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Neisseria gonorrhoeae

Complement activation

Survival

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Complement inhibition
C4BP-IgM protein as a novel therapeutic approach to treat *Neisseria gonorrhoeae* infections

Serena Bettoni¹, Jutamas Shaughnessy², Karolina Maziarz¹, David Ermert¹, Sunita Gulati², Bo Zheng², Matthias Mörgelin³, Susanne Jacobsson⁴, Kristian Riesbeck¹, Magnus Unemo⁴, Sanjay Ram² and Anna M. Blom¹

¹Department of Translational Medicine, Lund University, Malmö, Sweden; ²Department of Medicine, Division of Infectious Diseases, University of Massachusetts Medical School, Worcester, MA, USA; ³Colzyx, Lund, Sweden; ⁴WHO Collaborating Centre for Gonorrhoea and other STIs, Department of Laboratory Medicine, Örebro University, Örebro, Sweden.

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Corresponding author: Sanjay Ram, Email: Sanjay.Ram@umassmed.edu

Department of Medicine, Division of Infectious Diseases, University of Massachusetts Medical School, Worcester, MA, USA
Abstract
Gonorrhea is a sexually transmitted infection with 87 million new cases per year globally. Increasing antibiotic resistance has severely limited treatment options. A mechanism that *Neisseria gonorrhoeae* uses to evade complement attack is binding of the complement inhibitor C4b-binding protein (C4BP). We screened 107 PorB1a and 83 PorB1b clinical isolates randomly selected from a Swedish strain collection over the last 10 years and noted that 96/107 (89.7%) PorB1a and 16/83 (19.3%) PorB1b bound C4BP; C4BP binding significantly correlated with the ability to evade complement-dependent killing ($r = 0.78; p<0.0001$). We designed two chimeric proteins that fused C4BP domains to the backbone of immunoglobulins IgG or IgM (C4BP-IgG; C4BP-IgM) with the aim of enhancing complement activation and killing of gonococci. Both proteins bound gonococci ($K_d$ C4BP-IgM = 2.4 nM; $K_d$ C4BP-IgG 981 nM), but only hexameric C4BP-IgM efficiently out-competed heptameric C4BP from bacterial surface resulting in enhanced complement deposition and bacterial killing. Furthermore, C4BP-IgM significantly attenuated the duration and burden of colonization of two C4BP-binding gonococcal isolates, but not a C4BP non-binding strain in the mouse vaginal colonization model using human factor H/C4BP transgenic mice. Our pre-clinical data present C4BP-IgM as an adjunctive to conventional antimicrobials for the treatment of gonorrhea.
Introduction

Gonorrhea is a sexually transmitted infection caused by *Neisseria gonorrhoeae* that infects both men and women. *N. gonorrhoeae* can establish infections in the urogenital tract, rectum and pharynx, is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (1). Complications from untreated gonococcal infections include ectopic pregnancy, infertility in women, and increased risk of HIV infection. Gonorrhea can also be transmitted from mother to neonate and cause blindness or life-threatening disseminated infection (2). Gonococci have become resistant to almost every conventional antibiotic currently in clinical use and we might be entering an era of untreatable gonorrhea (3) (4) (5) (6). Therefore, the need for new treatment options has become a pressing issue.

An emerging approach to control microbial infections is to target bacterial virulence mechanisms (7) (8). Pathogens have evolved various strategies to escape the innate immune response, including killing by the complement system (9) (10). The complement pathway represents one of the most ancient innate immune systems that has been conserved through evolution, which protects the host against infections. Invading pathogens activate complement either because of differences in surface composition that are recognized by the host as foreign or ‘non-self’ (alternative and lectin pathways), or through antibody binding (classical pathway). This leads to the initiation of activation; sequential proteolytic cleavage results in the formation of central C3 convertases and opsonization of the target with iC3b, which leads to phagocytosis, release of pro-inflammatory anaphylatoxins (C5a, C3a) that attract white blood cells, and finally formation of a lytic membrane attack complex (MAC) that directly kills Gram-negative pathogens (11). To protect the own body from unwanted complement activation and damage, the complement system is tightly regulated. C4b-binding protein (C4BP) is one of the major
soluble complement inhibitors, which blocks complement cascade at the level of C3-convertases (12) (9).

Several pathogens have developed strategies to escape from complement mediated killing by recruiting complement inhibitors such as C4BP to their surface, resulting in decreased activation of complement cascade favoring bacterial survival (13) (14, 15) (16). The exclusively human pathogen *N. gonorrhoeae* binds C4BP through its major outer membrane protein, porin B (PorB) (17), which dampens classical pathway activation and mediates resistance to complement. PorB is an approximately 34-37 kDa transmembrane protein that is essential for survival of the organism and functions as a selective anion channel (18). PorB proteins are encoded by two mutually exclusive alleles of *porB*; based on the PorB molecule expressed, gonococcal strains may be classified either as PorB1a or PorB1b (previously referred to as Por1A and Por1B, respectively). We previously documented that C4BP binds both PorB isoforms with a small preference for PorB1a (8 strains out of 11 tested) versus PorB1b (8 strains out of 18 tested) (17). PorB1a strains are frequently associated with disseminated infection, while PorB1b usually cause local infections of genital tract (19). Surveillance data suggest that PorB1a strains are less prevalent than PorB1b strains (20) (21) (22).

Because of the global threat of antimicrobial resistant gonorrhea, we targeted the ability of gonococci to bind C4BP by developing a novel antimicrobial fusion protein, which combines the domains of C4BP that are required for binding to gonococci, with the Fc domain of IgG and IgM to promote MAC-mediated killing of the pathogen. Since complement inhibitory activity of C4BP requires the third CCP domain (23) these fusion proteins should not inhibit complement, while they should preserve the ability to deposit on bacteria. Previous work by our groups have shown that a chimeric protein that comprises the microbial binding domains
of another complement inhibitor, factor H (FH), fused to Fc is efficacious in vitro and in vivo against Streptococcus pyogenes (24), N. gonorrhoeae (7), N. meningitidis (25) and Haemophilus influenzae (26) and provided the rationale for targeting N. gonorrhoeae-C4BP interactions.
Results

C4BP binds *Neisseria gonorrhoeae* and protects the bacterium from serum-mediated killing

Prior work has shown that selected strains of *N. gonorrhoeae* bind human C4BP (17). We confirmed the previous results using 6 laboratory strains of *N. gonorrhoeae* (C4BP-binding *N. gonorrhoeae* 15253, FA1090, 1291 and MS11, and the non-C4BP-binding *N. gonorrhoeae* F62 and 252) either with purified, fluorescently labelled C4BP or with 10% of NHS as a source of C4BP (Fig. 1A-B). All C4BP-binding gonococcal strains survived in normal human serum (Suppl. Fig. 1A) suggesting a role for C4BP in protecting bacteria from complement-mediated lysis. However, some C4BP non-binders may possess other serum resistance mechanisms such as FH recruitment (for example, strain 252; Suppl. Fig. 1D). Of note, when gonococci were incubated with heat-inactivated human serum (HI NHS), C4BP-binding decreased suggesting that the protein binds not only to PorB but possibly also to complement C3/C4 fragments deposited on the bacterial surface after complement activation, because C4BP does not lose binding capacity and activity at 56°C (Suppl. Fig. 1B-C) (27).

Some serum sensitive *N. gonorrhoeae* strains become serum resistant *in vivo* as a result of sialylation (28), which leads to binding of FH that similarly to C4BP blocks the complement cascade at the level of C3. Here we show that sialylation of gonococci does not affect C4BP binding (Fig. 1C) while it increases FH deposition on the surface of the pathogens resulting in increased survival, a phenomenon that was expected (Suppl. Fig. 1D-E).

We measured C4BP binding to 190 recently isolated gonococcal clinical strains – 107 PorB1a and 83 PorB1b isolates – using flow cytometry (Table II). Laboratory strains 15253 and 252 were used as positive and negative controls, respectively for C4BP binding. The cut-off for positivity was fixed at geometrical mean fluorescence intensity (gMFI) + 3xSD of unspecific
background of signal obtained for strain 252 that does not bind C4BP. Ninety-six (89.7%) of
107 PorB1a isolates bound C4BP, while only 16 (19.3%) of 83 PorB1b strains bound C4BP
(Fig. 1D). Similar to previously published data, the majority of gonococci binding C4BP belong
to the PorB1a subclass (17). Furthermore, no association was found between C4BP binding and
anatomical site of isolation (Fig. 1E).

Serum resistance of clinical isolates was thereafter tested in the presence of 10% NHS, which
reflects complement activity in mid-cycle cervical secretions (29). A strong correlation was
observed between survival of gonococci and their ability to bind C4BP (Spearman analysis r =
0.7825, p<0.0001, n=189; Fig. 1F). Interestingly, only 8 of 106 serum resistant isolates did not
bind C4BP (shown as grey dots in Fig. 1F); 4 of these strains (strains 26, 107, 120, 185) bound
FH (Suppl. Fig. 1F). Similarly, the laboratory strain 252, which does not bind C4BP, survived
in NHS (Suppl. Fig. 1A) and also bound FH. The mechanism(s) by which gonococcal strains
53, 112, 167, 188 resist serum killing in the absence of C4BP or FH binding remains unclear.

Expression of C4BP-IgG and C4BP-IgM

To promote complement-mediated elimination of serum-resistant *N. gonorrhoeae* strains taking
advantage of their ability to bind C4BP, we created the two fusion proteins C4BP\DeltaCCP3-IgG
and C4BP(CCP1/2)-IgM by cloning C4BP domains on the backbone of a constant portion of
IgG or IgM (Fig. 2A-B). C4BP-IgG and C4BP-IgM constructs were prepared in the expression
vectors pClaire and pTorsten, respectively. Both fusion proteins were expressed recombinantly
in eukaryotic cells and purified using affinity chromatography. Purity and size of the obtained
proteins were verified by SDS-PAGE using silver staining (Fig. 2A-B). The reduced monomer
of C4BP-IgG is about 75 kDa, which assembles as a dimer of 150 kDa. C4BP-IgM has a
molecular mass of approximately 52 kDa when reduced, while it during non-reducing
conditions appears as a multimer with double bands larger than 500 kDa, similarly to the double band of human control IgM. Since C4BP-IgM is a fusion with IgM CH2-CH4, which commonly assembles in multimers, we analyzed it by electron microscopy. Similarly to human IgM, C4BP-IgM exists as either pentamers or hexamers; the latter was the prevalent form and constituted approximately 80% of recombinantly expressed C4BP-IgM (Fig. 2C).

Only C4BP-IgM outcompetes C4BP binding to bacteria

Both C4BP-IgG and C4BP-IgM fusion proteins contain CCP1-2 domains of C4BP, which are required for the binding to gonococcal PorB. Using flow cytometry, we confirmed that both fusion proteins bound the laboratory strains that also bound native C4BP, with the exception of MS11 which bound C4BP-IgM but not C4BP-IgG (Fig. 3A-B). Of note, sialylation (SA) of bacteria, which occurs in vivo and did not affect native C4BP binding, also did not affect C4BP-IgG or C4BP-IgM binding to the bacteria (Fig. 3D-E). For gonococcal strain FA1090 we then estimated the binding affinity for the two fusion proteins showing that C4BP-IgM has higher affinity than C4BP-IgG (Kd 2.4 nM versus 981 nM) (Fig. 3C-F). This is likely a result of the fact that the multimeric rearrangement of C4BP-IgM results in up to 12 binding sites compared to 2 of the dimeric C4BP-IgG. Analysis of binding to a selection of gonococcal clinical isolates (n=75) using flow cytometry showed a strong correlation between binding of C4BP and that of fusion proteins (Spearman correlation analysis: C4BP-IgM r = 0.8978, p<0.0001; C4BP-IgG r = 0.7915; p<0.0001; Fig. 3G-H).

We next investigated the competition between native C4BP and C4BP-fusion proteins at the gonococcal surface. Bacteria were mixed simultaneously with human serum supplemented with fluorescent labelled C4BP and increasing concentrations of either C4BP-IgM or C4BP-IgG, and the ability to recruit C4BP was thereafter evaluated in flow cytometry. As shown in Fig.
2I, C4BP-IgM but not C4BP-IgG efficiently inhibited binding of serum C4BP to gonococci in a dose-dependent fashion indicating a direct competition between the two proteins. These data indicate that the fusion proteins and C4BP may share common binding sites on the surface of bacteria. Inhibition of C4BP binding by C4BP-IgM was incomplete, possibly because some C4BP remains bound to C3/C4 fragments deposited on bacterial surface. In contrast, dimeric C4BP-IgG could not compete with the heptameric C4BP likely due to lower avidity binding to the target bacterium. Therefore, we selected C4BP-IgM for subsequent experiments.

C4BP-IgM triggers complement activation

C4BP-IgM immobilized on microtiter wells efficiently induced complement C1q and C3b deposition from human serum in a dose-dependent manner and to a similar extent as positive controls IgM and aggregated IgG, indicating that the fusion protein efficiently activated the classical pathway of complement system in vitro. Neither bovine serum albumin (BSA) nor alpha-1-antitrypsin (α1AT), that were used as negative controls mediated complement deposition (Fig. 4A-B). Furthermore, C3b deposition was also observed using mouse serum (Fig. 4C) confirming that C4BP-IgM also activated murine complement, an important consideration for subsequent testing of the fusion protein in mice.

To determine whether C4BP-IgM also enhanced complement deposition on intact bacteria, we incubated 5 laboratory strains of *N. gonorrhoeae* with 10% NHS with or without 20 µg/mL C4BP-IgM, and measured surface deposition of complement proteins by flow cytometry. Of note, the concentration of serum used here is 4-fold higher than that used in competition assays (Fig. 3I), therefore inhibition of C4BP binding was not as evident (Fig. 4D). Despite only modest inhibition of C4BP binding to gonococci, C4BP-IgM significantly enhanced deposition of both C3b fragments (Fig. 4E) and MAC complexes (Fig. 4F) on gonococci compared with
NHS alone. Furthermore, we examined the effect of C4BP-IgM on complement deposition on human cells. Jurkat apoptotic cells and human erythrocytes were incubated with 5% NHS in the presence or in the absence of 20 µg/mL C4BP-IgM, and C3 deposition was detected by flow cytometry. Similar to the irrelevant negative control protein hIgM, C4BP-IgM did not enhance complement deposition either on human dying cells or on human erythrocytes, whereas the anti-Jurkat and anti-erythrocyte antibodies used as positive controls efficiently sensitized the cells to C3 deposition (Fig. 4G-H).

C4BP-IgM enhances complement-mediated killing of gonococci

We then determined whether increased complement deposition on bacteria, mediated by C4BP-IgM, promoted complement-dependent killing of *N. gonorrhoeae* by human serum. C4BP-IgM enhanced in a dose-dependent manner killing of all 4 laboratory strains (15253, FA1090, MS11 and 1291) (Fig. 5A-D). Of note, MS11 and 1291 strains were incubated with 5% NHS because these strains were intrinsically more sensitive to complement than 15253 and FA1090; the latter two strains were tested using 20% NHS. Accordingly, killing of MS11 and 1291 occurred in the presence of only 0.25 µg/mL of C4BP-IgM, while a higher concentration (12.5 µg/mL) was required to obtain killing of 15253 and FA1090 over baseline survival in NHS alone. Of note, 20 µg/mL C4BP-IgM promoted significant killing of bacteria even when they were sialylated (FA1090 and MS11), while no effect for F62 which did not bind the protein (Fig. 5E-G).

However, sialylation decreased the ability of C4BP-IgM to kill bacteria, at least in part because sialic acid increases FH recruitment to the surface of bacteria (Fig. 5E-F; Suppl. Fig. 1D-E). Indeed, C4BP-IgM binding to gonococci did not affect FH recruitment to the bacterial surface both in the presence or in the absence of LOS sialylation (Suppl. Fig. 1G-H). We also evaluated the efficacy of C4BP-IgM against 26 recent clinical quinolone-resistant isolates of *N. gonorrhoeae* that all bound C4BP (Table II). All but two of the isolates (isolates 9 and 53, the
latter a FH-binder) were efficiently killed by 20 µg/mL of C4BP-IgM (> 1 log_{10} reduction in CFU/mL) (Fig. 5H). These results show efficacy of C4BP-IgM against a majority of current *N. gonorrhoeae* clinical isolates that bound C4BP. Certain strains that possess additional mechanisms to evade the complement-mediated attack resisted killing by C4BP-IgM.

C4BP-IgM enhances clearance of infection in a mouse model of gonorrhea

We next evaluated the efficacy of C4BP-IgM against *N. gonorrhoeae* MS11, FA1090 and F62 in a mouse vaginal colonization model. Gonococci bind to FH and C4bp-binding protein (C4BP) in a human-specific manner (30, 31). Thus, complement activation on gonococci in wildtype mice is unimpeded because mouse FH and C4BP do not bind to *N. gonorrhoeae*, which would overestimate the efficacy of C4BP-IgM. We therefore used FH/C4BP Tg mice to provide a more rigorous platform to test the efficacy of C4BP-IgM in the context of a more ‘humanized’ complement system. The selected strains differ in their ability to bind C4BP and to resist serum-mediated killing *in vitro*: MS11 and FA1090 are C4BP binders and serum resistant (in the unsialylated state), while F62 does not bind C4BP and is serum sensitive. A purified plasma IgM from a patient with myeloma was used as a negative control and did not bind to or promote killing of *N. gonorrhoeae* laboratory strains (Suppl. Fig. 2A-B). Treatment of mice infected with either MS11 or FA1090 (6 animals per group) with 5 µg/day of C4BP-IgM intravaginally daily significantly reduced the duration of infection (*p*<0.0001 versus control groups) in both cases, clearing the bacteria from 50% and 67% of the animals at day 3 for MS11 and FA1090 infection, respectively (Fig. 6A). Moreover, C4BP-IgM treatment of mice infected with either *N. gonorrhoeae* MS11 or FA1090 had a drastically reduced bacterial burden over time with complete clearance of bacteria on day 4 (Fig. 6B). Comparison of the area under the curve (AUC) as a measure of cumulative burden of infection over time revealed that C4BP-IgM treatment significantly reduced the bacterial loads in both groups of mice infected by *N.
gonorrhoeae MS11 or FA1090 compared to non-treated controls ($p = 0.0034$ and $p = 0.0049$, respectively; Fig. 6C). Infection with strain F62 that does not bind C4BP was unaffected by C4BP-IgM treatment. In parallel, the myeloma IgM did not affect the course of infection in all groups, indicating that the antibacterial efficacy was related to bacteria-bound C4BP-IgM.
This study identifies C4BP-IgM as a novel antimicrobial fusion protein against *N. gonorrhoeae*. We tested 190 clinical gonococcal isolates cultured in Sweden in the last 10 years, and we observed that 96/107 (85.7%) PorB1a and 16/83 (19.3%) PorB1b bound C4BP. Binding of C4BP is a strategy used by several strains of *N. gonorrhoeae* to evade complement, which may provide them a survival advantage in their human host. We have targeted this bacterial immune evasion mechanism by creating a chimeric protein composed of C4BP CCP1-2 and Fc of IgM, which binds to bacteria and promotes complement activation on the microbial surface. We showed that C4BP-IgM fusion protein outcompeted C4BP and efficiently promoted MAC-mediated killing of gonococci. Finally, we demonstrated efficacy of C4BP-IgM in clearing infection of in the gonococcal mouse vaginal colonization model.

Gonorrhea is a sexually transmitted infection caused by the obligate human pathogen *N. gonorrhoeae*. In 2016, the WHO reported 87 million cases occurring worldwide (2) (32). Current treatment of gonococcal infections is based on dual therapy with third-generation cephalosporin (for example, ceftriaxone) in combination with azithromycin. However, this treatment is already ineffective in some patients (5) (33). Considering that untreatable gonorrhea cases might soon become a reality, identifying new prevention and treatment options are crucial. In addition to new antibiotics or novel combination of the currently used ones, new therapies have been suggested to prevent recurrence of the infection: interleukin-12 to promote Th1-driven adaptive immune response (34); vaginal lactobacillus strains to counteract gonococcal growth (35); fatty acid derivatives for a fast anti-gonococcal activity targeting membrane disruption (36). Here we present a novel therapeutic approach that targets a virulence mechanism of bacteria – its ability to bind the complement inhibitor C4BP.
Data obtained from China and Europe approximately a decade ago suggest that about 30% and 70% of gonococcal isolates express PorB1a and PorB1b, respectively (20, 21). Based on our data and previous studies (17), if we assume that 90% of PorB1a isolates and about 20% of PorB1b isolates bind C4BP, we expect C4BP-IgM to provide about 45% strain coverage.

Initially we created two chimeric proteins combining α-chain domains of C4BP with two types of immunoglobulins, IgG and IgM. In C4BP-IgG full length of C4BP α chain was used with the intent to target different pathogens which possibly bind various domains of C4BP. In contrast, when IgM portion was used we considered only the first two domains of C4BP, essential for C4BP binding to *N. gonorrhoeae*, since the IgM structure alone is already relatively large. Only C4BP-IgM, but not the C4BP-IgG fusion protein, could compete with the multimeric C4BP for binding to the bacterial surface, even though the inhibition is not complete. We previously documented that the monomeric α-chain of C4BP did not bind to PorB, indicating a requirement for the polymeric form of C4BP to stably interact with PorB (17). Therefore C4BP-IgM was prioritized for further pre-clinical studies. However, it is worth noting that both C4BP fusion proteins could kill gonococci in vitro, although the IgM derivative was more effective on a molar basis (Suppl. Fig. 2C). C4BP-IgM proved effective in the mouse vaginal colonization model using human FH/C4BP transgenic mice – these mice provide gonococci with human FH and C4BP and raise the threshold for complement-dependent killing by immunotherapeutics as would be encountered in humans. Treatment with C4BP-IgM significantly reduced the duration of infection and bacterial load in animals challenged with the two C4BP-binding gonococcal strains, but not the non-C4BP binding strain, confirming the selectivity of action of C4BP-IgM on gonococci able to bind C4BP, and not because of non-specific activation of complement in solution.
Interestingly, we documented that binding of C4BP is not affected by sialylation of bacteria, the latter commonly happening after bacterial colonization in vivo and resulting in FH recruitment and protection against complement attack. Likewise, also C4BP-IgM binding is not affected by the presence of sialic acids; however, bactericidal activity of the chimeric protein reduces but it is still significantly effective in killing the sialylated pathogens, suggesting that the major activity promoted by the protein is to induce a potent complement activation on the surface of bacteria which counteracts the presence of complement inhibitors. A similar strategy had been successfully demonstrated in a recent study showing the effectiveness of a chimeric antigenococcal antibody which recognizes the lipooligosaccaride epitope and amplifies complement activation through a modification in Fc portion (37). Besides, previous studies hampering FH recruitment by gonococci using two types of FH-IgG chimeric proteins showed to resolve gonococcal infection in the same mouse model used here (7, 38). Thus, both strategies may be useful preventive for antimicrobials currently in use in clinic. In addition, combination of the two C4BP-IgM and FH-IgG chimeric proteins may represent a rational strategy to target simultaneously a broad range of strains at the same time, which might be capable to bind either C4BP or FH, or both proteins. C4BP-IgM targets PorB, FH domains 18-20 fused to IgG Fc binds sialylated LOS and PorB (39, 40), while FH domains 6 and 7 fused to Fc targets gonococcal NspA (41). Combination treatment directed against distinct molecules that serve important immune evasion functions (complement inhibition) or are essential for gonococcal viability (e.g., PorB) will make the development of resistance unlikely. It is important to note that PorB is essential for gonococcal viability and is constitutively expressed on the surface of gonococci (42) (43). PorB ‘escape mutants’ that may develop on treatment with FH-IgG Fc or C4BP-IgM are likely to lack the ability to bind to these complement inhibitors and incur a considerable fitness cost; inability to evade complement would enable the host to rapidly clear such mutants that resist these immunotherapeutics.
The use of fusion proteins as treatment might have some advantages. Firstly, the chimeric proteins may reduce the use of antibiotics and development of antibiotic resistance. Excessive antibiotic use is also associated with destruction of resident commensal microflora. Indeed, using C4BP-IgM as a treatment, in contrast to antibiotic therapy, would selectively target against those pathogens that developed the ability to escape complement attack by binding C4BP, and that most likely are pathogenic for our body. Therefore, endogenous microflora such as lactobacilli which are not able to recruit the complement inhibitor would not be affected (44). Moreover, C4BP-IgM lacks CCP3 domain which contains all the features responsible for binding to C4b fragments deposited on cell surface after complement activation, therefore C4BP-IgM deposition on C4b – decorated cells will be also excluded. Additionally, C4BP-IgM is not recruited by apoptotic cells because it is lacks β-chain of C4BP that is associated with protein S, which in turn recognizes the exposed phosphatidylserine molecules on dying cells (45). Accordingly, C4BP-IgM did not deposit complement on human erythrocytes or apoptotic cells.

In conclusion, we have created a novel therapeutic to kill gonococci by targeting a key immune evasion mechanism. C4BP-IgM fusion protein represents an innovative strategy to trigger complement-mediated elimination of Gram-negative pathogens, with a goal to improve the treatment and combat antimicrobial resistance. Besides, the study of the efficacy of this new class of antibacterial proteins can be expanded to a larger spectrum of pathogens capable to recruit the complement inhibitor C4BP on their surface, such as Neisseria meningitidis (46), Haemophilus influenzae (47), Bordetella pertussis (48), Moraxella catarrhalis (49), and Candida albicans (50). Notably, the ability to bind complement inhibitors has been exploited by microbes through evolution. The use of such chimeric proteins to selectively activate
complement on pathogens might offer a novel therapeutic avenue against multidrug-resistant bacterial infections.
Methods

Bacterial strains

Strains 15253 (51), FA1090 (52), 1291 (53), F62 (54), MS11 (55), and 252 (39) have all been described previously and Table I summarizes their characteristics. Genotypic/phenotypic characteristics of clinical *N. gonorrhoeae* isolates cultured in 2010-2018 in Sweden (but patients infected in >20 additional countries) and obtained from the World Health Organization Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections, Department of Laboratory Medicine, Microbiology, Örebro University Hospital, Örebro, Sweden are listed in Table II. All *N. gonorrhoeae* isolates were stored frozen at -80°C in trypticase soy broth (TSB) containing 20% glycerol.

Proteins and antibodies

Chocolate agar plates were prepared from GC agar base (Difco, 228950) following the published procedure (56). 5’-cytidinemonophospho-N-acetylneuraminic acid (CMP-Neu5Ac) from Sigma-Aldrich (C8271) was used as “sialic acid” substrate for gonococci by spreading 500 µL of the compound (25 µg/mL in sterile H₂O) on the plate at least 30 min prior to inoculating bacteria.

Human C4BP was purified from human citrate plasma by affinity chromatography using anti-C4BP antibody MK104 (57). Plasma purified C4BP and recombinant C4BP-IgM and C4BP-IgG fusion proteins were fluorescently labelled using AlexaFluor-647 (A30009), and AlexaFluor-488 (A10235) microscale labeling kits from Molecular Probes, respectively. For flow cytometry assays, bacteria were stained with either Carboxyfluorescein succinimidyl ester (CFSE) obtained from Fluka (21888) or CellTracer Calcein Violet from Thermo Fisher (C34858) and re-suspended in Hanks Balanced Salt Solution containing 0.15 mM CaCl₂ and 1 mM MgCl₂ (HBSS⁺⁺) from Gibco (14025092). Binding of C4BP from NHS was detected with
anti-C4BP antibody MK67 (specific for CCP4 domain of the α-chain of C4BP) (57).

Antibodies used for detection of C4BP-IgM and C4BP-IgG were anti-IgM AF647 from Sigma-Aldrich (SAB4600436) and anti-hIgG AF488 from Invitrogen (A11013), respectively. To detect biotinylated FH purified from NHS in house Streptavidin AF647 conjugate was used (Thermo Fisher, S32357). Antibodies used for detection of complement deposition were as follows. In plate: polyclonal anti-human C1q (Dako, A0136) and monoclonal anti-human C3d (Quidel, A207), followed by HRP swine anti-rabbit (Dako, P0399); FITC-conjugated goat to mouse C3 (ICN, 55500). On the surface of bacteria: monoclonal anti-human C3d (Quidel, A207) and monoclonal anti-human neo epitope C9 (Hycult Biotech, HM2167-IA), followed by Alexa Fluor 647 goat anti-mouse (Thermo, A21235). Polyclonal human IgM from Nordic Biosite (OAMA04116) was tested for binding to N. gonorrhoeae. Irrelevant IgM from human myeloma plasma (Athens Research and Technology; 16-16-090713-M) was used as negative control in selected in vitro and in vivo experiments.

Normal Human Serum (NHS)

NHS was obtained from whole blood collected from 11 normal healthy adult volunteers whom provided written consent according to the recommendations of the local ethical committee in Lund, Sweden (permit 2017/582) and the Declaration of Helsinki (58). After clotting of whole blood at 25°C for 30 min, NHS from each donor was separated by centrifugation at 1500 x g for 20 min at 4°C, pooled together, divided into aliquots, and stored at -80°C. Human serum inactivated by heat at 56°C for 30 min (HI-NHS) was used as negative control of complement activation.
Expression and purification of C4BP-IgM fusion protein in CHO cells

DNA sequence encoding for CCP1 and 2 domains of C4BP and for CH2-4 constant portion of IgM were cloned in frame with signal peptide sequence into the expression vector pClaire. For C4BP-IgG fusion protein, the construct was prepared cloning the sequence of C4BP lacking CCP3 domain into the expression vector pTorsten; both plasmids were kindly provided by Dr. Brad Spiller (Cardiff University). Chimeric proteins were stably expressed in adherent Chinese hamster ovary cells (CHO; Life Technologies) cultured in serum-free OptiMEM Glutamax at 37°C, 5% CO₂ collected every second day and replaced for one day with complete medium supplemented with 100 µg/mL hygromycin to maintain the selection of transfected clones.

C4BP-IgM was purified from cell culture supernatants by affinity chromatography using anti-C4BP antibody MK104 coupled to Affi-Gel 10 (Bio-Rad) as described (23). C4BP-IgG was purified using protein A column (GE Healthcare). Bound proteins were eluted using glycine and 6 M guanidinium chloride. Protein eluate was dialysed against PBS at 4°C and molecular mass and purity were evaluated by silver staining of proteins separated by SDS-PAGE.

Electron microscopy

The multimeric form of C4BP-IgM molecules was analyzed by negative staining and electron microscopy as described previously (59). Human IgM was tested in parallel. Solutions of 1 micromolar of proteins were prepared in PBS. Aliquots of 5 µL were adsorbed to carbon-coated grids for 1 min, then washed with two drops of water, and then stained with two drops of uranyl formate. Glow discharge at low pressure in air was used to render the grids hydrophyllic. The grids were analyzed using a Jeol JEM 1230 electron microscope operated at a 60kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 CCD camera.
Complement activation assay

Microtiter plates (MaxiSorp BreakApart; Nunc) were coated with C4BP-IgM (10 µg/mL), human IgM or aggregated human IgG (5 µg/mL; Kiovig, Baxalta) as positive controls, and a1AT (10 µg/mL) or Bovine Serum Albumin (1%) as negative controls, all diluted in PBS. The plates were incubated overnight at 4°C and washed four times with washing buffer (50 mM Tris [pH 8], 150 mM NaCl, 0.1% Tween 20), and blocked with 1% BSA in PBS for 1 hour at 37°C. Increasing concentrations of NHS diluted in GVB++ (5 mM veronal buffer [pH 7.3], 140 mM NaCl, 0.1% gelatin, 1 mM MgCl₂, and 5 mM CaCl₂) were incubated for 45 or 20 minutes at 37°C to detect C1q or C3d deposition, respectively. Complement deposition was detected with anti-C1q or anti-C3d both followed by swine anti-rabbit antibody all diluted in the blocking buffer. Color was developed using OPD tablets (Kem-En-Tec Diagnostics) and signal was measured at OD₄₉₀ (Cary 50 MPR microplate reader; Varian).

Flow cytometry assays for binding of C4BP-IgM/C4BP-IgG and complement proteins to bacteria

Bacteria were cultured on chocolate agar plate overnight and sub-cultured on new plates for at least 5 hours with or without CMP-Neu5Ac, the latter in case of sialylated gonococci. Harvested bacteria were then diluted to OD 2 in HBSS++ and stained with 10 µM CFSE or 1 mM Calcein Violet. Binding of C4BP and C4BP-IgM/C4BP-IgG to bacteria was measured by flow cytometry after incubation at 37°C for 30 min with the purified AlexaFluor 647 or 488-labelled proteins respectively, diluted in HBSS++. To verify the stability of C4BP after heat inactivation of NHS (HI NHS), purified C4BP was treated at 56°C for 30 minutes (C4BP (56°C)) and then tested as above. In some experiments, binding to un-labelled proteins was measured using anti-C4BP MK67 followed by AF647 anti-mouse, AF647 anti-hIgM or AF488 anti-hIgG, all diluted
in HBSS++. For competition assays, increasing concentration of C4BP fusion proteins were added to 2.5% NHS supplemented with 5 µg/mL of AlexaFluor 647-labelled C4BP and with OmCl (23 µg/mL) used as C5-inhibitor preventing cell lysis (60) in GVB++ followed by incubation at 37°C for 30 minutes with bacteria. In parallel experiments, complement activation on bacteria was induced by incubation of gonococci with 10% NHS diluted in GVB++ with or without C4BP-IgM (20 µg/mL). Serum incubation time was 15 min for C9 (MAC formation), and 30 min for C3d, the latter in the presence of OmCl (23 µg/mL); both reactions at 37°C. After incubation, deposited complement proteins on the surface of gonococci were detected by flow cytometry using the specific antibodies listed above. In additional experiments FH binding was measured by incubation of Calcein-Violet stained bacteria with biotinylated FH purified from NHS in house and followed by Streptavidin AF647 conjugate. To explore the competition between FH and C4BP-IgM for the binding to bacterial surface, selected strains (30, 35, 36 and FA1090) were incubated with both biotinylated FH and un-labelled C4BP-IgM and binding of the proteins was measured using Streptavidin AF488 conjugate and AF647 anti-IgM antibody. For analysis, bacteria were identified as CFSE- or Calcein Violet-positive events and geometric mean of fluorescence intensity was calculated with FlowJo software.

Complement deposition on human erythrocytes or apoptotic cells

Red blood cells were isolated from whole blood of healthy volunteers collected in tubes containing lepirudin (Refludan 50, Celgene). Apoptosis of Jurkat cells was induced by incubation with 0.5 µM of staurosporine for 14 hours at 37°C, 5% CO₂. Erythrocytes and apoptotic Jurkat cells were then incubated with NHS 5% diluted in GVB++ in the absence or in the presence of 20 µg/mL C4BP-IgM. Human IgM (20 µg/mL) from myeloma plasma was used an irrelevant protein. Rabbit anti-erythrocyte antibody (Abcam,197770) or rabbit anti-Jurkat antibody (produced in-house, (61)), both diluted in HBSS++ to 25 or 50 µg/mL respectively,
were used to sensitized the cells prior incubation with serum, and were used as positive controls.

C3 deposited on human cells was detected by flow cytometry using the FITC-labelled anti-C3c antibody (Dako, F0201). Apoptotic cells were gated on positive population for APC-AnnexinV (ImmunoTools, 31490016).

Serum bactericidal assay

Bactericidal assays with NHS in the presence of C4BP-IgM fusion protein were performed similarly to that described previously (38). Briefly, bacteria were cultured on chocolate agar plate overnight and sub-cultured on new plates for at least 5 hours with or without CMP-Neu5Ac, the latter in case of sialylated gonococci. Approximately 2x10^5 CFU of harvested N. gonorrhoeae in GVB++ were incubated with 5%, 10% or 20% human complement (NHS or IgG-/IgM-depleted NHS, both diluted in GVB++) in the presence or the absence of C4BP-IgM or C4BP-IgG (concentration indicated for each experiment) diluted in GVB++ in a final volume of 100 µL.

Aliquots of 25 µL reaction mixtures were collected at the beginning of the assay (t0) and plated onto chocolate agar in duplicate in 10^3 dilution. After incubation at 37°C for 30 min (t30) another aliquot of 25 µL was collected, diluted in PBS and plated onto chocolate agar in duplicate (10^1 or 10^2 or 10^3). Survival was calculated as the number of viable CFU/mL at t30, while CFU/mL at t0 is referred as starting number of bacteria in each graph. In some cases, survival was calculated as percentage of the number of viable colonies at t30 relative to t0. Serum resistant strains were defined as having survival greater than 50%.

Mouse vaginal colonization model

Use of animals in this study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health. The
protocol was approved by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School. Female transgenic BALB/c mice that expressed human FH and human C4BP (15) 6–8 weeks of age in the diestrus phase of the estrous cycle were started on treatment (that day) with 0.1 mg Premarin (Pfizer; conjugated estrogens) in 200 µl of water given s.c. on each of 3 d: −2, 0, and +2 (2 d before, the day of, and 2 d after inoculation) to prolong the estrus phase of the reproductive cycle and promote susceptibility to *N. gonorrhoeae* infection. Antibiotics (vancomycin, colistin, neomycin, trimethoprim, and streptomycin) ineffective against *N. gonorrhoeae* were also used to reduce competitive microflora. Mice were infected on day 0 with either strain MS11, FA1090, or F62 (inoculum specified for each experiment). Mice were treated daily with 5 µg of either C4BP-IgM or human irrelevant IgM intravaginally from day 1 until the conclusion of the experiment. In the experiments with FA1090 and MS11, we also included a group of mice that were given a corresponding volume of PBS (vehicle controls). Bacterial load was determined by counting CFU growth from vaginal swab daily collected.

**Statistics**

Statistical analyzes were performed using GraphPad Prism v8.0. Mann-Whitney test was used to analyze the difference in C4BP binding between subgroups PorB1a and PorB1b. Spearman correlation analysis was performed for comparison of C4BP binding versus serum survival or versus C4BP-fusion proteins binding. Two-way ANOVA with Sidak’s multiple comparisons or Tukey’s multiple comparison tests and one-way ANOVA with Dunnett’s multiple comparisons test were used to examine the differences between experimental results in complement deposition and serum killing assays. Significant differences are indicated with asterisks *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001. For animal experiments:
clearance of gonococcal infection over time was analyzed with Kaplan-Meier curves, and

groups compared using Log-rank (Mantel-Cox) test; burden of bacteria, expressed as Log_{10} CFU over time was evaluated as mean values ± SEM; the cumulative CFU as area under the curve (AUC) over time was analyzed as mean of AUC(Log_{10}CFU)/mouse over time and groups were compared using the nonparametric Kruskal-Wallis rank test.
Author contributions

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Figure 1. C4BP binds to Neisseria gonorrhoeae. A-B) Binding of C4BP from Normal Human Serum (NHS; 10%) or purified AlexaFluor 647-labelled C4BP (20 μg/mL) to 6 laboratory strains of N. gonorrhoeae. C) Binding of AlexaFluor 647-labelled C4BP to 4 laboratory strains of N. gonorrhoeae in the presence (w SA) or in the absence (w/o SA) of sialylation. D-E) Binding of AlexaFluor 647-labelled C4BP (20 μg/mL) to 190 clinical isolates of N. gonorrhoeae. The isolates are grouped according to their expressed subclass of PorB and anatomical site of isolation. In D statistic p value was performed by Mann-Whitney test (p < 0.0001). F) Spearman correlation analysis of survival of gonococcal clinical isolates in 10% NHS versus their C4BP binding (r = 0.7825, p < 0.0001; n = 189). Serum-resistant isolates incapable to bind C4BP are highlighted in grey. In A, B, C bars display mean ± SD, with circles indicating independent repeats. Dotted line refers to gMFI average value in the absence of protein. In D, E, F dotted line refers to the cut-off for positivity (300 gMFI) calculated as gMFI mean value + 3SD of unspecific background of signal obtained for strain 252 that does not bind C4BP; bars display median and circles correspond to the mean for each sample from two independent experiments performed in duplicates.
Figure 2

**A**

C4BP-IgM

Pentamer

Hexamer

**B**

C4BP-IgG

Dimer

**C**

overall IgM

overall C4BP-IgM

Figure 2. C4BP-IgM and C4BP-IgG fusion proteins. Schematic representation and silver stained SDS-PAGE gels of C4BP-IgM (A) and C4BP-IgG (B). C) Scanning electron microscopy of C4BP-IgM and IgM. Left panels are overviews of IgM and C4BP-IgM respectively. Scale bar: 50 nm. Right panels are high magnification examples of pentamers and hexamers of IgM and C4BP-IgM, respectively. Scale bar: 25 nm. Under each high magnification particle there is a representation in pseudocolours (red IgM, yellow C4BP).
Figure 3. C4BP-IgM and C4BP-IgG fusion proteins bind to *Neisseria gonorrhoeae*. Binding of C4BP-IgM (A) and C4BP-IgG (B) (20 μg/mL) to 6 laboratory strains of *N. gonorrhoeae*. Bars display mean ± SD, with circles indicating independent repeats. Dotted line refers to gMFI average value in the absence of protein. Binding curves of C4BP-IgM (C) and C4BP-IgG (F) to *N. gonorrhoeae* FA1090. Dotted line refers to nonlinear fitting of binding curves using multiple site binding model in GraphPad. Values for estimated Kd and r squared are reported in each graph. Binding of C4BP-IgM (D) and C4BP-IgG (E) (20 μg/mL) to 3 laboratory strains of *N. gonorrhoeae* with or without sialylation (SA). Bars display mean ± SD, with circles indicating individual replicates. Dotted line refers to gMFI average value in the absence of protein. Spearman correlation analysis of AlexaFluor 488-labelled C4BP-IgM (G) or C4BP-IgG (H) binding versus AlexaFluor 647-labelled C4BP binding of gonococcal clinical isolates (r = 0.8978, p < 0.0001; r = 0.7915; p < 0.0001; respectively, n = 75). Vertical dotted line refers to the cut-off for positivity for C4BP binding. I) Competition between AlexaFluor 647-labelled C4BP (5 μg/mL in NHS 2.5% + OmCI 23 μg/mL) and C4BP fusion proteins (0 - 40 μg/mL; 1:2 dilutions) for the binding to *N. gonorrhoeae* FA1090. Horizontal dotted line indicates the baseline value for gMFI measured in the absence of AlexaFluor 647-labelled C4BP. In graphs C, F and I, each dot represents the mean ± SD of three independently performed experiments.
Figure 4. C4BP-IgM promotes complement activation selectively on bacterial surface. A) C1q deposition in plate coated with C4BP-IgM fusion protein or human aggregated IgG (hAgg_IgG), or human IgM (hIgM), or alpha-1-antitrypsin (α1AT), or bovine serum albumin (BSA). B-C) C3 deposition from either human (NHS) or mouse serum on C4BP-IgM fusion protein or hAgg_IgG, or hIgM, or α1AT, or BSA immobilized on plate. Each dot represents mean ± SEM of three independently performed experiments. D-F) C4BP binding and complement deposition on the surface of 5 laboratory strains of N. gonorrhoeae using 10% NHS with or without C4BP-IgM 20 μg/mL. Bars display mean ± SD, with circles indicating independent repeats. Statistics p values were performed by two-way ANOVA with Sidak’s multiple comparisons test. G-H) C3 complement deposition on the surface of apoptotic Jurkat cells or human erythrocytes. Each bar represents the mean ± SD of four independently performed experiments. Differences were analyzed using one-way ANOVA with Dunnett’s multiple comparisons test. *p < 0.05, ***p < 0.005, ****p < 0.0001 as indicated, or compared with NHS.
Figure 5. C4BP-IgM promotes complement-mediated killing of Neisseria gonorrhoeae. A-D) Complement dependent bactericidal activity of C4BP-IgM on 4 laboratory strains of N. gonorrhoeae. Strains MS11 (A) and 1291 (B) were incubated with 5% of NHS, while strains 15253 (C) and FA1090 (D) were tested with 20% of NHS. Heat inactivated NHS (HINHS) was used as negative control. One-way ANOVA with Dunnett’s multiple comparisons test was performed considering NHS as reference. E-G) Complement-mediated bactericidal activity of 20 μg/mL C4BP-IgM in 10% NHS on FA1090, MS11 and F62 in the presence (w SA) or in the absence (w/o SA) of sialylation. Statistics p values were performed by two-way ANOVA with Tukey’s multiple comparison of Log_{10} (CFU/mL). H) Complement-mediated bactericidal activity of 20 μg/mL C4BP-IgM on 26 clinical isolates of N. gonorrhoeae in the presence of 10% NHS. Statistics p values were performed by two-way ANOVA with Sidak’s multiple comparisons test. In all graphs horizontal dotted line refers to the starting amount of bacteria used in the assay, and bar represents the mean ± SD of at least three independently performed experiments. *p < 0.05, **p < 0.01, ***p < 0.005, ****p < 0.0001 as indicated, or compared with NHS.
Figure 6

Treatment with C4BP-IgM decreases infection with *N. gonorrhoeae* strains MS11 and FA1090 in the mouse vaginal colonization model. Premarin (conjugated estrogen)-treated transgenic human FH/C4BP mice in a BALB/c background (n = 6 per group) were challenged with 4.1 x 10^7 CFU/10 μL for strain MS11, 3.6 x 10^7 CFU/10 μL for strain FA1090 and 3.2 x 10^7 CFU/10 μL for strain F62, all at day 0. Mice were then treated intravaginally daily with 5 μg/d of C4BP-IgM (solid black line with opened circles), or 5 μg/d of ‘nonspecific’ IgM (solid grey line with triangles) or 10 μL PBS (vehicle control; dotted line with black squares) from day 1 to day 8. Vaginas were swabbed daily to enumerate CFU. A) Kaplan-Meier analysis of time to clearance of infection. Group comparison was done using Log-rank (Mantel-Cox) test. Significance for MS11 and FA1090 were set at 0.0167 (Bonferroni’s correction for 3 groups). B) Log_{10} CFU versus time (days). Symbols indicate mean values ± SEM. C) Bacterial burdens consolidated over time (area under the curve of Log_{10} CFU analysis) for the three groups. Median values are shown for each group. For strains MS11 and FA1090 treatment groups were compared using the non-parametric Kruskal-Wallis equality of populations rank test. The χ² with ties (two degrees of freedom) for MS11 and FA1090 were 11.56 (p = 0.0004) and 11.42 (p = 0.0005), respectively. Pairwise comparisons across groups were made with Dunn’s post-hoc test (p = 0.0034 and p = 0.0049 C4BP-IgM treatment versus PBS, for MS11 and FA1090 respectively). AUC comparisons across the two treatment groups with strain F62 was made with the two-sample Wilcoxon rank-sum (Mann-Whitney) test.