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Utilizing complement evasion strategies to design complement-based antibacterial immunotherapeutics: lessons from the pathogenic *Neisseriae*

Sanjay Ram^{*}, Jutamas Shaughnessy, Rosane B. DeOliveira, Lisa A. Lewis, Sunita Gulati, and Peter A. Rice

Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abstract

Novel therapies are urgently needed to combat the global threat of multidrug-resistant pathogens. Complement forms an important arm of innate defenses against infections. In physiological conditions, complement activation is tightly controlled by soluble and membrane-associated complement inhibitors, but must be selectively activated on invading pathogens to facilitate microbial clearance. Many pathogens, including *Neisseria gonorrhoeae* and *N. meningitidis*, express glycans, including N-acetylneuraminic acid (Neu5Ac), that mimic host structures to evade host immunity. Neu5Ac is a negatively charged 9-carbon sugar that inhibits complement, in part by enhancing binding of the complement inhibitor factor H (FH) through C-terminal domains (19 and 20) on FH. Other microbes also bind FH, in most instances through FH domains 6 and 7 or 18-20. Here we describe two strategies to target complement activation on *Neisseriae*. First, microbial binding domains of FH were fused to IgG Fc to create FH18-20/Fc (binds gonococci) and FH6,7/Fc (binds meningococci). A point mutation in FH domain 19 eliminated hemolysis caused by unmodified FH18-20, but retained binding to gonococci. FH18-20/Fc and FH6,7/Fc mediated complement-dependent killing in vitro and showed efficacy in animal models of gonorrhea and meningococcal bacteremia, respectively. The second strategy utilized CMP-nonulosonate (CMP-NulO) analogs of sialic acid that were incorporated into LOS and prevented complement inhibition by physiologic CMP-Neu5Ac and resulted in attenuated gonococcal infection in mice. While studies to establish the safety of these agents are needed, enhancing complement activation on microbes may represent a promising strategy to treat antimicrobial resistant organisms.

Keywords

Complement; Immunotherapeutics; *Neisseria*; factor H; sialic acid

^{*}Corresponding author: Sanjay Ram, Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Lazare Research Building, Room 322, 364 Plantation Street, Worcester MA 01605, USA. Tel: +1-508-856-6269. Fax: +1-508-856-8447. sanjay.ram@umassmed.edu.

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Introduction

Complement deficiencies have long been recognized as risk factors for certain infections (Figuerola, et al., 1993, Figuerola and Densen, 1991, Ram, et al., 2010) or as the cause of conditions such as paroxysmal nocturnal hemoglobinuria (PNH), for example, in which the loss of GPI-anchored membrane complement inhibitors CD55 and CD59 on erythrocytes leads to hemolysis (Nicholson-Weller, et al., 1985, Pangburn, et al., 1983). Over the past two decades, the role of complement dysregulation in various pathologic states has been recognized increasingly (de Cordoba, et al., 2012, Hajishengallis, et al., 2015, McHarg, et al., 2015, Schramm, et al., 2014, Thurman and Holers, 2006). Loss-of-function mutations in molecules that inhibit complement such as FH, membrane cofactor protein (MCP; CD46) and factor I (FI), or gain-of-function mutations in molecules that activate complement such as C3 and factor B (FB) all lead to an overactive alternative pathway and are associated with atypical hemolytic uremic syndrome (aHUS), a condition characterized by thrombotic microangiopathy and renal failure (de Cordoba and de Jorge, 2008, Esparza-Gordillo, et al., 2005, Esparza-Gordillo, et al., 2006, Fremeaux-Bacchi, et al., 2008, Hofer, et al., 2014, Kavanagh and Goodship, 2010, Liszewski and Atkinson, 2015, Loirat and Fremeaux-Bacchi, 2011, Nester, et al., 2015, Pickering and Cook, 2008). A common polymorphism in domain 7 of human FH (402H) reduces the ability of FH to bind to malondialdehydes in drusen (the retinal lesions seen in age-related macular degeneration), which is associated with increased alternative pathway activation and accelerated vision loss (Edwards, et al., 2005, Haines, et al., 2005, Klein, et al., 2005, Weismann, et al., 2011). Excessive complement activation may also play a role in neurological conditions such as Alzheimer's disease and schizophrenia (Hong, et al., 2016, Sekar, et al., 2016).

Complement-based therapeutics that are currently in clinical use or in pre-clinical trials all inhibit the complement cascade (reviewed in (Reis, et al., 2015)). Purified C1 inhibitor is indicated for the treatment of hereditary or acquired C1 inhibitor deficiency. A humanized monoclonal antibody, eculizumab, has been used for several years to treat paroxysmal nocturnal hemoglobinuria (PNH) and more recently has been used successfully in several cases of shiga-toxin associated hemolytic uremic syndrome (Delmas, et al., 2014, Dinh, et al., 2015, Lapeyraque, et al., 2011). Other products in various stages of development include: antibodies or fragments of antibodies directed against C5, factor D, C1s or MASP-2; small molecules that block factor D, C5aR or C3 or soluble complement inhibitors of complement (CR1 or fragments of FH). All these agents are being evaluated for a variety of conditions where complement inhibition may be beneficial (Reis, et al., 2015).

In contrast to blocking the various complement pathways as described above, the goal of a complement-based anti-infective immunotherapeutic or prophylactic is to selectively activate the cascade on the microbial surface without causing collateral damage to normal host tissue. This is usually accomplished by molecules that specifically bind to invading pathogens and initiate complement activation. Antibodies that are elicited following natural infection or by immunization are historically the best appreciated initiators of the classical pathway, although the roles of lectins and ficolins in marking pathogens for subsequent complement activation are now well established (Degn and Thiel, 2013, Thiel and Gadjeva, 2009).

Immune antibodies are highly effective in preventing infections but their specificity can be a limitation. Extensive antigenic diversity even within a pathogenic species is a major challenge. As an example, over 90 distinct capsule types have been identified in *Streptococcus pneumoniae* (Kamerling, 2000), of which only 13 or 23 are targeted by conjugate or polysaccharide vaccines, respectively. Antigenic variation is a major hurdle in the development of vaccines against bacteria such as nontypeable *Haemophilus influenzae* and *Neisseria gonorrhoeae*. Protective epitopes often are encoded by several alleles and/or expression these epitopes may be regulated by phase-variable genes (Barnett, et al., 2015, Hill, et al., 2010, Lipsitch and O'Hagan, 2007, Telford, 2008). Antibodies against more conserved epitopes sometimes are not broadly protective and may even be subversive ('blocking' antibodies) (Ray, et al., 2011, Rice, et al., 1986, Schweinle, et al., 1989). Broad spectrum immunotherapeutics that target common pathogenic mechanism(s) across several pathogens would permit empiric treatment while awaiting a specific microbiologic diagnosis.

Mimicry of host glycans by pathogens

Several microbes evade host immunity by expressing glycans that mimic host sugars. Capsular polysaccharides produced by group B *N. meningitidis*, *Escherichia coli* K1, *Mannheimia haemolytica* and *Moraxella nonliquefaciens* all comprise $\alpha(2,8)$ -linked Neu5Ac, which is identical to human neural cell adhesion molecule (NCAM) (reviewed in (Cress, et al., 2014)). *E. coli* K4, *Pasturella multocida* type F and *Avibacterium paragallinarum* (genotype I) all produce chondroitin sulfate capsules. Capsules containing heparosan are produced by *E. coli* K5, *P. multocida* (type D), *A. paragallinarum* (genotype II), *Streptococcus pyogenes*, *S. equi* ssp. *zooepidemicus*, *S. dysgalactiae* ssp. *equisimilis*, *S. uberis*, *S. equi* ssp. *equi*, *P. multocida* (type A) and *A. paragallinarum* (Cress, et al., 2014).

Host-like glycans are also expressed by lipooligosaccharides (LOSs) of *N. gonorrhoeae*, *N. meningitidis*, *Campylobacter jejuni* and *H. influenzae* (Aspinall, et al., 1994, Houlston, et al., 2011, Mandrell and Apicella, 1993, Mandrell, et al., 1988, Yuki, et al., 2004, Yuki, et al., 1993, Mandrell, 1992). Relevant to this review, two 'host-like' structures expressed by Neisserial LOS structures include lacto-*N*-neotetraose (LNnT; Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc), identical to the terminal tetrasaccharide of paragloboside, a precursor of the major human blood group antigens (Mandrell, et al., 1988), and globotriose (Gal α 1-4Gal β 1-4Glc) that is identical to terminal globotriose trisaccharide of the P^K-like blood group antigen (Mandrell, 1992). The LNnT structure is found in eight LOS immunotypes of *N. meningitidis* (Tsai and Civin, 1991); the P^K-like structure is also referred to as the L1 immunotype. Host-like glycan structures expressed by microbes do not elicit robust antibody responses and therefore enable pathogens to evade the immune response.

The role of sialic acid in Neisserial complement evasion

In 1970, Ward et al reported that gonococci recovered directly from male urethral secretions (not sub-passaged onto routine culture media) were fully resistant to killing by complement in normal human serum (NHS), a property termed serum resistance (Ward, et al., 1970). However, even a single passage of most isolates on routine culture media resulted in serum

sensitivity, which suggested that in vivo, gonococci acquired a host factor that conferred complement resistance that was lost in vitro. A series of elegant and detailed studies by Harry Smith and his colleagues culminated in identification of cytidinemonophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) as the host molecule responsible for gonococcal serum resistance (Nairn, et al., 1988, Parsons, et al., 1993, Parsons, et al., 1994, Parsons, et al., 1988, Smith, et al., 1992). Neu5Ac is a negatively charged 9-carbon backbone sugar that is an example of a sialic acid (Sia). Sias include derivatives of neuraminic acid and ketodeoxynonulosonic acid and are part of a larger family of carbohydrates called nonulosonates (NulOs). Addition of purified CMP-Neu5Ac to gonococcal growth media converts strains otherwise sensitive to killing by NHS to a serum-resistant phenotype (Nairn, et al., 1988, Emond, et al., 1995, Wetzler, et al., 1992). The only molecule on gonococci that is modified by growth in media that contains CMP-Neu5Ac is LOS (Mandrell, et al., 1990, Parsons, et al., 1989). Sialylation of gonococcal LOS requires an exogenous source of CMP-Neu5Ac, however groups B, C, W and Y meningococci can synthesize CMP-Neu5Ac and therefore sialylate their LOS endogenously (Mandrell, et al., 1991, Swartley, et al., 1997, Blacklow and Warren, 1962, Warren and Blacklow, 1962, Frosch, et al., 1989).

Both, the L_{NnT} and P^K-like LOS species in *Neisseriae* can be substituted with Neu5Ac (Pavliak, et al., 1993, Wakarchuk, et al., 1998). L_{NnT} is expressed by *Neisserial* LOS more frequently, particularly in *N. gonorrhoeae*, than the P^K-like structure and therefore L_{NnT} is represented in most studies of LOS sialylation. An important difference in modification of these two LOSs by Neu5Ac is linkage specificity – Neu5Ac forms α 2-3 bonds with the terminal Gal residues of L_{NnT} LOS while α 2-6 bonds are formed with terminal Gal residues of P^K (Pavliak, et al., 1993, Wakarchuk, et al., 1998, Gulati, et al., 2005). Linkage specificity has implications in the extent of complement resistance as discussed below.

Studies of the interaction between sialic acid and the complement system have focused predominantly on the alternative pathway. Almost 40 years ago, it was recognized that sialic acid on cell surfaces enhances the affinity of FH for cell-surface associated C3b almost 10-fold (Fearon, 1978, Pangburn and Muller-Eberhard, 1978). Desialylation of complement non-activator surfaces such as sheep erythrocytes renders them susceptible to lysis by homologous complement (Fearon, 1978). The exocyclic substitutions (carbons 7, 8 and 9) of sialic acid are critical for alternative pathway regulation. Removal of the 9-carbon results in loss of 90% of complement inhibition by Sia (Michalek, et al., 1988). The extent of 9-O-acetylation of Sias on mouse erythrocytes correlates directly with the susceptibility of erythrocytes to lysis by the alternative pathway (Varki and Kornfeld, 1980). Similarly, increased expression of 9-O-acetylated sialoglycans on the red cells of individuals with visceral leishmaniasis is associated with greater alternative pathway activation (Chava, et al., 2004). Removal of C8 and C9 carbons from Sia with NaIO₄ treatment renders sheep erythrocytes susceptible to lysis by the alternative pathway (Fearon, 1978).

Certain other polyanions such as highly sulfated heparin, heparin sulfate, dermatan sulfate, chondroitin sulfate A and carrageenan (types III and IV) also enhance the affinity of FH for surface-bound C3b and promote complement inhibition (Carreno, et al., 1989, Kazatchkine, et al., 1979, Meri and Pangburn, 1990, Meri and Pangburn, 1994). Kajander et al proposed a model in which FH domains 19 and 20 interacted with C3 fragments and cell surface

polyanions, respectively (Kajander, et al., 2011). Subsequently, Blaum et al showed remarkable specificity of the N-acetyl neuraminic acid (Neu5Ac) linkage on host cell surfaces and the interaction with FH – only $\alpha(2,3)$ linked Neu5Ac interacted with FH domain 20; $\alpha(2,6)$, $\alpha(2,8)$ or $\alpha(2,9)$ linked Neu5Ac did not interact (Blaum, et al., 2015). The linkage specificity of Sia involved in alternative pathway regulation should be emphasized – to date, there is no evidence for enhanced FH binding or function on bacteria that possess capsular sialic acid in the $\alpha(2,6)$ (e.g., groups W and Y *N. meningitidis*), $\alpha(2,8)$ (e.g., group B meningococci and *E. coli* K1) or $\alpha(2,9)$ (e.g., group C *N. meningitidis*) linkage configuration. Paradoxically, upregulated alternative pathway activation is seen on groups W and Y meningococci and these capsules themselves bind C3 fragments (Ram, et al., 2011). These findings provide strong evidence that self-nonspecific discrimination results from recognition of highly specific glycans rather than non-specific charge interactions.

Unencapsulated bacteria such as nontypeable *H. influenzae* and *N. gonorrhoeae* interact with complement exclusively via their somatic antigens. Substitution of the terminal Gal of gonococcal LNT LOS with Neu5Ac, which occurs through an $\alpha(2-3)$ linkage, enhances the binding of FH to *N. gonorrhoeae* (Ram, et al., 1998). Binding of FH to sialylated gonococci has been localized to the three C-terminal domains of FH (Ram, et al., 1998, Ngampasutadol, et al., 2008) (Fig. 1A). However, sialylation of meningococcal LNT LOS does not enhance binding to the C-terminus of FH (Lewis, et al., 2012). This is because the interaction between FH and sialylated gonococci also requires gonococcal PorB; replacement of gonococcal PorB with meningococcal PorB does not increase FH binding (Madico, et al., 2007) (Fig. 1B). Conversely, replacing meningococcal PorB with gonococcal PorB, results in a ‘gonococcal phenotype’ – i.e., enhanced FH binding upon LNT LOS sialylation (Madico, et al., 2007). Thus, a stable interaction between FH and sialylated gonococci is mediated by concomitant engagement of FH by both LOS Neu5Ac and gonococcal PorB. Based on studies of Blaum et al (Blaum, et al., 2015) and Kajander et al (Kajander, et al., 2011), it is likely that, in *N. gonorrhoeae*, FH domain 20 interacts with the $\alpha(2-3)$ linked Neu5Ac on LNT LOS, while domain 19 may bind to PorB (or to C3 fragments, as occurs on meningococci). In the case of meningococci, sialylation of LNT LOS increases binding of the FH domains 18-20 to bacteria only when C3 fragments are also deposited on the bacterial surface (Lewis, et al., 2012), which simulates complement inhibition by FH on host cells (Kajander, et al., 2011, Blaum, et al., 2015). Thus, microbes have evolved to mimic their human hosts in the manner they recruit FH. Meningococci express at least four additional ligands for FH, all of which bind domains 6 and 7 in FH (discussed below). Only Neu5Ac, that is $\alpha(2,3)$ linked to gonococcal LNT LOS, increases FH binding; no increase in FH binding is seen with sialylation of gonococci that express only P^K-like LOS, to which Neu5Ac is $\alpha(2,6)$ linked (Gulati, et al., 2005) (Fig. 1C). Finally, the increase in FH binding to sialylated gonococci is restricted to human FH; replacing human domain 20 in FH18-20/Fc with the chimpanzee counterpart abrogated binding (Shaughnessy, et al., 2011) (Fig. 1D). Arg at position 1203 in human FH domain 20 is critical for human FH binding specificity; the human-to-chimpanzee Arg→Asn mutation abrogated binding and the converse chimp-to-human Asn→Arg mutation in the background of chimp domain 20 restored binding (Shaughnessy, et al., 2011).

Elkins and colleagues showed that sialylation of gonococcal LOS decreased binding of antibodies to the gonococcal surface (Elkins, et al., 1992). This effect was specific for mAbs against porin B (PorB), but not for mAbs against another outer membrane protein called opacity protein (Opa) (Elkins, et al., 1992). Subsequent studies did not confirm a reduction in binding of anti-PorB Abs (Wetzler, et al., 1992, de la Paz, et al., 1995), although differences in Abs used and sensitivity of the assays employed may have accounted for differences in results. As expected, LOS sialylation reduced binding of anti-LOS mAbs (de la Paz, et al., 1995). Binding of IgG present in pooled NHS was also decreased by LNT LOS sialylation (Gulati, et al., 2015). LOS sialylation decreases C4 deposition on organisms that are incubated with NHS (McQuillen, et al., 1999, Zaleski and Densen, 1996), which suggests inhibition of the classical pathway. The lectin pathway can also enhance C4 deposition, however its role in activating complement on gonococci remains controversial. Purified MBL can bind to gonococci and deposit C4, but this process is also inhibited by LNT LOS sialylation (Devyatyarova-Johnson, et al., 2000, Gulati, et al., 2002). Further, the MBL pathway may not be effective in the context of serum that contains C1 inhibitor and α 2-macroglobulin; both inhibit the lectin pathway on gonococci (Gulati, et al., 2002). Unsialylated Neisserial LOS is a target for C4b deposition (Lewis, et al., 2008), a process that may be blocked by sialylation. In sum, Neisserial LOS sialylation may mask select Ab epitopes and/or prevent C4b deposition, and thereby limit classical pathway activation.

Fragments of FH fused to Fc as anti-infective immunotherapeutics

The observation that most microbes bind FH through domains distinct from the complement-inhibiting domains of FH (N-terminal domains 1 through 4) (Table 1) renders these pathogen-binding domains as attractive therapeutic targets. We reasoned that fusing the microbial binding domains of FH to the Fc region of antibody could result in broad-spectrum “anti-pathogen immunoadhesins” with multiple possible modes of action (Fig. 2). In the case of *Neisseriae* and *Haemophilus*, antibody is critical for bacterial killing, thus highlighting the importance of Fc (Ingwer, et al., 1978, Lewis, et al., 2009, Steele, et al., 1984, Tarr, et al., 1982). Further, the dimeric nature of FH binding to bacteria in an Fc fusion protein (Fig. 2) would permit greater avidity than native/physiologic human FH (and factor H-like protein 1 (FHL-1) in the case of domains 6 and 7) and thus not be outcompeted by these monomeric molecules in the physiologic state.

Development of FH18-20/Fc against *N. gonorrhoeae*

In an initial proof-of-principle experiment, we showed that FH domains 18-20 fused to mouse IgG2a Fc could mediate complement-dependent killing of *N. gonorrhoeae* (Shaughnessy, et al., 2011). Importantly, killing was observed at FH/Fc concentrations that were below levels expected to block the binding of FH present in human serum to bacteria. As discussed above, bacteria have evolved to mimic their hosts and use similar mechanisms to scavenge FH. Thus, a molecule that comprises the C-terminus of FH (e.g., domains 18-20) fused to Fc would require modification of the FH fragment to avoid toxicity such that the fusion protein binds only to bacteria, but not to host cells.

Atypical hemolytic uremic syndrome (aHUS) is a disease caused by over-activity of the alternative pathway of complement (de Cordoba, et al., 2012, Nester, et al., 2015, Rodriguez de Cordoba, et al., 2014, Rodriguez, et al., 2014). A cause(s) of aHUS is 'loss of function' mutations in FH (de Cordoba, et al., 2012); some of these mutations interfere with the interaction of FH with host cells (Ferreira, et al., 2009). FH is important to protect human RBCs from hemolysis by homologous serum when RBCs are treated with function-blocking anti-CD59 antibodies. In this system, increasing concentrations of a recombinant protein comprising only FH domains 19 and 20 blocks binding of endogenous FH and results in dose-responsive lysis of RBC (Ferreira, et al., 2009). Ferreira et al introduced aHUS mutations into recombinant FH 19-20 to determine if these interfered with inhibition of FH in the lysis of anti-CD59-treated RBCs. Four mutant proteins were identified that did not otherwise interfere with FH function and therefore did not result in lysis of RBCs: D1119G (domain 19), R1182S, W1183R and R1215G (the latter three are in domain 20) (Ferreira, et al., 2009). Guided by these findings, we introduced each of these four mutations separately into FH18-20/Fc. Of the four mutant molecules, the greatest binding and functional serum bactericidal activity was seen with FH18-20/Fc that bore the D1119G mutation, henceforth called FHD1119G/Fc (Fig. 3A). While FH18-20/Fc (FH domains unmodified) caused lysis of anti-CD59-treated human RBCs by homologous human serum, no lysis was noted with FHD1119G/Fc (Fig. 3B). Based on its superior efficacy and the lack of hemolysis, FHD1119G/Fc was chosen as the lead molecule for further study.

Complement-mediated serum bactericidal activity (SBA) confers protection against meningococcal disease – a SBA titer of 1:4 is a surrogate of protection (Borrow, et al., 2006, van Alphen and van den Dobbelsteen, 2008) but the in vitro correlates of protection against gonococcal infection remain undefined. We have shown previously that monoclonal antibody 2C7 (mAb 2C7) that is directed against an LOS epitope expressed by >95% of gonococcal isolates in vivo also shows SBA and promotes opsonophagocytosis by human neutrophils (Gulati, et al., 2012, Gulati, et al., 1996, Gulati, et al., 2013). Thus, it is reasonable to speculate that the ability of an antibody (or in this case, FH/Fc) to either mediate SBA against or support opsonophagocytosis of *N. gonorrhoeae* may correlate with protection.

In the presence of 20% human complement (NHS depleted of IgG and IgM), FHD1119G/Fc showed bactericidal activity (defined as <50% survival following 30 min of incubation at 37 °C) against 11 of 16 strains of *N. gonorrhoeae*, including three ceftriaxone-resistant isolates (Shaughnessy, et al., 2016). C3 fragments deposited on bacteria can engage receptors on phagocytes such as CR3 and facilitate killing through opsonophagocytosis. FHD1119G/Fc enhanced C3 deposition on each of the 5 strains that resisted SBA at least 10-fold, which was sufficient to facilitate opsonophagocytic killing of gonococci by PMNs (Shaughnessy, et al., 2016). Using the BALB/c mouse vaginal colonization model developed by Jerse and colleagues (Jerse, et al., 2011) we showed that daily intravaginal treatment with FHD1119G/Fc significantly decreased the duration and burden of gonococcal infection (Shaughnessy, et al., 2016) (Fig. 4).

LOS sialylation confers survival advantage to gonococci in vivo. Following infection of human male volunteers with a LOS phase variant of a gonococcal strain that expressed

predominantly lactose from HepI (gonococci do not sialylate lactose), Schneider et al found that bacteria recovered later when subjects had become symptomatic had phase varied their LOS and now expressed LNnT (sialylatable) from HepI (Schneider, et al., 1991). Gonococcal mutants that do not sialylate because of insertional inactivation of their LOS sialyltransferase (*Ist*) gene are less virulent in mice (Lewis, et al., 2015, Wu and Jerse, 2006). Therefore, natural selection that resulted in resistance to FHD1119G/Fc, if it were to occur, would require loss of sialylation thereby incurring considerable cost to fitness.

Activity of FH6,7/Fc against *N. meningitidis* and *Haemophilus influenzae*

Ligands for human FH on *N. meningitidis* include factor H binding protein (FHbp) (Madico, et al., 2006), Neisserial surface protein A (Lewis, et al., 2010), Porin B2 (PorB2) (Lewis, et al., 2013) and select PorB3 molecules (Giuntini, et al., 2015). In each instance, binding occurs through FH domains 6 and 7. The FHbp-FH interaction has been characterized in elegant studies by Schneider and colleagues showing that amino acid side chains of meningococcal factor H binding protein (FHbp) mimicked the interactions of charged sugars with FH domains 6 and 7 (Schneider, et al., 2009). All *N. meningitidis* strains express PorB2 or PorB3 (the two are allelic variants) and NspA (Lewis, et al., 2010); with a few exceptions (Lucidarme, et al., 2011), almost every isolate also expresses FHbp. These ligands all bind FH domains 6 and 7; thus a chimeric protein comprising FH domains 6 and 7 fused to Fc (FH6,7/Fc) is likely to bind to most meningococcal strains. Nontypeable *H. influenzae* (NTHi) also bind FH, mainly through domains 6 and 7 (measured using FH6,7/Fc); some strains show weak binding to domains FH18-20/Fc. NTHi P5 is an acceptor for FH, although some isolates bind FH through a distinct ligand(s) (Wong, et al., 2016).

FH6,7/Fc possesses SBA against *N. meningitidis* and is effective in decreasing bacteremia in the infant Wistar rat model of meningococcal bacteremia (Shaughnessy, et al., 2014) (Fig. 5A and 5B). Similarly, FH6,7/Fc mediates complement-dependent killing of NTHi and causes a significant reduction in CFU counts in the mouse lung model (Wong, et al., 2016) (Fig. 5B and 5D). In conclusion, FH fragments fused to Fc can bind to microbial surfaces, activate complement and promote complement dependent killing, either through membrane attack complex formation and/or through opsonophagocytosis.

Because FH plays a key role in regulating complement on host surfaces, we acknowledge that considerable work will be needed to establish the safety of FH/Fc, especially if used systemically. The lack of hemolysis of human RBCs appears promising, but the effect of FH/Fc on kidneys and ocular tissue need careful study.

Sialic acid analogs as anti-gonococcal immunoprophylactics

The importance of gonococcal LOS sialylation in establishing infection in humans and in the mouse vaginal colonization model has been discussed above. Modifications of Neu5Ac – particularly in the exocyclic side chain (corresponding to carbons 7, 8 and 9; Fig. 6) – often result in loss of complement-inhibiting activity (Fearon, 1978, Michalek, et al., 1988, Varki and Kornfeld, 1980, Chava, et al., 2004). Thus, if sialic acid analogs that do not inhibit complement were available as CMP-nonulosonates (CMP-NulOs) and incorporated and expressed by gonococci, they could prove effective as prophylactic or adjunctive agents in

the treatment of multidrug-resistant gonorrhea. Such analogs would counteract the 'complement dampening' physiological form of sialic acid that ordinarily is scavenged from humans (i.e., Neu5Ac).

Six CMP-NuLOs were added to gonococcal growth media and bacteria were examined for incorporation of the NuLO into LNT LOS: CMP-Neu5Gc (glycolyl); CMP-Neu5Ac9Ac (acetyl); CMP-Neu5Ac9Az (azido); CMP-Neu5Gc8Me (methyl); CMP-Leg5Ac7Ac (legionaminic acid) and CMP-Pse5Ac7Ac (pseudaminic acid). The structures of neuraminic, legionaminic and pseudaminic acids are shown in Fig. 6. With the exception of Pse, all other NuLOs were incorporated into gonococcal LOS (Gulati, et al., 2015). CMP-Pse5Ac7Ac differs from the other CMP-NuLOs stereochemically at carbons 5, 7 and 8, and was not anticipated to be utilized by gonococcal sialyltransferase.

Serum bactericidal assays were performed to determine whether the Sia analogs that were linked to LOS affected killing by NHS. As shown in Fig. 7, CMP-Neu5Gc fully protected gonococci (>100% survival of organisms) at a serum concentration of 10%, similar to human CMP-Neu5Ac. Neu5Ac9Ac and Neu5Gc8Me incorporation conferred full protection of organisms (>100% survival) only in 3.3% serum, but did not protect bacteria (<10% survival) against higher serum concentrations (Gulati, et al., 2015). The addition of Neu5Ac9Az and Leg5Ac7Ac to LOS permitted killing of gonococci at all serum concentrations tested. As expected, Pse5Ac7Ac, which does not incorporate into LOS, did not influence serum killing.

High levels of human FH binding to gonococci, similar to that seen with native Neu5Ac-substituted LNT LOS, was restricted; weak FH binding was conferred by Neu5Ac9Ac substituted LOS and barely any binding was observed when other NuLOs were incorporated (Gulati, et al., 2015). These findings confirm prior observations that underscore the importance of exocyclic substitutions (e.g., C8 and C9) of Sia (Fearon, 1978, Michalek, et al., 1988, Varki and Kornfeld, 1980, Chava, et al., 2004). The data also are consistent with findings of Blaum et al, who showed that the O8- and O9-positioned hydroxyl groups on the glycerol chain of Neu5Ac that was α 2–3-linked to lactose, formed hydrogen bonds with the amide and carbonyl groups, respectively, of the W1198 residue in FH domain 20 (Blaum, et al., 2015). Alternatively, the single oxygen atom difference between Neu5Ac and Neu5Gc at the 5-position does not affect killing by NHS. Collectively, these findings further affirm the high degree of binding specificity between FH and glycosaminoglycans – the interactions are not merely related to charge (Blaum, et al., 2015).

Encouraged that Neu5Ac9Az and Leg5Ac7Ac expressed by gonococcal LNT LOS did not enhance resistance even to 3.3% serum, we compared the deposition of classical and alternative pathway components on the bacteria 'capped' with Neu5Ac, Neu5Ac9Az and Leg5Ac7Ac (Fig. 8). In contrast to Neu5Ac, which effectively inhibited the classical and alternative pathways, activation of both complement pathways on gonococci that expressed either Neu5Ac9Az or Leg5Ac7Ac was unimpeded (Gulati, et al., 2015).

To function as a therapeutic, the CMP-NuLOs must compete with the complement inhibiting function of CMP-Neu5Ac on humans (or both CMP-Neu5Ac and CMP-Neu5Gc in lower

animals). To address this in vitro, increasing concentrations of CMP-Neu5Ac9Az or CMP-Leg5Ac7Ac were added to growth media 15 min after the addition of CMP-Neu5Ac and bacterial survival in 10% human serum was measured. As shown in Fig. 9 (note that only data with CMP-Leg5Ac7Ac is shown; CMP-Neu5Ac9Az showed similar results (Gulati, et al., 2015)), the competing NulO, even at a 100-fold lower concentration, reversed resistance to killing by NHS, normally mediated by CMP-Neu5Ac. Quite apart from the role that Neu5Ac plays in augmenting resistance to killing by NHS, both pathways of complement were activated in the presence of the competing NulO, resulting in gonococcal killing (Fig. 9).

In our experiments, the amount of Neu5Ac incorporated into LOS exceeded the amount of the competing analog, thus a paucity of Neu5Ac does not explain high levels of complement activation when both Neu5Ac and Leg5Ac7Ac together were added to media (Gulati, et al., 2015). Why CMP-NulO (e.g., Leg5Ac7Ac) exerts a ‘dominant-suppressive’ effect over Neu5Ac remains unclear. Another interesting and yet unexplained observation is that despite near maximal levels of FH binding when Neu5Ac and Leg5Ac7Ac were both expressed by LOS, high amounts of FB (FB here represents the Bb fragment that is part of C3 and C5 convertases) were associated with bacteria. Surfaces that bind high levels of FH do not activate the alternative pathway and therefore are usually expected to be associated with low amounts of FB – i.e., an inverse correlation exists between FH and FB binding, as seen on the unsialylated bacteria and bacteria expressing only Neu5Ac (clear and solid black bars, respectively, in Fig. 9). However, surface-bound FH did not effectively inhibit the alternative pathway in the presence of both Neu5Ac and Leg5Ac7Ac (hatched bars in Fig. 9). Of note, gonococci coated with Neu5Ac alone also inhibit IgG binding and subsequent classical pathway activation; the simultaneous presence of Leg5Ac7Ac did not inhibit the classical pathway. The importance of the classical pathway in killing gonococci has been well documented (Ingwer, et al., 1978, Lewis, et al., 2009, Shaughnessy, et al., 2016). Previous reports suggest that C3b associated with IgG effectively amplifies the alternative pathway and may resist inactivation by FH (Fries, et al., 1984, Jelezarova and Lutz, 1999, Jelezarova, et al., 2000). Thus, uninhibited classical pathway activation may be a driving force behind killing of bacteria coated with both Leg5Ac7Ac and Neu5Ac.

Finally, the efficacy of intravaginally administered CMP-Leg5Ac7Ac was tested in the mouse vaginal colonization model of gonorrhea. Topical daily treatment was carried out to simulate the use of CMP-NulOs as a prophylactic microbicide. As shown in Fig. 10, CMP-Leg5Ac7Ac significantly reduced the duration and burden of infection with multidrug-resistant gonococcal strain H041 (Gulati, et al., 2015).

Important to consider in the development of CMP-NulOs as therapeutics will be to ensure that NulOs are not incorporated into host structures. Certain mammalian sialyltransferases, including human ST6Gal1, can utilize CMP-Leg5Ac7Ac as a substrate to modify host glycolipids and glycolipids (Watson, et al., 2015). However, CMP sugars do not traverse cell membranes, thus reducing the likelihood that they will be expressed on host cells.

Concluding remarks

Antibiotic resistant microbes pose a threat to human health globally (CDC, 2013). There is an urgent need to develop novel antimicrobials and safe and effective vaccines to combat multidrug-resistant infectious diseases such as gonorrhea (Unemo and Shafer, 2014). In the case of diseases such as gonorrhea, humans are the only natural reservoir and person-to-person spread is vital for its persistence. The simplest model of STD dynamics states that $R_0 = \beta Dc$, where R_0 is the average number of secondary cases of infection generated by a primary case in a susceptible population, β is the probability of transmission within a sexual partnership, c is the number of new sex partners the infected person has in a unit of time and D is the duration the person is infectious (Anderson and May, 1991, Garnett and Anderson, 1996). An $R_0 > 1$ implies that an infection will persist, while an $R_0 < 1$ predicts elimination of infection. Thus, even a modest decrease in the duration and burden of *N. gonorrhoeae* infection will profoundly impact transmission.

Over the past several years, numerous insights have been gained on the structural basis of complement activation and inhibition. The central role of excessive complement activation in several pathological conditions has been elucidated. This has led to the development of several candidate molecules to inhibit complement. Because of the central role of complement in fighting infections and increasing knowledge of the functions of the cascade, we believe that manipulation of complement on microbial surfaces to facilitate their elimination is a challenging and exciting area of investigation that holds considerable promise.

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Nonstandard abbreviations

NuIO	nonulosonate
LNnT	lacto-N-neotetraose
LOS	lipooligosaccharide
Lst	LOS sialyltransferase
FH	factor H

FB	factor B
FD	factor D
MASP-2	Mannan binding lectin associated serine protease 2
FHL-1	Factor H-like protein 1
FHR-1	Factor H-related protein 1
Por	Porin
NHS	normal human serum
SBA	serum bactericidal activity
NTHi	nontypeable <i>Haemophilus influenzae</i>

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Fig. 1.

Characterization of the interactions of FH with sialylated *N. gonorrhoeae*. **A.** Sialylated gonococci bind human FH domains 18-20. *N. gonorrhoeae* F62 was grown in media containing CMP-Neu5Ac to sialylate its LNnT LOS. Sialylated bacteria were incubated with FH/Fc fusion proteins (the fragments of FH are indicated to the right of the graph) and bound FH/Fc was detected by flow cytometry. Only the FH/Fc molecules that contained FH domains 18-20 bound sialylated gonococci. X-axis, fluorescence of FH binding on a log₁₀ axis; Y-axis, counts. Adapted from Ref. (Ngampasutadol, et al., 2008) with permission. Copyright 2008. The American Association of Immunologists, Inc. **B.** Binding of FH to sialylated gonococci requires concomitant expression of gonococcal PorB. *N. gonorrhoeae* (*Ng*) that expressed either its own PorB (*Ng*(*Ng* PorB)) or meningococcal PorB (*Ng*(*Nm* PorB)). Organisms were grown in the presence (Sia+) or absence (Sia –) of CMP-Neu5Ac and binding of human FH to bacteria was measured by flow cytometry. Only sialylated gonococci that expressed *Ng* PorB, but not *Nm* PorB, bound FH. Axes are as in A. Adapted from Ref. (Madico, et al., 2007) with permission. Copyright 2007. The American Association of Immunologists, Inc. **C.** Binding of FH to sialylated gonococci is restricted to sialylation of lacto-*N*-neotetraose (LNnT) LOS. LOS phase variants of a gonococcal strain that expressed either the LNnT LOS (left graph) or the PK-like LOS (right graph) were grown in CMP-Neu5Ac to sialylate LOS; binding of FH was measured by flow cytometry. FH binding was enhanced upon sialylation of the LNnT LOS expressing variant (Neu5Ac α (2-3)-linked to Gal-GlcNAc-Gal-Glc-HepI) but not upon sialylation of the PK-like LOS

expressing variant (Neu5Ac α (2-6)-linked to Gal-Gal-Glc-HepI). Axes are described in A. Similar data have been shown in Ref. (Gulati, et al., 2005). **D.** Binding of FH to sialylated gonococci is human specific. Only human FH18-20/Fc (HufH18-20/Fc), but not chimpanzee FH18-20/Fc (ChFH18-20/Fc) binds to sialylated *N. gonorrhoeae*. Replacing human with chimpanzee FH domain 20 to create HuFH18-19/ChFH20/Fc abrogated binding. Reproduced from Ref. (Ngampasutadol, et al., 2008) with permission. Copyright 2008. The American Association of Immunologists, Inc.

Fig. 2.

FH structure and the proposed mechanisms of FH/Fc activity. **A.** Schematic representation of the domain structure of human FH. The short consensus repeat (SCR, also called complement control protein or CCP) domains that are required for complement inhibition, binding to C3b, glycosaminoglycans (GAGs) and most pathogens are indicated. **B.** Proposed mechanism of action of a FH/Fc fusion protein. The microbial binding domain of FH is fused to IgG Fc. Upon binding to microbes, adjacent Fc fragments engage the C1 complex, which activates C4 and deposits C4b on the microbe. Subsequent complement activation deposits C3b and could result in membrane attack complex (C5b-9) insertion into the membrane. Conversion of C3b to iC3b can enhance uptake of the microbe by professional phagocytes through complement receptors such as CR3, in conjunction with engagement of Fc receptors (FcRs) by the Fc portion of FH/Fc. Blocking binding of host FH to the microbial surface constitutes a third possible mechanism of action of FH/Fc (left side).

Fig. 3.

In vitro activity and safety of FHD1119G/Fc. **A.** Selection of FHD1119G/Fc as the lead anti-gonococcal FH/fc molecule. Sialylated *N. gonorrhoeae* strain F62 was incubated with varying concentrations (X-axis) of each of the FH/Fc that contained FH domains 18-20 that were unmodified (wild-type (WT)) or that contained single amino acid mutations in domain 19 (D→G at position 1119) or domain 20 (R→S, W→R, or R→G at positions 1182, 1183 or 1215, respectively), followed by the addition of human complement (NHS depleted of IgG and IgM). Survival of bacteria at 30 min relative to bacterial counts at the beginning of

the assay (t_0 min) is shown on the Y-axis (mean of at least 2 independently performed experiments). **B.** Introducing the D→G mutation at position 1119 of FH18-20/Fc abrogates hemolysis of anti-CD59-treated human RBCs by homologous serum. Increasing concentrations (indicated on the X-axis) of wild type FH18-20/Fc or FHD1119G/Fc were added to anti-CD59-treated human RBCs, as described previously (Ferreira, et al., 2009). The OD_{410nm} (Y-axis) indicates the degree of hemolysis. (Data are adapted from Ref. (Shaughnessy, et al., 2016) with permission). Copyright 2016. The American Association of Immunologists, Inc.

Fig. 4. FHD1119G/Fc reduces the duration and burden of gonococcal infection in the murine vaginal model of gonococcal colonization. Two groups of Premarin[®]-treated wild-type BALB/c mice were infected with 1.5×10^6 CFU of *N. gonorrhoeae* strain F62 and given either 12 μ g FHD1119G/mouse IgG2a Fc (n=14) or a corresponding volume of PBS (n=12) as a vehicle control, daily for the duration of the experiment. Vaginal swabs were obtained daily to quantify Ng CFUs. **A.** Kaplan-Meier analysis of time to clearance. **B.** Colonization of bacteria (log₁₀ CFU) measured daily. **C.** Bacterial burdens consolidated over time (Area Under the Curve [log₁₀ CFU] analysis) for the two groups. Reproduced from Ref. (Shaughnessy, et al., 2016) with permission. Copyright 2016. The American Association of Immunologists, Inc.

Fig. 5. Activity of FH6,7/Fc against *N. meningitidis* and non-typeable *Haemophilus influenzae* (NTHi). **A.** FH6,7/Fc enhances complement-dependent killing of *N. meningitidis*. Bacteria were incubated with 20% human complement (IgG and IgM-depleted NHS) alone or with complement plus increasing concentration of FH6,7/Fc; percent survival at 30 min (relative to bacterial counts at the beginning of the assay (t_0 min)) was measured in a serum bactericidal assay. The mean (SEM) from 3 independent experiments are shown. *, $P < 0.05$, and **, $P < 0.01$ (ANOVA), compared to the value for bacteria plus complement alone (0 µg/ml FH6,7/HuFc). **B.** FH6,7/Fc decreases meningococcal bacteremia in the infant rat model. Infant Wistar rats (5 to 6 days old) were treated with PBS alone ($n = 20$), with one of two doses of FH6,7/Fc (150 µg/rat or 45 µg/rat; $n = 15$ in each group), or with FH18-20/Fc

(45 µg/rat; $n = 15$), which does not bind to the bacteria. Positive controls included groups of rats given an anti-group C capsule Ab (10 µg/rat; $n = 10$) or anti-PorA MAb P1.2 (10 µg/rat; $n = 10$). Two hours later, the animals were challenged IP with 730 CFU of group C strain 4243. Rats were sacrificed between 8 and 9 h post-infection, and the CFU per 100 µl of blood were quantified. The horizontal dotted line indicates the limit of detection of the assay (1 CFU/100 µl). Statistical comparisons between groups used the Mann-Whitney test. ****, $P < 0.0001$. **C.** FH6,7/Fc enhances C-dependent killing of NTHi. Strain NT127 and its P5 deletion (P5) mutant were incubated with 20% complement alone, or 20% C plus either FH6,7/Fc or FH18-20/Fc each at a final concentration of 20 µg/ml; percent survival of bacteria (Y-axis) was measured in a serum bactericidal assay. Each bar represents the mean (range) of two separate experiments. *, $P < 0.05$; **, $P < 0.01$ (one-way ANOVA). **D.** FH6,7Fc decreases burden of NTHi in the lung model (Wong, et al., 2016). 10^7 CFU NTHi NT127 and 50 µg of FH6,7/Fc protein (control animals received PBS) were co-inoculated intranasally into C57BL/6 mice; lung CFU were determined 20 h post-inoculation. Bars represent the geometric means. Comparisons between the two groups used the Mann-Whitney test. Panels A and B are reproduced with permission from Ref. (Shaughnessy, et al., 2014), and panels C and D are reproduced with permission from Ref. (Wong, et al., 2016).

Fig. 6. Schematic of the structures of CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac), CMP-diacetyl legionaminic acid (CMP-Leg5Ac7Ac) and CMP-diacetyl pseudaminic acid (CMP-Pse5Ac7Ac). 2C_5 chair chemical structures of the CMP-nonulosonates are shown. Neu and Leg have the same stereochemistry (*D*-glycero-*D*-galacto configuration), whereas Pse has an *L*-glycero-*L*-manno configuration, differing stereochemically at carbons 5, 7 and 8. For reference, the 9 carbon atoms of the NulOs are numbered.

Fig. 7.

Incorporation of select NulOs increases resistance of gonococci to killing by NHS. Serum sensitive *N. gonorrhoeae* F62 was incubated with ~30 μ M of the CMP salts of each of the indicated NulOs and serum bactericidal assays were performed using 3.3% or 10% NHS. The percent survival of bacteria at 30 min relative to bacterial counts at the beginning of the assay (t_0 min) is shown on the Y-axis. Only Neu5Ac and Neu5Gc incorporation into LOS rendered the bacteria resistant to killing by 10% NHS; Neu5Ac9Az and Leg5Ac7Ac incorporation did not confer resistance even to 3.3% serum. Pse5Ac7Ac is not incorporated into LOS and served as a negative control. Adapted from Ref. (Gulati, et al., 2015) with permission.

Fig. 8.

Incorporation of Neu5Ac9Az or Leg5Ac7Ac does not inhibit complement activation on *N. gonorrhoeae*. Bacteria were grown in media with or without 20 µg/ml of each of the CMP salts of the indicated NuLOs, then incubated with 10% NHS; IgG, C4, C3 and FB amounts that had deposited on the bacteria were measured by ELISA. FH binding was performed by flow cytometry following incubation of bacteria with purified FH (20 µg/ml). Adapted from Ref. (Gulati, et al., 2015) with permission.

Fig. 9.

CMP-Sia analogs prevents serum resistance and complement inhibition mediated by CMP-Neu5Ac. *N. gonorrhoeae* were grown in media alone, or media containing 20 μg/ml CMP-Neu5Ac. After 15 min, the indicated concentrations of CMP-Leg5Ac7Ac were added to media and bacteria were grown for an additional 2 h. Resistance to killing by NHS was measured by serum bactericidal assay. The percent survival of bacteria at 30 min relative to bacterial counts at the beginning of the assay (t_0 min) is indicated on the Y-axis. IgG and complement deposition were quantitated by ELISA or flow cytometry for FH. ****, $P < 0.0001$ by one-way ANOVA compared to all other groups. Adapted from Ref. (Gulati, et al., 2015) with permission.

Fig. 10.

Treatment with CMP-Leg5Ac7Ac decreases the duration and burden of infection with multidrug-resistant *N. gonorrhoeae* strain H041 in the mouse model of gonorrhea. Wild-type BALB/c mice were infected intravaginally with 9×10^5 CFU of strain H041. One group (n = 10) received CMP-Leg5Ac7Ac 10 µg/ml intravaginally daily; the other group (n = 10) received saline (vehicle control). Vaginal swabs were obtained daily to quantify *N. gonorrhoeae* CFUs. **A.** Kaplan Meier analysis of time to clearance. **B.** Bacterial burdens consolidated over time (Area Under the Curve [log₁₀ CFU] analysis) for the two groups. Adapted from Ref. (Gulati, et al., 2015) with permission.

Table

Microbes that bind human FH

Microbe	Binding region(s) in FH ^A	Ref.
Bacteria		
Gram-negative		
<i>Neisseria meningitidis</i>	6-7	(Schneider, et al., 2009, Shaughnessy, et al., 2009)
<i>Neisseria gonorrhoeae</i>	6-7; 18-20	(Ram, et al., 1998, Ngampasutadol, et al., 2008, Shaughnessy, et al., 2011, Lewis, et al., 2012)
<i>Pseudomonas aeruginosa</i>	6-7; 18-20	(Hallstrom, et al., 2012, Kunert, et al., 2007, Meri, et al., 2013)
<i>Yersinia pseudotuberculosis</i>	5-7; 19,20	(Ho, et al., 2012)
<i>Bordetella pertussis</i>	19-20	(Meri, et al., 2013, Amdahl, et al., 2011)
<i>Salmonella typhimurium</i>	5-7; 19-20	(Ho, et al., 2010)
Non-typeable <i>Haemophilus influenzae</i>	6-7	(Wong, et al., 2016)
<i>Haemophilus influenzae</i> types b and f	6-7; 18-20	(Meri, et al., 2013, Fleury, et al., 2014, Hallstrom, et al., 2008)
<i>Moraxella catarrhalis</i>	?	(Bernhard, et al., 2014)
<i>Acinetobacter baumannii</i>	?	(Kim, et al., 2009)
<i>Yersinia enterocolitica</i>	6-7	(Biedzka-Sarek, et al., 2008)
<i>Francisella tularensis</i>	?	(Ben Nasr and Klimpel, 2008)
<i>Pasteurella pneumotropica</i>	?	(Sahagun-Ruiz, et al., 2014)
<i>Fusobacterium necrophorum</i>	5-7; 19-20	(Friberg, et al., 2008)
<i>Histophilus somni</i>	?	(Inzana, et al., 2012)
Gram-positive		
<i>Streptococcus pneumoniae</i>		
PspC	8-11; 19-20	(Meri, et al., 2013, Hammerschmidt, et al., 2007)
PspC	6-10	
PspC	13-15	(Dave, et al., 2004)
Hic	8-11	(Duthy, et al., 2002)
		(Jarva, et al., 2002)
<i>Staphylococcus aureus</i>	?	(Sharp, et al., 2012)
Group A streptococci	6-7	(Pandiripally, et al., 2003, Sharma and Pangburn, 1997)
Group B Streptococcus	?	(Maruvada, et al., 2009)

Microbe	Binding region(s) in FH ^A	Ref.
<i>Streptococcus suis</i>	?	(Pian, et al., 2012)
Spirochetes		
<i>Borrelia burgdorferi</i>	5-7; 18-20	(Meri, et al., 2013, Bhattacharjee, et al., 2013, Bhide, et al., 2012, Hellwage, et al., 2001, Kraiczy, et al., 2001)
<i>Borrelia afzelii</i>	FHL-1	(Wallich, et al., 2005)
<i>Borrelia spielmanii</i>	FHL-1; FHR1	(Seling, et al., 2010)
<i>Borrelia parkeri</i>	FHR1	(Schott, et al., 2010)
<i>Borrelia hermsii</i>	FHR1	(Rossmann, et al., 2007)
<i>Borrelia recurrentis</i>	?	(Meri, et al., 2006)
<i>Borrelia duttoni</i>	?	(Meri, et al., 2006)
<i>Leptospira interrogans</i>	5-7; 18-20	(Castiblanco-Valencia, et al., 2012)
<i>Treponema denticola</i>	FHL-1	(McDowell, et al., 2005)
Fungi		
<i>Aspergillus fumigatus</i>	1-7; 20	(Behnsen, et al., 2008)
<i>Candida albicans</i>	6-7; 19-20	(Meri, et al., 2013, Poltermann, et al., 2007)
Viruses		
West Nile virus	?	(Chung, et al., 2006)
HIV-1	?	(Stoiber, et al., 1995)
Parasites / protozoa		
<i>Plasmodium falciparum</i>		
Gametocytes	5-7	(Simon, et al., 2013)
Schizonts	5; 20	(Rosa, et al., 2015)
<i>Onchocerca volvulus</i>	8-20	(Meri, et al., 2002)

^A Recombinant fragments of human FH were used to define binding to the indicated specific domain(s). In some instances binding to Factor H-like protein 1 (FHL-1) or Factor H related protein 1 (FHR-1) has been defined. FHL-1 is an alternatively spliced version of FH and comprises domains 1-7 plus four unique C-terminal amino acids (SFTL). FHR1 comprises five domains; the three C-terminal domains bear 100%, 100% and 97% identity with FH domains 18, 19 and 20, respectively. By inference, binding to FHL-1 and FHR1 localizes the binding regions in FH to domains 1-7 and 18-20, respectively.