to our lab strain RH4, regardless of their UspA type (**Figure 6A**). This binding of FH6–7/IgG fusion protein correlated with increased serum killing of a majority of the *M. catarrhalis* clinical isolates independently of their UspA type, when fusion protein and serum were added simultaneously (**Figures 6B–D**). This shows that FH6–7/IgG could constitute a promising therapeutic strategy in a large number of *M. catarrhalis* infections.

C4BP1–2/IgM Binds a Broad Panel of *M. catarrhalis* Clinical Isolates Resulting in Enhanced Serum Killing

Similarly, we next investigated the activity of C4BP1–2/IgM against a collection of genetically diverse *M. catarrhalis* clinical isolates. Surprisingly, all clinical isolates bound C4BP1–2/IgM much less than our reference strain RH4 (**Figure 7A**). To test whether this lower binding could be due to the fact that these isolates bind C4BP less than RH4, we assessed the binding of C4BP from serum to these isolates. With the exception of KR510, we observed significant correlation between the binding of C4BP1–2/IgM and C4BP from serum (**Figure 7B**). In addition all clinical isolates bound C4BP less than the laboratory strain RH4 (**Figure 7B**). Although KR510 bound the fusion protein poorly, it bound C4BP very strongly, suggesting that it may bind C4BP through domains other than CCP1 and 2, the only C4BP domains present in the fusion protein. Based on the following observations; (1) C4BP1–2/IgM cannot prevent the binding of C4BP from serum; (2) *M. catarrhalis* clinical isolates bound C4BP1–2/IgM more poorly than RH4; and (3) C4BP1–2/IgM was less efficient at enhancing RH4 serum killing when simultaneously added (**Figure 4B**); we investigated serum survival of clinical isolates pre-treated with C4BP1–2/IgM. Although clinical isolates bound C4BP1–2/IgM more poorly than RH4, we observed an increased killing of the majority of

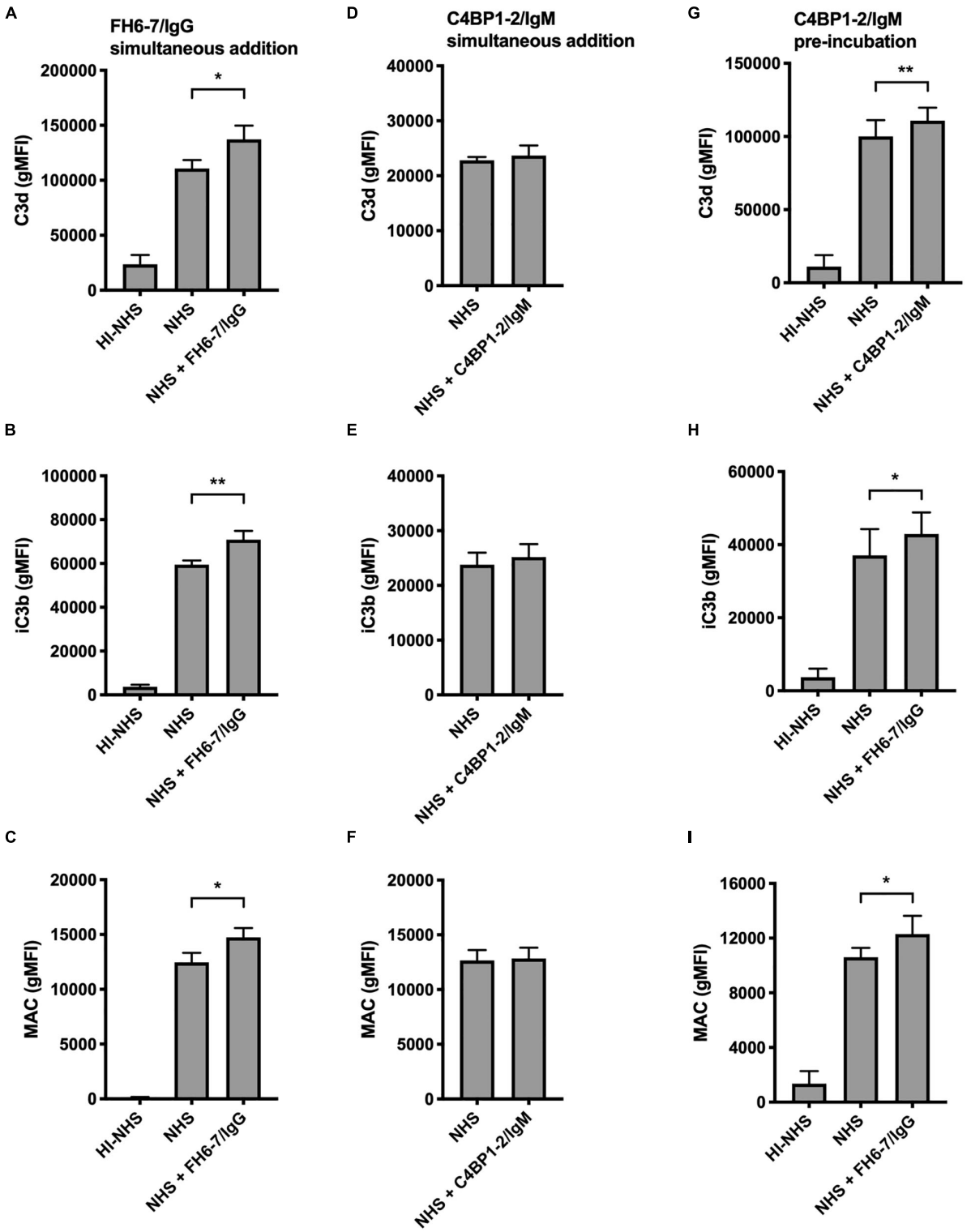

FIGURE 5 | Fusion proteins increase complement deposition on the bacterial surface. Flow cytometry was used to measure C3d, iC3b, and MAC binding at the surface of *M. catarrhalis*. **(A–C)** FH6-7/IgG and serum were added simultaneously to bacteria. Bacteria were incubated with 5% OmCI-treated serum for 30 min for C3 deposition, or 10% NHS for 20 min for MAC deposition. Heat-inactivated serum was used as a negative control of complement activation. **(D–F)** C4BP1-2/IgM and serum were added simultaneously to bacteria. **(G–I)** Bacteria were pre-incubated with C4BP1-2/IgM before addition of serum. Mean (±SD) from three **(A–F)** or four **(G–I)** independent experiments are shown. Statistical significance of differences was calculated using one-way ANOVA with Dunnett's post-test; $^{*}p < 0.05$; $^{**}p < 0.01$.

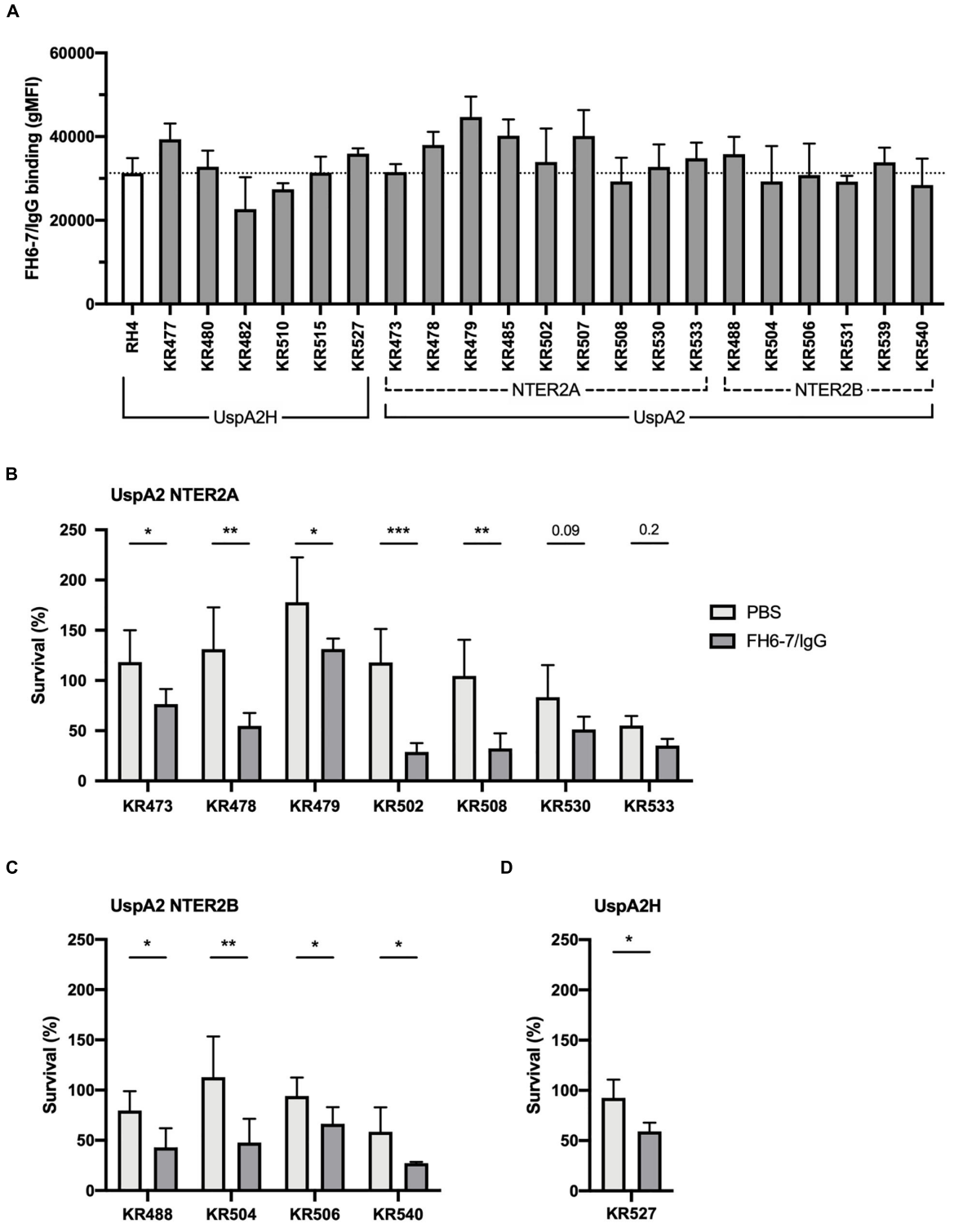

FIGURE 6 | FH6-7/IgG binds to and increases serum killing of a large majority of *M. catarrhalis* clinical isolates. **(A)** 21 clinical isolates and the laboratory strain RH4 were tested for FH6-7/IgG binding by flow cytometry. In the absence of FH6-7/IgG, bacteria incubated with detection antibody had a similar gMFI for all isolates (average 151, standard deviation 45). **(B–D)** Twelve clinical isolates were treated with 50 μg/ml of FH6-7/IgG or PBS, at the same time as 15% NHS, and their survival after 30 min was assessed by plating bacteria and counting remaining colony forming-units (CFU). The remaining nine isolates were not included because their survival in serum in the absence of fusion protein was less than 5%. **(B)** UspA2 NTER2A strains, **(C)** UspA2 NTER2B strains, **(D)** UspA2H strains. Mean (±SD) from three **(A–C)** or four **(D)** independent experiments are shown. Statistical significance of differences was calculated using two-way ANOVA with Holm–Sidak's post-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

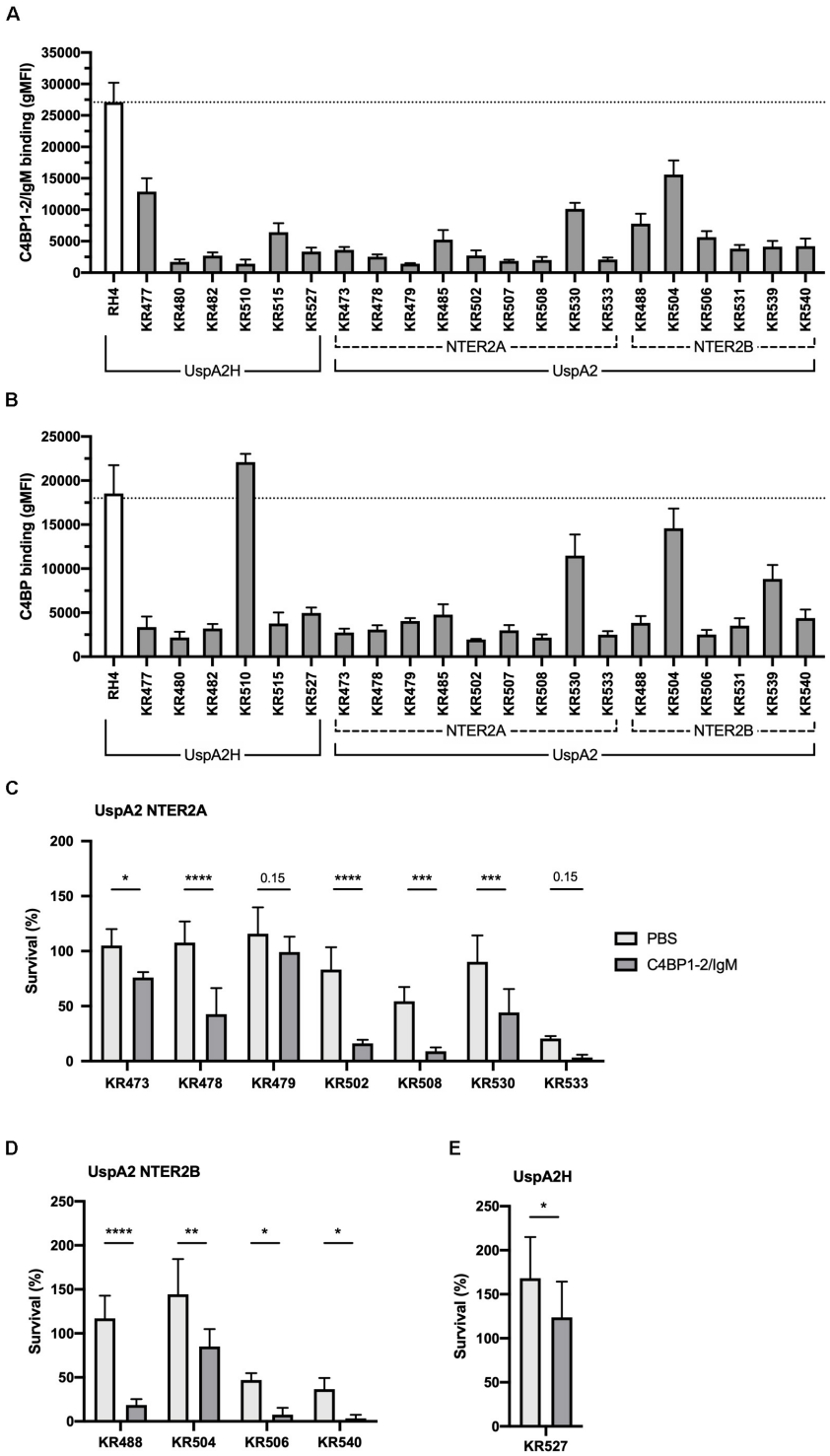

FIGURE 7 | C4BP1-2/IgM binds to and increases serum killing of many of the *M. catarrhalis* clinical isolates tested. **(A)** 21 clinical isolates and the lab strain RH4 were tested for C4BP1-2/IgM binding by flow cytometry. The gMFI for control without C4BP1-2/IgM was similar in all isolates (average 152, SD 75). **(B)** Clinical isolates were tested for C4BP binding from NHS by flow cytometry. Moraxella isolates were treated with 5% NHS + OmCI for 30 min. The gMFI for control without NHS was similar in all isolates (average 207, SD 132). **(C–E)** Twelve clinical isolates were treated with 50 μg/ml of C4BP1-2/IgM or PBS, before addition of as 15% NHS, and their survival after 30 min was assessed by plating bacteria and counting remaining CFU. The remaining nine isolates were not included because their survival in serum in the absence of fusion protein was less than 5%. **(C)** UspA2 NTER2A strains, **(D)** UspA2 NTER2B strains, **(E)** UspA2H strains. Mean (±SD) from three **(A)** or four **(B–E)** independent experiments are shown. Statistical significance of differences was calculated using two-way ANOVA with Holm–Sidak's post-test; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

M. catarrhalis isolates when they were pretreated with C4BP1–2/IgM (**Figures 7C–E**).

DISCUSSION

The common respiratory pathogen *M. catarrhalis* causes significant morbidity and economic burden because it is responsible for otitis media in children and exacerbations of chronic obstructive pulmonary disease in older individuals (2, 3). Currently, there is no vaccine to prevent *M. catarrhalis* infection. Given that otitis media is the most common reason for which children receive antibiotics (35), treatment of *M. catarrhalis* disease with antibiotics promotes antibiotic resistance and also disrupts normal commensal microbiota (1). Therefore, the development of novel adjunctive anti-infective immunotherapeutics against opportunistic pathogens such as *M. catarrhalis* would be highly beneficial.

Complement is present and active in both anatomical regions in which *M. catarrhalis* causes disease. In the ear, complement cleavage activation fragments of C3, C4, and Factor B have been detected in patients with otitis media with effusions (36). In a separate study, the cytolytic activity and presence of the MAC was observed in middle ear effusions obtained from persistent otitis media infections (37). In addition, C3-coated bacteria were identified in a small number of middle ear exudates originating from children suffering from acute otitis media (38). The role of complement in preventing middle ear infection has been demonstrated. Complement depletion by use of cobra venom factor restored the capacity of two avirulent NTHi strains to cause otitis media in a chinchilla model of infection (39). In the lungs, local synthesis of complement occurs; pulmonary alveolar type II epithelial cells secrete complement proteins C2, C4, C5, and Factor B (40), while bronchiolar epithelial cells generate C3 and membrane bound complement regulatory proteins (41). Complement components have been detected in bronchial secretions isolated from guinea pigs. Importantly, enhanced titers of complement proteins were observed following intranasal infection of guinea pigs with mycoplasma, indicating a role for complement in tackling lung infections (42). Moreover, intranasal administration of FH6–7/IgG enhanced clearing of NTHi in a mouse model of lung infection highlighting the therapeutic potential of this fusion protein (20).

In this study, we showed that fusion proteins, FH6–7/IgG and C4BP1–2/IgM, constitute novel antimicrobial strategies effective in eliminating *M. catarrhalis*. In particular, FH6–7/IgG bound all tested *M. catarrhalis* clinical strains taken from a cohort of genetically diverse isolates. Importantly, FH6–7/IgG significantly increased serum killing of the vast majority of tested isolates. Our data suggests that enhanced killing was due to a combination of (i) preventing the recruitment of the soluble complement inhibitor FH to the bacterial surface via displacement, while (ii) simultaneously promoting complement deposition by activation of the classical pathway via the exposed IgG Fc region.

Incubation of *M. catarrhalis* with C4BP1–2/IgM also resulted in decreased bacterial survival, although this was less efficient than FH6–7/IgG when added simultaneously with NHS. This is most likely due to the inability of C4BP1–2/IgM to outcompete and displace C4BP recruited from serum. We showed that C4BP1–2/IgM interacts with *M. catarrhalis* through UspA2, similarly to C4BP and FH. C4BP is a large glycoprotein containing seven identical alpha chains housing CCP domains, which are integral for complement inhibition (43). Previous work has illustrated that the binding region responsible for UspA2-C4BP interaction is localized to the CCP2, CCP5, and CCP7 domains of the alpha chain (24). We show that *M. catarrhalis* has the capacity to concomitantly bind C4BP and C4BP1–2/IgM. As a result, bound C4BP can continue to inhibit complement activation in the presence of C4BP1–2/IgM, thus preventing significant bacterial killing. Our results suggest that the bactericidal activity of C4BP1–2/IgM depends on which factor, C4BP1–2/IgM or serum derived C4BP, interacts first with the pathogen *in vivo*, as pre-incubation of the bacterium with C4BP1–2/IgM significantly accelerates serum killing.

Importantly, previous work has shown using *ex vivo* assay and *in vivo* animal models that these fusion proteins do not cause unwanted complement activation or tissue damage. Bettoni et al. (19) confirmed that C4BP1–2/IgM does not deposit complement on human erythrocytes or apoptotic cells. Importantly, this fusion protein lacks the CCP3 domain of C4BP which is required for binding to C4b fragments on the cell surface and therefore interaction of C4BP1–2/IgM with C4b-decorated cells is prevented. Furthermore, the authors showed that C4BP1–2/IgM was effective in preventing gonococcal colonization in a mouse vaginal colonization model using human FH/C4BP transgenic mice. Importantly, no adverse side effects following administration of C4BP1–2/IgM to mice was observed over the duration of the experiment. Blom et al. (22) confirmed that FH6–7/IgG does not enhance classical or AP activation measured via sensitized sheep and rabbit erythrocyte hemolysis assays. In addition, using a platelet aggregation assay, FH6–7/IgG did not activate platelets and no degree of coagulation activation was observed. In addition, FH6–7/IgG was effective in reducing mortality in a streptococcal sepsis model of infection using human FH/C4BP transgenic mice and showed no adverse side effects following administration. Specifically the authors noted no enhanced complement deposition in tissues sections taken from the eyes and kidneys of FH6-7/IgG treated and untreated animals, either infected or not with *S. pyogenes* (22). Lastly, Shaughnessy et al. (44) also observed no short term renal (increases in creatinine) or hematologic (increases in lactate dehydrogenase or decrease in hematocrit) or adverse side effects following systemic administration of FH6–7/IgG. Although this data is encouraging, future toxicology assessments are required and will take place during the preclinical development of these fusion proteins.

Alternative treatments such as fusion proteins could reduce the use of antibiotics, which are associated with many adverse effects such as the development of antibiotic resistance and the destruction of normal respiratory tract microbiota. Importantly, developing new treatment approaches based on components used by bacteria to confer serum resistance will ease or eliminate the selective pressure required to drive resistance against these therapeutics. We show that binding of both fusion proteins (FH6–7/IgG and C4BP1–2/IgM) to *M. catarrhalis* relies heavily

on the expression of Usp proteins, particularly UspA2. UspA2 is a non-fimbrial surface protein (45), highly conserved across disease isolates (46) and expressed during *in vivo* infection (47). Resistance to fusion proteins would require the emergence of UspA2 null mutants or UspA2 mutants unable to interact with FH6–7 and/or C4BP1–2 domains of circulating FH and C4BP, respectively, resulting in the reduced ability to recruit these soluble regulators. UspA2 plays even more roles in complement evasion as in addition to FH and C4BP, it also binds vitronectin, a multifunctional glycoprotein which can prevent the formation of MAC on the bacterial surface (32), and plasminogen, a zymogen which when converted to plasmin can degrade central complement components (33). Interaction of UspA2 with these glycoproteins adds extra pressure to maintain the WT UspA2 protein sequence and/or conformation. An inability to bind C4BP, FH, vitronectin and plasminogen due to loss of UspA2 would pose a significant fitness disadvantage promoting eradication by complement activity (24, 32, 48, 49).

Moraxella catarrhalis is both a normal commensal and a pathogen of the respiratory tract (50), and although little is known about the differences between the two or how it switches from one to the other, preserving the healthy microbiota when targeting pathogenic bacteria is critical. Microbial community composition has a profound impact on human health, protecting against the growth and invasion of respiratory pathogens (51), helping with the development and maintenance of a healthy immune system (52), but also preventing neurological diseases such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis (53). *M. catarrhalis* strains isolated from patients with bronchopulmonary infection were more resistant to complement-mediated killing than strains harbored by healthy carriers (89% vs 41.5%) (54). Furthermore, clinical studies have revealed that the increased prevalence of *M. catarrhalis* infections in the winter is due to the exposure to reduced environmental temperature in the nasopharynx (cold shock response), which increases the expression of virulence factors, including UspA2 (55). Cold-shocked bacteria therefore can bind more C4BP and FH through UspA2 and have an increased resistance to complement. Targeting strains that are able to bind complement inhibitors may thereby select between pathogenic strains and strains part of healthy microbiota.

The development of complement-activating antibodies targeting bacteria represents a promising strategy for antibacterial therapies but is limited by the need to raise specific antibodies against conserved, abundant surface proteins, which are expressed during infection. Such ubiquitous protective epitopes are very difficult to identify, because of the considerable antigenic and phase variability between strains within a species. Making monoclonal antibodies that recognize a wide array of strains and are functional is therefore extremely challenging. The fusion proteins outlined in this study bypass this restriction by behaving as soluble complement inhibitor decoys, which are bound by the majority of clinical isolates. Noteworthy, binding of complement inhibitors is an evasion strategy developed by many different pathogens, from fungi such as *Aspergillus* spp. and *Candida albicans* (56–58), parasites such as *Plasmodium falciparum* (59), and many bacterial species. Among these bacteria, FH6–7/IgG fusion protein successfully enhanced the killing of *H. influenzae* (20), *N. meningitidis* (18), *N. gonorrhoeae* (21), and *Streptococcus pyogenes* (22), while C4BP1–2/IgM strongly enhanced the killing of *N. gonorrhoeae* (19). Therefore, fusion proteins can prove very useful in the treatment of large variety of infections with this study highlighting their effectiveness in enhancing serum killing of *M. catarrhalis*.

CONCLUSION

In conclusion, we have shown that fusion proteins combining bacteria-binding sequences of complement inhibitors and Fc parts of immunoglobulins constitute a novel therapeutic approach against the human pathogen *M. catarrhalis*. Targeting such a key immune evasion strategy evolved by bacteria allows us to treat infection without triggering antibiotics resistance, and can be extended to a large variety of human pathogens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the ethical committee in Lund (Permit 2017/582). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML, LC, SB, DE, SR, and AB designed the experiments. ML, LC, SB, and KM conducted the experiments. ML, LC, DE, and AB analyzed the data. KR provided clinical isolates and mutants. ML, LC, and AB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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Tuning the Functionality by Splicing: Factor H and Its Alternative Splice Variant FHL-1 Share a Gene but Not All Functions

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The alternative pathway regulator Factor H-like protein 1 (FHL-1) is composed of the first 7 N-terminal complement control protein domains of Factor H (FH) and protects host surfaces from uncontrolled complement attack. Although FHL-1 shares the N-terminal regulatory domains with FH, it was thought to be a weaker regulator. Recently, the regulatory activity of FHL-1 was shown to be comparable to FH. Nonetheless, the question remained whether FHL-1 is an indispensable, unique regulator. The discovery that FHL-1 is the predominant regulator on Bruch's membrane, a critical site for the onset and progression of age-related-macular degeneration (AMD), showed that FHL-1 is essential for complement regulation. A common single nucleotide polymorphism in FH/FHL-1 that predisposes for AMD underlines the important role of FHL-1 in this context. Reports that some cancer tissues specifically upregulate FHL-1 expression, thereby evading immune surveillance, suggests a pronounced regulatory activity of the splice variant. Several microorganisms specifically recruit FHL-1 to evade complement attack. From a phylogenetic point of view, FHL-1 appears much later than other complement regulators, which could imply a specific role that is possibly not systemic but rather tissue specific. This review focuses on the current knowledge of FHL-1 and its physiological and pathophysiological roles.

Keywords: complement system, factor H, Factor H-like protein 1, cell protection, regulatory selectivity

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INTRODUCTION

The complement system is an essential part of the innate immune system protecting against infections and helping in maintaining tissue homeostasis. While the classical and lectin pathways are activated specifically, the activation of the alternative pathway (AP) occurs indiscriminately by spontaneous hydrolysis of C3, yielding C3(H_2O) (1). C3b produced by any initiating pathway in turn becomes amplified further by the AP amplification loop. This indiscriminate generation and surface deposition of C3b necessitates precise regulation to specifically downregulate AP amplification on self-surfaces. Therefore, only foreign, dangerous or impaired host surfaces allow unlimited or under-regulated AP activity. To protect themselves from AP-mediated attack, human cells and surfaces are equipped with preformed regulators of defense, that is, complement regulatory

proteins (2). These are membrane-bound regulators and soluble plasma proteins that normally control consumptive complement activation in the fluid phase. In addition, the soluble regulators are equipped with domains that specifically recognize polyanionic surface markers that are specific for host structures. Thereby, these soluble regulators intensify the complement regulation by the membrane-bound regulators. In particular, the basement membranes in the eye and the kidney, which are exposed to the blood stream at the fenestration of the endothelium, appear particularly vulnerable to AP attack (3). Because basement membranes lack plasma-membrane bound regulators, their only means to protect themselves from AP attack is to recruit soluble regulators *via* exposing polyanionic host surface markers that attract the soluble regulators.

Factor H (FH) and Factor H-like 1 (FHL-1) are the only known negative fluid-phase regulators of the AP. FHL-1 was discovered by Schwaeble et al. (4) as a short transcript that was constantly expressed in the human liver and secreted into the blood stream. Because of the similarity to the FH N-terminus, it was assumed from the beginning that FHL-1 should share regulatory functionalities with FH. Later, it was clarified that this truncated form of FH with a molecular weight of 49 kDa originates from the *FH* gene by alternative splicing (5–7). The FH gene is located on chromosome 1q32 and is part of the Regulation of Complement Activation gene cluster (8, 9). FHL-1 is composed of the first seven N-terminal complement control protein (CCP) domains of FH but lacks the remaining 13 CCP domains (**Figure 1**). At its C-terminus, FHL-1 contains four unique amino acids (SFTL), which are derived from the alternative splicing and are encoded on exon 10 (6, 7). Remarkably, in mice, no evidence for an alternative FH splice variant was found (10).

With the discovery of the splice variant FHL-1 in humans, several questions arose as to the physiological relevance of this truncated FH version, the possible existence of new functional features in FHL-1, or the functional consequences on AP regulation introduced by the deletion of the 13 C-terminal FH domains (4, 11). However, to date, the physiological role of FHL-1 remains the subject of controversial discussion. This review considers the possible physiological origin of this molecule and summarizes the current knowledge of its regulatory activities as well as its physiological and pathophysiological roles.

MOLECULAR INSIGHTS INTO THE REGULATORY ACTIVITY OF FHL-1 IN COMPARISON TO FH

FH is known to display several functional properties, which are: binding C3b, competition with Factor B for C3b binding, co-factor activity (CA) for Factor I-mediated cleavage of C3b, decay accelerating activity (DAA) for the C3 convertase C3bBb, and host recognition through binding to specific polyanionic markers on the surface of host cells. C3b binding is a precondition for all complement regulatory activities of FH. Binding to C3 activation fragments was initially described with the discovery of FHL-1 and later assessed again by testing binding properties of

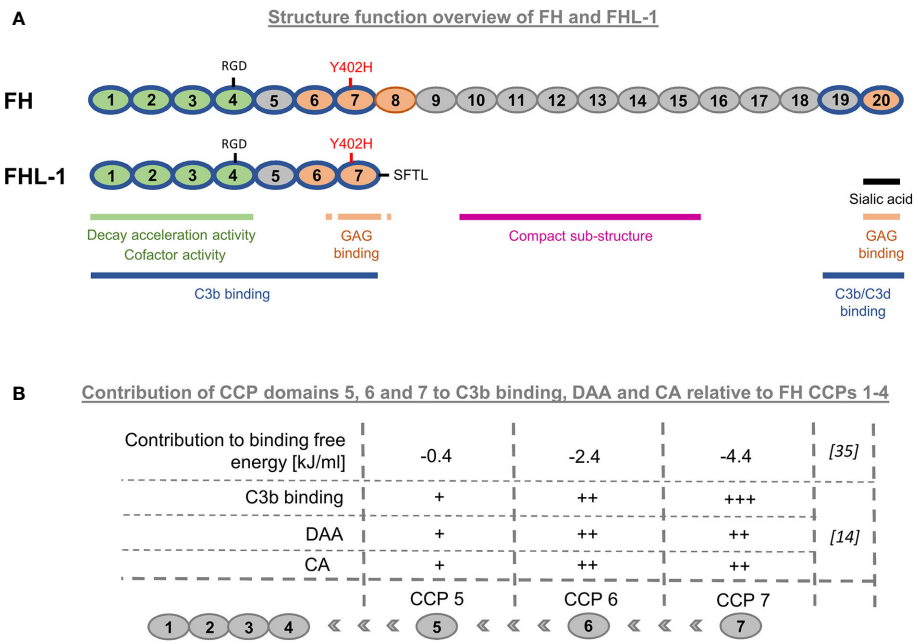

FIGURE 1 | Structure-function overview of Factor H-like-1. **(A)** Comparison of FHL-1 and FH. FH and its splice product FHL-1 share the amino acid sequence and functionalities of the first seven N-terminal complement control protein (CCP) domains. FHL-1 lacks important host recognition properties which are located in the C-terminal CCP domains 19–20 of FH. **(B)** Impact of individual CCP domains 5, 6 and 7 to CCP 1–4 for binding on C3b, decay accelerating activity (DAA), and cofactor-activity (CA).

recombinant FHL-1 and several fragments thereof to C3(H_2O) (4, 12). CCP1–4 were sufficient for C3(H_2O) binding, while further addition of the CCP domains 5, 6, and 7 increased the binding strength. This is in agreement with recent studies using surface plasmon resonance (SPR) for quantification of the binding affinity to C3b. The addition of the remaining CCP domains 5, 6, and 7 to CCP1–4 increased the affinity for C3b approximately 15 times, with an equilibrium dissociation constant of FHL-1 for C3b in the low micromolar range (~1 μM for FHL-1) (13, 14). By comparison, full-length FH displayed higher affinity for C3b (~0.6 μM), which is unsurprising, given that FH contains a second C3b binding patch within its two C-terminal domains 19–20 (15, 16). Some studies report a weak, third binding site for C3b located towards the middle of FH, while others could not detect it (15, 17). The difference between the binding affinity of FH and FHL-1 for C3b (and thus two *versus* one strong C3b-binding patch) appears less than expected. This may be explained by a complex structural model of FH, in which the C3b binding site in the C-terminal CCP domains is blocked or shielded by the N-terminal domains and only becomes accessible upon binding to polyanionic host surfaces marker. Several lines of evidence support the notion of such a structural FH model (13, 18–22). Through an absence of CCP domains 19–20, FHL-1 loses not only the second strong C3b binding site, but also the most critical host recognition site located in CCP20, which enables specific binding to glycosaminoglycans (GAG) and certain sialic acid-linked moieties (23–25). Therefore, the specificity for AP regulation on host surfaces for the splice variant FHL-1 could only derive from the GAG binding site located in CCP domain 7 (15, 26–29). On host-like sheep erythrocytes only FH CCPs 19–20 (carrying sialic acid and GAG binding properties), but not FH CCPs 6–8 (entailing only GAG binding functionality) exhibited functional competition with FH (24). A direct analysis of FH and FHL-1 regulatory capacity and surface specificity (host *versus* foreign) in FH/FHL-1-depleted serum revealed that both regulators protect foreign cell surfaces from complement attack at high concentrations, but FH was significantly more efficient in differentiating between foreign and host surfaces known to contain sialic acid moieties (14). This is in agreement with previous reports showing increased FH binding to sialylated surfaces (30, 31). These results show that the sialic acid (and GAG) binding site in CCP20 is much more important for self *versus* non-self discrimination than the GAG binding site in CCP7.

Misasi et al. (32) isolated FHL-1 from human plasma and demonstrated that it indeed harbors CA for the cleavage of C3b to iC3b (32). The precise determination of the involved CCP domains was demonstrated with experiments using FHL-1 and truncated fragments, with the result that the minimal functional unit to contain CA was CCP1–4 (33, 34). Similar to C3b binding, the successive addition of CCP domains to CCP1–4 enhanced the activity, while the addition of CCP6 to CCP1–5 had the largest impact on overall regulatory function (14, 35, 36). However, already in the fluid phase, when sialic acid binding does not even have a role in direct comparison to FH, FHL-1 appears to be a significantly weaker cofactor for Factor I-mediated C3b degradation (13, 14). Remarkably, this is also observed for the engineered versions of FH like miniFH. Although miniFH contains the N-terminal four and C-terminal two CCP domains of FH and thus exhibits a similar affinity for C3b as FH, miniFH is also a substantially weaker cofactor than FH (13, 14). This indicates that C3b affinity does not translate proportionally to CA. The structural complex of C3b with miniFH and Factor I revealed that only the two FH CCPs 2 and 3 directly contact Factor I (37). Processing of C3b by Factor I only occurs in ternary complexes of C3b with Factor I and a cofactor. Speculatively, FH with its two C3b binding patches is able to form a more stable ternary complex with C3b and Factor I than can FHL-1 or miniFH. This may be particularly true for the second cleavage of $iC3b_1$ into $iC3b_2$, because it is envisaged that the CUB (C1r/C1s–Uegf–Bmp1) domain of C3b begins to become partially unfolded upon consecutive Factor I cleavages (38).

Regarding the DAA of FHL-1, the literature is contradictory. FHL-1 was shown to act approximately 100 times weaker as a decay accelerator in comparison with FH when sheep erythrocytes deposited with C3 convertases were exposed to FH, FHL-1 or N-terminal fragments of FHL-1 (12). Nevertheless, it was shown unequivocally that FH CCPs 1–4 are the minimal requirement for fulfilling DAA. A recent SPR-based study, in which C3 convertases were formed on-chip, investigated the ability of FH, FHL-1 and FH-fragments to cause the decay of convertases. The outcome does not mirror the previous finding, because FHL-1 exhibited comparable DAA as full-length FH (14). The different outcomes could be attributed to differences in the assay. In the first report, the assay was performed on convertase pre-coated cells rather than in more defined (but less physiological) conditions on the SPR chip surface. However, because the solubility of FHL-1 in aqueous buffer at physiological pH and salt concentrations is rather limited, assays that require higher concentrations of FHL-1 stock solutions to be diluted into the assay conditions may need to be optimised for FHL-1 solubility (13, 14). In addition to specialized assays that investigate CA or DAA, recently the activities of FH and FHL-1 were directly compared in several serum assays. In these assays, the AP regulatory activities were nearly identical between the two protein variants when either FH or FHL-1 had been added to serum dilutions and consequently exposed to lipopolysaccharide-coated microtiter plates or complement vulnerable paroxysmal nocturnal haemoglobinuria cells (13). However, when FH and FHL-1 were compared on different self and non-self surfaces in FH/FHL-1-depleted serum, small difference in regulatory activity could be observed. FH was a slightly better regulator on self and self-like surfaces and a slightly worse regulator on foreign surfaces when compared to FHL-1. Additionally, although overall regulatory activity did not differ dramatically, it is obvious that FH does actively discriminate between self and non-self, whereas FHL-1 appears void of this capacity, at least for the surfaces tested (14).

To conclude, FHL-1 and FH share most of their complement regulatory features because they contain the first seven N-

terminal CCP domains. FHL-1 lacks the important host recognition site at the FH C-terminus that recognises sialic acid structures [and does not contain another sialic acid binding moiety within its seven domains (24)], explaining the lack of differentiation between host and foreign surfaces tested. Overall, as evaluated in several serum assays, FHL-1 exhibits comparable activity to FH in protecting host cells (14).

PHYSIOLOGICAL ROLE OF FHL-1

Continuous low-level activation of the AP by probing all surfaces requires constant readiness for action of the solvent based negative regulators, FHL-1 and FH, to avoid wasteful consumption of the complement components in the fluid phase and self-directed damage on host surfaces. The regulators must satisfy two major requirements: displaying regulatory activity and being sufficiently abundant. Although FH is more selective and has slightly higher activity on host surfaces than FHL-1, both display overall comparable regulatory activities (discussed above). Therefore, the answer to the question as to which of the variants is the main AP regulator lies in the relative abundance of the two proteins. The determination of the plasma FHL-1 concentration proved to be difficult because to date there is no (commercially) available monoclonal antibody which specifically detects FHL-1 but not FH. FH was consistently reported to occur in the circulation at a concentration of ~2–3 μM (39–41), whereas the blood FHL-1 concentration varied in several reports over a large concentration range. Friese et al. used an indirect enzyme-linked immunosorbent assay-based subtraction method in which the FHL-1 concentration was determined by detecting either both, FH and FHL-1, or only FH with appropriate polyclonal antisera. This resulted in a FHL-1 serum concentration of ~1 μM (42). In a recent report, the FHL-1 plasma concentration was determined using western blot analysis (14). Briefly, for semi-quantitative comparison, standards were prepared adding purified FH/FHL-1 proteins into FH/FHL-1-depleted serum to achieve different defined concentrations. The strength of these "reference" FH/FHL-1 bands was then semi-quantitatively compared with FH/FHL-1 bands derived from different donor sera. This determination method delivered serum concentrations for FHL-1 of approximately 0.04 μM (14) (**Table 1**). Schwaeble et al. reported similar FH and FHL-1 mRNA levels in the liver, but plasma protein concentrations are also driven by clearance from the circulation (4). Indeed, pharmacokinetic analysis of human FH and FHL-1 applied intravenously into mice demonstrated the splice variant to be cleared much more rapidly from the murine circulation (14). The rapid plasma clearance of FHL-1 and its resulting low plasma concentration indicate that FH is the major systemic regulator of the AP, although the regulatory activities are not dramatically different, making it increasingly unlikely that FHL-1 plays a major role in systemic AP control (14). These observations may also argue that the physiological role of FHL-1 lies in the protection of specific tissues. Although located on the same gene and sharing the same promotor as well as transcription start site, both molecules displayed distinct expression patterns in some tissues and cell lines (42–45). In accordance with this, a different molecular FH/FHL-1 ratio was also observed in Bruch's membrane, a layer of extracellular matrix positioned in the eye between the retinal pigment epithelium (RPE) and choroid blood vessels (46). FHL-1 was identified as the predominant AP regulator expressed by RPE cells. In contrast to FH, the splice variant is also able to diffuse from the choroid through the Bruch's membrane. In addition to the higher expression levels by the RPE, FHL-1 also appears to be supplied to the eye from the systemic blood compartment *via* diffusion through the Bruch's membrane, strongly indicating FHL-1 to be the major regulator at the RPE/Bruch's membrane interface (46, 47). It is envisaged that FHL-1 can localize to certain host surface structures *via* its GAG binding site in CCP7, thus, preventing uncontrolled complement activation on such surfaces (8, 48). These findings on FHL-1 are particularly relevant because Bruch's membrane is the site where drusen formation and tissue damage occur that are associated with the progressive eye disease age-related-macular

TABLE 1 | Comparison of FH and FHL-1.

Property	Protein		Reference
	FH	FHL-1	
Blood concentration [μM]	~2-3	0.04 (or up to ~1, see text)	(14, 40–43)
ß-phase plasma halftime of human proteins in mice [h]	18.3 ± 3.7	2.9 ± 0.5	(14)
K_D for C3b binding [μM]	~0.6	~1	(13, 14))
Cofactor activity (fluid phase)	++	+	(13, 14)
Decay accelerating activity (on SPR chip)	+	++	(13, 14)
Sialic acid binding (NMR saturation transfer technique)	+	none	(23, 24)
IC_{50} for PNH RBC protection [μM] (when added to FH/FHL-1-depleted serum)	1.4 ± 0.2	2.2 ± 0.5	(14)
IC_{50} for desialylated PNH RBC protection [μM] (when added to FH/FHL-1-depleted serum)	3.4 ± 0.8	2.2 ± 0.6	(14)
Deregulation by FHR-1	++	0 to +	(14)

degeneration (AMD)—the major cause of blindness in the older subjects in the western world (49). A polymorphism in CCP domain 7 (Y402H) is directly associated with an increased risk for developing AMD (50–53). Although the overall tertiary structure remains similar, the single nucleotide polymorphism (SNP) diminishes binding capability to heparan sulfate, which is the major GAG on Bruch's membrane and, therefore, the major interaction site for FHL-1 (8, 27, 28, 46, 48, 54). Of note, the same Y402H SNP within full length FH displayed none or only a marginal impact on GAG binding, whereas when within FHL-1, dramatic differences occurred, which can be explained by two *versus* just one GAG binding site being present, respectively (27). The proven loss and desulfation of heparan sulfate GAGs on Bruch's membrane with age and the resulting loss of binding sites for FHL-1 further supports the pathophysiological role of FHL-1, providing an explanation for the fact that particularly older people are affected (46, 55, 56).

PATHOPHYSIOLOGICAL ROLE OF FHL-1

In the further course of investigating the pathophysiological role of the Y402H SNP in CCP 7, several further binding partners have been implicated, but it appears that no uniformly accepted conclusion has to date been reached. Functional consequences of the Y402H SNP have been described for, including, among others, C-reactive protein, pentraxin-3, oxidation end products, and zinc ions (57–60). Knowing the importance of FHL-1 on Bruch's membrane, the question arose whether other membranes with a similar composition to that of Bruch's membrane, like the kidney glomerular basement membrane, are also predominantly controlled by FHL-1. However, it was shown that the GAG binding site in CCP19–20 of FH is mainly involved in the interaction within the kidney, indicating that different GAG signatures can exist at different basement membranes, thus questioning a role for FHL-1 at the kidney basement membrane (61).

In contrast to proteoglycan layers, which constitute the basement membranes and heavily rely on the soluble complement regulators in plasma, human cell plasma membranes express a mix of membrane-bound complement regulators, including, for example, CR1 (CD35), MCP (CD46), DAF (CD55) and/or CD59, and hence the soluble complement regulators function 'only' as an important addition to the membrane tethered regulators on cellular surfaces (2, 62, 63). However, some tumor cell lines were reported to use primarily FH/FHL-1 for complement evasion. H2-glioblastoma cells were shown to upregulate FH and FHL-1 expression, with overall higher expression levels of the splice variant (64). Increased amounts of FHL-1 were also synthesized by the ovarian cell lines SK-OV-3 and Caov-3 and were detected in their direct microenvironment (65). Increased FHL-1 levels could increase local control of complement activation.

Soluble complement regulators are also attractive targets of pathogens that recruit regulators to their surface to evade the complement immune surveillance mechanism. Different bacteria, fungi, and parasites have been identified to specifically capture FH [reviewed in (66)]. Remarkably, some pathogens preferentially or even exclusively recruit FHL-1 although the amino acid sequence is identical to that of FH, except for the unique four amino acid patch at the FHL-1 C-terminus. One example is the M-protein of some group A *Streptococcus* strains, which was shown to enable binding to the CCP-7 domains of FH/FHL-1 with higher affinity for FHL-1 (11, 67, 68). Other pathogens that recruit or even preferentially recruit FHL-1 for immune evasion are *Plasmodium falciparum* and the spirochete *Borrelia spielmanii*, respectively (69, 70). Moreover, McDowell et al. identified a small surface protein-exposed on *Treponema denticola*, a bacterium involved in periodontal disease, which appears to preferentially bind FHL-1 (71, 72). However, they demonstrated an FI-independent cleavage of C3b and suggested that the purpose of FHL-1 being recruited by *Treponema denticola* is adherence to human cells rather than immune evasion. Such specific adhesive properties for FHL-1 were reported previously by Hellwage et al. (73). Both, FH and FHL-1 bear the amino acid sequence RGD in CCP domain 4, a sequence that is also found in adhesive proteins, including vitronectin and fibronectin (74). In contrast to FH, only FHL-1 could act (when coated on a 'chamber slide') as a matrix for adherence and spreading of the tested cell lines by binding to integrin receptors, potentially allowing effectors cells to bind via their integrin receptors to the RGD motif of FHL-1 bound to C3b-opsonized surfaces bridging the humoral and cellular immune responses (73).

DISCUSSION

Over time, the perception of FHL-1 has changed because several studies showed that the splice variant is a unique molecule with many shared but also some unique properties compared to FH. But even now, the benefit of producing a truncated form of FH is not completely clear. For some specialized tissues, FHL-1 could be a tailor-made fluid phase regulator with specialized properties, for example, being able to diffuse through certain basement membranes. The importance of FHL-1 as a tissue-specific regulator may be supported by its relatively late appearance in evolution, indicating a possible coevolution of this splice variant with a specialized tissue that relies on the protection of FHL-1. An *in-silico* gene analysis revealed that similar gene structures to FH that allow for alternative splicing of a truncated FH version are not found prior to the order of old-world monkeys (14). Another topic of ongoing research, which directly relates to specialized FHL-1 functions, touches on the deregulation functionality of Factor H-related proteins (FHR). Because it lacks the C-terminal CCP domains of FH, FHL-1 was thought to be less prone to deregulation by FH-related proteins than FH. Additionally, although some competition between FHR molecules and FHL-1 for selected functions have been observed (75), in AP serum assays on host cells, the deregulation by FHR-1 was considerably less for the splice variant FHL-1 than for FH (14). However, future studies are

needed to further clarify the impact of deregulation of FH and its splice version by different FHRs. Future insights into how and why certain tissues modulate the splicing rates, and hence the relative expression levels of FH and FHL-1 will be fundamental in understanding the precise physiological role of FHL-1. As to why certain cancer types favor the relative expression of FHL-1 over FH, it can be speculated that by expressing FHL-1 an almost identical level of AP regulation can be achieved by using up much fewer resources, such that the energy for the production of 13 CCPs can be spared, which, however, comes at the expense of selectivity between self and non-self surfaces. To better define the role of FHL-1, it will also be important to identify further compartments that display altered FH/FHL-1 ratios compared to that in systemic circulation. To date, the synovial fluid expression levels of FH and FHL-1 in the settings of rheumatoid arthritis and the Bruch's membrane have been determined (46, 76). Other interesting body fluids in specialized compartments (which may not require as stringent a selectivity for AP regulation as the systemic circulation and hence might benefit from higher FHL-1/FH ratios) might, for example, be the cerebrospinal and the pleural fluids. However, research of FHL-1 remains challenging. Its low solubility in phosphate-buffered saline complicates the daily handling of many standard laboratory assays (13, 14). The absence of FHL-1 in mice and thus the lack of the opportunity to investigate the functional consequences of engineered FHL-1 knock-out mice further complicate the characterization and importance of the FH splice variant FHL-1. However, the recent findings that FHL-1 is almost as active in down-regulating the AP as FH, albeit being less selective for host tissues, in conjunction with FHL-1 being selectively expressed at higher levels in certain tissues, underline the unique role for the splice variant FHL-1, which in the future is expected to be more intensely studied and thus be better understood.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Function and Dysfunction of Complement Factor H During Formation of Lipid-Rich Deposits

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Complement-mediated inflammation or dysregulation in lipid metabolism are associated with the pathogenesis of several diseases. These include age-related macular degeneration (AMD), C3 glomerulonephritis (C3GN), dense deposit disease (DDD), atherosclerosis, and Alzheimer's disease (AD). In all these diseases, formation of characteristic lipid-rich deposits is evident. Here, we will discuss molecular mechanisms whereby dysfunction of complement, and especially of its key regulator factor H, could be involved in lipid accumulation and related inflammation. The genetic associations to factor H polymorphisms, the role of factor H in the resolution of inflammation in lipid-rich deposits, modification of macrophage functions, and complement-mediated clearance of apoptotic and damaged cells indicate that the function of factor H is crucial in limiting inflammation in these diseases.

Keywords: apoE, C-reactive protein, adiponectin, HDL, amyloid-beta- protein

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INTRODUCTION

A major function of the complement system is to handle invading microbes and clear debris, but without sufficient regulation it can attack and destroy our own cells and tissues. It can be activated through three pathways: the classical, alternative, and lectin pathways. The alternative pathway is constantly active in human plasma and responsible for amplifying all the complement activation cascades. To prevent potentially harmful complement attack toward host tissues the amplification pathway of complement is regulated by membrane inhibitors CD35, CD46, and CD55 and by soluble complement factor H and its alternatively spliced product factor H-like protein (FHL-1) (1). Factor H functions not only in the fluid phase to keep complement activation under control but also on surfaces to prevent attack against host targets. FHL-1 lacks the the ability to discriminate between self and nonself targets.

Factor H recognizes specific host markers directly, such as sialic acids and glycosaminoglycans, or indirectly *via* C-reactive protein (CRP) present or bound on self cell surfaces or apolipopoprotein E (apoE) on high-density lipoprotein particles (2–4). Binding to CRP is mediated *via* domains 6-8 and 19–20 of factor H, while domains 5–7 interact with apoE. Factor H usually binds to these structures in the context with surface-deposited C3b and acts as a cofactor for factor I-mediated inactivation of C3b to iC3b (1). From the 20 domains of factor H, the domains 7 and 19–20 mediate surface recognition, while domains 1-4 are required for regulatory activity (**Figure 1**). Several known mutations and polymorphisms in factor H and anti-factor H antibodies directed against these domains are associated with diseases that can be harmful for the carrier. This indicates that full function of factor H is essential in keeping the spontaneous alternative pathway activation in check and in preventing complement attack against self-structures (5–7).

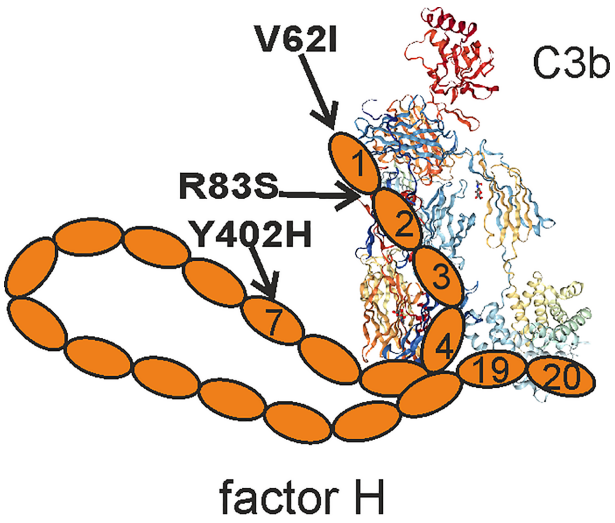

FIGURE 1 | Schematic presentation of factor H domain structure. Factor H domains 1–4 bind to C3b and regulate alternative pathway activation, while domains 19–20 are responsible for surface recognition. The positions of the disease-causing mutations discussed in this manuscript are indicated. C3b crystal structure is from PDB 5FO7.

Due to its ability to bind CRP and control the alternative pathway complement factor H has a central role in the non-inflammatory clearance of extracellular deposits and dying cells in areas of tissue damage (8, 9). Much of the material to be cleared includes various types of phospholipids from cell membranes. Failure in this clearance mechanism may lead to excessive inflammation because of complement activation and overstimulation of macrophages. As a consequence, macrophages can release free radicals that can oxidize lipids and other materials and eventually harm the local tissue. The role of complement is not only restricted to clear microbes and dead cells from human tissues, but it also has an important role in lipid metabolism. For example, complement expression levels are significantly elevated in visceral adipose tissue, and increased expression levels of *CFB* gene have been suggested to associate positively with triglyceride levels and negatively with high-density lipoprotein (HDL) levels in plasmas of obese individuals. This indicates that complement system is involved in the metabolic consequences associated with increased visceral fat mass (10, 11) and in diseases characterized by the presence of lipid-rich deposits.

DISEASES WITH LIPID-RICH DEPOSITS AND FACTOR H ASSOCIATION

Age-related-macular degeneration (AMD), C3 glomerulopathy (C3G) encompassing two different syndromes: C3 glomerulonephritis (C3GN) and dense deposit disease (DDD; previously called membranoproliferative glomerulonephritis type II; MPGN-II), atherosclerosis (AS) and Alzheimer's disease (AD) have similar histopathological features. They all are associated with accumulation of lipid-rich deposits in the retina (AMD), subendothelially in kidney glomeruli (C3GN), glomerular basement membrane (DDD), arterial intima (AS), or brain (AD). These deposits are called drusen in AMD, dense deposits in DDD, plaques in atherosclerosis, and senile plaques in Alzheimer's disease. Despite the different names the deposits share common features such as the presence of oxidized lipids and proteins, cholesterol and other lipids and apolipoproteins (12–14) (**Figure 2**). These diseases are also often affecting the same patient. As an example, individuals with DDD often develop ocular drusen in the macula, whose histopathology is indistinguishable from the drusen in AMD (15). Moreover, patients with atherosclerosis are at higher risk to develop Alzheimer's disease (16) similarly as AMD patients are at higher risk to develop atherosclerosis (17). These findings indicate that the development of the lipid-rich deposits could share a common pathophysiological mechanism. For example, alterations in vascular glycosaminoglycans that bind the complement inhibitor factor H could lead to both lipid accumulation and complement damage in atherosclerotic lesions (18). Finally, these diseases have been studied for their genetic association with the factor H Y402H polymorphism that is located in the domain seven of this molecule. This polymorphism was first found to associate with AMD (19–21). It confers a five-fold increased risk of developing the disease (22). Interestingly, the AMD risk variant 402H also associates with DDD, and possibly also with Alzheimer's disease and atherosclerosis, although conflicting results on the latter two have been found in different genetic studies (23–27).

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the most common cause of visual loss in the elderly people in industrialized countries. Accumulating evidence suggests that a defect in complement regulation by factor H and other abnormalities in the alternative pathway amplification process play a crucial role in the pathogenesis of the disease. The hallmark of early AMD is formation of drusen between the basal surface of the retinal pigmented epithelium (RPE) and Bruch's membrane. This leads later to necrosis of the RPE cells. Apart from the the genetic background, aging and smoking are the main risk factors for developing AMD (28).

Atherosclerosis

Atherosclerosis is a multifactorial disease driven by inflammation and vascular changes. It is caused by accumulation of lipids, immune cells and fibrous elements in the subendothelium of arteries. The thickening and hardening of the arterial wall may lead to a total obstruction of the blood vessel because of plaque rupture. A major critical site is the coronary arteries of myocardium, where a local infarction may occur. Rupture of coronary artery plaques has been shown to be associated with complement activation in myocardial infarction (29). Similarly, atherosclerosis may lead to cerebral infarction or be involved in the rupture of carotid aneurysms (30). The hallmark of early atherosclerotic lesion is the formation of fatty streaks composed of cholesterol-laden macrophages, which are called foam-cells. The foam cells are formed through macrophage-mediated phagocytosis of oxidized and modified low density lipoproteins (LDLs) that accumulate in the subendothelium of arteries. The local inflammation induces differentiation of monocytes into proinflammatory M1 type macrophages that play a crucial role in the pathogenesis of atherosclerotic plaques (31).

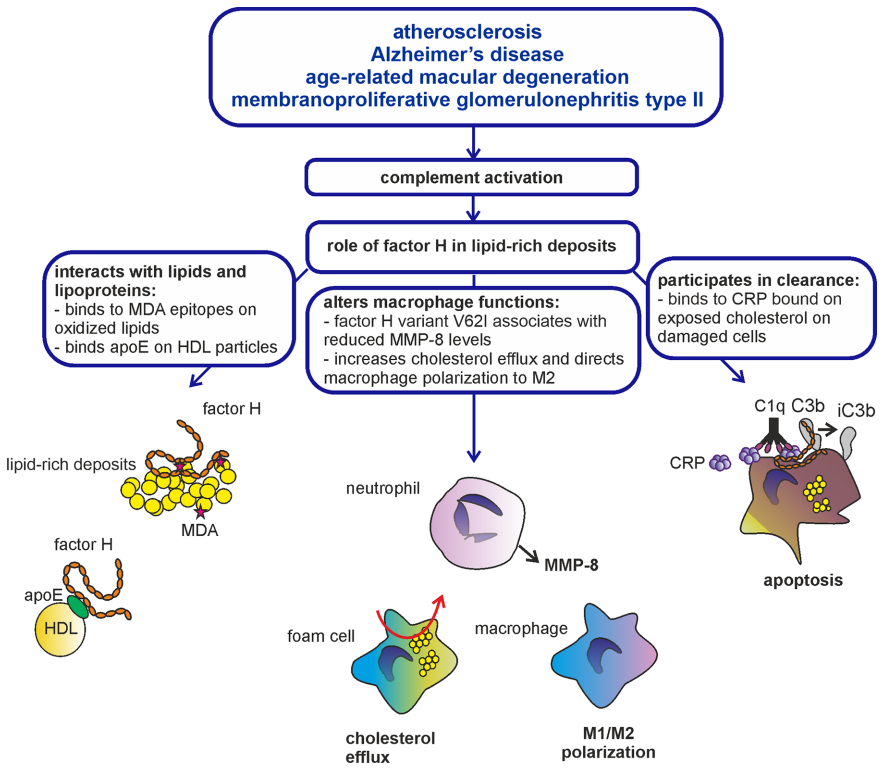

FIGURE 2 | Role of factor H in lipid-rich deposits. Atherosclerosis, Alzheimer'd disease, AMD, and C3G are diseases characterized by formation of lipid-rich deposits and complement-mediated inflammation. Complement regulator factor H is known to bind modified lipids and lipoproteins, interact with macrophages and participate in the clearance of damaged and apoptotic cells through acting as a cofactor for inactivation of C3b to iC3b. Therefore, factor H is likely involved in the resolution of inflammation in lipid-rich deposits accumulated in the arteries, brain, eyes, and kidneys.

It seems like the complement system and especially the alternative pathway of complement has a pro-inflammatory role in in atherosclerosis as it serves as an amplification mechanism for C3b generation (32).

Alzheimer's Disease

Alzheimer's disease is characterized by accumulation of amyloid-β, formation of neuronal plaques and neuroinflammation in the brain. Impaired clearance of amyloid-β by microglial cells leads to accumulation of senile plaques in the brain. These can activate the complement system that triggers the development of an inflammatory phenotype in microglia, the phagocytic cells in brain (33). The activated microglial cells trigger astrocytes, which amplify the pro-inflammatory signals and neurotoxic effects (34). Activated microglia are the main source of complement components such as C1q in the brain (35). Interestingly, the classical pathway of complement with its components C1q and C4 appear to contribute to the complement- and microglia-mediated loss of brain synapses in early Alzheimer's disease (36). Moreover, binding of C1q to apoE has been suggested to reduce C1q-mediated activation of the classical pathway implicating that apoE, the major factor related to Alzheimer's disease, acts as a complement inhibitor (37). It has, however, been suggested that the alternative pathway of complement could be responsible for the pro-inflammatory effects in the pathogenesis of Alzheimer's disease (38). Alzheimer's disease, AMD and atherosclerosis are all degenerative age-dependent diseases, where complement-mediated inflammation most likely plays a crucial role, as several markers of complement activation have been found in the diseased tissues (39, 40).

C3 Glomerulopathy

C3 glomerulopathy (C3G) entails two different diseases, where complement activation, and especially the alternative pathway, has a central role in disease pathogenesis (41). In DDD, C3b deposits accumulate in the glomerular basement membranes and can be seen as dense deposits by electron microscopy. In C3 glomerulonephritis (C3GN), no dense deposits are seen, but C3b accumulation occurs subendothelially and mesangially. In a proportion of cases, C3GN is associated with immunoglobulin paraproteins (42). In DDD, autoantibodies have been detected against against the C3bBb convertase, so called C3 nephritic factors (43) and against the N-terminus of factor H (6). C3GN and DDD are rare chronic nephritic diseases (44). DDD is more common in children. The inflammation in C3G is mediated *via* hyperactivation of complement that is triggered because of complement dysregulation in the fluid phase. DDD sometimes occurs in association with partial lipodystrophy (PLD). It is characterized by loss of subcutaneous fat from the upper part of the body. It has been suggested that complement-mediated

lysis of factor D expressing adipocytes is induced by C3 nephritic factor (C3Nef) in this disease (45). Moreover, in one study, a family with DDD and PLD was found to carry an R83S mutation in factor H in the interface region between domains one and two causing a defect in complement regulation. This indicated a role for alternative pathway dysregulation in the pathogenesis of this disease (46). Several complement components are expressed by adipose tissue including C2, C3, C4, C7, factor B, factor D (D, adipsin), FH, and adiponectin (10, 11, 47, 48). It has been suggested that complement is involved in mediating inflammation in the adipose tissue, but this is maintained only at a reasonable level due to expression of complement regulatory proteins, including factor H (49). Therefore, although not yet shown, a defect in factor H mediated complement regulation could also be involved in the loss of adipose tissue in DDD-related PLD.

LIPID-RICH DEPOSITS

According to current knowledge, inflammation is the key driver in the formation of lipid-rich deposits in AMD, C3G, atherosclerosis, and Alzheimer's disease. However, the initial trigger has not yet fully been defined except in rare cases, where carrying a mutation can be directly linked to the disease in the family (46). Conversely, lipid deposits may also undergo changes and modifications that convert them into promoters of inflammation. The particles may become proinflammatory themselves or in the context of their microenvironment.

Inflammatory Markers

Accumulation of complement components and inflammatory markers such as CRP around lipid-rich deposits indicate that the deposits have triggered inflammation (50–52). It is known, for example, that surface-exposed cholesterol binds CRP that activates the classical pathway of complement and triggers inflammation in the tissue (53). In atherosclerotic lesions CRP binds to phosphocholine in modified low-density lipoproteins (LDL) and colocalizes with LDL in human atherosclerotic lesions (54). In this context factor H plays a crucial role in limiting alternative complement activation through simultaneous binding to CRP and C3b (8). Inactivation of C3b to iC3b promotes noninflammatory clearance of certain lipid particles and dying cells through interaction with CR3 and CR4 receptors on macrophages (9).

In addition to CRP, there are other pentraxins that have been suggested to play a role in the pathogenesis of at least AMD, atherosclerosis, and Alzheimer's disease. Pentraxin 3 (PTX3) is expressed by RPE cells and glial cells *in vitro* and may be involved in oxidative stress-mediated cell injury (55, 56). Moreover, PTX3 is a marker of disease severity in cardiovascular diseases and may exhibit atheroprotective effects (57, 58). Similarly to CRP, PTX3 opsonizes apoptotic and damaged cells and interacts with factor H (59). The involvement of PTX3 also in C3G is possible, because factor H-related protein 5 (FHR5), which is implicated in DDD and FHR5-related glomerulopathy, enhances complement activation by inhibiting binding of factor H to PTX3 (60).

Matrix metalloproteinase 8 (MMP-8) is a pro-inflammatory marker secreted by neutrophils. Elevated plasma and serum levels of MMP-8 associate with conditions such as peritonitis, rheumatoid arthritis and cardiovascular diseases. A recent study showed a significant association between the V62I polymorphism in domain one of factor H and neutrophil MMP-8 levels suggesting that this variation, with increased regulatory activity, could be involved in suppressing complement activation, MMP-8 expression, and inflammation in cardiovascular diseases (61, 62). Interestingly, this same factor H V62I variant is also associated with decreased susceptibility to AMD (63), further strengthening the hypothesis that the function of factor H is crucial in limiting inflammation in these diseases.

Danger-Associated Molecular Patterns

Danger-associated molecular patterns (DAMPs) indicate structures that are normally hidden but exposed during tissue injury. These can be recognized by multiple different types of receptors that often recognize also structures found on microbes. The receptors for DAMPs include Toll-like receptors (TLRs) and Nod-like receptors (NLRs) that can lead to activation of the inflammasome structures inside cells, for example in macrophages. Depending in the nature of interactions, the consequences can be inflammatory or anti-inflammatory. Also, the complement system can be activated on DAMPs or DAMP-like structures, which are generated by tissue injury, protein misfolding. or mislocalization. Such structures include, for example, mitochondria, membrane phospholipids, oxidized lipids in atherosclerosis, amyloid-β in brain, or oxidized bisretinoids on retinal pigment epithelial (RPE) cells in the macular area of the retina (64–66).

Polyunsaturated fatty acids are vulnerable to free radical attack caused by activated immune cells or cell apoptosis. As a consequence, oxidation-specific epitopes, such as oxidized phosphocholine, 4-hydroxynonenal, isolevuglandin, and malondialdehyde (MDA), are formed. The roles of oxidized lipids and MDA adducts in the pathogenesis of AMD, atherosclerosis and Alzheimer's disease have been studied in more detail (67, 68). Interestingly, in AMD, factor H binds to both oxidized lipids and bisretinoids in drusen. Here, the common factor H variant 402Y has a higher affinity for oxidized lipids than the AMD risk allele 402H (69). Also the MDA-epitopes are recognized by factor H. Both the Y402H polymorphism in domain seven, and atypical hemolytic uremic syndrome (aHUS) associated mutations in domains 19–20 impair this interaction (70, 71). Therefore, the found association of factor H Y402H polymorphism with AMD, DDD and possibly also with atherosclerosis and Alzheimer's disease could be related to the reduced binding of 402H to CRP and oxidized lipids compared to 402Y (19–21, 72). Reduced control of the amplification pathway of complement could thus contribute to the inflammatory pathology in these diseases.

Gangliosides are highly sialylated glycosphingolipids and abundantly expressed in the human body (73). Cells lacking terminal sialic acids, for example, due to oxidative damage, ischemic, senescent, necroptotic or apoptotic cell death become "DAMPs" for the complement system. Therefore, they serve as signals for complement activation and phagocytosis (2, 74).

A distictive feature of the alternative pathway of complement is that its activators are structures that lack the ability to bind factor H (75). Thus, nearly any structure without suitable polyanions (sialic acids, glycosaminoglycans, and negatively charged phospholipids) or membrane complement regulators can act as complement activators as long as they provide binding sites for C3b molecules (76).

Because factor H interacts with cell surface sialic acids and surface deposited C3b, it has a crucial role in protecting self cells from complement attack. The presence of anti-ganglioside antibodies in atherosclerosis and Alzheimer's disease patient sera has been previously observed (77, 78). It is therefore possible, although not shown, that these antibodies could interfere with factor H binding to sialic acids present on gangliosides and thereby contribute to inflammation. The antibodies could have a double-negative effect: They could activate the classical pathway and promote alternative pathway activation by preventing binding of factor H to cell surface gangliosides. A documented role for anti-ganglioside antibodies has been demonstrated in the neurological diseases Guillain-Barré and Miller Fisher syndromes [reviewed recently by Cutillo et al. (73)].

Apolipoprotein E

Apolipoprotein E (ApoE) is the central molecule responsible for cholesterol metabolism in the liver, blood and brain. ApoE has been shown to be involved in amyloid-β clearance in the central nervous system. It promotes anti-atherosclerotic activity by regulating lipoprotein metabolism and promoting cholesterol efflux by the so called reverse cholesterol transport. ApoE also modulates macrophage polarization into the anti-inflammatory M2 phenotype (79, 80). ApoE has three allelic isoforms (apoE2/E3/E4) of which apoE4 is strongly associated with Alzheimer's disease (81). ApoE4 is also associated with atherosclerosis, nephrotic glomerular disease in children and AMD, of which the latter has the strongest association with FH polymorphism Y402H (26, 81–85).

ApoE is found abundantly in the lipid-rich deposits of AMD, DDD, atherosclerosis and Alzheimer's disease patients (12, 86–88). However, its function in plaque formation or clearance is not well known. *In vivo* apoE is mainly associated with lipids, such as HDL particles in plasma or with small HDL-like components in brain. However, a small portion of apoE is found in complex with lipid-free or lipid-poor proteins. Of these, especially the apoE4 variant is likely susceptible for self-aggregation and misfolding (89). Factor H binds both lipid-free and high density lipoprotein (HDL) associated apoE *via* domains 5–7 (4). On HDL particles factor H regulates the alternative pathway of complement but its role in binding to lipid-free apoE is not known. The single amino acid difference in residues 112 and 158 between the apoE isoforms is responsible for the structural differences between these proteins (90). Moreover, knowing that FH interacts with apoE *via* the domain 7, where the Y402H polymorphism is located, it is possible that these genetic variations could also affect binding between apoE and FH.

Phagocytic Cells

Phagocytic cells are closely associated with disease progression in AMD, DDD, atherosclerosis, and Alzheimer's disease. In atherosclerosis, macrophages play a crucial role in the phagocytosis of modified LDL particles and cholesterol efflux, while microglia are involved in amyloid-β phagocytosis. Increased intake of cholesterol by macrophages and reduced cholesterol efflux capacity leads to formation of foam cells in the arterial intima, while an increased intake of amyloid-β by microglial cells induces microglial activation and neurotoxicity (91). In microglia, the increase in free radical generation has been suggested to be related to the binding of amyloid-β to complement receptor type 3, CR3. CR3 is a phagocytic receptor that is involved in complement-mediated clearance of iC3b-coated particles and suppression of inflammation. It interacts with several different ligands, including factor H. Factor H interacts with CR3 on several cell types (92) and possibly has a direct effect on phagocytic functions. In cholesterol-loaded macrophages factor H has been shown to simultaneously promote cholesterol efflux, reduce transcription of proinflammatory genes and increase transcription of antiatherogenic genes such as ABCA1 and PPAR-α (93). PPAR-α transcription factor is known to induce the expression ABCA1, the intracellular ATP binding cassette transporter that regulates cellular cholesterol homeostasis (94). Binding of factor H to CR3 has been shown to reduce acute subretinal inflammation in mice indicating that factor H could be involved in suppressing inflammation in human AMD as well (95).

Necrotic and Apoptotic Cells

Formation of necrotic and apoptotic cells is involved in the pathogenesis of AMD, C3G, atherosclerosis and Alzheimer's disease. As mentioned earlier, complement system is involved in the non-inflammatory clearance of apoptotic and necrotic cells that is initiated through recognition of CRP by C1q that activates the classical pathway of complement (96, 97). Here, binding of factor H to CRP is crucial, as it is involved in suppressing activation of the alternative and terminal pathways at the site of tissue damage and during local inflammation (8, 98, 99).

While CR3 is the receptor of iC3b, complement receptor type 1 (CR1 and CD35) is the receptor that has a higher affinity toward C3b (100, 101). CR1 is a cell surface complement regulator that acts as a cofactor for factor I in the inactivation of C3b (similarly to factor H) and further in the cleavage of iC3b to C3c and C3dg. In addition to phagocytic cells CR1 is also found on red blood cells, where it participates in the clearance of immune complexes by transporting them for elimination in the spleen or liver (102, 103). Factor H blocks binding of CR1 to C3b (104) as it competes for the same binding site with CR1 on C3b. This competitive binding leads to decreased C3c/C3dg formation as only CR1, but not factor H, acts as a cofactor for factor I in the cleavage of iC3b to C3c and C3dg. As C3d/C3dg is recognized by CR2 on B-cells and thus links the innate and adaptive immunity, it is possible that inhibition of C3d formation by factor H could have consequences for the adaptive immunity and formation of autoantibodies that has been described at least in atherosclerosis and C3G (46, 105–107).

CONCLUSIONS

AMD, C3G, atherosclerosis and Alzheimer's disease seem to be unrelated to each other because of the different

locations of the affected tissues. However, current knowledge on the role of complement-mediated inflammation in the development of these diseases indicates that they partially share common pathophysiology. Current genetic and biochemical data indicate that complement regulator factor H participates in the modification of both complement activation and cell responses and that defects in complement regulation by factor H play an important role in the pathogenesis of these diseases. More knowledge is, however, needed to understand the exact molecular mechanisms whereby factor H protects the eyes, the kidneys, the brain, and arteries from inflammation that eventually leads to formation of lipid-rich deposits in AMD, C3G, Alzheimer's disease, and atherosclerosis.

AUTHOR CONTRIBUTIONS

SM and KH have been involved with original research leading to concepts described in this work. They both wrote the review. All authors contributed to the article and approved the submitted version.

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Factor H Autoantibodies and Complement-Mediated Diseases

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Factor H (FH), a member of the regulators-of-complement-activation (RCA) family of proteins, circulates in human plasma at concentrations of 180–420 mg/L where it controls the alternative pathway (AP) of complement in the fluid phase and on cell surfaces. When the regulatory function of FH is impaired, complement-mediated tissue injury and inflammation occur, leading to diseases such as atypical hemolytic uremic syndrome (a thrombotic microangiopathy or TMA), C3 glomerulopathy (C3G) and monoclonal gammopathy of renal significance (MGRS). A pathophysiological cause of compromised FH function is the development of autoantibodies to various domains of the FH protein. FH autoantibodies (FHAAs) are identified in 10.9% of patients with aHUS, 3.2% of patients with C3G, and rarely in patients with MGRS. The phenotypic variability of FHAA-mediated disease reflects both the complexity of FH and the epitope specificity of FHAA for select regions of the native protein. In this paper, we have characterized FHAA epitopes in a large cohort of patients diagnosed with TMA, C3G or MGRS. We explore the epitopes recognized by FHAAs in these diseases and the association of FHAAs with the genetic deletion of both copies of the *CFHR1* gene to show how these disease phenotypes are associated with this diverse spectrum of autoantibodies.

Keywords: factor H, autoantibodies, complement, C3 glomerulopathy, atypical hemolytic uremic syndrome, monoclonal gammopathy of renal significance

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INTRODUCTION

Complement factor H (FH), a 155 KDa glycoprotein comprised of 20 short consensus repeat (SCR) domains, circulates in the blood at concentrations of 180–420 mg/L. It functions as the major regulator of the alternative pathway (AP) of complement in the fluid phase and on cell surfaces. Fluid-phase complement regulation is mediated by the four N-terminal SCRs of FH through two different mechanisms—decay accelerating activity (DAA) and co-factor activity (CA). DAA refers to the ability of FH to promote displacement of the Bb fragment of factor B (FB) off C3 convertase through SCR1-2, thereby accelerating the irreversible decay of C3bBb to C3b and Bb. CA refers to the role of FH as a facilitator of factor I (FI)-mediated proteolytic cleavage of C3b to an inactivated

form of C3b called iC3b (1). In both scenarios, FH SCR1-4 interacts with the MG ring, CUB domain and TED domain on the C3b molecule (2). On cell surfaces, FH protects host surfaces from complement-mediated damage primarily through the two C-terminal SCRs, which recognize and bind to sialic acids, glycosaminoglycans (GAG), heparins, and a site on the C3b-cleavage fragment C3d (3, 4). These binding events ensure that the DAA function of FH is targeted to host cell surfaces, thereby protecting these cells from indiscriminate complement amplification that might otherwise be associated with surface deposition of C3b (5).

Genetic variation and/or acquired autoantibodies are the two major factors that impair FH function, and therefore are the primary drivers of two complement-mediated renal diseases, atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G). The former, a type of thrombotic microangiopathy (TMA), is characterized by hemolytic anemia, thrombocytopenia and acute renal injury (6). TMA itself is an overarching term used to describe any condition characterized by thrombocytopenia and microangiopathic hemolytic anemia (MAHA) with varying degrees of organ damage in the setting of normal clotting parameters. Although tissue diagnosis, most commonly in the form of a kidney biopsy showing abnormalities in arterioles and capillaries with microvascular thrombosis, is required, TMA is often inferred from the observation of thrombocytopenia and MAHA in the appropriate clinical setting. Complement-mediated aHUS occurs primarily on host cell surfaces and leads to acute vascular endothelial injury and thrombosis. When left untreated, the likelihood of renal failure and mortality are high (7). The second disease associated with impaired FH function, C3G, is a chronic glomerulopathy characterized by predominant C3 deposition in renal glomeruli. Classic findings of glomerulonephritis (hematuria, proteinuria and variable degrees of renal impairment) result from fluid-phase complement dysregulation (8). Renal survival is about 10 years in up to 50% of affected individuals and following transplantation, approximately 50% of patients experience disease recurrence with allograft loss.

FHAAs have been identified as drivers of complement dysregulation in both aHUS and C3G (9). They are more common in aHUS, being detected in ∼10% of patients in European cohorts and up to 50% in an Indian aHUS cohort (10–12). In C3G cohorts, FHAAs are present in ~3% of patients (13, 14).

Interestingly, in aHUS, the presence of FHAAs is often associated with a common genetic variation known as a copy number variation (CNV) in the *CFH-CFHR* genomic region. The *CFH-CFHR* gene family includes, in addition to *CFH*, five complement factor H-related (*CFHR*) genes located directly 3' of the *CFH* gene in the order of *CFHR3, CFHR1, CFHR4, CFHR2*, and *CFHR5*. The *CFHR* genes arose as a result of genomic duplication and because of the high sequence homology, the region is prone to non-allelic homologous recombination, a process that can result in gene deletion, duplication and rearrangement.

Non-allelic homologous recombination gives rise to CNVs. Absence of both copies of *CFHR1* due to homozygous deletion of *CFHR3-CFHR1* (del*CFHR3-1*) or less commonly compound heterozygous deletion of *CFHR3-CFHR1* and *CFHR1-CFHR4* or homozygous deletion of *CFHR1-CFHR4* is associated with an increased relative risk for aHUS as a consequence of the development of FHAAs, referred to as DEAP-HUS, *DE*ficiency of CFHR1 plasma proteins and *A*utoantibody *P*ositive *H*emolytic *U*remic *S*yndrome (15). It is important to note, however, that the increase in relative risk is small as homozygous del*CFHR3-1* is common. About 3% of European-Americans do not have any copies of *CFHR3-CFHR1*, a percentage that varies significantly by ethnic group (16, 17). The mechanism underlying the development of FHAAs in association with FHR1 deficiency is not well understood but may reflect structural differences between FHR1 and the carboxy terminus of FH (18). To date, there has been no such kind of association observed in C3G. In fact, FHAAs identified in C3G patients are frequently associated with the presence of C3 nephritic factors in children and with monoclonal gammopathy of renal significance (MGRS) in adults (13, 14, 19, 20).

Herein, we report the prevalence and immunological features of FHAAs in cohorts of aHUS and C3G patients from the USA.

METHODS

Patients

Patients with either C3G (n=589) or aHUS (n=448) referred to the Molecular Otolaryngology and Renal Research Laboratories (MORL) from 2013-'19 for FHAA testing were included in this study. Serum and plasma samples were collected using our standard operating procedure (SOP), aliquoted, and stored at -80°C prior to testing (21). Control sera and plasma (n=300) were collected using the same SOP. The study was approved by the Institutional Review Board of Carver College of Medicine at the University of Iowa.

Anti-Factor H Autoantibody Assay

FHAAs were detected as previously described (22, 23). Briefly, purified human FH (Complement Technology Inc, Tyler, TX) was coated in 1X PBS (pH=7.4) at a concentration of 10 μg/mL on a 96-well micro-titer plate, which was then kept overnight at 4°C. After washing three times with 1X PBST (1X PBS containing 0.1% Triton-X), free reactive sites were blocked with Ultrablock (AbD Serotec, Raleigh, NC) for 30 min at room temperature. Patient serum (1:50 dilution) was added for a 1-hour incubation at room temperature, after which plates were washed and incubated for another hour at room temperature with a horseradish peroxidase-labeled goat anti-human IgG antibody specific for the γ chain. After final washings, enzymatic activity was measured using OPD (o-phenylenediamine dihydrochloride) and absorbance was read at λ490.

A standard curve (4-parameter logistic regression) was generated for each run by serial dilutions of a positive sample (aHUS49, 3,000 arbitrary units (AU) at 1:50). The value was calibrated to a positive sample kindly provided by Dr. Marie Agnès Dragon-Durey (Georges Pompidou hospital, Paris, France).

Epitope Mapping and Isotyping

To map binding epitopes, recombinant FH fragments of SCRs1-6, 6-8, 8-15, 15-18, 18-20 and mini-FH (1-4 and 19-20) were produced as previously described (3, 5, 24), and used as capturing/coating proteins in the aforementioned protocol. Similarly, recombinant FHR1, 2, and 5 with 6X HIS tag were used as capturing/coating proteins for testing FHAA cross-reactivity to FHR1, 2 or 5.

To determine IgG subclass and light chains, the protocol was repeated with mouse anti-human IgG1, IgG2, IgG3, IgG4, kappa and lambda antibodies (all from Millipore Sigma) used at a dilution of 1:1,000–1:2,000 as detecting antibodies.

Other Autoantibody Detection

FB autoantibodies (FBAAs) were measured by ELISA and C3/C4/C5 nephritic factors were measured by cell-based hemolytic methods as previously described (25, 26).

M-Protein Detection

All FHAA-positive patients were screened for M-proteins using serum protein electrophoresis and immunofixation electrophoresis (IFE) on a SPIFE Touch System (Helena Laboratories, Beaumont, TX).

Complement Assays

Serum levels of C3 were measured by ELISA (Hycult Biotech Inc., Uden, Netherlands). C4 was measured using radial immunodiffusion (The Binding Site Inc., Birmingham, UK). Soluble C5b-9 and FH levels were measured using ELISA kits (Quidel Corporation, San Diego, CA).

Genetic Analysis

Genomic DNA was extracted from peripheral blood using the Gentra Puregene Kit (Qiagen Inc., Valencia, CA) and integrity was evaluated by 1% agarose gel electrophoresis. Absorbance at 230:260:280 was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) to ensure DNA samples met quality metrics of 1.8 for 260/280 and 260/230 ratios. DNA concentration was determined using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA). Samples were then screened using the Genetic Renal Panel focused on complement gene abnormalities, as previously described (27). To interrogate the *CFH-CFHR* region, multiplex ligation-dependent probe amplification (MLPA) was performed using MRC Holland SALSA kit (Amsterdam, Netherlands) and in-house designed probes (28).

Western Blotting for FHR1

Serum or plasma (1:40 diluted) in Laemmli buffer was separated on 4–15% gel followed by in-house produced polyclonal rabbit antibodies to the first SCR of FHR1 and FHR2.

Statistical Analyses

Statistical analysis was performed using GraphPad (version 8.2). The Student *t*-test or Mann-Whitney *U* Test was used to compare groups. $P < 0.05$ was considered statistically significant.

RESULTS

FHAAs in C3G and aHUS

Nineteen patients (3.2%) in the C3G cohort and 49 patients (10.9%) in the aHUS cohort were positive for FHAAs. In both cohorts, both genders were equally affected (**Tables 1** and **2**), however FHAA-positive aHUS patients were significantly younger than FHAA-positive C3G patients (median age, 10.2 vs 38.3, respectively; $P < 0.001$, **Figure 1A**). FHAA titers were also significantly higher in patients with aHUS as compared to patients with C3G (median, 4787 AU *vs* 1149 AU, respectively; $P < 0.05$, **Figure 1B**).

FHAA Targeting Epitopes and FHR1 Deficiency

FHR1 deficiency as determined by either MLPA to detect homozygous deletion of the *CFHR1* gene or Western blotting to detect absence of the FHR1 protein was observed in 37 of 49 (76%) aHUS patients and 2 of 19 (11%) C3G patients (**Tables 3** and **4**).

In aHUS, FHAA titers were higher in patients deficient as compared to patients replete in FHR1 (median 5,841 vs 3,217, respectively) although the difference was not statistically significant (P=0.131, Mann-Whitney *U* Test, **Figure 1C**). 37/37 (100%) of aHUS patients deficient in FHR1 carried FHAAs that primarily targeted the C-terminus of FH. In four-fifths of these patients (30/37, 81%), the FHAAs cross-reacted with FHR1, while in one patient (aHUS31), cross-reactivity to FHR2 was also seen. In these patients, the addition of recombinant FHR1 blocked binding of FHAAs to FH. Multiple epitopes of FH were recognized in six of the 37 FHR1-deficient aHUS patients, including four patients who were co-positive for FHAAs against N-terminus SCRs and two patients who were co-positive for FHAAs against mid-SCRs 8-15.

Of the 12 aHUS patients who express FHR1, six (aHUS41-46) had FHAAs that bind to the C-terminus of FH only, but in none of these patients did the FHAAs show cross-reactivity with FHR1. Two patients (aHUS38, 39) had FHAAs that bind to the N-terminus of FH only, while three patients (aHUS47-49) were co-positive for FHAAs that recognized N- and C-terminal epitopes of FH. In two of these three patients, there was cross-reactivity with FHR1. In one patient (aHUS40), FHAAs recognized an epitope in SCRs 8-15 of FH.

Only two patients with C3G were FHR1 deficient. In one patient (C3G1), FHAAs targeted the N-terminus (SCRs1-6) alone while in the other patient (C3G18), FHAAs reacted with the C-terminus of FH and also cross-reacted with FHR1. Of the 17 other C3G patients, all of whom express FHR1, 16 had FHAAs that recognized specific FH epitopes: four patients (C3G2-5) had FHAAs that bind to FH SCRs 1-6; two patients (C3G6, 7) had FHAAs that bind to FH SCRs 6-8; two patients (C3G8, 9) had FHAAs to both fragments; one patient (C3G10) had FHAAs that bind to FH SCRs 8-15; and four patients (C3G13-15, 17) had FHAAs that bind to SCRs 19 and 20. There were three patients (C3G12, 16, 19) whose FHAAs reacted with both N- and C-terminal SCRs of FH.

TABLE 1 | Demographic and genetic data for aHUS patients.

Patient	Sex	Age range	Ethnicity	Genetic findings	Copies		
aHUS				(rare variant MAF<0.01%)	*CFHR3*	*CFHR1*	*CFHR4*
aHUS1	M	6–10	Caucasian	N/A	N/A	N/A	N/A
aHUS2	M	6–10	Caucasian	no variants	0	0	2
aHUS3	M	11–15	Caucasian	no variants	0	0	2
aHUS4	M	6–10	Caucasian	no variants	1	0	1
aHUS5	F	6–10	Caucasian	N/A	0	0	2
aHUS6	M	6–10	Caucasian	no variants	0	0	2
aHUS7	F	16–20	Caucasian	no variants	0	0	2
aHUS8	F	1–5	Caucasian	no variants	0	0	2
aHUS9	F	6–10	Caucasian	N/A	N/A	N/A	N/A
aHUS10	M	16–20	African American	no variants	0	0	2
aHUS11	M	31–35	Caucasian	no variants	0	0	2
aHUS12	F	6–10	Caucasian	no variants	0	0	2
aHUS13	M	1–5	African American	*CFHR5* c.427A>C, p.Thr143Pro	0	0	2
aHUS14	F	6–10	Hispanic	no variants	1	0	1
aHUS15	M	11–15	Caucasian	no variants	0	0	2
aHUS16	M	11–15	Asian	no variants	0	0	2
aHUS17	F	11–15	Hispanic	no variants	0	0	2
aHUS18	M	6–10	Caucasian/African American	no variants	0	0	2
aHUS19	F	6–10	African American	no variants	0	0	2
aHUS20	M	6–10	Hispanic	N/A	N/A	N/A	N/A
aHUS21	F	6–10	Caucasian	*CFH* c.3644G>A, p.Arg1215Gln	0	0	2
aHUS22	M	11–15	Caucasian	no variants	0	0	2
aHUS23	M	16–20	Caucasian	no variants	0	0	2
aHUS24	M	61–65	Caucasian	no variants	1	0	1
aHUS25	F	11–15	Caucasian	no variants	0	0	2
aHUS26	M	11–15	Arabic	no variants	0	0	2
aHUS27	M	11–15	Caucasian	no variants	1	0	1
aHUS28	M	1–5	Caucasian	no variants	0	0	2
aHUS29	F	11–15	Caucasian	no variants	1	0	1
aHUS30	M	6–10	Hispanic	no variants	0	0	2
aHUS31	F	6–10	Caucasian	no variants	0	0	2
aHUS32	F	41–45	Caucasian	no variants	1	0	1
aHUS33	F	16–20	African American	no variants	0	0	2
aHUS34	F	6–10	Asian	no variants	0	0	2
aHUS35	F	11–15	Asian	N/A	0	0	2
aHUS36	M	6–10	Hispanic	no variants	0	0	2
aHUS37	M	6–10	Caucasian	no variants	0	0	2
aHUS38	M	1–5	African American	no variants	1	1	2
aHUS39	M	46–50	Caucasian	*CFH* c.3536T>C, p.Ile1179Thr	2	2	2
aHUS40	M	51–55	Caucasian	no variants	2	2	2
aHUS41	M	16–20	Hispanic	no variants	2	2	2
aHUS42	F	6–10	Hispanic	no variants	2	2	2
aHUS43	F	6–10	Caucasian	no variants	2	2	2
aHUS44	F	11–15	Asian	no variants	2	2	2
aHUS45	F	6–10	Hispanic	no variants	2	1	1
aHUS46	M	71–75	Caucasian	no variants	1	1	2
aHUS47	M	16–20	Caucasian	no variants	2	2	2
aHUS48	M	1–5	Arabic	no variants	2	2	2
aHUS49	F	1–5	Caucasian	no variants	N/A	N/A	N/A

N/A, not available.

No patients in this study carried FHAAs that recognized FH SCRs15-18 or had cross reactivity to FHR5.

IgG Subclasses and M-Proteins

The distribution of IgG subclasses was similar in the two disease cohorts and is listed in **Tables 3** and **4**. In the aHUS cohort, the prominent subclass was IgG3 (35/49, 71%), with most patients (29/35, 83%) having a restriction of either lambda or kappa, although six patients (6/35, 17%) were co-positive for lambda and kappa. Another six patients (6/49, 12%) were positive for only IgG1 with either lambda or kappa restriction; two of these patients had MGRS (one each of IgG κ and λ). Seven patients (7/49, 14%) were co-positive for IgG1 and IgG3 and one patient (1/49, 2%) was co-positive for IgG1, IgG3 and IgG4. No patient was positive for IgG2. With respect to light chains, 25 patients (25/49, 51%) were positive for lambda only, 14 patients (14/49, 29%) for kappa only, and 10 patients (10/49, 20%) for both.

In the C3G cohort, the prominent subclass was also IgG3 with either lambda or kappa restriction (11/19, 58%). There were two patients with MGRS in this group (one each of IgG κ and λ). Five

TABLE 2 | Demographic and genetic data for C3G patients.

Patient	Sex	Age range	Ethnicity	Genetic findings	Copies		
C3G				(rare variant MAF<0.01%)	*CFHR3*	*CFHR1*	*CFHR4*
C3G1	M	61–65	Caucasian	no variants	1	0	1
C3G2	M	26–30	Caucasian	no variants	1	1	2
C3G3	F	71–75	Caucasian	no variants	2	2	2
C3G4	M	26–30	Caucasian	N/A	N/A	N/A	N/A
C3G5	F	71–75	Asian	*C3* c.3214C>T, p.Arg1072Trp	2	2	2
C3G6	F	46–50	Caucasian	no variants	2	2	2
C3G7	F	51–55	Caucasian	no variants	3	3	2
C3G8	F	21–25	Caucasian	no variants	2	2	2
C3G9	M	36–40	Caucasian	no variants	2	2	2
C3G10	M	81–85	Caucasian	no variants	2	2	2
C3G11	M	66–70	Caucasian	no variants	2	2	2
C3G12	F	61–65	Caucasian	no variants	2	2	2
C3G13	F	6–10	Caucasian	*CFH* c.1854A>G, p.Asp619Gly	2	2	2
C3G14	M	21–25	Hispanic	no variants	2	2	2
C3G15	F	26–30	Caucasian	N/A	N/A	N/A	N/A
C3G16	M	11–15	Hispanic	N/A	N/A	N/A	N/A
C3G17	M	16–20	Hispanic	no variants	2	2	2
C3G18	F	36–40	Caucasian	no variants	0	0	2
C3G19	F	51–55	Caucasian	N/A	N/A	N/A	N/A

N/A, not available.

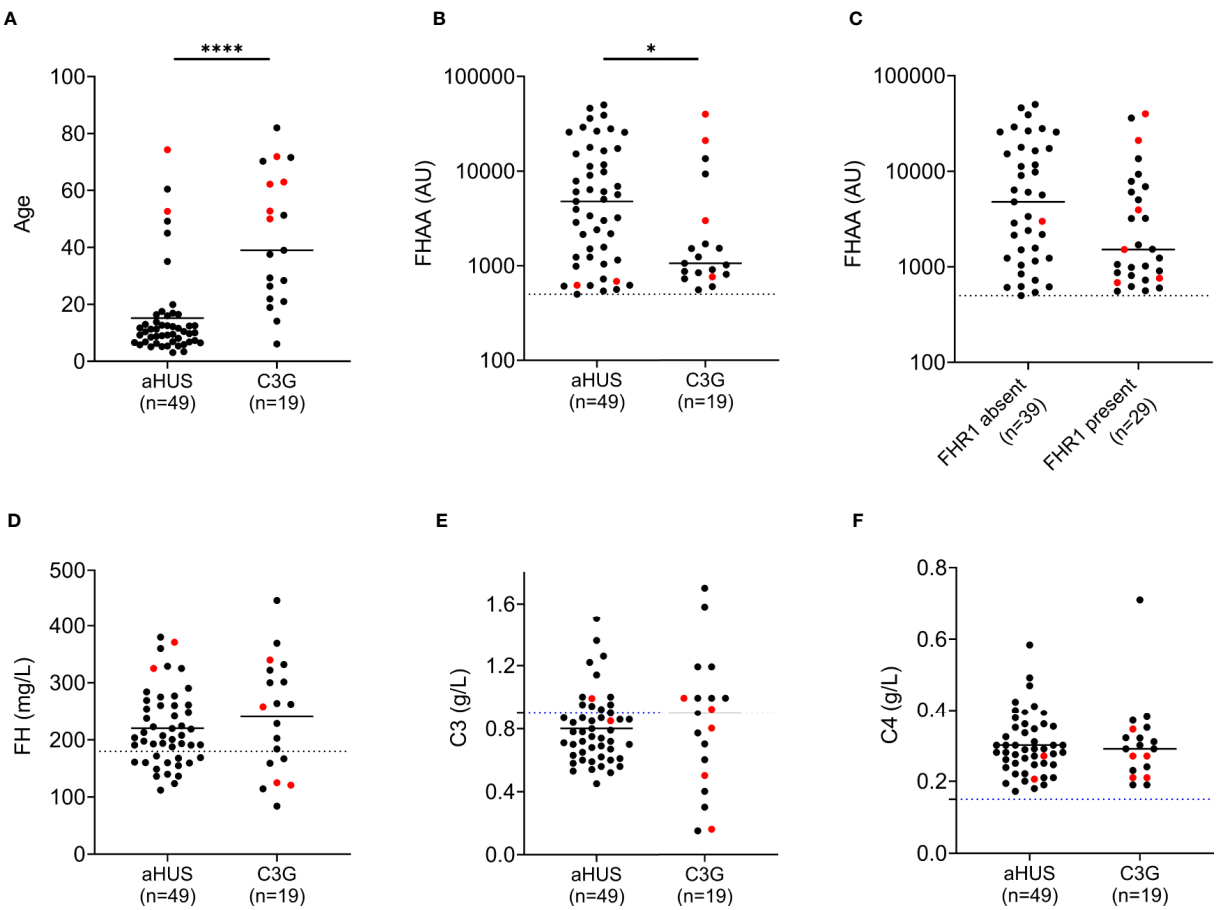

FIGURE 1 | FH autoantibodies (FHAAs) and key biomarkers at the age-of-onset of disease in patients with aHUS and C3G. Patient with M- proteins are in red. **(A)** Age; **(B)** FHAA titers in aHUS and C3G; **(C)** FHAAs (C3G and aHUS) with and without FHR1; **(D)** FH levels; **(E)** C3 levels; **(F)** C4 levels. Solid lines: medians. Dashed lines: normal cutoff based on results from 300 healthy individuals. *$P < 0.05$ and ****$P < 0.0001$ by Mann-Whitney *U* test.

TABLE 3 | FHAAs in patients with atypical hemolytic uremic syndrome.

Patient	FHR1^	FHAA (AU)	FH Epitope					Mini-FH	FHR1	FHR2	M-Spike	FHAA	
			1–6	6-8	8–15	15–18	18–20					IgG subclasses	Light chains
aHUS1	absent	2875					+	+				IgG3	λ
aHUS2	absent	2140					+	+				IgG3	λ
aHUS3	absent	540					+	+				IgG3	k
aHUS4	absent	9828					+	+				IgG3	λ
aHUS5	absent	500					+	+				IgG3	λ
aHUS6	absent	724					+	+				IgG3	λ
aHUS7	absent	618					+	+				IgG3	λ
aHUS8	absent	3362					+	+	+			IgG1	λ
aHUS9	absent	25690					+	+	+			IgG3	λ
aHUS10	absent	4787					+	+	+			IgG1+IgG3	k
aHUS11	absent	2180					+	N/D	+			IgG3	λ
aHUS12	absent	17290					+	+	+			IgG3	k
aHUS13	absent	11220					+	+	+			IgG1+IgG3	λ
aHUS14	absent	1230					+	N/D	+			IgG3	λ
aHUS15	absent	609					+	+	+			IgG3	k
aHUS16	absent	46180					+	+	+			IgG1+IgG3	λ+k
aHUS17	absent	27990					+	+	+			IgG3	λ+k
aHUS18	absent	29120					+	+	+			IgG3	λ
aHUS19	absent	39000					+	+	+			IgG3	k
aHUS20	absent	1041					+	N/D	+			IgG3	λ+k
aHUS21	absent	1148					+	+	+			IgG3	λ
aHUS22	absent	1230					+	+	+			IgG3	k
aHUS23	absent	6041					+	+	+			IgG3	λ
aHUS24	absent	5640					+	+	+			IgG3	λ
aHUS25	absent	2401					+	+	+			IgG1	λ
aHUS26	absent	25750					+	+	+			IgG3	λ+k
aHUS27	absent	6361					+	+	+			IgG3	λ
aHUS28	absent	26450					+	+	+			IgG3	λ
aHUS29	absent	15220					+	+	+			IgG3	λ
aHUS30	absent	9018					+	+	+			IgG3	k
aHUS31	absent	50070					+	+	+	+		IgG3	λ
aHUS32	absent	11680	+				+	+	+			IgG1+IgG3+IgG4	λ+k
aHUS33	absent	1505	+				+	N/D	+			IgG1+IgG3	λ+k
aHUS34	absent	1571	+	+			+	+	+			IgG1+IgG3	λ
aHUS35	absent	16360	+	+	+		+	+	+			IgG3	λ+k
aHUS36	absent	17830			+		+	+	+			IgG3	λ
aHUS37	absent	622			+		+	N/D	+			IgG1+IgG3	λ
aHUS38	present	3217	+					+				IgG3	λ+k
aHUS39	present	621	+					+				IgG3	λ
aHUS40	present	3948			+						IgG λ	IgG1	λ
aHUS41	present	5019					+	+				IgG3	k
aHUS42	present	35990					+	+				IgG3	k
aHUS43	present	562					+	N/D				IgG3	k
aHUS44	present	6895					+	+				IgG1	k
aHUS45	present	7823					+	+				IgG3	k
aHUS46	present	683					+	+			IgG k	IgG1	k
aHUS47	present	986	+	+			+	+				IgG1	k
aHUS48	present	6053	+				+	+	+			IgG1+IgG3	λ+k
aHUS49	present	3000	+	+			+	+	+			IgG3	λ+k

^*Determined by MLPA or the Western if no DNAs.*

Blank, Negative; N/D, not done.

patients (5/19, 26%) were positive for IgG1, with four patients showing lambda or kappa restriction, consistent with subclasses of M-spikes found in three patients (two IgG κ and one IgG λ); one patient was positive for both light chains. One patient (1/19, 5%) showed co-positivity for IgG1 and IgG3 with lambda restriction and one patient (5%) showed co-positivity for IgG1 and IgG4 with reactivity to both light chains. One patient (1/19, 5%) was positive for IgG4 only with kappa restriction. No patient was positive for IgG2.

Other Acquired Drivers of Disease

No other autoantibodies were detected in aHUS patients positive for FHAAs however one patient in the C3G cohort was co-positive for FBAA (C3G8) and two patients had C3 nephritic factors (C3G13, 18) (**Table 4**).

Complement Dysregulation With FHAAs

Low plasma FH levels were detected in 14 of 48 (29%) and 6 of 19 (32%) patients with aHUS and C3G, respectively (**Figure 1D**).

TABLE 4 | FHAAs in patients with C3 glomerulopathy.

Patient	FHR1^	FHAA (AU)	FH Epitope					Mini-FH	FHR1	FHR2	M-Spike	FHAA	
			1-6	6-8	8-15	15-18	18-20					IgG subclasses	Light chains
C3G1	absent	2990	+					+			IgG k	IgG1	k
C3G2	present	9315	+					+				IgG1+IgG4	λ+k
C3G3	present	1059	+					+				IgG4	λ
C3G4	present	13550	+					+				IgG1+IgG3	λ
C3G5	present	21010	+					+			IgG k	IgG1	k
C3G6	present	1514		+							IgG λ	IgG1	λ
C3G7	present	39980		+							IgG λ	IgG3	λ
C3G8#	present	555	+	+				+				IgG3	k
C3G9	present	809	+	+				+				IgG3	k
C3G10	present	1017			+							IgG1	λ
C3G11	present	869	+	+	+		+	+				IgG1	λ+k
C3G12	present	759	+	+			+	+			IgG k	IgG3	k
C3G13*	present	1525					+	+				IgG3	k
C3G14	present	601					+	+				IgG3	k
C3G15	present	725					+	+				IgG3	λ
C3G16	present	1697		+			+	+	+	+		IgG3	λ
C3G17	present	1238					+	+	+	+		IgG3	k
C3G18*	absent	843					+	N/D	+			IgG3	k
C3G19	present	908	+				+	+	+			IgG3	k

^*Determined by MLPA or the Western if no DNAs.*

**C3G13 and C3G18 are positive for C3Nefs.*

#*C3G8 is also positive for FBAAs.*

Blank, negative; N/D, not done.

There was no correlation between FH levels and FHAA titers. C3 levels were low in 12 of 20 (60%) patients with aHUS and 7 of 16 (44%) patients with C3G (**Figure 1E**). C4 levels were normal in all patients (**Figure 1F**). Soluble C5b-9 was elevated in 15 of 19 patients (79%) with aHUS not on Eculizumab and in 11 of 16 C3G patients (also not on Eculizumab; 69%), consistent with uncontrolled activity of the terminal complement pathway.

Genetic Findings

Genetic testing was completed in 45 aHUS and 15 C3G patients. Ultra-rare genetic variants (defined as a minor allele frequency (MAF) <0.01% and resulting in a nonsynonymous amino acid change) were identified in five patients (**Tables 1** and **2**). Three patients carry rare variants in the *CFH* gene (patients aHUS21, 39 and C3G13). The variants found in the two aHUS patients were in SCR20 of FH. One, FH p.R1215Q (in aHUS21), affects surface regulation by impairing surface heparin binding (3, 29); for the second variant, FH p.Ile1179Thr (in aHUS39), functional data are unavailable. The C3G patient (C3G13) carries FH p.Asp619Gly in SCR10, again a variant for which functional data are lacking. Another C3G patient (C3G5) carries C3 p.Arg1072Trp. In addition, one aHUS patient (aHUS13) carries a rare variant of unknown significance in the *CFHR5* gene.

DISCUSSION

Herein, we report a retrospective study of FHAAs in aHUS and C3G patient cohorts from North America. Overall, FHAAs were found in 10.9% of aHUS and 3.2% of C3G patients, respectively, consistent with prior reports in populations of European decent (13, 30). The high prevalence of FHR1 deficiency in association with FHAAs is seen only in the aHUS cohort and not in the C3G cohort (**Tables 3** and **4**).

In more than 80% of patients with FHR1 deficiency, FHAAs bind to both the carboxy-terminus of FH and FHR1. In these patients, FHAA titers tend to be extremely high in the acute phase of disease (**Figure 1B**, data collected in acute phase) but drop during remission, suggesting that the presence of FHR1 plays an important role in suppressing auto-immunogenicity of FH when a trigger is present. Consistent with this hypothesis, in these patients recombinant FHR1 can compete off FH for FHAA binding.

The last two SCRs of FH are essential for self-surface recognition and have ligand-binding sites for heparan sulfate, sialic acid and the complement cleavage product, C3d. Importantly, sequence homology between SCRs 19 and 20 of FH and SCRs 4 and 5 of FHR1 is very high, with amino acid differences only at two positions (S1191 and V1197 on FH *vs* L290 and A296 on FHR1) (31). It has been postulated that subtle conformational changes at residues 1,182–1,189 (due to S1191) in FH SCR20 occur during infections and may be auto antigenic in the absence of FHR1 (18). Our data appear to support this hypothesis since >92% of FHAA patients with FHR1 deficiency were pediatric cases with a median age of 10 (IQR 6.9–12.7), suggesting that the development of FHAAs is associated with common school-related infections.

The consequence of FHAAs that impair C-terminal function of FH is dysregulation of complement control on host cell surfaces. FHR1 deficiency alone, however, is not sufficient to trigger the generation of FHAAs. The deletion of 79.4 kb on

chromosome 1 (gnomAD ID: MCNV_1_81) that includes two *CFH*-related genes, *CFHR3* and *CFHR1*, is a common CNV in the human genome with homozygous deletion of both copies of the *CFHR3-CFHR1* genes present in 4.1% of Europeans, 16.2% of Africans, 1.9% of Latinos and 0.3% of East Asians (data based on the reported non-diploid CN frequency in the gnomAD). In fact, FHR1 deficiency has been reported in FHAA-*negative* aHUS patients at a frequency that is higher than that in an ethnically matched control population (32).

In our aHUS cohort, in contrast, after excluding patients with FHAAs, the frequency of FHR1 deficiency was comparable to that found in a control population of European decent (nine of 255 patients, 3.5%). In addition, we commonly detected FHAAs in patients who express *CFHR1*. Of the 29 patients with FHR1 who were positive for FHAAs, there were 12 cases of aHUS (24% of all FHAA-positive cases of aHUS) and 17 cases of C3G (89% of all FHAA-positive cases of C3G). In about half of these cases, the antibody recognized carboxy-terminal SCRs of FH; however, in no case was there cross-reactivity to FHR1 and the addition of recombinant FHR1 *ex vivo* has no influence on FHAA binding results.

In seven of 19 (37%) C3G patients (C3G1-5, 8, 9) and two of 49 (4%) aHUS patients (aHUS38, 39) with FHR1, the FHAAs reacted only with SCRs at the N-terminus (SCRs1-6). Epitope reactivity was confirmed using mini-FH (SCRs1-4+19-20). The N-terminal SCRs1-4 is the site of DAA and CA. Blocking these two major regulatory functions of FH would be predicted to lead to complement dysregulation in the fluid phase as well as on cell surfaces. Why some patients develop a C3G phenotype and others develop an aHUS phenotype is not clear but may reflect differences in the degree of residual DAA or CA, as well as factors that determine complement control in local microenvironments like the glomerulus. We speculate that in general C3G patients have better control of AP activity on cell surfaces as compared to aHUS patients due to the presence of complement regulators (*i.e.* CD46, CD55) or due to FH itself. In this study, for example, we identified two aHUS patients (aHUS21, 39) carrying pathogenic or likely pathogenic variants in *CFH* as genetic drivers of disease. With ongoing dysregulation primarily in the fluid phase, C3G patients present with chronic phenotypes such as proteinuria and hematuria, while aHUS patients present with acute phenotypes like endothelial cell damage and complement-mediated coagulopathy.

None of the aHUS patients had other acquired drivers of disease while in C3G, two patients had C3Nefs and one patient had FBAAs ($P < 0.05$ by Fisher exact test). This finding suggests that while aHUS patients are more likely to have FHAAs as a sole acquired driver, C3G patients may be co-positive for other acquired drivers of disease such as nephritic factors, perhaps implying that FHAAs play a secondary role in C3G.

Interestingly, in 37 of 49 (76%) aHUS patients and 16 of 19 (84%) C3G patients, the circulating FHAAs demonstrated monoclonal characteristics (one subclass of heavy chains + one type of light chains) with IgG3 followed by IgG1 being the most common heavy chain isotypes. These data are consistent with most previous reports (9, 33–35), but are at odds with the findings

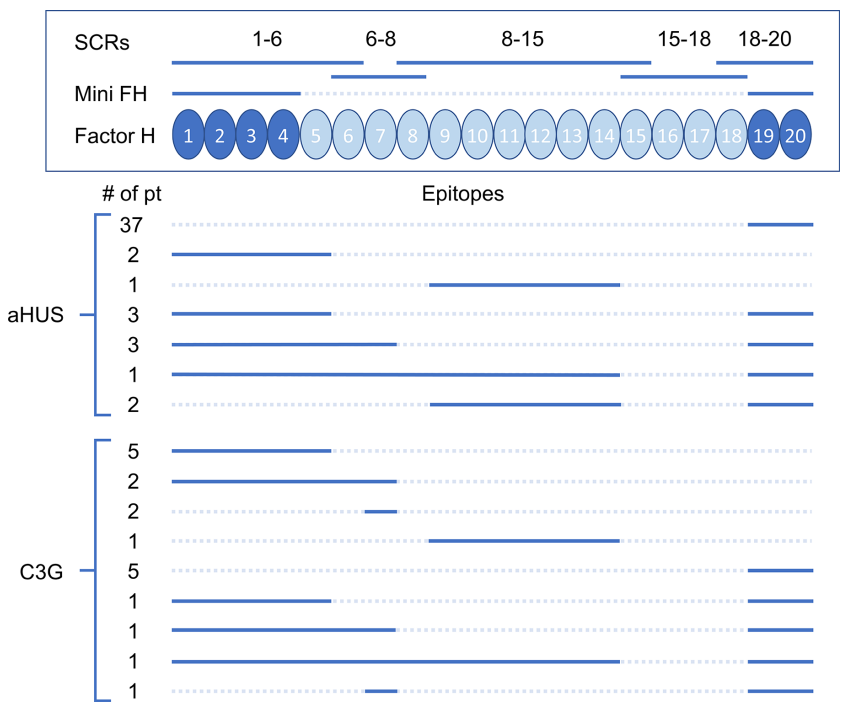

FIGURE 2 | Epitopes of factor H autoantibodies (FHAAs) targeting domains on factor H (FH). Recombinant FH fragments (SCRs) and the mini FH construct are shown above a schematic of FH. Below are shown the epitope mapping results for aHUS (n=49) and C3G (n=19) patients (solid lines = positive FHAA results, dash lines = negative FHAA results).

described by Guo *et al.*, who showed that FHAAs in the Chinese population are typically polyclonal (36). The presence of the lambda light chain was more dominant in the aHUS cohort while both light chains had equal presence in the C3G cohort.

Albeit many FHAAs appeared monoclonal, we only observed seven M-proteins by IFE (the most sensitive method for detecting circulating M-proteins) in both cohorts, two aHUS patients (aHUS40, 46) and five C3G patients (C3G1, 5–7, 12). These patients were all over 50 years of age and while monoclonal gammopathy has emerged as an underlying cause of C3G in the elderly (13, 14, 37), its association with aHUS has not been previously reported.

MGRS is a recently defined disease entity, in which the underlying pathogenesis for the renal disease is associated with circulating monoclonal immunoglobulins or M-proteins that drive renal injury. The malignant clonal B cell clones do not meet criteria for overt multiple myeloma/B-cell proliferation. M-proteins can directly deposit in the glomeruli and activate complement through the classical pathway resulting in immune complex glomerulonephritis, or alternatively directly activate the AP in the fluid phase or block complement regulators resulting in M-protein induced C3G or aHUS (13, 14, 19, 20).

As compared to C3G (5/19, 26%), the prevalence of M-proteins in aHUS (2/49, 4%) is rare. Patient aHUS46 has a monoclonal IgG1 kappa directed against the C-terminus of FH (without FHR1 reactivity) that impairs the surface regulation of the AP, consistent with aHUS phenotype. However, in patient aHUS40, FHAAs (also IgG1 but lambda light chain) only bind to the mid portion of FH (SCRs 8–15). In this patient, plasma FH is normal, C3 is borderline low, and sC5b-9 is slightly elevated, findings consistent with ongoing complement dysregulation. Interestingly, C3G patient C3G10 is also positive for FHAAs that target only SCRs 8-15 of FH with no apparent M-proteins and has a similar biomarker profile (normal FH, borderline low C3, slightly elevated sC5b-9). Additional research is warranted in these two cases, as it would be of great interest to clarify the underlying mechanisms by which FHAAs that target the mid-portion of FH impact complement control.

Finally, two C3G patients (C3G6, 7) circulate monoclonal FHAAs (IgG1, kappa and lambda, respectively) that target only SCR7 of FH. Recently, Li, et al. described a Chinese C3G patient with FHAAs that bind to SCR7. Functional studies showed that in this patient, the SCR7-recognizing FHAAs inhibited FH binding to C3b and accelerated formation of C3 convertase (37). In addition, FHL-1, a truncated version of FH containing the first 7 SCRs of FH, has regulatory activity that involves domains SCRs5-7 (38). Thus, compromising SCR7 function may play a role in the pathogenesis of MGRS-C3G.

In conclusion, we provide a comprehensive analysis of FHAAs in patients with aHUS and C3G (**Figure 2**). In aHUS, the absence of FHR1 is associated with a high incidence of FHAAs in patients age under 20 years of age; in patients over 50 years of age, FHAAs may be associated with MGRS. In C3G, FHAAs are more likely to be co-positive with other autoantibodies and the likelihood of MGRS in older patients is higher. Our data highlight the value of epitope mapping and isotyping in patients who are positive for FHAAs as a method of refining the underlying pathophysiology of complement dysregulation in the fluid phase and/or on cell surfaces.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of Carver College of Medicine at the University of Iowa. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YZ and RS designed the research, analyzed and interpreted data, and wrote the manuscript. NG, DS, AD, MJ, NM, GP, and AT performed experiments and participated in data analysis. CN and CS provided crucial conceptual input. CS contributed essential reagents. All authors contributed to the article and approved the submitted version.

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Functional Characterization of Rare Genetic Variants in the N-Terminus of Complement Factor H in aHUS, C3G, and AMD

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Membranoproliferative glomerulonephritis (MPGN), C3 glomerulopathy (C3G), atypical haemolytic uraemic syndrome (aHUS) and age-related macular degeneration (AMD) have all been strongly linked with dysfunction of the alternative pathway (AP) of complement. A significant proportion of individuals with MPGN, C3G, aHUS and AMD carry rare genetic variants in the *CFH* gene that cause functional or quantitative deficiencies in the factor H (FH) protein, an important regulator of the AP. *In silico* analysis of the deleteriousness of rare genetic variants in *CFH* is not reliable and careful biochemical assessment remains the gold standard. Six N-terminal variants of uncertain significance in *CFH* were identified in patients with these diseases of the AP and selected for analysis. The variants were produced in *Pichia Pastoris* in the setting of FH CCPs 1–4, purified by nickel affinity chromatography and size exclusion and characterized by surface plasmon resonance and haemolytic assays as well as by cofactor assays in the fluid phase. A single variant, Q81P demonstrated a profound loss of binding to C3b with consequent loss of cofactor and decay accelerating activity. A further 2 variants, G69E and D130N, demonstrated only subtle defects which could conceivably over time lead to disease progression of more chronic AP diseases such as C3G and AMD. In the variants S159N, A161S, and M162V any functional defect was below the capacity of the experimental assays to reliably detect. This study further underlines the importance of careful biochemical assessment when assigning functional consequences to rare genetic variants that may alter clinical decisions for patients.

Keywords: complement factor H, age-related macular degeneration, aHUS, C3G, MPGN

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INTRODUCTION

Atypical haemolytic uraemic syndrome (aHUS) (1), membranoproliferative glomerulonephritis (MPGN), C3 glomerulopathy (C3G) (2) and age-related macular degeneration (AMD) (3) are all diseases that have been associated with dysregulation of the alternative pathway (AP) of complement.

The hallmark pathological lesion in aHUS is thrombotic microangiopathy within the kidney, characterized by the clinical features of microangiopathic haemolytic anaemia, thrombocytopenia and acute kidney failure (1). Conversely, MPGN and C3G are a group of conditions in which deposition of complement activation products within the glomerulus occurs, resulting in nephrotic/nephritic syndrome and chronic progressive renal failure (2). Specifically, MPGN is a pattern of glomerular injury, involving thickening of the glomerular capillary wall and an increase in mesangial components. C3G is the current preferred term when the glomerular staining is predominantly C3 positive by immunofluorescence. Dense deposit disease is a special sub-type of C3G, characterized by prominent intramembranous laminar deposits that are visible on electron microscopy, associated historically with MPGN (2).

AMD is the leading cause of irreversible vision loss in elderly Caucasian populations. It is characterized by lipoprotein-rich drusen deposits that form in the subretinal space and the recruitment of immune cells, such as microglia and macrophages (4, 5). Degeneration and geographic atrophy of the retinal pigment epithelium and photoreceptors occur in the macula of the retina in advanced dry AMD, while advanced wet AMD involves angiogenesis in the choroid and subsequent choroidal neovascularization (6).

Genetic studies have linked common (7–11) and rare genetic variants (12–17) in the complement factor H gene (*CFH*) to all of these conditions. Complement factor H (FH) is an abundant fluid phase regulator of the AP, and functions as a cofactor for factor I (FI) in the cleavage of the activated central molecule of the pathway, C3b (18, 19). Screening for rare genetic variants in *CFH* has been established in clinical practice to determine a possible genetic cause of disease. Knowledge of *CFH* variants and their functional significance plays an important role in understanding prognosis (20) and determining the risk of disease recurrence after renal transplantation (21) in aHUS. Similar genotype-phenotype correlations have not yet been established in MPGN/C3G. In all of these diseases, common risk haplotypes also associate with pathology and play a role in genetic susceptibility (7–11).

The functional consequences of genetic variants identified in individual patients needs to be carefully considered as they could influence the approach to clinical management of the condition. Because each genetic variant is rare, there may not be any literature reports on its functional impact. There is thus a good case for a robust and consistent procedure to be established for assessing the likelihood that a rare variant in *CFH* is functionally detrimental and hence could be contributing to disease. Functional testing although time-consuming remains the gold standard for attributing significance to a rare genetic variant and has been shown to be contradictory to *in silico* analysis of variants in *CFH* (22).Most rare *CFH* genetic variants in aHUS reported to date have been found to code for amino acids in the C-terminal recognition domain, comprising complement control proteins (CCPs) 19–20 (23). Many variants have been extensively studied in the laboratory and most demonstrate functional significance (14, 24). Variants have also been reported in the N-terminal regulatory domain, comprising CCPs 1–4 and there is significant enrichment of N - terminal variants in AMD (25). To date, there have been functional studies of 10 variants in CFH CCPs 1-4, namely; R53C (16, 22), R53H (15, 26, 27), R53P (22), R53S (22), S58A (22), I62V (15, 27, 28), R78G (15), R83S (13), D90G (16) and Del K224 (29), and in many cases there was a severe loss of function.

The objective of this study was to expand this knowledge pool by testing the functional significance of other variants of uncertain significance identified in the N-terminal region of FH in patients with aHUS, MPGN, C3G, and AMD.

MATERIALS AND METHODS

Selection of N-Terminal *CFH* Variants for Functional Study

At the time of study inception, the literature was reviewed for N-terminal rare genetic variants described in the complement mediated diseases: MPGN/C3G; aHUS, and AMD. Six N-terminal heterozygous variants known to result in normal serum FH levels and for which no prior functional data were available were selected as follows: G69E (17, 25), Q81P (30), D130N (25, 31), S159N (25, 32, 33), A161S (25, 30, 31, 33, 34) and M162V (35).

Production and Purification of Proteins

Clones of *Pichia pastoris* strain KM71H producing wild-type (WT) and mutant (G69E, Q81P, D130N, S159N, A161S, and M162V) protein in the setting of CCPs1–4 were generated as described previously (15). In brief, each point mutation was generated in a pPICZαB (Invitrogen) vector containing residues 19–263 of FH (which encodes CCPs 1–4 of mature FH; residues 1–18 are the mammalian signal peptide), with a C-terminal 6x His tag and an N-terminal myc tag (EQKLISEEDL) using QuikChange site-directed mutagenesis kit (Stratagene). Fidelity was confirmed by bi-directional Sanger sequencing. KM71H cells were transformed using electroporation, selected for by zeocin, and screened for protein expression.

Protein expression was carried out in a 3L BioFlo 115 Biofermenter (New Brunswick). A starter culture was transferred into 1L of basal fermentor salts (0.095%) (w/v) calcium sulphate, 1.82% (w/v) potassium sulphate, 1.5% (w/v) magnesium sulphate heptahydrate, 0.42% (w/v) potassium hydroxide, 2.7% (v/v) phosphoric acid and 2.5% (v/v) glycerol) enriched with 1% (w/v) casein amino acids, 0.5% (w/v) PTM1 salts and 0.5% (v/v) antifoam A (Sigma). After the initial batch fed glycerol was exhausted, software-controlled glycerol feeds were maintained for 24 h at 30°C. The cells were allowed to

starve for 4 h before recombinant expression was induced with 0.75% methanol containing 0.5% (w/v) PTM1 salts. After three days at 15°C with software-controlled methanol feeds, cells were spun out and the supernatant was removed, filtered and its pH adjusted to 7.4.

The supernatant was then applied to a 5ml His-Trap FF column (GE-healthcare) at 4°C and the protein eluted with 500 mM imidazole followed by size exclusion chromatography using a HiLoad® 16/600 Superdex® 200 pg column (GE Healthcare) (**Supplementary Figure 1**). Protein concentrations were calculated using absorbance at 280 nm and a calculated extinction coefficient (478,70M cm^{-1}).

Binding Affinity for C3b by Surface Plasmon Resonance

The binding affinities of the WT and mutant FH1-4 proteins to C3b were monitored by SPR using a Biacore X100 instrument (GE Healthcare) (14). A total of 300 resonance units of human C3b (CompTech) were immobilized on a Biacore series S-CM5 sensor chip (GE Healthcare) using standard amine coupling. The reference surface of the chip was prepared by performing a mock coupling in the absence of any protein. Experiments were performed at 25°C and 30 µl/min flow rate. Duplicate injections (concentrations up to 40 µM) were performed in 10 mM HEPES-buffered saline, 3 mM EDTA, 0.05% (v/v) surfactant p20 (GE Healthcare). A contact time of 45s was used (sufficient for achieving steady state conditions for most samples) followed by a dissociation period of 90 s. Chips were regenerated between sample injections, with one 45 s injections of 1 M NaCl, pH 7.0. Data were processed using the BIAevaluation 4.1 software (GE Healthcare). Data from the reference cell and a blank (buffer) injection were subtracted and dissociation constants calculated using a steady-state affinity model from the background-subtracted traces.

Measurement of Decay Acceleration Activity by Surface Plasmon Resonance

Decay accelerating activity (DAA) was measured in real-time using a Biacore X100 instrument as described previously (15). Briefly, 300 resonance units of C3b were immobilized using standard amine coupling to the CM5 sensor chip. Subsequently, a mixture of FB (250 nM) and FD (30 nM) was flowed (10 µl/min) over the surface for 120s to form the AP convertase.

The convertase was allowed to decay naturally for 210s and then FH1-4 (WT or variants at 166nM) [in running buffer, HEPES-buffered saline containing 0.5% (v/v) surfactant P20 and 1 mM $MgCl_2$, Temp 25°C] was flowed across the surface for 60s, and convertase decay was visualized in real time. Between injections, surfaces were regenerated using a 45s injection of 1µM purified FH (CompTech) followed by a 45s injection of 1M NaCl, pH7.0. Data were evaluated using BIAevaluation 4.1 (GE Healthcare).

Cofactor Assay in the Fluid Phase

In vitro fluid phase assays were used to measure cofactor activity (CA) for FI-mediated proteolytic cleavage of C3b (14). FI and C3b were purchased from CompTech. An aliquot of 3µL of C3b (5.68 µM), 4.5 µL of FI (0.114 µM) and 5 µL of FH1-4 (0.75 µM) were made up to a final volume of 15µL in phosphate buffered saline (PBS). For the negative control 5 µL of PBS was used. The mixture was incubated at 37°C for 15 min, and the reaction stopped by the addition of 2x Laemmli reducing buffer to a final volume of 30 µL and heated to 95°C for 5 min. The products of the reaction were then separated by SDS-PAGE and revealed using Coomassie Blue staining.

Measuring Decay Acceleration on Sheep Erythrocytes

C3b-coated sheep erythrocytes (EA-C3b) were prepared as described previously (13). Cells were resuspended to 2% (v/v) in AP buffer (5 mM sodium barbitone, pH 7.4, 150 mM NaCl, 7 mM $MgCl_2$, 10 mM EGTA). The AP convertase was formed on the cell surface by incubating 50µL of cells with an equal volume of AP buffer containing FB (40µg/ml) and FD (0.4 µg/ml; CompTech) at 37°C for 15 min. Cells (100 µl) were incubated with 50 µl of a concentration range of FH1–4 (WT or variants) in PBS/20 mM EDTA for 15 min. Lysis was developed by adding 50 µl of 4% (v/v) normal human serum depleted of FB and FH (NHSΔBΔH) (13) in PBS/20 mM EDTA and incubating at 37°C for 60 min. To determine the amount of lysis, cells were pelleted by centrifugation, and hemoglobin release was measured at 410 nm (A410). Controls included 0% lysis (buffer only) and 100% lysis (0.1% (v/v) Nonidet P-40), Percentage of inhibition from lysis was calculated by the formula (A_{410}[buffer only]-A_{410}[FH])/A_{410}[buffer only]*100%.

Measuring Cofactor Activity on Sheep Erythrocytes

To test CA (13), washed EA-C3b cells were resuspended to 2% (v/v) in AP buffer and incubated with an equal volume of a range of concentrations of FH1–4 (WT and variants) and 2.5 µg/ml FI (CompTech) for 15 min at 25°C. After three washes in AP buffer, a 50 µl aliquot of cells (2%) was mixed with 50 µl AP buffer containing FB (40 µg/ml) and FD (0.4 µg/ml) and then incubated for 15 min at 25°C to form AP convertase on the remaining C3b. Lysis was developed by adding 50 µl of 4% (v/v) NHSΔBΔH in PBS/20 mM EDTA and incubating at 37°C for 10 min. Percentage of inhibition from lysis was calculated by the formula (A_{410}[buffer only]-A_{410}[FH])/A_{410}[buffer only]*100%.

RESULTS

Expression of Recombinant FH1-4 Variants

The rare genetic variants, G69E, Q81P, D130N, S159N, A161S, and M162V were analyzed in the setting of a construct containing the first four CCP domains of FH responsible for C3b binding, DAA, and CA (**Figure 1**). This allowed interrogation of the functional consequences of variants in the N-terminal region of FH without the confounding presence of the C-terminal region, CCP19-20, which contains a second,

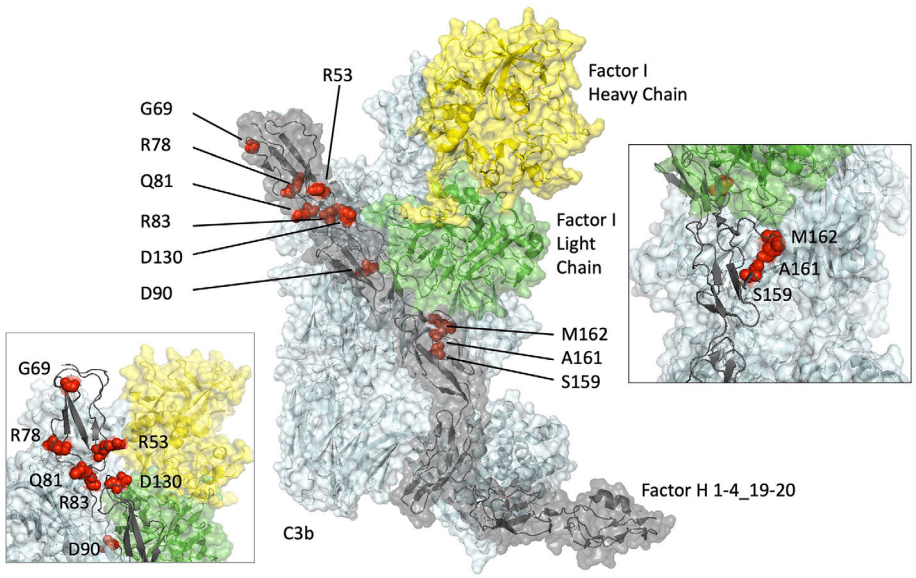

FIGURE 1 | Structural modelling of rare genetic variants in an x-ray–derived co-crystal structure of Factor H CCP1-4_19-20/C3b/Factor I displayed using PyMOL (V2.0.6, Schrödinger, LLC). The amino acids altered by the rare genetic variations analyzed in this study (G69, Q81, D130, S159, A161, M162) and previously (R53 (15, 16, 22), R78 (15), R83 (13), D90 (16)) (red spheres) are shown within a co-crystal structure of Factor I (heavy chain: yellow, light chain: green), Factor H (grey) and C3b (cyan). (Protein Database ID code 5O32) (36).

stronger, C3b binding site. We have previously validated this strategy in N terminal *CFH* variants by demonstrating equivalent results in the setting of both full-length recombinant FH and the CCP1-4 recombinant FH construct used in this study (15, 27).

In keeping with the normal FH serum levels reported for these variants, all six of them were produced as folded, soluble proteins by genetically modified *Pichia pastoris*. Following purification, all FH1-4 samples were free of aggregates and degradation (**Supplementary Figure 1**).

Effect of Variants on C3b Binding

Surface plasmon resonance (SPR) was used to measure the binding interaction between FH1-4 and immobilized C3b. The plots of maximum binding response (RUs) versus concentration, used to estimate K_D values, are shown for FH1–4 WT (**Figures 2A, B**); FH1–4 G69E (**Figure 2C**); FH1–4 Q81P (**Figure 2D**); FH1–4 D130N (**Figure 2E**); FH1–4 S159N (**Figure 2F**); FH1–4 A161S (**Figure 2G**); FH1–4 M162V (**Figure 2H**).

The variant FH1-4 Q81P showed no detectable binding to C3b at the highest concentration available (40 μM Q81P in the solution flowed over the chip). All the other variants bound to C3b with a strength comparable to that of WT FH1-4. As a ratio of the K_D for C3b of WT FH1-4, K_D values were 1.2-fold for G69E, 1.4-fold for D130N, 1.2-fold for S159N, 0.97-fold for A161S and 1.2-fold for M162V (**Table 1**).

Co-Factor Activity of FH1-4 to C3b

To assess the effect of these sequence variations on co-factor activity, fluid-phase CA assays were initially undertaken. These demonstrated that FH1–4 Q81P has minimal CA, judging by the relative strength of the intact α' substrate band of C3b on an SDS-polyacrylamide gel, and minimal formation of the α1 cleavage products (**Figure 3**). In these semi quantitative assays, the remaining variants demonstrated co-factor activity similar to that of WT FH1-4.

The activity of each rare variant as a co-factor in the FI-mediated proteolytic cleavage of surface-bound C3b was then assessed on sheep erythrocytes. Again, Q81P FH1-4 exhibited the poorest co-factor activity (IC_{50} 11x > that of WT, **Figure 4B**). The other variants tested had surface CAs comparable to WT CA (**Figures 4A, E, F**), although D130N (IC_{50} 1.66x greater than WT) (**Figure 4C**) and S159N (IC_{50} 1.54x greater than WT) (**Figure 4D**), showed small reductions in function.

Decay Acceleration Activity of FH1-4 to C3b

The effect of sequence variants on the ability of FH1-4 to accelerate decay of the C3 convertase (C3bBb) assembled on the surface of sheep erythrocytes was also analyzed. These assays revealed an approximately 25-fold reduction in activity of the FH1–4 Q81P compared with FH1–4 WT (**Figure 5B**). FH-D130N displayed a small decrease in surface DAA activity (IC_{50} 2x WT) (**Figure 5C**). The remaining variants displayed still smaller differences (IC_{50}: G69E 1.3x WT; S159N 0.9X WT; A161S 1.3X WT; M162V, 1.02X WT) (**Figure 5**).

The effect of the six sequence variations in FH 1-4 on DAA was also analyzed in real time on an SPR chip surface (**Figures 6A–F**). In this assay the FH1–4 Q81P variant demonstrated only minimal if any effect on the rate of C3bBb decay (**Figure 6B**). Consistent with DAA measurements on erythrocytes, D130N

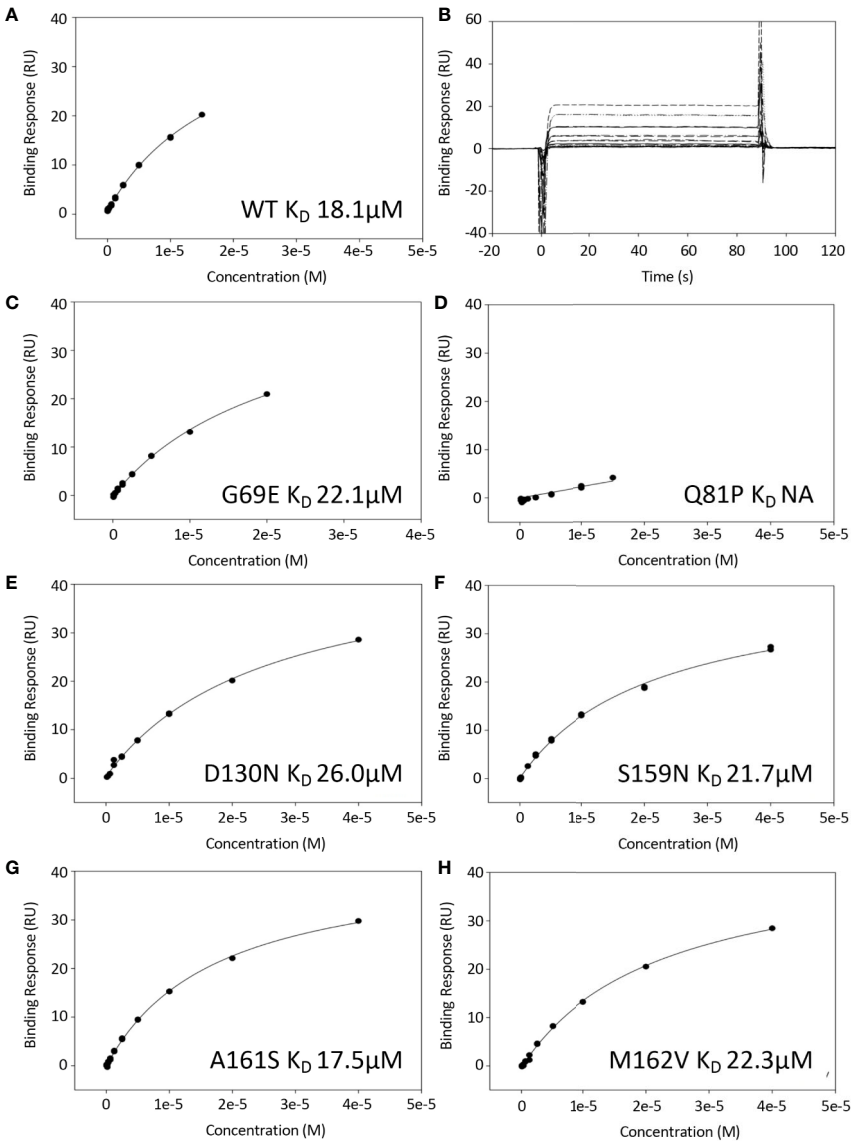

FIGURE 2 | Binding of FH1-4 to C3b using surface plasmon resonance. C3b was immobilized to a CM5 sensor chip (300RU) and FH1-4 (WT and variants) were injected across (concentrations up to and including 40uM). **(B)** Sensorgrams from WT experiment demonstrating rapid association/disassociation of FH1-4 to with/from C3b. This is representative of interactions from all variants. The steady state response was plotted against concentration for all variants and shown in **(A, C–H)**. All have similar K_D except for Q81P where there was minimal binding at the concentrations used.

TABLE 1 | Summary of the functional effects of each N-terminal *CFH* variant.

Variant	Disease	Effect of variant on				
		Affinity to C3b (Kd xWT)	Decay on SPR Assay	Decay on Sheep E (IC_{50} xWT)	Co-factor Fluid Phase	Co-factor on Sheep E (IC_{50} xWT)
G69E	AMD	1.2	↓	1.3	↔	1.1
Q81P	aHUS	↓	↓	25.1	↓	11.0
D130N	C3G, AMD	1.44	↓	2.0	↔	1.66
S159N	aHUS, AMD	1.20	↔	0.9	↔	1.54
A161S	aHUS, C3G, AMD	0.97	↔	1.3	↔	1.26
M162V	aHUS	1.23	↔	1.02	↔	1.48

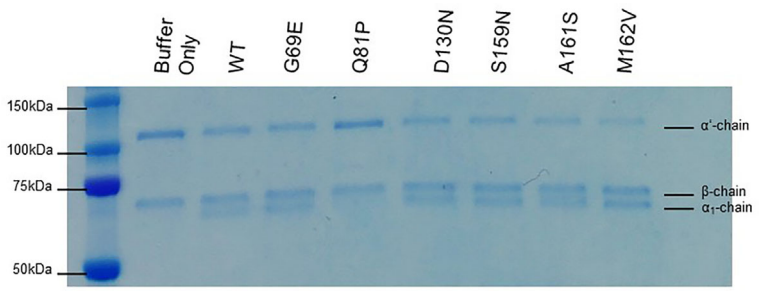

FIGURE 3 | Fluid phase co-factor activity. C3b, FI, and FH1-4 were incubated for 30 min at 37°C before the addition of 2x Laemmli to stop the co-factor reaction. The products of the reaction are visualized following separation by SDS-PAGE and staining with Coomassie blue. Q81P clearly has minimal co-factor activity in the fluid phase. All other variants have detectable co-factor activity by the loss of the α' band and the appearance of the α1 band. The β chain is the internal control. Data representative of at least 3 repeats.

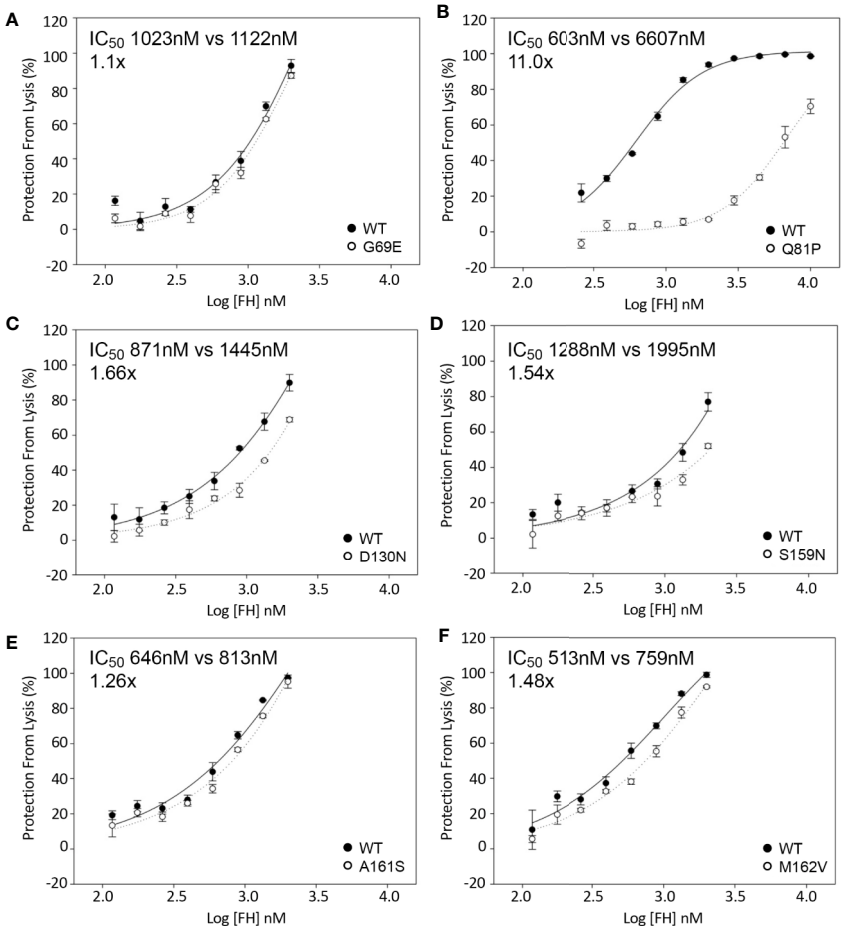

FIGURE 4 | Inactivation of C3b on the surface of sheep erythrocytes. FH1-4 and FI was incubated with sheep erythrocytes with preformed surface C3b for 15 min at 25°C. Following wash steps, C3 convertase was formed on the remaining intact C3b before the instigation of lysis by the addition of ΔBHNHS. Protection from lysis was calculated as [A_{410}(buffer only)–A_{410}(FH)/A_{410}(buffer only)*100]. **(B)** Q81P has large effect on co-factor activity compared to WT (IC_{50} 11x). **(C)** D130N demonstrated a slight loss of activity (IC_{50} 1.66x). The remaining variants demonstrated similar activity **(A, D–F)**.

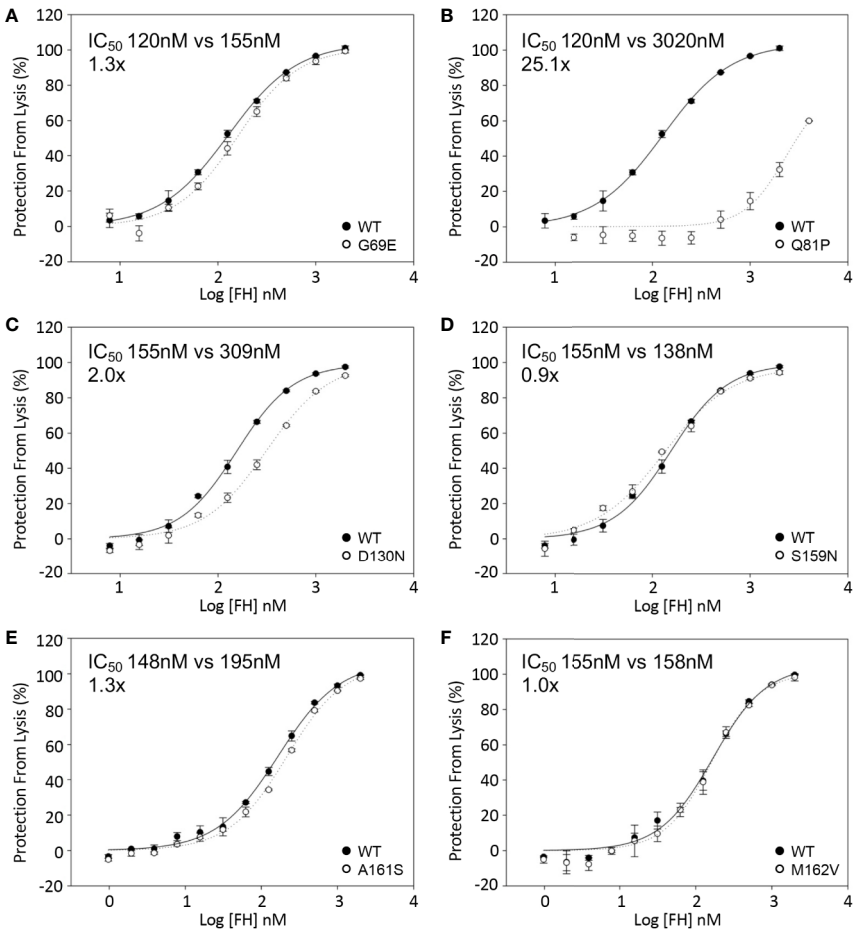

FIGURE 5 | Decay acceleration of C3 convertase formed on surface of sheep erythrocytes. FB and FD were incubated with sheep erythrocytes with preformed surface C3b. The cells were then incubated with FH1-4 for 15 min at 25°C before the addition of ΔBHNHS to instigate lysis. Protection from lysis was calculated as [A_{410}(buffer only)–A_{410}(FH)/A_{410}(buffer only)*100]. **(B)** Q81P has a profound effect of DAA with an IC_{50} 25-fold greater than wild type. The IC_{50}' s relative to WT were **(A)** 1.3x, G69E **(C)** 2x, D130N **(D)** 0.9x, S159N **(E)** 1.3, A161S **(F)** 1x, M162V.

FH1-4 had slightly less activity than WT (**Figure 6C**), as does G69E FH1-4 has less DAA than WT (**Figure 6A**).

DISCUSSION

The cost and speed of next-generation sequencing has now reached the point where this evolving technology can be used to alter clinical management in real time. While the results of sequencing results may be available rapidly, their interpretation can be difficult.

While the functional significance of large gene rearrangements, or frameshift mutations is clear-cut and the use of curated locus specific databases (e.g. https://www.complement-db.org/home.php) may provide data on previously described genetic variants (37), many variations in *CFH* consist of missense mutations or potential splice site changes not seen before. Interpreting these variants of unknown significance (VUSs) is particularly challenging (37).

In silico prediction of the consequences of amino acid changes may be attempted but in at least one *CFH* (22) case, these have proved unreliable. In the current study we provide detailed functional analysis of six rare genetic variants in the N-terminus of *CFH* that have been described in aHUS, C3G/MPGN and AMD.

The only one of these variants that had a profound effect on the complement regulatory functions of FH1-4 was Q81P, described in aHUS. It is apparent from the SPR results that its affinity for C3b is very weak compared to that of WT FH. The low affinity observed between Q81P and C3b agrees with inferences based on a co-crystal structure of C3b and CFH1–4 (36, 38) (**Figure 1**), which displays that Q81 is at the surface of the FH1-4 molecule, and is close to putative binding sites on C3b, similar to R83, a change to which (R83S) we have shown to be highly deleterious (13). In keeping with its inability to bind well to C3b, Q81P had a profound negative (25.1-fold and 11.0-fold) effect on DAA and CA, respectively. The proximal section of CCP2 and the CCP1-CCP2 linker region within FH interact through hydrophobic

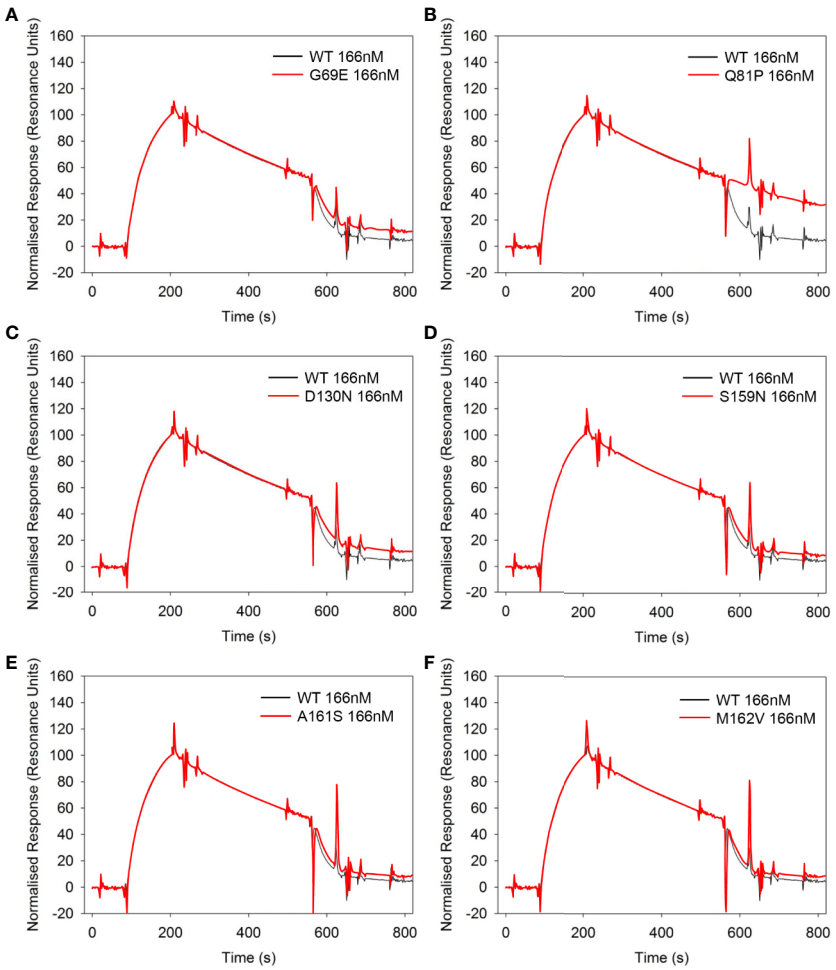

FIGURE 6 | Decay acceleration of C3 convertase in real time using surface plasmon resonance. FB and FD were injected over the surface of immobilized C3b (300RU) to form a convertase as shown by an initial increase in resonance units. The convertase was allowed to decay naturally for 120s before an injection of 166nM of each FH1-4 variant **(A–F)**. All variants show the ability to accelerate decay of the AP C3 convertase apart from Q81P **(B)**. Compared to WT, G69E **(A)** and D130N **(C)** appear to be slightly less effective. Data representative of at least 2 repeats. Variant curves highlighted in red.

interactions and salt bridges to the α' NT and MG7 domain of C3b. Q81 occupies this interface along with R78 and R83 that (when substituted by G and S respectively) also display markedly reduced affinity for C3b with consequent loss of DAA and CA (**Figure 1**).

This analysis is particularly interesting as it demonstrates that 3 variants in the same region of FH all lead to a profound defect in C3b binding with consequent abrogation of complement regulation yet cause different diseases: aHUS (R78G (15); Q81P) and C3G (R83S (13)). This suggests that the phenotype associated with these regulatory defects can be modified by genetic and environmental factors as demonstrated by Recalde et al. (39).

The G69E variation, also in CCP 1, was described in AMD. Unsurprisingly, given its location on the opposite face from the FH/C3b interface (**Figure 1**), the affinity for C3b was unaltered and there was no or only a very minor defect in CA (in the fluid phase and on cell surfaces) and there was only a subtle defect in DAA. In CCP2, D130N is predicted to be in a FH/C3b groove that allows binding of FI and subsequent cleavage of C3b, but only a small effect on co-factor activity (IC_{50} 1.66x) was observed for this variant in the sheep erythrocyte assay. Furthermore, the D130N variant produced a small but consistent effect on decay acceleration as observed on the SPR-based and cell lysis assay: such an effect on decay without loss of C3b binding has been reliably demonstrated in studies of R53H in aHUS and R53C, a variant that has been reported in association with aHUS, MPGN, C3G and AMD (16, 22, 25, 31, 32, 40) In these studies, however, there was a profound loss of DAA, suggesting the critical role of R53 in DAA. Whilst we have shown that there is an effect due to the D130N and G69E variants, the magnitude of the effect is much smaller than in causative N-terminal variants described in aHUS. This would be consistent with a minor overactivation of the AP of complement in a process that is thought to occur in

more chronic disease, as observed in both of these rare variants (G69E, AMD and D130N, C3G).

Of the other variants, none displayed a detectable loss of function. Within CCP3; S159N; A161S; and M162V do sit at the interface with C3b but they displayed similar affinities for C3b as WT FH1-4 (**Figure 1**). They also displayed DAA and CA of a similar order of magnitude to WT FH1-4. The S159N and A161S variants have been demonstrated to occur in excess in AMD-case cohorts (versus control) (25) and it is possible that even the very small variations versus WT contribute to a chronic low-level complement overactivity leading to slow accumulations of complement-mediated damage over a long period of time. The minor differences that were observed were not reproducible across different assays for CA or DAA and therefore we conclude that any difference is below the detection capability of our assays.

As with previous studies *in silico* analysis here proved unreliable (22) with the profoundly perturbed Q81P variant classified as "tolerated" or a "polymorphism" in some analyses while the S159N variant (with normal function) was classified as "possibly damaging" in other analysis (**Supplementary Table 1**). As such *in silico* analysis may still be applied but should be interpreted with great caution especially when used in clinical management of disease.

In summary, we identify significant abrogation of function in an N-terminal variant of FH, Q81P, which is likely to be causative of aHUS. Two variants, G69E and D130N, demonstrated minor defects in complement regulation, which could conceivably over time lead to disease progression of more chronic diseases i.e. in C3G and AMD. Conversely, in the S159N, A161S, and M162V any functional defect was below the capacity of the experimental assays to reliably detect. This study further underlines the importance of careful biochemical assessment of disease-associated variants in complement proteins through a battery of functional assays.

DATA AVAILABILITY STATMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.602284/full#supplementary-material

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A Dimerization Site at SCR-17/18 in Factor H Clarifies a New Mechanism for Complement Regulatory Control

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Complement Factor H (CFH), with 20 short complement regulator (SCR) domains, regulates the alternative pathway of complement in part through the interaction of its C-terminal SCR-19 and SCR-20 domains with host cell-bound C3b and anionic oligosaccharides. In solution, CFH forms small amounts of oligomers, with one of its self-association sites being in the SCR-16/20 domains. In order to correlate CFH function with dimer formation and the occurrence of rare disease-associated variants in SCR-16/20, we identified the dimerization site in SCR-16/20. For this, we expressed, in *Pichia pastoris*, the five domains in SCR-16/20 and six fragments of this with one-three domains (SCR-19/20, SCR-18/20, SCR-17/18, SCR-16/18, SCR-17 and SCR-18). Size-exclusion chromatography suggested that SCR dimer formation occurred in several fragments. Dimer formation was clarified using analytical ultracentrifugation, where quantitative *c(s)* size distribution analyses showed that SCR-19/20 was monomeric, SCR-18/20 was slightly dimeric, SCR-16/20, SCR-16/18 and SCR-18 showed more dimer formation, and SCR-17 and SCR-17/18 were primarily dimeric with dissociation constants of ~5 μM. The combination of these results located the SCR-16/20 dimerization site at SCR-17 and SCR-18. X-ray solution scattering experiments and molecular modelling fits confirmed the dimer site to be at SCR-17/18, this dimer being a side-by-side association of the two domains. We propose that the self-association of CFH at SCR-17/18 enables higher concentrations of CFH to be achieved when SCR-19/20 are bound to host cell surfaces in order to protect these better during inflammation. Dimer formation at SCR-17/18 clarified the association of genetic variants throughout SCR-16/20 with renal disease.

Keywords: analytical ultracentrifugation, complement factor H, inflammation, molecular modelling, X-ray scattering

INTRODUCTION

The complement system is an enzymatic cascade in the innate immunity which acts against damaged cells or invading pathogens before they can cause infection. In the alternative pathway of complement activation, non-active complement C3 is spontaneously hydrolyzed in a tickover mechanism to C3u [also known as C3(H_2O)], which is conformationally similar to active C3b. C3u leads to the amplification of C3 cleavage through the C3 convertase, which now hydrolyses C3 to form active C3b. C3b binds to exposed cell surfaces, targeting them for immune destruction. Complement Factor H (CFH) prevents complement-mediated host cell destruction through the interaction of its C-terminus with surface-bound C3b on anionic host cell surfaces (1, 2). Thus CFH acts as a cofactor for Factor I which cleaves C3b to inactive iC3b (3).

CFH is a 154 kDa glycoprotein composed of 20 short complement regulator (SCR) domains, each containing approximately 61 amino acids, and linked to each other by three to eight amino acids (**Figure 1A**) (4). There are nine N-linked glycosylation sites of which eight are occupied (5). Molecular structure determination for full length CFH is difficult due to its size, glycosylation, interdomain flexibility, and self-association. Nonetheless, high resolution structures are available for 12 SCR domain fragments of CFH solved by X-ray crystallography and for seven SCR domains solved by NMR spectroscopy (6). This leaves SCR-9, SCR-14 and SCR-17 as the only domains without high resolution structures (**Figure 1A**); however molecular models of these are available through standard homology modelling. Early electron microscopy and small angle scattering methods showed that full length CFH possesses a folded-back SCR domain structure through either its N- or C- terminals (6–8). The CFH C-terminal SCR-19 and SCR-20 domains in SCR-19/20 interact with C3b and its thioester domain C3d (9, 10). SCR-20 interacts with the cell surface through anionic interactions (11). Furthermore, CFH self-associates weakly, and that CFH forms dimers alongside higher oligomers that are directly observed as distinct peaks by analytical ultracentrifugation (AUC) (12). One of the two CFH self-association sites is localized to the five-domain fragment SCR-16/20 which exists in a monomer-dimer equilibrium, as shown using both AUC and small angle X-ray scattering (SAXS), although it was unclear from that study where the dimerization site was located in SCR-16/20 (13).

Atypical hemolytic uremic syndrome (aHUS) is a rare disease which is characterized by damage to the endothelial cells of the kidney through impaired complement regulation. It leads to end-stage renal failure and is often fatal (14). aHUS has been strongly associated with mutations in CFH (15–17). CFH-associated genetic variants cause loss of function which impairs the protection of the endothelial surfaces of the kidney, and causes complement activation on these surfaces (18). The most recent survey of CFH variants reported that there were 190 disease-associated variants in CFH (17, 19). Of these, 83 were located in the five C-terminal SCR domains of CFH. The web database (https://www.complement-db.org/) currently indicates six variants in SCR-16, four in SCR-17, ten in SCR-18, seven in SCR-19 and 37 in SCR-20 (**Figures 1B, C**). The majority of these variants are located in SCR-20 which has binding sites for C3b, C3d and anionic surfaces, demonstrating that these variants will directly perturb the ability of CFH to recognize and protect host cells. A further group of CFH variants involves 29 of the 40 disulphide bridges in CFH in which a single Cys residue is replaced, meaning that disease would be caused by protein misfolding of the SCR domain in question and the destabilisation of the CFH protein structure (19). Other complement-associated renal diseases include C3 glomerulopathy (C3G) (17).

In order to identify the CFH self-association site in SCR-16/20 and to clarify the involvement of the aHUS-associated variants in SCR-16/20 on the protein structure, we expressed seven recombinant fragments of these five C-terminal SCR domains. Using a combination of size exclusion chromatography, AUC and SAXS in both 137mM NaCl (physiological salt) and 50 mM NaCl (low salt) buffers, we identified the C-terminal dimer site in CFH to be within the double-domain SCR-17/18 region. From our AUC and SAXS results we propose that the dimer is formed by a side-by-side association of the SCR-17/18 domains, and confirmed this by recourse to recently-modelled solution structures for full-length CFH (6). We discuss the implications of our self-association results for CFH function and how genetic variants may compromise the function of CFH.

MATERIALS AND METHODS

Expression and Purification of the CFH Fragments

In order to locate the self-association site in the SCR-16/20 region of CFH, between one to five domains in the SCR-19/20, SCR-16/20, SCR-18/20, SCR-16/18H, SCR-17/18H, SCR-17H, and SCR-18H constructs were expressed and purified for this study, where the suffix H indicated the presence of a His tag (13). The N- and C-terminal sequences of the expressed SCR domains depended on the fragment. For all seven SCR fragments, the N-termini contain the sequence EAEAF corresponding to the α-factor secretion signal and the EcoRI restriction enzyme site. The SCR-18H, SCR-17H, SCR-17/18H, and SCR-16/18H C-termini contain the first four amino acids of the next linker region followed by the myc tag and His tag sequences ALEQKLISEEDLNSAVDHHHHHH (**Figure 1C**). The SCR-16/18 and SCR-16/20 fragments contained the last three residues while the SCR-19/20 fragment contained the last four residues of the linker at its N-terminal. The SCR domains were cloned into the *Pichia pastoris* expression plasmid pPICZαA and transformed into wild-type X33 cells. Expression was carried out according to Invitrogen guidelines. Briefly, transformants were selected using zeocin given that pPICZαA encodes a zeocin

Abbreviations: C3, complement component 3; CFH, Factor H; SCR, short complement regulator.

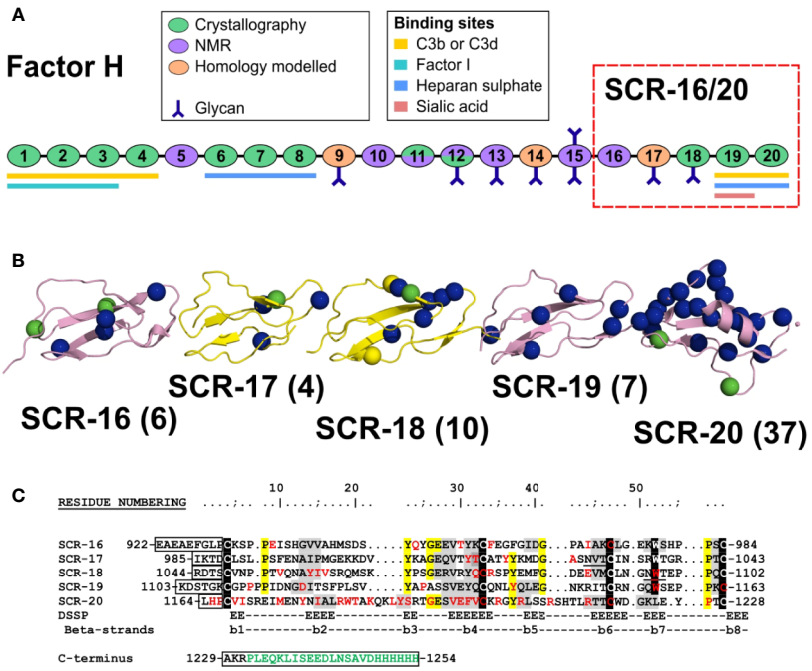

FIGURE 1 | Domain structure and sequences for the Factor H C-terminal domains. **(A)** Schematic diagram of the 20 SCR domains in Factor H, showing their functional significance, knowledge of their protein structures, and their glycan chains. **(B)** Schematic view of the SCR-15/16 protein structures with their disease-associated mutations as blue spheres (aHUS), green spheres (aHUS and C3G) and yellow spheres (C3G). The number of mutations in each domain is bracketed beside the domain label. **(C)** The five domain sequences are shown, with the five conserved Trp and Cys residues highlighted in black, and other conserved residues in yellow. The two glycosylation sites are underlined. The inter-SCR linkers are boxed. Residues highlighted in grey have β-strand secondary structures. The disease-associated residues are colored in red. If expressed with a hexaHis tag, the C-terminal sequence in green will be present. The N-terminal sequence EAEAEF is the α-factor signal and the EcoRI site.

resistant gene. Cell growth was maintained in media containing 2% glycerol for four days. Recombinant protein expression was induced using 0.5% methanol and was maintained every 24 h for four days. Cells were removed by centrifugation and the supernatant containing the secreted SCR domains were concentrated using a 5 kDa molecular weight cutoff membrane.

Fragments with the C-terminal hexa-histidine tags were purified using a 5 ml HiTrap Nickel column (GE Healthcare). The supernatant was dialyzed against 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0 (wash buffer), and loaded onto the column using an AKTA purifier system (GE Healthcare) which had been equilibrated with wash buffer. The column was washed with five column volumes of wash buffer to remove any non-specifically bound protein. Protein was eluted using 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0. For the non-His-tagged SCR domains, ion exchange chromatography was used. SCR-19/20, SCR-16/20 and SCR-18/20 have theoretical isoelectric points of 9.05, 8.04 and 7.69 respectively, thus cation exchange chromatography was used. The supernatant was dialyzed against 50 mM Tris-HCl pH 7.4, 25 mM NaCl, 1 mM EDTA and loaded onto a SP FF column (GE Healthcare) which had been pre-equilibrated with the same buffer. After loading, the column was washed with five column volumes of buffer. Protein was eluted using a salt gradient up to 1 M NaCl. In all seven purifications, protein elution was monitored using the absorbance at 280 nm. Fractions were pooled and concentrated using Amicon ultra centrifugal filters with a molecular weight cutoff membrane of 10 kDa or 3 kDa depending on the SCR domain. Size exclusion chromatography removed any remaining impurities and aggregation (**Figure 2A**). Protein samples were injected onto a Superdex 75 (GE Healthcare) column which had been equilibrated with 10 mM Hepes, 137 mM NaCl, pH 7.4. Molecular weight standards were from BioRad (BioRad Gel Filtration Standard, Hertfordshire, UK). SDS-PAGE monitored sample purity (**Figure 2B**).

Analytical Ultracentrifugation of the CFH Fragments

Analytical ultracentrifugation (AUC) data were collected for each SCR fragment in two buffers, namely 10 mM Hepes pH 7.4, 137 mM NaCl and 10 mM Hepes pH 7.4, 50 mM NaCl. Experiments were carried out in two sector cells (buffer and sample) with column heights of 12 mm. Data were collected in a concentration series between 0.2 – 3 mg/ml for each fragment. Sedimentation velocity experiments were carried out at 20 °C using an AnTi50 rotor at 50,000 rpm in a Beckman-Coulter Proteome XL-I analytical ultracentrifuge. Interference and absorbance optics at 280 nm were used for detection depending on concentrations, the absorbance data being saturated at higher concentrations. Size distribution *c(s)*

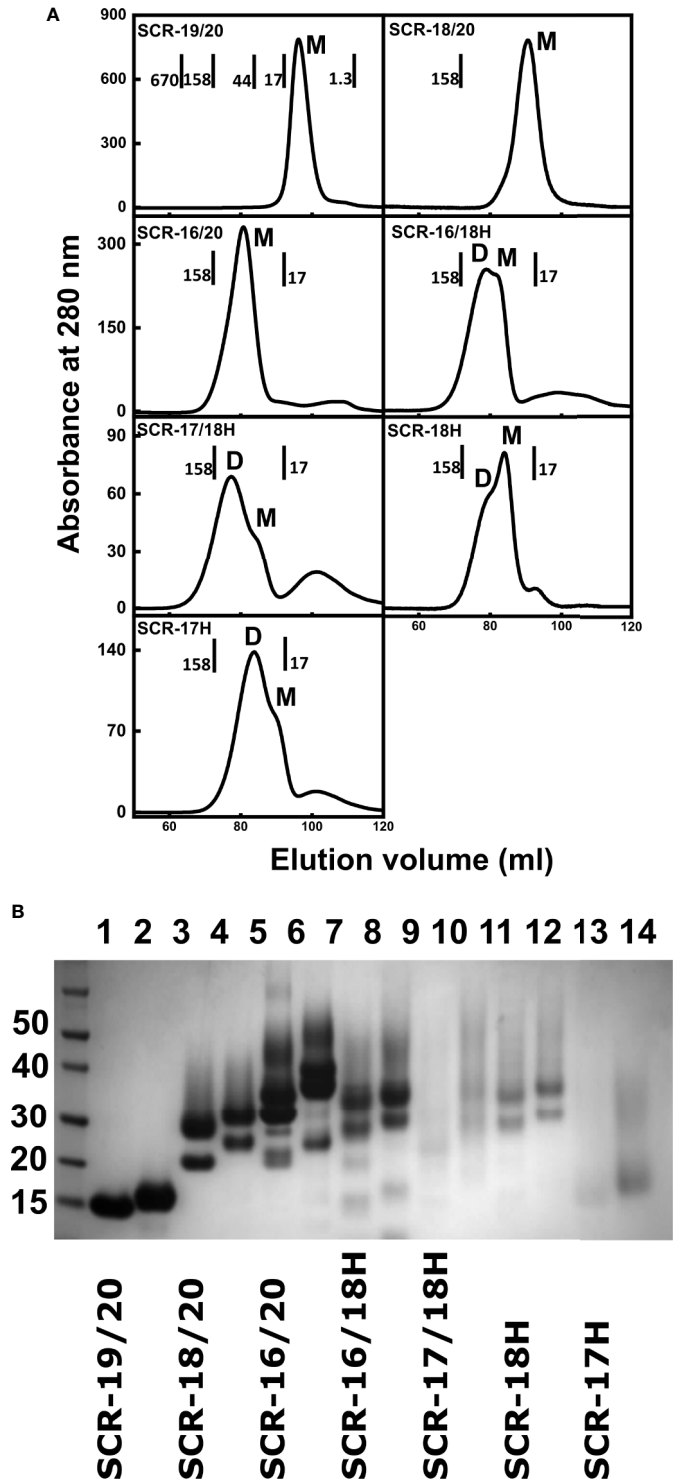

FIGURE 2 | Protein purifications for seven C-terminal domains of Factor H. **(A)** Size exclusion chromatography elution profiles of each of the seven SCR fragments. The molecular mass of the calibration standards are shown in full for SCR-19/20 and in part for the others. The assignment of the peaks as monomer or dimer is denoted by M and D respectively. **(B)** SDS-PAGE analysis of each of the seven SCR fragments. Lanes 1 and 2, SCR-19/20 non-reduced and reduced respectively; lanes 3 and 4, SCR-18/20 non-reduced and reduced; lanes 5 and 6, SCR-16/20 non-reduced and reduced; lanes 7 and 8, SCR-16/18H non-reduced and reduced; lanes 9 and 10, SCR-17/18H non-reduced and reduced; lanes 11 and 12, SCR-18H non-reduced and reduced; lanes 13 and 14, SCR-17H non-reduced; and reduced. Molecular weight standards in kDa are shown to the left.

analyses of the sedimentation boundaries were fitted using SEDFIT according to the Lamm equation (version 14.6) to give the sedimentation coefficients *s* which were corrected to standard $s_{20,w}$ values to allow for the density of water and 20°C (20, 21). Totals of 80-100 boundaries were used with the frictional ratio (f/f_0), meniscus and baseline all floated in the final analyses. The *c(s)* plots were converted to molar mass distribution *c(M)* in order to assess the molecular mass of each sedimenting species.

Small Angle X-Ray Scattering of the CFH Fragments

Small angle X-ray scattering (SAXS) experiments were performed on each of the SCR domains in both 10 mM Hepes, 137 mM NaCl, pH 7.4, and 10 mM Hepes, 50 mM NaCl, pH 7.4 buffers between concentrations of 0.5–2 mg/ml. Data were collected on the bioSAXS beamline BM29 at the European Synchrotron Radiation Facility, Grenoble, France (22). The X-ray wavelength was 0.09919 nm. All experiments were carried out at 20°C. An automated capillary flow sample changer was used on BM29 in which the buffer backgrounds were measured before and after each protein sample (23). Sample volumes of 50 µl were used, collecting 10 frames at a rate of one frame per second. Frames that showed no radiation damage or aggregation were averaged, and the averaged buffer frames were subtracted from the protein scattering curves. EDNA software provided automatic data processing in which the intensities *I(Q)* were automatically scaled by concentration (24). The Biosaxs Customized Beamline Environment (BsxCuBE) software was used for control of the automatic sample changer, and the sample settings were loaded from the Information System for Protein Crystallography Beamlines database (ISPyB) (22, 25).

Guinier analyses at low *Q* (where $Q = 4\pi \sin\theta/\lambda$; 2θ is the scattering angle and λ is the wavelength) were then performed according to the Guinier equation (26).

$$ln\, I(Q) = ln\, I(0) - R_G{}^2Q^2/3$$

Initial data subtraction and Guinier analyses were carried out using the software Primus (27). The radius of gyration R_G was calculated, which monitors the overall elongation of the protein in a given solute-solvent contrast if the internal inhomogeneity of scattering densities within the glycoprotein has no effect. The R_G value was calculated from the linear portion of the Guinier plot ($\ln I(Q)$ v Q^2) within an upper $Q.R_G$ limit of 1.5, together with the forward scattering intensity at zero angle *I(0)*. The program GNOM was used to transform the scattering curves in reciprocal space (*I(Q)*) into real space *via* an indirect Fourier transform to give the distance distribution *P(r)* function (28):

$$P(r) = \frac{1}{2\pi^2}\int_0^\infty I(Q)Qr\sin(Qr)dQ$$

The *P(r)* curve corresponds to the distribution of distances *r* between volume elements in the molecule. The *P(r)* curve yields the R_G value in real space together with *L*, the maximum dimension of the molecule, and *M*, the most frequently observed interatomic distance in the molecule.

Molecular Modelling of the Seven CFH Fragments

The most recent scattering modelling of the 20 SCR domains in CFH used a combination of MODELLER v9.14 and monomer Monte Carlo (SASSIE-web) (91) to build a starting CFH model from previously-known NMR and crystal structures for 17 SCR domains and three SCR homology models for SCR-9, SCR-14 and SCR-17 (6, 29, 30). Eight biantennary disialylated glycans were added to this CFH model (5). In four Monte Carlo simulations based on conformationally varying the inter-SCR linkers, 510,000 full-length CFH models were created, of which many were discarded for reason of steric clashes between the SCR domains to result in a library of 29,715 physically-realistic CFH models for SAXS curve fitting (6). A theoretical scattering curve was generated from each model for comparison with the experimental CFH scattering curve using the *R*-factor (31):

$$R = \sum_{Q_i}\frac{\left|(I_{exp}(Q_i) - I_{model}(Q_i))\right|}{\left|I_{exp}(Q_i)\right|} \quad (1)$$

where Q_i is the *Q* value of the *i*-th data point, $I_{\mathrm{exp}}(Q_i)$ is the experimental scattering intensity and $I_{\mathrm{model}}(Q_i)$ is the theoretical modelled scattering intensity. The *R*-factor vs R_G graphs for 29,715 CFH models were filtered on both the R_G value and *R*-factor. The best-fit 100 models were identified by ranking the filtered models by their *R*-factors. The Tyr402His polymorphism had no effect on the curve fits, leading to an *R*-factor difference of only 0.0003%, thus only the Tyr402 CFH models were used in the present study. These best-fit models are available from the **Supplementary Materials** of our earlier study (6); they are not available in the small angle scattering biological data bank (SASBDB) because this data bank is not suited to the deposition of atomistic scattering models.

In order to evaluate whether the seven CFH fragments of this study could be fitted to monomer models for their structures, each of the 100 best fit CFH Tyr402 models were edited to generate their seven fragments. Those for SCR-19/20, SCR-18/20 and SCR-16/20 were unchanged from those found in the full-length CFH models. Those for SCR-16/18H, SCR-17/18H, SCR-18H, and SCR-17H were modified by the addition of the C-terminal Histag sequence ALEQKLISEEDLNSAVDHHHHHH to the SCR models edited from the full-length CFH models (**Figure 1C**). This additional structure was added to each SCR fragment using MODELLER version 9.14. Because MODELLER does not handle glycans, the two biantennary disialylated glycan chains were reinstated on SCR-17 and SCR-18 by superimpositions using PyMol. CHARMM-GUI software was used to generate the CHARMM force field and PSF inputs for energy minimization in SASSIE-web (6). Once the two glycans were added to the SCR model and accepted by GlycanReader, bash scripts were used to finalize the nomenclature and numbering of the glycan and protein atoms in order to match the experimental protein.

For the AUC modelling, the theoretical $s^\circ_{20,w}$ values for the seven FH fragments were calculated directly from the atomic coordinates with the default value of 0.31 nm for the atomic

element radius for all atoms to represent the hydration shell by using the HYDROPRO shell modelling program (32, 33).

The sequence alignment of CFH SCR-17/18 with the SCR-1/2 domains of complement Factor H related-1 protein (FHR1) was carried out for the Uniprot KB sequences using the EMBOSS water sequence pairwise alignment tool (34). The SCR-17/18 domains were structurally aligned with the FHR1 SCR-1/2 domains using PyMol. This used the homology model for SCR-17 from the solution structure of CFH SCR-16/20 (13), SCR-18 from the crystal structure of CFH SCR-18/20 (35) and the crystal structure of the FHR1 SCR-1/2 dimer (PDB code: 3ZD2) (36). Alignment was carried out using the core residues of the β4 strand of each SCR domain, where SCR-17 was aligned with SCR-1, and SCR-18 was aligned with SCR-2.

RESULTS

Purification of the Seven SCR Fragments of CFH

The non-tagged SCR-19/20, SCR-18/20 and SCR-16/20 fragments were successfully purified from the *P. pastoris* growth media supernatant by cation exchange chromatography (13). Size exclusion chromatography, which separates molecules based on their size and shape, was used as the final purification step. Molecular weight standards were used to estimate the molecular weight, and therefore oligomeric state, of each of the SCR fragments. Elution was monitored by absorbance at 280 nm (**Figure 2A**). SCR-19/20 eluted as a single symmetrical peak with an apparent mass of 10 kDa, which is comparable to 14.7 kDa expected for the monomer (M). Both SCR-18/20 and SCR-16/20 eluted with a single broad peak with a small shoulder peak on the left. SCR-18/20 showed an estimated mass of 77 kDa, and SCR-16/20 showed a mass of 71 kDa, both of which were much larger than the expected masses of 24 kDa and 38 kDa respectively. The discrepancies between the observed and expected molecular masses were attributed to the elongated shapes of the three fragments, in distinction to the molecular weight standards used for the column calibrations which were a set of globular proteins of compact shapes.

Four additional fragments containing one-to-three domains and His-tagged C-termini, namely SCR-16/18H, SCR-17/18H, SCR-17H, and SCR-18H, were likewise purified from the yeast supernatant using nickel affinity chromatography. SCR-16/18H, SCR-17/18H, SCR-18H, and SCR-17H eluted with two overlapping peaks that were assigned to dimer (D) and monomer (M) (**Figure 2A**). SCR-16/18H, SCR-17/18H, and SCR-17H showed more dimer than monomer, while SCR-18H showed more monomer. For SCR-16/18H, even though the predicted mass from the sequence was 29 kDa, peaks D and M showed masses of 141 kDa and 120 kDa respectively. For SCR-17/18H, even though the predicted mass from the sequence was 22 kDa, peaks D and M showed masses of 102 kDa and 84 kDa respectively. SCR-17H with a predicted mass of 10 kDa showed apparent molecular masses of 48 kDa and 23 kDa for peaks D and M respectively. SCR-18H with a predicted mass of 11 kDa showed apparent molecular masses of 79 kDa and 48 kDa for peaks D and M respectively. The discrepancies between the observed and expected molecular masses in the latter cases were attributed to the presence of both glycan chains and extended His-tag structures.

Protein purities were assessed by SDS-PAGE (**Figure 2B**). As SCR-17 and SCR-18 contained N-linked glycan chains, six of the SCR fragments (**Figure 1B**) showed streaking on the gel which is characteristic of glycosylated proteins, and seen previously for SCR-16/20 (13). SDS does not bind sufficiently to glycan chains, resulting in a non-uniform net charge in SDS-PAGE. As expected, only SCR-19/20 showed one band. Multiple bands were observed for the glycosylated fragments and attributed to variations in the glycosylation pattern which is often observed for glycoproteins expressed in *P. pastoris* (37). The purity and consequently the identity of each of the constructs used in this study was confirmed by Western blots using an anti-FH polyclonal goat antibody. With the exception of SCR-18, all bands that were present on the SDS-PAGE gel were confirmed to be FH. For SCR-18, MALDI-TOF mass spectroscopy analysis was carried out to confirm its mass as 11,180 Da, in agreement with the sequence. Mass variations of ± 700 Da were observed corresponding to the differential glycosylation pattern in SCR-18.

AUC of the Seven CFH Fragments in 137 mM NaCl

AUC quantitatively separates macromolecules according to their size and shape, this method being superior to the qualitative estimates from size-exclusion chromatography (38). Different molecular species within a sample are detected from the peaks in the size distribution *c(s)* analyses (**Figure 3**, right) that are calculated from the sedimentation boundaries (**Figure 3**, left). The sedimentation coefficient $s_{20,w}$ at 20°C and corrected for the density of water gives the frictional ratio f/f_o. This measures the protein shape, with a compact globular protein typically having a f/f_o ratio of 1.2 where f is the observed frictional coefficient and f_0 is the frictional coefficient for a spherical protein with the same mass. The f/f_o ratio will indicate the degree of elongation upon protein dimerization. The relative percentages of monomer and dimer in the sample are calculated from peak integrations in the *c(s)* analyses. These integrations give the dissociation constant K_D for dimer formation. Conversion of the *c(s)* peaks to the corresponding mass distributions *c(M)* gives the molecular mass for each species present.

Interference optics were used for the three non-His tagged fragments SCR-19/20, SCR-18/20 and SCR-16/20, where good boundary fits were obtained in all cases (**Figure 3A**). For this work, buffers with 137 mM NaCl were used that correspond closely to the ionic strength of blood plasma.

(i) For SCR-19/20, only one peak was visible in the *c(s)* plot for four concentrations, indicating that only monomer was present with an $s_{20,w}$ value of 1.6 S (**Figure 4**). The *c(M)* analysis gave a molecular mass of 15–18 kDa, which was in good agreement with the sequence-calculated mass of 15 kDa. The f/f_o ratio for SCR-19/20 was 1.1 showing that it had a relatively compact shape (**Table 1**).

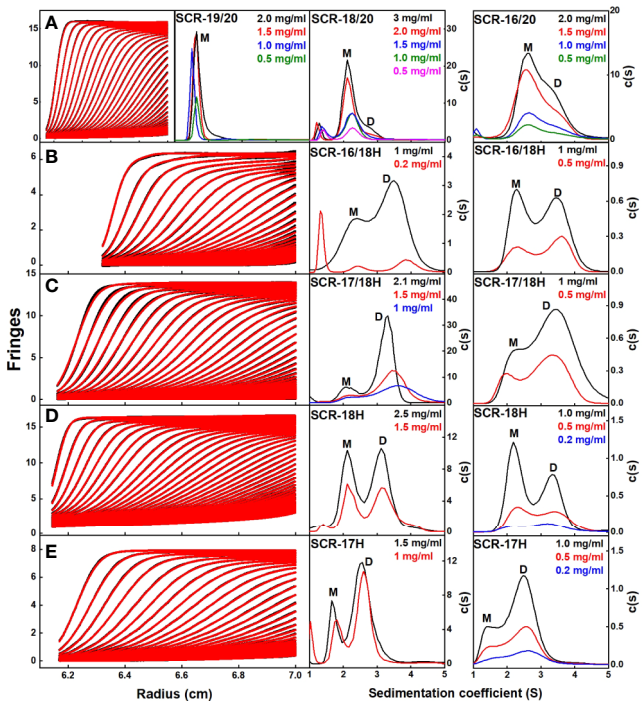

FIGURE 3 | Sedimentation velocity *c(s)* distribution analyses of the C-terminal SCR fragments. In the left panels, 60–80 scans and boundary fits are shown using interference optics. Only every seventh to tenth scan is shown. In the right panels, the resulting *c(s)* distributions are shown, in which the peaks for the observed *s* values of the monomer, dimer and tetramer are denoted by M, D, and T respectively. The concentrations are shown in each panel, highlighted according to their colors in the *c(s)* distributions. **(A)** For SCR-19/20, the *c(s)* analyses showed a single monomeric peak. For SCR-18/20, the *c(s)* analyses showed mostly monomer and some dimer. For SCR-16/20, the *c(s)* analyses showed two partly resolved peaks corresponding to monomer and dimer. **(B)** For SCR-16/18H, the *c(s)* distribution on the left shows interference optics while the right shows absorbance optics. The two peaks M and D correspond to monomeric and dimeric SCR-16/18H. **(C)** For SCR-17/18H, the two peaks M and D correspond to monomer and dimer. **(D)** For SCR-18H, the two peaks M and D correspond to monomer and dimer. **(E)** For SCR-17H, the two peaks M and D correspond to monomer and dimer.

(ii) SCR-18/20 showed one peak in the *c(s)* plot with an $s_{20,w}$ value of 2.4 S corresponding to the monomeric protein. At higher concentrations of 2–3 mg/ml, a small shoulder peak was evident with an $s_{20,w}$ of 3.1 S which was attributed to a low amount of dimer formation (**Figure 3A**). Integration showed that this shoulder peak accounted for 10–18% of the sample for 2–3 mg/ml (**Figure 4B**). From this, the dissociation constant K_D for the SCR-18/20 dimer was estimated to be 590 ± 150 μM. The molecular mass for the monomer was determined to be 26–34 kDa, in accord with the sequence-calculated monomer mass of 24 kDa, and 58 kDa for the shoulder peak to confirm that this was dimer. SCR-18/20 showed a f/f_0 ratio of 1.1 for the monomer and 1.6 for the dimer peak, showing that the dimer was more elongated than the monomer (**Table 1**).

(iii) The *c(s)* curve for SCR-16/20 showed the presence of monomer and dimer with two partially merged peaks corresponding to a monomer-dimer equilibrium, with the monomer $s_{20,w}$ value at 2.6 S and the dimer $s_{20,w}$ value at 3.6 S (**Figure 3A**), as reported previously (13). The 60–40% ratio of monomer-dimer did not significantly change with concentration (**Figure 4B**), and resulted in a K_D value of 31 ± 14 μM for dimer formation. The molecular masses were determined to be 41–48 kDa for the monomer and 65–66 kDa for dimeric SCR-16/20, as expected from the sequence-calculated monomer mass of 38 kDa. The f/f_0 ratio was 1.5 for the monomer and 1.7 for the dimer, indicating that the dimer was slightly more elongated than the monomeric protein (**Table 1**).

In order to locate the dimerization site more precisely, four His-tagged SCR fragments were available, based on SCR-16, SCR-17, and SCR-18, and denoted by H suffixes. AUC data for these were based on both interference and absorbance optics for which again good boundary fits were obtained (**Figures 3B–E**):

(iv) For SCR-16/18H, the *c(s)* plots showed well-resolved monomer and dimer peaks, even at low concentrations of 0.2 mg/ml (**Figure 3B**). Interference optics gave $s_{20,w}$ values of 2.6 S and 2.3 S for the monomer and 4.0 S and 3.6 S for the dimer. Absorbance optics gave $s_{20,w}$ values of 2.5 S and 3.6 S for the monomer and dimer respectively. The *c(M)* analyses gave 21–32 kDa for the monomer and 40–55 kDa for the dimer, in good accord with the sequence-calculated monomer mass of 29 kDa. Integration showed that SCR-16/18H was 40% monomer and 60% dimer (**Figure 4B**), giving a K_D value of 6 ± 5 μM for dimer

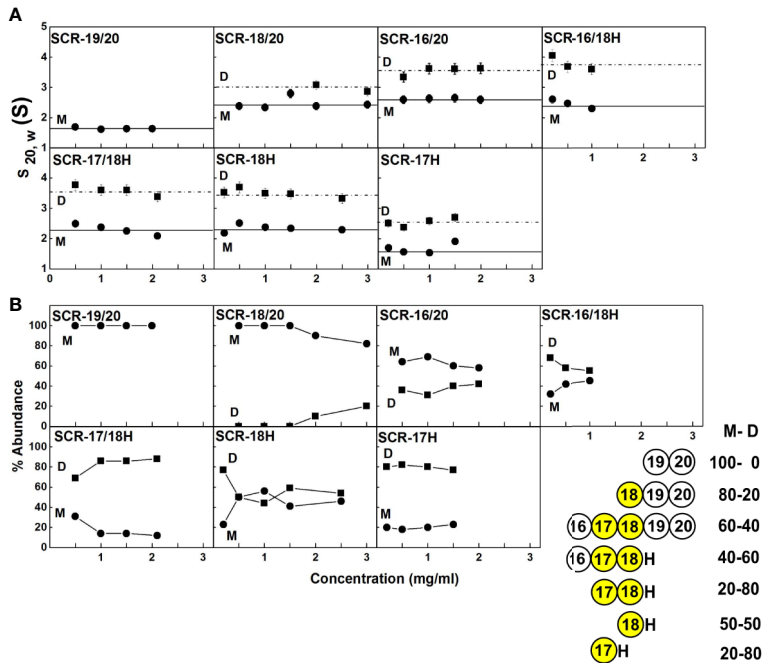

FIGURE 4 | Concentration-dependence of the sedimentation data for each of the SCR fragments. **(A)** The sedimentation coefficients $s_{20,w}$ corrected for buffer density and temperature for the monomer (M) is denoted by filled circles, and for the dimer (D) with filled squares. Statistical error bars are shown where visible. **(B)** The relative percentage of monomer and dimer present in the *c(s)* analyses. The inset to the right summarizes the relative percentages of monomer (M) and dimer (D) from the AUC analyses. SCR-17 and SCR-18 are highlighted in yellow as the dimer site.

TABLE 1 | AUC and SAXS parameters for the seven SCR fragments in 137 mM and 50 mM NaCl.

Domains	$s^0_{20,w}$ (S) 137 mM	$s^0_{20,w}$ (S) 50 mM	f/f_o 137 mM	f/f_o 50 mM	R_G (nm)[a] 137 mM	R_G/R_O 137 mM	R_G (nm)[a] 50 mM	R_G/R_O 50 mM	L (nm) 137 mM	L (nm) 50 mM
SCR-19/20 monomer	1.64	1.8	1.1	1.04	2.4 ± 0.09	1.7	2.2 ± 0.1	1.6	8.2 ± 0.4	8.2 ± 0.4
SCR-18/20 monomer	2.4	2.4	1.1	1.1	3.2 ± 0.06	2	3.4 ± 0.1	2	12.2 ± 0.6	11.7 ± 0.6
SCR-18/20 dimer	2.9	2.9	1.6	1.6	3.62 ± 0.03	2.2	3.6 ± 0.03	2.2	12.7 ± 0.3	12.3 ± 0.6
SCR-16/20 monomer	2.59	3.1	1.5	1.3	4.7 ± 0.06	2.4	4.8 ± 0.1	2.5	16.8 ± 0.8	17.5 ± 0.8
SCR-16/20 dimer	3.63	4.2	1.7	1.5	6 ± 0.1	3.1	6.1 ± 0.1	3.2	21 ± 1	24 ± 0.2
SCR-16/18H monomer	2.47	2.2	1.3	1.5	4.7 ± 0.04	2.7	4.4 ± 0.1	2.5	17 ± 0.8	15.5 ± 0.8
SCR-16/18H dimer	3.68	3.5	1.4	1.5	4.7 ± 0.06	2.7	4.4 ± 0.01	2.5	17 ± 0.5	15.9 ± 0.8
SCR-17/18H monomer	2.49	2.2	1.3	1.3	2.7 ± 0.2	1.7	2.7 ± 0.1	1.4	9 ± 0.5	9.7 ± 0.5
SCR-17/18H dimer	3.77	3.8	1.3	1.3	2.8 ± 0.01	1.7	2.8 ± 0.01	1.4	10.2 ± 0.5	9.7 ± 0.2
SCR-18H monomer	2.51	2.32	1.2	1.3	2.2 ± 0.03	1.4	2.2 ± 0.03	1.5	8 ± 0.4	7.9 ± 0.4
SCR-18H dimer	3.49	3.69	1.3	1.3	2.2 ± 0.01	1.4	2.2 ± 0.02	1.5	8 ± 0.1	7.9 ± 0.3
SCR-17H monomer	1.56	1.82	1.2	1.7	3.5 ± 0.03	2.2	3.2 ± 0.01	2	12 ± 0.6	11.9 ± 0.6
SCR-17H dimer	2.37	2.86	1.6	1.6	3.4 ± 0.01	2.2	3.2 ± 0.1	2	12 ± 0.1	11 ± 0.6

[a]*For the SAXS results, the monomer is taken as the value recorded at the lowest concentration (i.e. lowest dimer percentage) and the dimer is taken as the highest concentration (i.e. the highest dimer percentage).*

formation. The f/f_o ratio was 1.3 for the monomer and 1.4 for the dimer, indicating that the protein became slightly more elongated upon dimer formation (**Table 1**).

(v) For SCR-17/18H, the interference and absorbance data showed monomer and dimer peaks (**Figure 3C**). The $s_{20,w}$ values were 2.1 S to 2.5 S for the monomer and 3.4 S to 3.6 S for the dimer (**Figure 4A**). The experimental molecular masses were 21–24 kDa for the monomer and 44–49 kDa for the dimer, in good agreement with a sequence-calculated monomer mass of 22 kDa. SCR-17/18H existed as 80% dimer (**Figure 4B**), giving a K_D for SCR-17/18H dimer formation of 3 ± 1 μM. The f/f_0 ratio was 1.3 for both the monomer and dimer, showing that both were relatively compact in their structures.

(vi) SCR-18H also showed two peaks in the *c(s)* distribution (**Figure 3D**). The first peak showed $s_{20,w}$ values of 2.2–2.5 S and the second peak showed $s_{20,w}$ values of 3.5–4.0 S. The two peaks were each approximately 50% in size (**Figure 4B**), giving a K_D value for SCR-18H dimer formation of 37 ± 27 μM. The *c(M)*

analyses gave molecular masses of 20–31 kDa for the first peak and 37–57 kDa for the second peak. Both values were double those expected from the sequence-calculated mass of 11 kDa for the monomer, thus it was not clear if the two peaks corresponded to monomer-dimer or dimer-tetramer. It is possible that the relatively large glycan chain on SCR-18H may affect the sedimentation results. Nonetheless the f/f_0 ratio was calculated to be similar at 1.2 for the first peak and 1.3 for the second peak assuming that these corresponded to monomer and dimer, showing that both were relatively compact in their structures.

(vii) For SCR-17H, two peaks were also evident in the *c(s)* distribution (**Figure 3E**). The first peak showed $s_{20,w}$ values of 1.5–1.9 S and the second peak showed $s_{20,w}$ values of 2.4–2.7 S. SCR-17H exists as 80% dimer (**Figure 4B**), from which the K_D value for dimer formation was 5 ± 4 μM. The *c(M)* analyses gave molecular masses of 17–23 kDa for the first peak and 30–41 kDa for the second peak. As found with SCR-18H, both values were double those expected from the sequence-calculated mass of 10 kDa for the monomer, thus it was not clear if the two peaks corresponded to monomer-dimer or dimer–tetramer. Nonetheless the $s_{20,w}$ values were in the expected range for monomeric and dimeric SCR-17H. The f/f_0 ratios were calculated for monomeric and dimeric SCR-17H to be 1.14 for the monomer and 1.6 for the dimer, indicating some elongation upon dimer formation.

With the exception of SCR-19/20 which was monomeric, the other six SCR fragments each showed two distinct *c(s)* peaks corresponding to monomer and dimer. The strongest dimerization with K_D values in the range of 3-6 μM was observed for the three smaller fragments when SCR-17H was present (inset, **Figure 4B**), thus it was confirmed that SCR-17H comprised the main C-terminal CFH dimer site. SCR-18H alone also showed self-dimerization. The three larger fragments showed weaker dimer formation with K_D values of 31 μM, 37 μM and 590 μM.

AUC of the Seven CFH Fragments in 50 mM NaCl

The same AUC analysis was carried out on each of the seven SCR fragments, but in low salt buffers containing 50 mM NaCl in order to act as a control for the above analyses that used 137 mM NaCl buffers. Low salt buffer will promote stronger interactions between charged groups if present. Interference and absorbance optics were used for all the SCR fragments except for SCR-16/20 when only interference optics were used (data not shown) (39).

(i) The *c(s)* distribution for SCR-19/20 again showed only one peak for monomer with an $s_{20,w}$ value of 1.8 S and 1.7 S between 0.5–2.0 mg/ml (**Table 1**).

(ii) The *c(s)* distribution for SCR-18/20 also again showed a major monomer peak in the *c(s)* distribution with an $s_{20,w}$ value of 2.2–2.3 S, together with a small shoulder peak at concentrations above 1 mg/ml with a $s_{20,w}$ value of 2.9 S. Because the monomer accounted for 80% of the protein, the K_D value for dimer formation was estimated at 180 ± 130 μM (**Table 1**).

(iii) SCR-16/20 again showed two partially resolved peaks corresponding to monomer and dimer. The monomer showed an $s_{20,w}$ value of 2.8–3.1 S for 0.5–2 mg/ml while the dimer showed an $s_{20,w}$ value of 4.2 S. The percentage of dimer increased with concentration from 11% to 30%, leading to a K_D value for dimer formation of 90 ± 20 μM (**Table 1**).

(iv) SCR-16/18H showed a monomer peak in the *c(s)* plot with an $s_{20,w}$ value of 2.2 S and a dimer peak with an $s_{20,w}$ value of 3.3–3.5 S. Monomer and dimer comprised 50% each, and the K_D value for dimer formation was of the order 10 μM.

(v) SCR-17/18H showed a smaller monomer *c(s)* peak at a $s_{20,w}$ value of 2.2 S and a larger dimer peak at 3.5–3.8 S. The percentage of dimer was 80% and this corresponded to a K_D value for dimer formation of 2 ± 1 μM.

(vi) SCR-18H exhibited a larger monomer *c(s)* peak at an $s_{20,w}$ value of 2.3 S and a smaller dimer peak at an $s_{20,w}$ value of 3.7 S. As for the 137 mM NaCl *c(s)* analysis, the $s_{20,\ w}$ and mass values were larger than expected for a 10 kDa protein. The percentage of monomer was 60% and the K_D value for dimer formation was of the order of 60 μM.

(vii) SCR-17H showed a smaller monomer *c(s)* peak at an $s_{20,w}$ value of 1.5–1.8 S, and a larger dimer peak at an $s_{20,w}$ value of 2.5–2.8 S, with 80% dimer. The K_D value for SCR-17H dimer formation was calculated to be 5 ± 3 μM. The $s_{20,w}$ and mass values were larger than expected for a 10 kDa protein.

Overall, the *c(s)* results for 50 mM NaCl buffer agreed with the *c(s)* results from the 137 mM NaCl buffer study above. The K_D values were similar in 50 mM NaCl buffer when compared to those for 137 mM NaCl buffer. Importantly, it was deduced that electrostatic interactions were not significant in CFH dimer formation. SCR-17H was again identified as the dimer site because the three smaller fragments with this SCR-17 domain showed the lowest (strongest binding) K_D values in a range of 2-10 μM. In contrast the three other K_D values were 60, 90 and 180 μM.

SAXS Results for the Seven CFH Fragments in 137 mM NaCl

SAXS yields size and shape information on macromolecules in solution (40). SAXS data with good signal to noise was obtained for the seven SCR fragments in 137 mM NaCl buffer. Guinier analysis were carried out on the subtracted curves to calculate the radius of gyration R_G which is a measure of the overall elongation of the molecule (**Figure 5A**) and the R_G of the cross section (R_{XS}) (**Figure 5B**). Successful linear Guinier analyses for each of the SCR fragments were carried out within satisfactory fit limits of the $Q.R_G$ and $Q.R_{XS}$ values, namely 0.6–1.2 and 0.4–1.0 respectively. The R_G/R_O ratio compares the elongation of the protein with respect to a sphere, where R_O is the R_G of a perfect sphere with the same volume as the hydrated protein. Typical globular proteins have a R_G/R_O ratio of 1.28 (41). Solution scattering represents an average of the species present in the sample, and monomer and dimer could not be distinguished as such. Nonetheless scattering provides an independent monitor of the extent of SCR dimerization to complement the AUC data.

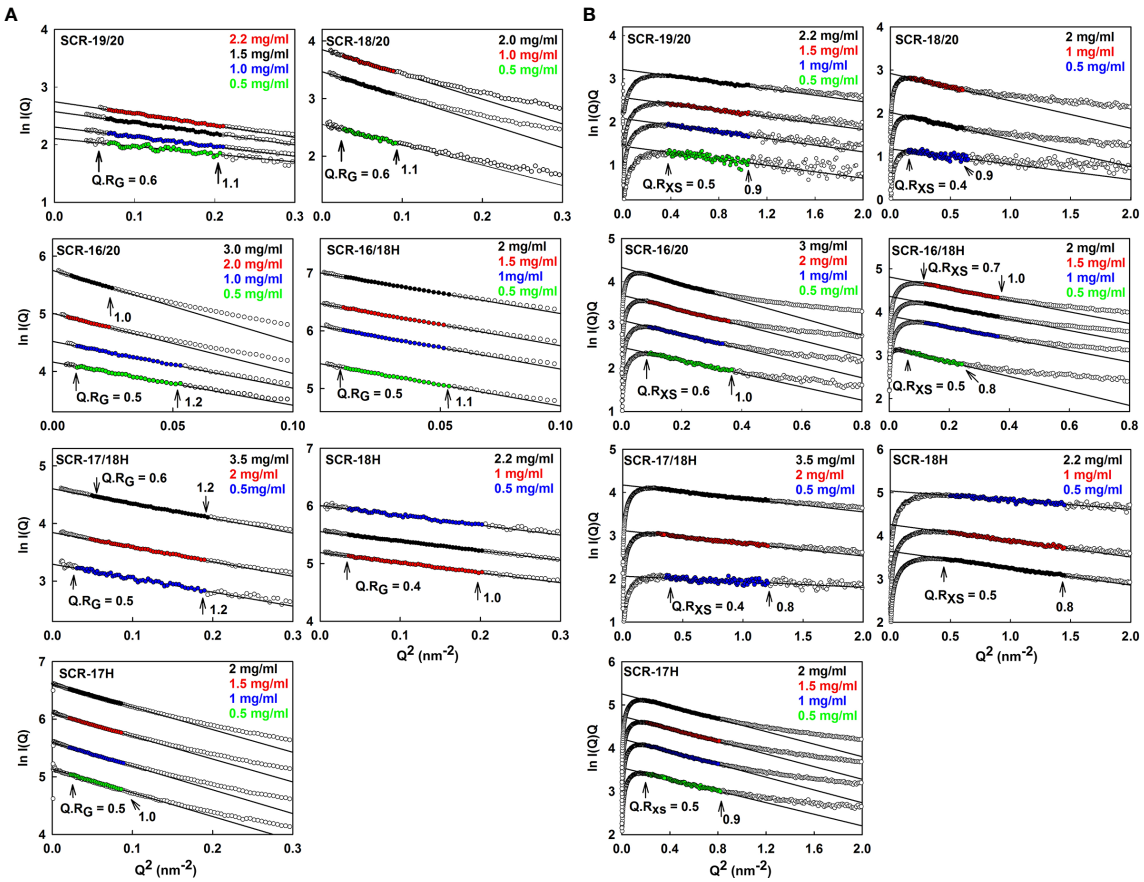

FIGURE 5 | Guinier R_G and R_{XS} analyses for each of the seven SCR fragments. The filled circles represent the experimental X-ray data points used to determine the R_G and R_{XS} values. Their values were measured within the satisfactory $Q.R_G$ and $Q.R_{XS}$ ranges shown in each panel. **(A)** The SCR fragment concentrations in 137 mM NaCl buffer are shown in the panels, where the colors correspond to the indicated concentrations. The filled circles correspond to the *I(Q)* values used to determine each R_G value. The *Q* ranges used for the R_G fits were 0.24–0.44 nm^{-1} for SCR-19/20, 0.14–0.3 nm^{-1} for SCR-18/20, 0.10–0.22 nm^{-1} for SCR-16/20 monomer and SCR-16/18H, 0.10–0.14 nm^{-1} for the SCR-16/20 dimer, 0.20–0.44 nm^{-1} for SCR-17/18H, 0.17–0.45 nm^{-1} for SCR-18H and 0.14–0.28 nm^{-1} for SCR-17H. **(B)** The corresponding R_{XS} analyses for the seven SCR fragments are shown, where the *Q* ranges used for the R_{XS} fits were 0.55–1.02 nm^{-1} for SCR-19/20, 0.39–0.79 nm^{-1} for SCR-18/20, 0.28–0.6 nm^{-1} for the SCR-16/20 monomer, 0.28–0.50 nm^{-1} for the SCR-16/20 dimer, 0.22–0.49 nm^{-1} for the SCR-16/18H monomer, 0.35–0.60 nm^{-1} for the SCR-16/18H dimer, 0.53–1.09 nm^{-1} for SCR-17/18H, 0.45–0.88 nm^{-1} for SCR-18H and 0.7–1.19 nm^{-1} for SCR-17H.

For the three non-His-tagged fragments SCR-19/20, SCR-18/20, and SCR-16/20, Guinier analyses were carried out:

(i) For SCR-19/20, the R_G value was 2.4 ± 0.1 nm and the R_G/R_O ratio was 1.7 indicating that it was elongated with respect to a globular protein of the same size. SCR-19/20 had an R_{XS} value of 0.86 ± 0.04 nm. Neither the R_G nor the R_{XS} changed significantly with respect to concentration as expected for the monomeric SCR-19/20 fragment (**Figures 6A, B**).

(ii) For SCR-18/20 the R_G value increased with concentration from 3.2 ± 0.1 nm to 3.6 ± 0.03 nm (**Figure 6A**). The R_G/R_O ratio increased from 2.0 to 2.2 which showed that SCR-18/20 had an elongated shape which became further elongated upon dimer formation. The R_{XS} value for SCR-18/20 increased from 0.9 ± 0.04 nm to 1.2 ± 0.1 nm (**Figure 6B**).

(iii) For SCR-16/20, the R_G value increased from 4.7 ± 0.1 nm to 6.0 ± 0.1 nm (**Figure 6A**). The R_G/R_O ratio increased from 2.4 to 3.1, with SCR-16/20 becoming more elongated upon dimer formation. The R_{XS} value increased with concentration from 1.6 ± 0.08 nm to 2.04 ± 0.1 nm (**Figure 6B**); the increase in R_{XS} compared to that of SCR-19/20 showed that dimerization occurred by a side-to-side association of the five SCR domains.

For the four His-tagged SCR fragments, Guinier analyses showed that the R_G values did not change significantly with concentration (**Figures 6A, B**).

(iv) SCR-16/18H had an R_G value of 4.7 ± 0.1 nm and an R_G/R_O ratio of 2.7. The R_{XS} value for SCR-16/18H was 1.6 ± 0.08 nm. These values resembled those for SCR-16/20.

(v) SCR-17/18H had an R_G value of 2.8 ± 0.1 nm and an R_G/R_O ratio of 1.7. SCR-17/18H had an R_{XS} value which increased at higher concentrations from 0.51 ± 0.03 nm to 0.73 ± 0.04 nm

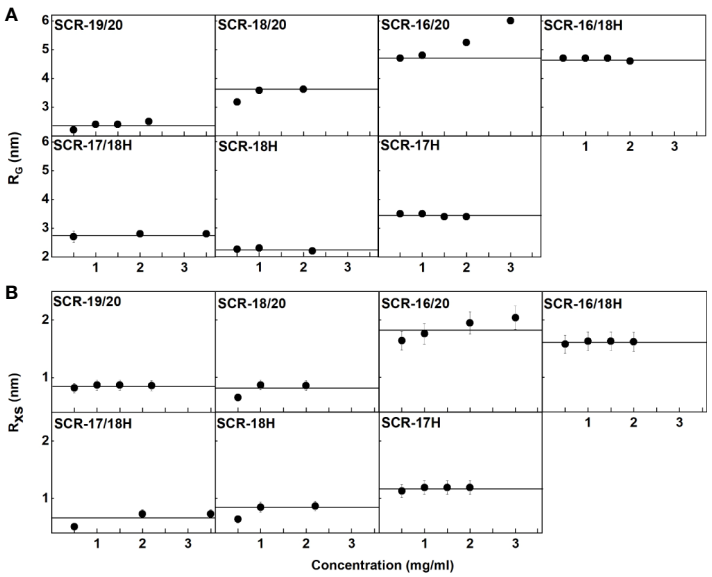

FIGURE 6 | Concentration-dependence of the R_G and R_{XS} value of the seven SCR fragments. **(A)** The X-ray R_G values are shown, where the lines denote the mean value. **(B)** The X-ray R_{XS} values are shown, where the lines denote the mean value.

(**Figure 6B**). Its R_G value was larger than that of SCR-19/20, this being attributed to its larger size and glycosylation.

(vi) SCR-18H had an R_G value of 2.3 ± 0.03 nm, an R_G/R_O ratio of 1.4, and an R_{XS} value of 2.3 ± 0.1 nm. The similarity of its R_G value to that of SCR-19/20 is consistent with SCR-18H existing as a mixture of monomer and dimer, although its glycosylation and His-tag will complicate this interpretation.

(vii) SCR-17H had an R_G value of 3.5 ± 0.02 nm, an R_G/R_O ratio of 2.2, and an R_{XS} value of 3.5 ± 0.2 nm. Its significantly larger R_G and R_{XS} values compared to SCR-19/20 and SCR-17/18H is attributed to its high level of dimer formation as well as its glycosylation and His-tag. This agrees with the 80% dimer seen in the AUC *c(s)* analyses.

The distance distribution function *P(r)* provides the maximum dimension of the macromolecule L and the most frequently observed interatomic distance M (**Figure 7**; **Table 1**). As a check, the *P(r)* analyses were found to give R_G values that agreed well with the Guinier R_G values.

(i) SCR-19/20 gave a length L of 8.2 ± 0.4 nm and an M value of 1.7 ± 0.1 nm, neither of which changed with concentration as expected for a monomer.

(ii) SCR-18/20 gave a length L of 12.2 ± 0.6 nm and an M value of 2.7 ± 0.1 nm at high concentration. The increase in L is as expected from the addition of an extra SCR domain of length 3.6 nm to SCR-19/20.

(iii) For SCR-16/20, the length L increased with concentration from 16.8 ± 0.8 nm to 21 ± 1 nm. The M values also increased from 4.0 ± 0.2 nm to 4.8 ± 0.2 nm. This indicated increases in its dimerization with concentration, this being consistent with the AUC data.

(iv) SCR-16/18H gave a length L of 17 ± 0.8 nm and an M value of 4.1 ± 0.2 nm with no observed concentration dependent changes. The additional length when compared to SCR-18/20 indicated that the His-tag is extended in its conformation.

(v) For SCR-17/18H, its length L was between 9 ± 0.5 nm to 10.2 ± 0.5 nm with M values of 1.7 ± 0.1 nm. Because these lengths are not much greater than that for SCR-19/20, this outcome suggested that dimerization occurred through a side-by-side association of SCR-17/18.

(vi) SCR-18H had a length of 8 ± 0.4 nm and an M value of 2.1 ± 0.1 nm. These relatively large values are consistent with dimer formation and the presence of glycosylation and the His-tag in its structure.

(vii) SCR-17H had an L value of 12 ± 0.6 nm with an M value of 2.6 ± 0.1 nm. These larger values compared to SCR-18H showed that more dimer was present in this case, as suggested by the AUC analyses.

Overall the SAXS analyses confirmed the AUC analyses that showed that SCR-19/20 was monomeric, and that dimerization occurred for the other six SCR fragments. The R_{XS} and L values suggested that dimer formation occurred as a side-by-side association and not as an end-to-end association.

SAXS Results for the Seven CFH Fragments in 50 mM NaCl

Similar SAXS analyses were performed on each of the seven SCR fragments in buffers containing 50 mM NaCl as a control to check the effect of low salt on protein dimerization (data not shown; **Table 1**) (39). The Guinier R_G and R_{XS} plots gave high quality linear fits, as exemplified in **Figure 5** for 137 mM NaCl buffer, within the satisfactory fit limits of the $Q.R_G$ and $Q.R_{XS}$

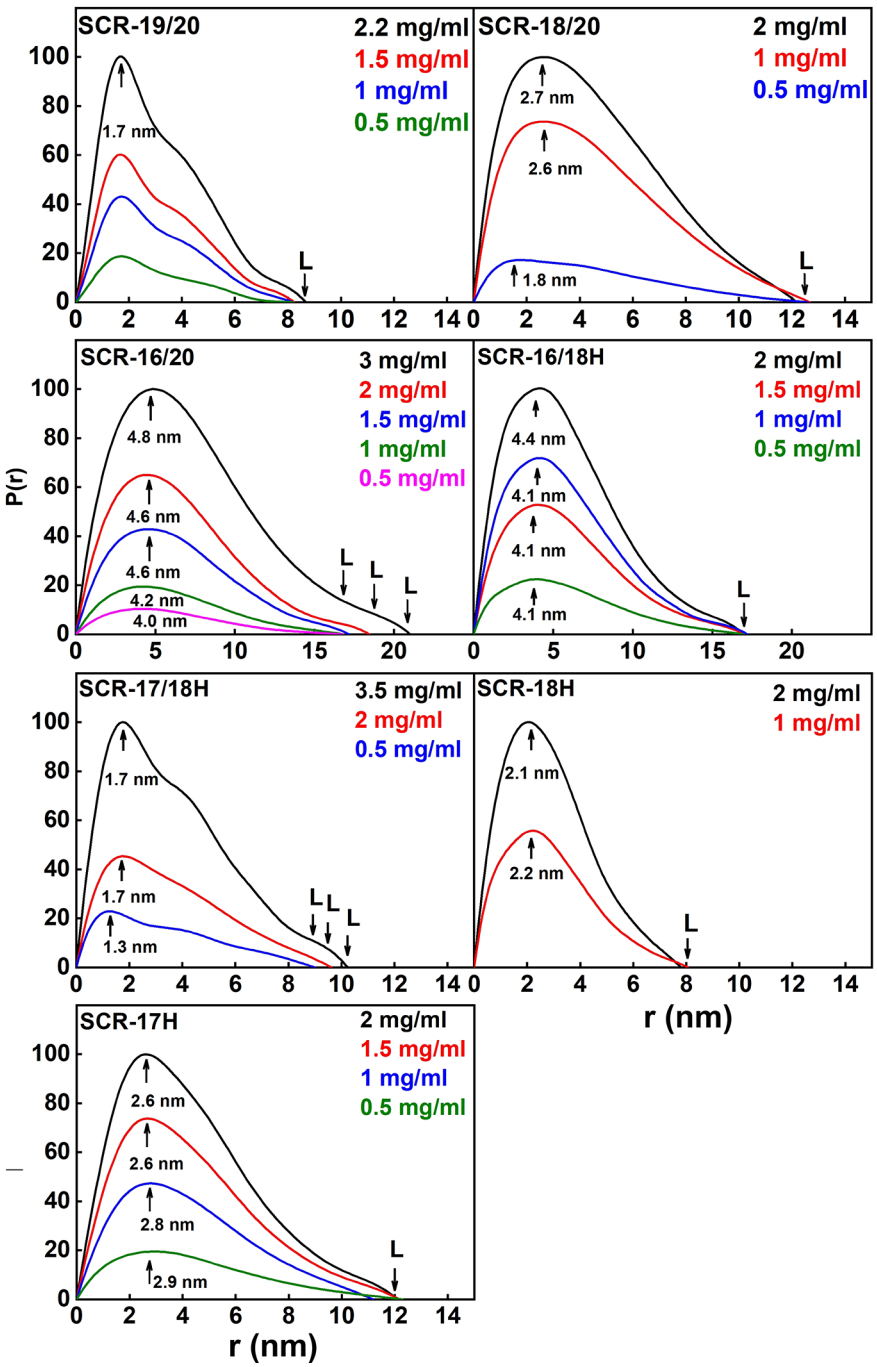

FIGURE 7 | Distance distribution function *P(r)* analyses for each of the seven SCR fragments. The arrow under each peak represents M, the most frequent distance within the protein, and L represents the maximum observed dimension of the SCR fragment.

values as above. For the seven SCR fragments, similar R_G and R_{XS} results to those in 137 mM NaCl were obtained (**Table 1**). The distance distribution *P(r)* curves for each of the SCR domains were likewise calculated for comparison with those obtained for 137 mM NaCl (not shown). Again, little difference in the lengths L was observed between 137 mM and 50 mM NaCl (**Table 1**). As for the AUC results, lowering the ionic strength of the buffer did not significantly alter the calculated SAXS parameters for the SCR fragments. This confirmed the above AUC results that electrostatic interactions were not significant in dimer formation.

Modelling of the SAXS Curves for the Seven CFH Fragments

Atomistic scattering modelling reproduces the SAXS scattering curves of a macromolecule by recourse to physically-realistic molecular models created from Monte Carlo and molecular dynamics simulations (42). To model the seven C-terminal SCR fragments in this study, we used the recently published scattering model of full-length CFH to create molecular structures for the seven individual CFH fragments (6). That study generated 29,715 physically-realistic conformationally-randomized structures for CFH, from which the 100 best-fit structures to the scattering curve of full-length CFH were identified, as well as a single best-fit median CFH structure. Starting from the best fit model of that study, the seven SCR fragments were created by edits of the full-length CFH structure. The C-terminal His-tags were modelled onto four of these fragments (**Supplementary Figure 1**). For the three non-His-tagged models, additional molecular structures for these fragments were extracted from the 100 best-fit CFH structures in order to assess the variability of the calculations between different structures in that set.

The scattering curve fits (**Figure 8**) confirmed the above AUC and SAXS results on the dimerization of the CFH fragments. Because the SAXS data represent an average of the species present in solution, the fits do not distinguish monomer and dimer, but rather the deviation of the fits from an assumed monomer structure. The modelling did not consider directly the occurrence of dimer formation because the molecular structures

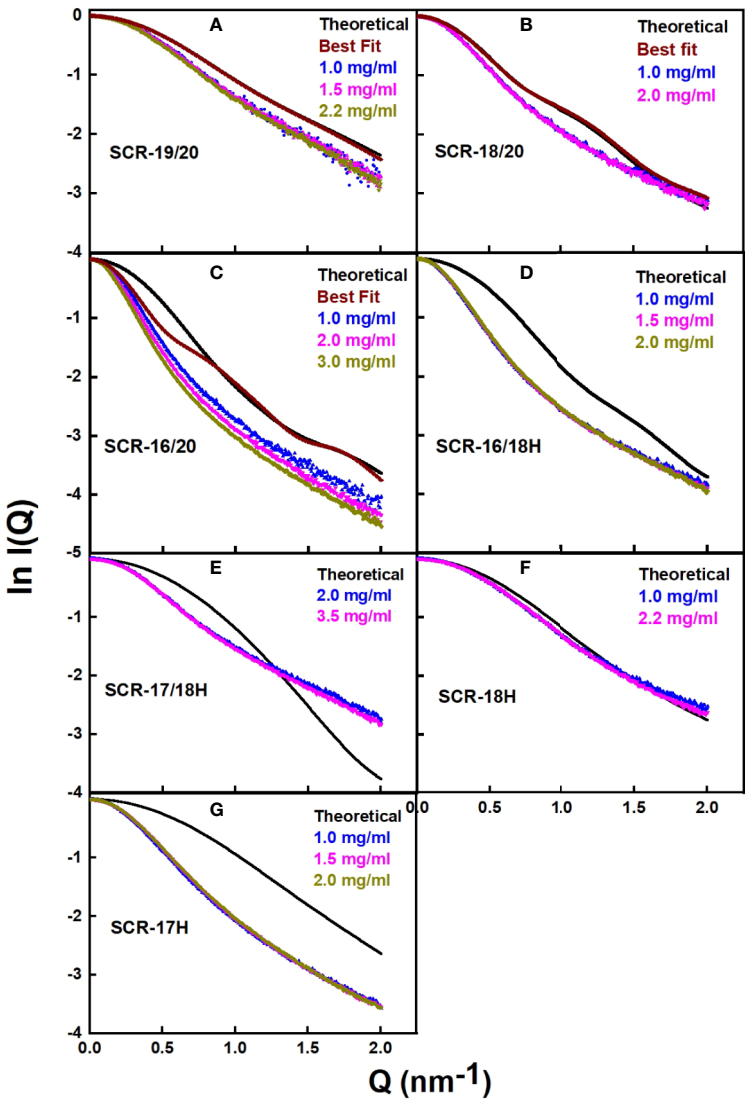

FIGURE 8 | Scattering curve fits for the atomistic models for each of the SCR fragments. **(A–G)** For each of the seven SCR fragments, the panels compare the experimental X-ray scattering and theoretical scattering curves. The theoretical curves are shown in black, and the experimental curves are shown in color to correspond to the different concentrations shown in the same panel. In **(A–C)**, curves from the 100 best-fit models are shown in brown.

of the dimers were unknown. However, by calculating the goodness-of-fit R-factor for each fragment model when compared with up to three different experimental curves, and by assuming that the monomer models would give good curve fits if the solution structure was monomeric, it was possible to assess the extent to which dimerization had perturbed the curve fits. As a bench mark, SCR-19/20 should give the best fits as this was determined to be monomeric by AUC and SAXS. The reasonable visual fits (**Figure 8A**) and R-factors of 14–17% (**Table 2**) indicated the type of fits that were obtained Relatively low amounts of dimer were seen for SCR-18/20 by AUC (**Figure 1B**) and SAXS; the visual fits (**Figure 8B**) and R-factors of 15% (**Table 2**) corroborated the relatively low amounts of dimer present for this fragment. For SCR-16/20, the presence of 40% dimers by AUC were confirmed by increases in the R-factors from 40 up to 64% as the concentration (and proportion of dimer) increased. For the four His-tagged fragments, while SCR-18H showed good agreements in the curve fits, the fits for SCR-16/18H, SCR-17/18H, and SCR-17H were poorer and gave R-factors as high as 68%. Taken together, the R-factors indicated that the primary location of CFH dimerization was again seen to be SCR-17, with some contribution from SCR-18.

TABLE 2 | The modelling of the AUC $s^0_{20,w}$ values and the best curve fit R-factors for the SCR fragments.

Fragment	$s^0_{20,w}$ (S) experimental	$s^0_{20,w}$ (S) modelled	R-factor 1.0 mg/ml	1.5 mg/ml	2.0 mg/ml	3.5 mg/ml
SCR-19/20	1.4	1.4	14.4%	15.0%	17.1%	
SCR-18/20	2.4	1.9	14.5%		15.2%	
SCR-16/20	2.7	2.7	40.2%		53.9%	64.4%
SCR-16/18H	2.3	1.9	51.2%	51.0%	49.5%	
SCR-17/18H	2.3	1.8			22.5%	23.0%
SCR-18H	2.4	1.1	6.4%		5.8%	
SCR-17H	1.7	1.2	68.0%	66.0%	65.2%	

The R-factor was calculated using the R-factor metric in the SASSIE package. For each SCR fragment, the R-factor was calculated at each of the concentrations in use from 1.0 mg/ml to 3.5 mg/ml.

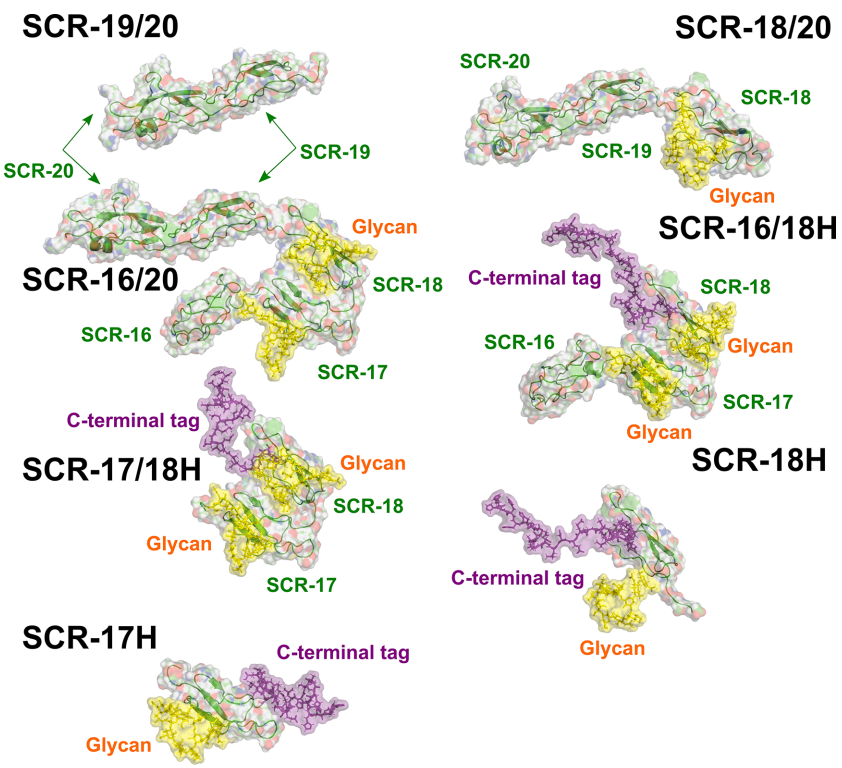

FIGURE 9 | The seven atomistic models for the SCR fragments. The structures of the C-terminal fragments of CFH used for the curve fits are shown. The individual SCR domains are labelled. Yellow surfaces represent the single glycan chains located on each of SCR-17 and SCR-18. The C-terminal tag is shown as a purple surface.

As another test of the scattering modelling, the $s^0_{20,w}$ values for the models of the seven monomeric CFH fragments (**Figure 9**) were calculated using HYDROPRO (**Table 2**). Given that the mean difference between the modelled and experimental values should be ± 0.21 S for related macromolecules (43), excellent agreements were obtained for monomeric SCR-19/20 and SCR-16/20, but less so for the SCR-18/20 monomer where the model appeared too elongated compared to the experimental value. For the four His-tagged fragments in **Table 2**, the calculations suggested that the models were too elongated compared to the experimental values. The simplest explanation of this is that the His-tag tails and glycans were extended in the models as shown in **Figure 9**. While these calculations corroborated the modelling for the monomeric fragments, overall the agreement was only qualitative.

Further experimental AUC analyses were made using the f/f_0 ratio which monitors the extent of how much the frictional coefficient of the glycoprotein deviates from that for a sphere of the same volume. The experimental f/f_0 ratio of 1.1 for monomeric SCR-19/20 defined a benchmark for two linearly-arranged SCR domains (**Table 1**; **Figure 9**). SCR-18/20 also showed an experimentally low f/f_0 ratio of 1.1 (**Table 1**). As deduced from the f/f_0 ratios of 1.3–1.5 (**Table 1**), the three SCR-16/20, SCR-16/18H, and SCR-17/18H monomers have similar but more elongated shapes. The similarity of the f/f_0 ratios of 1.3 for SCR-16/18H and SCR-17/18H indicated that the three-domain SCR-16/18H structure possessed a bent back solution structure of similar elongation to the two-domain SCR-17/18H structure. The sedimentation properties of the single SCR-17H and SCR-18H domains are likely to be perturbed by their relatively large glycan and His-tag groups, thus no further interpretation of their values was made here. It was however interesting that the f/f_0 ratios for the dimers of SCR-16/20, SCR-18/20, SCR-16/18H, SCR-17H, and SCR-18H showed that they became more elongated in their dimers compared to their monomers (**Table 1**). However, that for SCR-17/18H was unchanged in its dimer, suggesting that this was formed by a side-by-side interaction.

Previously SCR dimers have been seen in the crystal structure of FHR1 SCR-1/2, in which two copies of SCR-1/2 formed an anti-parallel dimer. This anti-parallel dimer structure was used to test whether the FH SCR-17/18 dimer could be formed from protein-protein contacts in the same way as the dimer interface seen in the FHR1 SCR-1/2 structure. Using the crystal structure of FHR1 as a template (PDB code 3ZD2), a model of the SCR17/18 dimer interface was constructed. A multiple sequence alignment between FHR1-SCR1/2 and SCR-17/18 of CFH gave a relatively low sequence homology of 41% between the two fragments. When CFH SCR-17 was aligned with FHR1 SCR-1, and SCR-18 was aligned with SCR-2 through their β4-strands, the resulting SCR-17/18 dimer model showed ill-fitting gaps at their interface. These observations argued against an antiparallel SCR arrangement for the C-terminal CFH dimer at SCR-17 and SCR-18. Accordingly, because each of SCR-17H and SCR-18H form dimers on their own (**Figure 4B**), it was concluded that a parallel arrangement of the SCR-17/18 domains is found in the SCR-17/18 dimer detected by AUC analysis.

DISCUSSION

Full length CFH forms weak dimers with an estimated range of 4–15% dimer present at typical CFH serum concentrations of 0.8–3.6 μM (0.116–0.562 mg/ml) (44). The dissociation constants K_D values for dimer formation ranged between 8–28 μM (45), thus CFH dimers are expected to co-exist with CFH monomers at physiological conditions in serum. The CFH self-association sites have previously been shown to be located in the SCR-6/8 and SCR-16/20 regions (12, 13, 46). Up to now, the more precise location of the SCR-16/20 dimerization site was not known, and this identification was addressed here. It was unlikely that the dimerization site would reside on either SCR-19 or SCR-20, because of the functional interaction of SCR-19 and SCR-20 with the C3d fragment of complement C3b and sialic acid as reported in crystal structures (47–51) (PDB codes 3OXU, 2XQW, 4ONT, 4ZH1, 5NBQ). This was confirmed in this study by showing that SCR-19/20 remained monomeric using a combination of AUC and SAXS experiments in 137 mM and 50 mM NaCl buffers, coupled with molecular simulations of the AUC and SAXS data based on the coordinates of our recent full-length CFH model (6). In contrast, using the same strategy, six other fragments containing SCR-17 and SCR-18 showed various degrees of dimer formation. The strongest dimer formation with K_D values of 3-6 μM was observed for SCR-17H, SCR-17/18H, and SCR-16/18H, in which SCR-17H showed higher dimer formation than SCR-18H. These data indicated that SCR-17H comprised the main C-terminal CFH dimer site. The presence of additional or alternative SCR domains as found in the SCR-16/20, SCR-18/20H, and SCR-18H fragments resulted in weaker dimer formation, indicating that SCR-18H made some contribution to this. Since full-length CFH showed extents of dimerization of 4–15% (12), while the smallest fragments showed dimerization of up to 80% (**Figure 4B**), the reduced dimer formation for larger CFH molecules is attributed to steric effects caused by the larger sizes of the CFH proteins in question. Their larger sizes are presumed to inhibit dimer formation. It should also be noted that the present study used a non-His tagged form of SCR-16/20, while our previous study used a His-tagged variant (13). Both studies gave similar AUC results, indicating that the presence of the His-tag made no difference on its dimerization.

The major complement regulator CFH functions to protect host cells from destruction through its C-terminal binding to C3b and anionic host cell surfaces mediated by the SCR-19 and SCR-20 domains. Our novel report of a CFH dimer site in SCR-17/18 may provide CFH with a functional mechanism through which CFH can become more concentrated on host surfaces during an inflammatory response. The SCR-17/18 dimer site is seen to be independent of the crystallographic-observed C3dg and anionic oligosaccharide binding sites on SCR-19/20 in the C-terminal region of CFH (**Figure 10**). Despite the presence of multiple binding sites for C3dg and polyanions, the schematic view of C3dg binding to SCR-19 and the dimer formation at SCR-17/18 in **Figure 10** indicated that dimer formation will still proceed when a single CFH molecule is bound to a host cell

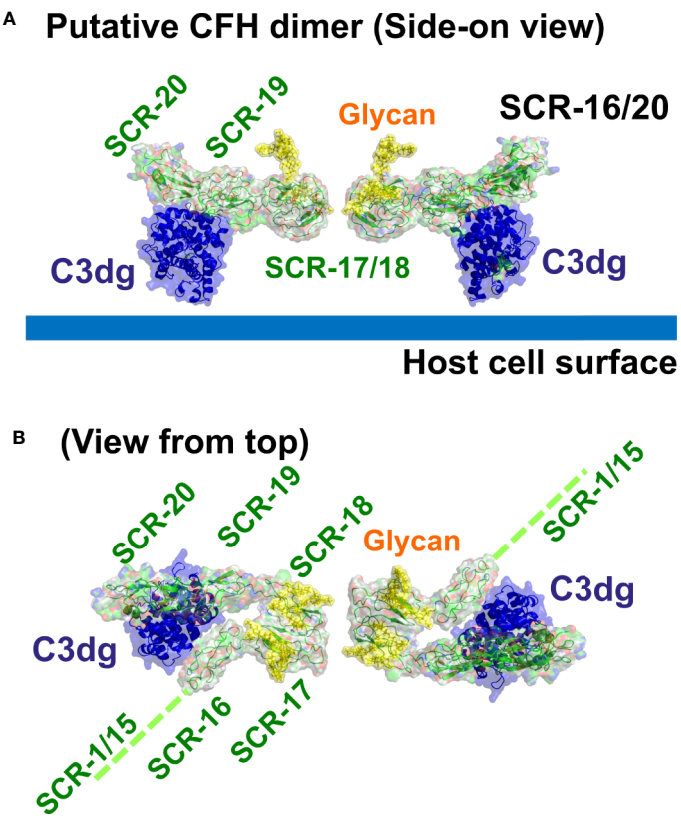

FIGURE 10 | Putative dimer of SCR-16/20 to show the crystallographic-observed complex between SCR-19/20 and C3dg. Molecular views of the SCR-16/20 fragment model (grey/green) determined from this study are shown as ribbons and surfaces to show how this binds to its ligand C3dg (blue) on the SCR-19 domain. To generate this complex, the SCR-16/20 model was superimposed onto the crystal structure of the SCR-19/20 and C3dg co-complex (PDB code: 5NBQ). The yellow surfaces show the two glycan chains located at each of SCR-17 and SCR-18. **(A)** The side-on view shows C3dg attached to the host cell surface (thick blue line) through its thioester group, as well as showing how SCR-17 and SCR-18 form a dimeric interface with another SCR-16/20 molecule (**Figure 4B**). **(B)** The same structure is viewed from the top to show the five individual SCR domains in each monomer of the SCR-16/20 dimer. This view corresponds to a 90° rotation about a horizontal axis compared to that in **(A)** The reminder of the CFH structure SCR-1/15 is denoted by a dashed green line.

surface through either cell-bound C3dg or polyanions. The single surface-bound CFH molecule will allow an additional CFH molecule to be recruited through a SCR-17/18 dimerization event to protect further the host surface under inflammatory conditions of excessive C3b deposition. In reflection of this topology, the 37 genetic variants throughout SCR-20 reported so far that lead to aHUS disease are comparatively abundant in CFH (**Figures 1B, C**) (17), indicating the importance of C-terminal CFH binding to host cell surfaces. The fewer variants reported to date for SCR-16 (six), SCR-17 (four), SCR-18 (ten) and SCR-19 (seven) might involve incorrect folding of the C-terminal domains if they alter any of the highly conserved Cys or Trp residues, or a reduction in dimer formation or C3dg binding (19). Visual inspection of the locations of these variants in the SCR-16/20 model (**Figures 1B, 10**) showed little further insight. Further experimental studies will identify the effect of the aHUS variants in SCR-17/18 on dimer formation in order to clarify the importance of C-terminal dimerization in CFH. Of interest was that no significant differences in dimer formation were observed between the 137 mM and 50 mM NaCl buffers, suggesting that electrostatic interactions were not significant in SCR-16/20 dimer formation (**Table 1**).

Besides CFH itself, another distinct SCR dimer has been observed in other members of the CFH gene family. The FHR proteins (52) including FHR1 with five SCR domains, FHR2 with four SCR domains, and FHR5 with nine SCR domains exist as dimers formed by an antiparallel pairing of their SCR-1/2 N-terminal domains, as opposed to the parallel pairing proposed for SCR-17/18. FHR dimerization confers avidity for their binding to complement activation fragments bound to host cell surfaces, and enables these FHR proteins to compete with CFH for binding (36). For FHR5, we showed that the antiparallel FHR5 dimer with 18 SCR domains has a compact domain structure that can bind bivalently to C3b when this is bound to host cells at a high enough surface density (53). However, sequence alignments between SCR-1/2 of FHR1 and SCR-17/18 of CFH showed that the three FHR1 residues (Tyr 34, Ser 36, and Tyr 39) essential for FHR1 dimer formation were not

conserved in SCR-17/18 which contains Thr residues at the equivalent positions. This difference makes it unlikely that SCR-1/2 of FHR1 would be a good model for the CFH dimer structure at SCR-17/18.

Our multidisciplinary approach to analyse the solution properties of the seven C-terminal SCR fragments showed consistent results from both the AUC and SAXS data sets. The main results showed that SCR-19/20 is monomeric, and that SCR-16/20 and SCR-18/20 became more elongated with dimer formation (**Table 1**). For the remaining fragments, the AUC data showed a range of dimer formation had taken place, although information from SAXS about shape or size changes associated with dimer formation was more limited because the scattering curves correspond to mixtures of monomer and dimer, and the AUC shape data were of limited precision. Nonetheless there was sufficient information in the datasets to indicate that SCR-17 and SCR-18 comprised the main dimerization site in the C-terminal region of CFH. Both the AUC and SAXS data sets were accessible to molecular modelling in order to clarify the significance of the experimental data sets. The application of modelling here confirmed that the largest deviations from the SAXS curve fits on the assumption of SCR monomers correlated with the greatest amount of dimer formation (**Table 2**), as well as showing that the CFH monomer models accounted well for the AUC $s_{20,w}$ values. In this analysis, the AUC and SAXS modelling outcomes extended our understanding of the proportions of monomer and dimer deduced from AUC (**Figure 4B**). The molecular modelling also provided a new functional explanation for the formation of SCR-17/18 dimers, and insight into why aHUS disease-associated genetic variants occur along the length of SCR-16/20 and not just in SCR-20 (**Figure 10**).

AUTHOR'S NOTE

Initial data from this work were presented at the 15th European Meeting on Complement in Human Disease, September 8–12, 2015, Uppsala, Sweden (Mol. Immunol. 2015, 67:136-136).

DATA AVAILABILITY STATEMENT

Requests to access the datasets should be directed to SP, s.perkins@ucl.ac.uk.

AUTHOR CONTRIBUTIONS

SP initiated and supervised the study at UCL. OD, RN, and SP designed the experiments. PA and DG provided the *Pichia* expression systems. OD expressed and purified the recombinant proteins with advice and support from MM and MH. VF supervised the study in Grenoble. XG and SP performed the computational modelling. OD, XG, and SP wrote the manuscript with the help of the other authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Hijacking Factor H for Complement Immune Evasion

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The complement system is an essential player in innate and adaptive immunity. It consists of three pathways (alternative, classical, and lectin) that initiate either spontaneously (alternative) or in response to danger (all pathways). Complement leads to numerous outcomes detrimental to invaders, including direct killing by formation of the pore-forming membrane attack complex, recruitment of immune cells to sites of invasion, facilitation of phagocytosis, and enhancement of cellular immune responses. Pathogens must overcome the complement system to survive in the host. A common strategy used by pathogens to evade complement is hijacking host complement regulators. Complement regulators prevent attack of host cells and include a collection of membrane-bound and fluid phase proteins. Factor H (FH), a fluid phase complement regulatory protein, controls the alternative pathway (AP) both in the fluid phase of the human body and on cell surfaces. In order to prevent complement activation and amplification on host cells and tissues, FH recognizes host cell-specific polyanionic markers in combination with complement C3 fragments. FH suppresses AP complement-mediated attack by accelerating decay of convertases and by helping to inactivate C3 fragments on host cells. Pathogens, most of which do not have polyanionic markers, are not recognized by FH. Numerous pathogens, including certain bacteria, viruses, protozoa, helminths, and fungi, can recruit FH to protect themselves against host-mediated complement attack, using either specific receptors and/or molecular mimicry to appear more like a host cell. This review will explore pathogen complement evasion mechanisms involving FH recruitment with an emphasis on: (a) characterizing the structural properties and expression patterns of pathogen FH binding proteins, as well as other strategies used by pathogens to capture FH; (b) classifying domains of FH important in pathogen interaction; and (c) discussing existing and potential treatment strategies that target FH interactions with pathogens. Overall, many pathogens use FH to avoid complement attack and appreciating the commonalities across these diverse microorganisms deepens the understanding of complement in microbiology.

Keywords: complement system, alternative pathway, Factor H, Factor H binding proteins, complement evasion, pathogen

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THE COMPLEMENT SYSTEM

Complement activates through a domino-like cascade comprising over 50 proteins, resulting in outcomes essential for innate and adaptive immunity. Activation of complement occurs through three pathways: classical, lectin, and alternative (**Figure 1A**), which converge on the cleavage of the central component, C3 [reviewed in (1)]. The classical pathway (CP) activates when C1q of the C1 complex (C1q, C1r, C1s) recognizes and binds pathogen- or cell-bound immunoglobulins, circulating immune complexes, or to pentraxins (e.g., C-reactive protein, pentraxin 3, serum amyloid P). When C1q binds a ligand, C1r is activated, which then activates C1s. C1s sequentially cleaves C4 and C2, resulting in the CP C3 convertase, C4bC2b [reviewed in (2)]. The lectin pathway (LP) activates when mannose-binding lectin (MBL), ficolins, or collectins recognize molecular patterns such as carbohydrates and other ligands on foreign surfaces. This leads to activation of MBL-associated serine proteases (MASPs), whereby MASP-2 cleaves C4 and C2 to form the LP C3 convertase, C4bC2b [reviewed in (3)].

Unlike the CP and LP, which are triggered upon recognition of distinct ligands, the alternative pathway (AP) is continuously active and initiates spontaneously on surfaces not protected by complement regulatory proteins. In blood, low levels of C3 undergo spontaneous hydrolysis ("tick-over") to form C3(H_2O). C3(H_2O) binds Factor B (FB) and circulating Factor D cleaves FB to Bb and Ba, resulting in formation of the fluid phase AP C3 convertase, C3(H_2O)Bb. C3(H_2O)Bb cleaves C3 to C3b and C3b binds covalently to nearby surfaces to form membrane-bound C3 convertase, C3bBb. The AP also contributes to a powerful amplification loop through activation of C3b, which in some cases contributes up to 80% of the total complement response, even after initiation by the CP and LP (4, 5). In fact, it is argued the AP is mainly an amplification mechanism with minimal contributions from the tick-over of C3 [reviewed in (6)].

C3 convertases derived from each pathway converge to cleave C3, generating C3a and C3b, which complexes on or near C3 convertases to form C5 convertases. Cleavage of C5 by C5 convertases generates C5a and C5b to initiate the terminal pathway. Sequential binding to C5b by C6, C7, C8, and multiple copies of C9 form the membrane attack complex (MAC, C5b-9). Outcomes of complement are numerous and include generation of pro-inflammatory mediators C3a and C5a, C3 fragments involved in opsonization and immune modulation, and cell lysis by the pore-forming MAC [reviewed in (7, 8)].

Regulation of all complement pathways protects the host from unwarranted complement-mediated attack. Complement negative regulators circulate in blood and include FH, Factor I (FI), C4 binding protein (C4BP), C1 inhibitor (C1-INH), clusterin, vitronectin, and Factor-H like protein 1 (FHL-1). Membrane-bound complement negative regulators include complement receptor 1 (CR1/CD35), decay accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46), CD59, and complement receptor of the immunoglobulin family (CRIg) [reviewed in (9)]. Importantly, FH is the primary regulator of the AP in the fluid phase and on cell surfaces and is essential for protecting the host from AP attack. Pathogens have developed survival strategies to evade the immune response including coopting FH from the host to avoid the AP. This phenomenon will be described in detail in this review.

FACTOR H IN THE HOST

FH, formerly known as β1H (10, 11), is abundantly found in plasma with a wide concentration range between 116 and 810 μg/ml [reviewed in (12, 13)]. However, recent studies reveal an average FH concentration of ~230 μg/ml, when measurement of FH family proteins FHL-1 and Factor-H related proteins (FHRs) are excluded [reviewed in (14)]. FH is constitutively expressed by hepatocytes (15, 16) and also produced by monocytes, fibroblasts, endothelial cells, platelets, retinal pigment epithelial cells, peripheral blood lymphocytes, myoblasts, rhabdomyosarcoma cells, glomerular mesangial cells, neurons, and glial cells [reviewed in (12, 17)]. FH is a 155-kDa glycoprotein (18, 19) encoded from a single gene, HF1/CFH, found within the regulator of complement activation gene cluster on chromosome 1q32 [reviewed in (20)]. FH consists of 20 homologous complement control protein modules (CCP) (21, 22), with each module containing ~60 amino acid residues (22) connected by short spaces of three to eight amino acid residues [reviewed in (17)]. Structural studies indicate FH may adopt a flexible folded back conformation in solution (23–25).

FH is the primary negative regulator of the AP in the fluid phase and at the cell surface. The three functions of FH include: (a) competing with FB for C3b binding (26); (b) accelerating the decay of surface-bound C3 and C5 convertases (10, 27, 28) and to a lesser extent, fluid-phase C3 convertase (29), by disassociating Bb from convertases; and (c) acting as a cofactor for FI-mediated cleavage of C3b into the inactive form, iC3b (28, 30, 31) (**Figure 1B**). These regulatory functions are carried out through CCPs 1–4 (32–35).

Upon binding to a surface, FH protects against AP activity. FH simultaneously recognizes C3 fragments and host cell markers to discriminate self (host, non-activators) from non-self (activators), which lack or have very low levels of surface polyanions. Several FH domains participate in binding C3 fragments and/or polyanions (**Figure 2**, top panel). CCPs 1–6 bind C3 and C3b and a weak binding site for C3b is recognized at CCPs 13–15 (36). CCPs 19–20 recognize iC3b, C3b, and C3d (36, 37). Host cell polyanions, such as sialic acids (38, 39) and glycosaminoglycans (GAGs), which include heparins (40, 41) and dextran sulfate (41, 42), serve as recognition markers for FH regulation on host cells. CCP 20 is the only known site to recognize sialic acid (43). Heparin binding sites include CCPs 6–8 and 18–20, with a possible weak binding site on CCPs 11–13 (36). CCPs 19–20 are the most important region of FH for binding to cell surfaces by recognizing both C3b and polyanions [reviewed in (44)]. FH has a 10-fold increase in affinity towards C3b in the presence of host sialic acid (38, 39, 41). In addition to recognizing host cell markers, FH acts as a ligand for annexin II, DNA, C-reactive protein, and pentraxin-3 to limit excessive complement activation during apoptosis [reviewed in (44)].

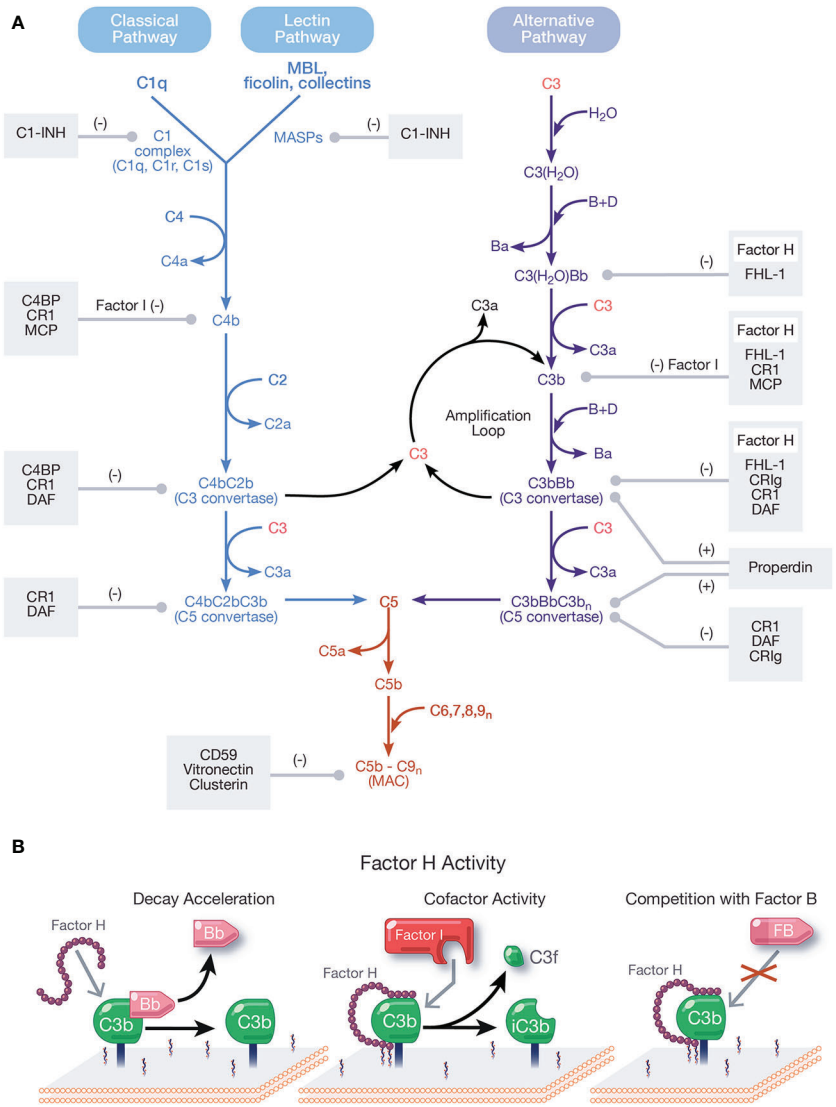

FIGURE 1 | Overview and regulation of the complement system. **(A)** Complement is activated by three pathways: the classical, lectin, and alternative pathways. The classical pathway (CP) activates when the C1 complex (C1q, C1r, C1s) recognizes and binds pathogen- or cell-bound immunoglobulins, circulating immune complexes, or pentraxins. The lectin pathway (LP) activates when mannose-binding lectin (MBL) ficolins, or collectins recognize molecular patterns such as carbohydrates and other ligands on foreign surfaces. CP and LP activation results in cleavage of C4, followed by cleavage of C2, forming the CP/LP surface bound C3 convertase, C4bC2b. The alternative pathway (AP) is spontaneously activated when soluble C3 hydrolyzes to $C3(H_2O)$. $C3(H_2O)$ can bind FB (labeled B) and recruit Factor D (labeled D) which cleaves FB to Bb (and Ba), resulting in the fluid phase AP C3 convertase, $C3(H_2O)Bb$. $C3(H_2O)Bb$ cleaves C3 to C3a and C3b. C3b then binds covalently to nearby surfaces to form membrane-bound C3 convertase, C3bBb. C3 convertases derived from all pathways cleave C3 to C3a and C3b. C3b combines with formed C3 convertases to form the CP/LP C5 convertase (C4bC2bC3b) and AP C5 convertase ($C3bBbC3b_n$). C5 is cleaved by C5 convertases to initiate the common, terminal pathway, which culminates in the formation of the membrane attack complex (MAC). C3b produced from the cleavage of C3 by C3 convertases from all pathways forms an amplification loop that contributes to the generation of additional AP C3 convertases. Positive (labeled +) and negative (labeled –) regulators of all complement pathways are shown. Membrane-bound complement negative regulators include complement receptor 1 (CR1/CD35), decay accelerating factor (DAF/CD55), membrane cofactor protein (MCP/CD46), CD59, and complement receptor of the immunoglobulin family (CRIg). Soluble negative regulators include Factor H (FH), FI, C4 binding protein (C4BP), C1 inhibitor (C1-INH), clusterin, vitronectin, and Factor-H like protein 1 (FHL-1). Positive regulators of complement include FH-related proteins (FHRs) -1, -4, and -5, and properdin. **(B)** Function of FH. FH accelerates the decay of AP C3 convertase by dissociating Bb (decay acceleration function); acts as a cofactor for Factor I-mediated cleavage of C3b into iC3b, an inactivated form that does not allow complement activity to progress (cofactor activity function); and competes with FB for binding to C3b to form the AP C3 convertase.

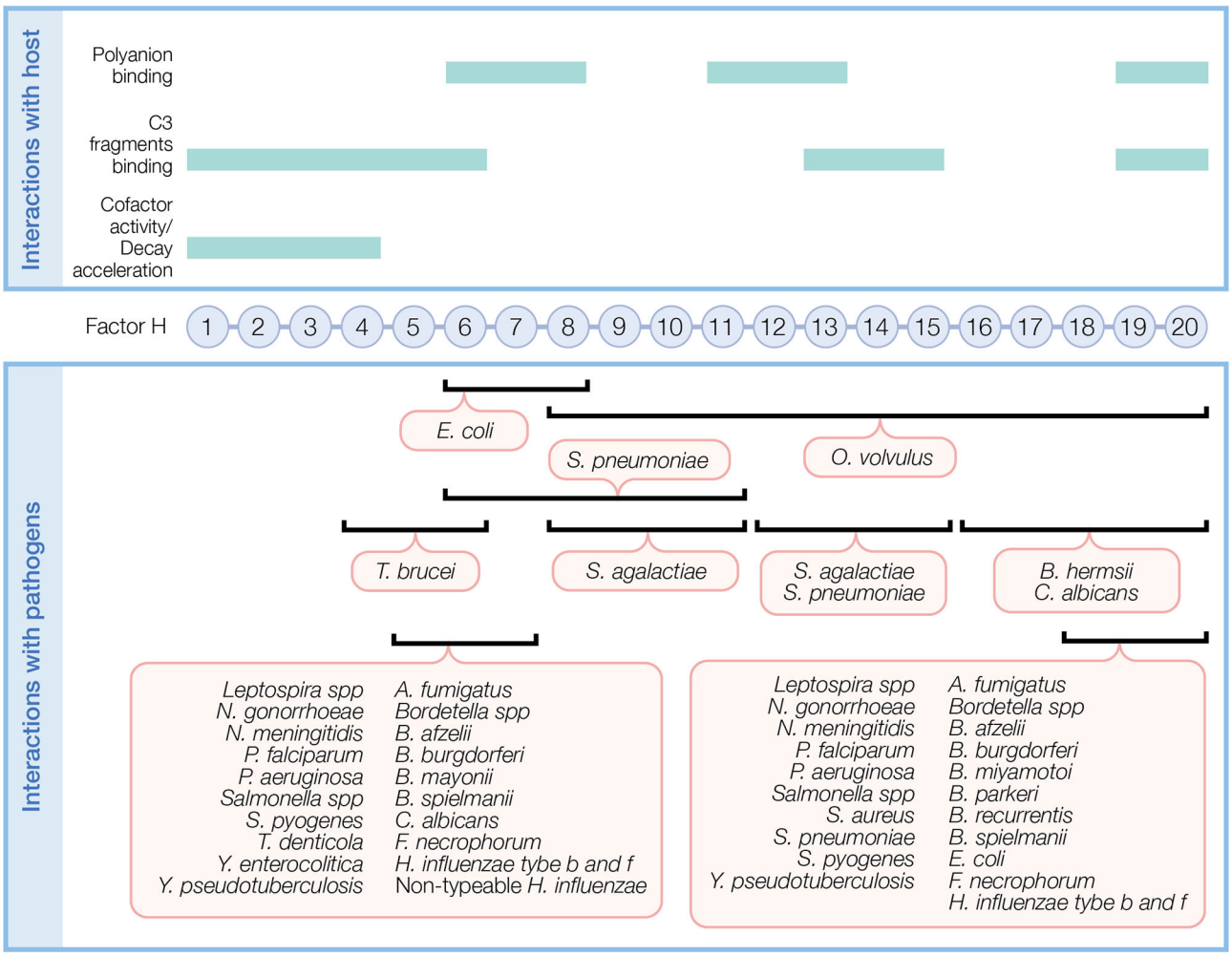

FIGURE 2 | Factor H domains involved in binding to the host and pathogens. FH contains 20 complement control protein modules (CCP) each with about 60 amino acids linked by three to five amino acids. In the top panel, FH domains necessary for its regulatory function and domains interacting with C3 fragments and polyanions are indicated. In the bottom panel, overlapping FH domains involved in binding pathogens are indicated. Refer to **Table 1** for specific FH domains involved in binding to individual pathogens and for pathogens binding FH through undefined domains.

FH belongs to a family of proteins including FHL-1 and five FHRs, reviewed elsewhere (14, 45), that are present at significantly lower concentrations than FH in blood [reviewed in (14)]. Briefly, FHL-1 is a result of alternative splicing of CFH and contains seven domains homologous to FH CCPs 1–7 plus four C-terminal amino acids. FHL-1 is a negative regulator of complement in the fluid phase [reviewed in (14, 45, 46)]. In contrast, FHRs do not share a gene with FH or FHL-1, but instead, have domains homologous to the center and C-terminal FH domains [reviewed in (14)]. Thus, due to a non-functional N-terminus, FHRs compete with FH, thereby acting as potential positive regulators of the AP, yet the roles of FHRs are not well defined [reviewed in (14, 45)].

In addition to the prototypical role of FH as a negative regulator of the AP, non-canonical roles of FH have emerged [reviewed in (45)]. FH is a ligand for complement receptor 3 (CR3; CD11b/CD18) on neutrophils and upon binding, results in release of hydrogen peroxide and lactoferrin (47). FH interaction with neutrophil CR3 also results in release of IL-8, prevention of neutrophil extracellular traps (NETs) formation, and production of reactive oxygen species (ROS) (48). Thus, FH may reduce host damage by inhibition of NETs and ROS (48). In particular, FH bound to *Candida albicans* modulates neutrophil function by interacting primarily with CR3, and to a lesser extent to complement receptor 4 (CR4; CD11c/CD18), leading to more effective killing of the pathogen (49). FH is also known as adrenomedullin-binding protein-1 and binds adrenomedullin (50), a vasodilator peptide hormone widely expressed in many human tissues [reviewed in (51)]. FH may protect adrenomedullin from proteolytic degradation and thus has therapeutic value in disease models of sepsis, wound healing, and hemorrhage [reviewed in (52)]. In addition to acting as a ligand, FH is internalized by early apoptotic cells, resulting in enhancement of intracellular C3 cleavage and increased iC3b

surface opsonization to promote uptake by monocytes [reviewed in (45)].

FACTOR H INTERACTIONS WITH PATHOGENS

Evasion of complement attack is key to pathogen survival in the host. In general, pathogens evade complement through numerous strategies, which include: (a) expression of proteins that mimic host surface-bound complement regulators; (b) secretion of proteases to digest complement fragments; (c) exploitation of complement opsonization to promote intracellular invasion; (d) secretion of complement inhibitory proteins; and (e) recruitment of fluid phase complement regulators, which includes FH family proteins [reviewed in (53–55)].

Pathogen evasion of the complement system has been described [reviewed in (54–58)], including strategies particular to bacteria [reviewed in (53, 59)]; fungi [reviewed in (60)]; parasites [reviewed in (61)]; viruses [reviewed in (62)]; and evasion mechanisms involving FH family proteins [reviewed in (17, 19, 63)]. Herein, we review how pathogens steal FH to outsmart the immune system. Specifically, we describe the implications of FH binding in pathogen evasion, mechanisms of binding, and therapeutic targeting of the FH-pathogen interface.

Binding of Factor H to Pathogens to Control Alternative Pathway Activity

FH is the primary target of pathogens for AP evasion. When sequestered from blood to the pathogen surface, FH retains its function as a negative regulator of complement, thus circumventing lysis by MAC, opsonization by C3 fragments, and pro-inflammatory consequences of complement cleavage products such as C3a and C5a [reviewed in (7, 8)].

Binding of Factor H to Pathogens for Purposes Other Than Alternative Pathway Evasion

Though FH is primarily a negative regulator of the AP, it has also been shown to regulate the CP [reviewed in (64)]. Moreover, FH binding to certain pathogens facilitates host cellular adherence and invasion. Binding of FH to pneumococcal surface protein C (PspC) on S*treptococcus pneumoniae* increases attachment to, and invasion of, host cells (65). Likewise, in *Mycoplasma hyopneumoniae*, FH binding increases adherence to epithelial cells (66) while FH interaction with influenza A virus promotes viral cellular invasion. As opposed to binding FH to evade complement attack, pathogens also cleave FH. Example of pathogens that cleave and inactivate FH include *Salmonella enterica* (67), *Yersinia pestis* (67), *C. albicans* (68), and *Treponema denticola* (68, 69). Inactivation of FH by pathogen proteases may result in unchecked AP activation, consequentially depleting complement proteins surrounding the pathogens, thus protecting them from attack. In addition, FH inactivation leads to complement dysregulation on host cells, which, as suggested by Riva et al. and Miler et al., may compromise tissue integrity to facilitate pathogen invasion (67, 70).

Binding of Factor H Family Members to Pathogens

FHL-1 and FHRs are also capable of binding pathogens (**Table 1**). Pathogen recruitment of FHL-1 serves the same purpose in complement evasion as binding to full-length FH. However, as suggested by Kunert et al., low serum FHL-1 titers, along with the increased binding affinity of full-length FH because of its additional C-terminal binding domains, may limit pathogen binding to FHL-1 (132). Interestingly, although most *Borrelia* species bind FH, *B. burgdorferi* Complement Regulator-Acquiring Surface Proteins (CRASP)-2 (Csp-Z) is shown to preferentially bind FHL-1 (183).

The benefits of pathogens binding FHRs are shown when Scl of *S. pyogenes* binds FHR-1 to inhibit the formation of the terminal complement pathway (157). However, to date, most studies suggest pathogen binding to FHRs are disadvantageous, because it outcompetes full-length FH for binding to pathogen surfaces and can enhance AP activity [reviewed in (45)]. In *S. pyogenes* (157) and *P. falciparum* (184), FHR-1 outcompetes FH for binding and impairs FH cofactor activity. Moreover, in the case of *P. falciparum*, FHR-1 binding also leads to decreased parasite viability (184). In *N. meningitidis*, FHR-3 outcompetes FH binding to result in complement-mediated bacteria lysis (185). *Borrelia* CRASP-3 (ErpP) and -5 (ErpA) bind FHR-1, -2, and -5 while only weakly binding FH (186). However, since FHRs binding does not provide any complement evasion benefits to *Borrelia* (186), the consequences of FHRs binding are unclear.

MECHANISMS OF HOW PATHOGENS BIND FACTOR H TO EVADE THE ALTERNATIVE PATHWAY

Pathogens capture FH by adapting their surfaces to mimic host cells (i.e., expressing host cell markers) and/or expressing specific FH binding receptors (FH binding proteins). Pathogens must bind FH using accessible domains that do not interfere with the N-terminal functions of FH. The sections below describe mechanisms used by pathogens to bind FH. **Figure 2** (bottom panel) illustrates regions of FH recognized by pathogens. Moreover, **Table 1** summarizes pathogens known to bind FH, FHL-1, and FHRs.

The Role of Sialylation in Factor H Recruitment

Pathogens evade complement by protecting their surfaces from complement attack using host cell markers. In the host, FH recognizes specific sialic acid capped glycans on host surfaces to distinguish self from non-self, which importantly restricts AP activation on self surfaces, while allowing AP activity to continue on non-self (pathogen) surfaces. However, pathogens bypass AP

TABLE 1 | Pathogen interactions with Factor H family proteins.

Pathogen	Factor H binding ligand (other name designations)	Binding domains of Factor H family proteins	References
	Bacteria		
Acinetobacter baumannii	AbOmpA	FH	(71)
Bacillus anthracis	BclA	FH	(72)
Bordetella parapertussis, pertussis	?	5–7 (FH, FHL-1); 19–20 (FH); FHR-1	(73, 74)
Borrelia afzelii	CspA (BaCRASP-1)	5–7 (FH, FHL-1)	(75–78)
	CspZ (CRASP-2, BBH06)	6–7 (FH, FHL-1)	(75, 77, 78)
	BaCRASP-3	6–7 (FHL-1)	(77)
	BaCRASP-4	19–20 (FH)	(77)
	BaCRASP-5	19–20 (FH)	(77)
	BAPKO_0422	FH	(79)
Borrelia burgdorferi	CspA (CRASP-1, BbCRASP-1, BBA68, FHBP)	5–7 (FH, FHL-1); 19–20 (FH)	(75, 77, 80)
	CspZ (CRASP-2, BbCRASP-2, BBH06)	7 (FH, FHL-1)	(77, 81)
	ErpP (CRASP-3, BbCRASP-3, BBN38)	19–20 (FH); FHR-1, -2, -5	(77, 82, 83)[#]
	ErpC (CRASP-4, BbCRASP-4)	FH; FHR-1, -2	(77, 83)[#]
	ErpA (CRASP-5, BbCRASP-5, ErpI, ErpN, BBP38, BBl39, OspE)	19–20 (FH); FHR-1, -2, -5	(74, 77, 83–85)[#]
Borrelia hermsii	BhCRASP-1 (FhbA, FHBP19)	16–20 (FH); FHL-1; FHR-1	(86–88)
Borrelia mayonii	CspA	5–7 (FH, FHL-1)	(89)
Borrelia miyamotoi	CbiA	20 (FH)	(90)
Borrelia parkeri	BpcA	19–20 (FH); FHR-1	(91)
Borrelia recurrentis	HcpA	19–20 (FH); FHR-1	(92)
	?	FH	(93)
Borrelia spielmanii	CspA (BsCRASP-1)	5–7 (FH, FHL-1)	(94–96)
	BsCRASP-2	FH; FHL-1	(94)
	BsCRASP-3	20 (FH); FHL-1; FHR-1	(94, 96)
Escherichia coli	Stx2	6–8 (FH, FHL-1); 18–20 (FH); 3–5 (FHR)	(97, 98)
	OmpW	FH	(99)
Francisella tularensis	?	FH	(100)
Fusobacterium necrophorum	?	5–7 (FH, FHL-1); 19–20 (FH); FHR-1, -4	(101)
Haemophilus influenzae type b and f	Protein H (PH)	7 (FH, FHL-1); 18–20 (FH)	(102, 103)
Histophilus somni	?	FH	(104)
Leptospira spp	LenA (LfhA, Lsa24)	18–20 (FH, FHR-1)	(105)
	Len B	FH	(106)
	LcpA	20 (FH)	(107)
	LigA, LigB	5–7 (FH, FHL-1); 18–20 (FH); FHR-1	(108, 109)
	Lsa23	FH	(110, 111)
	LIC11966/ErpY-like lipoprotein	FH	(112)
	EF-Tu	FH	(113)
	?	FH	(114)
	Enolase	FH	(115)
Moraxella catarrhalis	OlpA	FH	(116)
Mycoplasma hyopneumoniae	EF-Tu	FH	(66)
Neisseria cinerea	FHbp	FH	(117)
Neisseria gonorrhoeae	PorB.1A (PorB1a, Por1A)	6 (FH, FHL-1); 18–20 (FH); FHR-1	(118, 119)
	PorB.1B (PorB1b, Por1B)	18–20 (FH)	(118–120)
	NspA	6–7 (FH, FHL-1)	(121)
Neisseria meningitidis	FHbp	6–7 (FH)	(122, 123)
	NspA	6–7 (FH, FHL-1)	(124)
	PorB2	6–7 (FH, FHL-1)	(125)
	PorB3	6–7 (FH)	(126)
	LOS sialylation	18–20 (FH)	(127)
Non-typeable *Haemophilus influenzae*	OmpP5 (P5)	6–7 (FH)	(128, 129)
Pasteurella pneumotropica	?	FH	(130)
Pseudomonas aeruginosa	Lpd	7 (FH, FHL-1); 18–20 (FH); 3–5 (FHR-1)	(131)
	Tuf	6–7 (FH, FHL-1); 19–20 (FH); 3–5 (FHR-1)	(74, 132)
Rickettsia conorii	OmpB β-peptide	FH	(133)
Salmonella spp	Rck	5–7, 19–20 (FH)	(134)
Staphylococcus aureus	SdrE	20 (FH)	(135, 136)
	Sbi	19–20 (FH); FHR-1	(137)
Streptococcus agalactiae	β protein (Bac, β C)	8–11, 12–14 (FH)	(138, 139)

(Continued)

TABLE 1 | Continued

Pathogen	Factor H binding ligand (other name designations)	Binding domains of Factor H family proteins	References
	Sht I and II	FH	(140, 141)
Streptococcus pneumoniae	PspC (CbpA, SpA, Hic, C3-binding protein)	6–10, 8–10, 9, 8–11, 19–20, 13–15 (FH)	(65, 139, 142–148)
	Tuf	6–7 (FH, FHL-1); 18–20 (FH); 3–5 (FHR-1)	(149)
	LytA	FH	(150)
Streptococcus pyogenes	Fba	7 (FH, FHL-1)	(151, 152)
	M protein family	7 (FH, FHL-1)	(153–155)
	Scl1	19–20 (FH); 3–5 (FHR-1)	(156, 157)
Streptococcus suis	Fhb	FH	(158)
	Enolase, EF-Tu, PK, GAPDH, FBA, FBPS, KAR, MRP1	FH	(99)
Treponema denticola	FhbB	7 (FH)	(68, 70, 159)
Yersinia enterocolitica	YadA	FH	(160)
	Ail	6–7 (FH)	(160)
Yersinia pseudotuberculosis	Ail	5–7, 19–20 (FH)	(161)
	Fungi		
Aspergillus fumigatus	AfEno1	6–7 (FH, FHL-1); 19–20 (FH)	(162)
	Aspf2	6–7 (FH, FHL-1); 19–20 (FH); 3–5 (FHR-1)	(163)
Candida albicans	Pra1	5–7 (FH, FHL-1); 16–20 (FH)	(164)
	Gpm1p (CRASP 1)	6–7 (FH, FHL-1); 19–20 (FH)	(165)
	Gpd2 (Gapdh)	7 (FH, FHL-1)	(166)
	Hgt1p	6–7 (FH)	(167, 168)
	Protozoa		
Trypanosoma brucei	FHR	4–6 (FH)	(169)
Trypanosoma cruzi	?	FH	(170)
Plasmodium falciparum	?	5 (FH, FHL-1); 20 (FH); FHR-1	(171)
	PfGAP50	5–7 (FH, FHL-1)	(172)
	Pf92	5–6 (FH, FHL-1)	(173)
	Helminths		
Toxoplasma gondii	?	FH	(174)
Onchocerca volvulus	?	(8–20) FH	(175)
Echinococcus granulosus	?	FH	(176)
Loa Loa	?	FH	(177)
	Viruses		
West Nile virus	NS1	FH	(178)
Human immunodeficiency virus-1	gp41, gp120	FH	(179–182)

[#]*Reviewed in.*

attack by expressing host glycans (187) which go on to capture FH.

α-N-acetylneuraminic acid (Neu5Ac) is a sialic acid species present on pathogens and humans. FH exclusively binds Neu5Ac species with α(2,3) linkages (43), discriminating against pathogens expressing other Neu5Ac linkages including α(2,6) and α(2,8) [reviewed in (188, 189)]. *Neisseria meningitidis* and *Neisseria gonorrhoeae* are Gram-negative bacteria restricted to humans that express α(2,3) linked Neu5Ac which participate in FH binding and other complement evasion tactics [reviewed in (188–190)].

On the activated host surface, FH domain CCP 20 interacts with GAGs and CCP 19 binds C3b (191). Sialylated *N. meningitidis* likely recreates this interaction on the pathogen surface in order to bind FH. To bind FH, *N. meningitidis* and *N. gonorrhoeae* sialylate lacto-N-neotetraose (LNnT) branches of lipooligosaccharides (LOS) with Neu5Ac [reviewed in (188)]. Neu5Ac sialylation of *N. meningitidis* LOS enhances FH binding to pathogen surfaces (127) (**Figure 3A**). This is postulated to occur when sialylation on the pathogen surface replaces host GAGs as the ligand for CCP 20, while maintaining the interaction between CCP 19 and deposited C3 fragments [reviewed in (188)]. Similarly, binding of bovine FH to *Histophilus somni* increases when bacteria are sialylated with Neu5Ac (104). While pathogen sialylation promotes FH binding to the cell surface, it still renders pathogens vulnerable to complement-mediated damage because a portion of the deposited C3 fragments involved in binding FH will likely form C3 convertases as opposed to binding FH [reviewed in (190)].

Pathogen sialylation also promotes FH binding in the absence of complement fragments. Increased binding of FH to *N. gonorrhoeae* occurs with sialylation of LNnT LOS (120, 192) only with the concomitant presence of gonococcal FH binding protein, porin B.1B (PorB.1B) (192) (**Figure 3B**). This phenomenon is speculated to occur through an interaction

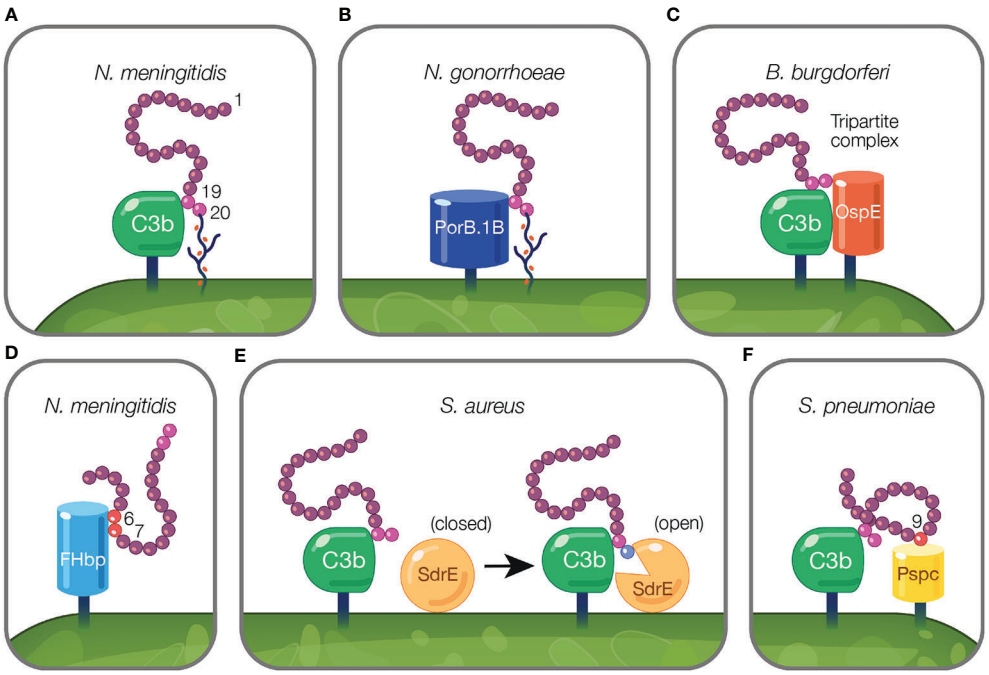

FIGURE 3 | Mechanisms of pathogens binding Factor H to evade the alternative pathway. **(A)** Pathogens evade complement by protecting their surfaces from complement attack using host cell markers. Neu5Ac sialylation of *N. meningitidis* replaces host GAGs and binds FH CCP 20 domain while maintaining the interaction between CCP 19 and deposited C3 fragments (127). **(B)** Pathogen sialylation promotes FH binding in the absence of complement fragments. *N. gonorrhoeae* FH binding protein, PorB.1B, binds FH CCP 19 domain replacing the interaction of C3b with FH CCP 19 [reviewed in (189)]. **(C, D)** Pathogen FH binding proteins mimic interactions between host and FH. **(C)** *B. burgdorferi* FH binding protein, OspE, forms a tripartite complex with FH where CCP 19 binds to C3 fragments and CCP 20 interacts with OspE. **(D)** *N. meningitidis* binds FH through its FH binding protein, FHbp, *via* FH CCPs 6–7. **(E, F)** Pathogen FH interactions that do not mimic mechanisms utilized by the host. **(E)** FH binding to deposited C3b on the pathogen surface results in a transformational change in *S. aureus* FH binding protein, SdrE, from a closed to open state. In the open state, SdrE facilitates the docking of CCP 20 into a ligand binding groove (136). **(F)** *S. pneumoniae* FH binding protein, PspC, *via* its tyrosine 90 residue acts like a key inserting into a hydrophobic lock formed by four hydrophobic residues of FH CCP 9 (146).

similar to what is described for *N. meningitidis*, but with PorB.1B replacing the interaction of C3b with FH CCP 19 [reviewed in (189)], thus eliminating the need for deposited C3 fragments.

Sialylation also increases binding of FH CCPs 6-7 to Neisserial surface protein A (NspA) of *N. meningitidis* (124). Here, bacterial sialic acid potentially acts as a docking station for FH, perhaps through binding CCP 20, but this has yet to be shown experimentally and may be structurally impossible, as suggested by Lewis et al. (124). In contrast, in *N. gonorrhoeae*, LOS sialylation impedes binding of FH CCPs 6-7 to NspA (121).

α-N-glycolylneuraminic acid (Neu5Gc) is a sialic acid variant not expressed by humans, but present in mice (193) and on the surface of sheep erythrocytes (194). Human FH binds Neu5Gc in a similar manner as Neu5Ac (195) and *N. gonorrhoeae* LNnT-LOS incorporation of Neu5Gc results in FH binding and serum resistance (196). As noted by Schmidt et al., this phenomenon may skew conclusions from animal research, including infection models using humanized FH transgenic mice, as FH binding may be affected in a manner not possible in humans (195).

Pathogen Factor H Binding Proteins

In addition to decorating their surfaces with host markers, certain pathogens capture FH directly using binding proteins alone. While many FH binding proteins have been identified along with the regions to which they bind on FH, the mechanisms explaining many of these interactions remain unresolved. Below, we describe two strategies employed by pathogen FH binding proteins to capture FH. In the first mechanism, FH binding proteins mimic binding interactions present in the host, but using protein-protein interactions instead of binding to polyanions on host surfaces. In these instances, FH binding proteins bind to the same domains on FH (CCPs 6–7 and 19–20) that are used to bind to the host. In the second mechanism, FH binding proteins capture FH *via* domains (i.e., amino acid residues) on FH that do not bind to the host.

Pathogen Factor H Binding Proteins Mimic Interactions Between Host and Factor H

In this section, we describe how FH binding proteins capture FH by binding to the regions on FH that would normally bind to the host. However, unlike host surfaces, which bind FH through expression of host cell markers (i.e., polyanions) in combination with C3 fragment, the binding of FH to the FH binding protein on the pathogen, constitutes solely a protein-protein interaction instead.

An excellent example of FH interactions with a FH binding protein was demonstrated for the FH binding protein of *N. meningitidis*, FHbp (**Figure 3D**). Crystallography studies revealed FHbp binds FH through extensive interactions between β-barrels of FHbp and CCP 6, and through minor contacts with CCP 7 (122). The site within CCP 6 utilized by FHbp (122) overlaps with a previously described binding location for sucrose octasulphate (SOS), a highly sulphated analog of GAGs (197). Similarly, the binding domain for FhbB from *T. denticola* also overlaps with the SOS binding domain (70). Together, these examples demonstrate how FH binding proteins mimic interactions between FH and host charged sugars through amino acid side chains (122).

Protein motif mimicry also involve pathogen interactions with FH C-terminal domains. FH binds host surfaces through dual recognition when CCP 20 binds GAGs and CCP 19 binds the C3d part of C3b (191). Similarly, in the process of mimicking host surfaces, FH binding proteins form a tripartite complex when FH CCP 19 binds C3 fragments and CCP 20 interacts with a FH binding protein (**Figure 3C**). This phenomena has been described for FH binding proteins of *Pseudomonas aeruginosa* (Tuf) (74), *Borrelia burgdorferi* (OspE) (74, 85, 198), *Borrelia hermsii* (FhBA) (74), and *Staphylococcus aureus* (Sbi) (137).

In *B. burgdorferi*, crystallography studies indicate OspE (a paralog of CRASP-3) forms a tripartite complex between FH and C3 fragments. This occurs when loops β2–4 and the interface between loops β5–6 interact with CCP 20 which includes FH amino acids overlapping with those involved in heparin binding, while deposited C3dg interacts with CCP 19 (85, 198). The majority of amino acid residues utilized by OspE for binding FH are conserved across the OspE protein family (85). Interestingly, ectopic expression of *B. burgdorferi* CRASP-3 in a serum sensitive *Borrelia* strain bound minute amounts of FH and did not confer serum protection (186). As suggested by Kolodziejczyk et al., the FH tripartite complex may not form when OspE is ectopically expressed, leading to inadequate complement protection (198). Alternatively, according to Siegel et al., additional complement binding proteins may be required to unfold FH to a conformation sufficient for interacting with binding proteins, which does not occur when ectopically expressed (186). While this study examined tripartite complex formation under conditions in which the complex is not surface-bound, this formation is possible under physiological conditions because the domains responsible for tethering OspE to the surface do not interfere with the binding site for FH and bound FH is orientated toward the surface to which C3b is bound *via* the thioester bond (85).

Pathogen Factor H Binding Proteins Sequester Factor H Using Interactions Not Found in the Host

Pathogen interactions with FH have been described that do not reflect mechanisms utilized by the host. In the model proposed by Zhang and colleagues, *S. aureus* surface protein serine–aspartate repeat protein E (SdrE) tightly binds a 21 amino acid region of CCP 20 not involved in the binding of C3d or of host cell markers (i.e., heparin, GAGs, and sialic acid) (85, 136). Upon FH binding to deposited C3b on the pathogen surface, SdrE undergoes a transformational change from a close to open state, which facilitates the docking of CCP 20 into a ligand binding groove (136). SdrE functions as a "clamp" to stabilize the SdrE-FH complex by locking and latching the FH tail into its ligand binding groove (136). The "close, dock, lock, and latch" mechanism is similar to strategies utilized by members of the microbial surface components recognizing adhesive matrix molecules, to which SdrE belongs (136). The described mechanism of SdrE is unique in the field of complement immune evasion [reviewed in (199)] (**Figure 3E**).

S. pneumoniae FH binding protein, PspC, also binds FH through a domain less utilized by other pathogens. PspC binds CCP 9 when the tyrosine 90 residue of PspC inserts like a key into a hydrophobic lock formed by four hydrophobic residues of FH (146) (**Figure 3F**). Studies using PspC lacking the residue important for interacting with the hydrophobic lock, showed PspC forms stable complexes with FH CCPs 8–10 (145). Here, PspC induces a functionally enhanced FH conformation that accelerates C3bBb decay 5-fold compared to FH alone, and increases the ability of FH to bind C3b 2-fold (145). Herbert et al. suggest a similar process may occur in the host in which host cell markers on host surfaces bind FH, and result in configurations of FH with enhanced regulatory activity.

ROLE OF FACTOR H BINDING PROTEINS IN PATHOGEN VIRULENCE

The necessity of FH binding proteins in pathogen virulence is questionable. While most of the described interactions between pathogens and FH demonstrate the advantage of binding FH in AP evasion through *in vitro* assays, *in vivo* studies are essential for further characterizing the role of FH binding in pathogen virulence. The importance of FH in pathogen virulence varies according to pathogen. For example, inhibition of FH binding is protective in several pathogens as supported by studies in which pathogen FH binding is blocked with recombinant FH proteins, or through vaccination with FH binding proteins (discussed in *Therapeutic and Preventive Strategies Targeting the Pathogen-Factor H Interface*). In addition, *in vivo* studies involving the FH binding protein, plasmodial transmembrane protein gliding associated protein 50 (PfGAP50) of *Plasmodium falciparum* was shown to be important in pathogen virulence when antibody neutralization of PfGAP50 reduced parasite transmission to the mosquito vector (172).

In vitro serum sensitivity of *Borrelia* species is associated with FH binding potential, considering almost all serum-resistant species recruit FH [reviewed in (200)]. However, *in vivo* studies demonstrate FH binding to pathogens is not required for infection. Numerous studies in *Borrelia* indicate FH may be dispensable for infection. For instance, FH-deficient mice, and mice without FB or C3 (no AP activity), were infected with *Borrelia* at levels similar to wild type animals (201). This finding suggests additional complement-dependent and -independent immune evasion strategies of *Borrelia* replace the benefits of FH capture for survival in the host (201). *B. garinii* is responsible for causing Lyme disease, but does not bind FH, highlighting that

FH may be dispensable for infection (202). The mechanisms by which *B. garinii* evades complement to establish infection in the host remain unknown [reviewed in (200)]. Similarly, CspA (CRASP-1) variants are not required for spirochete survival at the mouse tick bite site, though it is expressed at this location (203). Additionally, FH binding protein, FhbA of *B. hermsii* is not required for murine infection or human serum resistance (204). *B. hermsii* does not express additional FH binding proteins, suggesting the presence of additional complement evasion strategies (204). While *in vitro* studies demonstrate BclA, a FH binding protein of *Bacillus anthracis*, downregulates complement activation and protects against cell lysis, inoculation of mice with a lethal dose of *B. anthracis* spores lacking BclA, did not affect animal survival or bacterial burden compared to inoculation with an isogenic wild type strain (72).

While some studies convincingly demonstrate FH binding is dispensable for pathogen virulence, others have led to inconclusive findings. An example of disputable findings regarding the effect of FH binding in pathogen virulence involves studies of *S. pyogenes*. *S. pyogenes* is a human-specific pathogen that binds human FH through three proteins, including M protein (153). To study the effects of *S. pyogenes* complement evasion *in vivo*, transgenic mice expressing human FH and C4BP were generated. The human FH and C4BP in the serum from these animals, bound to *S. pyogenes in vitro* (205). Compared to wild type animals, transgenic mice expressing FH and C4BP were more susceptible to fatal infection by a *S. pyogenes* strain (AP1 strain) that binds human FH and C4BP through protein H (205), a member of the M protein family (206, 207). This demonstrates the additive virulence effect of binding more than one complement regulator (205). However, infection with a different *S. pyogenes* strain that does not bind FH or C4BP resulted in the same degree of mortality between transgenic and wildtype animals (205). Furthermore, pathogen inoculation with an isogenic *S. pyogenes* strain that does not express protein H, did not affect animal survival (205). Altogether, these results demonstrate recruitment of complement inhibitors by *S. pyogenes* exacerbates infection when FH and C4BP are present, but have no effect on disease if the strain cannot capture the regulatory proteins (205). However, in another study, FH binding to *S. pyogenes* did not modulate pathogen virulence. In this study, transgenic mice expressing chimeric FH (containing human FH CCPs 6–8) did not show an increased susceptibility to infection with a *S. pyogenes* strain capable of binding FH when compared to wild type animals (155). As suggested by Ermert et al., the discrepancy in findings may be due to differences in mouse strains, bacterial strains, and infection route (205).

FACTORS AFFECTING PATHOGEN FACTOR H BINDING PROTEIN EXPRESSION

FH binding proteins are not always constitutively expressed, and instead, expression is governed by numerous regulatory mechanisms described in the following section.

Environmental Stimuli Influence Factor H Binding Protein Expression

Pathogens are exposed to a wide variety of conditions within the host including transitions between hosts, nutrient supplies, and temperature, all of which have been shown to influence expression of FH binding protein.

FH binding protein expression is associated with nutrient availability within the pathogen environment. For example, expression of the FH binding protein, high-affinity glucose transporter 1 (Hgt1p) in *C. albicans* is highest at low, physiological, glucose concentrations compared to high glucose concentrations (168), which is likely due to the canonical role of Hgt1p in glucose metabolism (208). In *Streptococcus agalactiae*, zinc availability influences expression of FH binding proteins, Sht and ShtII (141). Purified Sht and ShtII proteins bind FH, suggesting a potential role in complement evasion. However, when bacteria survival was assessed in whole blood assays, no protective effect from Sht family proteins was observed. Though Sht and ShtII can bind FH, the primary role of these proteins is bacterial zinc acquisition. Thus, under conditions of low bioavailable zinc, Sht and ShtII expression is activated. In serum, adequate zinc levels repress Sht family gene expression, thus FH binding proteins may only participate in complement evasion under conditions of low zinc.

In some instances, FH binding protein expression is temperature dependent. In *N. meningitidis*, FHbp functions as a thermosensor when its expression increases with temperature (209, 210). *N. meningitidis* resides as harmless flora in the upper airway and enters the bloodstream during invasive disease where it encounters the complement system. Bloodstream temperature is warmer than that of the upper airway which, as suggested by Loh et al., may explain the upregulation of FHbp expression under conditions of increased temperature (210). Likewise, according to Kraiczy et al., temperature dependent upregulation of CRASPs occurs in cultured *Borrelia* (77), which may reflect differential regulation of CRASPs between mammals (higher temperatures) and ticks (lower temperatures) (77).

Similarly, binding outcomes of FH differ between *N. meningitidis* and *N. gonorrhoeae* to accommodate their respective environmental niches [reviewed in (211)]. *N. meningitidis* is an encapsulated organism, which enables high-level complement evasion (212). In contrast, *N. gonorrhoeae*, residing in the genitourinary tract, is not capsulated, and thus relies on host complement regulators for serum resistance. *N. gonorrhoeae* limits complement activity on its surface more effectively *via* FH binding through PorB.1B compared to FHbp of *N. meningitidis* (123). As noted by Shaughnessy et al., the functional differences in binding FH may, in part, be explained by the distinct niches in which these organisms inhabit. FH is very abundant in blood and therefore, likely easily recruited by *N. meningitidis*. In comparison, FH is present in low levels in the genitourinary tract, demanding efficient recruitment by *N. gonorrhoeae* (123).

Pathogen Life Cycle Affects Factor H Binding Protein Expression

Some pathogens reside in numerous hosts as part of a life cycle and must adapt accordingly to the changing host environment by

modulating gene expression, including regulation of FH binding proteins. For example, *Borrelia* participate in an enzootic life cycle between *Ixodes* tick vectors, and mammal, bird, and reptile hosts [reviewed in (213)]. Interestingly, expression of CRASPs reflects the needs of the spirochete in response to its changing environment. CspA (CRASP-1) is produced by *Borrelia* during tick-to-mammal and mammal-to-tick transmission, but is not expressed during established infection (214) or when *Borrelia* is cultured in environments mimicking host conditions (215). In contrast, CspZ (CRASP-2) expression increases in *Borrelia* at the tick bite site in the mammal and is maintained throughout infection, but is undetectable in the tick (214). CspZ gene and protein expression is also induced when *Borrelia* are treated with human blood and its expression is required for *Borrelia* bacteremia and tissue colonization in mice (216). In this particular study, even though mice were infected using spirochetes previously subjected to human blood, which does not reflect the natural life cycle of *Borrelia*, it nevertheless exemplifies the influence of human blood on *Borrelia* gene expression (217).

In another example, *Borrelia turicatae* mRNA expression of the gene for the FH binding protein, FhbA, was lower in *Borrelia* from fed ticks compared to *in vitro* culture; however the functional role of FhbA in infection remains to be determined (218). Finally, OspE paralogs (CRASPs 3–5) are expressed during all stages of mammalian infection [reviewed in (83, 219)]. Concurrent expression of CRASPs during the spirochete infection cycle confounds interpretation of the role each protein plays in complement evasion [reviewed in (83)]. Instead of contributing separately to complement evasion, CRASPs may work synergistically to carry out their functions as suggested by Bykowski et al. (214).

Some pathogens including protozoans differentiate into unique morphological life stages and FH binding capabilities vary concordantly. *P. falciparum*, a protozoan parasite, recruits FH using unique FH binding proteins according to the parasite life stage (172, 173). The invasive blood-stage merozoite found in humans uses Pf92 to bind FH (173), while extracellular gametes residing in the mosquito midgut, recruit human FH from a blood meal using a different FH binding protein, PfGAP50 (172). In *Trypanosoma brucei*, the proliferating slender form expresses the lowest levels of FH binding protein, FH receptor (FHR), followed by the quiescent stumpy form, and finally, the procyclic form expresses the highest levels of FHR (169). Both the slender and stumpy forms are present in mammalian blood, whereas the procyclic form is present in the midgut of the tsetse fly vector [reviewed in (220)]. In a related example, the FH binding proteins of *C. albicans*, Pra1, is upregulated upon switching from yeast to hyphal growth, which may explain, in part, why the hyphal form is more invasive than the yeast (164).

Pathogen Strain and Passage Number Affects Factor H Binding Protein Expression

Expression of FH binding proteins is subject to variability amongst pathogen strains. For example, PspC expressed by *S. pneumoniae* undergoes sequence variation leading to changes in FH binding capacity (221). Antibodies raised against a particular PspC variant successfully prevented FH binding and *in vivo* opsonophagocytic bacterial killing, but did not cross-react with *S. pneumoniae* that contained different sequence variations of PspC (221). Similarly, in *S. pneumoniae*, serotype invasiveness correlates with FH binding (222). Additionally, *S. pyogenes* bind FH through expression of M proteins. M proteins exhibit sequence variation between strains, and hence, vary in the ability to bind FH (155). Furthermore, analysis of FHbp expression across a panel of serogroup B meningococcal strains revealed the level of FHbp expression varies by at least 15-fold, and that variant 1, which is a component of meningococcus vaccines (discussed later), expresses significantly more protein than variant 2 or 3 strains (223). Expression of FH binding proteins Pra1 and Gpm1 is conserved across clinical isolates of *C. albicans*; however, expression levels vary, and higher expression levels correlate with FH binding (224). Moreover, FH binding varies from 5-42% across patient strains of *Fusobacterium necrophorum*, and strains which bound FH strongly result in more severe infections (101).

Culture passage number also influences FH binding expression. *Leptospira* strains with few passages display higher FH binding expression compared to culture attenuated strains, suggesting pathogens lose the ability to bind FH in culture (225). Similarly, passage number also decreases expression of CRASP-1, -2, and -5 in *Borrelia afzelii* (77). Passage number is postulated to suppress CRASPs expression in *B. garinii* because isolates from patients with neuroborreliosis bind FH, whereas strains with prolonged growth *in vitro*, do not (226).

Localization of Factor H Binding Proteins

FH binding proteins assume strategic spatial positioning to promote efficient evasion of the AP. This is evidenced by PspC of *S. pneumoniae*. Pneumococci divisional septa are insufficiently protected by the bacterial capsule, especially at the site where cell separation is initiated, permitting entry of serum factors and allowing complement activity (227). PspC compensates for the breach of protection by localizing to the cell septa to control lateral complement amplification (227).

While most FH binding proteins are surface bound, some are secreted. For example, Pra1 of *C. albicans* is secreted and regulates FH in the fluid phase (164). Pra1 is also surface expressed and localizes primarily at the tip of cells, which suggests an important role of Pra1 upon contact with host tissues and surfaces during infection (164). Likewise, Hgt1p of *C. albicans* is present on the cell wall, cell membrane, and intracellular and extracellular vesicles (168).

THERAPEUTIC AND PREVENTIVE STRATEGIES TARGETING THE PATHOGEN-FACTOR H INTERFACE

Acquiring host complement negative regulators, such as FH, allow pathogens to bypass complement attack and persist in the

host. Hence, strategies directed against the FH-pathogen interface hold therapeutic value. The following section describes existing and potential approaches to target pathogen binding of FH (**Figure 4**).

Interference of Pathogen Sialic Acid Expression

In addition to the role of sialylation in pathogen FH acquisition, it also modulates other pathways of complement, as well as phagocytosis and epithelial invasion [reviewed in (189)]. The importance of sialylation in pathogenesis renders it an enticing therapeutic target (**Figure 4A**). In the case of gonococci that are becoming increasingly antibiotic resistant, this approach is timely and reviewed elsewhere (188, 189, 228).

Gonococci incorporate sialic acid in surface glycans (LOS) resulting in FH binding [reviewed in (189)]. A strategy that manipulates pathogen sialylation to disable complement evasion, involves supplying gonococci with sialic acid analogs that do not bind FH. When such analogs are incorporated into pathogen glycans, it renders bacteria susceptible to complement and substantially reduces bacterial survival in normal human serum (196, 229). Intravaginal administration of a sialic acid analog (CMP-Leg5,7Ac_2) to transgenic mice capable of expressing sialic acid compounds found in humans protects against multidrug-resistant gonococci (229). Moreover, this sialic acid analog was stable at vaginal pH and temperature and did not incorporate into glycans on host surfaces, which is noteworthy because incorporation into host glycans may cause an immunogenic response against host tissue (229). The stability and limited side effects of sialic acid analog indicate an effective treatment option (229).

Inhibitors to Functionally Disable Factor H Binding Proteins or Its Interaction With Factor H

Inhibitors that block the interaction between FH and FH binding proteins can be considered as potential therapeutics (**Figures 4B, D**). As described previously, FHbp of *N. meningitidis* binds FH using a site shared by SOS, a highly sulphated analog of GAGs (122). The addition of SOS inhibits FH interaction with FHbp *in vitro*, thus as suggested by Schneider et al., provides support for small molecule competitive inhibitors in disabling pathogen evasion (122). However, competitive inhibitors binding FH may also interfere with host regulation of complement activity.

The binding of FH to SdrE of *S. aureus* is enhanced after a conformational change in the binding protein from a resting, locked state, to an open state (136). Small molecules inhibitors may have a potential in preventing conformational changes of

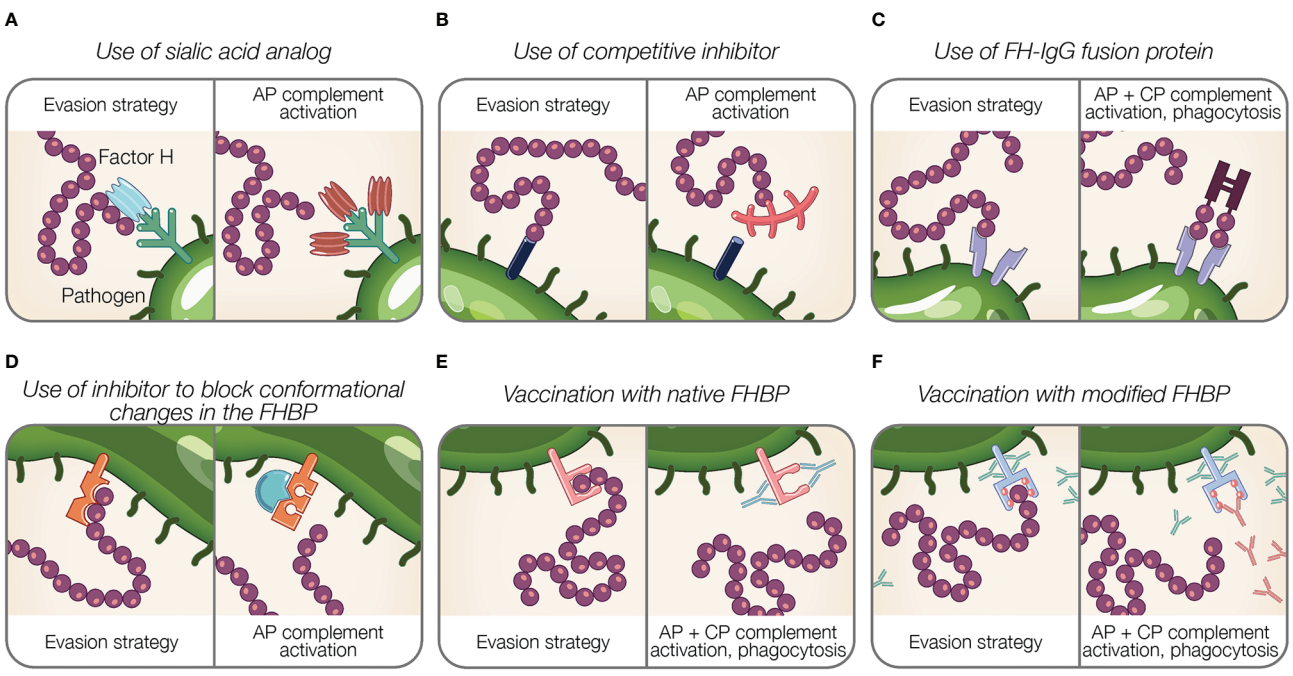

FIGURE 4 | Potential therapeutic and preventive strategies targeting the interaction between Factor H and pathogens. **(A–F)** Theoretical representations of pathogen evasion strategies targeted for therapy and prevention are shown. For each box, the left panel illustrates a pathogen evasion strategy and the right panel represents the intended result of therapeutic intervention, which includes AP and CP activation, and phagocytosis. **(A)** Sialic acid species (shown in light blue) are replaced by analogs unable to recognize FH (shown in brown). **(B)** Competitive inhibitors for FH binding proteins (FHBP) prevent FH interaction with the pathogen surface. **(C)** FH fusion proteins containing the Fc portion of IgG fused to FH binding fragments (CCPs 6–7, 19–20) bind FHBP to activate the CP *via* antibody recognition, facilitate Fcγ receptor-mediated phagocytosis, and prevent FH binding to the pathogen surface. **(D)** Inhibitors prevent conformational change of FHBP required to enhance their binding to FH. **(E)** Vaccination with native FHBP. **(F)** Vaccination with modified FHBP unable to bind FH, reveals FHBP epitopes (red balls) to improve immunogenicity.

FH binding proteins required to bind FH, similar to what occurs for SdrE [reviewed in (199)].

Factor H-Fc Fusion Proteins

Targeting the pathogen-FH interface is accomplished using fusion proteins in which FH domains essential for surface recognition (CCPs 18–20 or 6–7) are fused to the Fc region of IgG. Fusion proteins are designated FHX-X/Fc, where X-X refers to the FH domains used in the fusion protein. The proposed mechanisms of fusion proteins are threefold: (a) prevent FH binding to the microbial surface; (b) activate the CP *via* antibody recognition; and (c) facilitate Fcγ receptor-mediated phagocytosis [reviewed in (189)] (**Figure 4C**). Fusion proteins are advantageous because identifying pathogen FH binding proteins or ligands is not required. However, determining FH domains involved in pathogen binding is necessary to design applicable fusion proteins.

Animal model studies demonstrate the efficacy of fusion proteins using FH CCP 6–7. In infant rats, administration of FH6-7/Fc prior to intraperitoneal inoculation of a serogroup C strain of *N. meningitidis* dose-dependently reduced bacterial blood burden (230). Similarly, concurrent intranasal administration of FH6-7/Fc with inoculation of non-typeable *H. influenzae* reduced mouse lung bacterial burden (129). In a model of *S. pyogenes* sepsis, intranasal FH6-7/Fc treatment of human FH transgenic mice resulted in decreased animal mortality (231). Additionally, fusion proteins with CCPs 18–20 bound to gonococci and resulted in complement-dependent bactericidal activity (119).

Given the importance of CCP 6–8 (232–236) and CCP 19–20 (237, 238) in discriminating self from non-self surfaces, fusion proteins containing these domains may outcompete full-length FH for binding host cells, resulting in unwanted complement-mediated attack. In order for fusion proteins utilizing CCP 19–20 to be effective therapeutic solutions, these domains must be modified to prevent host cell interaction. This was accomplished by the generation of a CCP 19–20 fusion protein containing a point mutation in CCP 19 (D1119G). This mutation results in lower affinity for C3b binding compared to wild type CCP 19–20 restricting its ability to bind human cells, even while maintaining normal binding affinity for polyanions (238). D1119G bound to highly sialylated gonococci in the presence of serum, resulting in a robust *in vitro* complement response (239). Moreover, therapeutic administration of D1119G reduced infection duration and burden in a mouse vaginal colonization model of *N. gonorrhoeae* without affecting lysis of human erythrocytes (239). Similarly, FH CCP 19–20 fragments containing the D1119G mutation also bound to whole *P. aeruginosa*, *H. influenzae*, *Bordetella pertussis*, *S. pneumoniae*, and *C. albicans* organisms (74), suggesting the therapeutic relevance of mutant FH fusion proteins against numerous pathogens.

Factor H Binding Proteins as Vaccines

A suitable vaccine candidate is (a) immunogenic; (b) conserved across different strains and genospecies of the respective pathogen; (c) expressed during human infection; (d) necessary for development of a clinical infection; (e) surface exposed; and (f) raises an immune response that neutralizes an important virulence determinant as proposed by Bhattacharjee et al. and Meri et al. (85, 240). FH binding proteins meet many of these requirements, and thus are viable targets for vaccine development. This section describes clinically approved (**Figure 4E**) and emerging vaccines (**Figure 4F**) utilizing FH binding proteins.

Clinically Approved Vaccines Utilizing Factor H Binding Proteins

FHbp is a successful vaccine target against *N. meningitidis*, the causative agent of invasive meningococcal disease. Five of the six serogroups of *N. meningitidis* express capsular polysaccharides that are effective vaccine targets. However, because the capsule of serogroup B is poorly immunogenic, alternative vaccine targets are necessary to protect against the high mortality and morbidity associated with meningococcus disease [reviewed in (241)]. FHbp is a viable therapeutic target because it is widely expressed across numerous meningococcus B isolates [reviewed in (241)]. Two licensed vaccines, 4CMenB and MenB-FHbp, both containing FHbp, are approved to protect 10–25 year old individuals against serogroup B meningococcus [reviewed in (241, 242)].

FHbp displays sequence variability with 3 main variants belonging to subfamilies A (variant 2 and variant 3) and B (variant 1) (243, 244). Consequentially, immunization with vaccines representing single variants do not offer protection against strains containing other variants (243–245). MenB-FHbp is a bivalent vaccine containing recombinant lipidated FHbp from subfamily A variant A05 and subfamily B variant B01 [reviewed in (242)]. Because FHbp from two variants are represented in this vaccine, MenB-FHbp offers broad coverage against multiple *N. meningitidis* FHbp variants (246). In contrast, 4CMenB is a multi-component vaccine where one of the components is a FHbp that represents only one variant (subfamily B) [reviewed in (247)].

Sequence variability limits the degree of protection against FHbp variants. For example, post immunization sera from mice injected with a single variant did not offer *in vitro* cross protection against other variants (245). Thus, sequence variability of FHbp poses difficulties in vaccine development. However, recent studies have introduced alternative vaccine candidates that elicit antibody responses against FHbp variants 1, 2, and 3 to offer broad protection against meningococcal disease. One such promising candidate is the Gonococcal homologue of meningococcal FHbp (Ghfp) that protects against *N. meningitidis* expressing any of the 3 FHbp variants (248). Vaccination with chimeric FHbp antigens is an additional approach for offering broad protection. Scarselli et al. developed a chimeric FHbp antigen in which the surface is modified to confer specificity against all three variants, resulting in cross protection in mice (249). Another example includes chimeric vaccines developed by fusing domains conserved across all FHbp variants and selective portions of domains from variant 1 FHbp and domains of variant 2 FHbp (250). These molecules were

found to elicit bactericidal activity against strains expressing the different variants (250). Chimeric antigens can also be generated by fusing FHbp with other immunogenic antigens such as the VR2 epitope from the integral membrane protein PorA that induces an immunogenic response against *N. meningitidis* in mice (251).

Though approved for clinical use, understanding the scope of FHbp-based antibodies remains an area of active research. One such area involves long-term risks of vaccination, such as the development of anti-FH autoantibodies. Interaction of FHbp (delivered as a vaccine) with FH may result in the development of autoantibodies against FH. A few studies have investigated this potential outcome. Sharkey and colleagues noted that 2.5% of individuals vaccinated with 4CMenB show an increase in anti-FH autoantibodies (a low level of anti-FH autoantibodies are present in some individuals (252)). However, this increase was transient, and no adverse effects were reported in individuals with higher anti-FH autoantibodies (253). In another study, 4CMenB immunization resulted in the development of anti-FH autoantibodies in Rhesus macaques (254). Since the long-term effects of FH autoantibodies are still not clear, further investigation is required to assess the risk of autoimmune disease in response to meningococcal vaccines, as suggested by Sharkey et al. (253).

Other avenues of research into FHbp vaccines include expanding applications and improving efficacy. Recently, antibodies raised in response to MenB-FHbp were shown to be effective against non-serogroup B meningococci in serum bactericidal assays (255), suggesting this vaccine may provide broad protection against meningococcal disease. Additionally, vaccine delivery with polyhydroxybutyrate beads engineered to display FHbp antigens, show promise in preclinical studies as means to overcome limitations of recombinant protein vaccines such as poor immunogenicity and adjuvant requirements (256). Finally, recent studies using vaccines containing mutant FHbp with less ability to bind FH combined with native outer membrane vesicles showed higher serum bactericidal activity than 4CMenB vaccination (254). This vaccination approach also generated less FH autoantibodies than 4CMenB (254).

Emerging Vaccines Utilizing Factor H Binding Proteins

Vaccination With Native Factor H Binding Proteins

While vaccines against *N. meningitidis* are the only clinically approved therapies targeting the pathogen FH interface, other examples are emerging. The FH binding protein, PspC of *S. pneumoniae*, is a promising vaccine target. Mice immunized with a PspC fragment containing the FH binding domain conferred protection when challenged with the same strain used for immunization (257). Antibodies from immunized animals enhanced CP activity, but also competed for human FH binding, suggesting interference with the AP (257). However, antibodies generated against PspC from immunized human sera do not recognize the FH binding site, suggesting FH masks epitope recognition (258).

Vaccination with FH binding proteins is also effective against *Leptospira interrogans*. Immunization with a multi-subunit, adjuvant vaccine comprising multiple FH binding proteins from *L. interrogans* had similar protective efficacy and survival rate in hamsters challenged with *L. interrogans* compared to monovalent vaccine administration containing only LigAc (259). Though results indicate LigAc is likely a superior vaccine antigen in the multi-subunit vaccine, only the multi-subunit vaccine reduced leptospiral renal colonization in surviving animals (259). Further investigation is needed to identify the FH binding proteins in this observed effect from multi-subunit vaccination (259).

Vaccination With Modified Factor H Binding Proteins

An innovative approach towards applying FH binding proteins for vaccination involves manipulating FH binding proteins. Modification of FH binding proteins from several organisms has been shown to disable FH binding (**Figure 4F**).

Murine immunization with a non-binding FH mutant of *N. meningitidis* FHbp resulted in antibodies with a higher bactericidal activity than native FHbp vaccination (260). This finding suggests binding of FH to the native FHbp vaccine can decrease a protective antibody response and that mutant FH binding proteins may serve as a superior vaccine (260). Importantly, these studies utilized transgenic mice expressing human FH because FHbp only binds human FH (261).

As discussed above, PspC vaccination fails to generate antibodies against the FH binding site (258). Thus, as suggested by Glennie et al., generation of a modified PspC unable to bind FH presents a viable vaccine opportunity given the role of this protein in complement evasion and host cell invasion (258).

A recent study in *Borrelia* eloquently demonstrates the ability of mutated FH binding proteins to confer protective immunization. CspZ (CRASP-2) belongs to a family of lipoproteins utilized by *B. burgdorferi*, a causative agent of Lyme disease, to evade complement. CspZ binds FH (77) though the degree of binding varies across an extensive panel of human Lyme disease isolates (262). CspZ is an enticing therapeutic target because it is immunogenic in humans (though titers vary between individuals (262)) and its expression is highly conserved among species associated with Lyme disease [reviewed in (219)]. However, CspZ on its own is not protective against *B. burgdorferi* infection. Immunization with recombinant CspZ did not impact *B. burgdorferi* infection (262, 263) or prevent Lyme disease pathology in a mouse model (263).

Nonetheless, studies by Marcinkiewicz and colleagues revealed the potential of CspZ as a therapeutic target by using a nonbinding mutant of CspZ (CspZ-YA). Because CspZ-YA cannot bind FH, new epitopes previously cloaked by FH binding are revealed (216). As a result, the immunogenicity of CspZ increases and prevents *Borrelia* colonization (216). CspZ-YA conjugated to virus-like particles (VLP-CspZ-YA) protects passively immunized mice from Lyme infection (216). Additional work in mice infected by *Ixodes scapularis* nymphal ticks carrying *Borrelia* demonstrated VLP-CspZ-YA protects against spirochete tissue colonization and arthritis development (264). These authors suggest anti-CspZ-YA antibodies bind CspZ at epitopes within the FH binding site, thus competing with FH binding (264). Furthermore, CspZ-YA

vaccination protected mice against a strain of *B. burgdorferi* that does not bind FH and immunized sera eliminated this strain *in vitro*, suggesting resulting antibodies may target CspZ through the CP (264).

POTENTIAL TARGETING OF FACTOR H IN OTHER INFECTIOUS DISEASES

While FH interactions among bacteria are well-described and applied in clinical therapy, further research in this field is warranted for other classes of pathogens including protozoa, helminths, viruses, and fungi. Closing this gap is relevant for developing treatments for diseases caused by pathogens known to evade complement. The following sections provides preliminary support for pathogens that interact with FH, but require further work to harness these findings for therapy.

Factor H Interactions With Protozoa

The protozoan, *Trypanosoma cruzi* is the causative agent of Chagas disease, which progresses from an acute, often undiagnosed, asymptomatic stage, to a deadly chronic stage of which 30–40% of chronically infected individuals develop cardiomyopathy, mega-syndromes, etc. [reviewed in (265)]. Currently, there are no vaccines for *T. cruzi* nor treatments available for the chronic, fatal stage of the disease. *T. cruzi* adopts various strategies to evade complement, including the AP [reviewed in (266)]. When the infective form of *T. cruzi* (trypomastigotes), which is normally resistant to complement-mediated killing, is treated with enzymes including sialidase and trypsin, the parasites become susceptible to complement-mediated lysis in human serum (267), suggesting parasites may be protected from the AP by FH that has been hijacked by surface-bound sialic acid. This notion is also supported by data showing trypomastigotes that are pre-opsonized with C3b bind FH (170). However, the molecular mechanisms involved in binding remain unknown. Likewise, as suggested by Sikorski et al., the mechanism by which *Toxoplasma gondii* captures FH may be through expression of sialic acid or heparan sulfated proteoglycans (174). In addition, *P. falciparum*, the causative agent of malaria, has developed resistance to all antimalarial drugs used against it [reviewed in (268)]. FH has been shown to bind *P. falciparum*, though not all receptors have been identified (171) and doing so holds promise for therapeutic avenues against drug resistance. Overall, interfering with the ability of infectious protozoa to bind FH represents a possible treatment strategy against diseases caused by these pathogens; however, more work is required to understand the molecular mechanisms involved in these interactions with FH.

Factor H Interactions With Helminths

Echinococcus granulosus, a zoonotic cestode, causes cystic echinococcosis in humans when larvae develop cysts in organs including liver, lungs, brain, and bones [reviewed in (269)]. FH binds *E. granulosus* through interactions involving the neutral, sugar-rich, laminated layer of the cyst wall (176). Myoinositol hexakisphosphate ($InsP_6$) is a protein abundantly expressed in the laminated layer and is shown to bind FH through *in vitro* studies with purified protein (270). However, studies representing physiological conditions suggest $InsP_6$ does not protect against AP-mediated attack and may instead, activate the CP through C1q recognition (270). Thus, the mechanism for FH interaction with *E. granulosus* remains largely unresolved and likely involves other components on the laminated layer other than $InsP_6$ [reviewed in (271)].

Onchocerca volvulus is a parasitic nematode causing onchocerciasis, or river blindness. Novel treatment options are necessary for this disease given the threat of drug resistance to the standard therapy, ivermectin [reviewed in (272)]. The pathogenic stage of *O. volvulus* (microfilariae) bind FH using CCPs 8–20 with preservation of cofactor activity, but the binding ligand remains unknown (175). *Loa Loa* is another parasitic nematode and the causative agent of loiasis, characterized by subconjunctival eye passage of the adult worm, angioedema, and pathology associated with the lungs, brain, heart, and kidneys [reviewed in (273)]. Patients with a high infectious load are at risk for severe complications from ivermectin treatment (274), thus alternative treatment options are important. Larval *L. Loa* acquires FH on the outermost sheath layer and retains its cofactor activity (177). However, FH CCPs involved in this reaction are not yet described, but likely does not involve heparin binding domains (177).

Factor H Interactions With Viruses

There are many examples of viruses evading complement [reviewed in (62)], but few associate complement evasion to pathogen interactions with FH. West Nile virus binds FH using non-structural protein (NS1); however, the mechanisms of this interaction is unknown (178). Interestingly, serum levels of NS1 correlate with disease severity and viremia [reviewed in (275)], which may be due in part, to the ability of NS1 to capture FH.

Human immunodeficiency virus-1 (HIV-1) is resistant to complement lysis in circulation even though HIV-1 and anti-HIV-1 antibodies activate complement [reviewed in (276)]. FH binds the HIV-1 envelope proteins gp41 and gp120 (179–182). More robust studies are required to understand the role of FH binding in HIV-1 complement evasion.

CONCLUSION

Numerous pathogens exploit host FH, the primary negative regulator of the AP of complement, as part of a robust complement evasion strategy. Characterizing interactions between pathogens and FH provides insight into the failure of FH to discriminate self from non-self, and/or the impressive resourcefulness of pathogens to outsmart complement regulation. The biological relevance of binding FH is demonstrated by established and emerging vaccines targeting the pathogen-FH interface and evidenced by the number of pathogens utilizing FH and expressing multiple FH binding proteins. Additionally, studying pathogen interactions with FH

may reveal more information about how FH operates in the host. The selective pressure of pathogens to maintain FH binding capabilities suggest undiscovered details about the role of complement in immune defense against pathogens.

AUTHOR CONTRIBUTIONS

SRM, SSM, and VF conceived and wrote the paper. CC critically reviewed and edited the paper. SRM, SSM, and CC designed the figures. All authors contributed to the article and approved the submitted version.

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Complement Genetic Variants and FH Desialylation in *S. pneumoniae*-Haemolytic Uraemic Syndrome

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Haemolytic Uraemic Syndrome associated with *Streptococcus pneumoniae* infections (SP-HUS) is a clinically well-known entity that generally affects infants, and could have a worse prognosis than HUS associated to *E. coli* infections. It has been assumed that complement genetic variants associated with primary atypical HUS cases (aHUS) do not contribute to SP-HUS, which is solely attributed to the action of the pneumococcal neuraminidase on the host cellular surfaces. We previously identified complement pathogenic variants and risk polymorphisms in a few Hungarian SP-HUS patients, and have now extended these studies to a cohort of 13 Spanish SP-HUS patients. Five patients presented rare complement variants of unknown significance, but the frequency of the risk haplotypes in the *CFH-CFHR3-CFHR1* region was similar to the observed in aHUS. Moreover, we observed desialylation of Factor H (FH) and the FH-Related proteins in plasma samples from 2 Spanish and 4 Hungarian SP-HUS patients. To analyze the functional relevance of this finding, we compared the ability of native and "*in vitro*" desialylated FH in: (a) binding to C3b-coated microtiter plates; (b) proteolysis of fluid-phase and surface-bound C3b by Factor I; (c) dissociation of surface bound-C3bBb convertase; (d) haemolytic assays on sheep erythrocytes. We found that desialylated FH had reduced capacity to control complement activation on sheep erythrocytes, suggesting a role for FH sialic acids on binding to cellular surfaces. We conclude that aHUS-risk variants in the *CFH-CFHR3-CFHR1* region could also contribute to disease-predisposition to SP-HUS, and that transient desialylation of complement FH by the pneumococcal neuraminidase may have a role in disease pathogenesis.

Keywords: factor H, *Streptococcus pneumoniae* (pneumococcus), Haemolytic Uraemic Syndrome, genetic variant, complement system

INTRODUCTION

Streptococcus pneumoniae (SP) infections can give rise to potentially life-threatening infections such as pneumonia, meningitis or sepsis, especially in children under 2 years of age (1, 2). In the last 20 years, the generalization of vaccination against several SP serotypes have dramatically reduced the incidence and morbidity/mortality of these conditions, but many serotypes are not covered by vaccination and some are antibiotic-resistant (3).

Invasive *S. pneumoniae* infections sometimes result in a form of Haemolytic Uraemic Syndrome (SP-HUS) with high morbidity/mortality (1, 4, 5). It is thought that SP-HUS results from desialylation of host cells by the pneumococcal neuraminidase, which result in the exposition of the *Thomsen-Friedenreich* antigen (TF) in erythrocytes, platelets and glomeruli and its subsequent interaction with natural anti-TF antibodies (6, 7), and/or in reduced protection of host cells against autologous complement (8, 9). On the assumption that this is the main pathogenic mechanism, it is generally accepted that complement genetic variants are not involved in predisposition to SP-HUS (10). Nonetheless, we have already described a few SP-HUS patients presenting rare genetic variants in complement genes (11, 12), and these findings suggested that the complement contribution to SP-HUS could be underestimated.

Human Complement can eliminate *S. pneumoniae* through different mechanisms (13). *S. pneumoniae* activates the complement classical pathway, as illustrated by the high incidence of infections in individuals with deficiency of C1q, C2, or C4 (14). Nonetheless, the lectin and alternative pathways also contribute to bacterial killing, which is mainly done through opsonophagocytosis, and to a lesser extent through inflammation. Assembly of the Membrane Attack Complex on the pathogen surface, on the contrary, is of little relevance because of the presence of the *S. pneumoniae* capsule, which is a very important virulence factor and the first barrier against the immune system proteins.

S. pneumoniae can also avoid elimination by human complement by expressing several proteins (PspC, SpsA, Hic, C3-binding protein) that interact with human Factor H (FH), the main regulator of the complement alternative pathway (15, 16). FH is a 150-kDa plasma glycoprotein that is essential to control complement activation on plasma and on cellular surfaces, thus preventing hypocomplementemia and self-damage (17). The complement regulatory activities of FH rely on its interaction with soluble or surface-bound C3b, and with negatively-charged molecules (mainly sialic acids and glycosaminoglycans) present on host's cells and tissues. The distribution of ligand-binding sites in FH is well-known. The N-terminal, SCRs 1-4 domains bind to soluble C3b, SCRs 6-7 recognize soluble or surface polyanions, and the C-terminal, SCRs19-20 domains recognize both C3b and polyanions on cellular surfaces (18, 19). Defective function of the N-terminal domains of FH provokes uncontrolled complement activation in plasma and deposition of C3b fragments on autologous cells and tissues, thus favoring renal pathologies such as membranoproliferative glomerulonephritis. Defective function of the C-terminal domains of FH, in the other hand, predominantly alter complement regulation on cellular surfaces, and contributes to the endothelial damage characteristic of the thrombotic microangiopathy atypical HUS (aHUS) (20). FH function could be modulated by their homologous FH-Related (FHR) proteins, a group of plasma proteins whose precise role on complement physiopathology is not fully understood (21, 22).

In this report, we extend our complement findings to a cohort of 13 Spanish SP-HUS patients. We confirm the presence of rare complement genetic variants in SP-HUS patients, and show that there is a high frequency of some FH and FHRs polymorphisms associated to aHUS. Moreover, we report for the first time the transient desialylation of FH and FHR proteins by the pneumococcal neuraminidase in plasma samples from a few Spanish and Hungarian SP-HUS patients, and present functional data suggesting that FH sialic acids have a certain role in complement regulation on cellular surfaces.

Abbreviations: aHUS, Atypical HUS; HUS, Haemolytic Uraemic syndrome; FH, Factor H, FHR, Factor H-Related, FI, Factor I, RCA-I, *Ricinus Communis Agglutinin I,* SNA, *Sambucus Nigra Agglutinin,* SP, *Streptococcus pneumoniae*.

MATERIALS AND METHODS

Blood Samples

Blood samples from 13 Spanish and 11 Hungarian SP-HUS patients were drawn during the acute episode or at remission. EDTA-plasma was aliquoted and stored at −20 and −80°C until use, to avoid repeated freezing and thawing; peripheral blood leukocytes (PBLs) were used to prepare genomic DNA by standard procedures. Blood samples were also obtained from healthy volunteers. Patients and controls provided written informed consent, as approved by the ethical committees from La Paz University Hospital or the Semmelweis University.

Genetic Studies

Mutational screening on the Spanish patients was determined by an in-house next generation sequencing (NGS) panel which includes all the complement genes relevant to aHUS (23). Copy number variation in the *CFH-CFHRs* region was analyzed by multiplex ligation-dependent probe amplification (MLPA) with the P236 A1 ARMD mix 1 (MRC-Holland, Amsterdam, The Netherlands). Genotyping of the *CFHR3*A/B* alleles was performed by Sanger sequencing of *CFHR3* exon 5 (24). Genetic analysis of the Hungarian patients was done as described previously (11).

WBs Analyses of Plasma Samples

Primary and Secondary Antibodies

Rabbit polyclonal antibodies recognizing FH and different FHRs were generated in-house, or kindly provided by Dr. Richard Pouw and Dr. Mihály Jozsi. The anti-FH monoclonal antibodies (mAb) OX24 and C18 were from ThermoFisher (MA170057 and GAU0180302); the anti-FHR-1/FHR-2 mAb JHD7 was from Hycult Biotech (HM2301); the anti-FHR-4 mAb (MAB5980) and the anti-FHR-5 mAb (MAB3845) were from R&D. Rabbit polyclonal anti-human transferrin antibody PA527306 was from ThermoFisher. HRP-conjugated goat anti–rabbit IgG and HRP-conjugated goat anti–mouse IgG were obtained from Santa

Cruz, and used as secondary antibodies. Primary and secondary antibodies were diluted in Tris/Tween buffer containing 2% ECL Advance blocking agent (GE Healthcare). Secondary antibodies solutions also contained the Streptactin reagent (Bio-Rad), to further visualize the molecular weight markers by chemiluminescence.

Western Blot Protocol

Plasma proteins (1-2 μL of EDTA-plasma) were separated on 10% polyacrylamide gels by SDS-PAGE under the following conditions: 50 mA/30 min; 75 mA/30 min; 100 mA/90 min. Molecular weight markers (WesternC blotting standards; Bio-Rad) were also loaded in every gel. Proteins were then transferred to nitrocellulose membranes (iBlot™ Transfer Stacks) using an iBlot Dry Blotting System (ThermoFisher), and blocked overnight at 4°C with 2% ECL Advance blocking agent in Tris/Tween. The membranes were incubated at room temperature with primary antibodies for 2 h, and with secondary antibodies for 30 min, and developed with a chemiluminescent substrate (ECL Advance Kit; GE Healthcare). Gel images were detected in a CCD camera (UVITEC Cambridge). Tris/Tween buffer was used for all washing steps.

Two-dimensional Western-blot analysis of FH/FHRs was done following our previously described protocol (25). Briefly, 200 μL of EDTA-plasma samples were adsorbed in heparin columns under low ionic strength, and 150 μg of the protein eluate (free from plasma albumin and immunoglobulins) were subjected to analytical Isoelectrofocusing (first dimension) using 7 cm-IPG strips of pH 3-10 or pH 4-7 (GE Healthcare). The IPG strips were then subjected to SDS-PAGE (second dimension) and Western-blot as described above.

ELISA Assay for FHR-5 Levels

A sandwich ELISA that uses two capture antibodies and was originally developed by Dr. Elena Goicoechea de Jorge (Department of Immunology, Complutense University of Madrid) was adopted with small modifications. 96-well microtiter plates were coated with 50 μL of goat anti-mouse IgG_{2a} (Southern Biotech, 1080-01, 1/5,000 in PBS) and incubated overnight at 4°C. Plates were washed twice with washing buffer (PBS-0.2% Tween 20), and blocked for 1 h at 37°C with 100 μL of blocking buffer (PBS-1% BSA). After three washes, the plates were incubated for 1 h at 37°C with 50 μL of an in-house monoclonal antibody which recognizes FHR-1, FHR-2, and FHR-5 (2C6, IgG2a isotype, 1/4,000 dilution). Plates were washed four times, and 50 μL of 1/800 and 1/1,600 dilutions of plasma samples were incubated for 1 h at 37°C. After 4 washes, 50 μL of a mouse anti-FHR-5 monoclonal antibody (MAB3845 from R&D, $IgG1_1$ isotype, 1/500 dilution) were added, and incubated at 37°C for 1 h. Plates were washed 4 times, incubated at 37°C for 30 min with peroxidase-conjugated goat anti-mouse IgG_1 (1/5,000 dilution), and washed five times. A colored reaction was developed by using O-phenylenediamine dihydrochloride as substrate, the reaction was stopped with 10% sulfuric acid, and the absorbance was measured at 492 nm. A plasma sample with known FHR-5 levels was used as a standard curve.

Neuraminidase Activity Assay

Neuraminidase activity in plasma samples was determined by using the Neuraminidase Activity AssayKit (MAK121; Sigma-Aldrich) following the manufacturer's protocol. Briefly, 20 μL of whole plasma or plasma dilutions (1/5, 1/10, 1/50) were loaded onto 96-well microtiter plates; upon addition of 80 μL of the reaction mix, the plates were incubated at 37°C for a total of 50 min, with absorbance readings at 570 nm at 20 min and 50 min. The absorbance increase from 20 to 50 min was used to calculate neuraminidase activity using a standard curve.

FH Desialylation and Lectin Blotting

Twenty-five μg of FH (CompTech) in a volume of 25 μL were added to 100 μL of 0.1 M sodium acetate, pH 5, 25 μL of 1% BSA and 25 μL of *Clostridium perfringens* Neuraminidase (Sigma-Aldrich). Upon incubation at 37°C for 4 h under gentle shaking, the reaction was stopped with 25 μL of 0.5 M sodium hydrogen carbonate, pH 9.8. The same amount of FH was incubated in parallel in the same conditions, but without neuraminidase. The two FH samples (native and neuraminidase-treated) were loaded in triplicate on a 10% polyacrylamide gel, and after SDS-PAGE the samples were transferred to a nitrocellulose membrane as described above. The membrane was cut into 3 sections, each containing native and neuraminidase-treated FH; one section was incubated with rabbit polyclonal anti-FH antibodies, and the other two sections were incubated with two lectins with different sugar specificity, as described below.

Lectin RCA-I (*Ricinus Communis Agglutinin I*), which preferentially binds β-D-galactose residues, and lectin SNA (*Sambucus Nigra Agglutinin*), which binds α(2–6)-linked sialic acids, were purchased from Vector Laboratories, and used for blotting as reported (26). Membranes were blocked with MAL buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 0.2% BSA, 0.2% Tween-20) for 1 h at room temperature, and incubated overnight with 10 mL of 1 μg/mL biotinylated SNA or RCA-I in SNA buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 1% BSA, 0.1% Tween-20, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$). Membranes were washed three times for 10 min with 10 mL of SNA buffer, and then incubated for 1 h with 10 mL of 1 μg/mL streptavidin coupled with horseradish peroxidase (HRP). After 3 additional washes, the membranes were developed with a chemiluminescent substrate (ECL Advance Kit; GE Healthcare).

Binding of FH and Desialylated FH (dFH) to C3b-Coated Microtiter Plates

The binding of FH and dFH to surface-bound C3b was determined according to our reported ELISA protocol (27), with a few modifications. 96-well polystyrene microtiter plates (Nunc MaxiSorp®) were coated overnight at 4°C with 0.4 μg of purified C3b in 100 μL of 0.1 M $NaHCO_3$, pH 9.5. Plates were washed three times in TNT buffer (50 mM Tris/ HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20), and the wells were blocked at 37°C for 1 h with 1% BSA-TNT buffer. After washing, 100 μL of serial dilutions (from 2 μg/mL to 0.0325 μg/mL) of FH or dFH in 1% BSA-TNT buffer were added in duplicate, and allowed to interact with the surface-bound C3b at 37°C for 1 h. After three washes, 100 μL of an in-house rabbit anti-human FH polyclonal antibody

which lacks reactivity against human C3b were added, and the plates were incubated at 37°C for 1 h. After three more washes, 100 μL of a 1/1,000 dilution of goat anti-rabbit immunoglobulin G antibody coupled with HRP (Santa Cruz) was added, and the plates were kept at 37°C for other 30 min. The plates were washed three times, and the enzymatic reaction was developed with ABTS (Merck), and stopped with 0.1% sodium azide. The binding of FH/dFH to the C3b-coated wells was determined by reading absorbance at 405 nm.

Proteolytic Assays of C3b by FI

The cofactor activity of FH and dFH in the proteolytic cleavage of C3b by FI in the fluid phase was determined basically as described (28). Purified C3b (750 ng), FI (125 ng), and FH/dFH (100 ng) were diluted in 25 μL of 10 mM HEPES buffer, pH 7.5, 0.02% Tween 20 in Eppendorf microtubes (final concentrations: 170 nM C3b, 57 nM FI, 26 nM FH/dFH). Proteins were incubated at 37°C during 2.5 or 12.5 min, and after addition of 5 μL of 5X SDS-sample buffer solution with β-mercaptoethanol, 3 μL-aliquots were subjected to 10% SDS-PAGE and Western-blot, as described above. An anti-C3 antibody generated in rabbits (ab200999, Abcam) was used as a primary antibody; this antibody recognizes the α′chain of C3b and the α45 fragment of iC3b, but not the β chain. Upon completion of the Western-blot protocol, the gel images were analyzed with the ImageQuant TL software (GE Healthcare). For every incubation time, the intensity of the C3bα′ band plus the intensity of the iC3bα45 band in the gel lane was set to 100%, and the amount of C3b cleavage was then calculated as the percentage of the remaining C3bα′ band.

To analyse the cofactor activity of FH/dFH in the proteolytic cleavage of C3b by FI in the solid phase, 600 ng of C3b in 30 μL of PBS were added to microtiter wells, and incubated 1 h at 37°C. After 3 washing steps with PBS, 30 μL of a solution containing 100 ng of FI and 80 ng of FH/dFH were added (final concentrations: 100 nM C3b, 38 nM FI, 17 nM FH/dFH). Five μL of 5X SDS-sample buffer solution with β-mercaptoethanol were immediately added to one of the wells (0 time point). The plate was then incubated at 37°C, and the proteolytic reaction in the other wells was stopped after 2.5, 12.5, or 22.5 min by addition of 5 μL of 5X SDS-Sample buffer solution. The well content was carefully mixed by hand, and analyzed by 10% SDS-PAGE and Western-blot, using the same protocol as for the proteolysis in the fluid phase.

ELISA Assay for C3 Convertase Decay-Accelerating Activity

The decay-accelerating activity of FH/dFH was analyzed by generating Properdin-stabilized C3bBb (C3bBbP) on microtiter plates. One hundred μL of 5 μg/mL C3b in PBS were immobilized overnight at 4°C on microtiter plates (Nunc Medisorb). Plates were washed three times with assay buffer (2.5 mM sodium barbitone, pH 7.4, 71 mM NaCl, 0.15% Tween, 1 mM $MgCl_2$, 1 mM $NiSO_4$) and blocked for 1 h at 37°C with 1% BSA-assay buffer. C3bBbP was then generated by adding 50 μL of a solution containing 2 μg/mL FB, 0.2 μg/mL FD, and 4 μg/mL Properdin, in 1% BSA-assay buffer. Increasing concentrations of FH/dFH (from 0.039 to 5 μg/mL) were then added, and incubated at 37 °C for 30 min. Plates were washed in assay buffer, and the remaining C3bBbP molecules were detected with a murine anti-Bb antibody (A227, Quidel; 1/500, 37°C, 1 h). After washing, a 1/2,500 dilution of a peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) was incubated for 1 h. A colored reaction was developed by using ABTS (Merck) as peroxidase substrate, and absorbance was read at 405 nm.

Haemolytic Assays on Sheep Erythrocytes

Lysis of sheep erythrocytes by a serum sample from an aHUS patient carrying the FH mutation W1183L was performed as described (29). The amount of patient's serum giving about 50% lysis was then chosen to compare the capacity of increasing concentrations of FH/dFH (from 2.5 to 20 μg/mL) to prevent lysis.

Lysis of sheep erythrocytes by a normal human serum was induced by adding different amounts of the FH monoclonal antibodies OX24 (recognizing SCR5) or C18 (recognizing SCR20), as already reported (30). The amount of each antibody capable to induce 60–70% lysis was then used to test the capacity of increasing concentrations of FH/dFH (from 1 to 15 μg/mL) to prevent lysis.

In all the experiments, sheep erythrocyte lysis was calculated by reading absorbance at 414 nm.

RESULTS

Complement Rare Variants and Risk Polymorphisms in the Spanish Cohort of SP-HUS

From 2006 to 2019 we performed complement studies in 13 Spanish HUS patients (seven males and six females) who were diagnosed in the context of an *S. pneumoniae* infection (**Table 1**). All the patients but one were younger than 3 years at disease onset. Genetic screening of *CFH*, *MCP*, *CFI*, *CFB*, *C3*, the five *CFHR* genes, and other complement genes was undertaken in nine patients; genetic screening could not be done in one patient, and was uncompleted in three patients.

Rare complement variants were found in heterozygosis in five patients (four males and one female). Patient H150 carries a rare *CFHR3* variant (c.796+1G>A) that alters normal splicing and results in a null allele, and he also carries the *CFHR3-CFHR1* deletion (*DelCFHR3-CFHR1* or $\Delta_{CFHR3-CFHR1}$); thus, the two variants generate homozygous FHR-3 deficiency in this patient (25). Patient H619 presents partial FHR-5 deficiency, and he has recently been described together with a glomerulonephritis patient carrying a very similar variant (12). The *CFI* intronic variant (c.1534+5G>T) found in patient H640 is located within the donor splicing site of exon 11, but the patient had normal FI levels. The *CFHR5* variant in patient H731 (c.368A>G; p. Asn123Ser) was predicted to be likely benign; nonetheless, this patient also carries another *CFHR5* variant

TABLE 1 | Complement findings in the Spanish SP-HUS cohort.

Patient code	Gender	Age at onset	Rare Variants	Common aHUS-risk variants				*DelCFHR3-CFHR1*	C3/C4 (mg/dL)
				MCPggaac	*CFH(H3)*	*CFHR3*B*	*CFHR1*B*		
H118	Female	2 y	No	No	HET	HET	HOM	No	178/27.2
H150[a]	Male	12 mo	*CFHR3 (c.796+1G>A)*	No	No	No	HET	HET	132/34
H171[a,b]	Female	3 y	No	No	HET	HET	HET	No	81.3/40.6
H201	Female	19 mo	Uncomplete screening	Not done	No	HET	HOM	No	111/42.3
H202	Female	2 y	Uncomplete screening	Not done	No	No	HET	No	145/33.1
H582	Female	47 y	No	No	HET	HET	No	No	150/46.1
H619[c]	Male	2 y	*CFHR5 (c.486_487insAA; p.E163Kfs*10)*	HET	No	No	HET	No	166/44.8
H640	Male	5 mo	*CFI (c.1534+5G>T)*	No	No	No	No	No	153/30.3
H678	Male	12 mo	Uncomplete screening	Not done	HET	HET	HOM	No	123/26.2
H731	Female	17 mo	*CFHR5 (c.368A>G; p.N123S / c.832G>A; p.G278S)*	HET	HET	HOM	HET	No	143/36
H837	Male	21 mo	No	No	No	No	HET	No	87.1/9.6
H859	Male	16 mo	*C1QB (c.223G>A p.G75R)*	No	HET	HET	HOM	No	146/60
H946	Male	21 mo	Genetic screening not done	Not done	HET	HET	HOM	No	58.9/9.56

*Demographic and complement data of the 13 SP-HUS patients of Spanish origin studied during 2006–2019. Genetic data include rare complement variants, the aHUS-risk haplotypes MCPggaac, CFH(H3), CFHR3*B, and CFHR1*B, and the common DelCFHR3-CFHR1 variant. C3 and C4 levels in the first plasma sample available are also shown; normal ranges were 75-135 mg/dL for C3, and 14-60 mg/dL for C4. [a]Described in (25). [b]This patient presented anti-FH autoantibodies. [c]Described in (12).*

TABLE 2 | Contribution of aHUS-risk haplotypes to SP-HUS.

Haplotype		Control	aHUS	SP-HUS	P-aHUS
MCPggaac	N	222[a]	662	18	44
	Frequency	0.333	0.414	0.111	0.432
CFH(H3)	N	186[a]	668	26	44
	Frequency	0.242	0.302	0.269	0.455
*CFHR3*B*	N	194[b]	634	26	26
	Frequency	0.242	0.355	0.346	0.462
*CFHR1*B*	N	204[b]	610	26	44
	Frequency	0.368	0.467	0.615	0.455

*Frequency of the aHUS-risk haplotypes MCPggaac, CFH(H3), CFHR3*B and CFHR1*B in the Spanish cohorts of aHUS (352 patients), SP-HUS (13 patients), and P-aHUS (22 patients), and in a total of 227 Spanish control individuals. [a]Frequencies of MCPggaac and CFH(H3) were determined in one cohort of 116 control individuals. [b]Frequencies of CFHR3*B and CFHR1*B were determined in a cohort of 111 control individuals. N represents the number of chromosomes analyzed.*

(c.832G>A; p.Gly278Ser) that is a null allele, generating FHR-5 haploinsufficiency in the patient. Finally, patient H859 carries a genetic variant in the *C1QB* gene (c.223G>A; p.Gly75Arg) that results in an amino acid change at position 75 of the C1qB chain; C1q levels in this patient were normal.

Analysis of the *MCPggaac* and *CFH(H3)* aHUS-risk haplotypes, and of the aHUS-risk alleles *CFHR3*B* and *CFHR1*B*, could be done in most patients. Two out of nine patients (22%) were carriers of the *MCPggaac* risk haplotype, while the *CFH(H3)* risk haplotype was found in 7 out of 13 patients (54%), six of whom also carried the *CFHR3*B* and *CFHR1*B* alleles. We then compared the frequency of these variants in the 13 SP-HUS patients with the frequencies observed in 22 pregnancy-associated HUS patients (P-aHUS) (31), 352 patients from our aHUS cohort (24), and a total of 227 Spanish control individuals. As it is shown in **Table 2**, the *MCPggaac* risk haplotype is less frequent in the SP-HUS cohort, while the frequencies of the *CFHR1*B* and *CFHR3*B* risk alleles are higher than in controls, and comparable to the frequencies observed in the aHUS and P-aHUS cohorts.

Complement Studies in Plasma Samples From Spanish and Hungarian Patients

We determined the complement profile (i.e., levels of C3, C4, FH, FI, and anti-FH autoantibodies) in plasma samples from all the Spanish SP-HUS patients. Most plasma samples were obtained between 1 month and 3 years after disease onset, and they had normal C3 and C4 levels. Low C3 and C4 levels, revealing complement activation by the classical pathway, were only detected in patient H946, and, to a lesser extent, in patient H837 (**Table 1**). Because these two plasma samples were obtained in the first week after HUS onset, it could not be excluded that complement activation had also happened in the other patients during active infection. Anti-FH autoantibodies were

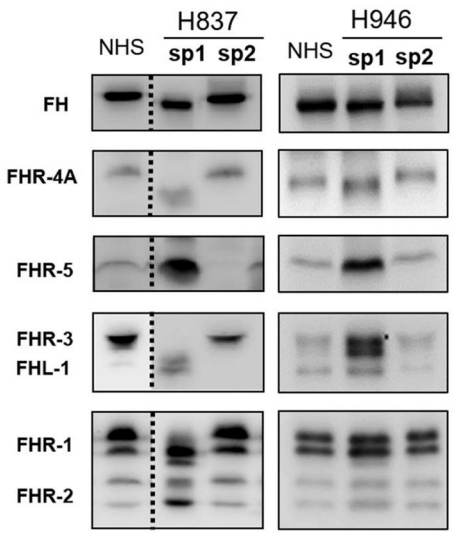

FIGURE 1 | 1D-WB analysis of FH/FHRs in Spanish patients H837 and H946. Two plasma samples from patients H837 and H946 drawn during the acute phase (sp1) or at remission (sp2) were analyzed by WB with polyclonal and monoclonal antibodies against FH and the FHR proteins. Samples from patient H837 were obtained 1 day (sp1) and 3 months (sp2) after SP-diagnosis. Samples from patient H946 were obtained 4 days (sp1) and 2 months (sp2) after SP-diagnosis. A plasma sample from a control individual was included in the same gel; the dotted lines denote that the control sample and the samples from patient H837 were not consecutive.

only detected in patient H171, who does not carry the *CFHR3-CFHR1* deletion.

All plasma samples were also analyzed by Western-blot with different sets of polyclonal and monoclonal antibodies recognizing FH and the FHRs. These analyses revealed that FH and the FHRs proteins in the plasma samples from patients H837 and H946, drawn at disease onset, presented a lower Molecular weight (Mw) than the control sample. We could analyse a second plasma sample from these patients, obtained at disease remission, and we observed that the Mw of FH and FHRs was normal (**Figure 1**). These findings suggested desialylation of FH/FHRs in patients H837 and H946 by the pneumococcal neuraminidase(s). Because sialic acid removal would also decrease the negative charge of the protein and increase its isoelectric point (pI), we performed 2D-Western blot analysis of plasma samples from patient H837 drawn at onset and at remission, following our reported protocol (25); in this kind of analysis, proteins are first separated according to their pI, and then according to its Mw. As it is shown in **Figure 2**, the characteristic 2D-pattern of FHR-3 and FHR-1 isoforms is drastically altered in the onset sample: there are fewer isoforms, with lower Mw and higher pI than in a control sample, or than in the remission sample. We interpreted this finding as a confirmation of transient desialylation of plasma glycoproteins by the pneumococcus. We then quantified neuraminidase activity in the two samples from patient H837 and in a control sample with an "*in vitro*" assay; a high neuraminidase activity (8.03 U/L) was detected in the onset sample, while no enzymatic activity was observed in the remission sample (0.01 U/L) and in the control sample (0.02 U/L). Neuraminidase activity (0.45 U/L) was also detected in the onset sample from patient H946, but not in the remission sample (0.01 U/L).

To determine whether the desialylation of FH/FHRs that we have observed in patients H837 and H946 was a general phenomenon in SP-HUS, we performed WB analyses in plasma samples drawn during the acute phase from 11 Hungarian SP-HUS patients. Patterns suggestive of desialylation (i.e., a lower Mw of FH and FHRs) were observed in four patients (**Figure 3**). WB analyses also revealed that the intensity of the FHR-5 band was higher in the samples drawn at disease onset than at remission; this was particularly evident for patient H837 (**Figure 1**) and patients HUN816 and HUN1869 (**Figure 3**). These differences were further confirmed by determining FHR-5 levels by ELISA (**Table 3**). All the samples showing FH/FHRs desialylation were also analyzed by WB with polyclonal antibodies recognizing human transferrin, a 77 kDa plasma glycoprotein; as it could be expected, the Mw of transferrin in those samples was lower than in the control sample (**Figure 3**), thus suggesting general desialylation of plasma glycoproteins by the pneumococcal neuraminidase.

A summary of demographic data, complement findings and neuraminidase activity in the samples from the six patients (two Spanish and four Hungarian) with transient desialylation is depicted in **Table 3**. Acute phase samples were drawn between 1 and 23 days after diagnosis, and they showed low C3 and C4 levels but no anti-FH antibodies. Remission samples were drawn between 42 days and 11 months after diagnosis, and presented normal C3 and C4 levels. Neuraminidase activity ranged from 0.17 U/L (remission sample from patient HUN1869) to 8.03 U/L (acute sample from patient H837). Nonetheless, a clear correlation between neuraminidase activity, days after diagnosis and desialylation was not observed in all the patients. Complement genetic variants were observed in three Hungarian patients. Patient HUN156 has a rare variant in *CFI* (c.148C>G, p.Pro50Ala; (11)); patient HUN2638 has a rare variant in *C3* (c.2852G>A, p.Arg951His), and patient HUN1869 presents homozygous FHR-3 and FHR-1 deficiency.

Functional Relevance of FH Desialylation

We wanted to know whether desialylation altered the functional activity of FH, but we could not purify it from any of the patients' samples drawn at disease onset because of limited sample volume. Therefore, to approach the potential relevance of FH desialylation, we generated dFH "*in vitro*" from commercially available FH, purified from human plasma. **Figure 4A** shows that dFH has the same Mw than FH in the onset sample from patient H837. We also checked sialic acid removal from purified FH by analyzing the binding of lectins RCA-I and SNA by Western-blot (**Figure 4B**). Lectin SNA binds predominantly to α(2–6)-linked sialic acids (present in FH but absent in dFH), while lectin RCA-I binds predominantly to β-galactose residues, which become fully accessible after desialylation. Thus, the preferential binding of lectin SNA to FH, and of lectin RCA-I to dFH confirmed "*in vitro*" FH desialylation.

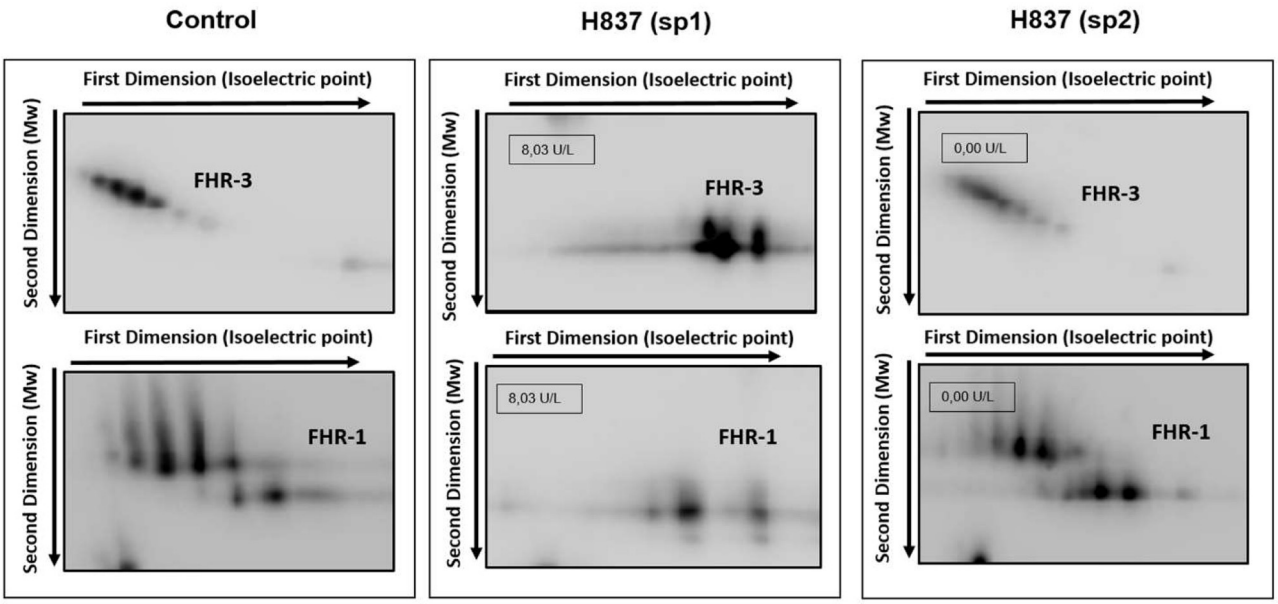

FIGURE 2 | Desialylation of FHR-1 and FHR-3 in patient H837 by 2D-WB analysis. FHR1 and FHR3 partially purified from sp1 and sp2 from patient H837 and from a control individual were subjected to 2D-electrophoresis and Western-blot with polyclonal antibodies. The characteristic ladder of FHR-3 spots (each having different Mw and isoelectric point) appeared as a few basic spots of lower Mw in the patient sample obtained 1 day after SP-HUS diagnosis (H837 sp1), and was recovered in the sample drawn 3 months later (H837 sp2). A similar situation applies to FHR-1. The small boxes within the gel images indicate the neuraminidase activity detected in samples sp1 and sp2 from patient H837.

We then compared the functional activity of FH and dFH in different experimental settings. We used an ELISA assay to analyse the binding of FH/dFH to surface-bound C3b, and observed that dFH bound more efficiently than native FH (**Figure 5**), suggesting that sialic acid removal favors the interaction of FH with surface-bound C3b. The higher binding of dFH, nonetheless, did not increase the cofactor activity of FH and dFH, either in the fluid phase or on surfaces. As illustrated in **Figure 6**, the cleavage of soluble or surface-bound C3b by Factor I to generate iC3b was not affected by using FH or dFH as cofactors. In the same way, no differences were observed when comparing the ability to dissociate solid-phase, preformed C3bBb(P) convertase, as similar decay-accelerating activities were observed with FH and with dFH (**Figure 7**).

To analyse the whole effect of FH desialylation on complement regulation on cellular surfaces, we used two different formats of haemolytic assays on sheep erythrocytes. The first format is our original assay of sheep erythrocyte lysis by the serum of an aHUS patient who carries the FH mutation W1183L (29). The second format is a modification of this assay, in which the addition of anti-FH monoclonal antibodies OX24 (targetting FH SCR4) or C18 (targeting FH SCR20) to a NHS renders it capable to lyse sheep erythrocytes (30). In both formats of haemolytic assays, we compared the capacity of exogenous FH/dFH to prevent haemolysis. Interestingly, we observed that FH desialylation clearly decreased its ability to prevent sheep erythrocytes lysis (**Figure 8**), suggesting the contribution of FH's own sialic acids on its regulatory activity on cellular surfaces.

DISCUSSION

An infrequent complication of *S. Pneumoniae* infections is the Haemolytic Uraemic Syndrome (HUS), a clinical entity characterized by the triad of thrombocytopenia, microangiopatic haemolytic anemia and acute renal failure (33). The contribution of complement pathogenic variants and risk polymorphisms in the atypical forms of HUS is very well-established, and screening of the complement genes *CFH, MCP, CFI, CFB, C3,* and *CFHRs* in these patients is mandatory (10). Complement studies in HUS associated to *S. Pneumoniae* (SP-HUS), however, are very limited because it has generally been considered that this is a secondary manifestation of the infection process (34).

We have performed complement genetic screening in 9 Spanish SP-HUS patients, and observed that five of them carry a total of six rare genetic variants. Three variants were null alleles in *CFHR3* (patient H150) or *CFHR5* (patient H619 and patient H731). FHR-3 competes FH binding to *Neisseria meningitidis* (35), thus decreasing bacterial survival. Although it is not known whether FHR-3 can also compete FH binding to *S. pneumoniae*, the lack of FHR-3 could be advantageous for the pneumococcus. This is difficult to determine, because isolated deficiencies of FHR-3 are very rare. Nonetheless, the combined deficiency of FHR-3 and FHR-1 as a consequence of the homozygous *DelCFHR3-CFHR1* deletion is relatively frequent, and there is no evidence that it predisposes to infections. The FHR-5 haploinsuficiency observed in patient H169 could decrease complement activation and increase infection susceptibility,

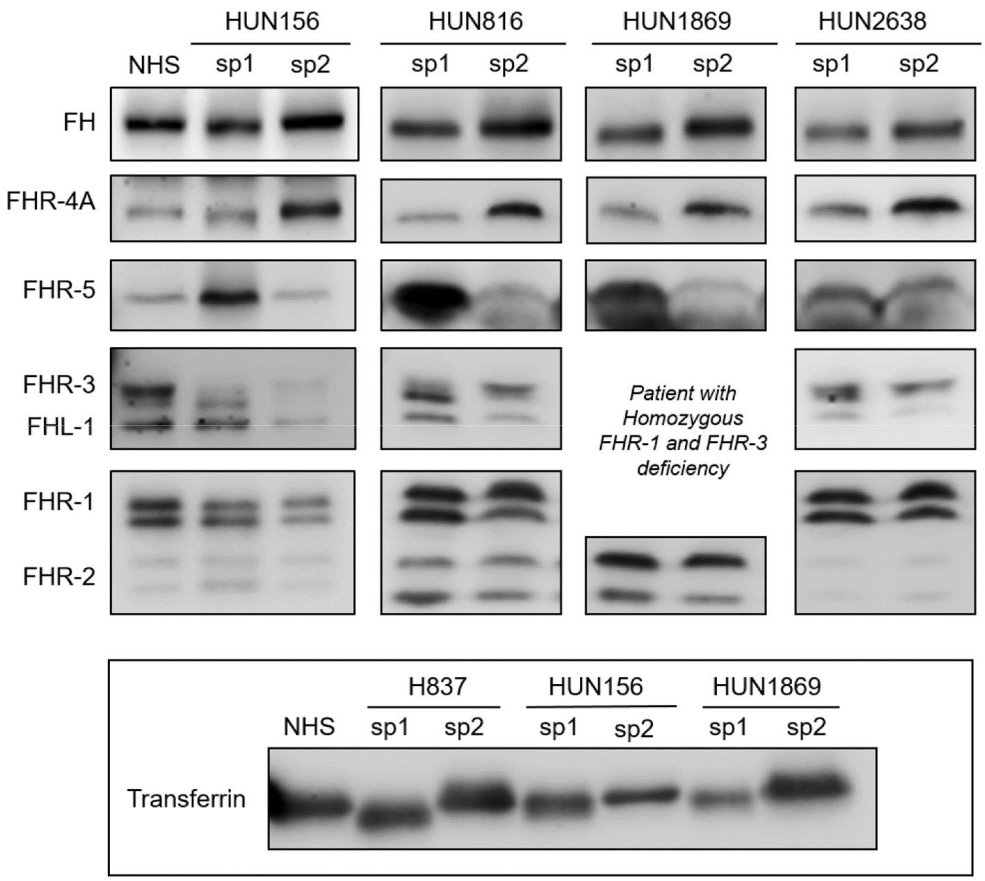

FIGURE 3 | Desialylated FH/FHRs in samples from Hungarian SP-HUS patients. WB analyses of FH/FHRs in two plasma samples (sp1 and sp2) from four Hungarian patients. Time between disease onset and extraction date was as follows: HUN156 (10 days and 11 months); HUN816 (9 days and 2 months); HUN1869 (5 days and 2 months), this patient is homozygous for FHR-3 and FHR-1 deficiency; HUN2638 (23 days and 43 days). WB analysis of human Transferrin (Mw 77 kDa) in sp1 and sp2 samples from patients H837, HUN156, and HUN 1869 is shown at the bottom inset.

although the clinical phenotype probably relies on additional, currently unknown risk factors (12).

The pathogenic relevance of the three other variants found in our SP-HUS patients is unknown. The *CFHR5* variant in patient H731 (c.368A>G; p.Asn123Ser), which abolishes one of the potential N-glycosilation sites in FHR-5, was predicted to be likely benign. The *C1QB* variant in patient H859 (c.223G>A; p. Gly75Arg) was reported in one individual with very early onset inflammatory bowel disease, and predictive tools suggested that it may alter the protein function (36). Thus, it is possible that this C1q variant has decreased capacity to activate the classical pathway and eliminate the pathogen, and/or that it binds the pneumococcal protein PepO with higher affinity, increasing bacterial adherence to the host's cells (37). The *CFI* intronic variant in patient H640 (c.1534+5G>T; rs114013791) has been described in an Italian aHUS patient (38), and in several patients from the Newcastle aHUS cohort (39), all of them having normal FI levels; the contribution of this variant, present in 1.55% of European controls, to the genetic predisposition to aHUS is thus uncertain. In conclusion, a significant proportion of the Spanish SP-HUS patients (five out of nine) carry rare genetic variants in complement genes, but their relevance to HUS predisposition is unknown.

The small sample size of our SP-HUS cohort (13 patients) does not allow to achieve statistically significant conclusions when comparing the frequency of the common genetic variants *MCPggaac*, *CFH(H3)*, *CFHR3*B*, and *CFHR1*B* with control individuals, or with the aHUS cohort (**Table 2**). Nonetheless, our analyses reveal that the *MCPggaac* haplotype is underrepresented in the SP-HUS patients, where it has a lower frequency than in our cohort of 352 aHUS patients (0.111 vs. 0.414), or that in 22 P-aHUS cases (0.111 vs. 0.432). The relevance of this observation would require analyses in more SP-HUS patients, but it suggests that the membrane regulator MCP is not an important player in SP-HUS pathogenesis. It is also interesting that the frequencies of the aHUS-risk variants *CFHR3*B* and *CFHR1*B* in our SP-HUS cohort (0.346 and 0.615, respectively) are higher than in control individuals (0.242 and 0.368), and comparable to the frequencies observed in the aHUS (0.355 and 0.467) and P-aHUS (0.462 and 0.455) cohorts. These findings suggest that

TABLE 3 | Transient desialylation of FH/FHRs in two Spanish and four Hungarian SP-HUS patients.

Patient code	Gender	Age at onset (mo)	Previous vaccination	Time after onset	Clinical status	Complement profile	FHR-5 Levels[a] (μg/mL)	FH/FHRs desialylation	Neuraminidase Activity (U/L)[b]	Genetic findings[c]
H837	Male	21	No info available	1 days	Onset	Low C4, FH, FI	3.61	YES	8.03	No pathogenic variants
				3 mo	Remission	Normal	0.03	NO	0	
H946	Male	21	Prevenar	4 days	Onset	Low C3, C4	1.97	YES	0.45	Non-available
				2 mo	Remission	Normal	0.80	NO	0	
HUN156[d]	Female	18	Pneumovax	10 days	Onset	Low C3,C4,FB,FI	1.51	YES	3.94	*CFI* (c.148C>G, p.Pro50Ala); *MCPggaac*
				11 mo	Remission	Low FI	0.48	NO	0	
HUN816	Male	36	No info available	9 days	Onset	Low C3, C4, FH	2.81	YES	0.54	Non available
				2 mo	Remission	High C3, FI, FB	0.86	NO	0.47	
HUN1869	Female	30	No info available	5 days	Onset	Low C3, C4, FI	1.19	YES	2.50	*DelCFHR3-CFHR1* (HOM)
				2 mo	Remission	Normal	0.42	NO	0.17	
HUN2638	Male	32	Prevenar	23 days	Onset	Low C3, C4, FH	1.08	YES	0.42	*C3 (c.2852G>A, p.Arg951His)*; *C3 (c.304C>G;R102G)*; C3 P314L; *CFH (c.184G>A; V62I)*; *CFH(H3); MCPggaac*
				42 days	Remission	Normal	0.86	NO	0.44	

Complement profile and neuraminidase activity in the two plasma samples drawn during disease onset and at remission. Anti-FH autoantibodies were negative in all samples. [a]Mean levels in controls: 1.98 ± 1.02 μg/mL. [b]Background level in controls: 0.50 U/L. [c]All genetic variants in patients HUN156 and HUN2638 are in heterozygosis. [d]Described in (11).

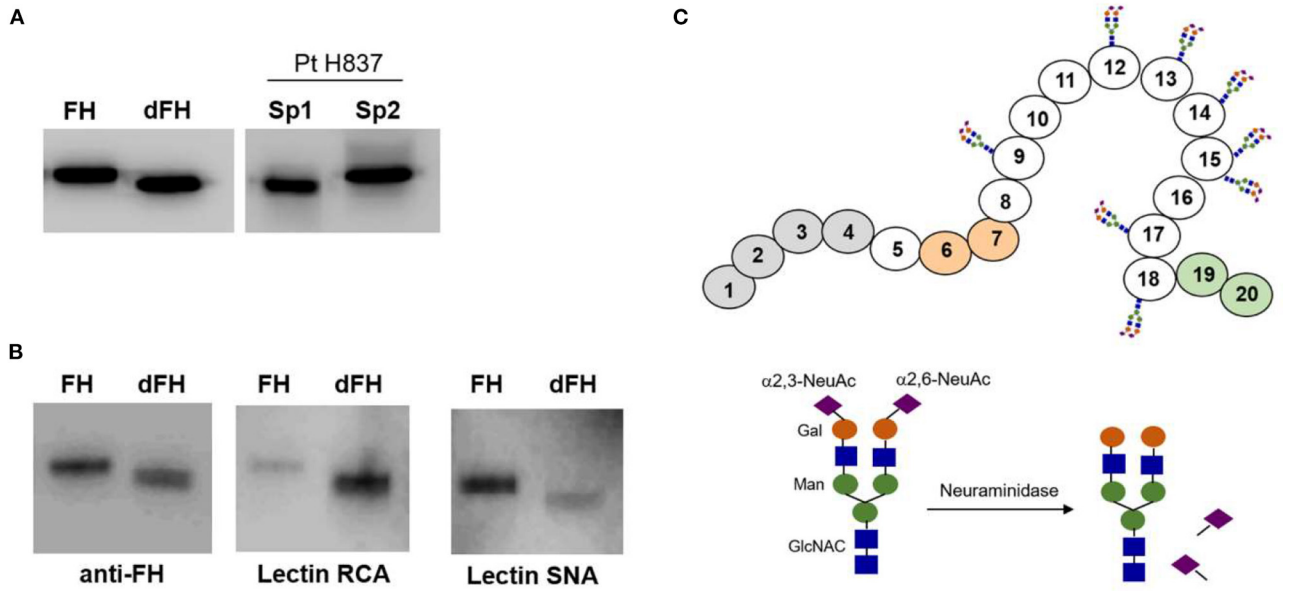

FIGURE 4 | Lectin recognition of FH and desialylated FH. **(A)** Western-blot analysis with polyclonal anti-FH antibodies. "*In vitro*" desialylated FH (dFH) has the same molecular weight than FH in a plasma sample from patient H837 obtained 1 day after disease onset (sp1), and lower than FH in the sample obtained at remission (sp2). **(B)** Differential recognition of FH and dFH by lectin RNA (which recognizes non-reducing terminal beta-D-galactose), and lectin SNA (which binds primarily to Neu5Ac(α2–6) Gal/GalNAc disaccharide sequences). **(C)** Schematics of FH showing the SCR domains with N-linked carbohydrates; the sugar composition of the carbohydrate molecule is based on Schmidt et al. (32).

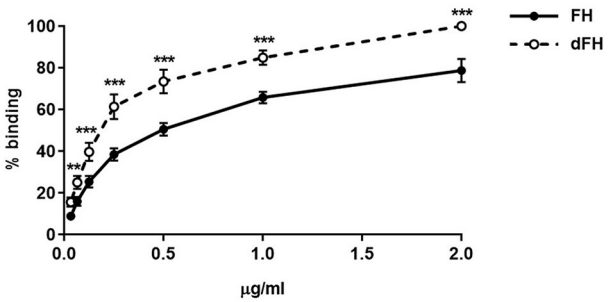

FIGURE 5 | Binding of FH and dFH to surface-bound C3b. Binding of increasing concentrations of FH and dFH to microtitre plates coated with 400 ng of C3b. Upon addition of a polyclonal antibody which recognizes FH and dFH, absorbance was read at 420 nm, and the maximum binding observed was set as 100%. The two curves represent the mean ± SD from four independent ELISA experiments. $^{**}P < 0.01$; $^{***}P < 0.001$.

*CFHR3*B* and *CFHR1*B* are predisposing factors to SP-HUS. These two variants frequently segregate in an extended *CFH(H3)-CFHR3*B-CFHR1*B* haplotype that associates with reduced FH levels and increased FHR-3 levels (24, 40), but whether a local imbalance of the FH/FHR-3 ratio predisposes to SP-HUS will require further investigation.

The contribution of FH and FHR proteins to the pathogenic mechanism of SP-HUS could also result from the transient removal of their sialic acids by the pneumococcal neuraminidase. It has been observed that the sequential action of pneumococcal neuraminidase, galactosidase, and NAcglucosidase reduce complement deposition on the pathogen surface and its subsequent phagocytosis by human neutrophils, but the complement glycoprotein(s) affected are unknown (41). FH is the complement protein with more N-Glycosylation sites (nine sites), followed by C2 (eight sites), and FI (six sites). FH deglycosylation decreases its Mw by 17.9 kDa, and eight of its nine N-glycosylation sites are occupied by complex, diantennary sialylated, non-fucosylated glycans, although a few triantennary structures are also present (42). Most FH sialic acids are alfa2-6-linked to the carbohydrate chains (32), but the functional consequences of FH desialylation are not fully understood. We here show desialylation of FH and FHRs in plasma samples from 6 SP-HUS patients (two from Spain and four from Hungary), that we attribute to the activity of the pneumococcal neuraminidase on human glycoproteins (**Figure 4**). Because FH and FHRs desialylation was most evident in a plasma sample drawn only 1 day after disease onset, we believe that this is a general finding that disappears upon infection resolution. We also think that the desialylation process is independent of the presence of rare complement genetic variants; in fact, it was more evident in patient H837, who does not carry any pathogenic variant.

To determine whether sialic acid removal had any consequences on FH function, we compared the regulatory activity of native and "*in vitro*" desialylated FH by using assays in the fluid phase and on surfaces, which we had already used to check FH mutants purified from aHUS patients (27). Sialic acid removal increased FH binding to C3b-coated microtiter plates (**Figure 5**). This result agrees with the enhanced binding of a partially deglycosylated and desialylated recombinant FH molecule in biosensor experiments (43).

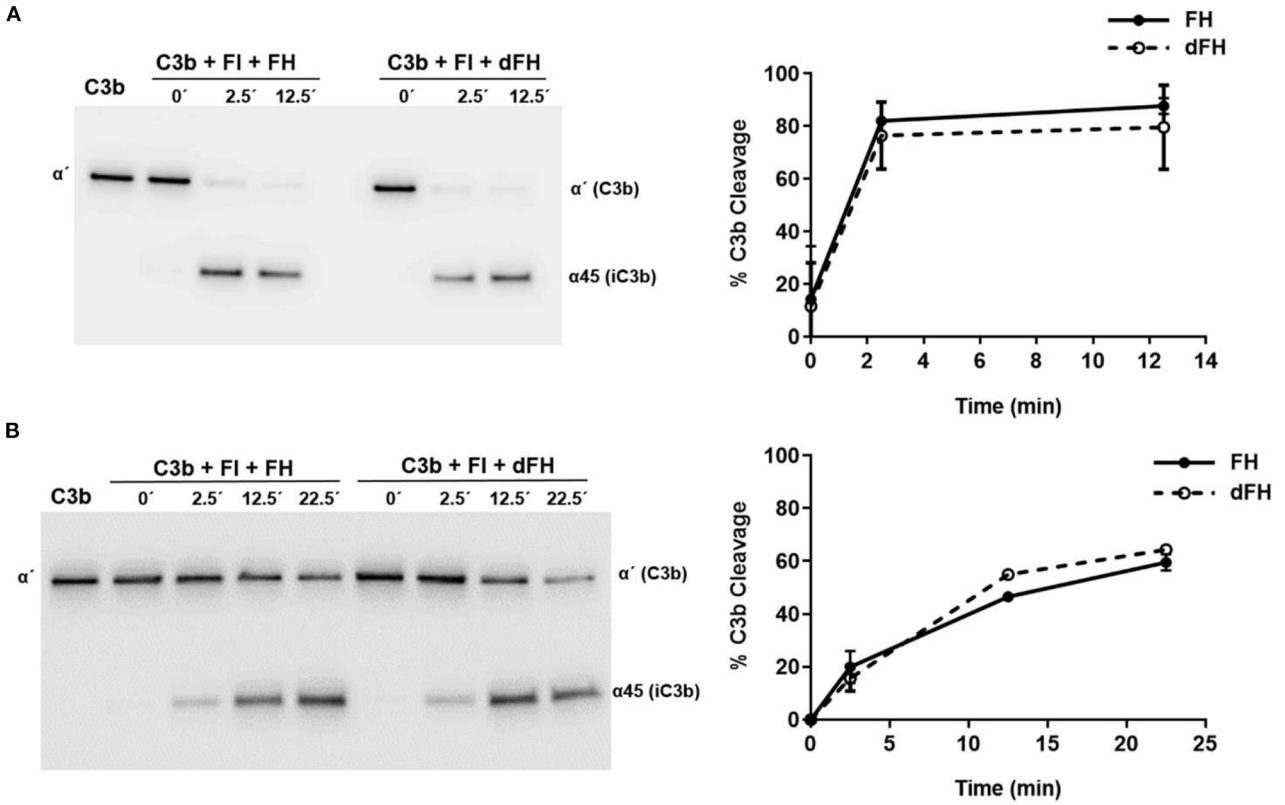

FIGURE 6 | Cofactor activity of FH and dFH in the proteolysis of C3b by Factor I. Proteolysis of C3b by FI in the fluid phase **(A)** or on microtiter plates **(B)**, by using FH or dFH as cofactors. The proteolytic activity at 2.5, 12.5, and 25 min was analyzed by Western-blot with an antibody which recognizes the α′ chain of C3b (substrate), and the α45 fragment of iC3b (product). Proteolytic cleavage was expressed as the relative intensity of the remaining C3bα′ chain at each time point. All curves represent the mean ± SD from three independent experiments.

FH desialylation, nonetheless, did not affect its capacity to act as a cofactor of FI in the proteolysis of C3b in the fluid phase or on surfaces, as no differences between native and desialylated FH were appreciated (**Figure 6**). The same observation was reported by Schmidt et al. (43), who analyzed cofactor activity in the fluid phase, and did not find differences between plasma FH and the partially deglycosylated and desialylated recombinant FH. In line with these results, the partial deglycosylation of FI to remove sialic acids and Galactose residues did not affect the proteolysis of C3(NH_3) (a structural C3b analogous) in the fluid phase (44). We conclude that in the proteolytic cleavage of C3b to iC3b, the sialic acid molecules of the enzyme (FI) or the cofactor (FH) do not play any relevant role.

We also observed that desialylated FH kept intact its capacity to dissociate preformed C3bBb(P) convertase (**Figure 6**). This result differs from the increased decay observed with the recombinant FH in biosensor experiments (43), and from the higher capacity of deglycosylated FH to dissociate properdin-stabilized C3bBb convertase preformed on the erythrocyte surface (45). These discrepancies could be due to the deglycosylation treatments of the FH molecules used in

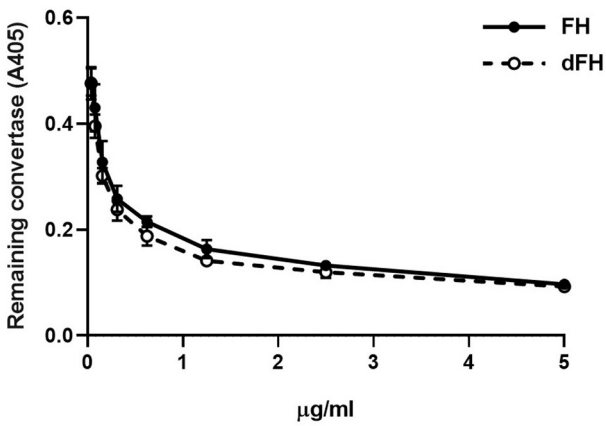

FIGURE 7 | Decay-accelerating activity of FH and dFH on surface-bound C3bBb(P) convertase. The alternative pathway C3bBbP convertase was built up on microtiter plates. After addition of increasing concentrations of FH or dFH, the remaining C3bBbP molecules were detected with a monoclonal anti-factor Bb antibody. Data are the mean ± SD from three independent ELISA experiments.

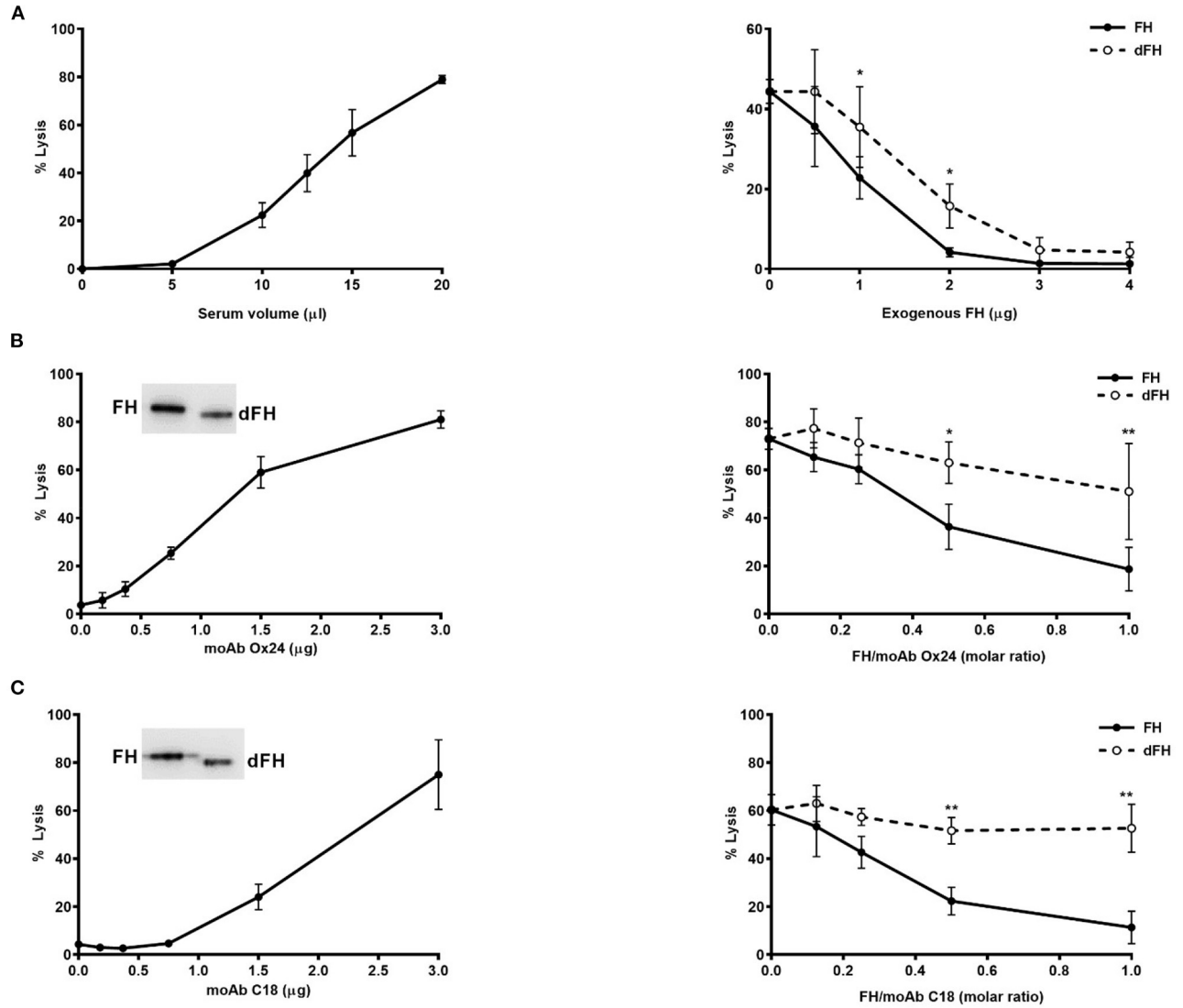

FIGURE 8 | Haemolytic assays on sheep erythrocytes. **(A)** Lysis of sheep erythrocytes by the serum from an aHUS patient carrying the mutation W1183L in FH (left panel). The lysis generated by 12.5 µL of patient's serum (about 50%) could be prevented by addition of exogenous FH or dFH, but dFH was less effective (right panel). **(B,C)** Lysis of sheep erythrocytes was induced by adding increasing amounts of monoclonal antibodies OX24 **(B)** or C18 **(C)** to 20 µL of NHS (left panels). MoAb OX24 recognizes FH SCR4 domain, and MoAb C18 recognizes FH SCR20 domain; both antibodies recognize similarly FH and dFH by Western-blot (insets). The lysis induced by 1.6 µg of OX24 (about 70%) or C18 (about 60%) could be inhibited by adding increasing concentrations of FH or dFH, although dFH was less effective (right panels). All curves represent the mean ± SD from three independent experiments. *$P < 0.05$; **$P < 0.01$.

these previous reports, while we have only removed FH sialic acids, leaving the other sugar residues in the native carbohydrate molecules unchanged.

Because our functional assays on microtiter plates did not take into account the relevance of surface polyanions for the FH regulatory activity, we performed two different kind of haemolytic assays with sheep erythrocytes, which have polyanionic molecules on their surface. In the first assay, sheep erythrocytes are "spontaneously" lysed by the serum from an aHUS patient whose mutated FH cannot bind to the sheep erythrocyte surface and protect them from complement attack (29). In the second assay, the addition of specific anti-FH monoclonal antibodies to a normal human serum abolishes FH binding to the sheep erythrocyte surface, rendering them susceptible to complement-mediated lysis (30). When we compared the capacity of exogenous FH and desialylated FH to prevent sheep erythrocytes lysis, we observed a lower activity of desialylated FH in the two kind of assays (**Figure 8**), suggesting that FH desialylation decreases its capacity to regulate complement activation on the erythrocyte surface. As the other functional assays do not suggest any role for FH sialic acids on C3 convertase dissociation or in the proteolytic cleavage of C3b, we think that FH desialylation could somehow alter its interaction with polyanionic molecules on the cellular

surface, and that this could result in decreased binding of desialylated FH to the cellular surface and decreased complement regulation. Further studies are required to determine the exact mechanism, and whether this transient dysregulation has a relevant role on SP-HUS pathogenesis, as already suggested (9, 46).

In summary, we here show that rare complement genetic variants in SP-HUS patients are more frequent than it could be expected, and that aHUS-risk polymorphisms in the *CFH-CFHR3-CFHR1* region likely contribute to SP-HUS. Based on these findings, we recommend complement genetic screening in patients who develop HUS in the context of *S. pneumoniae* infections, as well as to analyse aHUS-risk variants in these patients. We also show desialylation of human FH and FHR proteins by the pneumococcal neuraminidase at SP-HUS onset, and provide functional evidence suggesting that desialylated FH has a lower capacity to regulate complement activation on cellular surfaces.

DATA AVAILABILITY STATEMENT

The genetic datasets presented in this article are not readily available due to ethical restrictions of research participants. Requests to access experimental datasets should be directed to PS-C, pilar.sanchez-corral@idipaz.es.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committees from La Paz University Hospital. Madrid, Spain, and from Semmelweis University. Budapest, Hungary. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

IG and FC performed Western-blots and functional studies, analyzed data, and prepared figures. PN was responsible for the analysis of the complement profile in plasma samples from Spanish patients. EA collected biological samples and was responsible for complement genetic screening from Spanish aHUS patients. AM, MM, and JB gathered clinical data. NV and ZP collected plasma samples and clinical and complement data from Hungarian patients. DC and ÁS performed genetic screening of Hungarian patients. PS-C designed the study, analyzed data, prepared figures and wrote the first draft of the manuscript. All the authors revised the data and contributed to the final version of the manuscript.

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REVIEW
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A Family Affair: Addressing the Challenges of Factor H and the Related Proteins

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Inflammation is a common denominator of diseases. The complement system, an intrinsic part of the innate immune system, is a key driver of inflammation in numerous disorders. Recently, a family of proteins has been suggested to be of vital importance in conditions characterized by complement dysregulation: the human Factor H (FH) family. This group of proteins consists of FH, Factor H-like protein 1 and five Factor H-related proteins. The FH family has been linked to infectious, vascular, eye, kidney and autoimmune diseases. In contrast to FH, the functions of the other highly homologous proteins are largely unknown and, hence, their role in the different disease-specific pathogenic mechanisms remains elusive. In this perspective review, we address the major challenges ahead in this emerging area, including 1) the controversies about the functional roles of the FH protein family, 2) the discrepancies in quantification of the FH protein family, 3) the unmet needs for validated tools and 4) limitations of animal models. Next, we also discuss the opportunities that exist for the immunology community. A strong multidisciplinary approach is required to solve these obstacles and is only possible through interdisciplinary collaboration between biologists, chemists, geneticists and physicians. We position this review in light of our own perspective, as principal investigators of the SciFiMed Consortium, a consortium aiming to create a comprehensive analytical system for the quantitative and functional assessment of the entire FH protein family.

Keywords: complement system, factor H (FH), factor H-related protein, factor H-like protein 1, challenges - development directions

"When you can measure what you are speaking about, and express it in numbers, you know something about it"
– William Thomson, 1st Baron Kelvin

INTRODUCTION: THE FACTOR H PROTEIN FAMILY

The complement system forms a major arm of innate immunity and is of importance to fight invading pathogens (1). It consists of over 50 proteins that activate each other in a fixed order *via* three distinct pathways; the classical (CP), lectin (LP) and alternative pathway (AP), which all lead to cleavage of C3 and C5. This results in labeling of pathogens with C3b, attraction of immune cells *via* the anaphylatoxins C3a and C5a, and formation of the membrane attack complex [reviewed in (2)]. While the complement system is traditionally seen as a plasma system, recent studies also describe its importance locally, perhaps even inside cells (3). In health, the complement system is tightly regulated to prevent unwanted activation, inflammation and tissue damage. It has long been known that complement dysregulation contributes to various inflammatory and autoimmune diseases (4–6). A number of membrane-bound and fluid phase regulators ensure that the complement system is well-controlled (reviewed in (7)). Here, we will focus on the main regulator of the alternative pathway, namely Factor H (FH). FH can distinguish between self and non-self, and prevents complement activation both on cellular surfaces and in the circulation (8). More specifically, FH can function as a co-factor for Factor I (FI)-mediated proteolysis of C3b into iC3b, a molecule that cannot further propagate pathway activation. FH can also compete with Factor B (FB) to inhibit formation of the $C3(H_2O)B$ fluid phase tickover complex. In addition, FH promotes the decay of existing C3bBb-complexes (i.e., the C3-convertase), as well as the C4bC2aC3b and C3bBbC3b-complexes (i.e., the C5 convertases). FH is composed of 20 repetitive units, called complement control protein (CCP) domains, in a "beads on a string" configuration. The CCPs are ~65 amino acids in length and contain two invariant disulfide bonds. The FH N-terminal (CCPs 1–4) is important for decay accelerating activity and co-factor activity, while the internal region (CCPs 6–8) and the C-terminal (CCPs 19–20) are needed for host/ligand recognition and thus also for complement regulation on host surfaces (9–11). The human gene for FH is located on chromosome 1 within the Regulators of Complement Activation (RCA) gene cluster. The RCA gene cluster contains more than sixty genes and includes a ~700 Kb region in which FH as well as the Factor H-Related (FHR) proteins are encoded (described below). The complement FHR genes (*CFHR*) contain several repeating regions believed to have resulted from large genomic duplication events leading to the production of FHR proteins with partly similar domains to FH (12).

FH like-1 (FHL-1) is an alternatively-spliced version of FH and shares the first 7 CCP domains of FH before terminating with a unique four amino acid C-terminal tail. FHL-1 contains the C3b binding and regulatory domains of FH and thereby retains the regulatory function of FH. Also, since FHL-1 contains the CCP domains 6–7 of FH, it is assumed that FHL-1 shares some of the FH ligands and the ability to regulate complement on certain surfaces (13–15). Indeed, FHL-1 has been shown to bind similar ligands as FH such as C-reactive protein (CRP), pentraxin 3, heparin and malondialdehyde epitopes (16–18). Nevertheless, clear differences exist between these two proteins such as the extra binding domains in FH (CCP 8–20), the distinctive three-dimensional conformation of both proteins, and the unique C-terminus of FHL-1. This suggests that FH and FHL-1 also bind to distinct ligands expressed in certain tissues. Moreover, it has been implied that FHL-1 has a local and tissue specific role instead of a systemic function like FH (13).

Humans also have five FHR proteins; FHR-1, FHR-2, FHR-3, FHR-4 and FHR-5, whose functions are poorly characterized (described in more detail in (8)). Yet, their importance is shown by the causal link between genetic alterations in *CFHR* and various diseases (i.e., IgA nephropathy (IgAN) (19–23), age-related macular degeneration (AMD) (24–28), invasive meningococcal disease (29–31), atypical hemolytic uremic syndrome (aHUS) (32) and C3 glomerulopathy (C3G) (33). All FHR proteins share a high degree of similarity with FH in their N-terminus (varying between 36 and 94%) and their C-terminus (varying between 36 and 100%) (34). Notably, the N-terminus of the FHR proteins resembles CCPs 6–8 of FH, while the C-terminus is similar to CCPs 19–20 of FH. FHR-5 is an exception to this, since FHR-5 shares homology to CCPs 6–7 as well as CCPs 10–14 and CCPs 19–20 of FH. The homology of the FHR proteins to the surface recognition domains of FH enables these proteins to bind similar ligands on surfaces including heparin and C3 activation fragments such as C3b or C3d (32). However, since all FHR proteins lack the domains of FH responsible for the regulatory activity, the FHR proteins will, unlike FH, most likely not provide protection to these surfaces against complement attack. The current belief is, therefore, that the FHR proteins antagonize the ability of FH to regulate complement activation (35). Furthermore, some FHR proteins can form dimers. FHR-1, FHR-2, and FHR-5 contain a dimerization motif in their N-terminal domains, while in FHR-3 and FHR-4 this motif is missing. This would enable FHR-1, FHR-2, and FHR-5 to form both homodimers and heterodimers. Accordingly, structural and sequence analyses suggested that, in addition to homodimers, FHR-1 can form heterodimers with

Abbreviations: aHUS, Atypical hemolytic uremic syndrome; AMD, Age-related macular degeneration; ANCA, Anti-neutrophil cytoplasmic antibody; AP, Alternative pathway; AU, Arbitrary units; BLAST, Basic local alignment search tool; C3G, C3 glomerulopathy; CCP, Complement control protein; *CFH*, Complement Factor H gene; *CFHR*, Complement Factor H-related genes; CP, Classical pathway; CRP, C-reactive protein; FB, Factor B; FH, Factor H; FHL-1, Factor H-like 1; FHR, Factor H-related protein; FI, Factor I; FISH, Fluorescence *in situ* hybridization; IgAN, Immunoglobulin A nephropathy; LP, Lectin pathway; mAb, Monoclonal antibody; mCFH, Murine complement Factor H gene; mCFHR, Murine complement Factor H-related gene; mFH, Murine Factor H; mFHR, Murine Factor H-related protein; NLRP3, NOD-, LRR- and pyrin domain-containing protein 3; PTX3, Pentraxin-3; RCA, Regulators of complement activation gene cluster; SLE, Systemic lupus erythematosus; TMA, Thrombotic microangiopathy.

FHR-2 and FHR-5, while FHR-2/FHR-5 heterodimers would only be formed in sera partially or totally deficient in FHR-1 (36). However, recently, another study suggested that only four dimers occur in circulation: homodimers of FHR-1, FHR-2, and FHR-5, as well as FHR-1/FHR-2 heterodimers (37). Further studies are therefore needed to confirm the exact nature of the dimer composition, as well as the precise function of these dimers.

BRIEF DESCRIPTION OF THE HISTORY

In hindsight, the earliest publication about the FH family was in 1965, when Nilsson and Muller-Eberhard initially isolated FH from human serum and identified this novel protein as β1H globulin (38). Yet, it wasn't until 1976 that two groups independently of each other discovered the C3b inhibitory activity of FH as well as its regulatory activity on the C3-convertase (39–41). In 1983, regulation of C5 convertases by FH was first described (42). Finally, in 1988, the genetic code of FH and its amino acid sequence were identified (43). This discovery was essential to uncover the structure of FH as discussed above. At around the same time of this breakthrough, Schwaeble *et al.* demonstrated the expression of an additional smaller truncated form of FH in the human liver, which we now know as FHL-1 (44). In 1989, the same group demonstrated that FHL-1 had FI-cofactor activity (45). In the end, in 1991, FH and FHL-1 were shown to be derived from the same gene by a process of alternative splicing (46, 47).

One of the earliest mentions on any of the FHR proteins was in a paper describing the isolation of murine FHR proteins by Vik et al. in 1990 (48). Due to the extensive number of large genomic duplications between the exons of *CFH* and the *CFHR* genes, determining the genomic positions of the human *CFHR* genes was challenging and was performed throughout the early to mid-1990's. In 1991 and 1992, mRNA transcripts encoding for FHR-1, FHR-2 and FHR-3 were revealed (47, 49–51). Expression of FHRs on protein level were characterized and described soon after (44, 50, 52). The position of *CFHR2* was the first of the FHR proteins to be determined when it was identified within the region between *CFH* and *Factor XIII* (53). In the next years, the other three *CFHR* genes were mapped within the RCA cluster between *CFH* and *CFHR2* (54). However, due to the high sequence resemblances between these genes, the determination of their exact positioning was not possible. The last *CFHR* gene discovered was for FHR-5, which was first described at protein level in 2001 in studies of immune-complex-mediated kidney diseases (55, 56). Finally, the genetic location of *CFHR5* was determined using fluorescence *in situ* hybridization (FISH), radiation hybrid mapping and BLAST alignment (57). Ultimately, it wasn't until 2002 that the genomic segment containing the *CFH* and *CFHR* gene family was confirmed and to have the gene positions from centromere to telomere: *CFH*, *CFHR3*, *CFHR1*, *CFHR4*, *CFHR2*, *CFHR5* (a schematic overview of the genomic organization of the *CFH* gene family is provided in (35)). Furthermore, nowadays other forms of the FH protein family have also been described, namely the alternatively spliced forms of *CFHR4* named FHR-4A and FHR-4B leading to a total of 8 proteins that are encoded by the human *CFH* and *CFHR* gene family (excluding the different glycosylation variants as well as the homo- and heterodimers) (an outline of the protein structure of the FH family is provided in (8, 35, 58).

FACTOR H AND THE RELATED PROTEINS IN DISEASE

In recent years, numerous conditions have been associated with mutations or polymorphisms in the *CFH* gene family [an overview is provided in (32, 35, 59)]. These findings support the notion that complement dysregulation due to alterations in the FH family are a unifying pathogenic feature of various pathologies. Deciphering the pathogenic mechanism by which this protein family leads to disease is crucial for establishing the right diagnosis and therapeutic interventions. Despite the association of genetic variants in the *CFH* gene family with diseases, little is known regarding the biological processes leading to inflammation and tissue injury. Understanding the molecular mechanisms behind these genetic associations is challenging and represents an area of intense research. Notably, the disease-associated genetic variants in the *CFHR1-5* are particularly difficult to interpret due to the lack of knowledge regarding the biological role of the FHRs. We and others have shown the existence of genotype-phenotype correlations between gene variants in the *CFH-CFHR1-5* and complement-mediated diseases demonstrating that, although the same genes associate with various diseases, the molecular mechanisms behind these associations are specific of each condition (15, 59–61). In this context, the studies performed with FH, the best-known member of the family, were the first ones to illustrate such genotype–phenotype correlations. Mutations causing plasma FH deficiencies were amongst the first *CFH* alterations described. When these *CFH* mutations are present in homozygosis or compound heterozygosis they lead to complete FH deficiency, which cause massive complement activation in fluid phase, and are commonly associated with C3G, a heterogeneous histopathological entity characterized by glomerular C3 accumulation (62). However, when the null *CFH* alleles are in heterozygosis they only lead to partial FH deficiencies, and are equally associated with C3G as well as other diseases such as aHUS, AMD and IgAN. In this scenario, the combination with other genetic, acquired and/or environmental risk factors that are specific for each disease determines the final phenotype outcome. Interestingly, missense mutations within the C-terminus of FH are prototypical of aHUS and cause an inappropriate regulation of complement on endothelial surfaces leading to tissue damage, but without altering complement regulation in the fluid phase (63–66).

In addition to *CFH*, strong associations between genetic modifications in *CFHR1-5* and pathologic outcome have also emerged (32). Amongst the disease-associated *CFHR1-5* variants, genomic rearrangements leading to deletions, duplications, or hybrid genes are the most remarkable and

informative ones (59). Perhaps, the most extensively studied is the deletion of *CFHR3* and *CFHR1*, which has a variable allele frequency between 0–55% in various ethnic groups (67). Moreover, this common gene variant is associated with protection against the development of AMD and IgAN, while it increases the susceptibility for aHUS (due to anti-FH autoantibodies) and systemic lupus erythematosus (SLE) (21, 24, 68, 69). These different disease associations highlight the relevance of the context in defining the effect of the FHR-1 and FHR-3 deficiency, illustrating those situations where the promotion of complement activation by the FHR proteins may be detrimental (i.e., Bruch´s membrane and mesangium) or where it may be beneficial (i.e., apoptotic cells). Another captivating type of a genomic rearrangement of the *CFHRs* is the duplication of the dimerization domains in FHR-1, FHR-2 or FHR-5. These *CFHR* variations are exclusively associated with C3G (33, 70–73). In this case, the resulting proteins are gain-of-function mutants that present an increased avidity for their ligands (36). Hence, these mutant proteins are postulated to out–compete the binding of FH to C3b deposited on surfaces and impair complement regulation more efficiently than the corresponding wild-type proteins (32, 36).

Besides genetic modifications, systemic levels of FHRs may also be crucial in disease processes. In anti-neutrophil cytoplasmic antibody (ANCA)-associated glomerulonephritis, increased systemic levels of FHR-1 were found compared to healthy controls (74). Additionally, FHR-1 levels were shown to weakly correlate with lower renal function and the percentage of relapses increased with growing FHR-1 concentrations. In IgAN, two groups independently of each other reported that plasma levels of FHR-1 and the FHR-1/FH-ratio are elevated in these patients and associate with progressive disease (75, 76). In contrast, plasma FHR-5 and the FHR-5/FH-ratio were not associated with progressive disease (76). However, higher FHR-5 levels in IgAN did associate with histological disease severity. In aHUS, plasma FHR-3 levels were demonstrated to be elevated compared to controls even when taking the *CFHR3* genotype into account (77). Also, the aHUS-risk *CFH–CFHR3–CFHR1* haplotype was shown to be associated with increased plasma levels of FHR-3, suggesting that an imbalance between FH and FHR-3 concentration may predispose individuals to aHUS. Recently, increased systemic FHR-4 levels were shown to be strongly associated with AMD (28). This is the first time that FHR-4 has been associated with a disease. A genome-wide association study revealed that an intronic variant in *CFHR4* correlated with systemic complement activation in AMD patients and associated with an increased risk of AMD development (26). A follow-up study demonstrated that the *CFHR4* variant was associated with higher levels of FHR-4 (28). Moreover, circulating FHR-4 levels and the FHR-4/FH-ratio were demonstrated to be elevated in AMD compared to controls, and the protein co-localized with complement activation products in choriocapillaris beneath the retina.

In addition to autoimmune diseases, the FH family has also been known to be involved in infections (78). Pathogens evade complement attack by recruiting complement regulators such as FH onto their surface, and it is suggested that the FHR proteins have evolved as decoys to reduce the amount of FH that is acquired by the microbes (35, 79). An illustrative example of this situation was described by Caesar et al., who showed that FHR-3 competes with FH for the binding of a FH-binding protein on *Neisseria meningitidis*, acting as a FH antagonist, which explains why the *CFH* haplotype 3, characterized by low FH and high FHR-3 plasma levels, is associated with lower susceptibility to meningococcal disease (78, 80). However, in contrast, the deletion of *CFHR3* and *CFHR1* was found to be associated with better survival in patients with bacterial meningitis (81). Altogether, this demonstrates the complex and multifaceted roles of the FH family in infections.

Altogether, the associations of the FH family with these diseases illustrate the relevance of the delicate balance between the different family members. Notably, the ratio between the levels of the regulator FH and the FHR proteins (i.e., FHR-1, FHR-3, FHR-4 and FHR-5) seems crucial in determining the outcome. Hence, either genetic or environmental factors altering the protein levels or the functionality of these proteins will have an impact on complement regulation and will define the susceptibility for the development of pathological conditions.

MAJOR CHALLENGES

In the last few decades, major strides have been made in our understanding of the FH protein family. From these findings an appreciation has emerged of the vast complexity of this group of proteins as well as of the monoclonal antibodies developed to specifically detect the different members of the Factor H protein family. Unfortunately, we still face multiple unmet challenges. Many of these involve the need for reagents and models to better understand the function of FHR proteins in health and pathology. Here, we will discuss four specific unmet challenges that need to be resolved (**Figure 1**).

Controversies in Functional Roles

The biological function of the FH protein family has been elusive. While it is clear that FH is a potent inhibitor of the complement system, and most data point towards a similar role for FHL-1, the functions of the more recently discovered FHR proteins are less well characterized and therefore remain uncertain. Initially, it was suggested that certain family members (e. g. FHR-1, FHR-3 and FHR-4) had no specific function or, at least, no essential function within the complement system. This rationale was largely based on the high frequency of *CFHR* gene deletions in the general population and the high homology among these proteins, suggesting some degree of functional redundancy. Instead, genetic studies revealed that alterations in the *CFHR* genes were indeed associated with pathology (described above), thereby providing the first piece of evidence that FHR proteins could be key pathogenic drivers of human disease. Since the pathogenesis of these diseases involve complement dysregulation, initial functional studies primarily focused on the potential regulatory functions of the FHR proteins. As a

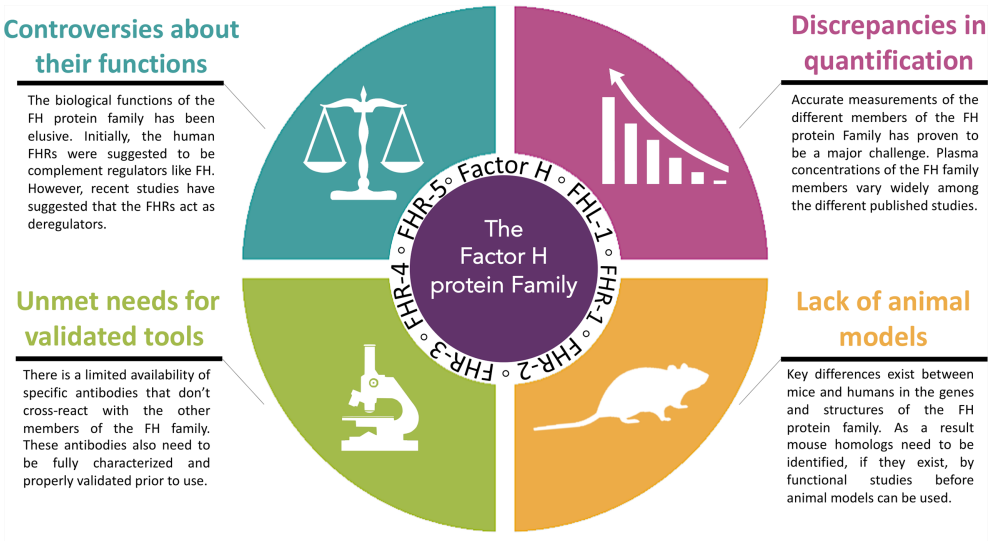

FIGURE 1 | Four specific unmet challenges for the Factor H protein Family. The major challenges ahead in this emerging area are: 1. the controversies about the functional roles of the Factor H protein family, 2. the discrepancies in quantification of the Factor H protein family, 3. the unmet needs for validated tools and 4. limitations of animal models.

result, considerable controversy exists over whether the FHR proteins have complement regulatory capacity or not. Results from these earlier studies indicated that the FHR proteins primarily functioned as complement regulators at specific steps of the cascade, while this concept was contested by later studies (82). Specifically, first the interaction of FHR proteins with C3 was investigated by functional studies, as an indicator of their potential complement inhibiting capacity, since the related proteins were assumed to be functional analogues of FH. Indeed, FHR-3 and FHR-4B were able to bind to the C3d region of C3b (83). Yet, when FHR-3 and FHR-4 were first studied for their effect on Factor I-mediated C3b inactivation, direct co-factor activity was very weak and only detectable at very high, and non-physiological, concentrations (i.e., 400 μg/ml). Moreover, the addition of FHR-3 and FHR-4 also enhanced the inhibitory activity of FH. A later study demonstrated a small inhibitory effect for FHR-3 in the hemolysis assays using FH-depleted serum (84). The regulatory effect of FHR-3 was shown to be based on cofactor activity, although supraphysiological concentrations were once again used. In contrast, others could not show any significant cofactor activity for FHR-4, even at high concentrations (i.e., 650 μg/ml) (85). In this study, FHR-4 did slightly enhance the inhibitory activity of FH. Later, FHR-2 was shown to bind C3b and C3d (86). While no cofactor or decay accelerating activity was found for FHR-2, it was shown to inhibit the activity of the C3bBb-convertase. For FHR-5, weak cofactor activity and fluid phase C3-convertase inhibiting activity were reported, once again at very high concentrations (87). FHR-5 was also found to inhibit both the C5-convertases of the CP and AP in an artificial, bead-based *in vitro* model (88). In these latter assays, the effective FHR-5 concentrations were close to serum levels measured in samples from healthy donors and patients with glomerulonephritis (37, 76, 87, 89). In conformity, FHR-5 produced by glioblastoma cells was also shown to act as a co-factor of Factor I and inhibit terminal pathway activation, although solely at high concentrations (90). Likewise, inhibition at the C5 level and/or the terminal pathway has also been reported for FHR-1, FHR-2 and FHR-3 (84, 86, 91). Fittingly, in a mouse model of a neurological autoimmune disease, injection of FHR-1 expressing neural stem cells ameliorated brain injury. Human FHR-1 was shown to protect astrocytes from complement activation by inhibiting the formation of the membrane attack complex (92). However, others have not been able to find any significant inhibiting activity of FHR-1 on the terminal pathway (36, 93–95).

Despite these initial studies, it has been very difficult to reconcile the reported regulatory activities of FHR proteins with their structures, especially considering the lack of structural homology of the related proteins with the regulatory domains of FH. In recent years, accumulating data strongly indicated a role for the FHR proteins as promoters of complement activation that stands in contrast to the regulatory function of FH and FHL-1. The study by Hebecker and Józsi was the first to challenge the paradigm, demonstrating that, by binding C3b, FHR-4 in fact enhances alternative pathway activation (85), a mechanism which was also suggested previously by Närkiö-Mäkelä et al. (96). This property of FHR-4 was recently exploited to overcome complement resistance of HER-2 positive tumor cells by applying FHR-4 based immunoconjugates (97). Studies by Tortajada et al. and Goicoechea et al. soon followed and described another mechanism, namely de-regulation by FHRs through competition with FH. FHR-1, FHR-2 and FHR-5 were shown to form homo- and hetero-oligomeric complexes, while a C3G-associated mutation in FHR-1 resulted in the duplication of the dimerization domain leading to the formation of unusually large

multimeric FHR complexes that exhibited increased avidity for C3 activation fragments (36, 71). Similarly, in IgAN, elevated levels of FHR proteins were shown to be associated with enhanced complement activation, while the absence of FHR-1 and FHR-3 was shown to decrease complement activation in AMD. These functional roles are opposite to that proposed in prior studies but are entirely consistent with the FHR structures, wherein the homologies of FHR proteins with FH are in the surface ligand-binding sites. Moreover, these studies strongly suggest that FHR proteins compete with FH (and FHL-1) mediated inhibition and thereby antagonize this key regulator of the complement system. Overall, FHRs were indeed shown to enhance complement activation both directly and indirectly (i.e., *via* competing with FH), thus emerging as "regulators of the regulator" (34). Competition between FHRs and FH has been described for several binding ligands. FHR-1, FHR-3, FHR-4 and FHR-5 were all shown to compete with FH for binding to C3b, to variable extent. Some of these differential effects may be related to the different avidities also determined by homo- or heterodimerization of FHR-1 and FHR-5 (36, 80, 84, 91). In addition, FHR-5 has been shown to strongly inhibit the binding of FH to the pentraxins (i.e., CRP and PTX3), as well as to extracellular matrix and malondialdehyde-acetaldehyde epitopes (98, 99). Subsequently, FHR-5 enhanced AP activation on these ligands. For FHR-1, FHR-4 and FHR-5 it has been shown that, by binding C3b, they can serve as a platform for the assembly of a functionally active C3bBbP convertase, consequently enhancing activation of the AP (85, 95, 98). Furthermore, FHR-5 can also induce AP activating by the recruitment of properdin *via* the CCPs 1-2 (99). Interestingly, in addition to modulation of the AP, FHR-1 and FHR-4 were both shown to activate the CP as well through the binding of CRP (i.e., FHR-1 the monomeric form, and FHR-4 the native, pentameric CRP) (85, 95, 100). More recently, FHR-1 and FHR-5 were shown to compete with FH for binding to DNA and thus promote AP activation, as well as to modulate both AP and CP activation on the surface of necrotic cells *via* interactions with monomeric CRP and PTX3 (101).

In conclusion, while complement inhibiting activity for some of the FHR proteins was described, the reported inhibitory activities were typically weak. More recent studies suggest that FHR proteins represent pattern recognition molecules that promote rather than constrain complement activation (35). To resolve the controversy, these functions need to be studied further, using physiological concentrations, and confirmed by independent research groups. The reported discrepancies may be related to the various sources of recombinant proteins used in the studies. In addition, the proteins may display different, context-dependent activities. Besides the function of the FH-family in the regulation of the complement system, non-canonical functions such as regulating cellular responses were described for FH, FHR-1 and FHR-3 (discussed in detail elsewhere (102)). Losse *et al.* reported that FHR-1 can bind to neutrophils *via* complement receptor 3 (CD11b/CD18), thereby resulting in enhanced antimicrobial activity (103). Recently, FHR-1 was shown to activate the NLRP3 inflammasome *via* the EMR2 receptor on monocytes and by binding to necrotic cells (74). Furthermore, these mentioned controversies raise an additional important question: How does the FH-family regulate inflammation, and what are their ligands and are there FHR protein receptors? Future studies need to define specific and shared ligands among members of this protein family, as well as conditions under which physiological or pathological functions are displayed, or competition occurs.

Discrepancies in Quantification

Accurate analysis of the FH protein family is of utmost importance for further deciphering of its function and role in complement-mediated diseases. Accurate information on physiological levels and the composition of the FH family members is also vital for functional studies, since supraphysiological levels can give misleading results. Yet, precise analysis of FH and other complement system components has proven to be challenging and systemic levels for FH family members vary widely among different studies (**Table 1**). These inconsistencies in levels are not only due to differences in sample type (plasma *vs* serum or different anticoagulants), storage (room temperature, 4°C *vs* -20°C or -80°C) and pre-analytical sample handling between studies, but also most likely caused by the use of different techniques (ELISA vs mass spectrometry), protocols and reagents (113). Another important explanation for the discrepancies in reported levels could be differences in characteristics of the blood donors. Age and gender have previously been demonstrated to significantly impact complement levels and functionality in the healthy population (114). Nevertheless, the impact of age and gender on the FH protein family has not been extensively studied. In healthy children, FHR-1, FHR-4A and FHR-5 levels were shown to be slightly lower in children compared to adults, but only FHR-5 levels were significantly associated with age (115). In addition, no gender differences were found. Levels of FH, FHR-2, and FHR-3 were similar to those found in adults (115). However, when corrected for genetic factors, an age-dependent increase of plasma levels of FH was seen for individuals aged 1 to 88 years (105). Furthermore, even when laboratories use the same technique, for instance ELISA, varying methods, reagents, calibrators and antibodies are used. Moreover, when antibodies are used, it is not always known whether these antibodies are truly specific for the target antigen or if cross-reactivity with other proteins may occur. Given that the FH protein family has a high degree of similarity in amino acid sequence, it is very well possible that antibodies against FH also cross-react with other FH family members. As a result, large discrepancies in levels for FH family members are observed between testing laboratories thereby hampering correct interpretation and hindering the comparison of results between studies. These inconsistencies in levels indicate the urgent need for well-characterized and standardized assays (116). Yet, validated and standardized assays for quantitative and functional analysis are not (widely) available for FH and its related proteins. Here, epitope mapping can be extremely valuable to predict (potential) cross-reactivity with other FH family members. The epitope location of a monoclonal antibody (mAb) can be determined using

TABLE 1 | Previously published systemic levels of the Factor H protein family.

Protein	Genotype	Levels (μg ml^{-1})	N	Reference
Total Factor H	N.D.	400 ± 62	1004	(104)
	N.D.	319.9 ± 71.4	358	(105)
	N.D.	233.2 ± 56.7	63	(106)
	N.D.	232.7 ± 74.5	1514	(107)
	N.D.	152.5 (95%-CI: 123 - 190)	161	(76)
	No ΔCFHR1/3 1*ΔCFHR1/3 2*ΔCFHR1/3	156 ± 39 168 ± 49 176 ± 39	44 24 8	(75)
	N.D.	349.0 (95%-CI: 339 - 359) 304.7 (95%-CI: 297 - 312)	214 308	(28)
Total FHL-1	N.D.	47 ± 11.3	2	(108)
Total FHR-1	N.D. 1**CFHR1* 2**CFHR1*	~2.33 (or ~0.04 μM) 61 ± 34 122 ± 26	3 24 44	(13) (75)
	N.D.	94 [IQR: 70.5 – 119.6]	161	(76)
	N.D.	1.63 ± 0.04	344	(109)
	N.D.	26.5 ± 2.3	55	(74)
	N.D.	70 – 100	?	(91)
FHR-1 homodimers	1**CFHR1* 2**CFHR1*	4.88 ± 1.33 14.64 ± 3.04	36 77	(37)
FHR-1/2 heterodimers	1**CFHR1* 2**CFHR1*	5.01 ± 1.49 5.84 ± 2.41	36 77	(37)
FHR-2 homodimers	0**CFHR1* 1**CFHR1* 2**CFHR1*	3.1 0.85 ± 0.41 0.65 ± 0.41	4 36 77	(37)
Total FHR-2	N.D.	3.64 ± 1.2	344	(109)
Total FHR-3	1**CFHR3* 2**CFHR3*	0.38 ± 0.23 0.83 ± 0.48	26 69	(77)
	2**CFHR3*A* 2**CFHR3*B*	0.55 ± 0.15 0.82 ± 0.08	16 4	(77)
	N.D.	1.06 ± 0.53	21	(110)
	N.D.	0.020 ± 0.001	344	(109)
Total FHR-4	N.D.	25.4 [IQR: 6.5 - 53.9]	11	(85)
	N.D.	2.42 ± 0.18	344	(109)
	N.D.	5.5 (95%-CI: 4.9 - 6.2) 6.0 (95%-CI: 5.6 - 6.3)	214 308	(28)
FHR-4A	N.D.	2.55 ± 1.46	129	(111)
FHR-4B	N.D.	Not detected	?	(111)
Total FHR-5	N.D.	5.5 [IQR: 3.4 – 10.1]	13	(89)
	N.D.	5.49 ± 1.55	344	(109)
	N.D.	2.46 [IQR: 1.79 – 3.67]	158	(76)
	N.D.	3.19 [IQR: 2.55 – 3.92]	153	(112)
Homodimers FHR-5	N.D.	1.66 ± 0.43	115	(37)

An overview of the published systemic levels of Factor H, Factor H-like 1 and the five Factor H-related proteins in healthy controls. Specific information is provided per study on the genetic background of the cohort, the number of subjects and whether they measured total levels, homo- or heterodimers. Data are presented as mean ± standard deviation (SD), mean with 95%-confidence interval (95%-CI) or median with interquartile range [IQR].

fragments consisting of CCP domains as previously described (111). It is important to note that in contrary to the FHR proteins, the functions of FH are well known and several functional assays for FH exist, some of which currently are being used in the clinic. FH-mediated decay-accelerating activity can be measured in both ELISA as well as surface plasmon resonance assays (117–119). The co-factor activity of FH can be determined in both fluid-phase as well as on the cell surface (120, 121). In addition, the full function of FH can be addressed on cell surfaces using cell-based assays (13, 117, 119).

In order to provide insight into the magnitude of the discrepancies in quantification of the FH protein family, we determined systemic FH levels in samples from healthy volunteers using seven commercially available ELISA's (**Table 2**). Levels of FH were evaluated in samples that were collected, stored and handled exactly the same way. Next to those 10 samples, we also included 2 samples obtained from the Complement EQA Group. Both samples consist of a pool of serum samples derived from 5 healthy individuals. The Complement EQA Group committee aims to standardize complement analysis by providing calibrator materials and collects, evaluates and compares levels of complement components from different testing facilities (see also below). It should be noted that within the FH protein family, only quantitative analysis of serum levels of FH is included in the standardization activities of the Complement EQA Group. In 2009, this group was formally recognized and became part of the IUIS (International Union of Immunological Societies) Quality Assessment and Standardization Committee (https://iuis.org/committees/qas/) (122). We chose plasma-EDTA samples, since coagulation enzymes can also cleave complement components with subsequent generation of activation products (122). EDTA blocks the *in vitro* activation of the complement system by Mg^{2+} and Ca^{2+} chelation. Citrate-based anticoagulants are less useful (123). Moreover, heparin-plasma should not be used since multiple members from the FH family are heparin-binding proteins, hence heparin could interfere with the measurements. All assays were performed in parallel by the same operator according to the manufacturer's protocols. In general, all %CV were ≤15%, indicating low variation.

First, human purified FH protein dissolved in PBS was measured in all seven assays (Cat# A137, Complement Technology Inc., TX, USA). None of the assays was able to 'pinpoint' this exact concentration (not corrected for the extinction coefficient). The assays from LSBio and USCN were not even able to detect purified FH protein in PBS (**Figure 2A**). Next, a FH depleted sample was measured as a negative control in all assays (Cat# A337, Complement Technology Inc., TX, USA). As expected, most assays did not detect FH in this sample. However, a FH concentration of 377 μg/ml was measured in the FH depleted sample using the USCN assay (**Figure 2B**). Subsequently, we assessed systemic FH levels in plasma-EDTA samples derived from 10 healthy controls. Results show large and significant differences in FH levels between the seven assays used ($P<0.0001$). No FH was detected with the LSBio assay (**Figure 2C**). In spite of these differences in absolute FH levels between the assays, moderate to high correlations were observed between the Abcam, Hycult, Quidel, R&Dsystems and Sanquin assays regarding the FH levels (**Figure 2D**). No correlation was observed between the USCN and the other assay. For the LSBio assay, no correlation could be calculated as no FH levels were detected with this assay. The FH levels in the serum pool samples obtained from the Complement EQA Group were comparable with the levels measured in the healthy control panel. Again, no FH was detected using the LSBio assay (**Figure 2E**). Lastly, calibrators were exchanged, except for the Abcam and Quidel ELISAs, as not enough calibrator was provided to be included in each assay as sample. Results show that the calibrators from Hycult, R&Dsystems and Sanquin were exchangeable, and yielded quantifiable and reliable levels. The calibrators from LSBio and USCN were not recognized in all other assays. In turn, the LSBio assay was not able to recognize/measure any of the calibrators except its 'own' calibrator. The USCN assay was able to recognize the calibrators from Hycult and Sanquin (**Figure 2F**). Overall, the assays from Abcam, Hycult, R&Dsystems and Sanquin were perfectly able to detect FH protein in the positive control and did not detect FH in the negative control. Additionally, the correlations between these assays were moderate to high. In contrast, the results obtained with the LSBio and the USCN assay suggest that these assays are not able to assess FH levels in samples in a reliable manner. Nevertheless, even in the reliable assays, FH levels obtained in the same sample set vary greatly. Given these discrepancies, we can conclude that the absolute FH levels determined with these assays, are probably not correct. Considering the lack of quantification, it is suggested to provide calibrators with these assays as arbitrary units (AU) only. In this instance, these assays would then be able to detect differences in FH levels between experimental groups (e.g., healthy *vs* disease), as long as the same assay is used for all analyzed groups. In the end, the fact that absolute FH levels vary greatly between assays may not be surprising as calibrators from different sources were used in their calibrations.

TABLE 2 | Human Factor H ELISA's included in the comparison analysis.

Company	Name	Cat#	Lot#	Website
Abcam	Human FH ELISA	ab137975	GR3261729-8	www.abcam.com
Hycult Biotech	Complement FH, human, ELISA kit	HK342	28643K0420	www.hycultbiotech.com
LSBio	LSbio CFH	LS-F21748	189699	www.lsbio.com
Quidel	MicroVue FH EIA	A039	184358	www.quidel.com
R&Dsystems	CFH duoset	DY4779	P240815	www.rndsystems.com
Sanquin	Human FH ELISA	No info*	No info*	www.sanquin.nl
USCN	CFH ELISA kit	SEA635Hu	L200831651	www.uscnk.com

**The assay of Sanquin is only available as service. Samples can be sent to Sanquin for analysis.*

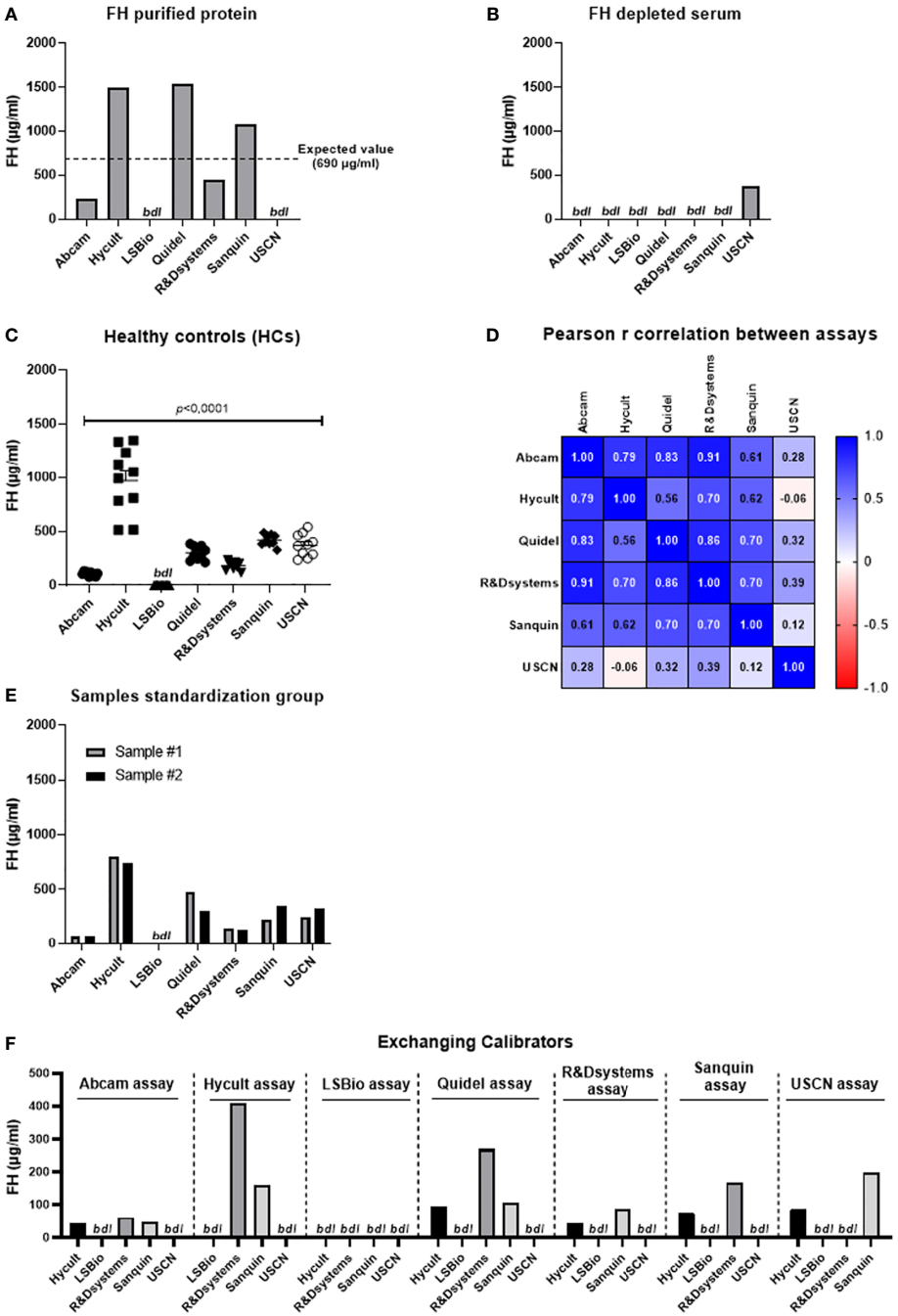

FIGURE 2 | Assessment of Factor H levels in samples using seven different assays. **(A)** Assessment of Factor H (FH) purified protein in PBS (expected value is 690 µg/ml). **(B)** Assessment of FH levels in FH depleted serum. **(C)** Assessment of systemic FH levels in samples derived from healthy controls (n=10). Data are represented as mean ± SEM. Data were analyzed using the one-way ANOVA (Graphpad Prism 8.4.2, San Diego, CA, USA). A p-value <0.05 was considered significant. **(D)** Pearson r correlation coefficient. Pearson coefficients range from +1 to -1, with +1 representing a positive correlation of FH sample values between assays, -1 representing a negative correlation of FH sample values between assays, and 0 representing no relationship. No correlation could be calculated for the LSBio assay as no FH levels were obtained using this assay. **(E)** Assessment of FH levels in 2 serum pool samples obtained from the Complement EQA Group. **(F)** Assessment of calibrators as sample in each assay. All calibrators were exchanged between the seven assays except for the Abcam and Quidel calibrator as not enough calibrator was provided with these kits to be included in each assay as sample. FH, factor H; HCs, healthy controls; bdl, below detection limit.

TABLE 3 | Published antibodies that have been proposed to be specific for each of the Factor H-related proteins.

	Characteristics	Validation steps	Use	Source	Ref.
FHL-1	Rabbit pAb IgG anti-human FHL-1	- Direct ELISA for FH and FHL-1. - Double staining with specific FH antibody as well as FH/FHL-1 antibody. - Preincubation of the pAb with FHL-1 prior to IHC - WB with recombinant FH and FHL-1 as well as tissue.	IHC, ELISA, WB.	Non-commercial	(127)
FHR-1	Mouse mAb anti-human FHR-1 (Clone JHD10)	- Preincubation of the mAb with FHR-1 prior to IHC. - IHC on material of patients with a combined CFHR1/3 deletion.	IHC, FC, WB.	Non-commercial	(74, 91)
	Mouse mAb anti-human FHR-1	- IHC on material of patients with a homozygous CFHR-1 deficiency.	IHC	(#3078-M01; Abnova, Taipei, Taiwan)	(128, 129)
FHR-2	Mouse mAb anti-human FHR-2	Unknown	IF	Non-commercial	(99)
FHR-3	Mouse IgG2 mAb anti-human FHR-3 (Clone: RETC-2)	- Direct ELISA for recombinant FH and all FHRs. - WB with recombinant FH, all FHRs and CFHR-3 deficient as well as normal serum. - Mass spectrometry analyses of the immuno-precipitation by the mAb.	IHC, ELISA, WB.	Non-commercial	(110)
	Mouse mAb anti-human FHR3 (Clone: HSL1)	- WB with normal human serum and CFHR3 deficient serum. - Direct ELISA for recombinant FH and all FHRs.	ELISA, FC, WB.	Non-commercial	(80)
FHR-4	Mouse IgG1 mAb anti-human FHR-4 (Clone 4.4)	- WB with recombinant FH and FHRs - WB with normal human serum and CFHR3 as well as CFHR4 deficient serum. - WB of the immunoprecipitation by the mAb.	ELISA, WB.	Non-commercial	(111)
	Mouse IgG mAb anti-human FHR-4 (Clone 4E9)	- WB with recombinant FHR-4 and normal human serum	ELISA, WB.	Non-commercial	(28)
	Mouse IgG mAb anti-human FHR-4 (clone 17)	- WB with recombinant FHR-4 and normal human serum	ELISA, WB.	Non-commercial	(28)
	Mouse IgG mAb anti-human FHR-4 (Clone 150)	- Preincubation of the mAb with FHR-4, FHL-1 prior to IHC.	IHC, WB.	Non-commercial	(28)
FHR-5	Rabbit pAb IgG anti-human FHR-5	- Preincubation of the pAb with FHR-5 prior to IHC. - Direct ELISA for C3c, iC3b and C3d with and without FHR-5.	IHC, ELISA.	(#81494-D01P; Abnova, Taipei, Taiwan)	(128, 129)
	Mouse mAb anti-human FHR-5 (Clone K2.254)	Unknown	IHC, ELISA	Non-commercial	(55)
	Mouse mAb IgG1 anti-human FHR-5	Unknown	IHC, ELISA, WB, FC.	(#390513, R&D Systems)	(99)
	Mouse mAb IgG1 anti-human FHR-5 (clone 5.1)	- WB with recombinant FH and FHRs - Direct ELISA for recombinant FH and all FHRs.	ELISA, WB	Non-commercial	(37)
	Mouse mAb IgG1 anti-human FHR-5 (Clone 5.4)	- WB with recombinant FH and FHRs - Direct ELISA for recombinant FH and all FHRs.	ELISA, WB	Non-commercial	(37)

An overview of the published antibodies that are proposed to be specific for one of the Factor H-related proteins. Specific information is provided for each antibody in regards to their characteristics, validation steps, application, source and the reference.

ELISA, enzyme-linked immunoassay; FC, flow cytometry; FH, Factor H; FHL-1. Factor H-like 1; FHR, Factor H-related protein; IF, immunofluorescence; IgG, immunoglobulin; IHC, immunohistochemistry; mAb, monoclonal antibody; pAb, polyclonal antibody; WB, Western blot.

For the future, we therefore recommend that a uniform protocol is used regarding sample collection, pre-analytical sample handling and storage. For the assessment itself, it is strongly recommended that standardized assays with a uniform calibrator are used. When antibodies are used for quantification, these must be characterized regarding specificity and cross-reactivity with other proteins (see also below in the next section). In this way, results can be produced that are robustly comparable between different studies.

Unmet Needs for Validated Tools

In 2016, Nature conducted a survey to understand scientist's view on the lack of reproducibility in research (124). When asked about the cause, a proportion pointed towards poorly-characterized tools leading to ambiguous findings, which results in an unstable knowledge foundation that is then built upon. Since this survey, different guidelines for *in vitro* and *in vivo* research have been suggested, issued and published (125, 126). Characterization of antibodies as well as validation of tools to quantify proteins is vital

for every field, but particularly for the FH protein family considering the high risk of cross-reactivity due to their homology. Yet, when looking at the specific antibodies that are currently used in peer-reviewed publications, results on validation of antibodies are not always provided (**Table 3**). Moreover, whenever present, results on antibody validation are usually in the supplementary data, a scientific habit that perhaps should be reconsidered. Safeguarding accurate antibody validation should be a main concern for all scientists, clinical end-users, industry, journal publishers and antibody-linked research alike. Every researcher has experienced research antibodies that don't live up to promises or expectations. Either because the antibody does not recognize the desired target, or because they recognize a different protein instead of, or as well as the desired target. Additional problems include functionality of the antibody being limited to certain applications. Too often the choice of an antibody is driven by the number of citations it has in the literature, as scientists falsely assume the antibody must have been validated previously, enabling self-perpetuating artefacts. In this regard, we offer a consensus report by the authors and hope to ignite further discussion among the community to establish recommendations for best practices to improve the reproducibility, validity and to help advance research into the FH-protein family. Obviously, these recommendations are not aimed at antibodies used for mere *in vitro* experiments with purified components, but rather for observational and intervention studies with a large sample size. First of all, it would be advised to (i) reference and (ii) validate the specific antibodies used for the FH protein family research field as detailed as possible (130). We further suggest a best practice protocol for the validation of all detection tools. Based on previous guidelines, we suggest the following 4-step validation protocol for antibodies against the FH protein family (131–134):

1. Show whole Western blot of target human/animals samples detected with the antibody.
   Value: Knowledge regarding the respective protein size(s).
   Cave: Only applicable if the antibody is reactive in Western blot.
2. Compare binding pattern of the antibody for ALL the different known FH-protein family members (e.g. ELISA, Western blot or Dot blot).
   Value: Characterization of intra-protein family specificity.
   Cave: Access to validation material can be restricted.
3. Test the antibody reactivity against either a.) non-depleted vs FH-protein family members depleted human serum/plasma (if available), b.) non-deficient cells vs target gene deficient cells (if available) or c.) wildtype vs knockout animals (e.g., ELISA, Western Blot or immunohistochemistry, if available).
   Value: Species specificity of the antibody can be determined independent from unknown protein modifications.
   Cave: Access to validation material can be restricted.
4. Characterize the specificity of an antibody using immunoprecipitation with subsequent mass spectrometry analysis from the respective target tissue.
   Value: Tissue specific cross reactions of the whole antibody (even the Fc-part) will be deciphered.
   Cave: Specialized collaborating laboratories are needed.

It would be preferred to perform this 4-step validation protocol in line with an already validated, antibody as a quality control (if available). A single protocol will not match all applications and researchers must ensure that the reported validation holds true for their specific use. Overall, we are convinced that validation of tools, together with transdisciplinary collaboration and transparency within the community will enable research to move forward and get one step closer towards deciphering the mode of action of the FH-protein family.

Limitations of Animal Models

Murine models have demonstrated high value for establishing fundamental principles of complement biology (135). The murine version of FH (mFH) was first identified in 1986 and is very similar in structure and function to the human FH (136). As its human equivalent, mFH has a molecular weight of around 155 kDa, has several glycosylation sites, is positioned on chromosome 1 and is primarily produced by the liver. The plasma concentration of mFH is estimated to be similar to human FH, but an exact concentration of mFH has not been determined yet (135, 136). Clear differences exist as well between mFH and human FH. For instance, in contrast to the human *CFH* gene, the *mCFH* gene does not have an alternative splicing variant and thus no murine equivalent of FHL-1 has been identified. Overall, The *mCFH* gene shares 63% of sequence identity with the human *CFH* gene (135). Despite the differences in genes and protein structures across mouse and human, it has been observed repeatedly that essential activation and regulatory functions of this system are retained across species. In accordance, genetically deficient animals have provided a powerful tool to help understand the function of FH.

The link between FH and disease is older than is frequently reported. Høgåsen et al. described more than 25 years ago that a hereditary deficiency of FH in pigs consistently led to the development of lethal renal disease, namely C3G (137). These findings were later confirmed in rodent models, demonstrating that mice deficient in FH had uncontrolled complement activation resulting in C3G (138). Interestingly, later studies revealed that aged $CFH^{-/-}$ mice also develop retinal abnormalities and visual dysfunction, resembling AMD (139). The broad outlines are therefore clear, dysfunction of FH leads to uncontrolled complement activation resulting in tissue injury and thus causing disease. However, it was not clear what then determines whether defects in FH cause one specific disease but not the other. Animal models have helped to attribute the different functions of FH to specific domains within the protein, and thereby reveal specific genotype–phenotype connections in FH that lead to either complement-mediated thrombotic microangiopathy (TMA) or C3G and AMD. A study by Pickering et al. uncovered that a homozygous FH deficiency in mice leads to defective function of FH in the fluid-phase triggering the development of C3G and AMD (138). In contrast, loss of the SCR 16-20 region of FH impairs the ability of FH to control complement activation on surfaces,

causing spontaneous TMA (60). In conclusion, the use of animal models has helped significantly to understand the function of complement proteins and their role in disease, especially for FH.

Animal models have barely been used to study the other members of the Factor H family. As mentioned before, no murine equivalent for the human FHL-1 exists. Furthermore, the *CFHR* genes have arisen during evolution through duplication events of the *CFH* gene (12). Extensive analysis of the human *CFH* and *CFHR* gene loci using Alu/L1 repeat dating established that these duplication events occurred after the separation of rodent and primate lineages and therefore no FHR orthologues exist in mice. More specifically, the *mCFHR* genes differ in structure, domain composition, and sequence from the human genes (32). Like the human FHR proteins, a total of five murine *CFHR* genes have been suggested (*mCFHR-A*, *mCFHR-B*, *mCFHR-C*, *mCFHR-D* and *mCFHR-E*) and evidence for four mFHR proteins (FHR-B, FHR-C, FHR-D and FHR-E) has been derived from mRNA transcripts isolated from mouse liver (48, 140–143). However, altogether, this suggests that direct comparisons between the human and mouse FHR proteins is not informative and, therefore, any mouse FHR homologs need to be identified, if they exist, by functional studies before rodent models can be used to further study the role of the FH protein family in human health and disease. Alternatively, genetic engineering approaches could be used to create a set of humanized transgenic mice to more closely mimic the human FHR situation. Until that is achieved, the lack of animal models remains a major barrier hindering the elucidation of disease mechanisms and drug development. More importantly, the absence of appropriate animal models stresses the importance of appropriate human assays to correctly identify and study the Factor H protein family in humans.

THERAPEUTIC VALUE OF FH AND DERIVATIVES IN COMPLEMENT-MEDIATED DISEASES

The various disorders linked to the FH family tend to be difficult to treat and some are even incurable. An obvious therapeutic strategy for these diseases could therefore be the administration of (purified or recombinant) FH to restore complement regulation. Indeed, both *in vitro* and animal studies have demonstrated the therapeutic value of FH (63, 137, 144–146). In $CFH^{-/-}$ pigs, a single dose of 5 mg/kg porcine FH resulted in normalization of plasma C3 levels and diminished systemic complement activation for almost 3 days (137). In $CFH^{-/-}$ mice, both purified mouse and purified human FH led to a rapid increase of plasma C3 levels and resolution of renal C3 deposition (144, 146). However, FH supplementation as a therapy would require large amounts of biologically active protein due to high circulating levels in healthy individual, making it labor and cost intensive. Various strategies have been tested to resolve these problems. Several groups have demonstrated successful production of high yields of recombinant FH in different expression systems (such as yeast and moss) as an alternative and economically viable method (147, 148). Others have created derivates or fusion proteins from FH with enhanced pharmacokinetic and pharmacodynamic properties. Smaller constructs of FH have been created by combining the regulatory domains (N-terminus) with the surface-recognition domains (C-terminus) (121, 149, 150, 158). These FH constructs can regulate complement *in vivo*, and effectively reverse renal C3 deposition and restore plasma C3 levels in $CFH^{-/-}$ mice. However, the short half-life of these constructs remains an important limitation. FH fusion protein have also been engineered as a therapeutic approach (151–153). Most extensively studied is the CR2-FH fusion protein, that links the C3d binding domain of complement receptor 2 (CR2) to the complement inhibitory domain of FH, thus ensuring targeted regulation by FH at sites of complement activation (154). Treatment with CR2-FH was beneficial in animal models of eye, kidney and autoimmune diseases (155–157). Finally, local injection of FH (or derivates) is another approach to circumvent the need for large amounts of biologically active protein. A clinical trial investigating the safety and effectivity of recombinant FH (GEM103) administered through intravitreal injections for the treatment of geographic atrophy secondary to dry AMD is currently on-going (ClinicalTrials.gov identifier, NCT04246866).

FUTURE PERSPECTIVE

A multidisciplinary approach is mandatory to overcome the challenges mentioned above, and is only possible through interdisciplinary collaboration between biologists, chemists, geneticists and physicians. But, where to start? As suggested by the quote of William Thomson, the authors of this paper believe that we should essentially begin with quantifying the levels and activity of the different members of the FH-family in health and disease. Detection of the FH-protein family will enable the scientific and clinical community to advance our understanding of the role of the FH-protein family in infectious, eye, kidney and autoimmune diseases, and potentially help treat these disorders.

CONCLUSION

As described, the FH-family, consists of FH, FHL-1 and the five FHR proteins which are important regulators of the complement system. Mutations and polymorphisms in the FH-family are involved in several diseases, indicating a potential crucial role of the FH-family in both health and disease. However, diagnosis and therapy of these partially incurable pathologies is to-date not related to the FH-protein family, due to a lack of fundamental knowledge of (i) the molecular mechanisms leading to disease, (ii) unknown functional, convincing principles of FH-protein family members, (iii) absent standardized diagnostics and (iv) missing suitable drug candidates. To overcome these challenges, an ardent multidisciplinary approach is required through interdisciplinary collaboration.

AUTHOR CONTRIBUTIONS

All authors contributed equally and the review was written together. All authors critically reviewed the manuscript prior to submission.

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Autoantibodies Against the Complement Regulator Factor H in the Serum of Patients With Neuromyelitis Optica Spectrum Disorder

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Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune inflammatory disease of the central nervous system (CNS), characterized by pathogenic, complement-activating autoantibodies against the main water channel in the CNS, aquaporin 4 (AQP4). NMOSD is frequently associated with additional autoantibodies and antibody-mediated diseases. Because the alternative pathway amplifies complement activation, our aim was to evaluate the presence of autoantibodies against the alternative pathway C3 convertase, its components C3b and factor B, and the complement regulator factor H (FH) in NMOSD. Four out of 45 AQP4-seropositive NMOSD patients (~9%) had FH autoantibodies in serum and none had antibodies to C3b, factor B and C3bBb. The FH autoantibody titers were low in three and high in one of the patients, and the avidity indexes were low. FH-IgG complexes were detected in the purified IgG fractions by Western blot. The autoantibodies bound to FH domains 19-20, and also recognized the homologous FH-related protein 1 (FHR-1), similar to FH autoantibodies associated with atypical hemolytic uremic syndrome (aHUS). However, in contrast to the majority of autoantibody-positive aHUS patients, these four NMOSD patients did not lack FHR-1. Analysis of autoantibody binding to FH19-20 mutants and linear synthetic peptides of the C-terminal FH and FHR-1 domains, as well as reduced FH, revealed differences in the exact binding sites of the autoantibodies. Importantly, all four autoantibodies inhibited C3b

binding to FH. In conclusion, our results demonstrate that FH autoantibodies are not uncommon in NMOSD and suggest that generation of antibodies against complement regulating factors among other autoantibodies may contribute to the complement-mediated damage in NMOSD.

Keywords: aquaporin (AQP) 4, complement, factor H, neuromyelitis optica spectrum disorder, autoantibody, autoimmunity, inflammation, central nervous system

INTRODUCTION

Neuromyelitis optica spectrum disorder (NMOSD) is a rare inflammatory disease of the central nervous system (CNS) with a prevalence of 0.7-10/100,000 worldwide (1) and is characterized by pathogenic complement-activating autoantibodies against aquaporin 4 (AQP4), the main water channel of the CNS (2, 3). Most commonly, relapsing and often bilateral optic neuritis, longitudinally extensive transverse myelitis, and brainstem symptoms characterize NMOSD (4–7).

Complement is an essential effector system of the humoral arm of innate immunity (8). The complement system can be activated *via* three main pathways, the classical, the lectin and the alternative pathways. It provides a first-line defense against infections, participates in the clearance of immune complexes and cellular waste, and influences adaptive immune responses (8–10). Complement gene mutations and polymorphisms that result in altered protein function and thus excessive activation, inappropriate regulation or failure in proper targeting of complement attack may lead to pathogenic complement activation, which by causing inflammation and tissue damage is implicated in the pathogenesis of several diseases (11). In addition to genetic alterations, autoantibodies to complement proteins can cause or contribute to diseases *via* binding to their target, which in turn may impair the function of the respective proteins and result in pathological complement activation (12–14).

Complement has also been implicated in the pathogenesis of NMOSD. CNS lesions are characterized by deposition of complement proteins along with IgG, IgM, and loss of astrocytic AQP4 (15–17). Patients with NMOSD have higher levels of complement activation products in the blood, and the three complement pathways are functionally abnormal even during the remission period (18–20). Autoantibodies to complement C1q were described in NMOSD patients (19). Autoantibodies can activate complement *via* the classical pathway when bound to their target proteins, which was recently described for AQP4-autoantibodies, as well (5, 21). AQP4 autoantibodies, also known as NMO-IgG, mainly belong to the IgG1 subclass, and astrocytes transfected by AQP4 are susceptible to cell death by IgG and IgM AQP4-antibodies in the presence of complement (22, 23). The pathogenic role of NMO-IgG in the presence of human complement is also supported by the results of passive transfer experiments (5, 21) and *in vivo* disease models, where complement inhibition was proven to be beneficial (24). Moreover, clinical experience also sustains the role of complement activation in disease pathogenesis, since treatment with the monoclonal anti-C5 antibody eculizumab reduced attack frequency, and stabilized or improved neurological disability of patients with NMOSD (25, 26). Thus, therapeutic complement inhibition is a promising strategy in the treatment of NMOSD (24, 27), and eculizumab has been approved for treating AQP4-IgG positive NMOSD.

Of the three major pathways of complement, the alternative pathway is particularly powerful because *via* the so-called amplification loop it can enhance complement activation initiated by any complement pathway (8, 28, 29). This is based on the generation of C3b and formation of the C3bBb alternative pathway C3 convertase (30). Therefore, proper regulation of the alternative pathway is essential in maintaining homeostasis. Antibodies to components of the alternative pathway may result in a wide spectrum of diseases. Antibodies to the C3 convertase, i.e. C3 nephritic factors (C3NeFs), may stabilize the convertase resulting in increased complement activation, which has been associated with C3 glomerulopathies (13, 14, 31). Autoantibodies to factor H (FH), the major soluble regulatory protein of the alternative complement pathway, are described in kidney diseases such as atypical hemolytic uremic syndrome (aHUS) and dense deposit disease, and are thought to cause pathogenic complement activation by blocking functional domains of this complement inhibitor protein (14, 32–34).

FH is a 155-kDa serum glycoprotein that upon binding to C3b inhibits the activation of the alternative pathway and the amplification loop. FH acts as a cofactor for the serum protease factor I in the enzymatic inactivation of C3b (cofactor activity), prevents assembly of the C3bBb convertase and accelerates its disassembly if already formed (decay accelerating activity) (35, 36). FH is composed of 20 homologous domains termed short consensus repeats (SCRs), of which SCR1-4 mediate the cofactor and decay accelerating activities of the protein, and SCR19-20 function as recognition domains for deposited C3b/C3d in the context of host surface glycans (37, 38). The physiological function of FH is critical for proper complement regulation. Altered FH activity caused by genetic changes and autoantibodies are associated with several inflammatory and autoimmune pathologies, such as age-related macular degeneration, C3 glomerulopathies and aHUS (36, 39).

Overactivation of the complement system was proven to be present in NMOSD; however, it is unclear what steps lead to complement activation in the pathogenesis of this disease. We

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AQP4, aquaporin 4; C3NeF, C3 nephritic factor; CNS, central nervous system; FB, factor B; FH, factor H; FHR-1, factor H-related protein 1; NMOSD, neuromyelitis optica spectrum disorder; SCR, short consensus repeat.

hypothesized that autoantibodies against complement proteins may contribute to abnormal complement activation in NMOSD. Therefore, the aim of this study was to evaluate the presence of antibodies against the alternative pathway convertase C3bBb, its components C3b and factor B (FB) as well as the regulator protein FH in the serum of patients with AQP4-seropositive NMOSD.

MATERIALS AND METHODS

Patients

Serum or EDTA-plasma samples of NMOSD patients were collected after informed consent in accordance with the Declaration of Helsinki. The study was approved by the National Ethical Committee (3893.316-12464/KK4/2010 and 42341-2/2013/EKU). Forty-five patients having NMOSD were included in this study and they all were seropositive for anti-AQP4 antibody determined by a commercially available cell-based assay (Euroimmune, Lübeck, Germany). The patients did not present other autoimmune diseases, cancer or infections. They were negative for antinuclear antibodies, except for patient #113; this was a single abnormality, no specific antigen was identified, and no other systemic autoantibodies (anti-dsDNA, anti-SSA, anti-SSB) were detected. Characteristics of the FH autoantibody-positive patients are summarized in **Table 1**.

Proteins, Sera and Antibodies

Purified human FH, FB, C3b, factor D, C1q, goat anti-human C1q antibody (Ab) and goat anti-human FH antibody (Ab) were purchased from Merck (Budapest, Hungary). Human serum albumin (HSA), bovine serum albumin (BSA), alpha1-antitrypsin, HRP-conjugated anti-human IgG, HRP-conjugated anti-human IgA, HRP-conjugated anti-human IgM, and monoclonal antibodies (mAbs) specific for IgG1, IgG2, IgG3, IgG4, Ig kappa and Ig lambda were purchased from Sigma-Aldrich (Budapest, Hungary). HRP-conjugated goat anti-mouse Ig and HRP-conjugated rabbit anti-goat Ig were purchased from DakoCytomation (Hamburg, Germany). HRP-conjugated goat anti-human C3 was from MP Biomedicals (Solon, OH). The anti-FH mAb A254 was purchased from Quidel (Biomedica, Budapest, Hungary), and the mAb C18 (40) was from Alexis Biochemicals (Lörrach, Germany). The anti-FH mAb IXF9 was described earlier (41).

Codon-optimized sequences of FHR-1, FHR-4B, FH SCRs 1-4, FH SCRs 8-14, FH SCRs 15-20 were synthesized (GenScript, Piscataway, NJ) and cloned into the pBSV-8His baculovirus expression vector, expressed in *Spodoptera frugiperda* Sf9 cells and purified by nickel affinity chromatography as described previously (42, 43). FH SCRs 19-20 and mutant 19-20 fragments were expressed in *E. coli* (44).

TABLE 1 | Characteristics of NMOSD patients with FH autoantibodies.

	Age at onset (y)	Diagnosis	AQP4-Ab
NMO64	23	relapsing ON and LETM	+
NMO84	41	relapsing ON and LETM	+
NMO113	54	relapsing ON and LETM	+
NMO210	64	LETM	+

All four patients positive for FH autoantibodies are female and have NMOSD for >5 years. All patients have anti-AQP4 antibodies (AQP4-Ab) in their serum as determined by a cell-based assay. ON: optic neuritis, LETM: longitudinally extensive transverse myelitis.

Microtiter Plate Assays

Microtiter plate wells were coated with 5 μg/ml FH, FB, C3b, or HSA as negative control antigen, for 1 h at 20°C. To measure autoantibody binding to solid-phase C3bBb convertase, the convertase was built up in microtiter plate wells as previously described (45). After blocking with 5% BSA and 0.1% Tween-20 in phosphate buffered saline (BSA-PBS), patients' serum samples diluted 1:50 in Dulbecco's PBS (DPBS; Lonza, Budapest, Hungary) were added for 1 h. Bound IgG was detected by incubating the wells with HRP-conjugated anti-human IgG for 1 h. Color reaction was developed with TMB (Kem-En-Tec Diagnostics, Taastrup, Denmark) and absorbance was measured at 450 nm. Antibody positivity was determined based on the reactivity with the specific antigen and the negative control protein; those having an OD value ≥ the double of that of the control protein were considered positive. The identified samples were analyzed in additional assays (see below) to confirm autoantibody positivity and characterize specific binding sites and potential functional effects of the autoantibodies.

To detect IgM and IgA autoantibodies, samples were preincubated with Protein G-agarose beads (Sigma-Aldrich) to deplete IgG, and these IgG-depleted samples were added to wells coated with 5 μg/ml FH. The presence of IgM and IgA autoantibodies was detected as described above, except for using the corresponding HRP-conjugated detection antibodies instead of anti-human IgG. In some assays, prior to immobilization FH was treated with 10 mM Tris(2-carboxyethyl)phosphine (TCEP; Sigma-Aldrich) to generate reduced FH (46). To this end, 20 mM TCEP dissolved in 0.4 M Tris pH 7.4 was mixed 1:1 with 1 mg/ml FH and incubated for 30 min at 20°C. FH was then diluted and immobilized on microplate wells, and used for autoantibody binding assay as described above. Autoantibodies against C1q were analyzed as described previously (45). Briefly, microtiter plates were coated with 2 μg/ml C1q and, as negative control antigens, HSA and α1-antitrypsin. After blocking and washing, serum samples diluted 1:50 in DPBS containing 1 M NaCl were added. Autoantibody binding was detected with HRP-conjugated anti-human IgG diluted in DPBS containing 1 M NaCl.

To map the antibody binding sites within FH, recombinant FH fragments were immobilized and autoantibody binding was detected as described above. For the characterization of IgG isotypes, FH and HSA were immobilized and, after blocking with BSA-PBS, the plates were incubated with patients' samples. For the detection, mAbs specific for IgG1, IgG2, IgG3, IgG4, Ig kappa and Ig lambda, followed by HRP-conjugated goat anti-mouse Ig, were used. To analyze the effect of anti-FH mAbs, the wells were incubated with the anti-FH mAbs prior to the addition of patients' sera as described (33). To determine the avidity of the FH autoantibodies, NaSCN as a chaotropic salt was used as

described (47, 48). Briefly, after incubation of the wells with the patients' sera, 0.5 M NaSCN was added for 15 min at 20°C and, after washing, the bound IgG was detected with HRP-conjugated anti-human IgG. Titers of the samples were calculated based on a standard curve and avidity index was calculated as the ratio of bound antibodies in the presence and absence of NaSCN. To calculate the avidity profile, various concentrations of NaSCN were used. To measure the inhibitory effect of autoantibodies on C3b binding, wells were coated with FH19-20 at 5 μg/ml. After blocking with BSA-PBS, the wells were incubated with 500 μg/ml purified IgG, then 2 μg/ml C3b was added. C3b-binding was detected by HRP-conjugated anti-human C3.

Western Blot

The presence of native FHR-1 was analyzed by Western blotting. To this end, 0.4 μl patient serum diluted in non-reducing sample buffer was run on 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane, and after blocking, the membrane was incubated with the anti-FH mAb C18, which recognizes both FH and FHR-1 (40), followed by HRP-conjugated goat anti-mouse Ig. The blot was developed using the ECL detection kit (Merck).

IgG Isolation and Analysis

10 μl serum diluted in DPBS was incubated with protein G beads (Life Technologies, Budapest, Hungary) for 2 h at 20°C. After washing, the bound IgG fraction was eluted with non-reducing sample buffer and analyzed for the presence of FH and FHR-1 by SDS-PAGE and Western blotting using the anti-FH mAb C18 followed by HRP-conjugated goat anti-mouse Ig for detection.

For epitope analysis of FH autoantibodies immobilized peptides were used. To this end, acetylated linear 15-mer peptides overlapping in 10 amino acids, and covering the FH SCRs 19-20 (amino acids 1107-1231) as well as their S1191L and V1197A modified peptides corresponding to the homologous FHR-1 sequence were designed. The peptides were prepared in duplicates on functionalized hydroxypropylmethacrylate non-cleavable gears of a nominal capacity of 66 nmol (Mimotopes, Clayton Victoria, Australia) by solid phase Fmoc/tBu peptide synthesis according to Geysen's method (49), as described earlier (50), with slight modifications. Briefly, the Fmoc protecting groups were removed by 2 v/v% piperidine/2 v/v% 1,8-diazabicyclo[5.4.0]undec-7-ene in N,N-dimethylformamide, the Fmoc-protected amino acid derivatives were coupled by N,N′-diisopropylcarbodiimide/1-hydroxybenzotriazole in N,N-dimethylformamide using ~200 eq reagents. After building up the peptide chains, the N-terminal α-amino group was acetylated and the side chain protecting groups were cleaved with TFA/thioanisole/phenol/water/EDT 82.5:5:5:5:2.5 (v/v/v/v/v).) The peptides remained covalently attached to the gears and were used in linear epitope mapping of the anti-FH autoantibodies.

Autoantibody binding to the synthetic peptides was detected using a modified ELISA described earlier (51). After blocking the non-specific binding sites with 0.5% gelatin in PBS, the gears were incubated with 150 μl of 1:600 diluted sera in PBS/0.5% gelatin/0.05% Tween-20 for 1 h at 20°C. Autoantibody binding was detected using HRP-conjugated rabbit anti-human IgG (DakoCytomation) and TMB detection system. Gears were used repeatedly after thorough cleaning by sonication in PBS containing 1% SDS and 0.1% 2-mercaptoethanol. The ODs were normalized by the following formula: OD_{sample}/OD_{min}, where OD_{sample} is the mean of duplicate OD values of the test samples and OD_{min} represents the mean binding to the negative control HSP 480-489 peptide, chosen based on our previous study (50). Data were further normalized to OD obtained with sera of healthy controls.

RESULTS

Identification of FH Autoantibodies in NMOSD Sera

In order to identify autoantibodies to complement proteins in sera of NMOSD patients, ELISA was performed using immobilized FH, C3b and FB, as well as solid-phase C3bBb convertase. Of the 45 samples analyzed, four were positive for FH autoantibodies (i.e., ~ 9%), and specific autoantibodies to C3b, FB or C3bBb were not detected in any of the samples (**Figure 1 and data not shown**). Some samples showed high background binding; these were considered negative for the autoantibodies if binding to all antigens, including HSA, was similar. In addition, we depleted IgGs from these autoantibody positive samples to facilitate the detection of IgM and IgA isotype FH autoantibodies, if present. There was no specific signal detected for these samples, except for the IgG-depleted serum of patient #210, where slight IgA positivity was observed (**Supplementary Figure 1**). We also tested the presence of autoantibodies to C1q; a few but none of the FH autoantibody positive NMOSD serum samples were positive for C1q autoantibodies (**Supplementary Figure 2**), confirming previous report (19).

Because FH autoantibodies are strongly associated with the deletion of the *CFHR1* gene in aHUS (52, 53), we investigated the sera of the above four patients for the presence of FHR-1 protein, since DNA samples were not available. The patients did not receive plasma treatment, thus exogenous origin of FHR-1 could be excluded. All four patients had both FHR-1 isoforms in their serum as detected by Western blot analysis (**Figure 2**).

We also analyzed the presence of FH-autoantibody immune complexes in the patients' sera. To this end, IgG was precipitated using Protein G beads, and the bound proteins were eluted and analyzed by Western blot using the anti-FH mAb C18. FH was detected in the sera of patients #64, #84 and #210, displaying stronger bands compared with the IgG of a healthy individual, used as control (**Figure 3**). In addition, FHR-1 was detected in the case of patient #210, indicating cross-reactivity of the FH autoantibody with FHR-1 (**Figure 3**).

Biological Features (Isotype, Titer, Avidity) of the FH Autoantibodies

Next, we determined the isotypes of the FH autoantibodies by ELISA. The FH autoantibodies were of the IgG3 isotype in all four patients, and had κ light chains, except for #210, who had λ light chains. The autoantibody titers were determined by applying serial serum dilutions on HSA- and FH-coated microtiter plate wells. The autoantibody titers of three of the

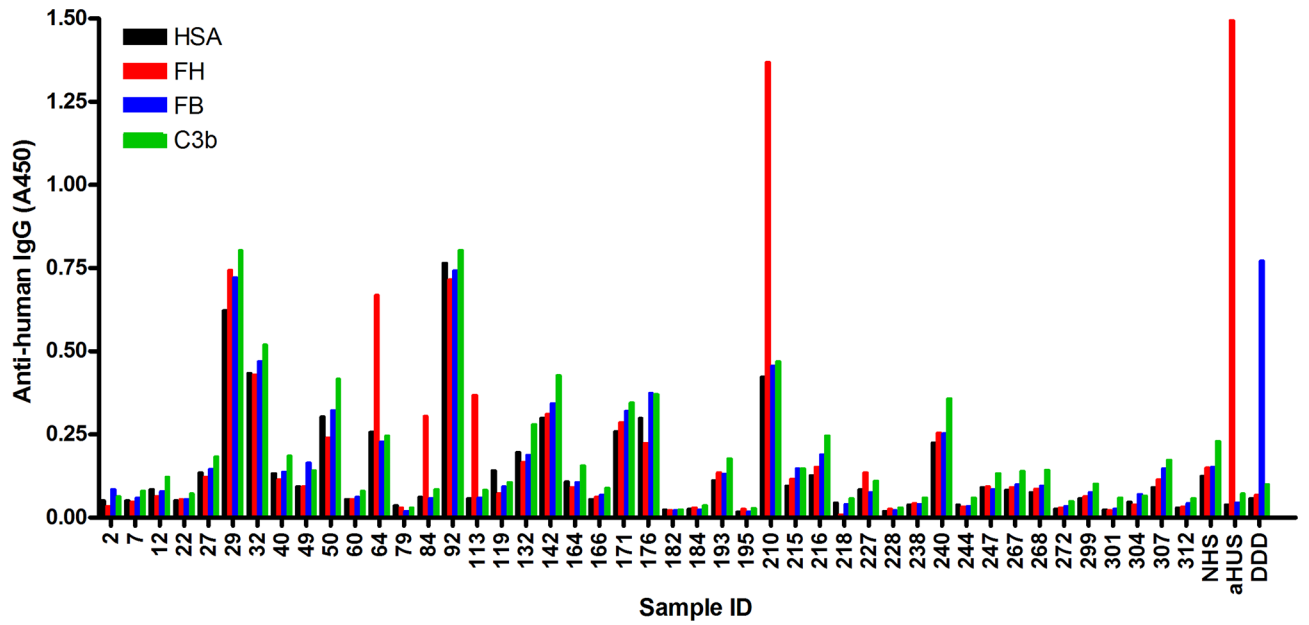

FIGURE 1 | Screening of NMOSD sera for autoantibodies by ELISA. Microplate wells were coated with human serum albumin (HSA), FH, FB and C3b, and after blocking, incubated with sera of 45 NMOSD patients and controls (all serum samples diluted 1:50 in PBS). Binding of autoantibodies to these antigens was detected using HRP-conjugated anti-human IgG. Serum sample of a patient with atypical hemolytic uremic syndrome (aHUS) and of a patient with dense deposit disease (DDD), positive for FH and FB autoantibodies, respectively, were used as positive controls. NHS: normal human serum. Data are means of two measurements. Some samples showed reactivity or high background with all four antigens, and these were considered autoantibody negative. The four samples #64, #84, #113 and #210 showing clearly stronger reactivity with FH compared to reactivity with HSA, FB and C3b, were considered autoantibody positive.

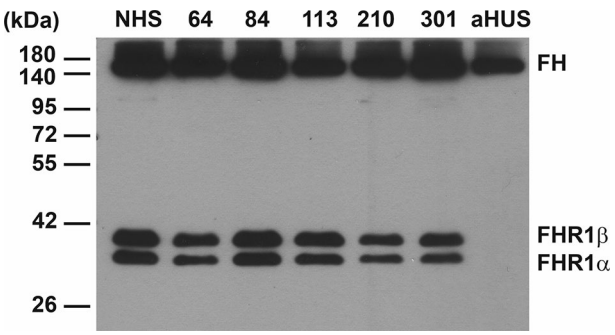

FIGURE 2 | Western blot analysis of NMOSD sera for FHR-1 protein. 0.4 µl of serum samples were run on 10% SDS-PAGE and the blot was developed using the anti-FH mAb C18. The two isoforms of factor H-related protein 1 (FHR-1) are seen in the 4 FH autoantibody positive NMOSD patients (64, 84, 113, 210), in an NMOSD patient (301) negative for FH autoantibody, and in a healthy control sample (NHS). FHR-1 is missing in a FH autoantibody positive aHUS patient, used as a control sample, as typical for ~90% of aHUS patients with FH autoantibodies. The blot is representative of two experiments.

patients were low (#64, 1:200; #84, 1:100, and #113, 1:200) and one (#210, 1:800) was higher, similar to a typical, autoimmune aHUS-associated high-titer anti-FH antibody, used as positive control.

The avidity of the autoantibodies was determined using NaSCN to dissociate the FH-bound autoantibodies. A relatively low NaSCN concentration (0.5 M) was sufficient to dissociate the majority of the autoantibodies (**Figure 4A**). Autoantibodies of #210 showed slightly higher avidity, since ~80% of the autoantibodies were able to bind to FH at 0.25 M NaSCN, in contrast to the other patients' autoantibodies. The small values of avidity indexes calculated at 0.5 M NaSCN (**Figure 4B**) indicated relatively low-avidity interaction between the autoantibodies of these NMOSD patients and FH.

NMOSD-Associated FH Autoantibodies Bind to the FH C Terminus

Recombinant deletion mutants of FH, recombinant FHR-1 and, as a control, FHR-4B protein were used to determine the binding domains within FH. All four autoantibodies bound to the C-terminal domains of FH and cross-reacted with FHR-1 (**Table 2**). However, the binding profiles were slightly different: the three samples with low autoantibody titers bound strongly to FH15-20, FH19-20 and FHR-1, and comparatively weaker to purified, full-length FH, whereas the sample of patient #210, which showed a high background in ELISA and had relatively higher autoantibody titer, showed very weak binding to FH15-20, but bound equally well to FH19-20, FH and FHR-1 (**Table 2**).

To confirm and further characterize the binding site of these FH autoantibodies, two C-terminally binding mAbs against FH were used in competition assays. The inhibition profiles were heterogeneous. The mAb C18 recognizing an epitope in SCR20 (44) caused ~30% and ~50% inhibition of autoantibody binding

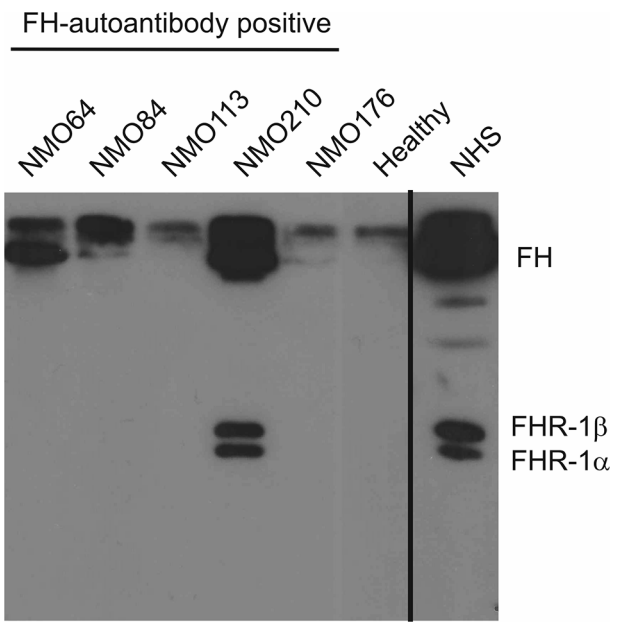

FIGURE 3 | Detection of FH–autoantibody complexes. Western blot analysis of the IgG fractions for FH/FHR-1 – IgG complexes. 10 µl of serum samples were incubated with Protein G beads. The bound proteins, eluted with SDS-sample buffer, were run on 10% SDS-PAGE, transferred to nitrocellulose membrane, and the blot was developed with monoclonal anti-FH recognizing also FHR-1 (mAb C18). FH is detected in the FH-autoantibody positive NMOSD patients (NMO64, NMO84, NMO210), but not in the FH-autoantibody negative (NMO176) or healthy control sample. In addition, FHR-1 is also seen in the NMOSD sample with the highest autoantibody titer (NMO210). Normal human serum (NHS) was run as a control. The blot is representative of three experiments.

to FH in the case of patients #113 and #210, respectively, whereas it had no significant inhibitory effect in the case of patients #64 and #84. The mAb IXF9 recognizing an epitope within FH SCR18-19 (41) inhibited autoantibody binding to FH by ~30% in the case of patients #64, #84 and #113, whereas its slight inhibitory effect did not reach statistical significance in the case of patient #210 (**Figure 5**). In the case of a control sample from an FH autoantibody positive aHUS patient, mAb IXF9 did not inhibit autoantibody binding to FH, but mAb C18 almost completely blocked autoantibody binding (**Figure 5**).

TABLE 2 | Summary of autoantibody binding sites on FH.

	NMO64	NMO84	NMO113	NMO210
FH	(+)	(+)	(+)	+
FH1-4	-	-	-	-
FH8-14	–	–	–	–
FH15-20	+	+	+	(+)
FH19-20	+	+	+	+
FHR-1	+	+	+	+
FHR-4B	–	–	–	–

The binding sites of the autoantibodies were determined using recombinant FH fragments containing domains 1-4 (FH1-4), 8-14 (FH8-14), 15-20 (FH15-20) and 19-20 (FH19-20), purified FH, the recombinant FH-related FHR-1 and FHR-4B proteins, the latter used as a negative control.
"(+)" indicates weak binding, "+" indicates prominent binding, "-" indicates no binding.

To further analyze the autoantibody binding sites, 14 recombinant FH19-20 fragments with different single amino acid exchanges were used. With this approach, in the case of patient #210 strongly reduced autoantibody binding (50% or less binding) to the R1182A, W1183L, K1186A, K1188A and E1198A mutants was found, indicating that these residues are included in the binding site of the autoantibody (**Figure 6**). This site is within the hypervariable loop of FH SCR20 and coincides with the autoantibody epitope identified for most aHUS patients, as well as with the binding epitope of mAb C18 (44, 47, 54). Using the three other patients' sera, no significant reduction in autoantibody binding to any of the tested mutants was found, except for ~25% or less inhibition of binding to the D1119G, K1186A and E1198A mutants in the case of #64, suggesting that their binding epitope lies elsewhere in SCR19 or SCR20.

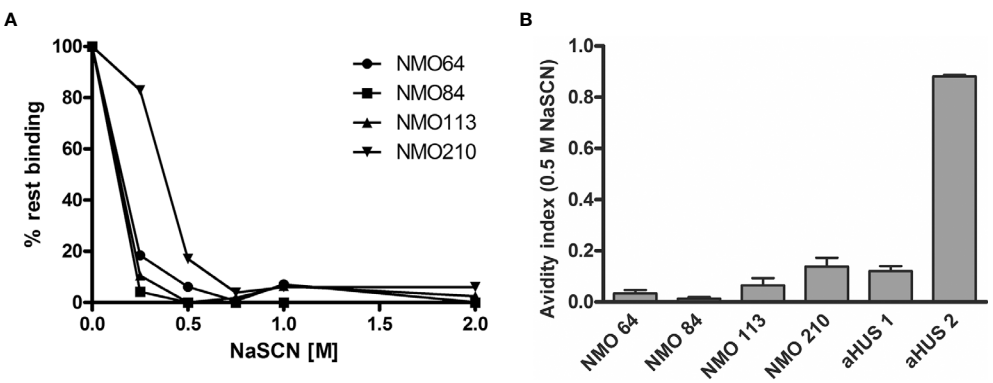

FIGURE 4 | Avidity of FH autoantibodies. Avidity of the FH autoantibodies was determined by ELISA using NaSCN as a chaotropic agent. **(A)** Avidity profile of the autoantibodies. Residual binding of the FH autoantibodies from serum was measured after applying various NaSCN concentrations. Data are normalized to binding in the absence of NaSCN (=100% binding). A representative experiment out of two is shown. **(B)** Avidity indexes calculated at 0.5 M NaSCN. Avidity index is calculated as the ratio of remaining bound autoantibody after NaSCN elution (AU/ml)/autoantibody bound without NaSCN elution (AU/ml). Data are means ± SD of three measurements.

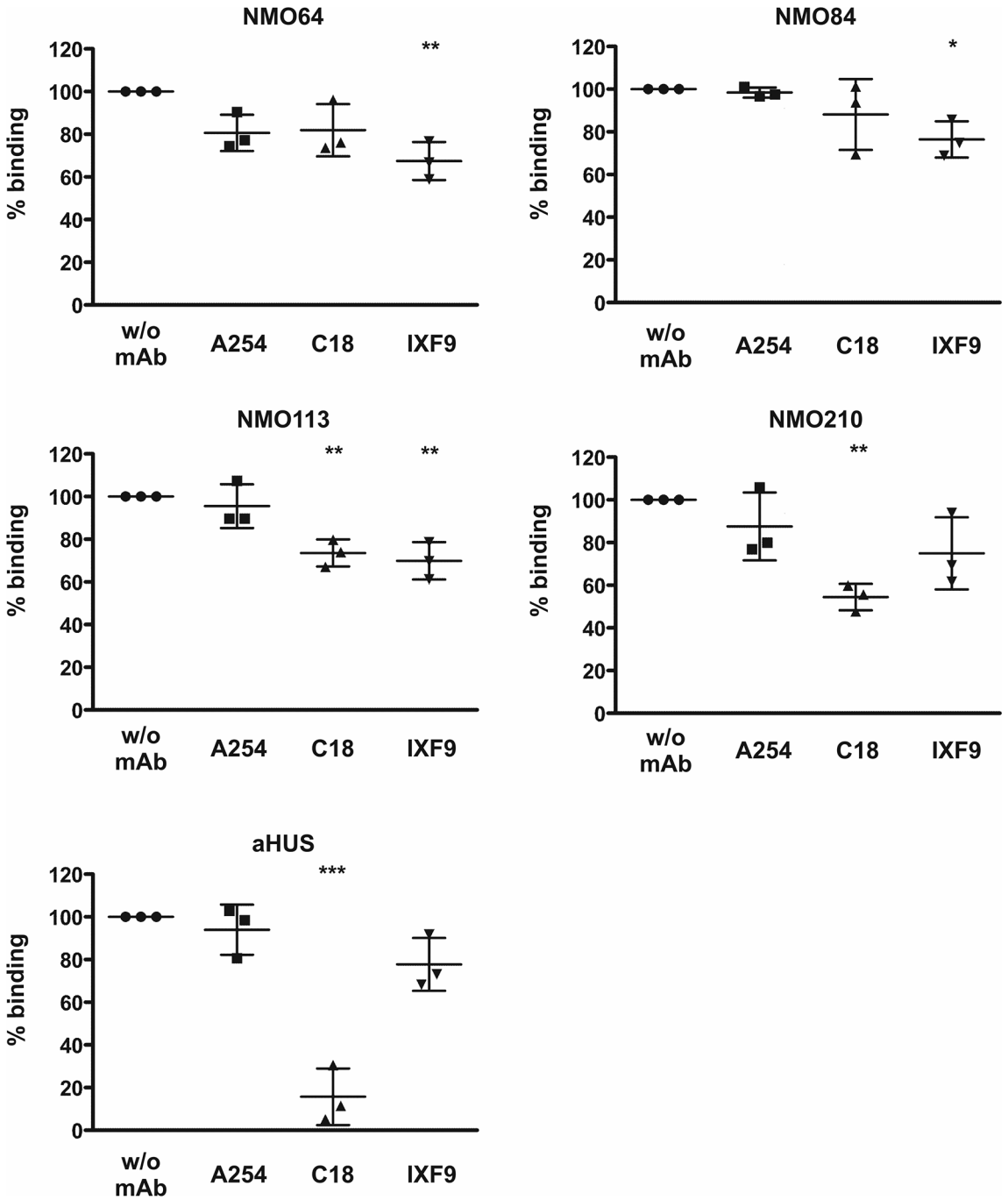

FIGURE 5 | Inhibition of autoantibody binding to FH by mAbs. Immobilized FH was preincubated with the anti-FH mAbs A254 (binding in SCR1), C18 (binding in SCR20) and IXF9 (binding in SCR18-19), then serum samples of the FH autoantibody positive four NMOSD patients and of an aHUS patient, used as control, were added to the wells. Autoantibody binding was detected by HRP-conjugated anti-human IgG. Data are normalized to autoantibody binding in the absence of mAb. Data are mean ± SD of three measurements. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, one-way ANOVA.

Linear epitope mapping of the autoantibodies was performed by peptide analysis using overlapping 15-mer peptides covering FH19-20. A heterogeneity of the binding sites of the autoantibodies was clearly detectable. Samples of #64 and #113 were positive for peptides derived from SCR19, the sample #210 reacted with peptides in SCR20, whereas autoantibodies of #84 bound to peptides of both SCRs (**Figure 7A**). Peptides corresponding to the differences in the FH SCR20-homolog domain of FHR-1, *i.e.* including the FH S1191L and V1197A amino acid exchanges, were also synthesized and analyzed. The

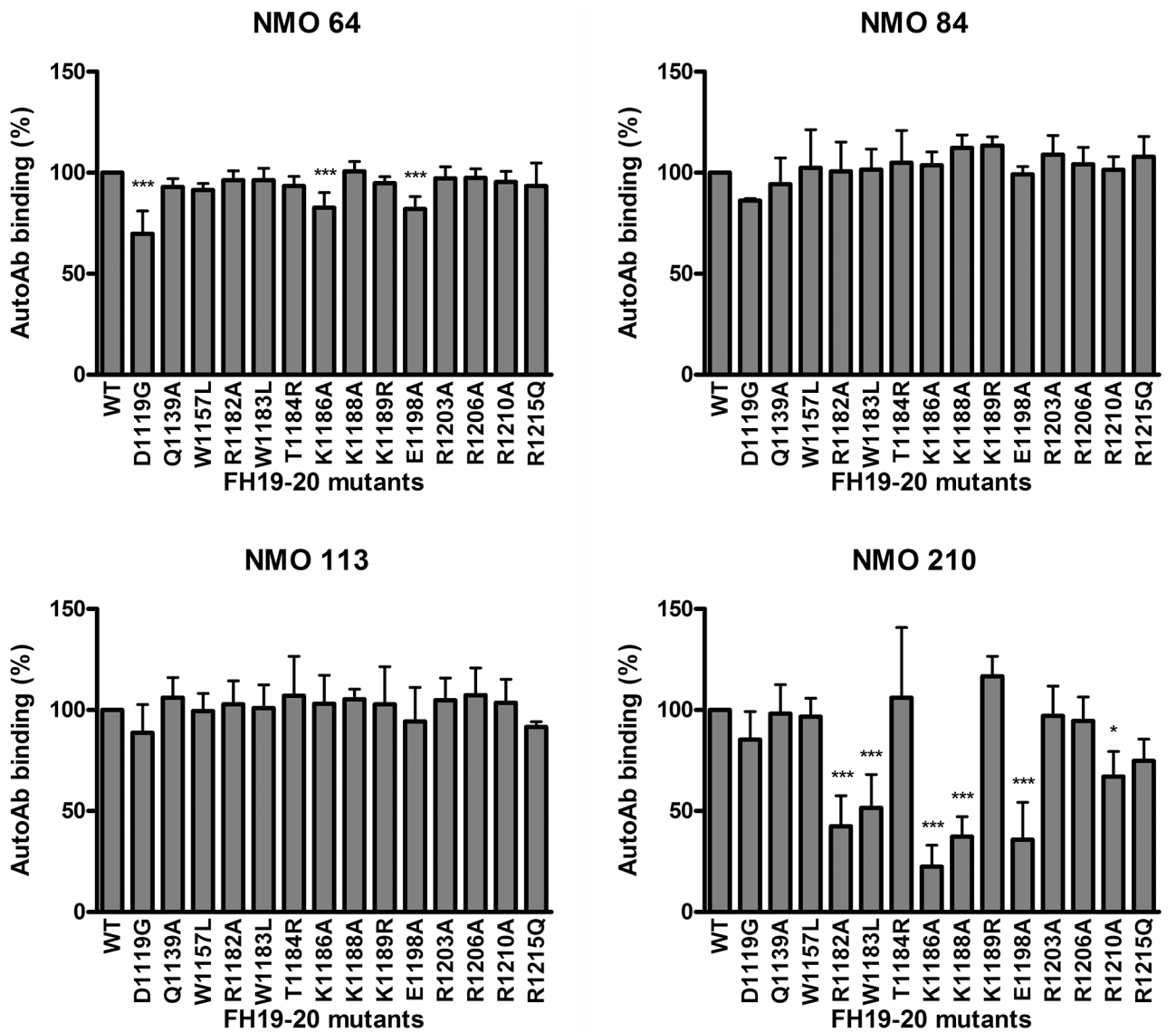

FIGURE 6 | Epitope mapping using mutant FH19-20 fragments. The wild type FH19-20 fragment and 14 mutants containing single amino acid exchanges were immobilized in microtiter plate wells and incubated with patient serum. Autoantibody binding was detected using HRP-conjugated anti-human IgG. Data are mean ± SD of three experiments. *$p < 0.05$ and ***$p < 0.001$, one-way ANOVA.

peptide reactivity by the autoantibodies confirmed the cross-reactivity of the NMOSD-associated FH autoantibodies with FHR-1; interestingly, the sample of patient #210 showed strongly increased binding to the FHR-1 peptide 286-300 in comparison with the corresponding FH peptide 1187-1201 (**Figure 7B**). The identified peptides are shown on the FH19-20 structure in **Figure 7C**.

The SCR19 peptide 1114-PIDNGDIT-1121 was previously identified as a binding site for FH-autoantibodies detected in patients with non-small cell lung cancer, and the autoantibodies recognized FH particularly when FH was reduced. To further characterize the NMOSD-associated FH autoantibodies in this regard, the binding of autoantibodies to FH and TCEP-treated, reduced FH was compared. In this assay, the three samples that showed SCR19 reactivity in the epitope mapping assays, #64, #84 and #113 showed markedly increased binding to reduced FH, whereas in the case of #210 and an aHUS patient sample with known SCR20-binding autoantibodies, reduction of FH did not result in enhancement of reactivity (**Figure 8**).

FH Autoantibodies of NMOSD Patients Inhibit the Interaction of FH With C3b

To assess whether FH autoantibodies of the NMOSD patients interfere with FH function, we analyzed the interaction of C3b with the FH19-20 fragment in the presence of the autoantibodies. IgG of patients #64 and #84 inhibited C3b binding to FH19-20 by

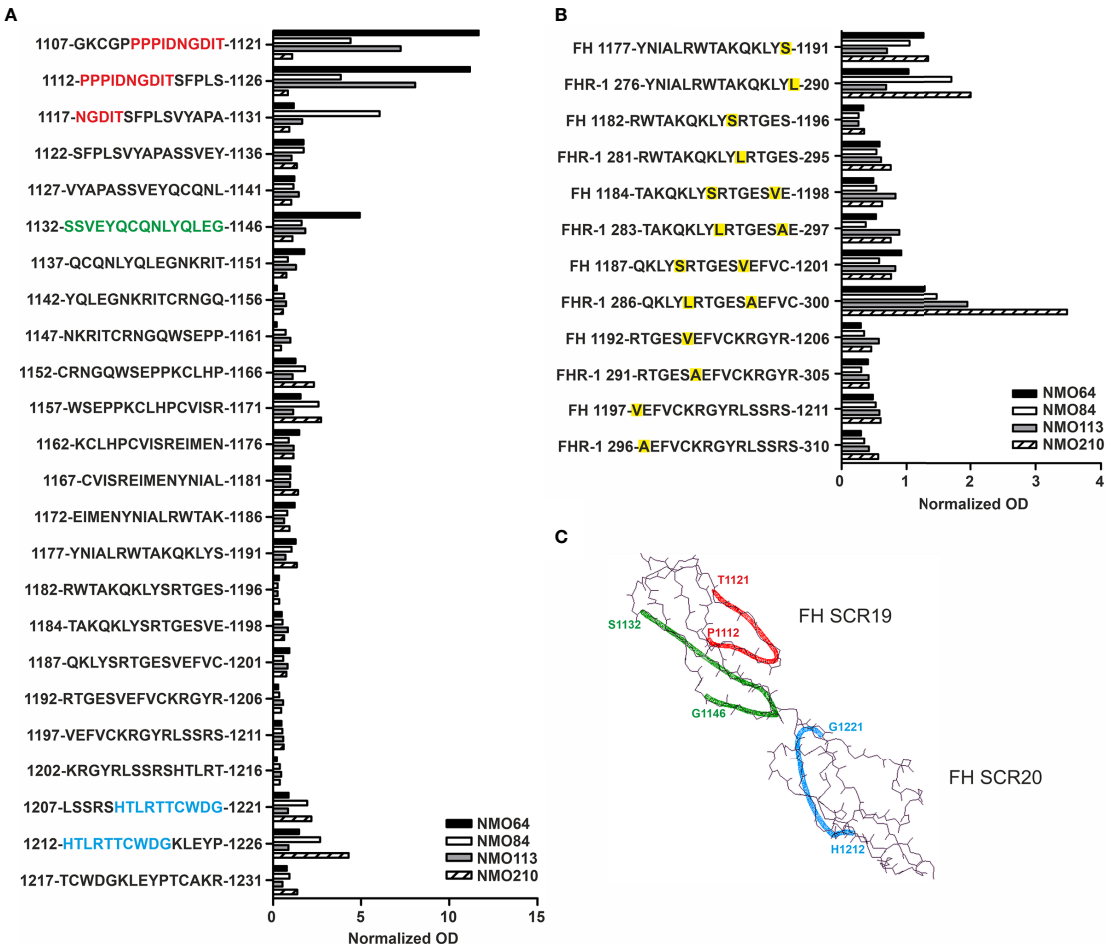

FIGURE 7 | Linear epitope mapping of the FH autoantibodies. Overlapping 15-mer solid phase peptides **(A)** covering the 19-20 domains of FH and **(B)** containing the FH S1191L and V1197A FHR-1 specific amino acid exchanges (indicated by yellow highlighting) were incubated with patients' sera. Autoantibody binding was detected using HRP-conjugated anti-human IgG, and is expressed as ratio of ODsample/ODmin, where ODsample is the mean of duplicate OD values of the patients' samples, while ODmin represents the mean antibody binding to the negative control HSP480-489 peptide. On the *y* axis the initial and final amino acid of each tested peptide is displayed with the single-letter amino acid sequence indicated in between. **(C)** The schematic picture of the FH C-terminal domains shows the identified epitopes highlighted in red (1112-1121), green (1132-1146) and blue (1212-1221), corresponding to the color codes of the one-letter amino codes in A.

~40%, IgG of patient #113 by ~30%, and that of patient #210 by ~70%. By contrast, IgG derived from healthy individuals or NMOSD patients without autoantibodies to FH did not affect C3b binding (**Figure 9**).

DISCUSSION

Anti-complement autoantibodies are involved in several different diseases (12–14). For most autoantibodies a directly pathogenic role is not proven and therefore a matter of debate, such as in the case of C3NeF. FH autoantibodies appear pathogenic in aHUS and dense deposit disease, as functional consequence of the presence of the autoantibodies was described in terms of interfering with the interaction of FH with C3b and host cells and with the cofactor activity of FH, respectively (33, 34). FH autoantibodies are reported in 8-25% of aHUS patients in different cohorts and are strongly associated with homozygous deletion of the *CFHR1* gene, but are more rarely reported in patients with C3 glomerulopathies including dense deposit disease (32, 53, 55). On the other hand, FH autoantibodies might have a protective role such as described in patients with non-small cell lung cancer (46, 56). FH autoantibodies were also reported in inflammatory, autoimmune diseases where their role is less clear (57, 58). In addition, autoantibodies against the C3bBb convertase and its components C3b and FB are described in diseases associated with alternative complement pathway dysregulation (14, 45, 59–61). Therefore, we studied whether autoantibodies against C3bBb, C3b, FB and FH occur in NMOSD, a spectrum disease characterized by pathological complement activation.

FH autoantibodies were detected in four out of 45 NMOSD serum samples (~9%), while no antibodies against C3b, FB and C3bBb were found in these samples (**Figure 1**). The relevance of C1q autoantibodies that were detected in some samples

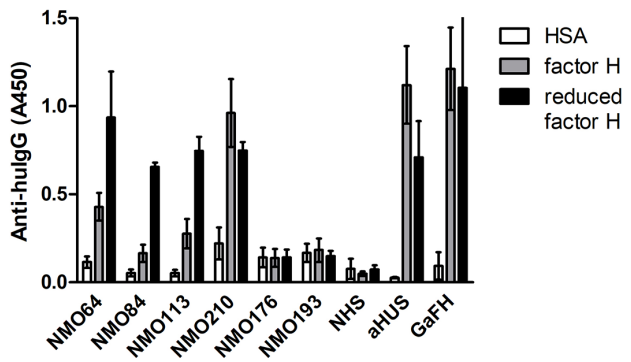

FIGURE 8 | Autoantibody binding to reduced FH. Microplate wells were coated with equimolar amounts of HSA, FH and TCEP-treated, reduced FH, and after blocking, incubated with patients' sera diluted 1:50 in PBS. Autoantibody binding was detected by HRP-conjugated anti-human IgG. As controls, sera of two FH autoantibody-negative NMO patients (#176 and #193), an aHUS patient and a healthy control (normal human serum, NHS) were used. Goat antiserum was used to prove that FH and reduced FH were immobilized on the plate. Data are mean ± SD of two experiments.

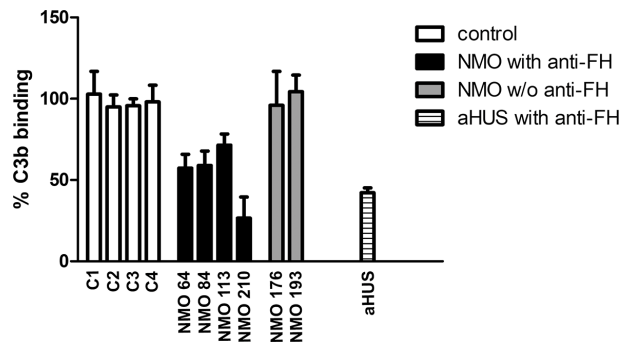

FIGURE 9 | Inhibition of C3b binding to FH19-20 by FH autoantibodies. The immobilized FH19-20 fragment was incubated with IgG purified from the sera of NMOSD patients, healthy human sera (C) or serum of an aHUS patient with FH autoantibodies, then C3b was added. C3b binding was detected using HRP-conjugated anti-human C3 antibodies. Data are normalized to binding in the absence of IgG (=100%) and are means + SD of three measurements.

(**Supplementary Figure 2**) should be investigated in the future. Three of the four identified FH autoantibodies were similar to each other by having low-titer, low-avidity autoantibodies and displaying identical binding profile to FH domains. The fourth autoantibody, that of patient #210, was clearly different and resembled more the aHUS-associated antibodies than the other three, by having high-titer FH autoantibodies and binding to the same hypervariable loop on SCR20 of FH (**Figures 5**–**9**) as the aHUS-associated autoantibodies (44). The autoantibodies bound to FH domains 19-20, and also recognized the homologous protein FHR-1, similar to FH autoantibodies associated with aHUS (44, 62, 63). However, in contrast to most autoantibody-positive aHUS patients, these four NMOSD patients did not lack the FHR-1 protein. The clear detectability of native FHR-1 in immune complexes of patient #210 could have been because of the higher autoantibody titer and avidity, as well as stronger reactivity with FHR-1 peptides in the case of this patient compared with the three other NMOSD patients (**Figures 3**, **4**, **7**). In both NMOSD and aHUS, the IgG3 isotype dominated among the FH autoantibodies, indicating infection- or inflammation-related generation of the autoreactive antibodies. Recently, based on the slight structural differences between the C termini of FH and FHR-1, and the lack of FHR-1 in most aHUS patients, we proposed a model for the generation of the aHUS-associated autoantibodies in the context of infection and induced neo-epitope due to slight structural change in the FH C terminus upon binding to microbial proteins (44). Collectively, the results of our experiments suggest a mechanism of autoantibody generation in the NMOSD patients different from that in the aHUS patients.

The FH19-20 and FHR-1 peptides recognized by the four NMOSD autoantibodies are in part similar to those described in aHUS patients (**Figure 7**) (54). These include the linear epitopes 1152-1171 in FH SCR19, which showed weak reactivity with the FH autoantibody positive NMOSD sera and strong reactivity with aHUS sera, and the peptides 1207-1226 in FH SCR20. The FH SCR19 peptides 1107-1131 and 1132-1146 showed reactivity only with the FH autoantibody positive NMOSD sera, but not with aHUS sera. The peptides FH SCR20 1177-1191 and the homologue FHR-1 276-290 showed only weak reactivity with the NMOSD sera compared with the strong reactivity of the autoantibody positive aHUS sera (**Figure 7**) (54). The FHR-1 peptide 286-300 showed strong reactivity with the serum of patient #210, and this peptide was non-reactive with aHUS sera. Interestingly, FH autoantibodies found in patients with non-small cell lung cancer recognize the peptide PIDNGDIT in FH SCR19, inhibit FH binding to lung carcinoma cells and cause increased C3-deposition when binding to FH that is already bound to the cancer cell surface (46). In our experiments, the linear epitope analysis showed the common recognition of epitope PPPIDNGDIT (SCR19 1107-1131) by FH autoantibodies of patients #64, #84 and #113, and these samples also showed enhanced reactivity with reduced FH (**Figures 7**-**8**). In the lung cancer study, the patients' sera reacted strongly with reduced FH compared with the non-reduced protein, suggesting a cryptic epitope and/or a cancer-specific, posttranslational modification of the protein that is recognized by the autoantibodies. Similarly, it is possible that in NMOSD lesions the ongoing inflammation and damage of glial cells cause a slightly reducing microenvironment that may influence the conformation of FH, and allow for inflammation-driven induction of autoreactivity against this complement regulator.

Although recognizing different epitopes, autoantibodies of all patients affected binding of FH to C3b, with that of patient #210 strongly inhibiting the FH-C3b interaction (**Figure 9**). Since the interaction of the FH C terminus with C3b is critical for docking FH to C3b-covered surfaces and allowing FH to act as a regulator at the surface (37, 38, 64), the presence of these interfering autoantibodies may contribute to ongoing complement activation and damage of host cell surfaces, e.g. on astrocytes, where complement activation was initially triggered by NMO-IgG.

It is important to note that these NMOSD patients, particularly #210, despite having FH autoantibodies with overlapping characteristics and similar, C-terminal binding sites as the aHUS-associated FH autoantibodies, did not have manifest kidney disease. This might also be related to the relatively low avidity of the NMOSD-associated FH autoantibodies or difference in the exact binding site, compared to FH autoantibodies from aHUS patients' sera. In addition, a fraction of the autoantibodies could bind FHR-1 instead of FH. However, it is theoretically possible that co-existence of AQP4-antibodies and FH-antibodies may contribute to subclinical impairment of kidney functions. Complement regulators are important in preventing peripheral organ injury in NMOSD patients and in the animal model of NMOSD (65, 66). However, the urine proteome and metabolome of NMOSD is different from multiple sclerosis (67, 68). Whether this reflects kidney alterations in a subgroup of patients with FH autoantibodies maybe worth investigating.

A characteristic feature of NMOSD is the increased frequency of associated autoantibodies and autoimmune diseases. Antibodies against gastrointestinal antigens may be present (69), and antinuclear antibodies were detected in 44% of patients with NMOSD (70). AQP4-antibodies were detected in patients with rheumatologic diseases in the presence of NMOSD-associated syndromes (71, 72), and temporal changes in SLE-associated antibody levels overlap with dynamics of AQP4-antibodies (73). Generation of autoantibodies against complement regulators, such as FH may be part of a co-existing condition in patients with susceptibility to multiple autoimmunity (72). We also describe that autoantibodies against the natural complement inhibitor FH in NMOSD patients impair the interaction of FH with C3b, which is the basis of its complement regulatory activity. This in turn may contribute to disease activity.

Limitations of our study include the low patient and sample number due to the rarity of the disease, and the lack of complement-active serial serum samples that restricted the breadth and the power of the analyses. At present, no clear conclusion on the correlation of the presence of FH autoantibodies with the clinical manifestation can be drawn. Analysis of additional patient cohorts and samples is expected to establish the frequency and the biological characteristics of the FH autoantibodies in NMOSD, and also whether and how these autoantibodies may contribute to the pathology of the disease and influence the clinical phenotype.

In conclusion, our results demonstrate that systemic FH autoantibodies are not uncommon in NMOSD, and they influence binding of FH to its main ligand, complement C3b. Our data also suggest that generation of autoantibodies against complement regulating factors among other autoantibodies may contribute to the complement-mediated damage in NMOSD.

AUTHOR'S NOTE

Parts of this work were presented at the 25th International Complement Workshop, September 14-18, 2014, Rio de Janeiro, Brazil (*Mol. Immunol.* 2014, 61: 227).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Ethical Committee (3893.316-12464/KK4/2010 and 42341-2/2013/EKU). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MJ initiated and supervised the study. BU and ZS performed autoantibody detection and characterization, Western blot and competition assays. KU designed and synthesized peptides. ET performed peptide binding assays. HN, ZI and ZP provided serum samples and patient data. SH and TJ provided recombinant mutant proteins. AE interpreted data. All authors contributed to the article and approved the submitted version. BU, ZI and MJ wrote the manuscript with the help of the other authors.

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SUPPLEMENTARY MATERIAL

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Mini-Factor H Modulates Complement-Dependent IL-6 and IL-10 Release in an Immune Cell Culture (PBMC) Model: Potential Benefits Against Cytokine Storm

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Cytokine storm (CS), an excessive release of proinflammatory cytokines upon overactivation of the innate immune system, came recently to the focus of interest because of its role in the life-threatening consequences of certain immune therapies and viral diseases, including CAR-T cell therapy and Covid-19. Because complement activation with subsequent anaphylatoxin release is in the core of innate immune stimulation, studying the relationship between complement activation and cytokine release in an *in vitro* CS model holds promise to better understand CS and identify new therapies against it. We used peripheral blood mononuclear cells (PBMCs) cultured in the presence of autologous serum to test the impact of complement activation and inhibition on cytokine release, testing the effects of liposomal amphotericin B (AmBisome), zymosan and bacterial lipopolysaccharide (LPS) as immune activators and heat inactivation of serum, EDTA and mini-factor H (mfH) as complement inhibitors. These activators induced significant rises of complement activation markers C3a, C4a, C5a, Ba, Bb, and sC5b-9 at 45 min of incubation, with or without ∼5- to ∼2,000-fold rises of IL-1α, IL-1β, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13 and TNFα at 6 and 18 h later. Inhibition of complement activation by the mentioned three methods had differential inhibition, or even stimulation of certain cytokines, among which effects a limited suppressive effect of mfH on IL-6 secretion and significant stimulation of IL-10 implies anti-CS and anti-inflammatory impacts. These findings suggest the utility of the model for *in vitro* studies on CS, and the potential clinical use of mfH against CS.

Keywords: factor H, complement activation/inhibition, cytokine release syndrome, whole blood assay, COVID-19, immune stimulation, zymosan, anaphylatoxins

INTRODUCTION

Cytokine storm (CS), the most intense manifestation of cytokine release syndrome (CRS), is a dysregulated hyperactive immune response characterized by the release of a variety of mediators including but not limited to interleukins, chemokines, interferons, tumor-necrosis factor and other white blood cell (WBC) mediators which, unlike in physiological inflammatory responses, can damage the host. They are also produced as a consequence of severe adverse effect of some monoclonal antibodies and CAR-T-cell therapies (1, 2), and came to the focus of world-wide attention as a contributor to the acute respiratory distress syndrome (ARDS) in Covid-19, as the major mechanism of severe, often fatal outcome of SARS-CoV-2 infection (3–5).

For these reasons modeling CRS/CS *in vitro* is important for better understanding of these adverse conditions and screening of medications against them. It is with this goal that we carried out the studies described here, using a PBMC-culture model of CRS/CS that was found to correlate with *in vivo* features of the disease (6–8). Activation of the first line of immune defense, the complement system, has been known to be a critical contributor to cytokine release by activated immune cells in blood (9, 10). However, the current PBMC-based immunoassays usually utilize culture media supplemented with heat inactivated serum, which excludes getting insights into the role of complement in cytokine release. To fill this gap in *in vivo* relevance, we modified the traditional protocol by supplementing the culture medium with autologous serum. As presented below, this "complement-sensitized" test system enabled the assessment of the role of complement activation in CS/CRS, also highlighting the possible utility of mini-factor H (mfH) against these conditions. In particular, our data suggest that the latter protein, a truncated, recombinant version of the natural complement inhibitor, factor H (fH) (11–15), may have three independent beneficial actions against CS/CRS; suppression of complement activation and complement-dependent IL-6 production, and, stimulation of IL-10 production, a cytokine with anti-inflammatory properties (16–19).

MATERIALS AND METHODS

Materials

For the experiments Dulbecco's phosphate-buffered saline (D-PBS), ethylenediaminetetraacetic acid (EDTA), lipopolysaccharide from Escherichia coli (LPS), Zymosan A from Saccharomyces cerevisiae and the components of complete Growth Medium (cGM, consisting of RMPI-1640 with glutamine, 0.1 mM non-essential amino acids, 50 μM β-mercaptoethanol, 1 mM pyruvate and penicillin/streptomycin) were from Sigma-Aldrich Ltd. (Budapest, Hungary). Ficoll-Paque was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). AmBisome was purchased from Gilead Sciences Ltd. (Paris, France). The content of the vial, after reconstituting with 12 ml sterile water for injection, contained hydrogenated soy phospholipid (HSPC), 17.75 mg/mL; distearoyl-phosphatidylglycerol (DSPG), 7 mg/ml, amphotericin B, 4.2 mg/ml; cholesterol, 4.3 mg/ml; tocopherol, 0. 05 mg/ml; Sucrose, 75 mg/ml; Sodium succinate, 2.3 mg/ml. The 96-well cell culturing plates (U plate) were obtained from Sarstedt (Nümbrecht, Germany).

Preparation of Mini-fH

Mini-fH, a polypeptide construct consisting of the 4 N-terminal, ∼60 amino acid-containing complement control protein modules (also known as short consensus repeats (SCRs or Suchi repeats) and the two C-terminal SCRs of factor H, was produced in insect cells as described in Refs. (20, 21).

Mononuclear Cell and Serum Preparation From Blood

Blood was collected from healthy volunteers under ethical protocol TUKEB 15576/2018/EKU and the National Cancer Institute-at-Frederick protocol OH9-C-N046 (in the Nanotechnology Characterization Lab., NCL). Blood anticoagulated with EDTA or Li-heparin (at NCL) was used to purify PBMC using Ficoll Paque gradient density centrifugation according to the procedure described previously (8). Serum was separated by centrifugation of the whole blood at 4°C. Part of the serum was heated at 56°C for 30 min to inactivate complement.

PBMC Culture

After removing the residual Ficoll and the majority of thrombocytes by washings, PBMCs were washed again with cGM, and 50% autologous serum which was used in the final step for cell suspension. Culturing of PBMCs were done in 250 μl volume in the inner wells of 96-well cell culturing plates (Sarstedt U plate for suspension cells), and each well composed of PBMCs (11-times more concentrated than the original blood, 2.5–5 × 10^6 cells/well), 50% of normal or heat-inactivated autologous serum and the specified immune activators and complement inhibitors. Plates were incubated in a CO_2 incubator at 37°C, (except 0-min samples) and samples were obtained in three time points (45 min.: 60 μl, 6 h: 50 μl and 18 h: 140 μl) to prepare supernatants by centrifugation. Aliquots of cell culture supernatants were stored at −80°C until complement or cytokine measurements. For 0-min sampling, cells in cGM and 50% autologous (auto-SE) or heat-inactivated sera (Hi-SE) were immediately processed without any incubation, after diluting them by the solvents of activators (D-PBS) and complement inhibitors (cGM). In another, independent experiment (done at NCL according to the protocol NCL ITA-10 (22) PBMC from 10 healthy donors were incubated for 24 h in cGM supplemented either with 10% heat-inactivated fetal bovine serum (Hi-FBS) or 20% autologous human serum (auto-HS) obtained from the same donor. Cells were stimulated with 20 ng/ml E. coli K12 LPS (PBS served as negative control) and culture supernatants were analyzed by multiplex ELISA for the presence of cytokines (Quansys Biosciences, Logan, UT, USA).

Complement/Cell Activators and Complement Inhibitors

AmBisome, zymosan and LPS were applied at 2 mg phospholipid/mL, 0.5 mg/ml and 0.5 μg/ml, respectively. To inhibit complement activation EDTA was applied at 20 mM and mfH at 1 μM. Heat inactivation of complement in sera was done by incubation at 56° C for 30 min. In the independent experiment presented in **Supplementary Figure 1**, in addition to the above stimulants, liposomal doxorubicin (Doxil), phytohemagglutinin and phorbol myristate acetate (PMA)/Ionomycin were applied at 2 mg/ml, 0.1 mg/ml, 5 and 500 ng/ml, respectively.

Complement and Cytokine Measurements

Complement activation in PBMC supernatant was assessed at 45 min, 6 h and 18 h after starting the incubation by measuring C3a, C4a, C5a, Ba, Bb, and sC5b-9 by a 8-plex chemiluminescence immunoassay (CLIA) (Quansys Biosciences Inc., West Logan, UT, USA), or by individual ELISAs. The levels of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-23, IFNγ, TNFα and TNFβ at 6 and 18 h was measured in the same supernatants by a 16-Plex Human Cytokine kit also from Quansys Biosciences Inc. (West Logan, UT, USA), according to the recommendation of the manufacturer. Data collection was done by "Imager LS" from Quansys, using Q-View Software 3.11 for analysis. The C5a, Bb and sC5b-9 ELISA kits were from TECO*Medical Inc.* (Sissach, Switzerland).

Data Analysis

The 18-h cytokine values (mean ± SD for n=3 different donors) were either given in absolute, or relative terms, by dividing the final concentrations with the respective (0 min) baselines. If values of 0 min measurements were below the quantification limit, the Lower Limit of Quantification (LLOQ) were used for normalization after correction with the dilution. The choice of statistical analyses was based on the fact that the immune activators we used showed substantial differences in activation levels, thus, although the assays were done at the same time, they had to be considered as independent experiments. This ruled out pooling data from the different activator groups for ANOVA. The application of ANOVA was also problematic within the treatment groups because the independent variables were "manipulated within the subjects" inasmuch as cytokine suppression by EDTA could result both from direct cytokine inhibition and indirect complement blockage. Also, we were not "interested" in comparing the complement inhibitors to each other but asked the question of whether the inhibition of cytokine induction was correlating with inhibition of complement, one by one. For these reasons, and because of the low n, we used paired t-test wherein the dependent variable was compared to baseline for each individual analyte and inhibitor within an activator group. The use of one or two-tailed t-tests depended on whether the direction of changes was predictable or not and is specified in the figure legends. The analysis was performed using GraphPad Prism software (San Diego, CA, USA).

RESULTS

Complement Activation by AmBisome, Zymosan, and LPS

We analyzed complement activation in 2 experimental series, applying individual ELISAs in the first and an 8-plex CLIA in the second. **Figure 1A** shows the results of the first experiment, indicating significant rises of C5a, Bb and sC5b-9 after 45 min incubation with zymosan, AmBisome and LPS. The simultaneous and correlating rises of C5a and Bb (**Figure 1B**) indicates that formation of the most effective anaphylatoxin is primarily due to complement activation via the alternative pathway in the case of zymosan and AmBisome. The second series confirmed these changes for zymosan (**Figure 1C**) and AmBisome (**Figure 1D**) with the additional information that C3a, C4a and Ba also increased and that the levels of most activation markers decreased after 6 h incubation, except C4a. The effect of 20 mM EDTA is shown for AmBisome (**Figure 1D**, dashed curves), indicating full suppression of the rise of all activation byproducts, except C4a.

Inhibition of C Activation in PBMC Cultures

Figure 2 shows the effects of heat inactivation, EDTA and mini-fH on complement activations by AmBisome, Zymosan and LPS in PBMC cultures, using C5a, Bb and sC5b-9 as endpoints. All these inhibition methods caused major reduction of all activation markers, most efficiently those triggered by zymosan (**Figures 2C,G,K**). Mini-fH in this case was equally effective as EDTA or heat inactivation (**Figures 2C,G,K**), exerting > 90% inhibition of complement activation in all three donor PBMC. Interestingly, heat inactivation tended to increase spontaneous C5a and Bb formation in the absence of complement activators (**Figures 2A,E**).

Cytokine Release by AmBisome, Zymosan, and LPS in PBMC Cultures: Time Course and Relative Differences

Among the tested cytokines (see section Methods) IL-2, -4, -15, -17, -23, IFNγ, and TNFβ did not show measurable response to the applied immune stimulations (not shown) even after 18h incubation, while 9 cytokines shown in **Figure 3** did respond with significant elevations to one or more stimulators. As shown in **Figure 3A**, the responses relative to 0 min baseline varied between ∼5 to ∼2,000-fold. Because the 6 h values were generally significantly lower than the 18 h values for all cytokines except TNFα (**Figure 3B**), 6 h was in the window of dynamic changes for most cytokines, while TNFα could reach plateau already at 6 h. On the other hand, the lack of difference between LPS and zymosan in inducing maximal increase of some cytokines at 18 h (**Figure 3A**) suggest that the rise of these cytokines reached plateau at this time.

While the cytokine inducing effects of LPS and zymosan were known from previous studies, the effect of AmBisome was surprising since non-PEGylated, highly negative phospholipid vesicles, such as AmBisome, have been known to activate complement but not immune cells for cytokine release. On the other hand, amphotericin B *per se*, can induce cytokines in innate immune cells (23), thus, the membrane-associated antifungal

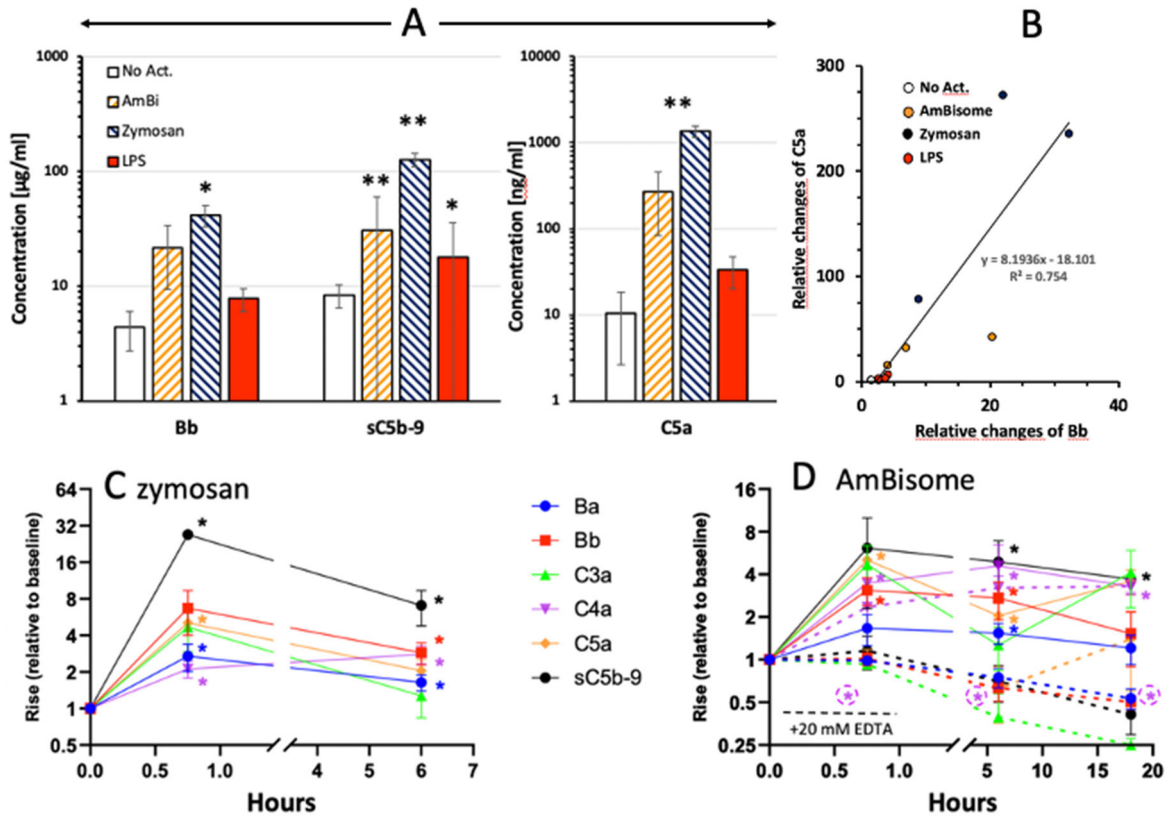

FIGURE 1 | Complement activation by liposomal Amphotericin B (AmBi, 1.98 mg PL/ml), Zymosan (0.5 mg/ml) and LPS (0.5 µg/ml) in PBMC cultures supplemented with autologous serum. **(A)** Columns and error bars represent mean ± SD (n=3); * and ** indicate statistically significant differences comparing to appropriate control (No Act. or baseline) groups, P<0.05 or 0.01, respectively. **(B)** Correlation between the individual relative rises (related to 0 min) of C5a and Bb in the samples plotted in **(A)**. Different groups of treatments are represented by different colors (empty: no activation, yellow: AmBisome, blue: Zymosan, red: LPS). Slope shows significant correlation (P=0.0002). **(C)** and **(D)**, Similar experiments as in **(A)**, except that the complement activation byproducts were measured by a chemiluminescence immunoassay.

agent might have played a role in the observed cytokine induction by AmBisome, particularly IL-6 and IL-8.

Interestingly, IL-10 was at baseline at 6 h during incubation with LPS (**Figure 3B**), although it rose to near maximum level at 18 h (**Figure 3A**). This implies retarded induction of a cytokine that has a negative feedback on the production of inflammatory cytokines (24). As discussed later, this effect may contribute to the strong proinflammatory effect of other stimulants. A further notable observation in **Figure 3A** is that LPS, whose complement activating effect was the smallest under these conditions, also led to robust cytokine release, just as zymosan, the strongest complement activator. This observation suggests that complement activation was not rate limiting in LPS-induced cytokine release, which is in keeping with differential influence of other controlling factors on the two processes, such as sCD14 and LPS-binding protein (LBP) in serum (25).

To explore the performance of our *in vitro* model at a lower level (10%) of autologous serum, we conducted an additional experiment using PBMCs of 10 healthy donors and tested their cytokine responses to the assay positive control (LPS). As control, we used complete cell culture media supplemented with 10% heat inactivated fetal bovine serum. This study also demonstrated variable, complement-independent induction of most cytokines by LPS except IL-1α and IL-1β, whose production was increased by 10% autologous serum (**Supplementary Figure 1**).

Taken together, these observations suggest differential regulation of cytokine secretion by complement activation byproducts, which can be studied by adding autologous serum to PBMC cultures. Another important finding in the present study is that PBMC cultures supplemented with autologous serum allow for analysis of cytokines that are known to rise in CRS/CS, including the syndrome observed in severe Covid-19 and immunotherapies such as CAR-T cells (26–28). Moreover, the *in vitro* system affords screening of inhibitory approaches, such as complement inhibition, as shown by the results below. The performance of this model is verified in two laboratories and demonstrates consistent results despite of the use of different percentages of autologous serum. Our study also contributes to the existing knowledgebase emphasizing the predictive capability of PBMC cultures in individualized screening of cytokine responses in human blood donors (29).

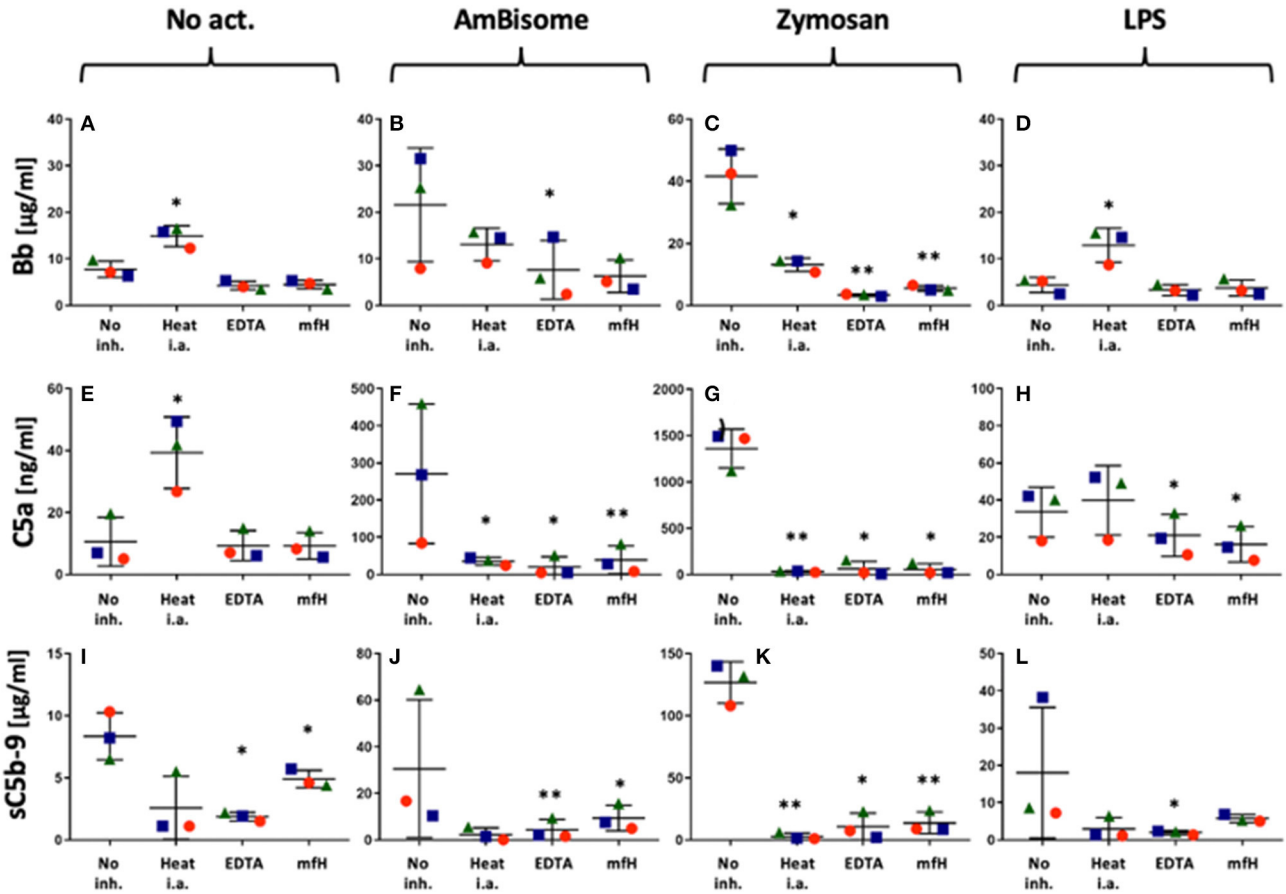

FIGURE 2 | Inhibition of AmBisome-, zymosan- and LPS-induced complement activation (ordered in columns) by different inhibition methods specified on the X axis. No act. and no inh. mean no added activator or complement inhibitor, respectively. On the X axes "Heat i.a." "EDTA" and "mfH" mean heat-inactivation of autologous serum at 56°C for 30 min, addition of 20 mM EDTA or 1 µM mfH, respectively. Different symbols specify the donors, the bars show the mean ± SD (n=3); *(P<0.05) and ** (P<0.01) indicate statistically significant differences using one-tailed T-test comparing the values to the No Act. group.

Differential Inhibition of Immune Activator-Induced Release of Cytokines by Different Approaches of Complement Inhibition

Figure 4 shows dot plots of individual responses of each responder cytokines following activation with 3 activators (stapled columns) for 18 h at 37°C with or without complement inhibition (inhibitors specified on the bottom axes). In order to show that the individual variation of cytokine responses, when ever seen, is due to differences in individual sensitivity of blood donors rather than measurement (random) error, the three PBMC donors are distinguished by different shapes and colors.

These data provide evidence that inhibition of complement activation can entail inhibition of some cytokines' release. This also means that complement activation contributes to the release of these cytokines, thus, the test system reproduces the clinical observations on the beneficial effects of complement inhibition in CRS/CS, including that observed in Covid-19. A repeat experiment using only AmBisome as stimulant and EDTA, as inhibitor, confirmed the complement-dependent response of IL-1α, IL-1β, IL-6, IL-10 and TNFα, as well as the lack of such response of IL-2 (**Supplementary Figure 2**).

Enhancement of Zymosan and LPS-Induced IL-10 Production by Mini-fH at 6 h

Figure 5, focusing on the inhibition of zymosan and LPS-induced cytokine release by mfH at 6 h, presents an unexpected significant stimulatory effect of this complement inhibitor on IL-10 induction on top of the effects of zymosan and LPS at this time.

DISCUSSION

Approaches of Complement Activation and Inhibition

The complement inducers used in this study represent different types of immune stimulants that act both in the humoral and cellular arms of the innate immune response. The liposomal

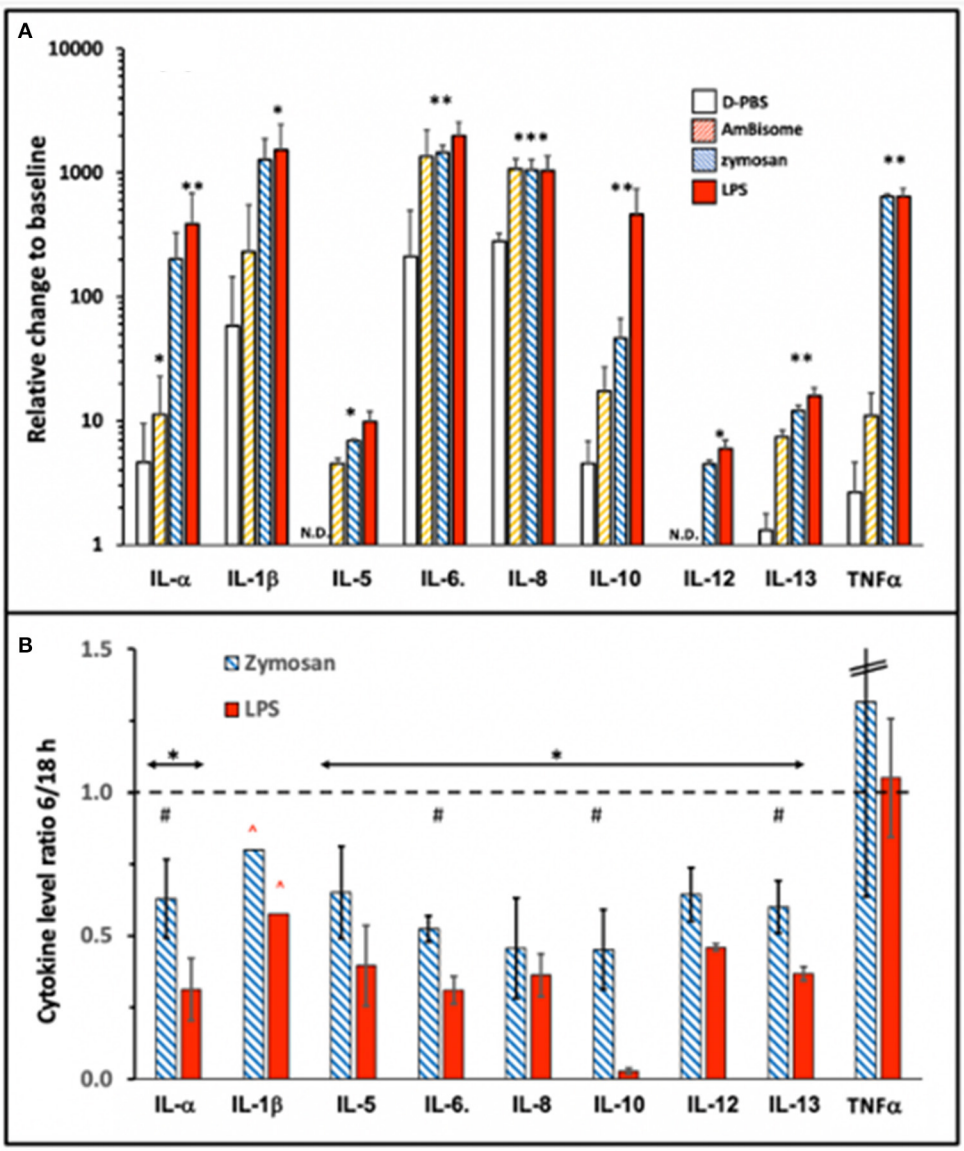

FIGURE 3 | Cytokine induction by immune stimulants that also activate complement. AmBisome, zymosan and LPS were applied at 1.98 mg phospholipid/ml, 0.5 mg/ml and 0.5 μg/ml, respectively, and the PBMC supernatants were analyzed for cytokine levels after 6 and 18 h incubation. **(A)** Shows the cytokine levels expressed as ratios, relative to 0 min baseline at 18 h, while **(B)** shows the ratios of 6 h readings relative to 18 h only for zymosan and LPS. Other details are the same as described for **Figures 1** and **2**. The bars show the mean ±SD (n=3); * and ** indicate statistically significant increases comparing to D-PBS; $P<0.05$ or 0.01, respectively; #, undetectable rises. **(B)**, * and # indicate statistically significant decrease using one-tailed T-test comparing to 1, or between the two columns, respectively ($P<0.05$); ^ indicates higher real value since data point(s) was/were out of the detection range of the assay.

drug AmBisome and the yeast glucan zymosan are potent complement activators whereas LPS is a weak trigger of complement. Both zymosan and LPS are also known for their ability to trigger cell activation via pattern recognition receptors expressed on the surface of immune cells. Specifically, zymosan has been described as a stimulant of TLR 2/6 (30, 31) and another transmembrane signaling receptor, Dectin-1, which collaborates with TLR-2 in NF-κB-mediated cytokine production (32, 33), whereas LPS activates proinflammatory signaling via the TLR4/MD2/CD14 receptor complex (25). It is currently unknown whether AmBisome can trigger the activation of pattern recognition receptors on the surface of the immune cells, although amphotericin B, alone, can do that (23).

Since there was a major difference between the complement activating powers of AmBisome and zymosan, using these two activators enabled us to dissect the significance of complement activating power in inducing cytokines.

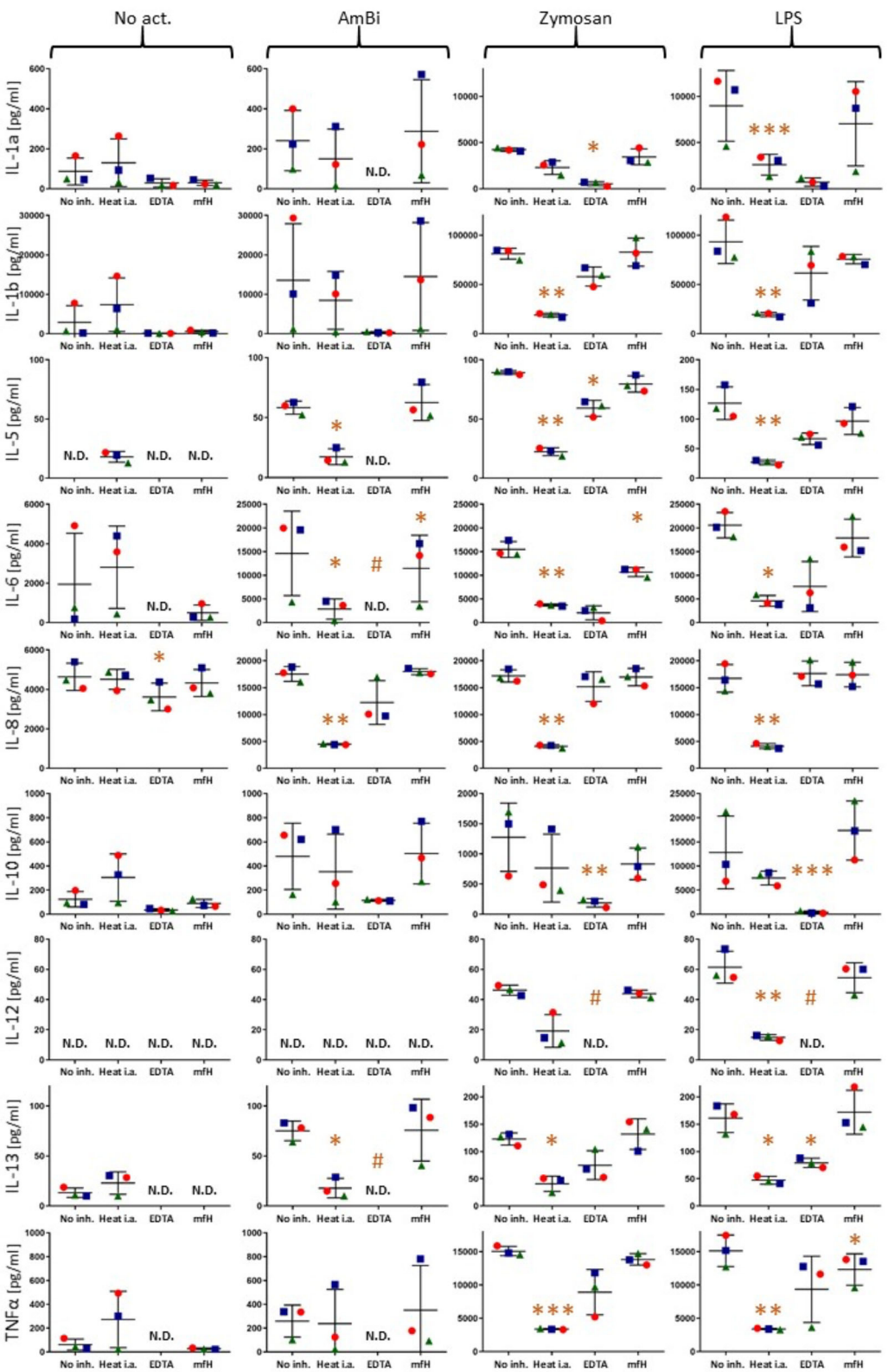

FIGURE 4 | Cytokine levels in PBMC culture supernatants after 18 h activation without any activator (No act.) or with AmBisome, zymosan or LPS, as specified on the top of the figure. No complement inhibition (No inh.), or complement inhibitions of sera by heat inactivation (Heat i.a.), 20 mM EDTA (EDTA), 1 mM mfH are shown on the X axes. Each panel presents data for different cytokines (Y axis labels). The colored spheres, triangles and rectangles specify the three different blood donors. N.D., *(Continued)*

FIGURE 4 | (non-detectable) means values below the limit of detection (< LLOQ). *($P<0.05$), **($P<0.01$), or ***($P<0.001$) imply significant inhibition compared to control (No inh.) by pairwise two-tailed *T* test, # indicates significant inhibition calculated with the LLOQ of the assay.

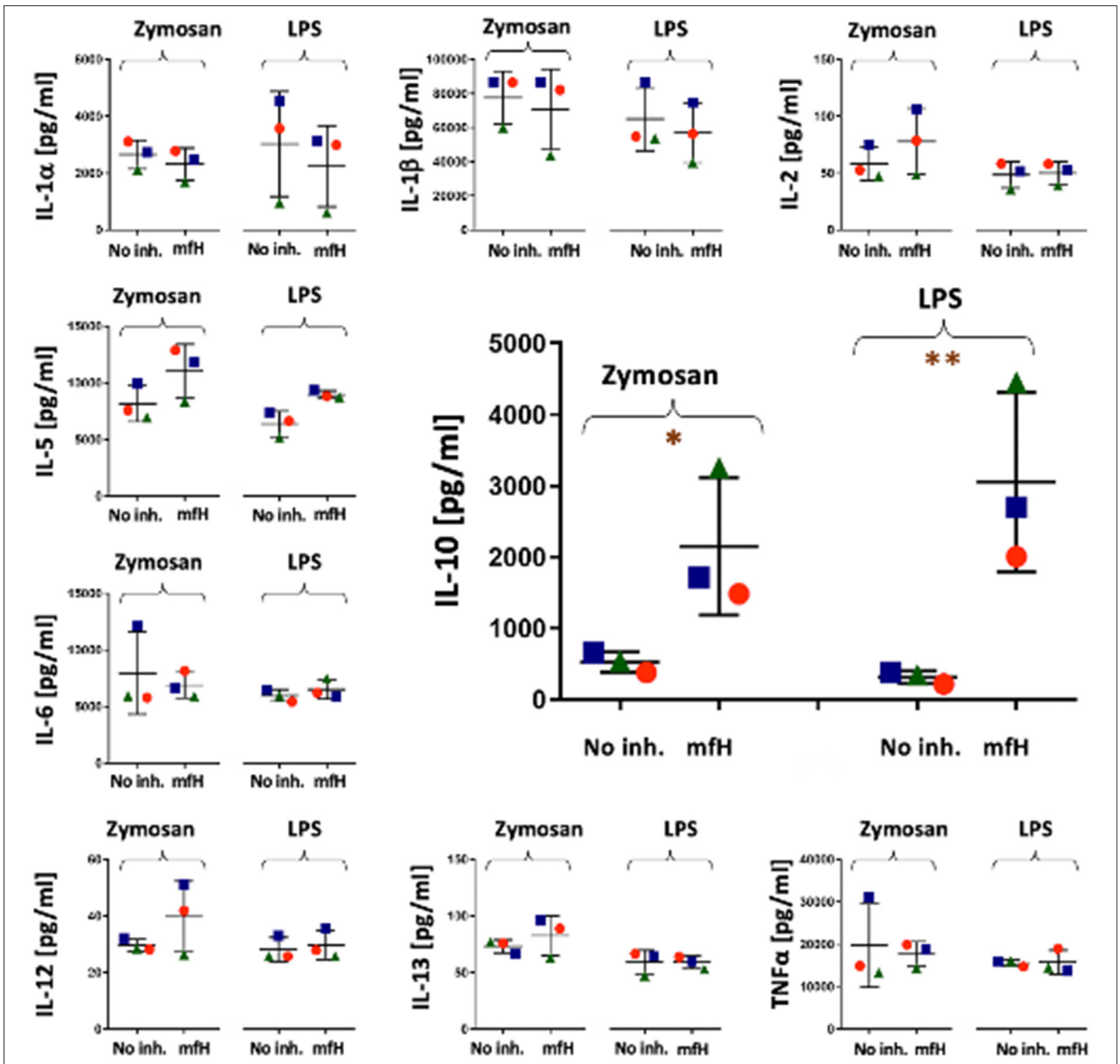

FIGURE 5 | Cytokine levels in PBMC culture supernatants after 6 h activation with zymosan and LPS in the presence and absence of mfH. Each panel presents data for different cytokines (Y axis labels). The colored spheres, triangles and rectangles specify the three different blood donors. The significant stimulatory effect of mfH on IL-10 is enlarged in the middle of the figure. *($P<0.05$), **($P<0.01$) imply significant enhancement compared to control (No inh.) by pairwise two-tailed *T* test.

Among the tested complement byproducts, C5a, a cleavage product of C5, is a potent proinflammatory anaphylatoxin in the fluid phase; Bb, a cleavage product of factor B whose rise in the fluid phase indicates the involvement of alternative pathway in complement activation; and sC5b-9, also in the fluid phase, provides an indirect measure of membrane attack complex (C5b-9) deposition on cell membranes, entailing cytotoxic pore formation.

The inhibition of complement activation in our study was achieved by EDTA, heat inactivation and mfH, each having

different mechanism of action. EDTA prevents the Ca^{++}/Mg^{++}-dependent buildup of classical and alternative pathway C3 convertases, heat treatment entails the formation of IgG and other protein aggregates and anti-complementary C1 and C1s (34–36) and mFH is a clinically relevant complement inhibitor, a ∼373 amino acid-containing, ∼42 kDa MW recombinant protein that contains 6 SCRs from fH, the most effective inhibitor of alternative complement activation in plasma (37). The first 4 SCRs on its N-terminal bind to C3b and exert decay accelerating activity on the alternative pathway C3 convertase (C3bBb) and cofactor activity for the C3b cleavage by factor I. The C-terminal 2 SCRs, corresponding to fH 19, 20, bind to C3b fragments (iC3b and C3d) and polyanions (glycosaminoglycans or sialic acid) on host cell membranes. This triple targeting provides a unique, therapeutically valuable defense against complement activation on host cells. Despite a 70% reduction in size relative to fH, mfH extends the functional spectrum of fH outperforming it in a model of paroxysmal nocturnal hemoglobinuria (12). Mini-fH was also shown to protect against experimental glomerulopathy (13, 14) and its phosphatidylinositol-derivative, anchored to endothelial cells, mitigates organ rejection in a porcine xenotransplantation model (11).

Complement Activation and Inhibition in PBMC Cultures

As expected, we obtained significant rises of all complement activation markers in the supernatant of PBMC cultures incubated with AmBisome, zymosan and LPS, validating the approach of supplementing the tissue culture medium with intact serum. The power of activation decreased in the order zymosan > AmBisome > LPS, although this order does not reflect on biological potency to activate complement since, being a pilot study, the concentrations of activators were chosen on the basis of literature data without attempt to achieve equipotency either in complement activation or cytokine release. Accordingly, the fact that LPS was the least effective complement activator at 0.5 μg/ml is in keeping with earlier data showing major complement activation by LPS (in rat serum) only at 0.5 mg/ml (38).

The effective suppression of all these complement cleavage products by all three approaches of complement inhibition also validates the model inasmuch as it shows that the applied 50% serum provided sufficient dynamic window for the changes to allow statistical analysis of inhibition. The comparison of the effect of 10% autologous serum vs. 50% for the case of LPS-induced IL-1α and IL-1β (**Supplementary Figure 1**) also confirmed the essential role of intact serum in cytokine release, and the increase in absolute amounts of these cytokins following LPS stimulation is consistent with the 5-fold greater amount of serum in the case of 50% serum (**Supplementary Figure 1** vs. **Figure 3A**). This proportionality suggests that cytotoxicity by intact (non-heat inactivated) autologous serum does not interfere with quantitative evaluation of cytokine induction, a presumption consistent with that heat inactivation of fetal calf serum is not required for *in vitro* measurement of lymphocyte functions (35).

There were also some unexplainable findings in our complement studies. One was the stimulation by heat-inactivated serum of Bb rise in LPS-treated serum (**Figure 2D**) and C5a rise in untreated serum (**Figure 2E**). These observations need confirmation and further studies to understand, just as the massive rise of Ba by zymosan (**Figure 1C**) and Ca^{++}-independent rise of C4a by AmBisome (failure of 20 mM EDTA to block it, **Figure 1D**). The biological relevance of the latter observations is not clear at this time, but based on available information, some of these changes may be beneficial, since C4a, the third anaphylatoxin (39) was shown to interfere with C5a actions and to have antimicrobial activity (71, 72), and Ba, too, has been shown to have indirect anti-inflammatory properties (40–42).

Complement-Dependent Cytokine Production in PBMC Cultures

PBMC is known to consist of lymphocytes, monocytes and dendritic cells, all expressing anaphylatoxin receptors (ATRs). Unstimulated T cells express C5a receptor (C5aR) only at a low basal level; the expression of this receptor is strikingly up-regulated upon activation of T-cells (43). It has also been shown that there is strong interaction between TLR and ATR signaling (43), mutually enhancing each other's cytokine inductive effects. In one example of such cooperation, Zhang et al. reported striking rise of plasma IL-6, TNFα and IL-1β in decay-accelerating factor (DAF)-deficient mice treated with LPS and zymosan. In this model, the lack of membrane complement inhibitor, DAF, sensitized the animals for anaphylatoxin liberation, and, hence, C3a-C5aR signaling (31). In another example, wild-type mice co-treated with TLR ligands and cobra venom factor, a potent complement activator, significantly increased cytokine production, which was accompanied by increased mitogen-activated protein kinase and nuclear factor-κB (NF-κB) activation in the spleen. These *in vivo* results suggest therefore synergistic ATR and TLR stimulation as an underlying mechanism of cytokine storm.

The efficacy of complement inhibition in attenuating cytokine induction in the present study was shown by near full suppression of IL-1β, IL-5, IL-6 and TNFα by EDTA and/or heat treatment. In case of IL-6 these results are in keeping with earlier observations on major IL-6 response to the infusion of liposome-encapsulated hemoglobin in rats (45), a treatment that led to massive complement activation under the applied conditions (46). Although Ca^{++} binding by EDTA could inhibit cytokine production independently from complement inhibition, the paralleling, and mostly correlating inhibition of these cytokines' secretion by the two fundamentally different approaches of complement blockade can most easily be rationalized by their common effect, complement inhibition.

The scheme in **Figure 6** illustrates the above delineated relationships among different activation pathways *via* which zymosan, AmBisome and LPS might have triggered the release of cytokines from responsive immune cells *via* ATRs and TLRs and other pattern recognition or danger signal receptors (47–49). The

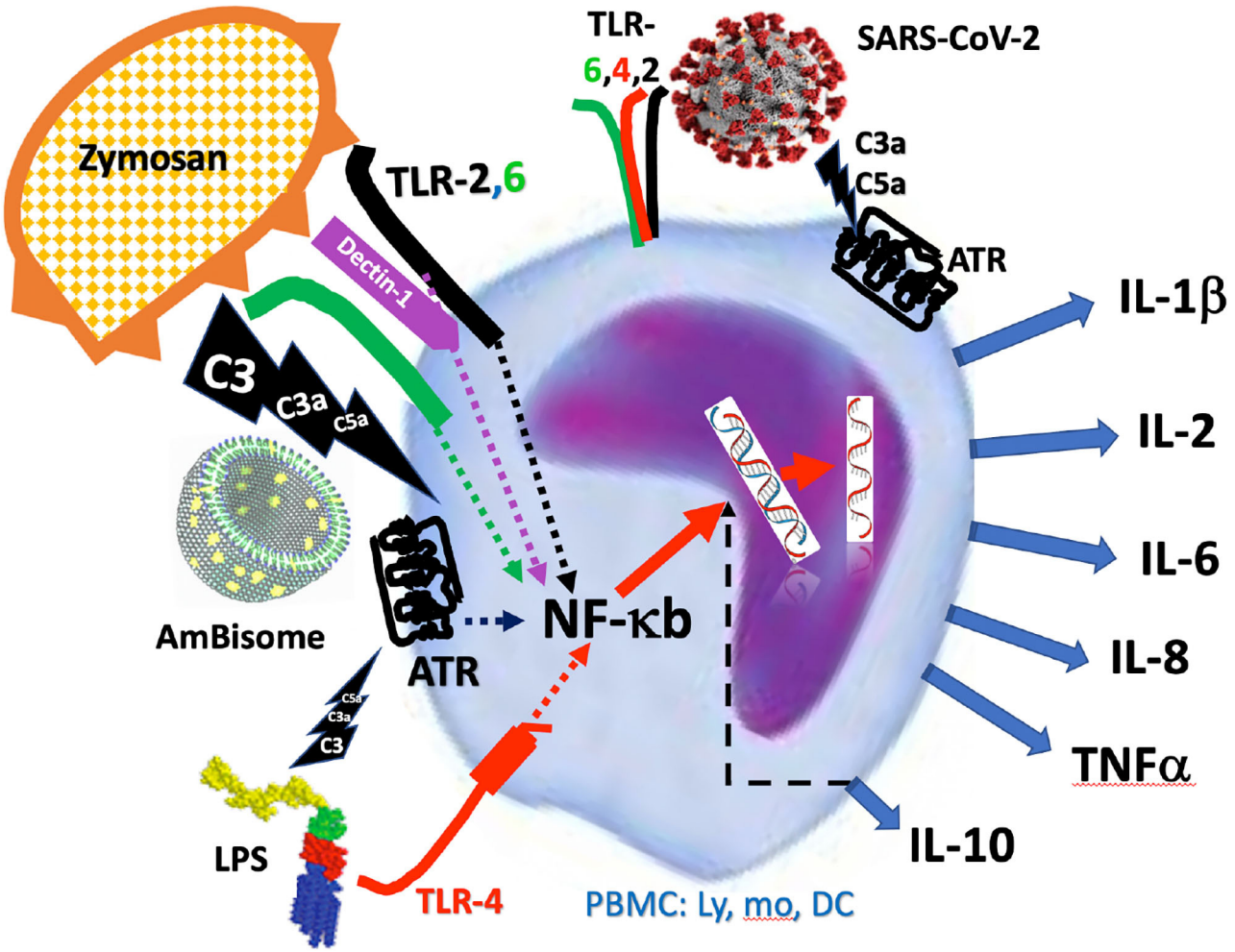

FIGURE 6 | Schematic illustration of activation of PBMC by zymosan, AmBisome and LPS *via* simultaneous engagement of anaphylatoxin and Toll-like receptors (ATR, TLR). The intracellular signaling of activator-receptor binding merges into NF-κb-mediated *de novo* transcription of inflammatory cytokines, explaining the complement inhibition-sensitive production of cytokines. The figure also reminds that the SARS-CoV-2 may also induce cytokine release *via* additive, or synergistic stimulation of both the ATR and Toll-like receptors, making the process complement- dependent.

fact that inhibition of complement also inhibited or reduced the production of some cytokines suggests that the ATR-mediated activation cooperates in these cytokines' release, permitting adding upon or synergizing with cell activation via other channels. However, if a trigger mechanism is overwhelming, there is no need for collaboration with other activation channels. In other words, the efficacy of signal transduction via these channels may represent a spectrum, depending on a variety of factors, and the cells' response may reflect a summation of all concurrent input signals. This "double hit" hypothesis, developed for nanoparticle-induced hypersensitivity reactions (50, 51), is illustrated in **Figure 6**. It shows that all three immune activators tested in this study trigger at least two activation channels with varying efficacy one being the ATR channel.

Relevance for COVID-19

Considering the mounting evidence of a critical role of complement activation and anaphylatoxins in the CS in Covid-19 and the efficacy of complement inhibitors in attenuating the disease (52–65), the complement dependence of cytokine release in our PBMC assay highlights the possible clinical relevance of the model for Covid-19 therapy. Infact, the cytokines that were found to be induced by the complement activators, particularly IL-6 and TNFα, are among those typically elevated in Covid-19 (66–69). The inhibitory effect of mfH on IL-6 release (**Figure 4**) looks promising, as mfH is a druggable protein. The finding is consistent with that mfH is an alternative pathway inhibitor and SARS-CoV-2 activates complement via the alternative pathway (65). The observation that AmBisome was an effective activator of cytokine release is

notable because it mimics viruses in terms of bilayer structure and size [80–90 nm], and it too activates complement via the alternative pathway (44). Therefore, it may represent a safe and simple model for studying the innate responses to CS-inducing viruses, such as SARS-CoV-2. **Figure 6** highlights the hypothesis that the SARS-CoV-2 may induce cytokine storm via additive, or synergistic induction of both ATR and TLR-mediated intracellular signaling.

OUTLOOK

Our experiments suggest the utility of non-heat inactivated autologous serum-containing PBMC assay in studying the mechanism and pharmacological sensitivity of CS in general, and, in Covid-19, in particular. Observations in this model point to the possible use of mfH, or similar SCR-based complement inhibitors against pathologies triggered by the excessive cytokine release. Although the inhibition of IL-6 by mfH was relatively small, this study was a pilot exploration of efficacy without attempt to establish dose-effect relationship or pursue other aspects of drug development. It should be noted in this regard that the stimulating effect of mfH on IL-10 at 6 h is another promising observation, since IL-10 is an anti-inflammatory cytokine known to limit tissue damage in chronic severe inflammations (16, 18, 19). Furthermore, the clinical efficacy of convalescent plasma has been suggested not to be due only to neutralizing antibodies, but also to the presence of innate inhibitors of inflammation, including soluble complement inhibitors, such as fH (70).

Being a small recombinant protein with proven efficacy in other diseases (11–15, 20), mfH offers a new strategy against CS in combination with other drugs and treatment modalities, obviously after intense preclinical analysis of efficacy and safety. Its use also draws attention to the potential use of fH and/or other SCR constructs in overcoming the fatality of diseases associated with CS, such as Covid-19.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.642860/full#supplementary-material

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Complement Factor H Family Proteins Modulate Monocyte and Neutrophil Granulocyte Functions

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Besides being a key effector arm of innate immunity, a plethora of non-canonical functions of complement has recently been emerging. Factor H (FH), the main regulator of the alternative pathway of complement activation, has been reported to bind to various immune cells and regulate their functions, beyond its role in modulating complement activation. In this study we investigated the effect of FH, its alternative splice product FH-like protein 1 (FHL-1), the FH-related (FHR) proteins FHR-1 and FHR-5, and the recently developed artificial complement inhibitor mini-FH, on two key innate immune cells, monocytes and neutrophilic granulocytes. We found that, similar to FH, the other factor H family proteins FHL-1, FHR-1 and FHR-5, as well as the recombinant mini-FH, are able to bind to both monocytes and neutrophils. As a functional outcome, immobilized FH and FHR-1 inhibited PMA-induced NET formation, but increased the adherence and IL-8 production of neutrophils. FHL-1 increased only the adherence of the cells, while FHR-5 was ineffective in altering these functions. The adherence of monocytes was increased on FH, recombinant mini-FH and FHL-1 covered surfaces and, except for FHL-1, the same molecules also enhanced secretion of the inflammatory cytokines IL-1β and TNFα. When monocytes were stimulated with LPS in the presence of immobilized FH family proteins, FH, FHL-1 and mini-FH enhanced whereas FHR-1 and FHR-5 decreased the secretion of TNFα; FHL-1 and mini-FH also enhanced IL-10 release compared to the effect of LPS alone. Our results reveal heterogeneous effects of FH and FH family members on monocytes and neutrophils, altering key features involved in pathogen killing, and also demonstrate that FH-based complement inhibitors, such as mini-FH, may have effects beyond their function of inhibiting complement activation. Thus, our data provide new insight into the non-canonical functions of FH, FHL-1, FHR-1 and FHR-5 that might be exploited during protection against infections and in vaccine development.

Keywords: complement, cytokine, neutrophil extracellular trap (NET), factor H (FH), factor H-related protein (FHR), monocyte, neutrophil granulocyte, extracellular DNA

INTRODUCTION

The complement system is a fundamental part of innate immunity, the main function of which is to discriminate self and non-self structures in order to keep immune homeostasis. Recognition of foreign particles and modified host cells by complement results in its activation *via* the classical, lectin or alternative pathways, leading to the cleavage of the central component C3 into C3a and C3b. C3 is the major effector of the complement cascade, causing inflammation by releasing the anaphylatoxin C3a or clearance of the target structures by opsonization with C3b and allowing for further propagation of the cascade, potentially leading to target cell lysis (1). Although complement activation is necessary to eliminate altered self and foreign structures, its over-stimulation might result in attack on the host itself and cause tissue injury. Hence a strict control of complement activation is necessary to maintain immune balance and preserve host integrity (2).

Factor H (FH) is the major regulator of the alternative pathway (AP) of complement by acting as a cofactor for Factor I mediated cleavage of C3b and accelerating the decay of AP C3 and C5 convertases either in solution or when bound to host surfaces (3). FH consists of 20 short consensus repeats (SCR): the N-terminal SCR1-4 domains bind to C3b and exert complement regulatory activity, while the C-terminal SCR19-20 domains ensure binding to host surfaces *via* recognizing highly sialylated molecules (4, 5). In addition, FH can recognize other polyanionic molecules, like proteoglycans, glycosaminoglycans and other components of the extracellular matrix (6–9), extending the FH mediated protection also to tissues and extracellular spaces. Moreover, to exploit the potential of FH as a therapeutic agent, an artificial mini-FH was generated that contains the regulatory (SCR1-4) and ligand binding (SCR19-20) domains of FH (10, 11). Besides acting as a complement regulator, FH also exerts non-canonical functions *via* binding to various immune cells, and modulating their activation and function (12). Among those are monocytes and neutrophils, two key components of innate immunity which, similar to complement proteins, circulate in the blood to detect potentially dangerous structures and regulate inflammatory processes (13).

Binding of FH to neutrophils has been reported to modulate a wide range of their functions, among them phagocytosis, ROS production and release of antimicrobial peptides, thus FH may directly influence the ability of the cells to kill pathogens (14–16). FH exerts the vast majority of these effects in immobilized form, serving as an adhesion ligand for the cells, however in soluble form, it is mainly ineffective. Indeed, the increased adherence observed on FH covered surfaces induces rapid polarization of neutrophils, elevation in intracellular Ca^{2+} levels followed by rapid spreading on FH-coated surfaces (14, 16). Similarly, immobilized FH was shown to enhance the release of IL-8 (16), secretion of the antimicrobial peptide lactoferrin and release of hydrogen-peroxide (14), as well as the uptake of *Streptococcus pneumoniae* (17). Additionally, FH itself also promotes neutrophil migration (16). Besides phagocytosis and intracellular killing, neutrophils can trap and eliminate microorganisms by releasing neutrophil extracellular traps (NETs) (18–20). NETs induce microbial killing by histones and granule-derived antimicrobial proteins, such as myeloperoxidase (MPO), neutrophil elastase and matrix metalloproteinases that associate with these chromatin fibers (20–23). In contrast to the enhanced activation and cytokine secretion, interestingly, the release of NETs by both phorbol-myristate acetate (PMA) or fibronectin plus β-glucan-stimulated neutrophils is decreased in the presence of immobilized FH (16). Similarly to neutrophils, FH was shown to influence a wide plethora of monocyte and macrophage functions too, such as chemotaxis (24), stimulation of respiratory burst (25) and cytokine secretion (26), mainly *via* the complement receptors CR3 (CD11b/CD18) and CR4 (CD11c/CD18) (14, 27). Later, FH binding to *Mycobacterium bovis* and *Candida albicans* has been shown to play a role in the evasion of the innate immune system and in parallel, to enhance the pro-inflammatory (IL-1β, IL-6, TNFα) cytokine response of macrophages (27, 28). Monocytes can also produce extracellular traps (MoETs), mainly to enhance killing of extracellular pathogens (29–31). It was shown that FH could bind to NET and MoET directly (29, 32) and bound FH decreases the secretion of the inflammatory cytokine IL-1β in monocytes (29). These data suggest that FH can exert both pro- and anti-inflammatory activities on monocytes and neutrophils, allowing fine-tuning of cellular functions for the regulation of inflammation.

FH belongs to a protein family that also includes the FH splice variant FH-like protein 1 (FHL-1) and five FH-related proteins (FHRs), FHR-1 to FHR-5 (33–35). Similar to FH, they are also exclusively composed of SCRs. Although the complement regulatory region SCR1-4 of FH is not conserved in the FHRs, their C-terminal domains show various degree of sequence identity with C-terminal SCRs of FH. This region represents the major cell surface binding- and C3b recognition part of FH, suggesting that the main function of FHRs is to compete with FH for surface binding and thus modulate complement activation (33, 36). Indeed, both FHR-1 and FHR-5 are competitive inhibitors of FH on different ligands such as C3b, C-reactive protein, pentraxin 3, DNA and extracellular matrix components (37–41). The FH/FHR encoding gene cluster is prone to genetic rearrangements, which are associated with distinct human disorders such as atypical hemolytic uremic syndrome, C3 glomerulopathy, age-related macular degeneration and other autoimmune/inflammatory diseases (33, 42, 43). Due to their role in various diseases, the interest in the FHR molecules has increased, both on the genetic and functional level. Despite the described role of FH in modulating neutrophil and monocyte responses, only a few data are available how FHRs influence the function of these cells. For example, FHR-1 was shown to enhance the adhesion of neutrophils to *Candida albicans*, causing increased lactoferrin and ROS generation (15),

Abbreviations: BSA, bovine serum albumin; FH, factor H; FHL-1, factor H-like protein 1; FHR, factor H-related protein; HSA, human serum albumin; FN, fibronectin; NET, neutrophil extracellular trap; SCR, short consensus repeat.

suggesting an FHR-1 mediated regulation of inflammation. Recently, FHR-1 was shown to activate the NLRP3 inflammasome in monocytes when bound to necrotic cells (44).

Considering that both the FHRs and neutrophils and monocytes are key players of inflammatory diseases, we aimed to investigate whether similar to FH, the related proteins FHL-1, FHR-1 and FHR-5, as well as the recently developed mini-FH, a potential complement therapeutic, are able to modulate neutrophil and monocyte functions.

MATERIALS AND METHODS

Proteins, Abs, Dyes and Reagents

Mini-FH was cloned into the pBSV-8His baculovirus expression vector (45), and produced in *Spodoptera frugiperda* (Sf9) cells and purified by nickel-affinity chromatography as described previously (10). FHL-1 protein, which was expressed recombinantly in *Pichia pastoris* and purified according to the methodology described previously (46, 47), was kindly provided by Dr. Christoph Schmidt (Ulm University, Germany). Recombinant human FHR-1 and FHR-5 were obtained from Novoprotein (Summit, NJ). Bovine serum albumin (BSA) was from Applichem (Darmstadt, Germany), and human serum albumin (HSA) and fibronectin (FN) were from Sigma-Aldrich (Budapest, Hungary), respectively. Purified human FH, iC3b, polyclonal goat anti-human FH antibody and polyclonal anti-histone H4 (citrulline 3) were purchased from Merck-Millipore (Burlington, MA). Mouse anti-human FH antibodies were purchased from Enzo (C18; Lausen, Switzerland) and Quidel (A254; San Diego, CA), respectively. Goat anti-human FHR-5 antibody was from R&D Systems (McKinley Place, MN). Alexa-488-conjugated rabbit anti-goat IgG, Alexa-488-conjugated donkey anti-mouse IgG and Alexa-647-conjugated goat anti-rabbit IgG were from Molecular Probes-Invitrogen (Carlsbad, CA). Phorbol-myristate acetate (PMA) and lipopolysaccharide (LPS) were from Sigma-Aldrich. Sytox Orange and Phalloidin Alexa-488 were from Thermo Scientific (Waltham, MA).

Cells

Human neutrophil granulocytes were isolated from whole blood and human monocytes from buffy coats of healthy blood donors according to standard procedures (16, 48). Samples were provided by the Hungarian National Blood Transfusion Service after acquiring written informed consent from all donors. The study was conducted in accordance with the respective national authority (TUKEB ETT, permission number 838/PI/12). Cell purity was investigated by flow cytometry using anti-CD14 and anti-CD16 antibodies (BD Biosciences, Germany) followed by the corresponding secondary antibodies, and was higher than 90% in each case.

The U937 human monocytic cells and U937 cells overexpressing CR3 (CR3+ U937) (49) were kindly provided by Drs. Karita Haapasalo (University of Helsinki, Finland) and Carla J. C. De Haas (University Medical Center Utrecht, the Netherlands). These cell lines were maintained in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% FBS (Euroclone) under humidified air with 5% CO_2 at 37°C. Cell viability was assessed regularly by trypane blue dye exclusion.

Flow Cytometry

To detect FHL-1, FHR-1, FHR-5 and mini-FH binding to neutrophil and monocyte cell surfaces, 5×10^5 monocytes and 1×10^6 neutrophils were first incubated with 300 nM of each protein for 20 minutes at 20°C in Ca^{2+} and Mg^{2+} containing DPBS (Lonza). After washing the cells with DPBS, Fc receptor blocking reagent (Miltenyi Biotec, Germany) was added to reduce the amount of nonspecific Ab binding. Bound proteins were detected *via* incubation with the polyclonal goat anti-human FH Ab in DPBS containing fetal bovine serum (FBS, 0.1%, EuroClone, Pero, Italy) for 15 minutes at 4°C, washed and labelled with Alexa-488-conjugated rabbit anti-goat antibody (Thermo Scientific, Waltham, MA) under the same conditions. Samples were measured on FACSCalibur flow cytometer (BD Biosciences, Mountain View, US) and analyzed by FCSExpress software, version 7 (BD Biosciences).

To detect the competition between FH and FHR-1 or FHR-5, we used Alexa-488 conjugated FH generated by the Alexa Fluor™ 488 Antibody Labeling Kit according to the manufacturer's instructions (Invitrogen). First, 5×10^5 neutrophils were incubated with FHR-1 or FHR-5 at increasing concentrations between 300 and 5,000 nM in Ca^{2+} and Mg^{2+} containing DPBS (Lonza) for 20 min at 4°C, followed by, without washing, 300 nM Alexa-488 conjugated FH for an additional 20 min at 4°C. After washing, bound FH was measured on FACSCalibur flow cytometer. The geomean of the FH sample without competitor was set as 100%.

To compare the expression level of CR3 between the U937 and U937 CR3+ cell lines, APC-conjugated mouse anti-human CD11b (Pharmingen) antibody was added to 5×10^5 cells at 5 μg/ml concentration for 30 min at 4°C. Fc-receptor blocking reagent (Miltenyi Biotec, Germany) was added to reduce the amount of nonspecific Ab binding.

To detect the binding of FH, FHR-1 and FHR-5 to the U937 cell lines, 5×10^5 cells were first incubated with 50 μg/ml FH or FHR-1, or 10 μg/ml FHR-5, for 30 min at 20°C in Ca^{2+} and Mg^{2+} containing modified Hanks's buffer, as described previously (16). After washing, Fc-receptor blocking reagent (Miltenyi) was added to reduce the amount of nonspecific Ab binding. Bound FH and FHR-1 proteins were detected by incubation with 1:250 diluted polyclonal goat anti-human FH Ab (Merck) for 30 min at 4°C, followed by 1:1000 diluted Alexa488-conjugated rabbit anti-goat Ig (Thermo Scientific) also for 30 min at 4°C. Bound FHR-5 was detected using goat anti-human FHR-5 antibody (R&D Systems) followed by the Alexa488-conjugated rabbit anti-goat Ig under the same conditions.

To detect the presence of C3-fragments on cell surfaces, 5×10^5 U937 and CR3+ U937 cells were left untreated or incubated with 20 μg/ml iC3b (30 min on ice). After washing, the cells were incubated with polyclonal anti-C3 (Cappel, West Chester, PA) or anti-C3c (Dako, Santa Clara, CA) antibodies, both diluted 1:100, for 30 minutes at 4°C, then washed and incubated with Alexa488-conjugated rabbit anti-goat Ig or Alexa-488-conjugated donkey anti-rabbit Ig (Invitrogen)

secondary antibodies, both diluted 1:500, under the same conditions. Samples were measured on Cytoflex flow cytometer (Beckman Coulter) and analyzed by FlowJo_V10 software.

Visualization of NET Formation

To visualize the effect of FH family members on PMA-induced NET formation, 300 nM of each protein (FHR-1, FHR-5, FHL-1, mini-FH or, as negative control, HSA) was immobilized on borosilicate chambered cover glass microplates (NUNC, Rochester, NY) overnight at 4°C. NET formation was induced by addition of 100 nM PMA in solution for 3 hours and labelled with Sytox Orange following previously described protocols (16). To confirm NET formation and exclude the potential detection of nuclear DNA, antibodies against histones [a well-accepted marker of NET formation (21)] were used. After Sytox Orange staining, samples were fixed with 4% PFA for 10 minutes, washed twice and blocked with PBS containing 5% BSA and Fc receptor blocking reagent (Miltenyi Biotec) for 30 minutes at 37°C. Thereafter citrullinated H4 histones were stained with rabbit polyclonal anti-H4 antibody (citrulline 3; 1:500 dilution in DPBS) for 1 h at 20°C and with the appropriate Alexa-488 conjugated goat anti-rabbit Ig secondary Ab (1:1000) for 30 minutes at 20°C in dark. After extensive washing, NETs were investigated with an Olympus FluoView 500 laser-scanning confocal microscope (Hamburg, Germany) equipped with argon ion laser (488nm) and two He-Ne lasers (with 543 and 632 nm excitation wavelengths, respectively). Fluorescence and DIC images were acquired using a 20× objective. Images were processed by ImageJ (http://rsbweb.nih.gov/ij) and Photoshop softwares.

Quantification of NET Production

To quantify NET production, neutrophils were incubated on FH/FHR-coated 96-well black plate (Greiner Bio-One, Kremsmünster, Austria) in the presence or absence of 100 nM PMA for 3 hours. After incubation, each well was treated with 1 U/ml Micrococcal Nuclease (Thermo Scientific) for 10 minutes at 37°C to release NETs attached to the cell surface. The reaction was stopped by applying 5 mM EDTA. After digestion, cells were pelleted and separated from the released NET by centrifugation at 1800 *g* for 8 minutes. The cell-free NET was then labeled with 5 mM Sytox Orange for 20 minutes at 37°C. Fluorescence was measured on a Fluoroscan Ascent FL microplate reader (Thermo Scientific) with excitation and emission filters of 543 nm and 592 nm, respectively. Maximal NET release was obtained after 100 nM PMA treatment, set as 100%. In case of each sample, relative fluorescence % was calculated compared to the 100% signal of PMA treatment.

Measurement of Neutrophil Spreading

Borosilicate chambered coverglass microplates were coated with 300 nM of each protein (FHR-1, FHR-5, FHL-1, mini-FH or, as negative control, BSA) in Ca^{2+} and Mg^{2+} containing DPBS (Lonza) at 4°C overnight. Neutrophil spreading was monitored by phalloidin Alexa-488 staining with an Olympus FluoView 500 laser-scanning confocal microscope as described earlier (16). The phalloidin staining was carried out according to the manufacturer's instructions. The contact zone areas were quantified from 150 cells in each experiment using ImageJ software with Analyze Particle tool.

Measurement of Cytokine Production

96-well cell culture plates (Greiner Bio-One) were coated with 300 nM of FHR-1, FHR-5, FHL-1, mini-FH or, as negative control, with FN in Ca^{2+} and Mg^{2+} containing DPBS (Lonza) at 4°C, overnight. After extensive washing, neutrophils or monocytes (2×10^5 cells/well) were added in 200 µl of RPMI 1640 (Invitrogen) supplemented with 10% FBS and gentamycin (Lonza) and cultured for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. As a positive control, cells were stimulated with 10 ng/ml LPS. To measure the combined effect of immobilized proteins (FHR-1, FHR-5, FHL-1 and mini-FH) and soluble LPS on cytokine production, LPS was added for 24 hours after starting culturing the cells on FH/FHRs coated surfaces. Cytokine production was measured after 24 hours of stimulation, using commercial sandwich ELISA kits (R&D Systems). ELISAs were carried out according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). A p value < 0.05 was considered statistically significant. The specific statistical tests applied are indicated in the Figure legends.

RESULTS

FH Family Members Bind to Human Neutrophils and Monocytes

The binding of FH to various immune cells, especially to monocytes and neutrophils, is known for long (14–17, 24, 25, 27, 28, 50–52). However, the binding of the FHR proteins to primary cells has only been poorly studied (15, 53, 54). To investigate whether other FH family members are able to bind to neutrophils and monocytes similar to FH, freshly isolated cells were incubated with the recombinant proteins and their binding was detected by flow cytometry. FHR-1, FHR-5, FHL-1 and mini-FH, as well as FH used as a positive control, bound to both monocytes and neutrophils with high efficiency (**Figure 1**). Many factors are likely to affect the binding of these investigated FH family members *in vivo*, thus different baseline signals were observed in donors, but binding of the recombinant FHRs could always be detected.

The Role of CR3 in Binding FH Family Proteins

Previously, CR3 was described to be a receptor for both FH and FHR-1 on human neutrophils based on inhibition of binding by CR3-specific mAbs (15), and also for FH on macrophages using siRNA (27). To study whether FHR-1 or FHR-5 can inhibit FH binding to the cells, human neutrophils were incubated with fluorescence-labelled FH in the absence or presence of increasing concentrations of recombinant FHR-1 and FHR-5. At higher

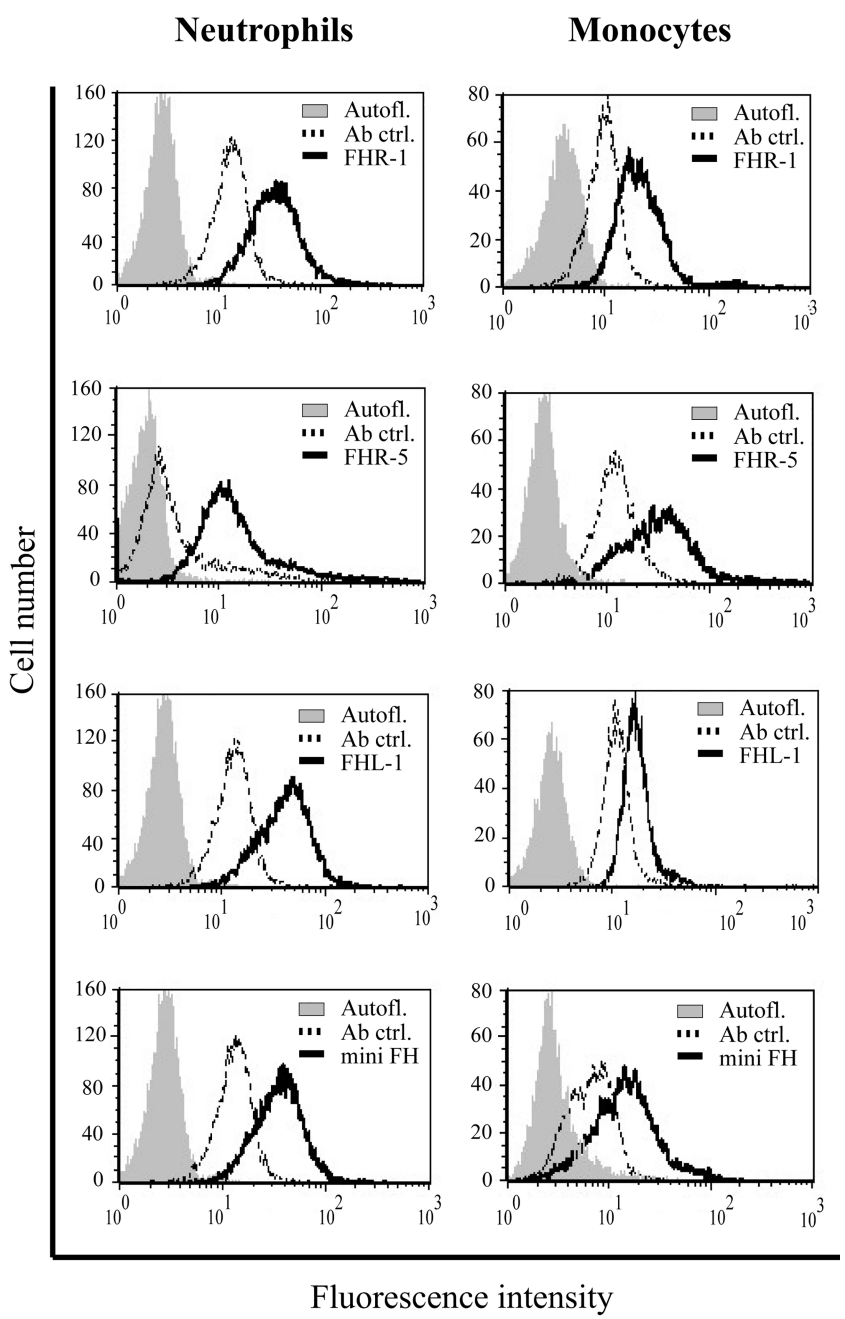

FIGURE 1 | FHR-1, FHR-5, FHL-1 and mini-FH bind to primary neutrophil granulocytes and monocytes. Binding of 300 nM of each protein (solid lines) to neutrophil granulocytes and monocytes in Ca^{2+} and Mg^{2+} containing DPBS was measured by flow cytometry. Filled histograms indicate autofluorescence, dotted lines show samples incubated without the proteins added (antibody controls). Binding of the added proteins (solid black line) was analyzed using polyclonal anti-FH Ab for FHR-1, FHL-1 and mini-FH detection, and monoclonal anti-FHR-5 for FHR-5 detection. A representative histogram out of three independent experiments is shown in each case.

FHR-1 concentrations a significant decrease of FH binding to neutrophils was detected, indicating at least partially overlapping binding sites of FH and FHR-1 on the cells, whereas FHR-5 was not able to inhibit FH binding (**Figure 2**).

Since FH family proteins all bind to various C3-fragments, and such C3-fragments might be present on the cells isolated from human blood, we wanted to exclude this potential factor influencing FH and FHR binding. Therefore, and to further study the role of CR3, we used the U937 cell line and also an U937 cell line that overexpresses CR3 (CR3+ U937) (49). Compared to the wild-type U937 cells, CR3+ U937 cells bound more FH and FHR-1; however, FHR-5 binding was not increased (**Figure 3**). Because cell-bound

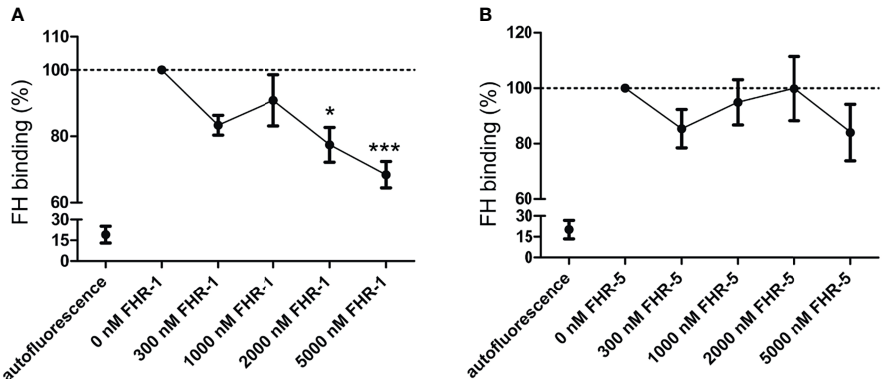

FIGURE 2 | Competition between FHR-1/FHR-5 and FH for binding to neutrophils. Neutrophils were preincubated with increasing concentrations of **(A)** unlabeled recombinant FHR-1 or **(B)** FHR-5, then fluorescence-labelled FH (50 µg/ml; 300 nM) was added to the cells. Bound FH was detected by flow cytometry. Data represent means ± SEM from five **(A)** and four **(B)** independent experiments. Differences with $p < 0.05$ were considered statistically significant and compared to the FH treated samples without competitor protein (one-way ANOVA with Dunnett's multiple comparison test, $^{*}p < 0.05^{***}p < 0.001$).

C3-fragments may be derived from intracellularly synthesized C3, such as shown for U937 cells particularly upon activation (55, 56), we detected the presence of C3-fragments on the cells used in our assays. There was little to no C3-fragments detectable on the cell surface by flow cytometry in the case of both U937 cells and CR3+ U937 cells (**Supplementary Figure 1**), further supporting the role of CR3 in the binding of FH and FHR-1 in our previous assays (**Figures 1** and **2**).

FH Family Members Modify PMA-Induced NET Formation

To investigate whether the binding of the studied FH family proteins to monocytes and neutrophils influence cellular functions, first we investigated how they influence NET formation of neutrophil granulocytes. Previously, we reported that FH alone does not influence NET production; however, after immobilization it significantly reduces the release of PMA induced extracellular DNA (16). Similar to FH, none of the FHRs alone had any effect on NET production (**Figure 4A**). PMA-induced DNA release, however, was slightly but significantly decreased on immobilized FHR-1, similar to FH (**Figures 4B, C**). FHR-5, FHL-1 and mini-FH however did not influence NET production even after PMA stimulation (**Figures 4B, C**).

FH Family Members Influence Neutrophil and Monocyte Spreading

It has previously been shown that FH, FHR-1 and FHL-1 serve as an adhesion ligand for neutrophils and that FH supports neutrophil spreading (14–17). Therefore, we investigated whether FHR-1, FHR-5, FHL-1 and mini-FH influence spreading of primary monocytes and neutrophils, an indispensable process of their extravasation and cell polarization (57). As shown in **Figure 5A**, neutrophil spreading was significantly increased on FH, FHR-1, FHL-1 and mini-FH coated surfaces compared to HSA, but FHR-5 had no effect. In the case of monocytes (**Figure 5B**), only FH, FHL-1 and mini-FH increased cell spreading significantly, whereas immobilized FHR-1 and FHR-5 did not alter the cellular morphology.

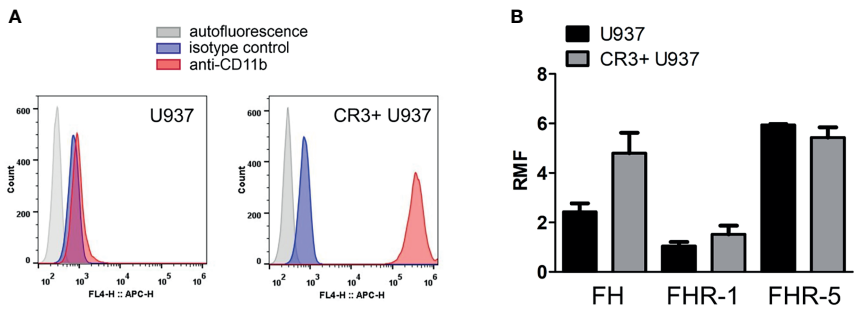

FIGURE 3 | Binding of FH, FHR-1 and FHR-5 to U937 and CR3+ U937 cells. **(A)** The expression of CR3 on U937 human monocytic cells and U937 cells overexpressing CR3 (CR3+ U937) was analyzed by flow cytometry using an anti-CD11b antibody. Representative histograms are shown. **(B)** Binding of purified FH (50 µg/ml) and recombinant FHR-1 (50 µg/ml) and FHR-5 (10 µg/ml) to U937 cells and CR3+ U937 cells was measured by flow cytometry. RMF, relative mean fluorescence. Data are means + SEM from three independent experiments.

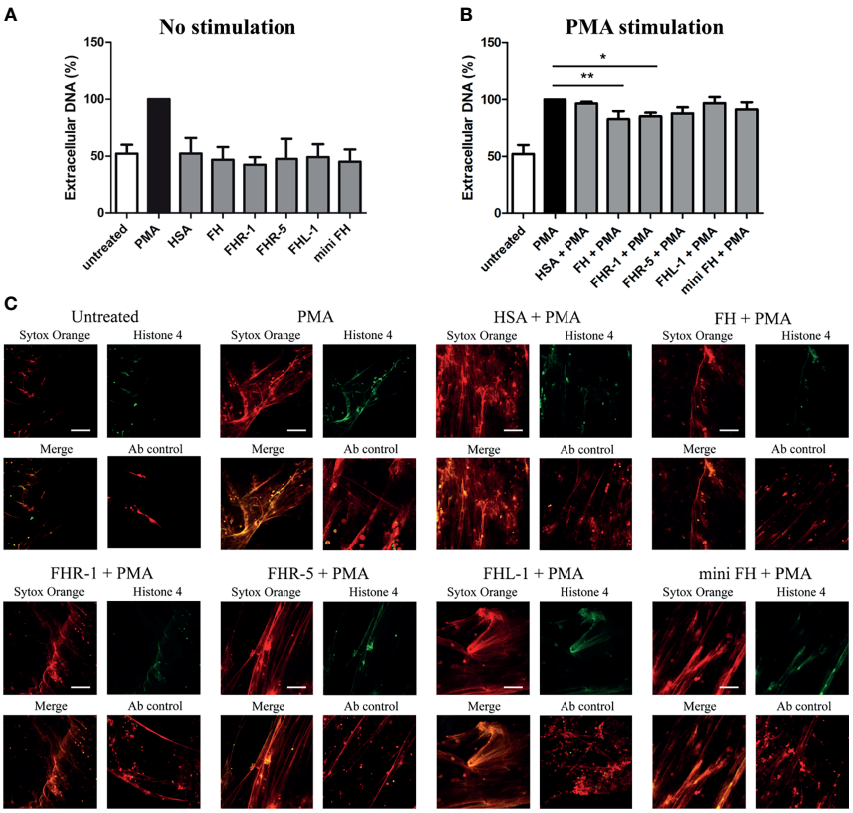

FIGURE 4 | FH and FHR-1 decrease PMA-induced NET formation. 300 nM FH, FHR-1, FHR-5, FHL-1 or mini-FH were immobilized and incubated with neutrophil granulocytes in the absence **(A)** or presence **(B, C)** of 100 nM PMA for 3 h. As negative control, HSA-stimulated cells were used. **(A, B)** Cell-free NET was quantified by fluoroscan measurement using Sytox Orange DNA staining. PMA-stimulated samples were set as 100%. Data are means + SD derived from three different donors. Differences with $p < 0.05$ were considered statistically significant and compared to PMA treated samples (one-way ANOVA with Dunnett's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$). **(C)** Representative microscopic images of NET formation in the presence of PMA on 300 nM immobilized FH, FHR-1, FHR-5, FHL-1 and mini-FH using a 20× objective. NET formation was visualized by 5 µM Sytox Orange staining and histone H4 labeling. Scale bars represent 100 µm.

FH Family Members Alter the Cytokine Profile of Resting Neutrophils and Monocytes

It has previously been shown that binding of FH to different surfaces influences the pro-inflammatory cytokine production of monocytes, macrophages and neutrophils (16, 27–29). To investigate whether FHR-1, FHR-5, FHL-1 and mini-FH are also able to modulate cytokine production of these cells, primary monocytes and neutrophils were incubated on surfaces where these proteins were immobilized, and cytokine secretion was analyzed after 24 hours. In the case of monocytes, only mini-FH enhanced IL-1β secretion significantly, however FH and FHR-1 also showed a tendency toward increased IL-1β production (**Figure 6A**). Additionally, FH and mini-FH caused increased TNFα release from monocytes, but only FH-treated samples reached statistical significance in the assay (**Figure 6B**). Interestingly, none of the proteins altered the anti-inflammatory IL-10 secretion significantly compared to the untreated sample (**Figure 6C**). In the case of neutrophils, immobilized FH increased IL-8 release, in agreement with our previous results (**Figure 6D**) (16). Similar to the case of IL-1β, the effect of FHR-1 and mini-FH on IL-8 production did not reach statistical significance, but followed a tendency to enhance the cytokine secretion.

FH Family Members Modify LPS-Induced TNFα Response of Monocytes

Since monocytes and neutrophils are able to bind FH family members and mini-FH, these proteins could influence the response of the cells when they are exposed to LPS from Gram-negative bacteria during infection. Therefore, we investigated the effect of FHR-1, FHR-5, FHL-1 and mini-FH on LPS-induced cytokine secretion of monocytes and neutrophils. None of the proteins affected IL-1β secretion compared to LPS alone (**Figure 7A**). However, when monocytes were incubated with immobilized FHR-1 or FHR-5 and simultaneously treated with LPS, a reduced secretion of TNFα compared to treatment with LPS alone was observed (**Figure 7B**). In contrast, FH, FHL-1 and mini-FH enhanced the amount of TNFα under the same conditions (**Figure 7B**). FHL-1 and mini-FH similarly enhanced IL-10 secretion compared to treatment with LPS alone (**Figure 7C**). In the case of neutrophilic granulocytes, none of

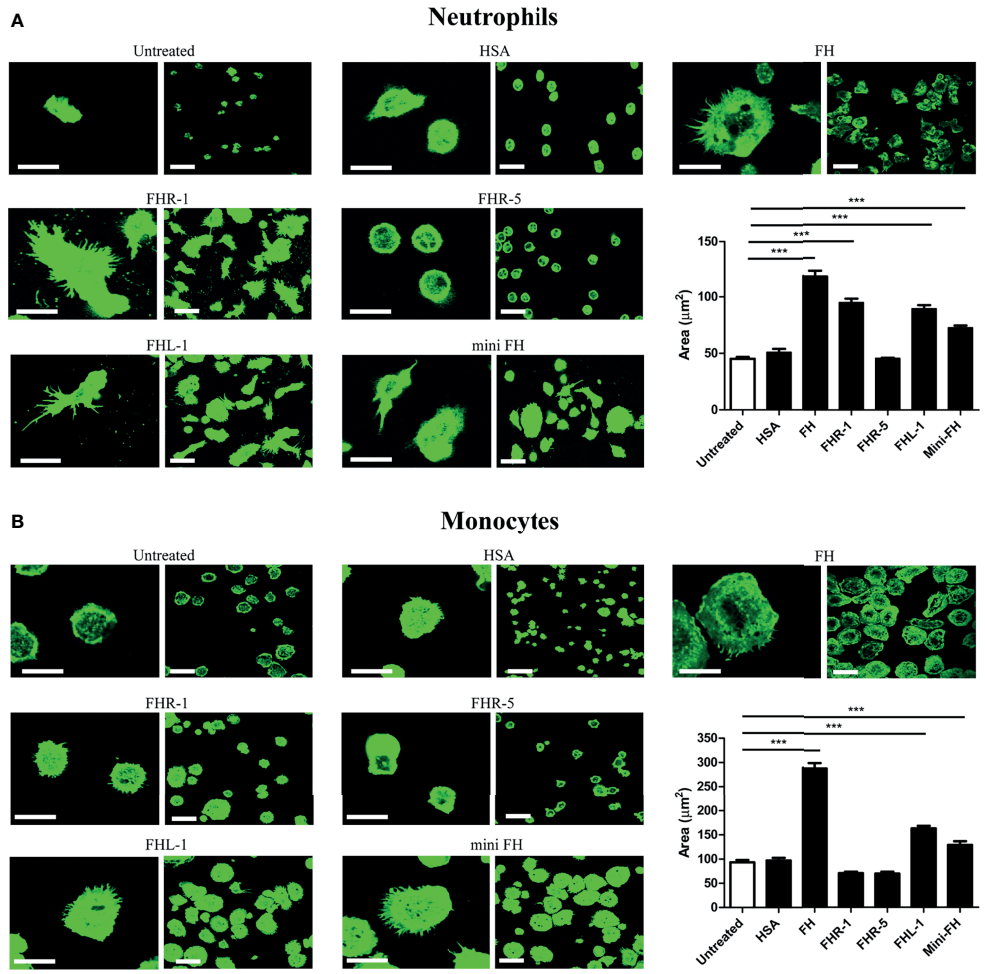

FIGURE 5 | FH family proteins influence spreading of neutrophil granulocytes and monocytes. Confocal laser scanning images of **(A)** neutrophils and **(B)** monocytes in response to 300 nM of immobilized proteins on chambered microplate wells. The contact surface of the cells was monitored by labelling F-actin with phalloidin-A488. Original scale bars, 20 µm (left columns) and 40 µm (right columns). Confocal images shown are representative of four (neutrophils) or five (monocytes) independent experiments. The contact zone areas of phalloidin-A488 labelled neutrophils and monocytes were quantified using ImageJ software from 150 cells in each experiment. Error bars represent means + SEM. Differences with $p < 0.05$ were considered statistically significant and compared to untreated samples (non-coated microplate surface) (one-way ANOVA with Bonferroni's multiple comparison test, $^{***}p < 0.001$).

the tested proteins influenced the LPS-induced, pro-inflammatory IL-8 release (**Supplementary Figure 2**).

DISCUSSION

Besides the canonical roles of FH family proteins in the modulation of complement activation, their non-canonical roles as modulators of cellular functions were also observed, but poorly examined yet (12, 33). Among blood cells, neutrophil granulocytes and monocytes were the most extensively studied in this regard as they are essential players in inflammation and innate immunity and they also influence the adaptive immune response. FH binding to neutrophils was first demonstrated in 1993 (58), and later it was shown to be mediated *via* CR3 (14–17) and to a lesser extent *via* CR4; the domains within FH responsible for this complement receptor binding were identified as SCR7 and SCR19-20 (15). These SCRs of FH show 100% amino acid sequence identity with the corresponding SCRs of FHL-1 (SCR7) or mini-FH (SCR19-20) (10, 33) (**Figure 8**). Therefore, FHL-1 and mini-FH have the potential to bind to the cell surface of monocytes and neutrophils similar to FH, which we confirmed by flow cytometry using recombinant proteins (**Figure 1**). FHR-1 binding to neutrophils was also shown before and is explained by its C-terminal domains that are highly similar to FH SCR19-20; FHR-1 binding could be inhibited by anti-CD11b and anti-CD18 specific mAbs but not by anti-CD11c (15). Moreover, binding of recombinant FHR-5, with less sequence similarity to FH, to the cells was also detected (**Figure 1**). These results are in agreement with our previous study where neutrophils were incubated with NHS and, in addition to FH, the binding of

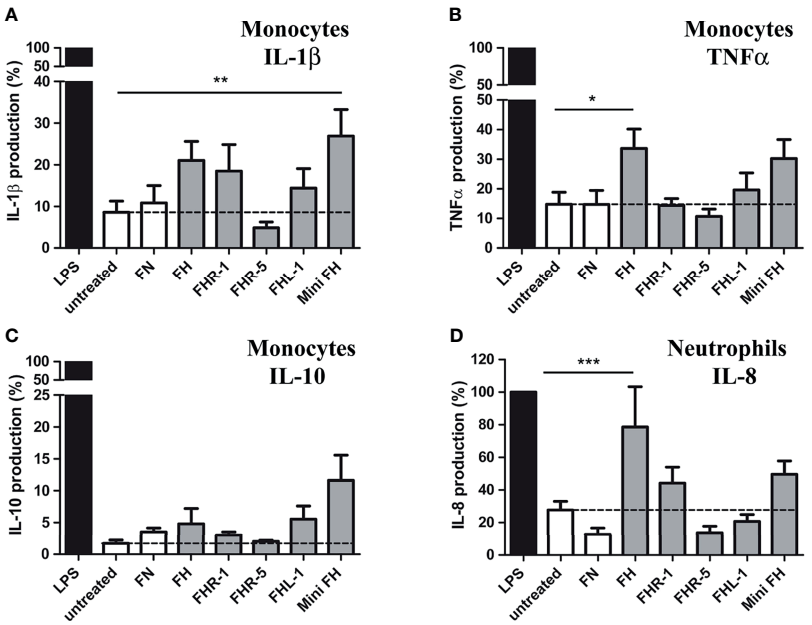

FIGURE 6 | FH family members influence the cytokine profile of monocytes and neutrophil granulocytes. Cells were incubated in the presence or absence (untreated sample) of 300 nM immobilized FH, FHR-1, FHR-5, FHL-1 and mini-FH for 24 h, and IL-1β **(A)**, TNFα **(B)**, and IL-10 **(C)** secretion of monocytes and IL-8 secretion of neutrophils **(D)** were investigated by ELISA. As a positive control, cells were activated with LPS; 300 nM fibronectin (FN) was also used as an additional immobilized control protein unrelated to the FH family. Data represent mean + SEM of nine independent donors. Differences compared to untreated samples with $p < 0.05$ were considered statistically significant (one-way ANOVA with Bonferroni's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

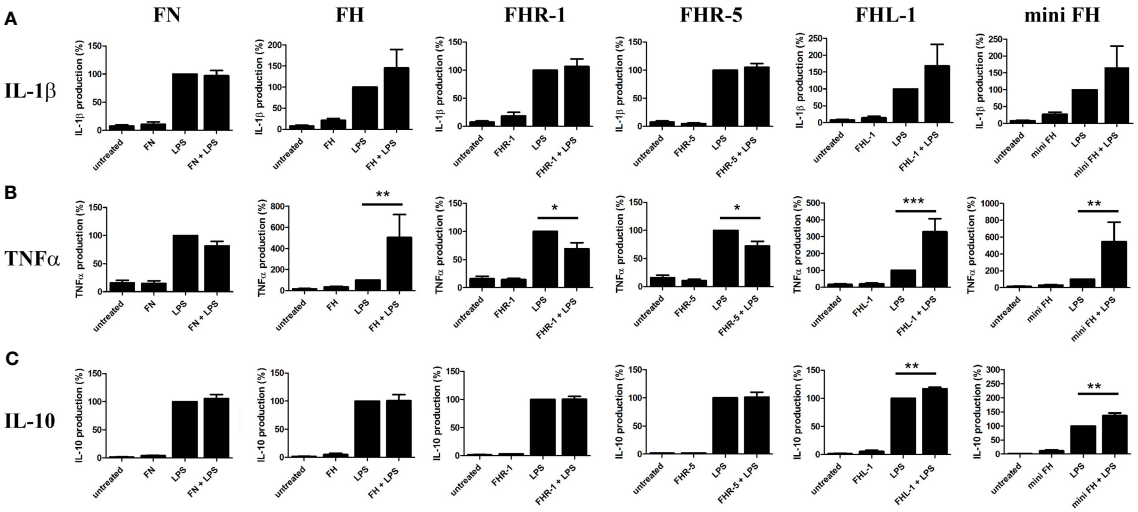

FIGURE 7 | Combined effect of immobilized FH, FHR-1, FHR-5, FHL-1 and mini-FH with soluble LPS on cytokine production by monocytes. Human monocytes were stimulated with 10 ng/mL LPS in the presence or absence of 300 nM immobilized FH, FHR-1, FHR-5, FHL-1 and mini-FH for 24 h, and IL-1β **(A)**, TNFα **(B)**, and IL-10 **(C)** secretion were measured by ELISA. As a control, FN-coated cells were used. Data represent mean + SEM of experiments with five independent donors. Differences compared to LPS treated samples with $p < 0.05$ were considered statistically significant (one-way ANOVA with Bonferroni's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

serum-derived FHL-1, FHR-1 and FHR-5 could be detected by western blot analysis using the cell lysates (15). The binding of FH to neutrophils could be inhibited by FHR-1 but not by FHR-5, further supporting the role of shared binding sites including CR3 for FH and FHR-1 on neutrophils (**Figure 2**). The relatively high amount of FHR-1 needed for significant inhibition of FH binding suggests that the binding of FH is stronger, e.g. because of the presence of at least two binding sites in FH *versus* a single

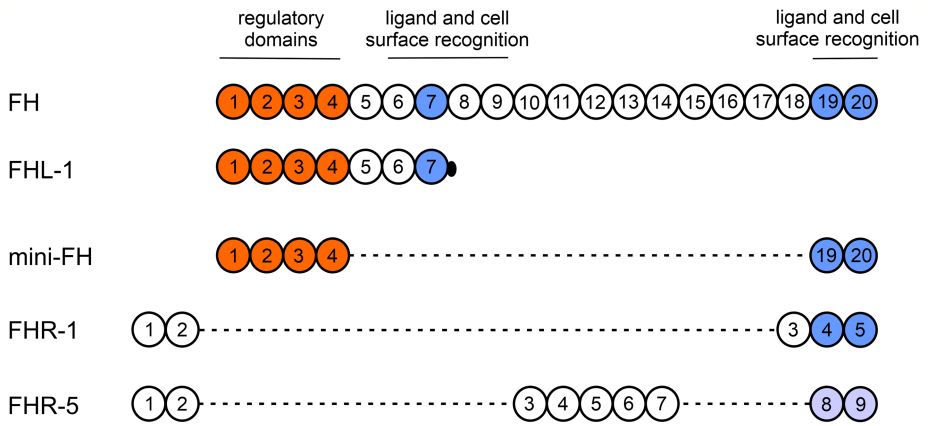

FIGURE 8 | Schematic structures of FH, FHL-1, mini-FH, FHR-1 and FHR-5. The individual SCR domains are indicated with numbered circles and are vertically aligned based on homology among the domains of the proteins. Major functional sites of FH are indicated above. SCRs marked orange are responsible for the complement regulatory functions, such as cofactor and decay accelerating activities and also binding to C3b. FH shows 100% amino acid sequence identity with the corresponding SCRs of FHL-1 and mini-FH, but FHL-1 includes a unique four aminoacid long sequence at its C terminus. SCRs marked blue are involved in binding to several ligands and cell surfaces and also have a role in receptor binding. A previous study using various FH fragments and receptor-specific antibodies suggests that the FH C terminus mediates binding to CR3, whereas SCR7 to both CR3 and CR4 (15). Note that except for the C-terminal domains of FHR-1, FHR-1 and FHR-5 show lower aminoacid sequence similarity to the respective FH domains. The N-terminal domains of FHR-1 and FHR-5 mediate dimerization of these proteins.

site in FHR-1, and is also explained in part by the binding of FH to CR4, as well.

In addition to binding to neutrophil granulocytes, FH, FHR-1 and FHL-1 were described to influence certain cellular functions, but these proteins were studied only in certain aspects (14–17). Here, we studied all these proteins in the same assays and included FHR-5 as well as the artificial construct mini-FH (**Table 1**). The similar functional effect of mini-FH on the cells compared with FH can be explained by the shared SCR19-20 that are involved in FH binding to CR3 on neutrophils. FHR-5, despite of its strong binding capacity to both neutrophils and monocytes, had no effect on the investigated cell functions except for the TNFα response when applied in combination with LPS, clearly indicating marked functional differences among the otherwise related members of the FH protein family (**Figure 8**).

Previously, we showed that neither soluble nor immobilized FH alone affected NET production. However, when NET formation was induced with PMA, immobilized but not soluble FH could significantly decrease the amount of extracellular DNA (16). In this study, FHR-1 but not the other FH family proteins or mini-FH had similar inhibitory effect on PMA-induced NET release as did FH (**Figure 4**). Prolonged or uncontrolled NET formation may enhance the development of inflammation or autoimmune diseases by providing autoantigens, such as DNA or histones. By inhibiting NET production, FH and FHR-1 may limit such undesirable reactions.

TABLE 1 | Summary of the effects of FH, FHR-1, FHR-5, FHL-1 and mini-FH on different cell functions.

	FH	FHR-1	FHR-5	FHL-1	Mini-FH
Neutrophils					
Cell binding	+	+	+	+	+
NET release	–	–	–	–	–
PMA-induced NET release	↓	↓	–	–	–
Cell spreading	↑	↑	–	↑	↑
IL-8 production	↑	–	–	–	–
IL-8 production with LPS	–	–	–	–	–
Monocytes					
Cell binding	+	+	+	+	+
Cell spreading	↑	–	–	↑	↑
IL-1β production	–	–	–	–	↑
IL-1β production with LPS	–	–	–	–	–
TNFα production	↑	–	–	–	–
TNFα production with LPS	↑	↓	↓	↑	↑
IL-10 production	–	–	–	–	–
IL-10 production with LPS	–	–	–	↑	↑

(+ means binding; ↑ means significant increase and ↓ means significant decrease of each cell function; – means no effect).

During inflammation caused by pathogens or other damage signals, extravasation of neutrophils and monocytes is essential. This is a multistep process including sequential rolling, firm adhesion/spreading and trans-endothelial migration (57, 59). Previous data suggested an adhesive function for FH in the case of neutrophils that is mediated *via* the complement receptor CR3 (14, 15). In addition, immobilized FH was shown to increase neutrophil spreading through the CR3 receptor, since in our previous study we found that specific antibodies against CD11b could inhibit this process (16), and to support the migration of both neutrophils and monocytes (15, 16, 24). Our present study confirmed the previously described increased neutrophil spreading on FH-covered surface (**Figure 5A**) and the same effect was observed on monocytes (**Figure 5B**). Immobilized FHL-1 and mini-FH had the same effect on both cell types (**Figure 5**). Interestingly, FHR-1 increased the spreading only in the case of neutrophils which suggests a different regulation process for the two cell types, and may be explained by the differential receptor expression (e.g., CR3 and CR4) on monocytes and neutrophils.

While all of the analyzed FH family proteins and mini-FH showed clear binding to monocytes and neutrophils, they also enhanced the attachment/spreading of the cells and influenced cytokine production, except for FHR-5. Since both cell types are key players in the initiation phase of the immune response, their cytokine production is essential during fighting against infections or can be an important factor in the pathogenesis of inflammatory diseases. We found significant increase in proinflammatory IL-1β secretion of monocytes cultured on mini-FH and slight increase when they were cultivated on FH, FHR-1 and FHL-1 coated wells. Similarly, TNF-α production by monocytes was significantly elevated on wells coated with FH and slightly on those with mini-FH. In the case of neutrophils, IL-8 production was monitored and immobilized FH was shown to enhance the secretion of IL-8 significantly, while FHR-1 and mini-FH induced only a slight increase (**Figure 6**). The direct role of FH family members in the cytokine production of different immune cells is not clear yet, since controversial findings have been described. Enhanced proinflammatory IL-8 and IL-1β production upon FH treatment was reported, however in most cases FH was applied in combination with other stimuli such as NET-derived DNA or *Candida albicans* (27, 28). Other studies support an anti-inflammatory role for FH, but again cells were treated with FH plus NET derived DNA, malondialdehyde acetaldehyde BSA or LPS and R848 as TLR ligands (29, 60, 61). We also tested the effect of the different FH family proteins and mini-FH on the cytokine production of LPS stimulated cells. In the case of TNF-α, FH, FHL-1 and mini-FH increased, whereas the FHR proteins FHR-1 and FHR-5 slightly decreased the level of this cytokine. In addition, FHL-1 and mini-FH slightly increased the secretion of IL-10. These results showing that the FH family proteins influence the LPS-triggered TNFα and IL-10 responses of monocytes may suggest their potential involvement in the regulation of inflammatory processes during infection. Interestingly, a recent study also found that mini-FH modulates complement-dependent IL-6 and IL-10 secretion by peripheral mononuclear cells in the presence of autologous serum; IL-10 release was increased by mini-FH when the cells were stimulated with LPS (62). When the effect of the FH family proteins was analyzed in isolated form, FH and FHR-1 treatment induced elevated IL-1β and IL-8 secretion (16, 26, 44) (**Figures 6** and **7**, **Supplementary Figure 2**). In our assays we applied immobilized FH family proteins, and in most other studies FH family proteins influenced cell functions only if they were immobilized on a surface of either a cell culture dish or yeast/bacteria particles. This supports the notion, that FH family members act as circulating molecular sentinels, that continuously monitor for altered structures or invading pathogens to which they can attach and then, in addition to modulating complement activation, they may bind to immune cells and modulate their function.

Soluble FH was found to be a direct modulator of monocyte differentiation since it can induce the differentiation of $CD14^+$ human monocytes into immunosuppressive macrophages; this FH effect was inhibited in the presence of autologous plasma, suggesting that this function likely occurs only in tissues (63). Moreover, soluble FH can also generate an anti-inflammatory and tolerogenic state in monocyte-derived dendritic cells (61). However, in these experiments applying several days of co-incubation with FH, the possibility that the added FH may eventually coats the plate surface and thus also acts in an immobilized form, cannot be ruled out. Integrin receptors can sense differences between immobilized and soluble ligands (64, 65), which may explain why we observed an elevated, although not significant IL-1β production upon LPS stimulation on FH coat, while Smolag et al. found a significantly decreased production upon LPS stimulation in FH-treated monocytes (63).

In our experiments we used purified proteins to compare differences in direct cellular functions without adding any other complement component or serum, but it has to be noted that *in vivo* their relative local concentration and surface bound forms could influence the surface and receptor binding capacity of FH and FHRs, and thus cellular responses. Several questions remain to be answered regarding the non-canonical role of FH family members. The complement receptor CR3 (CD11b/CD18) and to a lesser extent CR4 (CD11c/CD18) were identified as the receptors for FH binding on immune cells; however, other candidates are also found, and the receptors for FHR proteins have not been clearly identified yet (14–16, 27, 58, 66). The similarity of FHR-1 domains 3-5 to FH domains 18-20 implies that the C-terminal SCR18-20 (or 19-20) domains are responsible for the binding and functional effect of FH and FHR-1 on immune cells. The identity of FHL-1 with FH SCR1-7 suggests, however, that SCR6-7 might play an important role as well in influencing immune cell functions. FHR-5 shows a lower degree of homology to both parts of FH, that may be the reason why we could not detect any direct functional effect of this molecule despite its prominent binding to the cells. These findings also suggest that FHR proteins could bind to other receptors than does FH.

While CR3 was identified as a FH receptor in the absence of deposited C3b (58) on human neutrophils and also on U937 monocytic cells (**Figure 3**, **Supplementary Figure 1**), the

interaction of FH with cell surfaces is a complex phenomenon and is influenced among others by cell surface glycosaminoglycans and deposited other ligands, such as complement C3 fragments. The C-terminal domains of FH bind surface bound C3b *via* SCR19, while SCR20 binds sialic acids and glycosaminoglycans present on self surfaces which is important in the regulation of complement activation on the cell surface by FH (67–69); in addition, receptor-bound FH also retains its cofactor activity for C3b inactivation (16). The same C-terminal domains are involved in binding to neutrophils *via* complement receptors and mediating the non-canonical functions of FH (15). FH and FH SCR19-20 were also shown to prevent the adhesion of sickle cell disease erythrocytes to P-selectin and/or to CR3 by blocking C3b-cell interactions (70). Potentially, receptor-bound C3-fragments could also modify the interaction of FH and FHRs with cells. Moreover, it is unknown where exactly FH binds to its receptors. CR3 and CR4 are known to interact with several ligands, and their major ligand that promotes phagocytosis is iC3b on opsonized microbes and other particles (71). However, in our fibronectin and β-glucan induced NET model, iC3b used in combination with FH could not inhibit the effect of FH (16). Because there is evidence for ligand contact outside the I domain of CD18 (71) and the incubation of neutrophils with FH did not activate CR3 (72), it is likely that FH and iC3b bind differently. Further studies are warranted to better understand these interactions and their *in vivo* relevance.

In summary, we identify variable and context-dependent effects of FH family proteins on the function of human monocytes and neutrophil granulocytes. Furthermore, we show that FH-based complement inhibitors such as mini-FH, in addition to their effect on complement activation, may potentially also affect inflammatory cells. Further studies are needed to identify the exact receptors and mechanisms by which these proteins exert their functional effects on immune cells. A detailed characterization of the non-canonical functions of FH family proteins could provide a better understanding of their role in the clearance of different pathogens or host-derived debris and in the modulation of the resolution of inflammation and the pathogenesis of inflammatory disorders.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Research Ethics Committee (TUKEB ETT, permission number 838/PI/12). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ÉK, MK, NS and MJ designed the experiments. ÉK, MK, NS, DH and AS performed experiments. MJ supervised the study. All authors discussed the data, revised and approved the manuscript. ÉK, MK, NS, AS and MJ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.660852/full#supplementary-material

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