Short Leucine-Rich Proteoglycans Modulate Complement Activity and Increase Killing of the Respiratory Pathogen Moraxella catarrhalis

Maisem Laabei,*1 Guanghui Liu,*1,2 David Ermert,* John D. Lambris,† Kristian Riesbeck,‡ and Anna M. Blom*

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 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. In this study, 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 suggest 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 ubiquitous surface protein A2/A2H in mediating binding to host SLRPs. A conserved immune evasive strategy used by 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. The Journal of Immunology, 2018, 201: 000–000.

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 APCs 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 and initiates the formation of the membrane attack complex (MAC), 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 serve as cofactors for the serum 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 (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 because of 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 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. Several SLRPs have been shown to interact with integrins (7). More recently, the role of SLRPs as complement modulators, both as activators and inhibitors (10). Both FMOD and OSAD interact with TLR, 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 sera**

A list of bacterial strains used in this study is shown in Table I. *M. catarrhalis* clinical and laboratory strains, *Haemophilus influenzae* type b strain RM804 and non-typeable *H. influenzae* strain 3655 were grown on chocolate agar plates for 24 h at 37°C with 5% CO2. Bacteria were subsequently streaked onto new chocolate agar plates for 6 h, scraped from plates, resuspended in 25% (v/v) brain–heart infusion broth (BHI) broth/glycerol, and stored in aliquots at −80°C. *Pseudomonas aeruginosa* ATCC 27853 and KR601 were grown in Luria–Bertani broth and adjusted to 0.3 M NaCl and 50 mM Tris–HCl (pH 8). The eluted proteins were analyzed by SDS-PAGE, diazylated against PBS, and stored at −80°C in aliquots. SLRPs were confirmed by Western blotting with polyclonal rabbit anti-bovine SLRPs Abs (home-made). The yield of protein from 1 l 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).

**Proteins, Abs, and sera**

Human recombinant SLRPs, including FMOD, OSAD, BGN, and 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 FMOD was a gift from Dr. S. Kalamajski (15) (Uppsala University, Uppsala, Sweden). 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). 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 Ni2+–NTA column equilibrated with 50 mM Tris–HCl (pH 8) with 0.3 M NaCl. After washing with 5 vol of 50 mM Tris–HCl (pH 8), the protein in the column was eluted with a linear gradient of 0–500 mM imidazole in 50 mM Tris–HCl (pH 8). The eluted proteins were analyzed by SDS-PAGE, diazylated against PBS, and stored at −80°C in aliquots. SLRPs were confirmed by Western blotting with polyclonal rabbit anti-bovine SLRPs Abs (home-made). The yield of protein from 1 l 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).

**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 × 106 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 (20 μM) after incubating 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 conjugate 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 because of C1q binding to the Ab column. C4BP, purified from the serum from which it was depleted, was replenished at physiological concentrations (200 μg/ml).

*Normal human serum* (NHS) was prepared from freshly drawn blood obtained from 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 × g 4°C for 8 min, serum fractions were collected, 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, Sweden (permit 2017/582) and the Declaration of Helsinki (18). To prepare C4BP-depleted NHS (C4BP-dpl), freshly pooled human serum from four donors was passed through a HiTrap affinity column coupled with the monoclonal C4BP Ab MK104. Resulting serum samples were verified to be C4BP-dpl 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 because of C1q binding to the Ab column. C4BP, purified from the serum from which it was depleted, was replenished at physiological concentrations (200 μg/ml).

**Resulting serum samples were verified to be C4BP-dpl 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 because of C1q binding to the Ab column. C4BP, purified from the serum from which it was depleted, was replenished at physiological concentrations (200 μg/ml).**
were incubated at 37˚C and 5% CO2, and at time 30 and 60 min neu-
mosium (22) on ice for 30 min or compstatin CP40 (20 μM) 
lement C5 inhibitor OmCI (10 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 Ab 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. Geometric mean fluorescence intensity was used to determine the amount of complement deposition. Heat-inactivated serum, primary isotype Ab, and secondary Ab only controls were used to assess specificity of Abs used. Stained and unstained bacteria were used for gating bacteria, and a minimum of 20,000 events were examined.

Neutrophil bactericidal assays

Human neutrophils were isolated using, first, a Histopaque-1119 (Sigma-Aldrich) separation of peripheral venous blood drawn from healthy volunteers and, second, 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 >95%. For neutrophil bactericidal assays, neutrophils (5 × 106) were incubated with M. catarrhalis (5 × 104 CFU) (multiplicity of infection 10) in the presence of 200 μg/ml SLRPs (4.5 μM FMOD, 3.9 μM OSAD, 4.7 μM BGN, and 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 and 5% CO2, 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% CO2. 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) in which 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 ECMs 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 SLRPs, 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 because of glycosylation. Next, we determined the binding of biotinylated SLRPs to major Gram-negative bacterial species important in respiratory infections, namely P. aeruginosa, H. 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 NHS. We found that SLRPs FMOD, OSAD, and BGN, when supplemented at 50 μg/ml, significantly decreased survival of both M. catarrhalis RH4 (Fig. 2A) and Bc5 (Fig. 2B) in NHS. Despite being not statistically significant, DCN led to a slight reduction in survival in the Bc5 strain compared with BSA, but no difference was observed in strain RH4, suggesting that DCN does not enhance complement-mediated killing of M. catarrhalis. Furthermore, inhibition of MAC formation by previous treatment of serum with the C5

Table 1. List of strains used in this study

<table>
<thead>
<tr>
<th>Clinical Isolate/Strain</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KR529</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR485</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>O35E</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR516</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR531</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR540</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR503</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR488</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR509</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR484</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR480</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>BKH18</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>G6E</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>CCUG353</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>KR483</td>
<td>Clinical isolate</td>
<td>(14)</td>
</tr>
<tr>
<td>Bc5</td>
<td>Laboratory strain</td>
<td>(34)</td>
</tr>
<tr>
<td>RH4</td>
<td>Laboratory strain</td>
<td>(35)</td>
</tr>
<tr>
<td>RH4ΔupsA1</td>
<td>RH4 devoid of Usp A1</td>
<td>(6)</td>
</tr>
<tr>
<td>RH4ΔupsA2</td>
<td>RH4 devoid of Usp A2</td>
<td>(6)</td>
</tr>
<tr>
<td>RH4ΔupsA1ΔupsA2</td>
<td>RH4 devoid of both Usp A1 and A2</td>
<td>(6)</td>
</tr>
<tr>
<td>RH4Δmid</td>
<td>RH4 devoid of IgD-binding protein (MID)</td>
<td>(36)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>ATCC27853</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>KR601</td>
<td>Clinical isolate</td>
<td>This study</td>
</tr>
<tr>
<td>H. influenzae</td>
<td>Type b strain</td>
<td>Clinical isolate, capsule-deficient</td>
</tr>
<tr>
<td>Nontypeable (NTHi) STRA</td>
<td>Strain 3655</td>
<td>Clinical isolate, encapsulated</td>
</tr>
</tbody>
</table>

ATCC, American Type Culture Collection; CCUG, Culture Collection University of Gothenburg.
inhibitor OmCI prevented killing of *M. catarrhalis* under any SLRP condition, illustrating that SLRPs enhance killing through complement-mediated lysis (Fig. 2A, 2B). Finally, no antimicrobial activity was observed when SLRPs were incubated with *M. catarrhalis* in GVB++ buffer in the absence of serum, confirming that the enhanced killing was mediated by complement.

To verify that excess unbound SLRPs were not causing a bystander complement activation effect and contributing to enhanced killing, we also measured the effect of washing bacteria following SLRP binding prior to incubation with serum (Supplemental Fig. 1). As in the above results, a significant decrease in survival was observed for FMOD, OSAD, and BGN but not DCN, indicating the SLRPs bound to the bacterial surface promoted enhanced bacterial killing in the presence of serum.

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

*M. catarrhalis* interacts with human proteins via major surface proteins such as OmpCD and Mha (4). Given that previous work has shown that UspA1, UspA2, and MID can interact with soluble ECM proteins, we investigated the interaction of wild-type (RH4) and isogenic mutants lacking the above surface proteins with biotinylated SLRPs through flow cytometry (Fig. 3A–D). We found that deletion of the *uspA2* gene resulted in a 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 either 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, 3F). 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 (*K_d = 8.96 ± 11 nM*); similar affinities were seen for FMOD (*K_d = 202 ± 21 nM*) and OSAD (*K_d = 231 ± 28 nM*), and the lowest affinity was seen for DCN (*K_d = 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 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 (Supplemental Fig. 2).

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

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

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

To confirm our results that SLRPs inhibit binding of C4BP and, thus, disrupt a major immune evasive strategy of *M. catarrhalis*, we depleted C4BP from NHS using an anti-C4BP mAb MK104-coupled column, which interacts with high affinity to the α-chain complement control protein (CCP) domain 1 of C4BP (17). C4BP-dpl resulted in increased killing of *M. catarrhalis* RH4 compared with NHS in the presence of both BSA and DCN (Fig. 5D). Increased survival comparable to NHS was observed following replenishment of purified C4BP to physiological levels (200 μg/ml) when BSA and DCN were present. In comparison, FMOD, OSAD, and BGN enhanced serum bactericidal activity in NHS compared with both BSA and DCN as observed previously (Fig. 2A, 2B). Importantly, no significant change in serum killing was observed between BSA/DCN and FMOD/OSAD/BGN in C4BP-dpl (Fig. 5D), confirming that prevention of C4BP binding by FMOD, OSAD, and BGN to the bacterial surface is responsible for the increased complement-mediated killing of *M. catarrhalis*.

**OSAD and BGN enhance neutrophil killing of *M. catarrhalis* in a complement-dependent and independent manner**

Neutrophils represent a critical phagocytic cell type in innate immunity, central to host defense against invading pathogens. Additionally, complement-mediated opsonization accelerates phagocytosis and removal of pathogenic bacteria. Considering that SLRP-bound bacteria had increased complement deposition in the presence of serum, we wanted to investigate whether this translated into increased killing in a neutrophil bactericidal assay. Interestingly, in the presence of human neutrophils and OmCl-treated serum, both OSAD and BGN significantly enhanced *M. catarrhalis* RH4 killing observed at both 30 and 60 min incubation periods (Fig. 6A). Incubation with FMOD or DCN did not significantly increase bacterial killing compared with BSA. Next, we wanted to investigate whether this enhanced neutrophil killing was dependent on complement opsonization or whether SLRPs themselves could serve as mediators of enhanced neutrophil killing. Using OSAD and BGN, we repeated the neutrophil bactericidal assays with either OmCl-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 OmCl-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 OmCl- and compstatin-treated sera compared with BSA. This suggests that the main mechanism of SLRP-dependently enhanced neutrophil killing is via complement activation. After a prolonged incubation time, however, SLRPs promote a bactericidal killing effect in concert with neutrophils that 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 Clq from serum in the presence of SLRPs (Supplemental Fig. 3). We observed no difference in binding of Clq to the bacterial surface when bacteria were incubated with FMOD or DCN compared with BSA. In contrast, a significant reduction in Clq 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 Clq binding.

Discussion

*M. catarrhalis* causes significant morbidity in children and chronic obstructive pulmonary disease patients and is responsible for a plethora of respiratory infections and occasionally systemic diseases (26). The exact molecular mechanisms governing *M. catarrhalis* pathogenicity are not fully understood. However, mounting evidence suggests that immune evasion, directed primarily at circumventing the complement system, is an essential feature of pathogenic strains (4–6, 14, 21, 27). Therefore, future treatment intervention directed at hampering complement inhibitor recruitment is a promising avenue of research. In this study, we highlight a novel antimicrobial role displayed by specific SLRPs, namely FMOD, OSAD, and BGN, and reveal in detail the molecular mechanisms resulting in enhanced innate immunity against *M. catarrhalis*. SLRPs, such as BGN and DCN, are considered bifunctional proteoglycans, acting both as central structural components of the ECM and danger-associated molecular patterns stimulating immune reactions (28). SLRPs are abundantly present in the ECM and distributed in numerous tissues throughout the body (7). Previous immunohistochemical analysis has shown that BGN and DCN are expressed in the human lung and bronchial tissue (29–31). Furthermore, mining of the Human Protein Atlas (www.proteinatlas.org) (32), a genome-wide analysis of RNA and protein expression from samples representing major tissues and...
organs, confirmed the expression of all SLRPs used in this study in lung tissue. RNA expression data generated from 320 individual tissue samples showed that for this set of SLRPs, BGN and DCN had the highest expression, followed by FMOD, with OSAD having the lowest expression (Supplemental Fig. 4). Combined, these expression data and previous immunohistochemical analysis indicate that these SLRPs are present in sites 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 tissues 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 nonsterile 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 TNF-α and MIP-2, contributing to the overall proinflammatory environment and increased SLRP expression (8). Furthermore, DCN expression was higher in cohorts of septic patients compared with healthy controls (9). Previous studies by this group also estimated another SLRP, proline/arginine-rich end leucine-rich repeat protein (PRELP), to be present at a similar range 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 proinflammatory cytokines stimulating macrophages and other immune and nonimmune 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 UspA1 and A2/2H interact with C4BP; however, UspA2/2H is more strongly expressed than UspA1 and, therefore, plays a more prominent role in C4BP binding and in conferring a complement resistant phenotype (6). Through mutational analysis, we determined that all four SLRPs bound to *M. catarrhalis* predominantly through UspA2/2H. This suggested that SLRP binding to UspA2/2H could competitively inhibit C4BP binding, resulting in reduced complement inhibition. Using flow cytometry, we illustrated that prior binding of FMOD, OSAD, and BGN but not DCN to *M. catarrhalis* effectively reduced C4BP binding, thus explaining the increased serum sensitivity.

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

Incubation of bacteria with SLRPs in the presence of serum resulted in significant opsonization with C3b/iC3b (Fig. 5A). As these opsonins are recognized by complement receptors present on neutrophils, we wished to examine where this resulted in enhanced neutrophil bactericidal killing. In this study, we observed that only OSAD and BGN effectively increased neutrophil killing of *M. catarrhalis* but not FMOD or DCN. This was surprising, considering that FMOD enhanced C3b opsonization in the presence of serum. Therefore, we checked whether this enhanced neutrophil killing was independent of complement by using compstatin-treated serum, effectively blocking complement at the C3 level. We observed that the majority of the neutrophil killing was complement (opsonization)-mediated (Fig. 6A). However, with prolonged incubation, both OSAD and BGN enhanced killing in a complement (opsonization)-independent manner. It is known that certain ECM proteins, such as the SLRP lumican, can enhance phagocytosis by interacting with both bacteria and phagocytes via surface-expressed integrins (33). Additionally, it has been shown that other SLRPs, such as BGN and DCN, can bind to TLR expressed on professional phagocytes and induce a proinflammatory response (8, 9). Two questions arise that require future molecular dissection: 1) Can FMOD and OSAD interact with professional phagocytes and induce an immune response analogous to BGN and DCN; and 2) Can the SLRPs in question mediate an interaction between bacteria and phagocytes that facilitates enhanced phagocytosis and subsequent killing. As such, future molecular characterization is underway to elucidate fully the mechanisms of SLRP-mediated neutrophil bactericidal activity.

Recent work by our laboratory has shown that respiratory pathogens, such as *M. catarrhalis*, can interact with ECM components whereby two opposing scenarios may result, namely attenuated or enhanced complement activity. *M. catarrhalis* interacts with cartilage oligomeric matrix protein (COMP), preventing complement deposition and interfering with complement-independent phagocytosis, enhancing survival (21). Conversely, *M. catarrhalis* can be bound by the SLRP proline/arginine-rich end leucine-rich repeat protein (PRELP), which disrupts C4BP binding, significantly augmenting complement-mediated lysis and neutrophil killing (14). To this complex interaction between complement, *M. catarrhalis*, and ECM components we introduce the newfound antibacterial role of FMOD, OSAD, and BGN, which through interaction with the surface-expressed UspA2/2H and in concert with complement, accelerate the eradication of an important respiratory pathogen. Finally, the elucidation of the molecular basis for SLRP-mediated–enhanced killing may provide novel research avenues to devise therapies to treat infection.
37˚C and 5% CO2. Following incubation, total viable bacteria were enumerated following lysis of neutrophils, and bacterial survival was calculated by dividing CFU at time 30 or 60 with that of time 0. Graphs are presented as the mean and SD of five independent experiments and analyzed using a two-way ANOVA with Bonferroni post hoc tests comparing SLRP/BSA condition to that of no protein control (w/o) (A) or SLRPs to BSA control (B). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Acknowledgments

We thank Dr. Sebastian Kalamajski (Uppsala University) for the kind gift of the pCEP4:FMOD plasmid and the Swedish Orphan Biovitrum for the OmCl vector and Dr. Sara Nilsson and Dr. Chrysostomi Gialeli (Lund University) for their helpful discussions on the manuscript.

Disclosures

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 for the treatment of diseases characterized by complement activation and inflammation and cancer through PDCD4 and MicroRNA-21. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. JAMA 310: 2191–2194.

References


