Short leucine-rich proteoglycans modulate complement activity and increase killing of the respiratory pathogen *Moraxella catarrhalis*

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Abstract

The respiratory pathogen, *Moraxella catarrhalis,* is a human-specific commensal that frequently causes acute otitis media in children and stimulates acute exacerbations in chronic obstructive pulmonary disease (COPD) patients. The exact molecular mechanisms defining host-pathogen interactions promoting pathogenesis are not clearly understood. Limited knowledge hampers vaccine and immunotherapeutic development required to treat this emerging pathogen. Here we reveal in detail a novel antibacterial role displayed by short leucine–rich proteoglycans (SLRPs) in concert with complement. We show that fibromodulin (FMOD), osteoadherin (OSAD) and biglycan (BGN) but not decorin (DCN) enhance serum killing of *M. catarrhalis*. Our results suggests that *M. catarrhalis* binding to SLRPs is a conserved feature as the overwhelming majority of clinical and laboratory strains bound all four SLRPs. Furthermore, we resolve the binding mechanism responsible for this interaction and highlight the role of the ubiquitous surface protein (Usp), A2/A2H in mediating binding to host SLRPs. A conserved immune evasive strategy used by *M. catarrhalis* and other pathogens is the surface acquisition of host complement inhibitors such as C4b-binding protein (C4BP). We observed that FMOD, OSAD and BGN competitively inhibit binding of C4BP to the surface of *M. catarrhalis*, resulting in increased C3b/iC3b deposition, membrane attack complex (MAC) formation and subsequently decreased bacterial survival. Furthermore, both OSAD and BGN promote enhanced neutrophil killing *in vitro*, both in a complement dependent and independent fashion. In summary, our results illustrate that SLRPs, FMOD, OSAD and BGN, portray complement-modulating activity enhancing *M. catarrhalis* killing, defining a new antibacterial role supplied by SLRPs.

Introduction

Evolutionary pressure has dictated the development of several key features to protect the mammalian host from infection from the billions of endogenous and exogenous microflora. The innate immune system governs the first response to any potentially infectious agent. Physical barriers lined with intricate detection and signaling systems, ancient elaborate effector pathways and responder phagocytic and antigen presenting cells mediate overall protection. One critical element of innate immunity in mediating this detection, response and subsequent elimination of foreign species is complement.

The complement system is composed of a multitude of soluble or surface expressed proteins with defined activators and inhibitors embroiled in a constant flux to maintain homeostasis. Complement components circulate in the blood and extracellular fluids. Microbial activation of complement occurs through various means but converges at the level of C3 activated through the formation of C3 convertases. These complexes instigate the cleavage of C3 into the anaphylatoxin and antimicrobial C3a peptide and major opsonin C3b/iC3b, responsible for mediating phagocytosis of foreign bodies by professional phagocytes. The next major step in complement activation is the formation of C5 convertases via binding of C3b to C3 convertases resulting in a new enzymatic platform directing the cleavage of C5 into C5a and C5b. Whereas C5a is a potent anaphylatoxin, C5b deposits onto the bacterial membrane initiates the formation of the membrane attack complex, resulting in lysis of susceptible cells, such as Gram-negative bacteria (1). To prevent host cell attack, complement inhibitors regulate complement activation in a strict manner. Two soluble inhibitors, factor H (FH) and C4b-binding protein (C4BP) (2) prevent formation of C3 convertase through binding of C3b and C4b respectively and serving as cofactors for

the serine protease, factor I.

Microbes, particularly bacteria, have evolved several mechanisms to inhibit complement activation and examples of bacteria targeting every feature of complement have been reported (3). The Gram-negative opportunistic respiratory pathogen, *Moraxella catarrhalis,* is no exception. *M. catarrhalis* is a human specific commensal and a recognized respiratory pathogen (4, 5). *M. catarrhalis* causes significant morbidity and economic burden as a common etiological agent of otitis media and exacerbations in patients with chronic obstructive pulmonary disease (COPD) (4, 5). One major immune evasion strategy employed by *M. catarrhalis* is the recruitment of the complement inhibitor C4BP (6). Inhibiting C4BP acquisition by *M. catarrhalis* may provide a novel therapeutic avenue to treat infections, which is urgently required given the increasing problem of failed therapy due to antibiotic resistance.

Short-leucine rich proteoglycans (SLRPs) such as fibromodulin (FMOD), osteoadherin (OSAD), biglycan (BGN) and decorin (DCN) are extracellular matrix (ECM) components containing a distinct central leucine – rich repeat region (LRR) flanked by disulphide bridges at the N- and C-termini (7). SLRPs are highly versatile molecules displaying differences in glycosylation of the core region and amino acid sequence and charge at the terminal ends. Classically, SLRPs function as important components in maintaining and regulating the ECM structure and cellular adhesion through interaction with integrins (7). More recently, the role of SLRPs, specifically BGN and DCN, as regulators of the innate immune system in response to tissue injury or cellular stress has been illustrated. Under normal physiological conditions matrix-bound SLRPs are not capable of immune activation, however in soluble form, following limited proteolysis of the ECM or secretion from macrophages, SLRPs act

as endogenous ligands of toll-like receptors triggering a rapid sterile inflammatory response (8, 9).

SLRPs also function as complement modulators, both as activators and inhibitors (10). Both FMOD and OSAD interact with the globular head domain of C1q stimulating activation of the classical complement pathway (11). In contrast, both BGN and DCN bind primarily to the stalk region of C1q, inhibiting classical pathway activation, presumably through inhibition of C1s/C1r activity (11, 12). Additionally, both FMOD and OSAD capture C4BP and FH and therefore may limit complement activation at early stages of the classical pathway (11, 13). Whether these SLRPs interact with *M. catarrhalis* and alter complement activity and bacterial elimination is currently unknown and provided the motivation for the current study.

Materials and Methods

Bacteria and culture conditions

A list of bacterial strains used in this study is shown in Table 1. *Moraxella catarrhalis* clinical and laboratory strains, *Haemophilus influenzae* type b (Hib) strain RM804 and non-typeable H. influenzae (NTHi) strain 3655 were grown on chocolate agar plates for 24 h at 37 °C with 5 % CO_2. Bacteria were subsequently streaked onto new chocolate agar plates for 6 h, scraped from plates, resuspended in 25 % (v/v) brain-heart infusion (BHI) broth/glycerol and stored in aliquots at - 80 °C. *Pseudomonas aeruginosa* ATCC27853 and KR601were grown in LB broth for 24 h at 37 °C with shaking.

Proteins, antibodies and sera

Human recombinant small leucine-rich proteoglycans (SLRPs) including fibromodulin (FMOD), osteoadherin (OSAD), biglycan (BGN), and decorin (DCN) were expressed with a hexa histidine tag from the pCEP4 vector in FreeStyle 293-F cells (Invitrogen) and purified using a similar protocol as described (14). The pCEP4 vector containing fibromodulin (FMOD) was a gift from Dr. Sebastian Kalamajski (Uppsala University, Sweden) (15). Briefly, FreeStyle 293 Expression Medium (Invitrogen) containing secreted SLRPs was collected and adjusted to 0.3 M NaCl and 50 mM Tris-HCl, pH 8.0. Medium was then filtered through a 0.45-μm membrane, and concentrated using a 10-kDa cellulose membrane in a stirred ultrafiltration system (Amicon). The concentrated medium was then applied to a Ni^{2+}-NTA column equilibrated with 50 mM Tris-HCl, pH 8.0 with 0.3 M NaCl. After washing with 5 volumes of 50 mM Tris-HCl, pH 8.0, the protein in the column was eluted with a linear gradient of 0-500 mM imidazole in 50 mM Tris-HCl, pH 8.0. The eluted

proteins were analyzed by SDS-PAGE, dialyzed against PBS, and stored at -80°C in aliquots. SLRPs were confirmed by Western blotting with polyclonal rabbit anti-bovine SLRPs Abs (homemade). The yield of protein from 1 liter of conditioned medium was 17 mg for FMOD, 10 mg for OSAD, 7 mg for BGN, and 14 mg for DCN. C4BP was purified from human plasma as described previously (16). Biotinylation of SLRPs was achieved using the EZ-Link™ Sulfo-NHS-LC-Biotinylation kit (ThermoFisher) as per manufacturers' instructions. Bovine serum albumin (A8806; Sigma) was used as control protein.

The following primary antibodies (Abs) were used for flow cytometric analysis of complement deposition on the surface of *M. catarrhalis*: polyclonal rabbit anti-human C1q (A0136, Dako), monoclonal mouse anti-human C4BP MK104 (homemade, (17)), mouse anti-human MAC (aE11, Hycult Biotech), and polyclonal rabbit anti-human C3d (A0063, Dako). Primary Abs were detected using fluorescently labeled secondary $F(ab')_2$ goat anti-rabbit AF647 (A21246, Invitrogen) or goat anti-mouse AF647 (A21235, Invitrogen). For the detection of biotinylated proteins, streptavidin AF647 conjugate (S21374, ThermoFisher) was used.

Normal human serum (NHS) was prepared from freshly drawn blood obtained form at least 10 healthy volunteers. Blood was allowed to clot for 30 min at room temperature and then incubated on ice for 1 h. Following two rounds of centrifugations at 700 x *g*, at 4 °C for 8 min, serum fractions were collect, pooled and stored immediately at - 80 °C. All healthy volunteers provided written informed consent according to the recommendations of the local ethical committee in Lund (permit 2017/582) and the Declaration of Helsinki (18). To prepare C4BP-depleted human serum (C4BP-dpl), freshly pooled human serum from four donors was passed through HiTrap affinity column coupled with the monoclonal C4BP antibody MK104.

Resulting serum samples were verified to be C4BP-depleted through ELISA analysis as described previously (19). Plasma-purified C1q was added (20 μg/mL) to restore C1q concentration to normal levels, as C1q is partially lost during C4BP depletion due to C1q binding to Ab-column. C4BP, purified from the serum from which it was depleted, was replenished at physiological concentrations (200 μg/mL).

Binding of SLRPs to bacteria

To screen binding of SLRPs to pathogenic bacteria, bacteria were grown on corresponding agar plates, washed, and suspended in PBS. After staining with 10 μM CFSE (Sigma-Aldrich), bacteria were resuspended into 1% (w/v) BSA/PBS. Bacterial suspension with 5×10^6 CFU in 50 μl was then mixed with an equal volume of 1% (w/v) BSA/PBS containing 100 μg/ml biotinylated FMOD (2.3 μM), OSAD (1.94 μM), BGN (2.35 μM), and 200 μg/ml biotinylated DCN (4.94 μM). After an incubation at 37°C for 1 h, bacteria were centrifuged at 5000 × *g* for 10 min, washed once with 1% (w/v) BSA/PBS, and incubated with streptavidin-AF647 at room temperature. After incubation for 1 h in the dark, bacteria were centrifuged, washed once with 1% (w/v) BSA/PBS, and bound SLRPs on bacteria were detected using a Cyflow space flow cytometer (Partec). To examine binding of SLRPs to clinical isolates of bacteria, binding assays were performed as described above, and bound SLRPs on bacteria were detected in a 96-well plate using a CytoFLEX flow cytometer (Beckman Coulter). Bacteria directly incubated with Streptavidin Alexa Fluor 647 conjugate were used as treatment control for background binding. $CFSE^+$ bacteria were detected based on their fluorescence signal and a gating region was set to exclude debris. Geometric mean fluorescence intensity (gMFI) was used to determine amount of SLRPs binding to bacteria.

To assess bacterial cell surface proteins responsible for binding of SLRPs, wild-type (RH4) and isogenic mutants of *uspA1*, *uspA2*, *mid* and double mutant *uspA1uspA2* and (Table 1) were grown, stained with CFSE and binding performed in identical fashion to the above conditions using a CytoFLEX flow cytometer.

To assess direct binding of SLRPs with UspA2, MaxiSorp microtiter plates (Nunc) were coated overnight at 4 °C with recombinant UspA2 (0.14 μM; 10 μg/mL), cloned from *M. catarrhalis* strain RH4 and expressed in *E. coli* as described previously (20). Plates were washed three times with 300 μL of wash buffer (50 mM Tris, 150 mM NaCl, 0.1 % Tween 20, pH 8). Plates were blocked to prevent non-specific binding by using 250 μL of quench (wash buffer containing 3 % fish gelatin) and incubated at room temperature for 2 h. Plates were further washed three times in wash buffer and biotinylated SLRPs were added at increasing amounts (0.012 – 1.98 μM; 0.6 – 80 μg/mL) in binding buffer (50 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) for 30 min at room temperature. Following incubation, plates were washed three times in wash buffer and wells were incubated with 50 μL streptavidin – HRP (1:200) in quench for 1 h at room temperature. Plates were further washed three times in wash buffer, developed using TMB substrate solution (ThermoFisher) and reaction stopped using 0.5 M H_2SO_4. Binding of SLRPs was detected using a Cytation 5 Cell Imaging plate reader (BioTek) at 450_{nm}.

Serum bactericidal assay

Serum bactericidal assay was performed as described previously (21). Briefly, *M. catarrhalis* (2.5 x 10^5 CFU) were incubated with either 5 or 50 μg/mL SLRPs or BSA at 37 °C for 30 min in GVB^{++} buffer (5 mM veronal buffer pH 7.3, 0.1% (w/v) gelatin, 140 mM NaCl, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$). Following incubation,

SLRPs/bacteria solution was washed in PBS or not and further incubated with pooled NHS to a final concentration of 10% (strain RH4) or 20% (strain Bc5) in GVB^{++} buffer. For calculation of bacterial survival, aliquots of bacteria were removed at time 0 and following incubation at 37°C for 30 min, diluted in PBS and spread onto BHI agar for colony enumeration. Serum treated with complement C5 inhibitor OmCI (10 μg/ml; 0.625 μM) (Swedish Orphan Biovitrum) (22) on ice for 30 min or compstatin CP40 (20 μM) (23) were used as serum controls. BSA at 50 μg/mL (0.75 μM), which has no effect on complement activation, was used as negative protein control. Bacteria were incubated with SLRPs alone at 37°C for 30 min in GVB^{++} buffer to determine whether SLRPs have antimicrobial activity.

Complement deposition assay

CFSE-labeled *M. catarrhalis* was incubated with pooled NHS in a 96-well plate in the presence of SLRPs, as described in the serum bactericidal assay. After incubation, bacteria were washed once with 1%-BSA/PBS, and deposited complement components were detected with primary Abs incubated at room temperature for 30 min at a dilution of 1:1000 in 1%-BSA/PBS. Bacteria were centrifuged and washed once in 1%-BSA/PBS followed by fluorescently labeled secondary Abs staining for 30 min at room temperature in the dark using a dilution of 1:1000. Bacteria were again centrifuged and washed once in 1%-BSA/PBS and finally resuspended in 150 μL of 1%-BSA/PBS. Deposited complement components were assessed using a CytoFLEX flow cytometer (Beckman Coulter). Geometric mean fluorescence intensity (gMFI) was used to determine amount of complement deposition. Heat-inactivated serum, primary isotype antibody and secondary antibody only controls

were used to assess specificity of antibodies used. Stained and unstained bacteria were used for gating bacteria and a minimum of 20,000 events was examined.

Neutrophil bactericidal assays

Human neutrophils were isolated using firstly Histopaque®-1119 (Sigma) separation of peripheral venous blood drawn from healthy volunteers and secondly a Percoll-based gradient method as previously described (24). Neutrophils were resuspended in RPMI 1640 plus 10 mM HEPES and viability was assessed by trypan blue staining typically yielding greater than 95%. For neutrophil bactericidal assays, neutrophils (5 x 10^5) were incubated with *M. catarrhalis* (5 x 10^6 CFU) (MOI 10) in the presence of 200 μg/mL SLRPs (4.5 μM (FMOD), 3.9 μM (OSAD), 4.7 μM (BGN), 4.9 μM (DCN)) or BSA with either 5% OmCI-treated or compstatin-treated serum in a final volume of 300 μL. Plates were incubated at 37°C, 5% CO_2 and at time 30 and 60 min neutrophils were lysed using 1 % saponin/PBS for 15 min on ice. Bacteria were diluted in PBS and plated onto BHI agar plates and incubated for 24 h at 37°C with 5% CO_2. Colonies were counted and intra- and extracellular bacterial survival was assessed by dividing CFU at time 30 or 60 min by CFU at time 0.

Statistical analysis

A one-way or two-way ANOVA was used to examine the difference between experimental results (GraphPad Prism v7.0) where a p value <0.05 was considered to be statistically significant. The p values reported in figure legends represent the post-hoc tests.

Results

SLRPs specifically bind M. catarrhalis

SLRPs have been shown to regulate various extracellular matrices and modulate cellular functions and innate immunity via interaction with cell surface receptors (7-9). We previously reported that SLRPs FMOD, OSAD, BGN and DCN could regulate complement activity through interaction with C1q, C4BP and FH (11, 13). However, whether these SLRPs play a role in modulating innate immune responses directed against pathogenic bacteria remains unclear. To understand the role of SLRPs in innate immunity, we expressed recombinant human SLRPs in eukaryotic cells and purified them using affinity chromatography. The purified SLRPs were estimated with a purity of ≥ 90% by SDS-PAGE under reducing conditions (Fig. 1A) and confirmed by Western blotting using our in-house rabbit anti-bovine SLPRs, which are highly similar to human SLRPs (Fig. 1B). Recombinant his-tagged FMOD, OSAD, BGN, and DCN are predicted be 44.0, 50.4, 40.6, and 38.7 kDa, respectively. However, all proteins are larger than the predicted molecular mass in SDS-PAGE gel due to glycosylation. Next, we determined the binding of biotinylated SLRPs to major Gram-negative bacterial species important in respiratory infections, namely *Pseudomonas aeruginosa*, *Haemophilius influenzae* and *M. catarrhalis*. We found that of these pathogens only *M. catarrhalis* (laboratory strains Bc5 and RH4) bound the four SLRPs (Fig. 1C-F).

FMOD, OSAD and BGN enhance complement-mediated killing of M. catarrhalis

As SLRPs can both regulate complement activity and bind *M. catarrhalis*, we aimed to determine whether SLRPs affect survival of *M. catarrhalis* in pooled normal human serum (NHS). We found that SLRPs FMOD, OSAD and BGN when

284 supplemented at 50μg/mL significantly decreased survival of both *M. catarrhalis*
285 RH4 (Fig. 2A) and Bc5 (Fig. 2B) in NHS. Despite being not statistically significant,
286 DCN led to a slight reduction in survival in the Bc5 strain compared to BSA, but no
287 difference was observed in strain RH4, suggesting that DCN does not enhance
288 complement-mediated killing of *M. catarrhalis*. Furthermore, inhibition of MAC
289 formation by previous treatment of serum with the C5 inhibitor OmCI prevented
290 killing of *M. catarrhalis* under any SLRP condition illustrating that SLRPs enhance
291 killing through complement mediated lysis (Fig 2A-B). Lastly, no antimicrobial
292 activity was observed when SLRPs were incubated with *M. catarrhalis* in GVB^{++}
293 buffer in the absence of serum, confirming that the enhanced killing was mediated by
294 complement. To verify that excess unbound SLRPs were not causing a by-stander
295 complement activation effect and contributing to enhanced killing, we also measured
296 the effect of washing bacteria following SLRP binding prior to incubation with serum
297 (Fig. S1). As in the above results, a significant decrease in survival was observed for
298 FMOD, OSAD and BGN but not DCN, indicating the SLRPs bound to the bacterial
299 surface promoted enhanced bacterial killing in the presence of serum.
300
301 *SLRPs interact directly with UspA2/2H of* M. catarrhalis
302 *M. catarrhalis* interacts with human proteins via major surface proteins such as
303 UspA1/A2, MID, and outer membrane porins such as OmpCD and Mha (4). Given
304 that previous work has shown that UspA1, UspA2 and MID can interact with soluble
305 extracellular matrix proteins we investigated the interaction of wild type (RH4) and
306 isogenic mutants lacking the above surface proteins with biotinylated SLRPs through
307 flow cytometry (Fig. 3A-D). We found that deletion of the *uspA2* gene resulted in a
308 significant decrease in binding of all SLRPs in question highlighting the importance

of UspA2 as a ligand for SLRP binding. No difference in binding was observed with neither the *uspA1* nor *mid* mutants.

To further elucidate the interaction between SLRPs and *M. catarrhalis* we employed a direct biochemical binding assay using immobilized recombinant UspA2, derived from strain RH4, and increasing concentrations of biotinylated SLRPs and BSA (Fig. 3E-F). In accordance with our binding results above employing wild-type and *uspA2* mutant, all four SLRPs bound UspA2, with the highest affinity observed for BGN (Kd = 89 ±11 nM), similar affinities seen for FMOD (Kd = 202 ±21 nM) and OSAD (Kd = 231 ±28 nM) and the lowest affinity seen for DCN (Kd = 293 ±32 nM).

SLRPs bind to the majority of clinical isolates of M. catarrhalis

To determine the clinical relevance of *M. catarrhalis* interaction with SLRPs we evaluated the binding capacity of a panel of clinical isolates (n=16) to all four SLRPs (Fig. 4A-D). These clinical isolates were chosen based on their respective differences in the N- terminal sequence motif of the UspA2 protein, in order to capture a significant diversity of important clinical *M. catarrhalis* strains. This domain is classified into the different groups 2A, 2B, 2C and 'nontypeable' based on the domain distribution and sequence similarity (25). We found that the overwhelming majority of clinical isolates bound all four SLRPs whereby there was a general trend for increased binding in the order of FMOD ≥OSAD >BGN >DCN. However, isolates that express UspA2/2H with different N-terminal repeats of head domains showed no significant difference in binding of SLRPs (Fig. S2).

334 *FMOD, OSAD and BGN increase C3b and MAC deposition by preventing C4BP*
335 *binding* to M. catarrhalis

336 To further understand how SLRPs regulate complement leading to the enhanced
337 serum sensitivity of *M. catarrhalis*, we measured deposition of complement
338 components on the bacterial surface in the presence of SLRPs, BSA or no added
339 protein, using flow cytometry. In agreement with decreased survival of *M. catarrhalis*
340 in serum, FMOD, OSAD and BGN significantly increased C3b deposition compared
341 to BSA (gray stars) whereas only OSAD significantly increased C3b deposition
342 compared to no protein control (Fig. 5A). Next, we looked at MAC deposition
343 following incubation for 20 min in serum, a shortened time to prevent significant
344 lysis. Complementing the serum killing and C3b deposition results, FMOD, OSAD
345 and BGN had significantly more MAC deposited on the bacterial surface compared to
346 BSA or DCN (Fig. 5B).

347 As acquisition of C4BP by *M. catarrhalis* is an efficient strategy to prevent
348 complement-mediated lysis and is facilitated through interaction with UspA1 and
349 UspA2 (6), with hypothesized that SLRPs FMOD, OSAD and BGN may
350 competitively inhibit binding of C4BP and thus render *M. catarrhalis* more
351 susceptible to serum killing. We measured C4BP binding following incubation in
352 OmCI-treated serum and showed a significant decrease when bacteria were
353 previously incubated with FMOD, OSAD and BGN, with again, no difference
354 observed with DCN or BSA (Fig. 5C).

355 To confirm our results that SLRPs inhibit binding of C4BP and thus disrupt a
356 major immune evasive strategy of *M. catarrhalis*, we depleted C4BP from NHS using
357 an anti-C4BP mAb MK104 coupled column, which interacts with high affinity to the
358 α-chain complement control protein (CCP) domain 1 of C4BP (17). Depletion of

359 C4BP from NHS (C4BP-dpl) resulted in increased killing of *M. catarrhalis* RH4
360 compared to NHS in the presence of both BSA and DCN (Fig. 5D). Increased
361 survival comparable to NHS was observed following replenishment of purified C4BP
362 to physiological levels (200 μg/mL) when BSA and DCN were present. In
363 comparison, FMOD, OSAD and BGN enhanced serum bactericidal activity in NHS
364 compared to both BSA and DCN as observed previously (Fig 2A-B). Importantly, no
365 significant change in serum killing was observed between BSA/DCN and
366 FMOD/OSAD/BGN in C4BP-dpl (Fig. 5D) confirming that prevention of C4BP
367 binding by FMOD, OSAD and BGN to the bacterial surface is responsible for the
368 increased complement-mediated killing of *M. catarrhalis*.
369
370 *OSAD and BGN enhance neutrophil killing of* M. catarrhalis *in a complement*
371 *dependent and independent manner*
372 Neutrophils represent a critical phagocytic cell type in innate immunity, central to
373 host defense against invading pathogens. Additionally, complement mediated
374 opsonisation accelerates phagocytosis and removal of pathogenic bacteria.
375 Considering that SLRP-bound bacteria had increased complement deposition in the
376 presence of serum, we wanted to investigate whether this translated into increased
377 killing in a neutrophil bactericidal assay. Interestingly, in the presence of human
378 neutrophils and OmCI-treated serum, both OSAD and BGN significantly enhanced
379 *M. catarrhalis* RH4 killing, observed at both 30 and 60 min incubation period (Fig.
380 6A). Incubation with FMOD or DCN did not significantly increase bacterial killing
381 compared to BSA. Next we wanted to investigate whether this enhanced neutrophil
382 killing was dependent on complement opsonisation or whether SLRPs themselves
383 could serve as mediators of enhanced neutrophil killing. Using OSAD and BGN, we

repeated the neutrophil bactericidal assays with either OmCI-treated serum (inhibiting complement at the C5 level) or compstatin-treated serum (inhibiting complement at the C3 level). At 30 min we observed only a decrease in bacterial survival in the OmCI-treated serum conditions and not in the presence of compstatin (Fig. 6B). Surprisingly, after 60 min we observed a statistically significant decrease in survival both with the OmCI- and compstatin-treated sera compared to BSA. This suggests that the main mechanism of SLRP-dependent enhanced neutrophil killing is via complement activation. After a prolonged incubation time, however, SLRPs promote a bactericidal killing effect in concert with neutrophils, which is independent of complement.

As compstatin-treated serum still contains C1q, which can act as an opsonin and promote phagocytosis, and as previous work has shown that SLRPs can interact with C1q (11, 12), we investigated the binding of C1q from serum in the presence of SLRPs (Supp Fig. 3). We observed no difference in binding of C1q to the bacterial surface when bacteria were incubated with FMOD or DCN compared to BSA. In contrast, a significant reduction in C1q binding was shown when bacteria were bound with OSAD and BGN. Therefore, these results indicate that enhanced neutrophil killing under compstatin-treated serum conditions in the presence of OSAD and BGN was not due to increased C1q binding.

403 **Discussion**

404 *M. catarrhalis* causes significant morbidity in children and COPD patients, and is
405 responsible for a plethora of respiratory infections and occasionally, systemic diseases
406 (26). The exact molecular mechanisms governing *M. catarrhalis* pathogenicity are not
407 fully understood. However, mounting evidence suggests that immune evasion,
408 directed primarily at circumventing the complement system, is an essential feature of
409 pathogenic strains (4-6, 14, 21, 27). Therefore, future treatment intervention directed
410 at hampering complement inhibitor recruitment is a promising avenue of research. In
411 this study, we highlight a novel antimicrobial role displayed by specific SLRPs,
412 namely FMOD, OSAD and BGN and in detail revealed the molecular mechanisms
413 resulting in enhanced innate immunity against *M. catarrhalis*.

414 SLRPs such as BGN and DCN are considered bi-functional proteoglycans, acting
415 both as central structural components of the ECM and danger-associated molecular
416 patterns (DAMPs) stimulating immune reactions (28). SLRPs are abundantly present
417 in the ECM and distributed in numerous tissues throughout the body (7). Previous
418 immumohistochemical analysis has shown that BGN and DCN are expressed in the
419 human lung and bronchial tissue (29-31). Furthermore, mining of the Human Protein
420 Atlas (www.proteinatlas.org) (32), a genome-wide analysis of RNA and protein
421 expression from samples representing major tissues and organs, confirmed the
422 expression of all SLRPs used in this study in lung tissue. RNA expression data
423 generated from 320 individual tissue samples showed that for this set of SLRPs, BGN
424 and DCN had the highest expression, followed by FMOD with OSAD having the
425 lowest expression (Suppl Fig 4). Combined, these expression data and previous
426 immunohistochemical analysis indicate that these SLRPs are present in sites
427 anatomically important for *M. catarrhalis* infection and therefore may play a role in

host innate immune defense. The exact concentrations of SLRPs present in human tissue and/or plasma are difficult to estimate. One reason for this is that SLRPs are present in higher concentrations following trauma, proteolysis of the ECM and under sterile and non-sterile inflammatory conditions. Previous work in the field has shown that both BGN and DCN expression is enhanced during experimental sepsis in murine models following LPS challenge (8, 9). Macrophages were observed to be the main secretory cell responsible for enhanced expression. Following stimulation with IL-1β and Il-6, macrophage increased BGN secretion, which in turn induced expression of TNFα and MIP-2, contributing to the overall pro-inflammatory environment and increased SLRP expression (8). Furthermore, DCN expression has been shown to be higher in cohorts of septic patients compared to healthy controls (9). In this study DCN concentration in plasma were estimated to be at 10 ng/mL in septic patients. Previous studies by this group also estimated another SLRP, PRELP, to be at a similar range present in bronchoalveolar lavage fluid (14). It is tempting to speculate that during infection and particularly sepsis, SLRP expression is increased as a result of secretion of pro-inflammatory cytokines stimulating macrophages and other immune and non-immune cells while at the same time the highly inflamed environment contributes to increased SLRP proteolysis from the ECM. Therefore during infection the local concentration of SLRPs may be higher than the surrounding environment which could influence complement and innate immune activity and bacterial survival.

Of the three main Gram-negative respiratory pathogens screened in this study, only *M. catarrhalis* was bound by SLRPs. *M. catarrhalis* expresses numerous surface proteins, which bind an array of ECM proteins, plasma and complement components permitting colonization and evasion of the host innate and adaptive immunity (4, 26). The major surface proteins of *M. catarrhalis* are the UspA1 and UspA2/2H. Both

453 UspA1 and A2/2H interact with C4BP, however UspA2/2H is more strongly
454 expressed than UspA1 and therefore plays a more prominent role in C4BP binding
455 and in conferring a complement resistant phenotype (6). Through mutational analysis
456 we determined that all four SLRPs bound to *M. catarrhalis* predominantly through
457 UspA2/2H. This suggested that SLRP binding to UspA2/2H could competitively
458 inhibit C4BP binding resulting in reduced complement inhibition. Using flow
459 cytometry we illustrated that prior binding of FMOD, OSAD, BGN but not DCN to
460 *M. catarrhalis* effective reduced C4BP binding thus explaining the increased serum
461 sensitivity.

462 Given the similarity between BGN and DCN it is surprising that BGN and not
463 DCN competitively inhibits C4BP binding. Both BGN and DCN are members of the
464 class I SLRP family, possessing significant homology at both the protein and genetic
465 level. BGN contains two N-terminal tissue-specific chondroitin/dermatan sulfate side
466 chains whereas DCN contains one, and both differ in the pattern and level of
467 glycosylation (7). These differences permit both SLRPs to perform different tasks in
468 terms of ECM maintenance and cell signaling and possibly binding to different
469 regions of UspA2, resulting in differential inhibition of C4BP. Our results show that
470 *M. catarrhalis* can bind both DCN and C4BP simultaneously. UspA2 is a trimeric
471 autotransporter adhesin, which interacts with C4BP specifically at the CCP2, CCP5
472 and CCP7 domains (6). UspA2 is composed of a globular head and stalk domain and
473 therefore it is feasible that DCN but not the other SLRPs bind to specific regions
474 within UspA2 that are not required for C4BP binding. Future biochemical studies are
475 required to fully confirm this hypothesis.

476 Incubation of bacteria with SLRPs in the presence of serum resulted in significant
477 opsonisation with C3b/iC3b (Fig. 5A). As these opsonins are recognized by

478 complement receptors present on neutrophils we wished to examine where this
479 resulted in enhanced neutrophil bactericidal killing. Here we observed that only
480 OSAD and BGN effectively increased neutrophil killing of *M. catarrhalis* but not
481 FMOD or DCN. This was surprising considering that FMOD enhanced C3b
482 opsonisation in the presence of serum. Therefore, we checked whether this enhanced
483 neutrophil killing was independent of complement by using compstatin-treated serum,
484 effectively blocking complement at the C3 level. We observed that the majority of the
485 neutrophil killing was complement (opsonisation) mediated (Fig. 6A). However with
486 prolonged incubation both OSAD and BGN enhanced killing in a complement
487 (opsonisation) independent manner. It is known that certain ECM proteins such as the
488 SLRP lumican can enhance phagocytosis by interacting with both bacteria and
489 phagocytes via surface expressed integrins (33). Additionally, it has been shown that
490 other SLRPs such as BGN and DCN can bind to toll-like receptors expressed on
491 professional phagocytes and induce a pro-inflammatory response (8, 9). Two
492 questions arise that require future molecular dissection: 1) Can FMOD and OSAD
493 interact with professional phagocytes and induce an immune response analogous to
494 BGN and DCN and 2) can the SLRPs in question mediate an interaction between
495 bacteria and phagocytes which facilitates enhanced phagocytosis and subsequent
496 killing. As such future molecular characterization is underway to elucidate fully the
497 mechanisms of SLRPs mediated neutrophil bactericidal activity.

498 Recent work by our lab has shown that respiratory pathogens such as *M.*
499 *catarrhalis* can interact with ECM components whereby two opposing scenarios may
500 result, namely attenuated or enhanced complement activity. *M. catarrhalis* interacts
501 with cartilage oligomeric matrix protein (COMP) preventing complement deposition
502 and interfering with complement-independent phagocytosis, enhancing survival (21).

503 Conversely, *M. catarrhalis* can be bound by the SLRP, Proline/arginine-rich end
504 leucine-rich repeat protein (PRELP), which disrupts C4BP binding, significantly
505 augmenting complement-mediated lysis and neutrophil killing (14). To this complex
506 interaction between complement, *M. catarrhalis* and ECM components we introduce
507 the newfound antibacterial role of FMOD, OSAD and BGN, which through
508 interaction with the surface expressed UspA2/2H and in concert with complement,
509 accelerate the eradication of an important respiratory pathogen. Finally, the
510 elucidation of the molecular basis for SLRP-mediated enhanced killing may provide
511 novel research avenues to devise therapies to treat infection.

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Conflict of interest

J.D.L. is the inventor of patents and/or patent applications that describe the use of complement inhibitors for therapeutic purposes; the founder of Amyndas Pharmaceuticals, which is developing complement inhibitors (i.e., next generation compstatins) for clinical applications; and the inventor of the compstatin technology licensed to Apellis Pharmaceuticals (i.e., 4(1MeW)7W/POT-4/APL-1 and its PEGylated derivatives) .

536 **Table 1: List of strains used in this study**

Clinical isolate / strain	Description	Reference
Moraxella catarrhalis		
KR529	Clinical isolate	(14)
KR485	Clinical isolate	(14)
O35E	Clinical isolate	(14)
KR516	Clinical isolate	(14)
KR531	Clinical isolate	(14)
KR540	Clinical isolate	(14)
KR503	Clinical isolate	(14)
KR488	Clinical isolate	(14)
KR509	Clinical isolate	(14)
KR484	Clinical isolate	(14)
KR480	Clinical isolate	(14)
BBH18	Clinical isolate	(14)
O46E	Clinical isolate	(14)
CCUG353	Clinical isolate	(14)
KR483	Clinical isolate	(14)
Bc5	Laboratory strain	(34)
RH4	Laboratory strain	(35)
RH4Δ*uspA1*	RH4 devoid of ubiquitous surface protein A1	(6)
RH4Δ*uspA2*	RH4 devoid of ubiquitous surface protein A2	(6)
RH4Δ*uspA1*Δ*uspA2*	RH4 devoid of both ubiquitous surface protein A1 and A2	(6)
RH4Δ*mid*	RH4 devoid of immunoglobulin D (IgD)-binding protein (MID)	(36)
Pseudomonas aeruginosa		
ATCC27853	Laboratory strain	ATCC
KR601	Clinical isolate	This study
Haemophilus influenzae		
type b strain RM804	Clinical isolate, capsule-deficient	(37)
non-typeable (NTHi) strain 3655	Clinical isolate, encapsulated	CCUG

537 Abbreviations: CCUG; Culture Collection University of Gothenburg, ATCC;

538 American Type Culture Collection

539

Figure legends

Figure 1. Small leucine-rich proteoglycans (SLRPs) interact with *M. catarrhalis*

Recombinant human SLRPs detected by **A)** reducing SDS-PAGE (5 μg of each protein) and **B)** Western blotting (0.5 μg). Biotinylated **C)** FMOD**, D)** OSAD, **E)** BGN and **F)** DCN were incubated with major respiratory pathogens *P. aeruginosa*, *H. influenzae* and *M. catarrhalis* and bound SLRPs were detected with fluorescently labeled streptavidin by measuring fluorescence intensity using a Cyflow space flow cytometer (Partec). Mean values and standard deviation (SD) of at least 3 individual experiments are shown. Statistical differences were calculated using a one-way ANOVA analysis with Bonferroni's post-test in comparison to control without SLRPs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. FMOD, OSAD and BGN enhance serum killing of *M. catarrhalis*

Bacterial survival in human serum was defined as the ratio (%) of the colony-forming units (CFU) at 30 min to time 0. Error bars represent SD of three independent experiments. Serum treated by C5 inhibitor OmCI was used as serum control, and 50 μg/ml BSA was used as a negative protein control. Statistical differences were calculated using a one-way ANOVA with Dunnett's post-test versus bacteria without SLRPs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 3. SLRPs interact directly with UspA2/2H of *M. catarrhalis*

Biotinylated SLRPs were incubated with *M. catarrhalis* mutants devoid of selected surface proteins UspA1, UspA2/2H, and MID that interact with various host proteins. Bound SLRPs were detected with fluorescently labeled streptavidin by measuring fluorescence intensity using a CytoFLEX flow cytometer (Beckman Coulter).

565 UspA2/2H mutant showed significantly reduced binding of all SLRPs (**A-D**).
566 Biotinylated SLRPs **E)** (FMOD and OSAD) and **F)** (BGN and DCN) bind
567 immobilized recombinant UspA2 with differing affinities. Error bars represent SD of
568 three independent experiments. Statistical differences were calculated using a one-
569 way ANOVA with Dunnett's posttest versus wild type (RH4) bacteria. ** $p < 0.01$,
570 *** $p < 0.001$, **** $p < 0.0001$.
571
572 **Figure 4. SLRPs bind to *M. catarrhalis* clinical isolates expressing UspA2/2H**
573 The highly diverse head domains of UspA2/2H are classified into N-terminal repeats
574 (NTER) 2A, 2B, 2C, and 2H, and non-typeable. All tested clinical strains bound
575 SLRPs at varying degrees. Negative control consisted of bacterial straining with
576 Streptavidin Alexa Fluor 647 in the absence of biotinylated proteins. Error bars
577 represent the SD of three individual experiments.
578
579 **Figure 5. FMOD, OSAD and BGN increase complement deposition through**
580 **inhibition of C4BP binding**
581 Deposition of complement components **A)** C3b **B)** MAC and **C)** C4BP on *M.*
582 *catarrhalis* RH4 was analyzed using flow cytometry. **D)** Bacterial survival in 5 %
583 C4BP depleted sera (C4BP dpl) with matched survival in 5 % NHS and C4BP dpl
584 replenished with C4BP (C4BP dpl + C4BP) at physiological concentrations. Error
585 bars represent SD of three (A-C) and 5 (D) independent experiments. Serum treated
586 by C5 inhibitor OmCI or heat-inactivated was used as serum control, and BSA was
587 used as a negative protein control. Grey stars (BSA) and black starts (without protein
588 w/o) indicate statistical calculations using a one-way ANOVA with Dunnett's post-
589 test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

Figure 6. SLRPs OSAD and BGN enhance neutrophil killing of *M. catarrhalis*

Human neutrophils were incubated with *M. catarrhalis* RH4 in the presence of **A)** OmCI-treated serum and SLRPs or BSA or **B)** OmCI or compstatin-reated serum and OSAD, BGN or BSA for 30 or 60 min at 37°C and 5%CO_2. Following incubation total viable bacteria was enumerated following lysis of neutrophils and bacterial survival was calculated by diving CFU at time 30 or 60 with that of time 0. Graphs are presented as the mean and SD of 5 independent experiments and analyzed using a two-way ANOVA with Bonferroni post-hoc tests comparing SLRP/BSA condition to that of no protein control (without, w/o) A) or SLRPs to BSA control B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Abbreviations: BHI, brain-heart infusion; BGN, biglycan; C4BP, C4b-binding protein; CCP, complement control protein; DCN, decorin; dpl, depleted serum; FH, factor H; FMOD, fibromodulin; MAC, membrane attack complex; NHS, normal human serum; NTER, N-terminal repeat; OSAD, osteoadherin; SLRP, short leucine-rich proteoglycan; Usp, ubiquitous surface protein.

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743

Figure 1

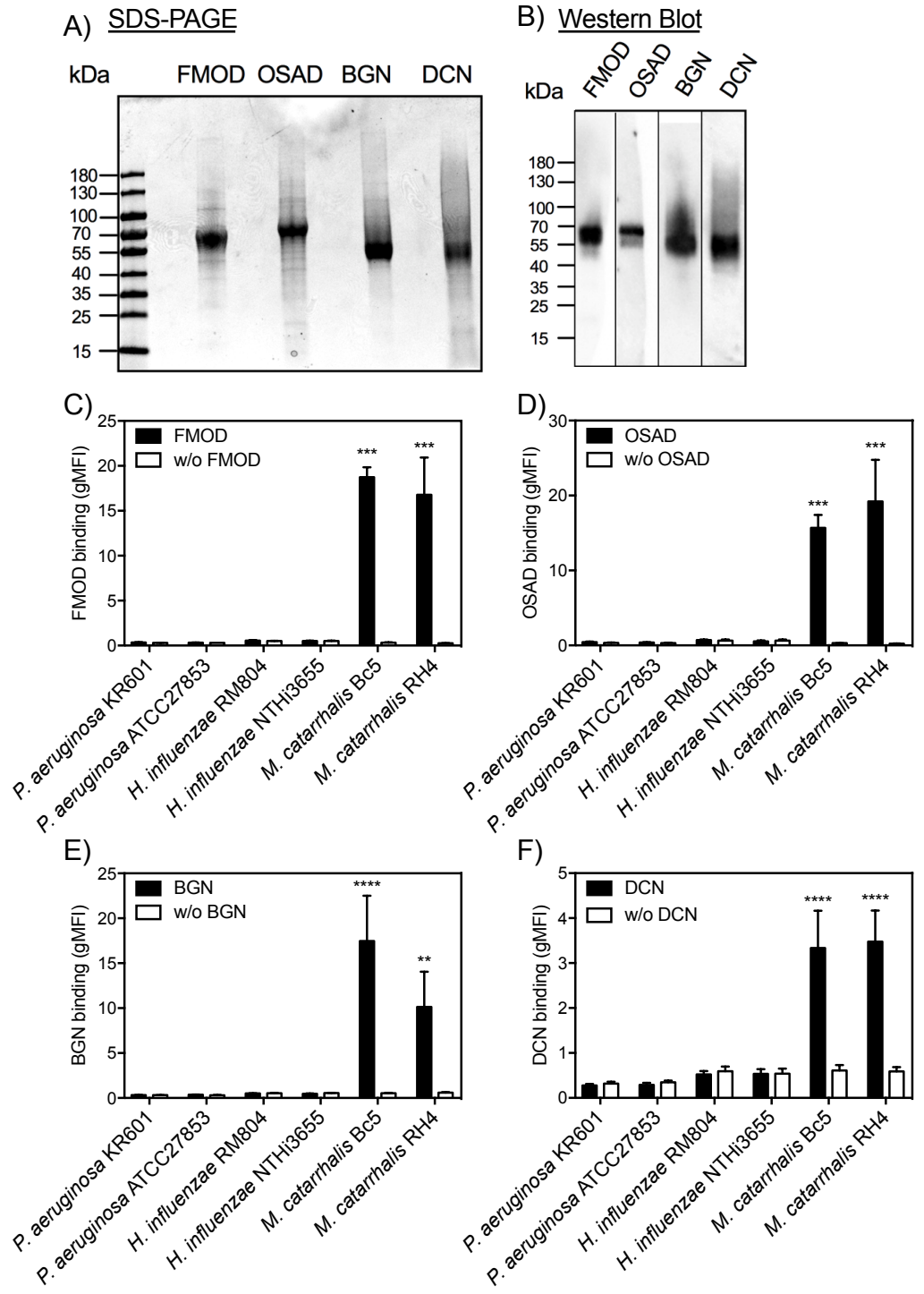

Figure 1. Small leucine-rich proteoglycans (SLRPs) interact with *M. catarrhalis* Recombinant human SLRPs detected by **A)** SDS-PAGE and **B)** Western blotting. 5 µg of each protein was loaded into wells for reducing SDS-PAGE, and 0.5 µg for Western blotting. Biotinylated **C)** FMOD**, D)** OSAD, **E)** BGN and **F)** DCN were incubated with major respiratroy pathogens *P. aeruginosa*, *H. influenzae* and *M. catarrhalis* and bound SLRPs were detected with fluorescently labeled streptavidin by measuring fluorescence intensity. Standard error of the mean (SEM) of at least 3 individual experiments is shown. Statistical differences were calculated using a one-way ANOVA analysis with Bonferroni's posttest in comparison to control without SLRPs. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2

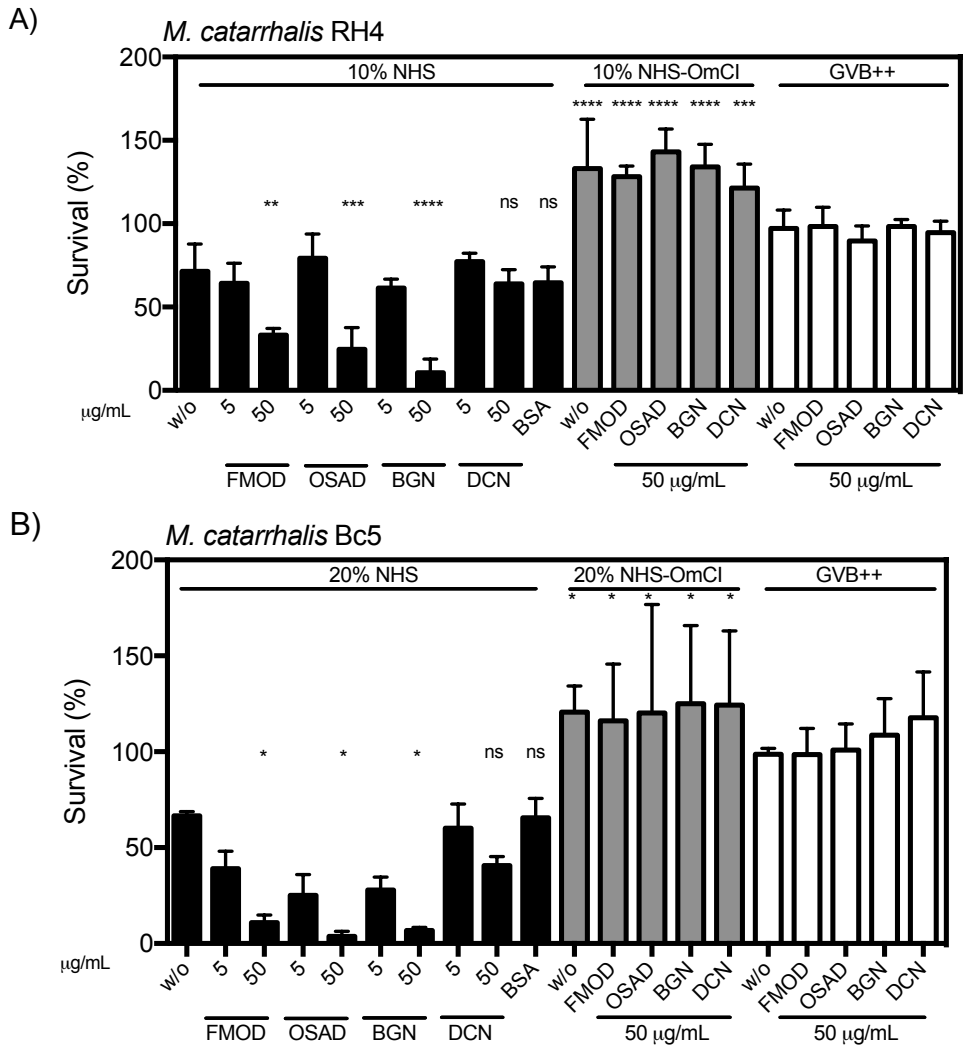

Figure 2. SLRPs enhance serum killing of *M. catarrhalis*
Bacterial survival in human serum was defined as the ratio (%) of the colony-forming units (CFU) at 30 min to time 0. Error bars represent SD of three independent experiments. Serum treated by C5 inhibitor OmCI was used as serum control, and 50 µg/ml BSA was used as a negative protein control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ versus w/o SLRPs, one-way ANOVA with Dunnett's posttest.

Figure 3

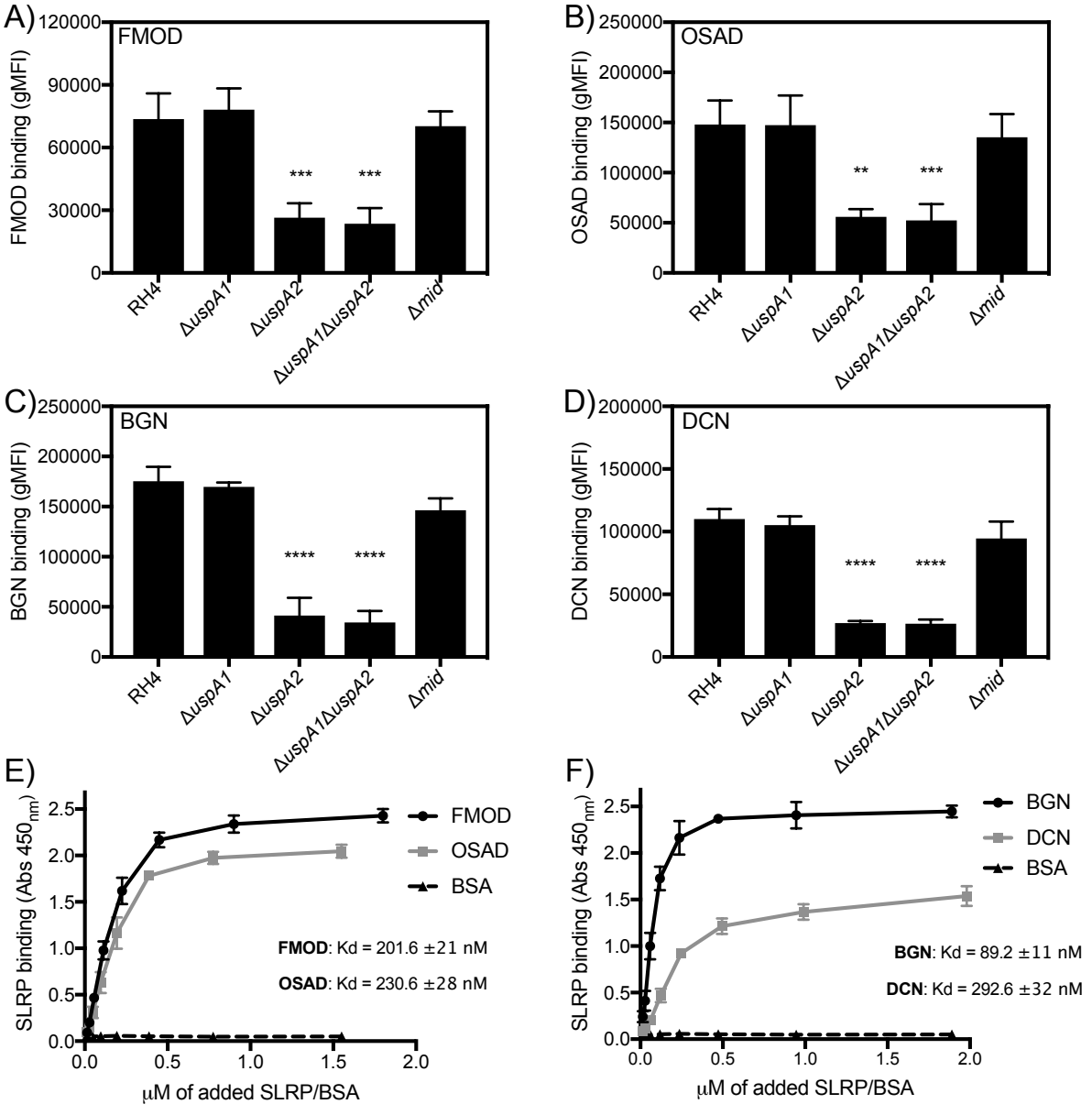
A)
FMOD
FMOD binding (gMFI)
120000
90000
60000
30000
0
***
***
RH4
ΔuspA1
ΔuspA2
ΔuspA1ΔuspA2
Δmid
B)
OSAD
OSAD binding (gMFI)
250000
200000
150000
100000
50000
0
**
***
RH4
ΔuspA1
ΔuspA2
ΔuspA1ΔuspA2
Δmid
C)
BGN
BGN binding (gMFI)
250000
200000
150000
100000
50000
0
****
****
RH4
ΔuspA1
ΔuspA2
ΔuspA1ΔuspA2
Δmid
D)
DCN
DCN binding (gMFI)
200000
150000
100000
50000
0
****
****
RH4
ΔuspA1
ΔuspA2
ΔuspA1ΔuspA2
Δmid
E)
SLRP binding (Abs 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
μM of added SLRP/BSA
FMOD
OSAD
BSA
FMOD: Kd = 201.6 ±21 nM
OSAD: Kd = 230.6 ±28 nM
F)
SLRP binding (Abs 450nm)
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
μM of added SLRP/BSA
BGN
DCN
BSA
BGN: Kd = 89.2 ±11 nM
DCN: Kd = 292.6 ±32 nM

Figure 4

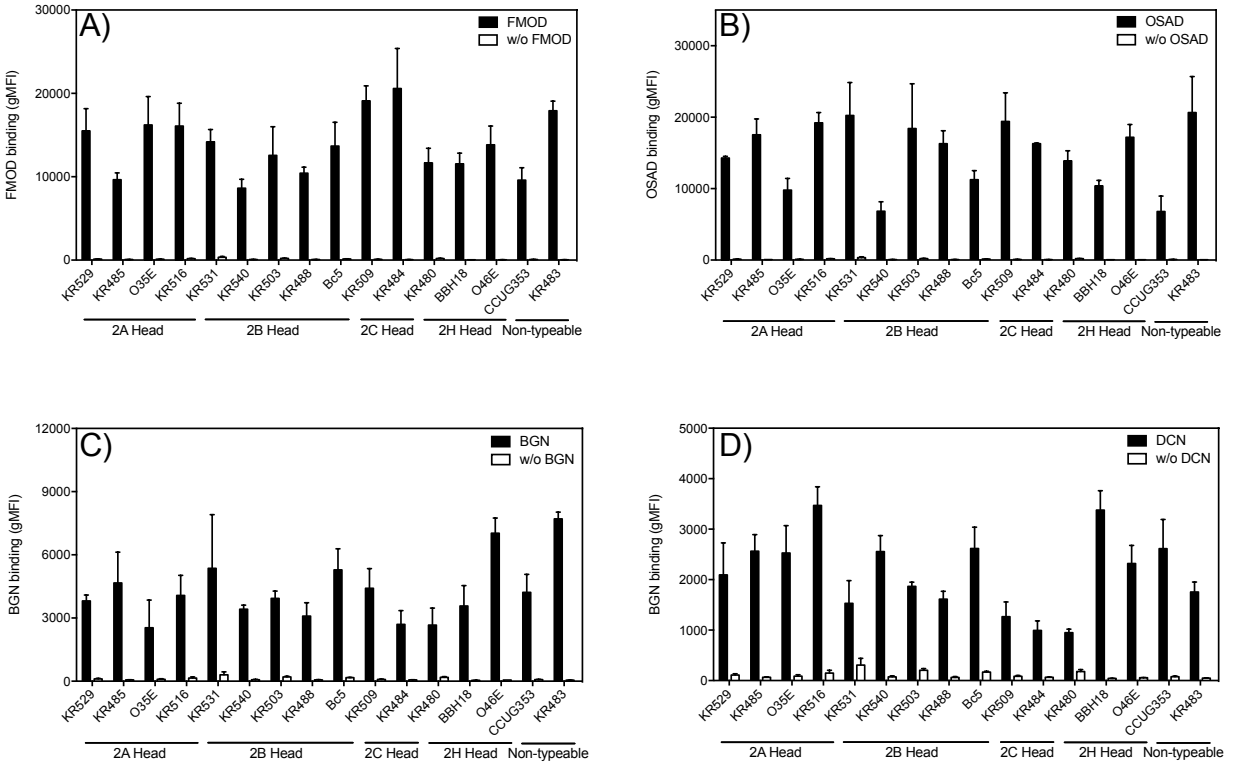

Figure 4. SLRPs bind to *M. catarrhalis* clinical isolates expressing UspA2/2H. The highly diverse head domains of UspA2/2H are classified into N-terminal repeats (NTER) 2A, 2B, 2C, and 2H, and non-typeable. All tested clinical strains bound SLRPs at varying degrees. Negative control consisted of bacterial straining with Streptavidin Alexa Fluor 647 in the absence of biotinylated proteins. Error bars represent the SD of three individual experiments.

Figure 5

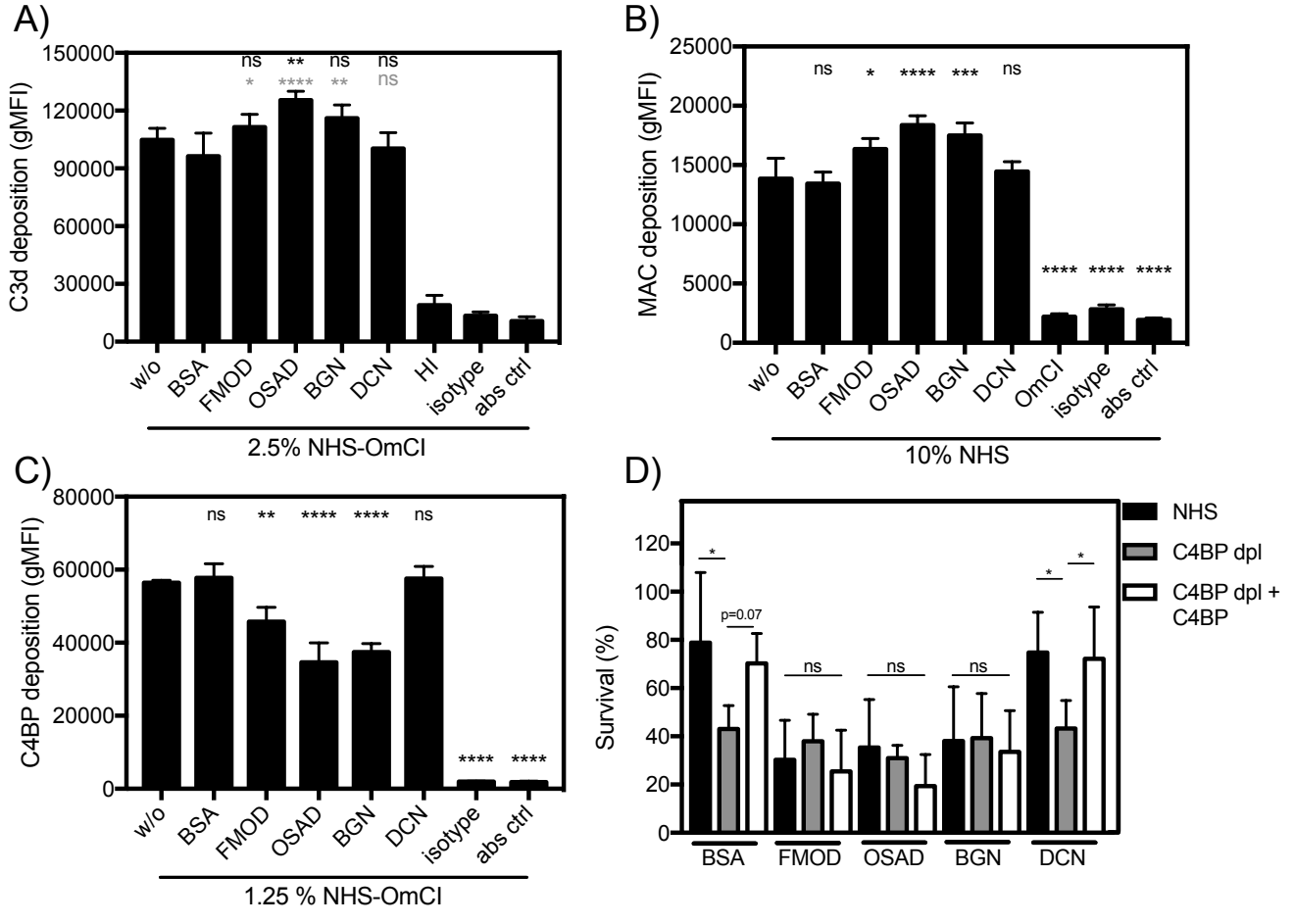

Figure 5. FMOD, OSAD and BGN increase complement deposition through inhibition of C4BP binding
Deposition of complement components **A)** C3b **B)** MAC and **C)** C4BP on *M. catarrhalis* was analyzed using flow cytometry. **D)** Bacterial survival in 5 % C4BP depleted sera (C4BP dpl) with matched survival in 5 % NHS and C4BP dpl replenished with C4BP (C4BP dpl + C4BP) at physiological concentrations. Error bars represent SD of three (A-C) and 5 (D) independent experiments. Serum treated by C5 inhibitor OmCI or heat-inactivated was used as serum control, and BSA was used as a negative protein control. Grey stars (BSA) and black starts (without protein w/o) indicate statistical calculations using a one-way ANOVA with Dunnett's posttest. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns, not significant.

Figure 6

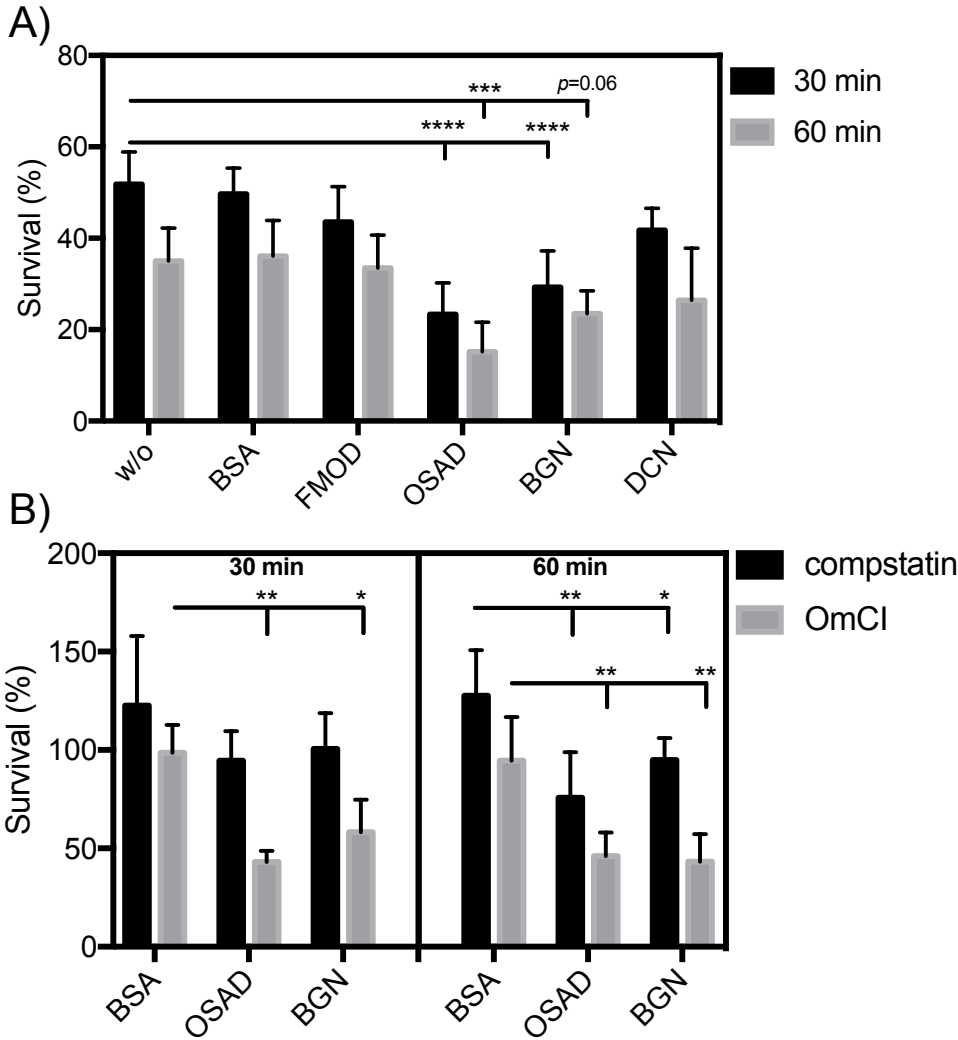

Figure 6. SLRPs OSAD and BGN enhance neutrophil killing of *M. catarrhalis*
Human neutrophils were incubated with *M. catarrhalis* in the presence of **A)** OmCI-treated serum and SLRPs or BSA or **B)** OmCI or compstatin-reated serum and OSAD, BGN or BSA for 30 or 60 min at 37°C and 5%CO_2. Following incubation total viable bacteria was enumerated following lysis of neutrophils and bacterial survival was calculated by diving CFU at time 30 or 60 with that of time 0. Graphs are presented as the mean and SD of 5 independent experiments and analyzed using a two-way ANOVA with Bonferroni post hoc tests comparing SLRP/BSA condition to that of no protein control (without, w/o) A) or SLRPs to BSA control B). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure 1

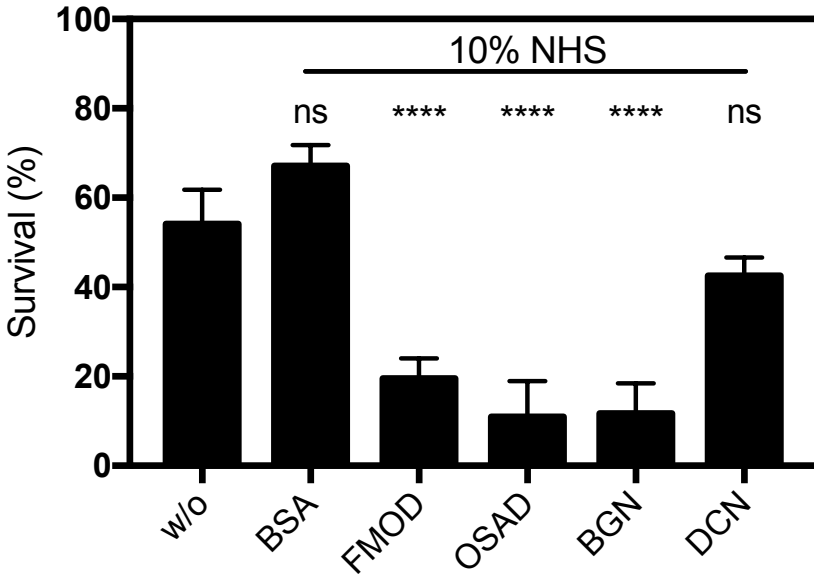

Figure S1. Removal of excess SLRPs does not impede serum killing

Serum killing of strain RH4 following washing of bacterial-SLRP solution after 30 min incubation, removing unbound SLRPs. Survival is calculated as the percentage of colony-forming units (CFU) at 30 min to time 0. Error bars represent SD of three independent experiments. 50 µg/ml BSA was used as a negative protein control. Statistical differences were calculated using a one-way ANOVA with Dunnett's posttest versus bacteria without SLRPs. **** $p < 0.0001$.

Supplementary Figure 2

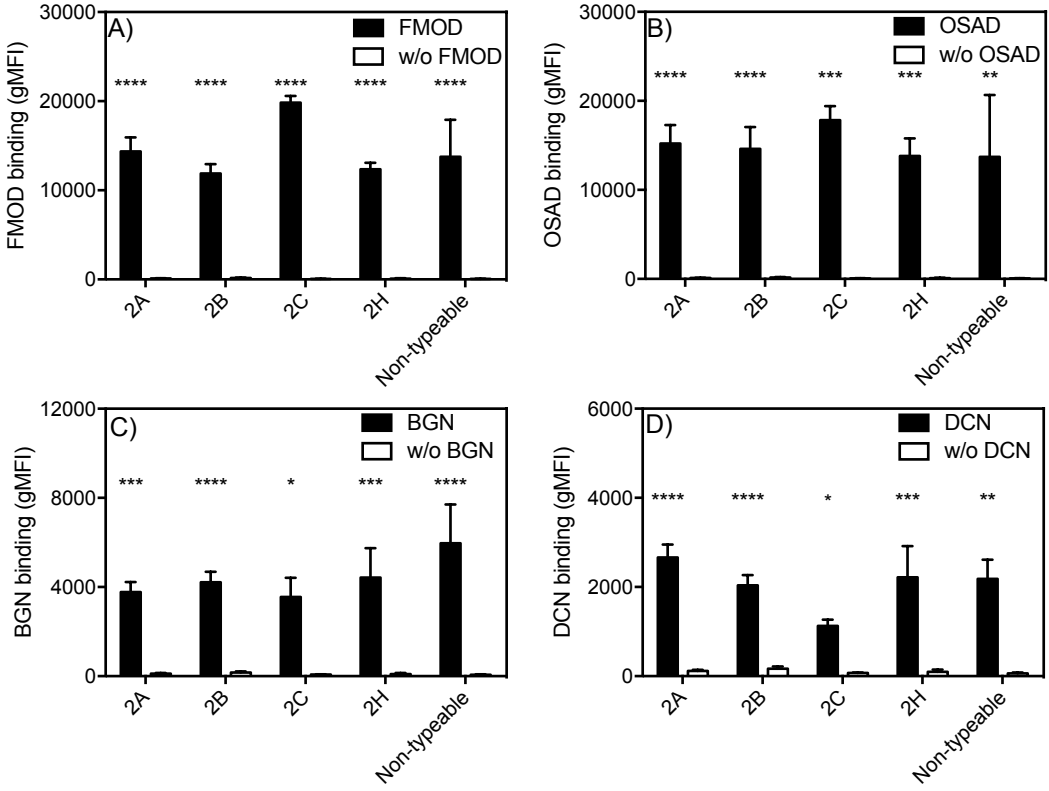

Figure S2. *M. catarrhalis* isolates of different N-terminal repeats (NTER) of head domains bind SLRPs in similar fashion

The SLRP binding capacities of *M. catarrhalis* clinical isolates expressing UspA2/2H were grouped according to their N-terminal repeat (NTER) domain; 2A, 2B, 2C, and 2H, and non-typeable. No significant difference in binding to SLRPs was observed between NTER groups and all NTER groups bound SLRPs in a statistically significant manner. Statistical differences were calculated using a one-way ANOVA with Dunnett's posttest. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Figure 3

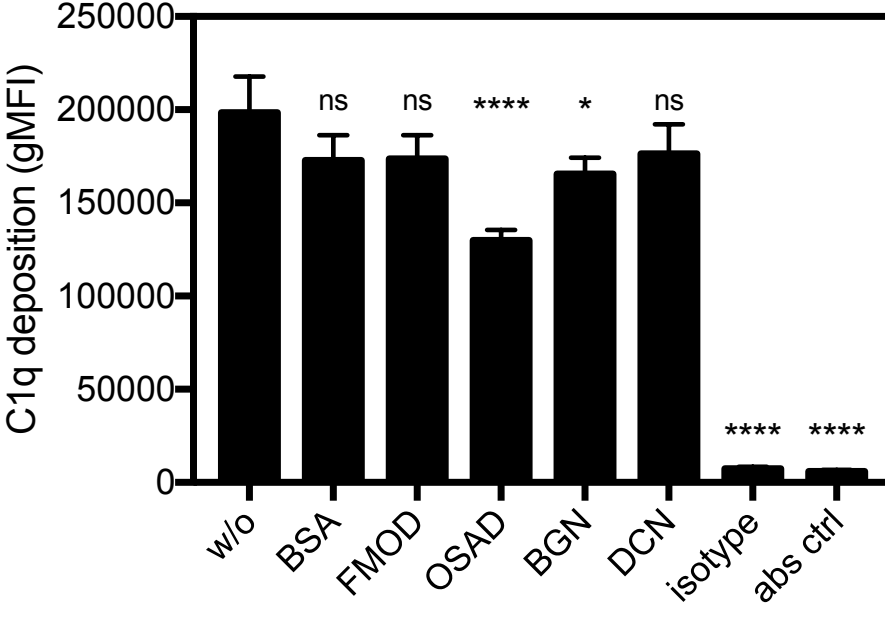

Figure S3. OSAD and BGN significantly reduce C1q binding on *M. catarrhalis*

Deposition of C1q on the surface of *M. catarrhalis* was analysed by flow cytometry using 1% OmCI-treated serum. Data illustrates the mean and SD of three independent experiments. Statistical differences were calculated using a one-way ANOVA with Dunnett's posttest. * $p < 0.05$, **** $p < 0.0001$.

Supplementary Figure 4

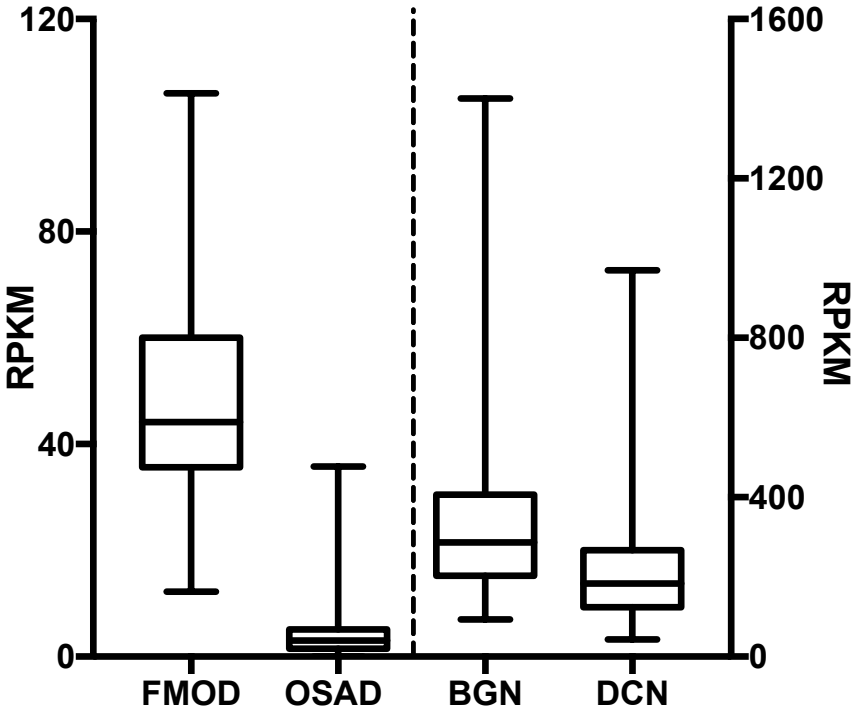

Figure S4. SLRP RNA expression from lung tissue

RNA expression of SLRPs from lung tissue samples (n=320) adapted from the Human Protein Atlas program (www.proteinatlas.org) (32). RNA sequencing data is reported in reads per kilobase per million mapped reads (RPKM).